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Research Article

Ex-situ conservation and cytotoxic activity assessment of native medicinal orchid: *Coelogyne stricta*

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Abstract Ex-situ conservation of the ornamental and medicinal orchid, *Coelogyne stricta*, was performed by mass propagation using seed culture. Propagation stages were optimized using full- and half-strength solidified MS medium with different phytohormones. Maximum seed germination (88 ± 0.5% over 6 weeks of culture) was achieved on half-strength MS medium supplemented with 15% coconut water. Maximum shoot numbers were found on full-strength MS medium supplemented with 1 mg/L BAP, 2 mg/L Kinetin, and 10% coconut water, while the longest root was developed on full-strength MS medium with 1.5 mg/L IBA. A 2:1:1 combination of coco-peat, pine bark, and sphagnum moss was found to be a suitable potting mixture resulting in 80% seedling survivability. The cytotoxic activity of extracts of both wild plants and in vitro-developed protocorms was determined using an MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay on a cervical cancer cell line. The wild plant extract inhibited the growth of 41.99% of cells, showing that this extract has moderate cytotoxic activity toward cervical cancer cells.

Keywords *Coelogyne stricta*, Conservation, HeLa cell line, Micropropagation, MTT assay

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Introduction

Orchidaceae is being the second-largest family in angiosperms with roughly around 28,484 species (Govaerts et al. 2017) that set up nearly 10% of the total flowering species (Roberts and Dixon 2008; Tsai et al. 2013). Orchidaceae is considered to be highly advanced as proven by their unique floral architecture, condensed pack of pollinia, habitat ecology, and their particular pollination mechanism (Jersáková et al. 2006; Park et al. 2018).

Orchids are famous among cut flowers and potted plants with most beautiful flowers leaving outstanding royalty in the horticulture industry (Murthy et al. 2018). Orchids are of considerable economic increasing indicator particularly in horticulture and floristry for their striking attractiveness and long-lasting blooming age but also they are equally important in the pharmaceutical and perfume industries (Pant 2013). Owing to high demand in the national and international market places, over-collection from its natural habitat and gentle growth rate in nature, their species are delimited only to narrow pocket areas in nature (Pant et al. 2016). Complex life cycle, seed without endosperm, specificity with fungus (Chand et al. 2020; Pant 2013; Shah et al. 2019), habitat-specific and specialized pollinators (Cozzolino and Widmer 2005), illegal and uncontrolled collection and trade, deforestation and defragmentation of habitat are measures for their rarity in nature (McCormick et al. 2004; Shefferson et al. 2007). The IUCN red list of threatened species has 3.3% of the estimated 28,484 orchid species worldwide (Govaerts et al. 2017), but already 56.5% of these were found to be threatened with extinction.

Due to the exposed borders with neighbouring countries, the illegal trade of raw orchid plants has fast-tracked their extinction process in Nepal. Thus, in vitro mass propagation technique is being applied to raise plants in the laboratory to save them from extinction (Pant et al. 2019; Pant et al.

2018) ever since the development of a protocol for non-symbiotic seed germination of orchids by Knudson (1951).

Plants can produce certain bioactive chemicals in response to the influence of their physical and chemical environments. They are used to overcome biotic and abiotic stresses by employing in defence and secondary metabolism (Andrew et al. 2007; Sudha and Ravishankar 2003). This facility of plants respond to physical and/or chemical stimuli can be used for the elicitation of pharmacologically active compounds by exposing an intact plant to stress factor/s (Chand et al. 2020; Kuzel et al. 2009). Therefore, in vitro techniques are very useful in safeguarding sustainable optimized sources of plant-derived natural products that can be used against vicious diseases like cancers. With the recent noteworthy anticancer activity of orchid based novel compounds like moscatilin, denbinobin, erianin, dendrochrysanene, fimbriatone, cirrohopetalanthrin (Chen et al. 2007; Heo et al. 2007; Paudel et al. 2020; Peng et al. 2007; Wu et al. 2006; Xia et al. 2005) made orchids become a plausible candidate in the battle against cancer.

Coelogyne stricta (D. Don) Schltr. (Fig. 1a and b), native orchid to Nepal, commonly known as 'The Rigid Coelogyne Pseudobulbs' is an epiphytic orchid found on trees along steep riverbanks in lower and upper montane forests and also on mossy rocks as lithophytes at elevations of 1400 to 2000 m in Nepal (Raskoti 2009; Rajbhandari 2015). The plant has sparse pseudobulbs on the rhizome, leaves are leathery and inflorescence hysteranthous and up to 10-flowers. Flowers are white, lip with yellow spots, lamellae tinged with red in apical part. It has high aesthetic value, is often used as an ornamental plant in many gardens, nurseries, hotels. *Coelogyne stricta* has been mentioned to be beneficial against headache and fever by using the paste of pseudobulb (Baral and Kurmi 2006; Cragg and Newman 2013; Newman et al. 2003; Pant and Raskoti 2013).

Protocorms cultures have been established for the mass propagation and the production of bioactive compounds of valuable orchid species (Park et al. 2000). In vitro grown protocorms could be a good source of bioactive compounds and possess good biological activity. We have reported for the first time the cytotoxic activity of in vitro grown protocorms and wild plants of *C. stricta* in the present paper. Also, an effective conservation strategy is optimized for this orchid through tissue culture.

Materials and Methods

Plant material for in vitro culture

The 8 months old capsules of *C. stricta* (Fig. 1c) were collected from Godawari Lalitpur. They are about 2.5 ~ 3.5 cm long and 1.5 ~ 2.0 cm wide, have a mass of dark brown-yellow seeds inside were used for in vitro seed culture materials.

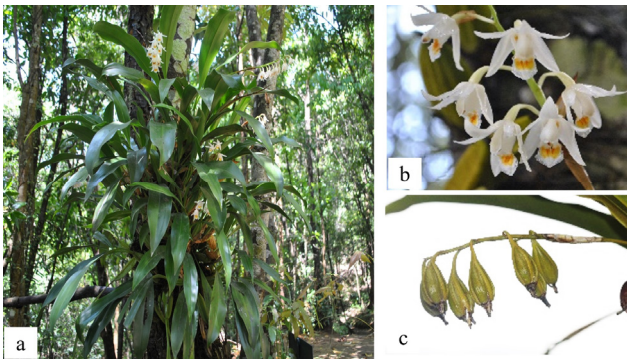

Fig. 1 *Coelogyne stricta*: habit (a), flowers (b), and fruits (c)

Sterilization of plant material

The capsule was washed in tap water with tween-20 detergent to remove the waste adherents. Then, inside the contamination-free chamber of laminar air hood, it was dipped in 0.6% sodium hypochlorite (Merck, India) for 10 min, 95% ethanol (Merck, India) for 1 min, and washed with sterile distilled water.

Culture medium

MS medium was selected to optimize the growth of *C. stricta* in vitro. A 30 g/L table sugar was used as a carbohydrate source and 0.01 g/L myoinositol as a vitamin in the MS medium (Hi-Media, India). Full and half strength's MS medium was additionally supplied with coconut water (CW) and Adenine sulfate (Ad). The medium was solidified with 8 g/L agar (Hi-Media, India). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl before autoclaving. The MS medium was autoclaved at 120°C and 15 lb for 15 minutes for effective sterilization.

Culture initiation

The capsule was cut longitudinally in a sterile petriplate with the help of a sterile No. 10 surgical blade. Seeds were inoculated on the above-mentioned culture medium for the initiation of culture. They were considered to have germinated upon the emergence of the embryo from the testa. Germination percentage of seeds in different culture medium was determined by examining the seeds microscopically after 60 days of culture.

Culture differentiation

After 8 weeks of inoculation, seeds gave enough mass of the green and fully developed protocorms, subcultures of them were performed for further development. The shoot proliferating medium was prepared by using different combination and concentration of plant growth hormones and additives in the full- and half-strength MS medium. Once the shoots reached about 2.5 cm long, they were further subculture on medium varied with auxin type and its concentrations.

Culture conditions

All inoculations were carried out under aseptic conditions in a laminar airflow chamber. The cultures were maintained at 25±2°C under white fluorescent light with a 16-h photoperiod with a light intensity of 3000 lux (fluorescent tubes 40 W, Philips, India). The number and length of seedlings in the culture medium were recorded. Each treatment consisted of at least six cultures replicas for an effective reproducibility check.

Acclimatization of plantlets

Plantlets above 2.5 cm in height with well-developed roots were removed from the medium, washed gently with tap water to remove the medium, and transplanted to appropriate plastic pots containing different substrates. The infected roots were treated with 2% of Bavistin. The pots were covered with plastic bags and its coverage was reduced weekly by 20% to decrease humidity. All the transplanted pots were maintained at greenhouse temperature (22 ~ 25 °C) under natural light for 3 months sprayed with water daily and external vitamins fortnightly.

Preparation of extract

The pseudobulbs of *C. stricta* (Fig. 2a) were collected from Godawari Lalitpur for the wild extract (CsW) preparation. For in vitro extract (CsI) preparation, in vitro developed protocorms (Fig. 2b) were used. The collected pseudobulbs and in vitro protocorms were washed, air-dried and powdered. A sonication extraction was done with methanol in the ratio of 1:10 of the weight of material and volume of methanol (w/v) three times until the powder broth becomes discolour. The extracts were concentrated in rotavapor (Eyela, Japan) to obtain dry extract and the extract was stored at 4°C for further use.

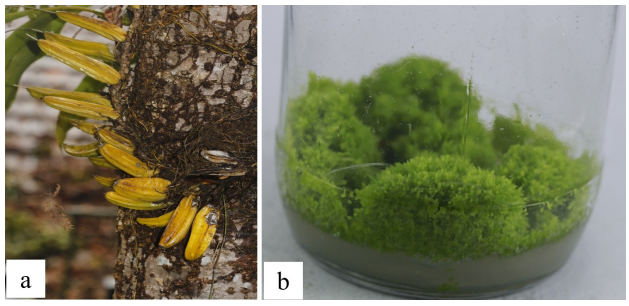

Fig. 2 Plant materials for extract preparation: pseudobulbs (a) and in vitro-developed protocorms (b)

Cytotoxicity assay

Cytotoxic activity of extracts was evaluated by using the MTT (3- [4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) colourimetric assay with slight modification. For this purpose, about 8000 HeLa cells (cervical cancer) were cultured in T-flasks containing Minimum Essential Medium Eagle (EMEM) medium (Caisson Lab, USA) supplemented with 10% of fetal bovine serum (FBS) (Caisson Lab, USA), 1% of penicillin/streptomycin (Caisson Lab, USA) and 1% L-Glutamine (Caisson Lab, USA). The culture was kept in 5% CO_2 incubator at 37°C (Mosmann 1983). Following the attachment and cell confluence, the cells were treated with different concentrations (50, 100, 200, and 400 µg/ml) of the plant extracts for 48 hours in the 96 well cell culture plate. Following 48-hour incubation supernatant was removed and 50 µL of 5mg/ml MTT (prepared in EMEM medium) was added to each well. Following 4 hours of incubation, a purple formazan product was produced. About 100 µL DMSO (2.5%) (Merck, India) was added to dissolve formazan crystals. The absorbance was measured in a microplate reader at 630 nm. The percentage of the cytotoxic activity was calculated by using the following formula:

$$\% \text{ cytotoxic activity} = (A_0\text{-}A_t)/ (A_0) \times 100$$

Where, A_0 is the absorbance of cells except plant extract, and A_t is the absorbance of extract-treated cells.

Statistical analysis

The average percentage of germinated seeds was taken after the eighth week of seed culture. Then, growth parameters were noted and analyzed every two weeks after the required subcultures. Data for the shoot and root development were presented as the mean of their respective numbers and lengths with ± standard error. The data of the percentage of cell growth inhibition was recorded as

the mean of triplicate. All the data were analyzed in Microsoft Excel 2019.

Results and Discussion

In vitro seed germination

The seeds of *C. stricta* (Fig. 3a and b) were inoculated on the different combinations of MS medium (FMS with 10% CW and 1mg/L adenine sulfate, half MS, half MS with 10% CW, and half MS with 15% CW). The inoculated seeds were started to swell up after 4 ~ 8 week of inoculation. After few weeks of incubation, green swelled seeds were changed into large globular protocorms. HMS medium with 15% coconut water was found suitable where the maximum seeds were germinated (Fig. 3c). Similarly, both combination formed with 10% coconut water in MS and HMS was also found quite responsive toward seed germination. In these conditions, more than 70% of seeds were germinated (Fig. 4). From all these data, we found that coconut water (CW) was found effective additives for the seed germination. This was supported by the findings of Reddy et al. (1992), who studied the seed germination and seedling growth in four different species of orchids (*Cymbidium aloifolium, Dendrobium crepidatum, Epidendrum radicans, and Spathoglottis plicata*) and found the seed germination after 5 weeks. It was also supported by Hoshi et al. (1994), who worked on the seed germination of four species of *Cypripedium* and Pradhan and Pant (2009) in *Dendrobium densiflorum*.

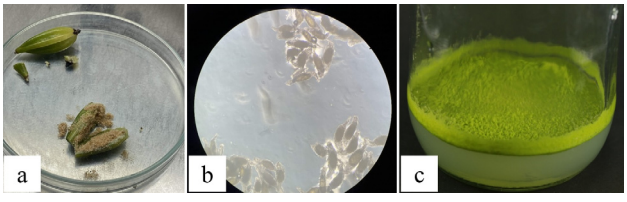

Fig. 3 Germination of seeds taken from healthy capsules: longitudinally cut capsule with brown, dusty seeds (a), seeds under a microscope (b), and maximum germinated seeds on half-strength MS medium with 15% coconut water (c)

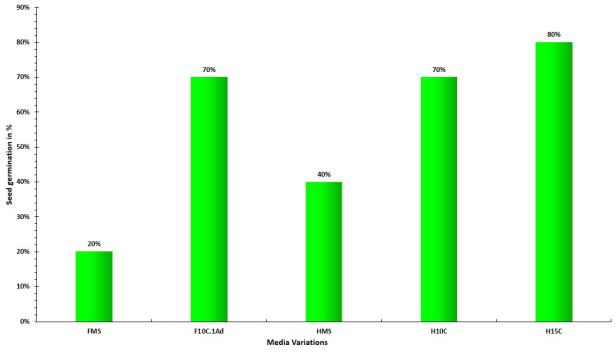

Fig. 4 Percentage of seed germination on different media

Development of protocorms into the shoots

The shoot proliferation from the protocorms was tested in various media (MS, MS with 10% CW, MS with 10% CW and 0.5 mg/L Adenine sulfate, MS with 15% CW, MS with 1 mg/L BAP with 2 mg/L Kinetin and 10% CW and HMS with 10% CW). Among all these tested medium, maximum shoot number was found in MS with 1 mg/L BAP and 2 mg/L Kinetin plus 10% CW medium (Fig. 5 and 6). For complete plantlet formation from 10 weeks old protocorms, it took 24 to 30 weeks. The present result was supported by Pant et al. (2011) in *Phiaus tancarvilleae* which took 24 weeks to develop into complete plantlets and Paudel and Pant (2012) in *Esmeralda clarkei* which took 25 weeks. Similarly, Parmar and Pant (2016) found that the MS medium with plant hormones (1 g/L NAA and 1 g/L BAP) were rather similar to this finding.

Development of roots on the shoots

Once the shoots reached about 2.0 ~ 2.5 cm long, they were cultured on full MS (FMS), full MS with 1 mg/L NAA (F1N) and full MS with 1.5 mg/L IBA (F1.5I) and full MS with 1 mg/L NAA and 1 mg/L adenine sulfate (F1N.1Ad). Among all these tested medium, the maximum root was

Fig. 5 Response of shoot formation from protocorms on full-strength MS medium (a), full-strength MS medium with 10% coconut water (b), and full-strength MS medium with 15% coconut water (c)

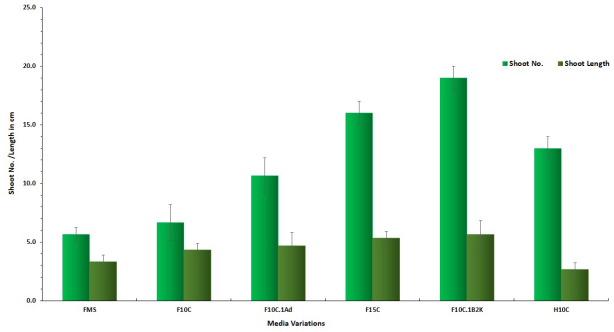

Fig. 6 Development of shoots on different media

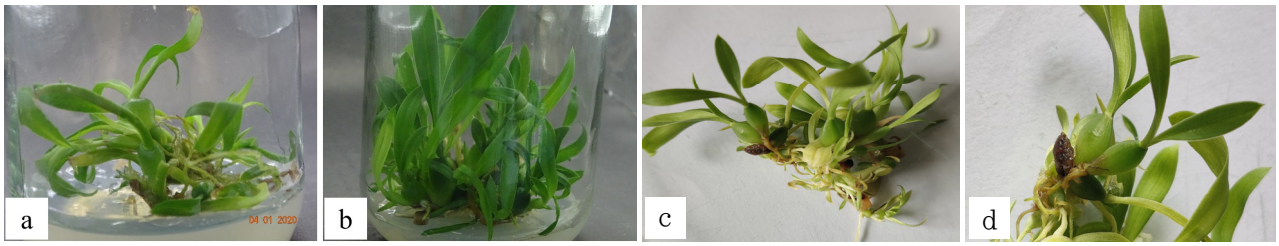

Fig. 7 Root formation response from shoots on different media: full-strength MS medium with 1.5 mg/L IBA (a), full-strength MS medium with 1 mg/L NAA (b), and full-strength MS medium with 1 mg/L NAA and 1 mg/L adenine sulfate (c), (d) pseudobulb development

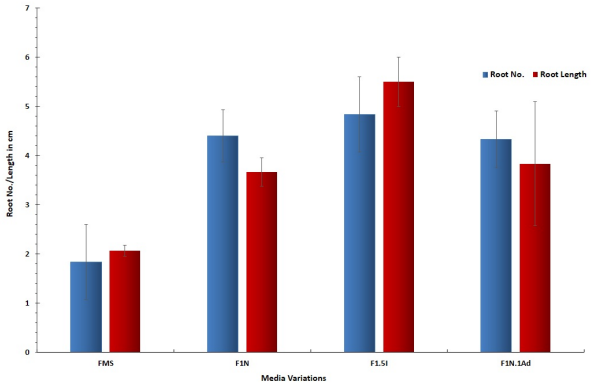

Fig. 8 Root development on different media

Fig. 9 Survivability of in vitro-developed plantlets in response to a 2:1:1 blended substrate of coco-peat, pine bark, and sphagnum moss (a, b)

found in MS with 1.5 mg/L IBA (Figs. 7 and 8). Whereas only the MS medium showed the lowest rate of root proliferation and development. Parmar and Pant (2015) found that MS medium with NAA was more suitable for root proliferation. Similarly, Basker and Bai (2006) found that the MS medium with NAA showed significant results in root initiation and development. However, in the present study, full strength's MS medium with 1.5 mg/L IBA was found more effective for root development.

Acclimatization of plantlets

Transplantation of the delicately raised in vitro plants had not yet been ill coped with the weather and necessary nutrients essential to develop resistance against physical, chemical, and biological factors. A good substrate has the optimum properties like water holding capacity, porosity, and drainage for the survivability of in vitro grown plantlets. In the present study, in vitro raised plantlets above 2.5 cm with well-developed roots were selected for acclimatization. They were transferred to the earthen pot containing different acclimatization substrates. The combination of coco-peat, pine bark, and sphagnum moss in ratio 2:1:1 was found to be a suitable potting mixture for hardening. Eighty percentage of plantlets were successfully survived under this condition. Hence, this result suggests that the mixture of coco-peat, pine bark, and moss will be favourable for the acclimatization of epiphytic orchid, *C. stricta* (Fig. 9).

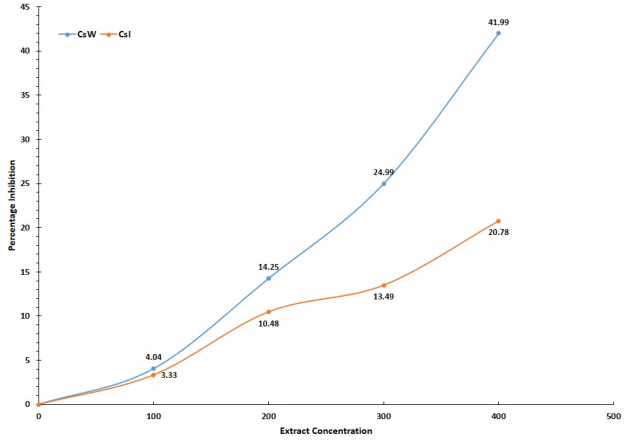

Fig. 10 Percent growth inhibition of HeLa cells by different extract concentrations (μg/ml) of wild pseudobulbs and in vitro-developed protocorms

MTT assay

The cytotoxic activity of wild plant (CsW) and in vitro protocorms (CsI) of *C. stricta* was assessed by MTT. The percentage of HeLa cells growth inhibition for wild plant's extract (CsW) was 41.99% and in vitro plant's extract (CsI) was 20.78% at 400 μg/ml (Fig. 10).

Previous studies made it clear that orchids are a potent source of anticancer agents. Several studies about the pharmacological properties of plant metabolites support the findings of this study. The majority of plant-based secondary metabolites like flavonoids, triterpenoids, and steroids (Gupta et al. 2004; Uddin et al. 2009; Wong et al. 2006) possess diverse pharmacological properties, including cytotoxic and cancer chemopreventive effects. In particular, they exert multiple biological effects due to their antioxidant and free radical-scavenging abilities (Gupta et al. 2004).

Many orchid species have shown cytotoxic activity against different human cancer cell lines, some examples like *Dendrobium nobile* has shown cytotoxic activity toward lung carcinoma, ovary adenocarcinoma, and promyelocytic leukemia cell lines (You et al. 1995), *D. chrysanthum* has shown cytotoxic activity against HL-60 cells (Li et al. 2001), *D. amoenum*, *D. longicornu*, *D. moniliforme* and *D. crepidatum* against cervical and brain cancer cell lines (Paudel and Pant 2017; Paudel et al. 2017, 2018 & 2019), *Bulbophyllum kwangtungense* against cervical and leukemia cell lines (Wu et al. 2006), and *B. odoratissimum* was found to have cytotoxicity against leukemia, human hepatoma, human lung adenocarcinoma and human stomach cancer cell lines (Chen et al. 2007). *Dendrobium transparens* and *Vanda cristata* have also shown the cytotoxic activity toward cervical cancer and brain tumour cell lines (Joshi et al. 2020). The previous findings of anticancer activity of the extracts support the cytotoxic activity of *C. stricta* toward HeLa cell line.

Conclusion

Plant cell and tissue culture offer an alternative source for the rapid propagation of medicinal plants for conservation and else. Protocorm cultures have established for the mass propagation of this orchid species. Half-strength's MS medium with 15% coconut water, full-strength's MS medium with 1 mg/L BAP, 2 mg/L kinetin and 10% coconut water, and full-strength's MS medium with 1.5 mg/L IBA were recorded as suitable for the different stages of propagation from seeds. The in vitro-developed plantlets were successfully acclimatized on the 2:1:1 ratio of coco-peat, pine bark and sphagnum moss. The in vitro-developed protocorms are enriched in bioactive metabolite is likely to be highly useful for commercial production. However, wild pseudobulbs' extract has shown more growth inhibition of cervical cancer cells as compared to in vitro-developed protocorms. The less cytotoxic activity of protocorms is may be due to the immaturity of protocorms where more bioactive compounds cannot be formed.

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