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PLANT TISSUE CULTURE

Assessment of genetic, biochemical fidelity, and therapeutic activity of *in vitro* regenerated *Hedychium coronarium*

Shashikanta Behera[1,2] · Subrat K. Kar[4] · Kumari Monalisa[1] · Soumyajit Mohapatra[1,5] · Rajesh K. Meher[2] · Durga P. Barik[1] · Pratap C. Panda[6] · Pradeep K. Naik[2] · Soumendra K. Naik[1,3]

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Abstract

Hedychium coronarium J. Koenig is a rhizomatous herb (Zingiberaceae family) and is well known for its uses in traditional systems of medicine for the treatment of various diseases. The plant has been over-exploited and enlisted as a threatened species in India. Thus, there is an urgent attention required for its conservation. Here, a callus-mediated *in vitro* plant regeneration protocol was developed using leaf sheath of *H. coronarium*. The optimal medium for callus induction (85.0%) and subsequent proliferation was found to be MS basal medium augmented with 3.0 mg L^{-1} N^6-benzyladenine (BA), 0.5 mg L^{-1} α-naphthaleneacetic acid (NAA), and 3.0% sucrose, and gelled with 0.5% agar. Optimum callus-mediated shoot organogenesis (78.3%; ca. 11.4 shoots/0.5 g of callus) was obtained on MS medium supplemented with 3.0 mg L^{-1} BA after 6 wk of culture. All the *in vitro* regenerated shoots were rooted (8.5 roots/shoot) successfully on plant growth regulator–free MS medium. About 90% plantlets were acclimatized on the planting tray filled with garden soil and sand (1:1). Transfer of these plants to larger pots containing garden soil and subsequent field transfer under full sun resulted in cent-percent survival. Monomorphic banding profile obtained using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) markers ascertained the clonal fidelity of the *in vitro* regenerated plants. Similarly, the biochemical fidelity of the *in vitro* regenerated plants vis-à-vis mother plant was ascertained by comparing the composition of essential oil through gas chromatography/mass spectrometry (GC/MS) analysis. Furthermore, the antioxidant activities estimated by DPPH and ABTS free radical scavenging assay and anticancer activities evaluated against two cell lines, *i.e.*, MCF 7 and MDA-MB 231, also confirmed comparable effectiveness of *in vitro* regenerated plants to that of the mother plant. Thus, the study has the potential to provide a platform to achieve sustainability by using the *in vitro* regenerated *H. coronarium* in place of naturally available population.

Keywords Anticancer activity · Antioxidant activity · Callus · Organogenesis · Essential oil

✉ Soumendra K. Naik
sknuu@yahoo.com

1 Department of Botany, Ravenshaw University, Cuttack 753 003, Odisha, India

2 Centre of Excellence in Natural Products and Therapeutics, Department of Biotechnology and Bioinformatics, Sambalpur University, Sambalpur 768019, Odisha, India

3 Center of Excellence in Environment and Public Health, Ravenshaw University, Cuttack 753 003, Odisha, India

4 Rivaara Labs, BS-3, B-Wing, Kamal Palace, Ramdaspeth, Nagpur 440010, Maharashtra, India

5 Central Institute of Medicinal and Aromatic Plants, CSIR, Lucknow 226015, Uttar Pradesh, India

6 Centre for Biotechnology, School of Pharmaceutical Sciences, Siksha 'O' Anusandhan (Deemed to be University), Bhubaneswar 751003, Odisha, India

Introduction

Zingiberaceae or the ginger family is the largest flowering family of the order Zingiberales. *Hedychium coronarium* J. Koenig, commonly known as butterfly ginger or butterfly lily, is a valuable multipurpose plant with medicinal, ornamental, food, and cosmetic values belonging to the Zingiberaceae family. Different parts of this plant, such as rhizomes, leaves, roots, and flowers, have been used in traditional systems of medicine for the treatment of various diseases, including fever, headache, infected nostrils, inflammation, insomnia, stomach disorders, pain caused by rheumatism, and tonsillitis (Bailly 2020; Tavares *et al.* 2020). These medicinal uses are due to the attributes of various bioactive compounds, *i.e.*, phenols, terpenoids, flavonoids,

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and glycosides (Zhou *et al.* 2021). Especially, labdane-type diterpene coronarin D has already been recognized as an important signature pharmaceutical bioactive compound in *H. coronarium*. Several reports have already confirmed the various pharmaceutical (*e.g.*, anticancer, anti-inflammatory, and antimicrobial) activities of coronarin D (Bailly 2020); Zhou *et al.* 2020. The essential oil derived from leaves, flowers, and rhizome of this plant also exhibits anti-inflammatory, antimicrobial, cytotoxic, antioxidant, and analgesic activities (Chan and Wong 2015; Costa *et al.* 2021).

H. coronarium is also used for ornamental purposes and planted both in pots and gardens because of its beautiful flowers with nice fragrance (Li and Fan 2007; Behera *et al.* 2020). Besides, the plant is known to be used in perfume industries (Chadha 2005; Behera *et al.* 2018a). Different parts of this plant have also been used as food source in different countries, including India, the USA (in Hawaii), and Japan (Chan and Wong 2015; Behera *et al.* 2018b). Due to demand associated with its medicinal and food values, the plant has already been over-exploited and enlisted in vulnerable or near threatened category (Ved *et al.* 2008). Thus, an efficient *in vitro* plant regeneration protocol is required to produce large number of plants for conservation, as source of food and utilization in pharmaceutical sector. *H. coronarium* is propagated vegetatively by separating its rhizomes, but it becomes dormant during summer and winter season which slowed down round the year propagation of the plant (Chadha 2005; Behera *et al.* 2020). For the better utilization of *H. coronarium*, an efficient *in vitro* plant propagation system could not only increase the propagation rate but also offer possibility for its sustainable utilization without influencing the wild population.

Various plant propagation methods have already been developed for *H. coronarium in vitro* (Mohanty *et al.* 2013; Verma and Bansal 2013, 2014; Behera *et al.* 2018b, 2019, 2020). However, callus-mediated plant propagation method for *H. coronarium* is lacking to date. A single preliminary report on callus culture for this plant has been reported, to date, by Parida (2018). They studied the effects of different plant growth regulators for callus induction, but no data has been provided about the regeneration of plants from callus. Therefore, one of the objectives of this study was to obtain an efficient plant regeneration protocol by investigating the effect of different factors, such as plant growth regulators and concentration of agar, on callus induction, proliferation, and subsequent callus-mediated plant regeneration. Evaluation of genetic and biochemical stability, and pharmaceutical potential of *in vitro* regenerated plants is always crucial for the success of a tissue culture–mediated plant regeneration protocol. In this backdrop, the next objective of this study was to check the genetic fidelity as well as biochemical stability of the callus-mediated field-established plants (henceforth called as *in vitro* regenerated plant(s)) vis-à-vis mother plant. Molecular markers such as Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) were used to check the genetic fidelity. Biochemical stability in terms of chemical composition of essential oil was tested through gas chromatography/mass spectrometry (GC/MS) analysis and antioxidant activity analysis by DPPH and ABTS free radical assays. Finally, the pharmaceutical activity, *i.e.*, anticancer activity, against two different breast cancer cell lines, *i.e.*, MCF 7 and MDA MB 231, of the *in vitro* regenerated plants vis-à-vis mother plant was tested. Overall, the objectives of the work were to standardize an efficient callus-mediated plant regeneration protocol and ascertain the genetic and biochemical stability as well as pharmaceutical efficiency of the *in vitro* regenerated plants vis-à-vis mother plant for sustainable utilization of *H. coronarium*.

Materials and methods

Plant materials and surface sterilization of explants Plants of *H. coronarium* were collected from the Botanical Garden (20° 18′ 09.0″ N, 85° 48′ 18.5″ E, 241 ft. above the sea level) of Regional Plant Resource Centre (RPRC), Bhubaneswar, India, and authenticated by Dr. P.C. Panda, Principal Scientist, Taxonomy and Conservation Division, Regional Plant Resource Centre (RPRC). The specimen voucher (12004/ RPRC) has been deposited in the institutional herbarium of RPRC.

The plants were subsequently maintained in the garden of the Department of Botany, Ravenshaw University, Cuttack, Odisha, India, and used as the source of explant. The healthy, young shoots of those plants (2 yr old) were collected and washed under running tap water to remove the dust particles. Leaves were excised and the shoots were cut into small pieces (3.0–4.0 cm), followed by 10 min treatment with aqueous solution of "Teepol," a liquid detergent (5%, v/v; Reckiit Benckiser Ltd., Gurgaon, India). Subsequently the explants were subjected to treatment with a fungicide (2%, w/v), namely "Bavistin" (BASF, Mumbai, India), for 5 min and then rinsed with double distilled water (3–4 times). Finally surface sterilization of shoot was carried out under a laminar hood with 0.1% (w/v) $HgCl_2$ (Hi-Media, India) for 6 min followed by proper washing in autoclaved double distilled water. Finally, leaf sheaths from the shoots were removed and cut into small pieces (1.5–2.0 cm) and used as explants for callus culture and subsequent organogenesis.

Optimization of callus induction and proliferation medium Influence of plant growth regulators on callus culture For callus induction and proliferation, Murashige and Skoog's (1962) (MS) medium was used as the basal medium. Sucrose (3.0%) and 0.7% (w/v) agar were used as the carbon source and gelling agent respectively. The pH of the medium was adjusted to 5.8 ± 0.1 prior to addition of agar and sterilized at 15 psi and 121°C for 17 min using an autoclave. The MS medium either free of plant growth regulator(s) or supplemented with various concentrations and combinations of plant growth regulators like N^6-benzyladenine (BA; 1.0–4.0

mg L^{-1}), α-naphthaleneacetic acid (NAA; 0.25–1.0 mg L^{-1}), and 2,4-dichlorophenoxyacetic acid (2,4-D; 1.0–5.0 mg L^{-1}) was used for induction and proliferation of callus.

Influence of agar, media pH, and sucrose on callus culture "Callus culture medium" (MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA + 0.7% agar) that showed the highest callus biomass was subsequently used to find out the influence of different concentrations of agar (0.1–1.0%) on callus growth and standardize the medium further for optimum agar concentration. The effects of different media pH (4.5–6.5) and concentrations of sucrose (1.0–5.0%) on the growth of callus were also evaluated using the earlier standardize medium (MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA + 0.5% agar).

The calli were separated from explants and sub-cultured to fresh culture medium at 4 wk of interval. The number of explants exhibiting callus formation was recorded to obtain the frequency of callus induction. Some of the calli were transferred to the medium for shoot regeneration. Other calli were maintained on the "optimum callus culture medium" to study their potential for morphogenesis after various passages of callus sub-culture. Growth of callus was estimated by fresh weight (FW) and dry weight (DW) measurements. Callus was dried at room temperature until a constant weight was obtained and weighed to find out the DW. Growth of callus was also recorded on the degree of callus formation based on observation through the naked eye.

Shoot organogenesis media Calli produced on "optimum callus culture (induction and proliferation) medium" (*i.e.*, MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA + 3.0% sucrose gelled with 0.5% agar at pH 5.8) were used to evaluate optimum medium for shoot regeneration from callus. Approximately 0.5 g of calli was transferred to either MS basal medium devoid of plant growth regulator(s) or containing different concentrations and combinations of BA (1.0–5.0 mg L^{-1}), zeatin (Z; 1.0–5.0 mg L^{-1}), *meta*-topolin (mT; 1.0–5.0 mg L^{-1}), and adenine sulfate (ADS; 25–100 mg L^{-1}) for shoot regeneration from callus. The shoots obtained per callus were recorded at 6 wk of culture.

Rooting of *in vitro* regenerated shoots derived from callus *In vitro* regenerated shoots (3.0–6.0 cm) were harvested individually from the callus and inoculated on growth regulator–free MS medium for rooting.

Acclimatization About 100 well-rooted *in vitro* shoots were removed from the culture vessels. The roots were thoroughly but carefully washed under tap water to clean the agar. These plantlets were planted in the small pots of planting trays containing autoclaved garden soil and sand (1:1) followed by field transfer under full sun as per the procedure described by Behera *et al.* (2019).

Morphological analysis and estimation of chlorophyll content The mother plant vis-à-vis *in vitro* regenerated plants (one and half years old field-established plants) was taken for morphological characteristic analysis. Morphological features, such as plant height, pseudo stem number/plant, leaf number/shoot, leaf size, and fresh and dry weight of rhizome, were compared between them. Furthermore, the total chlorophyll content was estimated using the protocol described by Behera *et al.* (2018b).

Genetic fidelity analysis through RAPD and ISSR markers Genetic fidelity analysis of the *in vitro* regenerated plants was carried out by using both Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers. Randomly, 10 plants (one and half year's old) were selected for genetic fidelity study. For genomic DNA isolation, fresh tender leaves were collected from the mother plant and 10 randomly selected *in vitro* regenerated plants. Isolation of genomic DNA was carried out by using the standard procedure developed by Doyle and Doyle (1990) with minor modifications. After isolation of genomic DNA, its purification and quantification were carried out as described earlier by Behera *et al.* (2018b). Randomly, 18 and 20 different RAPD and ISSR primers (Eurofins, India) respectively were chosen for preliminary screening to check the genetic fidelity of the *in vitro* regenerated plants. Out of these, seven primers for RAPD and nine primers for ISSR were used for final genetic fidelity analysis. The basis of this selection was their clear and distinct banding pattern with reproducibility. Amplification of DNA by polymerase chain reaction (PCR) was carried out as reported previously by Behera *et al.* (2020) and the sizes of amplicons were estimated using a 100-bp plus DNA ladder (3 kb) (Thermo Scientific, USA).

Essential oil extraction and GC/MS analysis For the analysis of chemical constituents of essential oil, 500 g fresh rhizome sample of both mother and *in vitro* regenerated plants (one and half years old plants after transfer to field under natural conditions) was collected, washed properly under running tap water, and chopped into small pieces. The essential oil was extracted by hydro-distillation method using Clevenger-type apparatus (Borosil, India). The flask having rhizomes was heated at 50°C for 8 h and the condensed vapor was separated in oil-water surface. The extracted essential oil was collected and dehydrated by using Na_2SO_4 and was kept in the refrigerator at 4°C for the study of GC/MS analysis and antioxidant activity.

The chemical constituents of essential oil were analyzed by GC/MS (Agilent Technologies 7980 A gas chromatograph system with the 5977 A mass selective detector). About 1 µL of diluted essential oil (oil:hexane; 1:10) was injected for GC/MS analysis. The column used was DB5-MS (dimension 30 m × 250 µm; film thickness 0.25 µm) for finding the peak

separation in the chromatogram. Helium (split ratio of 10:1; flow rate of 1 mL min^{-1}) was used as the carrier gas. The running condition for the sample was programmed as described earlier by Behera *et al.* (2022b). Data was processed by the MSD Chemstation F.01.01.2317 software. For compound identification, NIST/EPA/NIH mass spectral library (version 2.0 g) was used (Agilent Technologies, USA).

Phytochemical analysis The mother plant of *H. coronarium* and *in vitro* regenerated plant rhizome sample was used for comparative phytochemical analysis. Phytochemicals, such as alkaloids, flavonoids, phenolics, tannins, and saponins content, were estimated following the protocol described by Behera *et al.* (2018b; 2022b).

Preparation of rhizome extract For antioxidant and anticancer activity experiments, the rhizome samples of both the mother plant and randomly ten *in vitro* regenerated plants (one and half years old plants) were selected and used for extract preparation. The extracts were prepared in aqueous, methanol, and acetone solvent system separately following the standard procedure described by Behera *et al.* (2022b). Finally, the extracts were dried and stored at 4°C for antioxidant and anticancer activity profiling.

Antioxidant activity of essential oil and rhizome extract

2,2-Diphenyl-1-picryhydrazyl (DPPH) scavenging assay This experiment was conducted to estimate the DPPH radical scavenging activity of rhizome essential oil and rhizome aqueous and methanol extract of the mother as well as *in vitro* regenerated plants using the procedure described by Borah *et al.* (2019) with minor modifications. One milliliter of plant sample (both essential oil and methanol extract) with different concentrations (0.05–2.4 mg mL^{-1}) was added to 1 mL of 0.15 mM DPPH methanol solution. The reaction mixture was mixed properly and incubated in dark condition for 30 min at room temperature. Then, the absorbance was taken at 517 nm using ascorbic acid as standard. The concentration of rhizome essential oil and rhizome aqueous and methanol extract that could scavenge 50% of the DPPH radicals was measured as IC_{50} value.

2,2-Azino-bis (3-Ethylbenzothiazoline) (ABTS) scavenging assay ABTS radical scavenging assay of rhizome essential oil and rhizome aqueous and methanol extract of the mother plant and *in vitro* regenerated plants was carried out as the procedure described by Panigrahy *et al.* (2017) with slight modification. Stock solution of ABTS was prepared by mixing 7.4 mM ABTS and 2.6 mM ammonium persulfate followed by incubation at room temperature for 16 h before use. About 1 mL of plant samples each (essential oil, aqueous and methanol extracts) with different concentrations (0.05–2.4 mg mL^{-1}) was added to 1 mL of ABTS solution, incubated at room temperature in dark for 2 h. Then, ascorbic acid was taken as standard, and the absorbance was recorded at 734 nm. The percentage of scavenging activity of ABTS radicals was expressed using the formula as described for the DPPH test. The concentration of rhizome aqueous extract, methanol extracts, and rhizome essential oil that could scavenge 50% of the ABTS radicals was measured as IC_{50} value.

Anticancer activity of rhizome acetone extract

Cell proliferative assay The anticancer activity of aqueous and acetone extracts of rhizome (both mother plant and *in vitro* regenerated plants) was evaluated by using two different breast cancer cell lines, *i.e.*, MCF 7 and MDA MB 231 of human. MCF 7 and MDA MB 231 cell lines were allowed to grow on Minimal Essential Medium (MEM) and Dulbecco's Modified Minimal Essential Medium (DMEM) culture medium respectively supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in humidified atmosphere with 5% CO_2. In a 96-well plate, cancer cells were seeded at a density of 4×10^3 cells per well. They were then treated for 72 h with increasing concentrations, *i.e.*, from 6.25 to 150 µg mL^{-1}, of acetone extracts of both mother rhizome and rhizome of *in vitro* regenerated plants. The cells were then stained by 0.56% of sulforhodamine B in 1% acetic acid. The residual stain was then washed with 1% acetic acid solution. Tris base (10 mM; pH 10.5) was added to the 96-well plate containing fixed cells with protein-bound stain and the absorbance was taken at 495 nm wavelength using a 96-well plate reader (Bio-Rad, India). The IC_{50} values for the extract were calculated from the plate reader data by using online IC_{50} value calculator (AAT Bioquest Inc., Sunnyvale, CA).

Apoptosis assay Apoptotic cells were visualized by different staining methods including 4,6-diamidino-2-phenylindole (DAPI), acridine orange (AO), and ethidium bromide (EtBr) and merged staining method separately with fluorescence microscopy (Nikon Eclipse Ts2R-FL). MDA MB 231 cells were grown on 6-well plates and were treated with 50 µg mL^{-1} and 94 µg mL^{-1} (IC_{50}) of acetone extract of both mother plant rhizome and rhizome of *in vitro* regenerated plants respectively for 72 h. After incubation, the 6-well plate was fixed in 3% formaldehyde and washed with phosphate buffer saline (PBS). For DAPI staining, 1 mg/mL of DAPI stain was used. For AO and EtBr staining, treated cells were separately stained with 3 mg mL^{-1} of AO and EtBr respectively. After 5 min of staining, stained cells were washed by using PBS to remove unbound stain. Images were captured using a fluorescent microscope (Nikon Eclipse Ts2R-FL). Identification of apoptotic cells was based on stain taken by the cells and morphology of cells, *i.e.*, membrane blabbing forming apoptotic bodies as well as nuclear condensation.

Statistical analysis of data For experiments related to callus culture, each treatment comprised of 10 culture flasks and each flask consisted of 2 explants (*i.e.*, 20 explants/treatment; total 2×10×3=60). For shoot culture, each treatment consisted of 20 flasks and 0.5 g callus inoculated per flask. All these experiments were repeated three times. Data were analyzed using one-way analysis of variance (ANOVA) for a completely randomized design (CRD). Duncan's new multiple range test (DMRT) (Gomez and Gomez 1984) was used to separate the mean values for significant effect. Essential oil samples were taken in biologically triplicates and in technically duplicates for GC/MS analysis and data were represented as means with standard deviation. Morphological and phytochemical analysis data were analyzed using *t*-test.

Results

Establishment of callus culture

Influence of plant growth regulators The explants inoculated on plant growth regulator–free MS medium failed to show callus induction. It was observed that augmentation of plant growth regulator(s) to MS medium was essential for callus induction and subsequent proliferation. In all the media augmented with plant growth regulator(s), callus was initiated at the cut end of the leaf sheath within 2 wk of culture. It was observed that callus culture, *i.e.*, induction of callus and its subsequent growth, was significantly influenced by plant growth regulator(s) (Fig. 1). About 73.3% of the explants responded for callus induction on MS medium augmented with 3.0 mg L^{-1} BA along with 0.5 mg L^{-1} NAA. On this medium, the highest degree of callus induction was also observed (Fig. 1).

Influence of agar, pH, and sucrose During initial experiments when the "callus culture medium" (MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA) was gelled with 0.7% agar, about 73.3% explants responded for callus culture. Subsequently, the influence of different concentrations of agar was tested using "callus culture medium" to evaluate the suitable concentration of agar for callus induction and growth. Best induction and growth of callus in terms of biomass were observed on callus culture medium gelled with 0.5% agar. On this medium, both the rate of explant showing callus induction (85.0%) and biomass of callus after 4 wk of subculture (6.75 g fresh weight; 0.373 g dry weight) were the highest. Very low concentrations and high concentrations of agar showed poor callus induction and subsequent growth (Fig. S1; Supplementary Data). "Revised callus culture

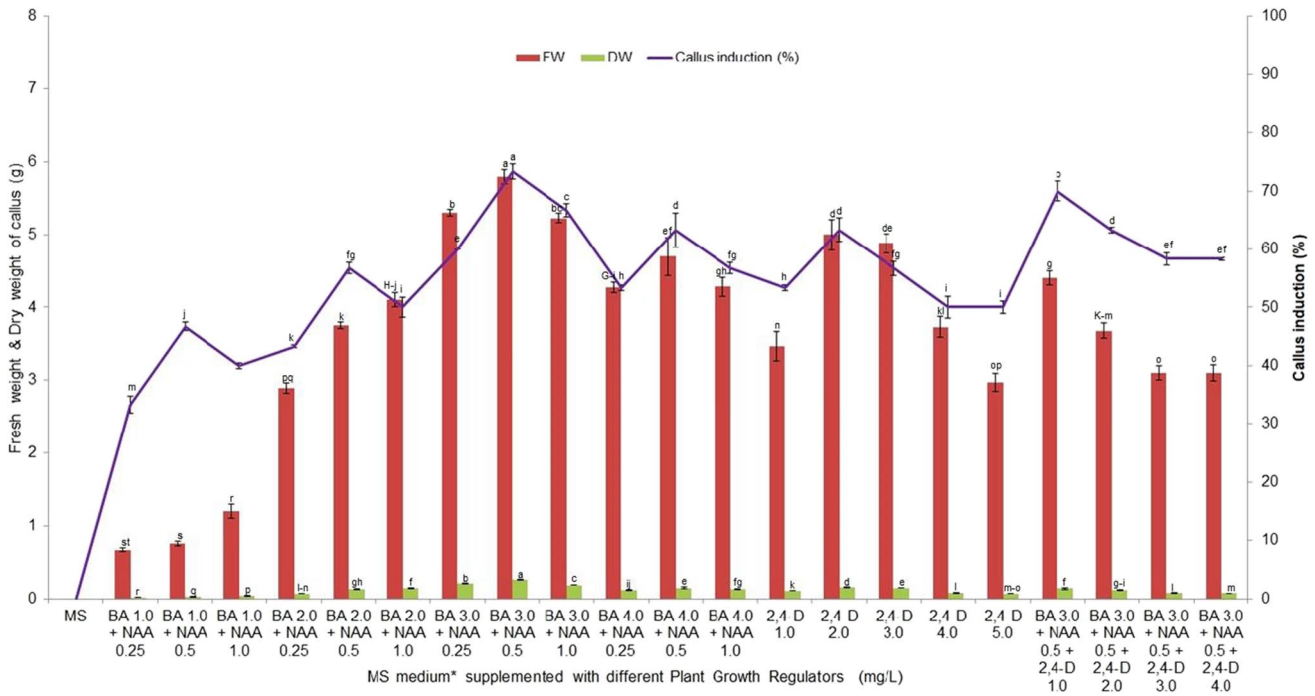
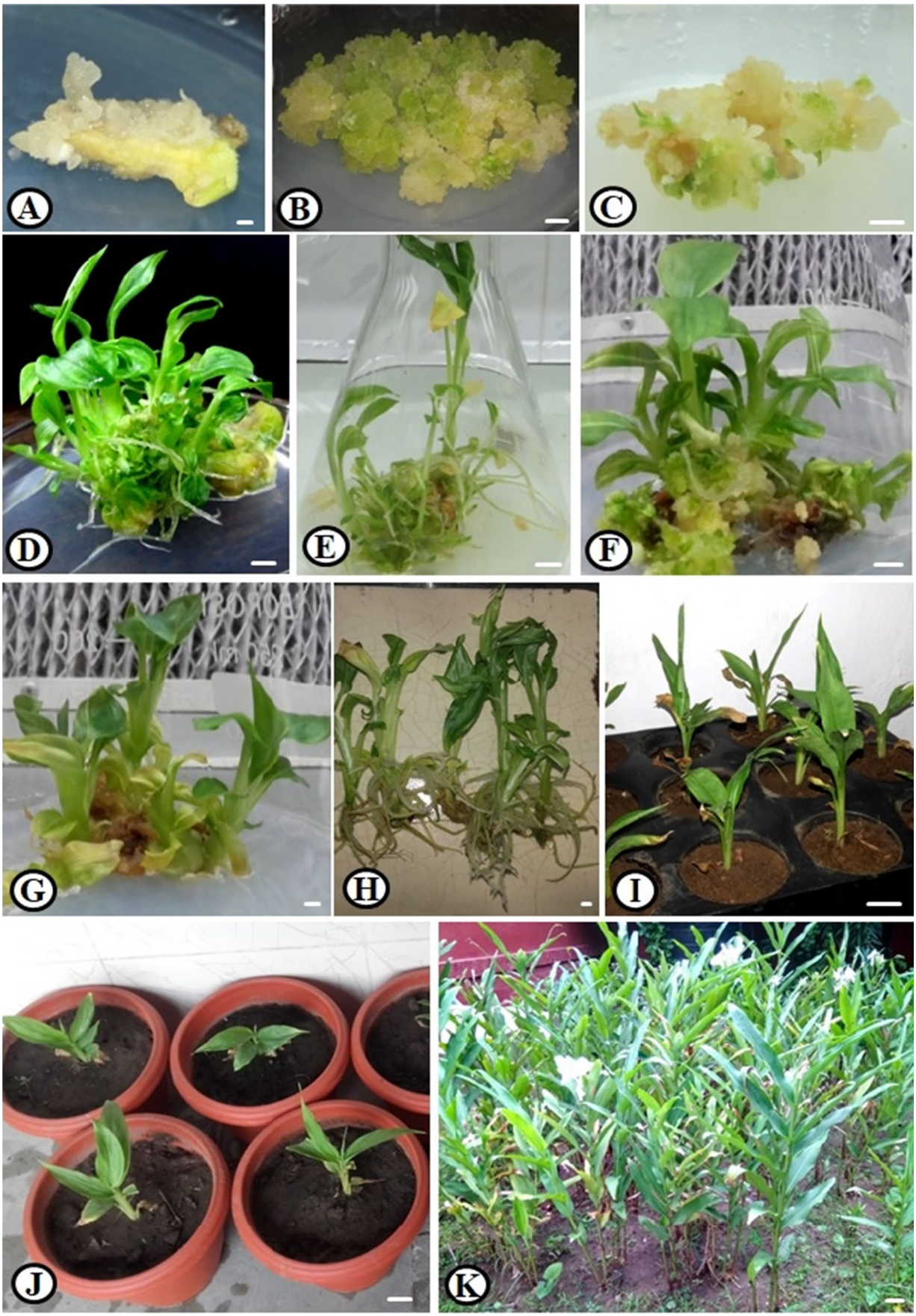


Figure 1. Influence of plant growth regulators on induction and proliferation of callus from leaf sheath explants of *H. coronarium*. *MS medium containing 3.0% sucrose, gelled with agar 0.7%, and pH adjusted to 5.8. Values represent means ± standard deviation (SD). *Bars* with different alphabets specify statistically significant different between means (one-way analysis of variance (ANOVA) with Duncan's new multiple range test, $p \leq 0.05$). BA, N^6-benzyladenine; NAA, α-naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; FW, fresh weight of callus (g); DW, dry weight of callus (g).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◂**Figure 2.** Callus-mediated plant regeneration *in Hedychium coronarium*: *A* Induction of callus from leaf sheath of *H. coronarium* on MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA + 3% sucrose gelled with 0.5% agar at pH 5.8 after 2 wk. *B* Proliferated callus after sub-culture on MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA + 3% sucrose gelled with 0.5% agar at pH 5.8 after 4 wk. *C* Emergence of multiple shoot buds from callus on MS + 3.0 mg L^{-1} BA (shoot regeneration medium). *D* Elongated multiple shoots from callus on MS + 3.0 mg L^{-1} BA after 6 wk. *E* Multiple shoot regeneration from callus on MS + 3.0 mg L^{-1} BA + 50 mg L^{-1} ADS. *F* Multiple shoot formation from callus on MS + 2.0 mg L^{-1} mT. *G* Multiple shoot formation from callus on MS + 3.0 mg L^{-1} Z. *H* Rooting of *in vitro* regenerated shoots on MS medium at 4 wk. *I* Acclimatization of *in vitro* regenerated plantlets in planting tray containing autoclaved garden soil and sand (1:1). *J* Acclimatized plants in the large pot containing garden soil. *K In vitro* regenerated plants established in the field. *Bars* = 1 cm.

medium" (MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA + 0.5% agar) was used to find out the influence of pH (4.5–6.5) on establishment of callus culture. Culture medium having pH 5.8 was found to be optimum for callus induction and proliferation (Fig S2; Supplementary Data).

Influence of sucrose at different concentrations (1.0–5.0%) was also tested using the "revised callus culture medium," *i.e.*, MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA gelled with 0.5% agar and having pH 5.8. The concentration of sucrose played a significant role for the establishment of callus culture. Best callus induction and growth were observed on culture medium containing 3.0% sucrose followed by 4.0% (Fig. S3; Supplementary Data). Finally, "MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA supplemented with 3.0% sucrose, gelled with 0.5% agar and having pH 5.8" was recorded as "optimum callus culture medium." On this medium, the highest callus induction (85.0%) as well as biomass of callus (0.370 g dry weight) was recorded.

The calli induced on the "optimum callus culture medium," *i.e.*, MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA supplemented with 3.0% sucrose, gelled with 0.5% agar, and having pH 5.8, were soft, friable, and cream-white in color (Fig. 2*A*). These calli were separated from the explant after 4 wk of explant inoculation and cultured on fresh callus induction medium of same composition for further proliferation of callus (Fig. 2*B*). With the increase in the duration of culture, the callus mass was also increased continuously irrespective of the media tested. The highest callus biomass was obtained from the explants cultured on "optimum callus culture medium" after 4 wk of sub-culture of callus (Figs. S2 and S3; Supplementary Data).

Shoot organogenesis from callus After 4 wk of sub-culture on the "optimal callus culture medium," the callus was transferred to either MS medium alone or MS medium supplemented with different concentrations and combinations of BA, Z, mT, and ADS for regeneration of shoots. After 2 wk of culture on the shoot regeneration medium, it was observed that the callus started turning green and shoot primordia developed on the surface of the calli irrespective of the media tested (Fig. 2*C*). Firstly, the leaves were emerged, and subsequently, shoots were elongated from callus. Interestingly, in this study MS medium devoid of any plant growth regulators also exhibited shoot regeneration. On MS medium, about 28.3% of shoot regeneration with an average number of shoots of 1.0 having average length of 2.0 cm was recorded per callus. However, the percentage and the number of shoots regenerated from callus were greatly influenced by the plant growth regulators and/or their combination present in the shoot regeneration medium (Table 1). The best response of callus-mediated shoot organogenesis was observed on MS medium augmented with 3.0 mg L^{-1} BA. About 78.3% of callus exhibited shoot regeneration on this medium and maximum number of shoots (11.4) per callus was recorded on this medium at 6 wk of culture. On this medium, average shoot length (5.3 cm) was also maximum (Fig. 2*D*; Table 1). BA (3.0 mg L^{-1}) supplemented with 25 mg L^{-1} and 50 mg L^{-1} ADS media also showed maximum frequency, *i.e.*, 78.3% of shoot regeneration from callus. On these two media, about 11.0 and 10.2 shoots were recorded respectively. However, the length of regenerated shoots (2.5 cm) on these two media was comparatively less than 3.0 mg L^{-1} BA–supplemented MS medium (5.3 cm) (Fig. 2*E*; Table 1). In this study, other cytokinins such as Z and mT exhibited poor results compared to BA in terms of frequency as well as the number of shoots regenerated (Table 1). At an optimum concentration of mT (2.0 mg L^{-1})–supplemented MS medium, about 73.3% of callus showed shoot regeneration. About 6.2 shoots with shoot length of 4.8 cm were found to be regenerated on this medium (Fig. 2*F*). On MS medium supplemented with optimum concentration of Z (3.0 mg L^{-1}), about 68.3% shoot regeneration with an average of 4.5 shoots per callus was observed (Fig. 2*G*). Development of roots simultaneously along with shoots from callus of *H. coronarium* on all shoot regeneration medium during shoot organogenesis was an interesting event noticed in this study.

Evaluation of regeneration potential of callus Well-developed calli grown on the "optimum callus culture medium" were used for shoot regeneration experiment as well as maintained on the same callusing medium by repeated sub-cultures. During each passage of sub-culture, the callus was also inoculated on the "optimum shoot regeneration medium," *i.e.*, MS + 3.0 mg L^{-1} BA, to study its retention of shoot organogenesis potential. It was observed that the callus retained its shoot regeneration potential until the 3rd sub-culture without significant loss (Fig. 3).

Rooting of *in vitro* regenerated shoots *In vitro* formed shoots were harvested from the callus and inoculated on MS medium for rooting. Rooting was observed in all the shoots

Table 1. Effect of plant growth regulators on regenerative response of callus derived from leaf sheath of *H. coronarium*

MS + plant growth regulators (mg L^{-1})				Frequency of shoot regeneration/0.5 g of callus (%)	Mean no. of shoots/0.5 g of callus	Shoot length (cm)
BA	Z	mT	ADS			
0.0	-	-	-	28.3 ± 0.5 ^{k}	1.0 ± 0.0 ^{p}	2.0 ± 0.2 ^{k}
1.0	-	-	-	60.0 ± 1.0 ^{g}	4.2 ± 0.2^{ij}	5.0 ± 0.5 $^{a-c}$
2.0	-	-	-	68.3 ± 0.5 ^{d}	7.8 ± 0.3 ^{d}	5.4 ± 0.4 ^{a}
3.0	-	-	-	78.3 ± 1.5 ^{a}	11.4 ± 0.4 ^{a}	5.3 ± 0.5^{ab}
4.0	-	-	-	71.7 ± 0.7 ^{c}	6.5 ± 0.5 ^{e}	5.0 ± 0.0 $^{a-c}$
5.0	-	-	-	65.0 ± 1.0 ^{e}	4.5 ± 0.2 ^{i}	4.0 ± 0.2^{ef}
-	1.0	-	-	58.3 ± 0.5 ^{h}	2.3 ± 0.0 ^{o}	2.5 ± 0.5 $^{h-j}$
-	2.0	-	-	65.0 ± 0.5 ^{e}	3.8 ± 0.3^{jk}	2.8 ± 0.3 ^{hi}
-	3.0	-	-	68.3 ± 0.8 ^{d}	4.5 ± 0.3 ^{i}	3.5 ± 0.3^{fg}
-	4.0	-	-	56.7 ± 1.2 ^{i}	4.2 ± 0.3^{ij}	3.0 ± 0.2^{gh}
-	5.0	-	-	48.3 ± 0.5 ^{j}	3.5 ± 0.1 ^{kl}	3.0 ± 0.0^{gh}
-	-	1.0	-	60.0 ± 0.0 ^{g}	3.3 ± 0.2 $^{k-m}$	4.0 ± 0.4^{ef}
-	-	2.0	-	73.3 ± 1.0 ^{b}	6.2 ± 0.3^{ef}	4.8 ± 0.3 $^{b-d}$
-	-	3.0	-	71.7 ± 0.3 ^{c}	5.5 ± 0.5^{gh}	4.3 ± 0.5 ^{de}
-	-	4.0	-	63.3 ± 2.0 ^{f}	4.2 ± 0.2^{ij}	3.5 ± 0.3^{fg}
-	-	5.0	-	56.7 ± 1.2 ^{i}	3.0 ± 0.0 $^{l-n}$	3.0 ± 0.2^{gh}
3.0	-	-	25	78.3 ± 0.9 ^{a}	10.2 ± 0.3^{c}	2.5 ± 0.2 $^{h-j}$
3.0	-	-	50	78.3 ± 0.6 ^{a}	11.0 ± 1.0^{ab}	2.5 ± 0.0 $^{h-j}$
3.0	-	-	75	68.3 ± 0.5 ^{d}	7.8 ± 0.5 ^{d}	3.0 ± 0.3^{gh}
3.0	-	-	100	65.0 ± 1.0 ^{e}	5.6 ± 0.2^{fg}	5.0 ± 0.3 $^{a-c}$

Data recorded at 6 wk of culture. Values represent means ± standard deviation (SD). In a *column*, means followed by the same *letters* in superscripts are not significantly different among each other (one-way analysis of variance (ANOVA) with Duncan's new multiple range test, $p \leq 0.05$)

BA, N^6-benzyladenine; *Z*, zeatin; *mT*, *meta*-topolin; *ADS*, adenine sulfate

after 1 wk of culture on MS medium. About 8.5 of roots with an average root length of 5.7 cm were recorded after 4 wk of culture (Fig. 2*H*).

Acclimatization About 90% of the plantlets survived after 3 wk from the start of acclimatization process (Fig. 2*I*). When these plants were transferred to larger pots having garden soil and sand (1:1), zero mortality was recorded at 3 wk of transfer (Fig. 2*J*). Finally, all the plants were successfully established in the field condition under full sun where they grew normally (Fig. 2*K*). Thus, about 24 wk time was required to complete the plant establishment in field starting from callus induction.

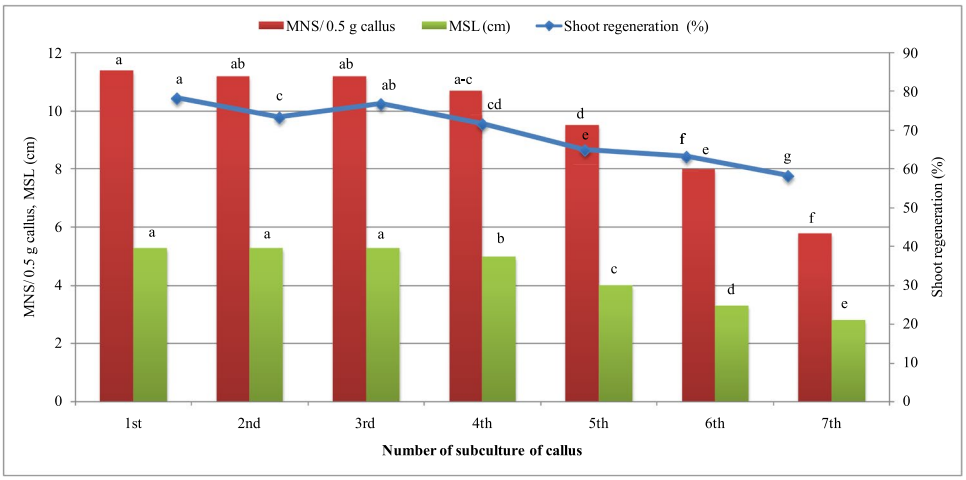

Figure 3. Evaluation of shoot regeneration potential of callus on optimum shoot regeneration medium after various subcultures. *Bars* with different alphabets specify statistically significant difference between means (one-way analysis of variance (ANOVA) with Duncan's new multiple range test, $p \leq 0.05$). MNS, mean number of shoot(s); MSL, mean shoot length.

Morphological analysis and estimation of chlorophyll content Morphological characteristics of *in vitro* regenerated plants were compared with those of the mother plant in field under natural conditions. The morphological characteristics, such as plant height, pseudo stem number/plant, leaf number/shoot, leaf size, and rhizome fresh and dry weight of *in vitro* regenerated plant, were on par with the mother plant (Table S1; Supplementary Data). Similarly, the chlorophyll content of both the mother plant (37.5 mg g^{-1} FW) and *in vitro* regenerated plants (38.9 mg g^{-1} DW) was found to be almost the same without a significant difference (Table S1; Supplementary Data).

Assessment of clonal fidelity of *in vitro* regenerated plants vis-à-vis mother plant using RAPD and ISSR markers Molecular markers including RAPD and ISSR primers were used for the analysis of clonal fidelity of *in vitro* regenerated plants. In the present study, seven RAPD primers and nine ISSR primers were selected for the assessment of genetic fidelity analysis based on reproducibility and clear banding pattern. The detail of amplified bands for each of RAPD and ISSR primers is presented in Tables 2 and 3 respectively. Seven RAPD primers produced a total of 27 scorable bands with an average of 3.85 bands per RAPD primer. The sizes of the amplified bands were from 400 to 2200 bp (Table 2). The highest numbers of bands (6) were produced by primer OPN 4. The lowest numbers of bands (3) were produced by three RAPD primers, namely OPA 10, OPC 2, and OPN 6 (Table 2). A total of 28 bands were produced by nine ISSR primers with an average of 3.11 bands produced per primer. The amplified bands range from 400 to 2000 bp (Table 3). The lowest number of band (1) was produced by three different primers, namely ISK 13, ISK 16, and ISK 24, whereas the highest number of bands (6) was obtained by primer ISK4. *In vitro* regenerated plants were found to be genetically similar to the mother plant as indicated by the presence of monomorphic banding profile generated in both RAPD and ISSR markers (Fig. 4*A*, *B*; Fig. 5*A*, *B*).

Table 2. Details of RAPD primers used for assessment of genetic fidelity of the *in vitro* regenerated field-established plants vis-à-vis mother plant of *H. coronarium*, number and approximate size range of the amplicons generated

Primers	Primer sequences (5′-3′)	No. of scorable bands	Approximate range of amplifications (bp)
OPA 3	AGTCAGCCAC	4	500–1300
OPA 10	GTGATCGCAG	3	500–750
OPC 2	GTGAGGCGTC	3	550–1400
OPD 5	TGAGCGGACA	4	750–2000
OPN 2	ACCAGGGGCA	4	400–1900
OPN 4	$(GACC)_2CA$	6	500–1500
OPN 6	GAGACGCACA	3	600–2200

GC/MS analysis of essential oil Essential oils from rhizome of *H. coronarium* mother plant and *in vitro* regenerated plants were extracted by hydro-distillation method and about 0.31% and 0.29% (v/w) of oil were recorded in these plants respectively. These plants' essential oils were used for GC/MS analysis to find their chemical compositions. GC/MS study revealed that thirty-six and thirty-eight compounds were present in the mother plant and *in vitro* regenerated plants respectively. The total peak area percent, *i.e.*, the concentration of total compound present in essential oil (%) of the mother plant and *in vitro* regenerated plants, was 99.75% and 99.95% respectively (Table 4). The major chemical constituents were eucalyptol; β-pinene; α-pinene; α-terpineol; 3-cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-; *p*-cymene; α-phellandrene; and ϒ-terpinene both in the mother plant and *in vitro* regenerated plants' rhizome essential oil. At the same time, eucalyptol was observed as the principal compound found in the rhizome essential oil of *H. coronarium* (Table 4). *In vitro* regenerated plants were found chemically stable with their donor mother plant.

Phytochemical analysis Phytochemical analysis in terms of alkaloid, flavonoid, phenolic, tannin, and saponin contents

Table 3. Details of ISSR primers used for the assessment of genetic fidelity of the *in vitro* regenerated field-established plants vis-à-vis mother plant of *H. coronarium*, number and approximate size range of the amplicons generated

Primers	Primer sequences (5′-3′)	Melting temperature (°C)	Annealing temperature (°C)	No. of scorable bands	Approximate range of amplifications (bp)
ISK 4	$(GA)_8G$	57.2	52.2	6	400–1200
ISK 5	$CA(GA)_7G$	57.2	52.2	5	500–1250
ISK 8	$T(GACA)_4$	54.8	49.8	2	550–1100
ISK 10	$(GACA)_4G$	57.2	52.2	4	500–1800
ISK 13	C(GATA)4	47.5	42.5	1	850
ISK 14	G(GATG)3GAT	54.2	49.2	4	450–1200
ISK 15	(CT)8	54.2	49.2	4	725–1200
ISK 16	$(CT)_8G$	57.2	52.2	1	550
ISK 24	$(CTGT)_4C$	57.2	52.2	1	2000

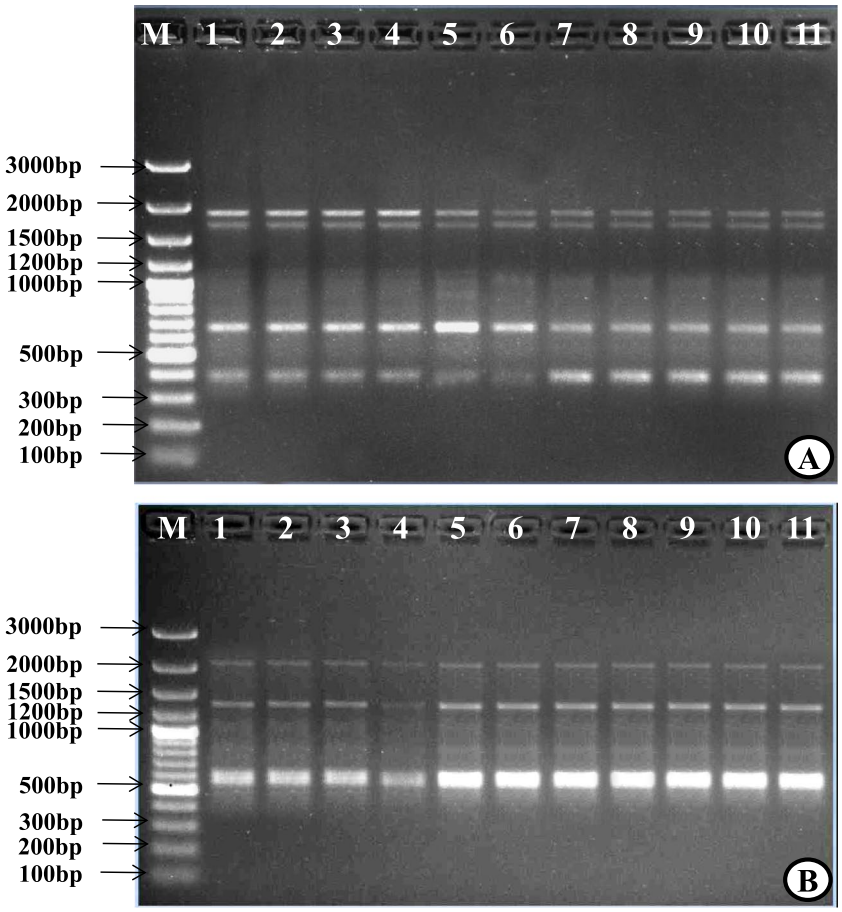

Figure 4. The DNA banding profile of both mother plant and *in vitro* regenerated plants of *H. coronarium* using RAPD primers: *A* OPN 2 and *B* OPN 6; *lane* 1: mother plant, *lanes* 2–11: *in vitro* regenerated plants. M is the marker (3kb).

was carried out for both the mother plant vis-à-vis *in vitro* regenerated plants. In this study, it was found that the phytochemical constituents of the mother plant and *in vitro* regenerated plants' rhizome were almost similar (Table S1; Supplementary Data). Among all the tested phytochemicals, saponin content was found to be maximum being 171.3 mg g^{-1} DW and 165.7 mg g^{-1} DW in the mother plant and *in vitro* regenerated plants respectively.

Antioxidant activity The antioxidant activities of *H. coronarium* essential oil and aqueous and methanol extracts of both mother plant and *in vitro* regenerated plants were analyzed using DPPH assay and ABTS assay and ascorbic acid was taken as standard. This study revealed that rhizome essential oil has more potential to scavenge free radicals than aqueous and methanol extract of rhizome (essential oil > aqueous extract > methanol extract) (Table 5). The IC_{50} value of the mother plant and *in vitro* regenerated plants' rhizome essential oil was found to be 1.14 mg mL^{-1} and 1.15 mg mL^{-1} for DPPH scavenging assay, while the IC_{50} value of rhizome essential oil of the mother plant and *in vitro* regenerated plants was 0.46 mg mL^{-1} and 0.49 mg mL^{-1} for ABTS scavenging assay. Essential oil, aqueous extracts, and methanol extracts of rhizome from both the sources showed less antioxidant activity in comparison to standard, *i.e.*, ascorbic acid (Table 5).

Anticancer activity The anticancer activity in terms of anti-proliferation activity and apoptosis assay was evaluated by using aqueous and acetone extracts of both the mother plant and *in vitro* regenerated plant rhizome against two different breast cancer cell lines, *i.e.*, MCF 7 and MDA MB 231. The percent of cell viability varies between the two cell lines with respect to different concentrations of aqueous and acetone extracts of rhizome (Fig. 6). Acetone extract was found to be more effective against the two breast cancer cell lines in comparison to aqueous extract (acetone extract > aqueous extract). Anti-proliferation activity results were expressed/calculated in terms of IC_{50} value. A lower IC_{50} value indicated more effectiveness against cancer cells. The IC_{50} values of acetone extracts for the mother plant and *in vitro* regenerated plants were recorded as 87.6 µg mL^{-1} and 97.4 µg mL^{-1} respectively for the MCF 7 cell line, whereas the IC_{50} values of acetone extracts for the mother plant and *in vitro* regenerated plants were found to be 49.6 µg mL^{-1} and 93.9 µg mL^{-1} for the MDA MB 231 cell line respectively

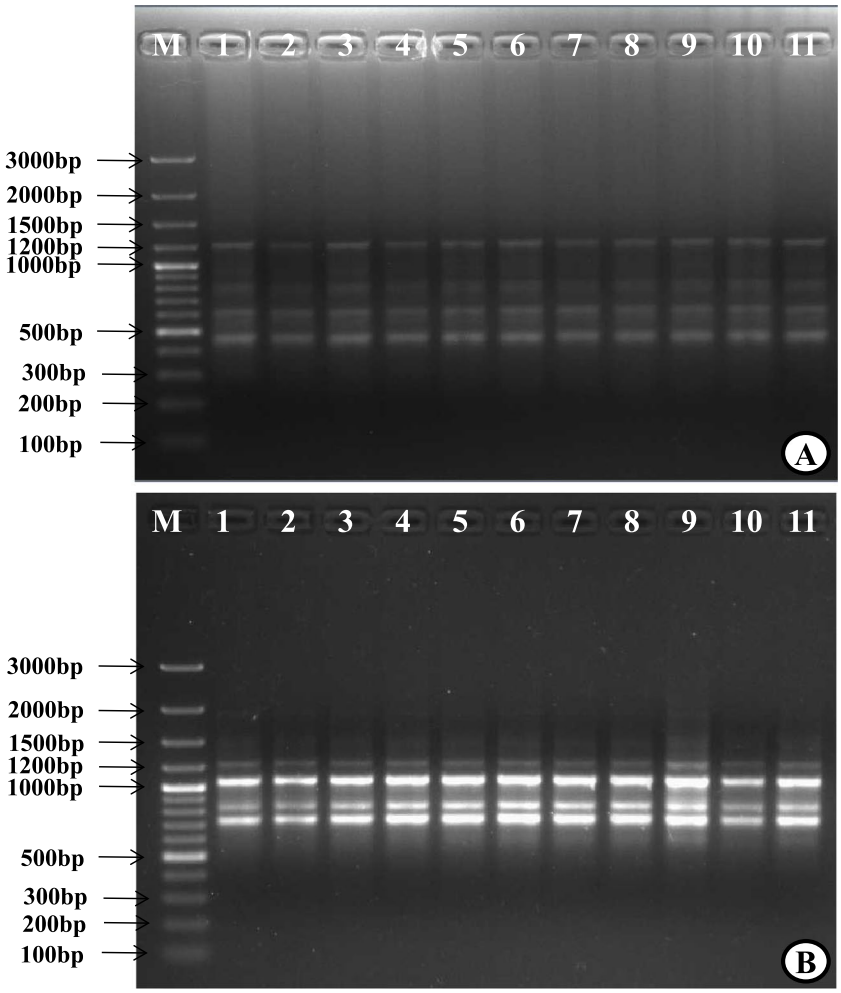

Figure 5. The DNA banding profile of both mother plant and *in vitro* regenerated plants of *H. coronarium* using ISSR primers: ***A*** ISK 14 and ***B*** ISK 15; *lane* 1: mother plant, *lanes* 2–11: *in vitro* regenerated plants. M is the marker (3 kb).

(Table 6). Acetone extract was found to be more effective against the two breast cancer cell lines in comparison to aqueous extract. The MDA MB 231 cancer cell line was used to determine the induction of apoptotic cell death by acetone extracts of both the mother plant and *in vitro* regenerated plants. Apoptotic cells were identified based on morphology of cell, *i.e.*, membrane blebbing forming apoptotic bodies as well as nuclear condensation (Fig. 7).

Discussion

Establishment of callus culture Establishment of callus culture and subsequent regeneration of plants (i.e., callus mediated plant regeneration) is one of the pathways of plant tissue culture to study the totipotency nature of plant cells (Li *et al.* 2012). Various factors are responsible for the development of an efficient callus-mediated plant regeneration protocol. The type of explants is one of the factors influencing the establishment of callus culture (Anjusha and Gangaprasad 2016). In the present study, leaf sheath was used as explant for induction of callus and subsequent plant regeneration through callus-mediated organogenesis of *H. coronarium*. Callus-mediated plant regeneration protocols have already been developed using leaf or leaf sheath as explant in several plants belonging to the Zingiberaceae family, including *Curcuma amada* (Raju *et al.* 2013), *Zingiber officinale* (Taha *et al.* 2013; Babu *et al.* 2016), and *Zingiber zerumbet* (Stanly *et al.* 2011). The percentage of callus induction and proliferation depends upon the exogenous supply of plant growth regulators (Jayaraman *et al.* 2014). In this study, the highest frequency (73.3%) of callus induction and maximum production of biomass was observed on MS medium supplemented with BA (3.0 mg L^{-1}) and NAA (0.5 mg L^{-1}). The result of this study corroborates with the earlier reports, where BA and NAA combination was used for callus induction and proliferation in plants including *Curcuma longa* (Salvi *et al.* 2001) and *Zingiber officinale* (Solanky *et al.* 2013). In contrast, Parida (2018) reported the use of MS medium supplemented with only a single auxin, *i.e.*, 2,4-D,

Table 4. GC-MS analysis of rhizome essential oil of mother plant and *in vitro* regenerated field-established plant

SL. no.	Name of the compounds	Concentration of compounds (%)	
		Mother plant	*In vitro regenerated* plant
1	Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	0.45 ± 0.009	0.44 ± 0.007
2	α-Pinene	11.63 ±0.193	11.39±0.234
3	Camphene	0.72±0.010	0.69±0.013
4	β-Pinene	24.0±0.416	23.54±0.453
5	β-Myrcene	0.76±0.016	0.76±0.013
6	α-Phellandrene	1.10±0.008	1.12±0.020
7	3-Carene	0.35±0.006	0.36±0.005
8	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	0.27±0.005	0.27±0.006
9	p-Cymene	1.78±0.031	1.8±0.029
10	Eucalyptol	42.68±0.468	43.16±0.489
11	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	ND	0.02±0.015
12	ϒ-Terpinene	1.10±0.019	1.12±0.012
13	(+)-4-Carene	ND	0.37±0.005
14	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	0.37±0.008	ND
15	1,6-Octadien-3-ol, 3,7-dimethyl-	0.69±0.018	0.70±0.016
16	Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl-	0.11±0.000	0.11±0.004
17	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, trans-	0.12±0.005	0.13±0.005
18	α-Campholenal	0.06±0.004	0.06±0.005
19	Bicyclo[3.1.1]heptan-2-one, 6,6-dimethyl-, (1R)-	0.06±0.000	0.06±0.005
20	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-	0.14±0.004	0.14±0.004
21	(+)-2-Bornanone	ND	0.02±0.020
22	Bicyclo[2.2.1]heptan-2-ol, 2,3,3-trimethyl-	0.06±0.012	0.07±0.000
23	Pinocarvone	0.11±0.005	0.12±0.006
24	endo-Borneol	0.94±0.022	0.97±0.030
25	Bicyclo[3.1.1]heptan-3-one, 2,6,6-trimethyl-,(1 α,2,5 α)-	0.04±0.018	0.03±0.025
26	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	3.17±0.031	3.23±0.068
27	α-Terpineol	6.87±0.036	7.03±0.154
28	Bicyclo[3.1.1]hept-2-ene-2-carboxaldehyde, 6,6-dimethyl-	0.19±0.005	0.19±0.007
29	Tricyclo[5.2.1.0(1,5)]dec-2-ene	0.08±0.005	0.08±0.000
30	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl)-, cis-	0.05±0.000	0.05±0.000
31	Phenol, 2-methyl-5-(1-methylethyl)-	0.07±0.004	ND
32	3-Methyl-4-isopropylphenol	ND	0.07±0.005
33	3-Cyclohexene-1-methanol, α, α, 4-trimethyl-, acetate	0.20±0.012	ND
34	(+)-4-Carene	ND	0.21±0.007
35	Caryophyllene	0.10±0.008	0.10±0.005
36	Caryophyllene oxide	0.12±0.007	0.12±0.007
37	1-Oxaspiro[2.5]octane, 5,5-dimethyl-4-(3-methyl-1,3-butadienyl)-	0.14±0.010	0.15±0.011
38	Longipinane, (E)-	0.06±0.005	0.06±0.004
39	Amiphenazole	0.14±0.011	ND
40	Longifolenaldehyde	ND	0.15±0.008
41	Bicyclo[6.1.0]nonane, 9-bromo-9-methyl-, (1α,8α,9α)-	0.24±0.018	ND
42	Trans, trans-2-ethylbicyclo[4.4.0]decane	ND	0.25±0.016
43	exo-Norbornanol, pentamethyldisilyl ether	0.77±0.071	ND
44	Estra-1,3,5(10)-trien-17-one, 3-methoxy-	ND	0.79±0.063
	Total area (%)	99.75	99.95

ND, not detected. Values represent means ± standard deviation (SD)

Table 5. Antioxidant activity of essential oil and aqueous and methanol extracts of *H. coronarium* rhizome

Plant samples/control		DPPH IC_{50} (mg mL^{-1})	ABTS IC_{50} (mg mL^{-1})
Control	Ascorbic acid	0.10±0.01^{g}	0.06±0.01^{g}
Rhizome essential oil	*In vitro* regenerated plant	1.15±0.03 ^{e}	0.49±0.01^{bc}
	Mother plant	1.14±0.02 ^{ef}	0.46±0.01^{d}
Aqueous extract of rhizome	*In vitro* regenerated plant	1.21±0.03 ^{cd}	0.43±0.02 ^{e}
	Mother plant	1.24±0.06 ^{c}	0.38±0.04 ^{f}
Methanol extract of rhizome	*In vitro* regenerated plant	1.36±0.04 ^{ab}	0.52±0.02^{a}
	Mother plant	1.37±0.02 ^{a}	0.50±0.01^{ab}

Values represent means ± standard deviation (SD). In a *column*, means followed by the same *letters* in superscripts are not significantly different among each other (one-way analysis of variance (ANOVA) with Duncan's new multiple range test, $p \leq 0.05$)

ABTS, 2, 2-azino-bis (3-ethylbenzothiazoline); *DPPH*, 2, 2-diphenyl-1-picryhydrazyl

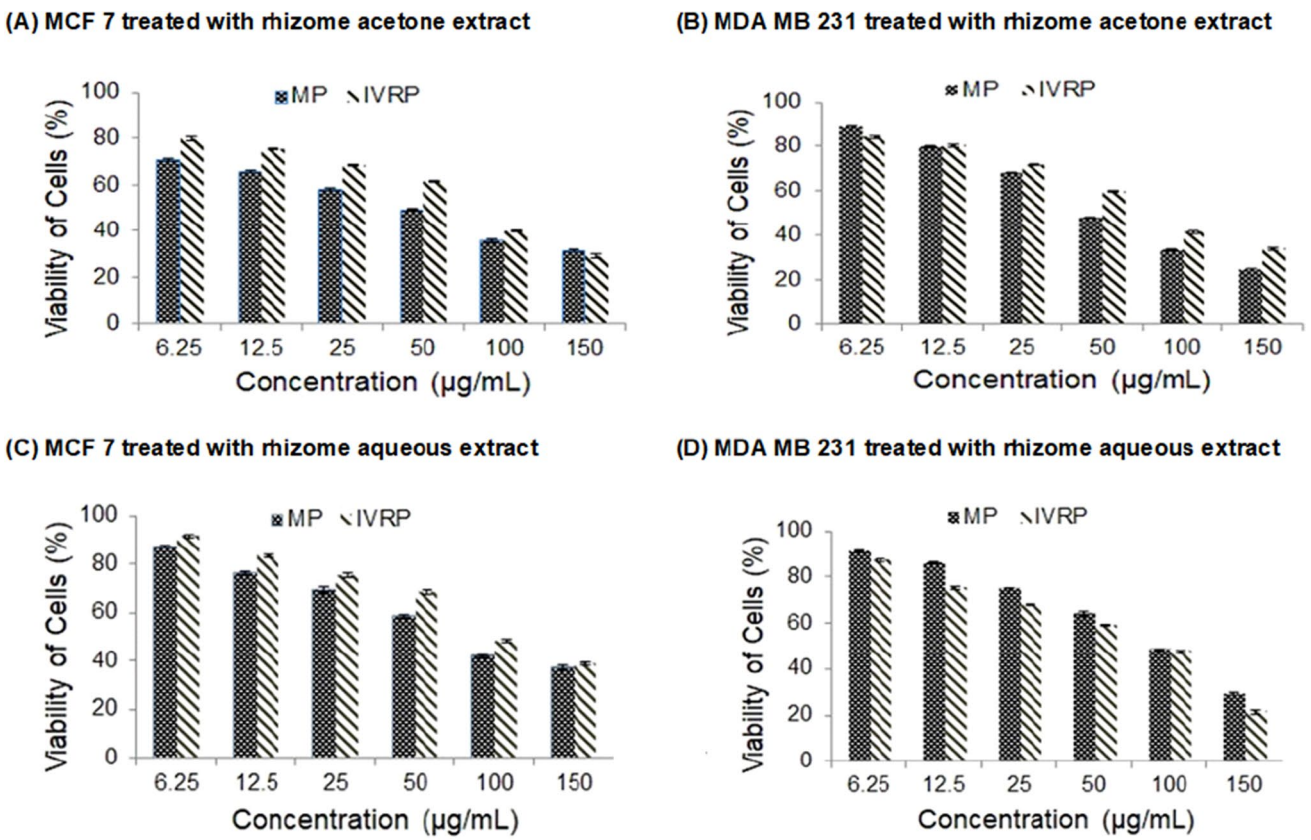

Figure 6. Cell anti-proliferative assay (%) of *H. coronarium* rhizome aqueous and acetone extract against two cell lines, *i.e.*, MCF 7 and MDA MB 231 cell lines, at 72 h of treatment. MP, mother plant; IVRP, *in vitro* regenerated plants. The difference in anti-proliferation activity between the MP and IVRP was not statistically significant at $p \leq 0.01$. All data represented in means ± standard deviation.

for the establishment of callus culture from rhizome explants of *H. coronarium*.

Besides the optimum concentrations and combinations of plant growth regulators, agar concentration and sucrose concentration of the medium are also major factors for establishment of callus culture. Generally, agar is added to the culture medium for solidification of the medium (Debergh *et al.* 1983; Suthar *et al.* 2011). As a result, the plant cell, tissue, or organ can easily be positioned on the nutrient medium. When the concentration of agar in the culture medium is more than the optimum, it could inhibit the growth of tissues/organs due to lack of water content and proper availability of plant growth regulators to the culture from the culture medium (Selby *et al.* 1989). When the medium is too poorly gelled with a very low concentration of agar, it failed to solidify which creates inconvenience for

Table 6. IC_{50} values for anti-proliferation activity of *H. coronarium* rhizome aqueous and acetone extracts against two breast cancer cells, *i.e.*, MCF-7 and MDA-MB-231

Rhizome extracts	Cell lines	IC_{50} (μg mL^{-1})	
		Mother plant	*In vitro* regenerated plant
Aqueous	MCF-7	89.85±2.3[b]	95.61±1.8[a]
	MDA-MB-231	67.91±1.9[b]	92.5±1.3[a]
Acetone	MCF-7	87.6±1.5[b]	97.4±0.6[a]
	MDA-MB-231	49.6±1.4[b]	93.9±1.2[a]

Values represent means ± standard deviation (SD). In a *row*, means followed by the same *letters* in superscripts are not significantly different among each other (one-way analysis of variance (ANOVA) with Duncan's new multiple range test, $p \leq 0.05$)

the proper positioning of explants in the culture medium. But, when an optimum concentration of agar is used in the culture medium, the gelling of medium usually facilitates adequate contact between the explant and culture medium. Thus, the explants can properly absorb nutrients from the medium by the process of diffusion from the cut end as well as surface of the explants (Casanova *et al.* 2008; Suthar *et al.* 2011) and assume proper growth and development. In the present study, it was found that 0.5% of agar showed best result for establishment of callus culture. At the same time, sucrose plays a key role by providing energy for *in vitro* culture, which are grown heterotrophically (Naik *et al.* 2010). Sucrose also acts as osmotic balance between the cell and external environment of the medium (Kaur *et al.* 2022). It also helps the cells to absorb the essential mineral nutrients present in the culture medium for cell growth (Jayaraman *et al.* 2014). The frequency of callus induction and proliferation usually varies depending on the concentrations of sucrose used in the medium. In this study, maximum frequency of callus induction and biomass production was recorded in medium containing 3.0% sucrose. In most of the earlier callus-mediated plant regeneration reports on different plant species, 3.0% sucrose was used for establishment of callus culture. However, reports on use of 2.0% sucrose were not scanty. In plants including *Bacopa monnieri* (Naik *et al.* 2010), 2.0% sucrose was optimum for callus-mediated shoot organogenesis. At the same time in plants like *Lilium longiflorum* (Ramsay and Galitz 2003), 4.0% sucrose was found to be beneficial for establishment of callus culture.

Shoot organogenesis from callus The types, combinations, and concentrations of plant growth regulators are known to influence the shoot organogenesis. For regeneration of shoots from callus, either cytokinins alone or combinations of auxin(s) and cytokinin(s) are necessary as they play crucial roles in cell division, growth, and differentiation of plant organs (Purkayastha *et al.* 2010; Li *et al.* 2012). In this study, it was found that 3.0 mg/L BA alone exhibited the highest shoot regeneration (11.4) per 0.5 g of callus. Other cytokinins including Z and mT as well as combination of BA and ADS showed poor results. This result agreed with other plant species including *Alpinia galanga* (Rao *et al.* 2011) and *Zingiber officinale* (Taha *et al.* 2013; Ibrahim *et al.* 2015) of the Zingiberaceae family where BA was reported to be optimum for callus-mediated shoot organogenesis. BA is reported as one of the most effective cytokinins among all other cytokinins for multiple shoot regeneration in plant cell, tissue, and organ culture due to the stability of ribosides and nucleosides (Behera *et al.* 2022a). It is slowly degraded and converted to a variety of conjugates, such as benzyladenosine-5-monophosphate, benzyladenosine-5-diphosphate, and benzyladenosine-5-triphosphate, as a result that contribute to a pool of BA metabolites required for multiple shoot formation (Auer and Cohen 1993). In contrast to this study, Parida (2018) reported the highest shoot proliferation from callus on MS medium supplemented with 3.0 mg L^{-1} kinetin (KIN) and 1.0 mg L^{-1} NAA in *H. coronarium*. However, no information has been provided by the authors regarding the number of shoots regenerated on the said medium.

Rooting, acclimatization of *in vitro* regenerated plantlets, and morphological characteristic analysis All shoots developed from callus successfully rooted on plant growth regulator–free MS medium may be due to the presence of endogenous auxin in this plant tissues, which is sufficient for root induction. MS medium for rooting was also reported in different plant species belonging to Zingiberaceae including *Curcuma caesia* (Jose and Thomas 2015) and *Zingiber officinale* (Ibrahim *et al.* 2015). Acclimatization is the last and important step in plant tissue culture (Naik and Chand 2011) and successful acclimatization procedure indicates the efficiency of the protocol for plant regeneration. In this study, 90% of *in vitro* regenerated plants were acclimatized successfully in the pots containing garden soil and sand (1:1) under culture room conditions. Subsequently, these plants were transferred to field with zero mortality. Morphological features of *in vitro* regenerated plants were analyzed with the mother plant and found that there is no variation between them. Our finding was in agreement with the results of Behera *et al.* (2018b) in *H. coronarium* that micropropagated field grown plants are morphologically identical with mother plants.

Genetic fidelity studies In recent years, various molecular marker techniques, including Amplified Fragment Length Polymorphism (AFLP), ISSR, RAPD, Restriction Fragment Length Polymorphism (RFLP), and Start Codon Targeted (SCoT), have gained popularity in assessing the genetic fidelity of the *in vitro* regenerated plants (Lata *et al.* 2016). These markers have their advantages and disadvantages. A

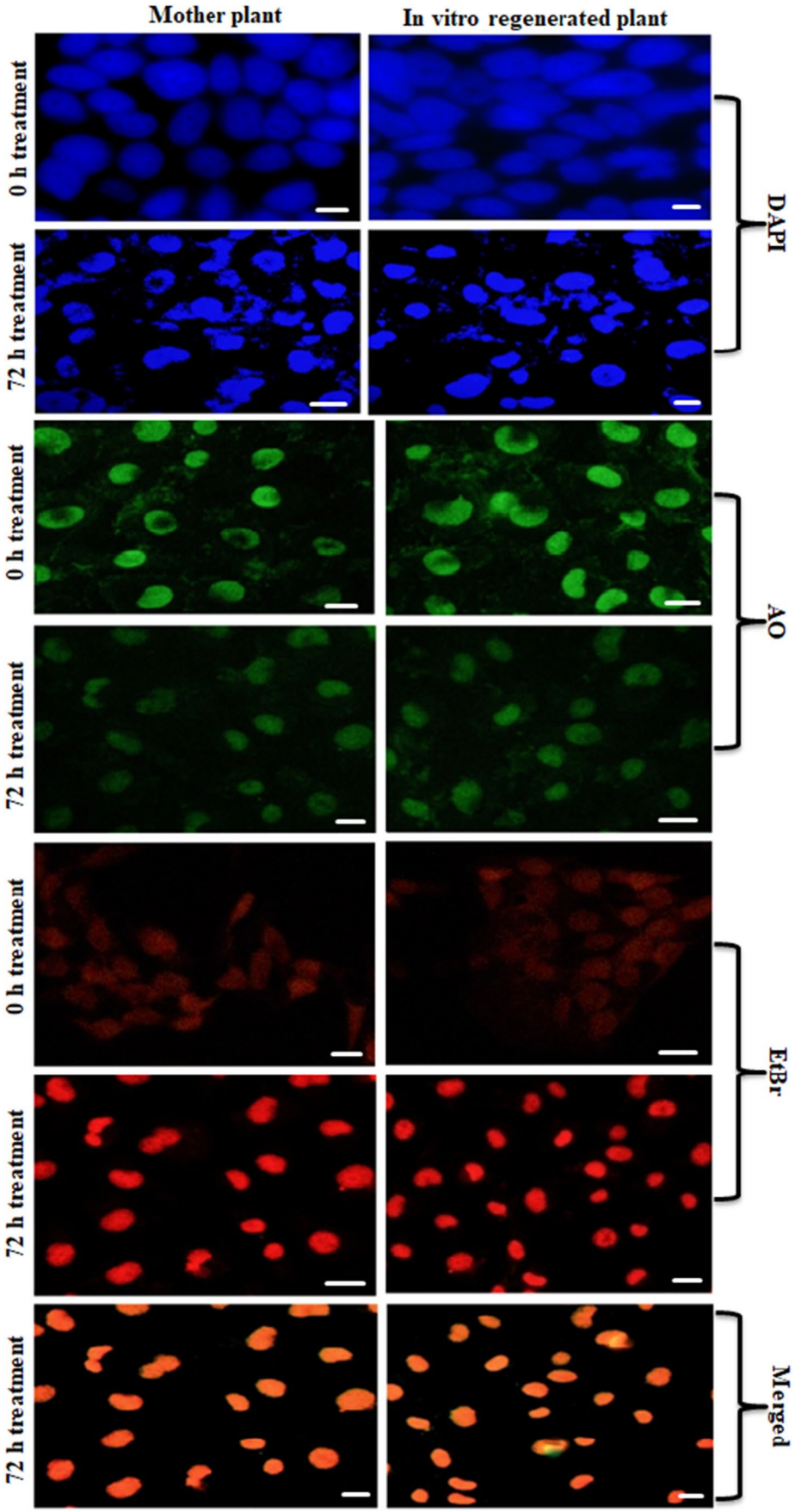

Figure 7. Apoptotic cells were identified by changes in morphological characteristics as chromatin condensation, blebbing of the plasma membrane, and the formation of micro, apoptotic entities of MDA MB 231 cells. The *panels* display the morphological characteristics of cells stained with 4,6-diamidino-2-phenylindole (DAPI), acridine orange (AO), ethidium bromide (EtBr), and merged of AO and EtBr channels. DAPI, AO, and EtBr stained with *upper left* and *right panel* contain 0 h treated (untreated) cancer cells, *lower left* and *right panel* treated with extract of mother plant (IC_{50}; 50 µg mL^{-1}) and *in vitro* regenerated plant (IC_{50}; 94 µg mL^{-1}) treated for 72 h. After 72 h of treatment of the extract, the cancer cells that had undergone apoptosis were visible in microscopy. *Bars* = 1 cm.

single molecular marker, RAPD, was used for the genetic fidelity analysis of the plants regenerated from callus-mediated organogenesis in *Curcuma longa* (Salvi *et al.* 2001) and *Zingiber officinale* (Taha *et al.* 2013). In these works, RAPD alone could detect 6.3% (*Zingiber officinale*: Taha *et al.* 2013) to 16.5% (*Curcuma longa*: Salvi *et al.* 2001) polymorphism. However, a single molecular marker alone may not always confirm the genetic fidelity of the *in vitro* regenerated plants (Mallon *et al.* 2010). It was also assumed that the use of more than one molecular marker is essential for the assessment of genetic fidelity of plants regenerated through callus-mediated organogenesis which is prone to produce variants. Thus, in the present study two molecular markers, namely RAPD and ISSR, were used for the analysis of genetic fidelity. These markers were used due to their simplicity, cost-effectiveness, and requirement of low amount of genomic DNA (Behera *et al.* 2020). Both the markers produced monomorphic banding profile for the mother plant as well as *in vitro* regenerated plants and thus confirmed the genetic fidelity of the *H. coronarium* regenerants produced from callus. This work was in agreement with the results of Jose and Thomas (2015) in *Curcuma caesia*, where they successfully used both RAPD and ISSR markers to validate the genetic fidelity among callus-mediated plant as well as the mother plant.

Biochemical fidelity studies According to Lata *et al.* (2016) "Consistency in the chemical profile and useful metabolites content of raw material is of utmost interest in any plant-based pharmaceutics." Furthermore, it is well known that callus-mediated organogenesis or indirect organogenesis may not always produce genetically and biochemically stable plants (Amoo *et al.* 2012). Like genetic stability, the secondary metabolite production including essential oil in tissue-cultured raised plants may also be affected by various factors during culture (Hussain *et al.* 2012). Thus, in this study of callus-mediated organogenesis biochemical fidelity study was essential for commercial utilization of *H. coronarium*.

Pharmaceutical properties of plants are mainly due to the presence of various secondary metabolites including essential oils (Chen *et al.* 2017; Lin *et al.* 2018). In *H. coronarium*, essential oils were found abundantly in its rhizomes (da Silva *et al.* 2021). Thus, the chemical constituent of rhizome essential oils of the mother plant and *in vitro* regenerated plants was analyzed and confirmed biochemical stability among them. Earlier reports indicated eucalyptol as the major component of the rhizome essential oil of *H. coronarium* (Joy *et al.* 2007; Bruna *et al.* 2010). Some other main compounds were reported to be β-pinene, α-pinene, and α-terpineol (Bruna *et al.* 2010; Ray *et al.* 2018; da Silva *et al.* 2021). The results of the present study corroborate with the earlier reports and eucalyptol was found as the principal compound in rhizome oil of both the mother plant and *in vitro* regenerated plants without any significant variations (42.68% in the mother plant and 43.16% in *in vitro* regenerated plants). This result was also in agreement with the earlier work reported by Parida *et al.* (2015). They also detected eucalyptol as the principal compound both in the mother plant and micropropagated plant of *H. coronarium*. However, comparative biochemical fidelity analysis of callus-mediated plants with that of the mother plant is rare in plants belonging to Zingiberaceae. Besides essential oils, the phytochemicals such as alkaloids, flavonoids, phenolics, tannins, and saponins were also analyzed in both the mother plant vis-à-vis *in vitro* regenerated plants' rhizome and it was observed that there is no any significant difference between mother and *in vitro* regenerated plants. The finding of this study is in agreement with the results documented by Behera *et al.* (2018b and 2019) in *H. coronarium* and Behera *et al.* (2022b) in *Curcuma amada*.

Antioxidant and anticancer activity *H. coronarium* is well known for its medicinal uses and pharmaceutical activities due to the presence of several important secondary metabolites and essential oils (Bailly 2020; Tavares *et al.* 2020). Thus, validation of pharmacological properties in terms of antioxidant and anticancer activities of *in vitro* regenerated plants is imperative before it is used as an alternative source in place of mother plant collected from wild habitat (Behera *et al.* 2022b). In this study, the antioxidant activities of *H. coronarium* rhizome essential oil, aqueous extract, and methanol extract of the mother plant and *in vitro* regenerated plants were carried out by DPPH and ABTS free radical scavenging assay. DPPH and ABTS free radical scavenging assays are widely used to assess antioxidant activity (Rather *et al.* 2012; Panigrahy *et al.* 2017). Free radicals of reactive oxygen species are sources of cell damage and cause several diseases, such as cancer and neurodegenerative diseases (Panigrahy *et al.* 2017). Antioxidant plays a key role to provide defense mechanism to the body against free radicals of reactive oxygen species (Gutteridge and Halliwell 2000). In this investigation, it was observed that both the mother plant and *in vitro* regenerated plants showed similar antioxidant activity.

Recently, researchers have focused on this plant due to its anticancer activity, because of the presence of coronarin D, which is a signature pharmaceutical important bioactive compound of *H. coronarium* (Liu *et al.* 2019; Bailly 2020). Hence, the anticancer activity was evaluated against two different cell lines, *i.e.*, MCF 7 and MDA MB 231, using aqueous and acetone extracts of both the mother plant and *in vitro* regenerated plants of *H. coronarium*. Both plant types showed anticancer activity against MCF 7 and MDA MB 231 cell lines. Thus, it was confirmed that *in vitro* regenerated plants have therapeutics potential so as its mother plant.

Earlier, the anticancer activity of natural *H. coronarium* plant has also been reported against several cancer cell lines, such as HeLa, MCF-7, A549, SK-N-SH, HUMEC, HepG2, and LNCaP (Bailly 2020). In this study, we are the first to report the anticancer activity of mother *H. coronarium* vis-à-vis *in vitro* regenerated field-established plants.

Conclusions

In the present study, an efficient plant regeneration protocol *via* callus-mediated organogenesis has been reported for *H. coronarium* using leaf sheath as explants. The *in vitro* regenerated plants were found to be genetically, phytochemically, and therapeutically stable. The present protocol could be useful for production of plants which may be used for conservation of *H. coronarium* by their reestablishment in natural habitat. At the same time due to their similarity, both genetic and biochemical, with the mother plant, they can be useful for commercial purposes in pharmaceutical and cosmetic industries in place of mother plant so that sustainability will be achieved. This study will also be used to develop procedure for the production of pharmaceutically important secondary metabolites through callus culture as well as cell suspension culture by precursor feeding, elicitors, and nanoparticle treatment.

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Author contribution SB and SKN conceptualized the study, designed the entire experiment, and wrote the paper. SB and KM carried out all the tissue culture experiments, data collection, and analysis. DPB analyzed the tissue culture data along with SB and KM. PCP guided the RAPD and ISSR experiments carried out by SB and SKK. GC/MS analysis was carried out by SB and SM. PKN guided anticancer activity studies carried out by SB and RKM. SKN supervised the entire research and edited the manuscript to the final version. All the authors read and approved the final version of the manuscript.

Data Availability All data generated or analyzed during this study are included in this published paper.

Declarations

Conflict of interest The authors declare no competing interests.

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