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Ex - situ multiplication of *Coelogyne ovalis* Lindl.: Nutrient optimization for asymbiotic seed germination and mass scale propagation of genetically stable plantlets

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***Abstract*: For conservation of an endangered, horticultural and medicinally important orchid *Coelogyne ovalis* Lindl., an efficient protocol for its propagation was developed via asymbiotic seed germination. Eight months-old capsules of *C. ovalis* were inoculated aseptically on different nutrient media *viz.,* Gamborg (B5), Knudson C (KC) and Mitra, with or without supplementation of plant growth regulators. Amongst the studied media, the best seed germination response (88.74%) was found on KC medium. The germination percentage was found to be enhanced in KC medium supplemented with 3 µM of BAP (6-Benzylaminopurine). For shoot induction, the seedlings were further cultured in medium supplemented with different concentration of BAP and α-Naphthaleneacetic acid (NAA). The best shooting was observed in medium augmented with 15 µM of BAP and 5 µM of NAA in combination. Optimum rooting frequency of regenerated shoots was achieved with 15 µM NAA and 30 µM phloroglucinol in the medium. The well-developed plantlets were hardened in a compost mixture. Genetic integrity of the micropropagated plantlets was ascertained using Start Codon Targeted polymorphism (SCoT) and Inter Simple Sequence Repeat (ISSR) markers. The present investigation on *in vitro* regeneration of *C. ovalis* ensures rapid propagation of plantlets for conservation of clonally stable plantlets.**

***Keywords: Coelogyne ovalis*, Asymbiotic, Conservation, horticultural, Genetic integrity**

I. INTRODUCTION

The family orchidaceae is scattered worldwide from tropics to high alpines [1]. Orchids, among the flowering plants occupy the top position due to their incredible array of diversity in size, shape and colour of their flowers. From the ancient times, the orchids are known for their colourful, long lasting beautiful flowers and are marketed as cut flowers, potted plants. The charming beauty and an extensive durable blooming phase of the flowers fetch an incredibly high cost in the international market amounting to approx. at $126 million [2], [3]. The tremendous orchid production demand as potted plants has increased in the countries namely China, Japan, Germany, United States of America, Taiwan, Netherlands and Thailand [4]. Among the Asian nations, Thailand exports about 50 million cut orchids, followed by Singapore, Malaysia and New Zealand [5], [6]. The uses of orchids are not only restricted to their ornamental value but are also known for the medicinal values [7], [8]. The cultivation and medicinal uses of orchids were first described by Chinese [9]. Orchids are known to be used since Vedic period in the preparation of various Ayurvedic medicines such as "Asthavarga" which is an important ingredient of "Chavyanprasa" containing four types of orchids *viz.*, *Habenaria edgeworthii*, *H. intermedi*, *Malaxis muscifera, M. acuminata* [10].

The genus *Coelogyne* is an epiphytic, evergreen, sympodial orchid, comprising approx. 200 species [11]. This genus is known for its long lasting fragrant flowers and floricultural importance as potted plants. Besides their horticultural value, many species of this genus are known for their ethnobotanical uses and various bioactive compounds have been identified

[12]. An important species of this genus, *Coelogyne ovalis* also known as "jeevanti", is used by the indigenous people of southern parts of India for healing of urinary infections, eye disorders and cough [13]. The plant has a fragrance smell with a high marked value as cut flowers. The orchids are fast depleting in nature due to the conversion of their habitats to agricultural, residential areas and also as a result of illegal collections. As a result, the orchids have become endangered and are listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2012) [14].

The *in vitro* propagation of orchids through asymbiotic seed germination was initially established by Knudson, (1922) [15]. Subsequently a number of nutrient media have been used for *in vitro* seed germination, development and mass scale propagation for conservation and sustainable utilization purposes [16], [17], [18]. The appropriate choices of nutrient media and plant growth regulators (PGRs) play crucial role in the germination and development of orchid seeds. Orchids are traditionally cultivated mainly by means of vegetative propagation. In nature, the orchid seeds that are produced (2-3 million per capsule) are non-endospermous, need fungal association for germination [19]. Less than 5% of orchid seeds germinate in nature. In spite of the various advantages of tissue-cultured plantlets, the occurrence of clonal variation among the regenerated plantlets is one of the major challenges. It is important to assess the genetic integrity within micropropagated plantlets as there is a high chance of getting somaclonal variations. Confirmation of genetic stability using polymerase chain reaction (PCR) - based single primer amplification reaction (SPAR) markers *viz.,* Start Codon Targeted polymorphism (SCoT), mini-satellite DNA region (DAMD), inter-simple sequence repeat (ISSR) is frequently practised [20].

The main objective of the present study was to optimize the nutrient requirement for *in vitro* propagation of *C. ovalis,* so as to obtain genetically stable regenerants for sustainable conservation and utilization of this horticultural and medicinally important orchid.

II. MATERIALS AND METHODS

Plant material and culture conditions

Plants of *C. ovalis* were collected from their natural habitat, Upper Shillong, Meghalaya, India and authenticated by North-Eastern Hill University (NEHU) herbarium, Department of Botany, NEHU, Shillong, Meghalaya with accession no. of NEHU-12051. The plants were maintained in the net house of Plant Biotechnology Laboratory, NEHU (Fig. 2A). Eight months - old capsules were taken for asymbiotic seed germination (Fig. 2B). These capsules were washed with detergent for 5 min, then surface sterilized with 70% ethanol for few seconds followed by flaming. The capsules were dissected longitudinally with sterile scalpel and approx. 100 mg of seeds were inoculated on different culture media viz., B5 [21], KC [15], [22] supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. The pH of the medium was adjusted using 1N NaOH or HCl. To test the effects of PGRs on seed germination, the preliminary screening was performed with different PGRs and finally the best PGRs were screened out. Further the best PGRs namely, 6- Benzylaminopurine (BAP) and 2, 4-Dichlorophenoxyacetic acid (2, 4-D) have been selected and incorporated in media with different concentrations (1, 3, 6 µM). Five replicates were taken for each treatment and cultures were incubated at 25 ± 2 °C. Initially, the inoculated seeds were kept in dark for 7 days, then subjected to 16 h photoperiod with 50 µmol $m^{-2}s^{-1}$ light intensity provided by cool- white fluorescent tubes. The seeds were considered as germinated upon emergence of the embryo from the testa [23].

Seedlings about 0.5 to 0.8 cm in length were used to check the effect of BAP and α-naphthaleneacetic acid (NAA) on the growth and development of *C. ovalis*. Data on growth parameters, namely regeneration frequency, shoot number, shoot length were recorded after 5 weeks of culture. Pseudostem segments with two expanded leaves (approx. 1cm in length) cut from the base of developed *in vitro*-raised seedlings were used as explants. Further growth and development of these segments were observed in KC medium supplemented with a 15 µM of BAP and 5 µM of NAA in a combination.

Rooting and acclimatization

Regenerated micropropagated shoots, after 6 weeks of culture were transferred for rooting in KC medium augmented with NAA (5 - 20 µM) singly or in combination with phenolic compounds (salicylic acid, SA and phloroglucinol, PG). The rooting percentage was recorded after one month of culture. Plantlets with approx. 4-5 cm of length with well developed roots were transplanted into thermocol pots containing compost mixture (vermiculite, decaying litter with chopped fine

bark, brick pieces, charcoal chunks in 2:2:1:1 a top layer of moss), and transferred to the net house. These plantlets were sprayed with 1/2 strength KC medium without sucrose on alternate days.

Genetic integrity analysis of regenerants

Genomic DNA was isolated from leaves of *in vitro* regenerated plantlets (mother and developed progenies after 12 months) by CTAB method [24]. Prior to hardening, 16 micropropagated plantlets were randomly taken for scrutiny of the somaclonal variations within the regenerants. The purity of isolated DNA concentrations were assessed by nanodrop (2000-C, Thermo Scientific, USA). The molecular markers, SCoT and ISSR were used for checking genetic integrity and preliminary 36 primers of SCoT and 20 primers of ISSR were screened. Based on their apparent and reproducible banding patterns, 10 primers from both SCoT and ISSR markers were finally used for analysis of genetic integrity. The PCR was performed in Veritti thermal cycler (Applied Biosystems, USA), with 15 µl final reaction mix containing 40 ng of template, 10 µM primers, 10X PCR buffer with 15 mM of magnesium chloride, 2.5 mM each dNTPs, 2.5 U taq polymerase (Takara, India). The PCR cycles consisted of initial denaturation at 94 °C for 4 min, 40 cycles of 40 °C for 1 min followed by 54 – 60 °C for 1 min, 2 min at 72 °C, and final extension of 72 °C for 7 min. The PCR products were resolved on 2 % of agarose gel and visualized under gel documentation system (Biostep DH – 20, Germany).

Data analysis

The experiments were carried out with five replicates each and repeated three times. The recorded data were subjected to analysis of variance (ANOVA) (SAS- JMP software, Cary, USA) and the significant differences among the means were determined by Tukey's Test.

For genetic integrity analysis only the clear and reproducible amplicons were taken into consideration based on the presence (1) and absence (0) of bands generated by ISSR and SCoT primers. Pairwise similarity matrices were constructed based on Jaccard's coefficient similarity using the software SIMQUAL format of NTSYS-pc [25] and dendograms were constructed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

III. RESULT AND DISCUSSION

Optimization of nutrient conditions for seed germination and seedling development

Asymbiotic seed germination have been used as an effective propagation technique for large scale production and conservation of orchid species [17], [26]. However, in nature the germination percentage of orchid seeds is very less as they lack specific nutritional and environmental conditions for their growth and development. Therefore, *in vitro* technique plays a valuable role in large scale production of the plantlets within a short span of time [27].

Seed germination and seedling development of orchids have been reported to be significantly influenced by different nutrient media used [17], [28]. Even the PGRs play an effective role on the seedling growth and proliferations [29]. In the present study, a method has been optimized for rapid mass propagation of *C. ovalis*, an endangered orchid species. The developed protocol can be used for regeneration of higher number of plants, within a short period and also help to conserve this exquisite orchid species. Three different nutrient media viz., KC, B5 and Mitra supplemented with BAP and 2, 4- D were used for seed germination of *C. ovalis*. The response of the seeds cultured on these nutrient media varied (Table 1, Fig. 1C, D, and E). The highest germination of 88.74% was recorded on KC medium supplemented with 3 µM of BAP (Table 1; Fig. 1E). The embryo bulged and emerged out from the seed coat as spherules within 6-7 weeks which finally developed into protocorms. The protocorm sizes were larger on KC medium compared to those on other nutrient media tested (Fig. 1F). Subsequently, the protcorms developed into PLBs (Fig. 1G). The lowest seed germination was observed in Mitra medium supplemented with 6 µM of 2, 4-D (31.01%). The differences in the responses in seed germination on different culture media might be due to the nutrient composition and the effects of PGRs [30]. Incorporation of either BAP or 2, 4-D at specific concentration in media, enhanced the seed germination percentage. The medium supplemented with BAP resulted in the highest seed germination compared to 2, 4-D incorporated medium. The effectiveness of BAP over 2, 4-D in the enhancement of seed germination has been reported in many orchids [14], [30]. In the present report, the KC medium was found to be effective medium for seed germination compared to other tested media. This could be due to the different constituent of macro- and micronutrients present in KC medium. The presence of nutrients $MnCl_2$, KI, KH_2PO_4 and H_3BO_4 in KC medium in larger concentration could have enhanced seed germination responses compared to the other tested media. For orchid species propagation, no specific culture medium has been

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proven to be universally accepted for all the genera of orchidaceae family [31], [14]. Since, the highest seed germination was found on KC medium, further growth and development of seedlings was optimized in KC medium in presence of BAP and NAA in different concentrations. The efficacy of KC medium on seed germination was also reported on other plants [32], [31].

In the present study, the efficiency of KC medium augmented with BAP and NAA on seedlings development is shown in Table 2. The seedlings inoculated in KC medium supplemented with BAP (15 µM) singly responded in the highest number of shoots (6.20) with a maximum length of 2.86 cm (Table 2). However, similar medium supplemented with BAP and NAA in combination the seedlings showed an improved shoot development and multiplication. The highest shoot number of 8.46 with shoot length of 4.70 cm was recorded in medium containing 15 µM BAP and 5 µM NAA (Table 2). These results are in agreement to those reported in *Geodorum densiflorum* [33]. The synergistic effect of BAP and NAA has been well documented [34]. *In vitro*-raised pseudostem segments approx. 1cm in length was used as explants source for micropropagated plantlets and cultured in KC medium augmented with 15 µM of BAP and 5 µM of NAA in a combination for further growth and development of *C. ovalis*.

Rooting and acclimatization

Shoots approx. 3 - 4 cm in height were transferred to a medium supplemented either singly with NAA (5 - 20 µm) or in combination with SA and PG (10 - 40 µM) (Table 3). Maximum regeneration frequency (80.64%) was achieved in KC medium supplemented with 15 µM of NAA and 30 µM of PG with an average of 16. 6 roots per explant were recorded (Fig. 1K) (Table 3). Whereas, the shoots transferred to a medium supplemented singly with NAA (15 µM) showed the regeneration frequency of 75% with 5.53 roots per explant (Fig. 1H, I). Furthermore, the shoots inoculated in the medium incorporated with NAA (10 µM) and SA (20 µM) in combination showed the regeneration frequency of 70.96 % with 8.53 roots per explant (Fig. 1J). The effectiveness of NAA in root induction has also been reported [35]. Phenolic compounds have been reported to be beneficial in orchids for root induction [36].

The roots of the well developed plantlets of 4 - 5 cm height were washed thoroughly with water for removing traces of agar and hardened in compost mix (vermiculite, decaying litter with chopped fine bark, brick pieces, charcoal chunks in 2:2:1:1 (Fig. 1L). Hardening is one of the most vital steps for *in vitro*-raised plantlets, it is necessary for their continuity and successful establishment. The previous reports on *in-vitro* raised plantlets revealed that upon the transfer of *in vitro* plantlets to *ex- vitro* conditions shows the high mortality rate due to the weak root system, non functional stomata, poorly developed cuticle, and are not capable to compete with soil microorganisms [37]. Therefore, in the present study the hardened plantlets were kept in the net house. Initially the plantlets were covered with polythene bags in order to maintain the high relative humidity. The survivability rate of the transferred plantlets was found to be 82%. The potential cause behind the high survivability of *in vitro*-raised plantlets might be due to the high moisture contain of vermiculite which permit the regular absorption of oxygen by the hardened plantlets, which is further beneficial for the acclimatization process. The presence of the top layer of moss helped in upholding moisture and humidity in the plantlets.

Genetic integrity analysis

In the present work, the developed micropropagated plantlets were used for genetic integrity analysis. The genetic integrity was assessed within nine months old *in vitro*-raised plantlets, which was considered as the mother plant. Subsequently developed plantlets from this mother were taken for genetic integrity analysis (12 months-old plantlets) prior to hardening. Two PCR-based molecular markers, SCoT (10 primers) and ISSR (10 primers) were used for genetic integrity analysis, the profiles generated by SCoT primers (S2 and S5) and ISSR primers (CH7 and CH8) are shown in Fig. 2. The banding profiles generated by both the markers showed the occurrence of monomorphic bands, except the primer S5 where one polymorphic band was found. Genetic variability within the micropropagated plantlets may occur due to the various factors such as explant source, use of PGRs, concentration used during growth, development and artificial conditions. These factors are the major obstacle that contributes towards the genetic variations and major complication towards the sustainable commercial exploitation [38]. Ten SCoT primers produced a total of 41 amplifiable bands whereas 10 ISSR primers produced 40 amplifiable bands. When the data from both the markers were compared, 2.43% of genetic variability was observed within the micropropagated plantlets and Jaccard's similarity index of 0.96 - 1.00 was observed (Table 4; Fig 4). The occurrence of the somaclonal variations within the micropropagated plantlets is the major obstacle; therefore more than one marker should be used for determining the variations in the regenerants [39]. Both the marker systems used in the present study are simple, cost effective and resolvable. The ISSR marker targets the

non coding region of the DNA wherein SCoT, targets the ATG codon within the plant gene and is considered to be more superior marker for the analysis of genetic variation [40], [41]. For the proper conservation of any important taxon it is mandatory to assess the variation within the *in vitro*-raised plantlets. Markers namely ISSR and SCoT have been effectively authenticated in *in vitro*-raised plantlets [42], [43]. The present investigation gives a detailed account of the nutrient requirement for the growth and development of clonally stable plantlets of *C. ovalis*, an orchid of importance.

Fig. 1- *In vitro* propagation of *C. ovalis* (A) Mature plant in greenhouse B) Eight months old capsule (C) Seed germination in B5 medium (60 days; bar = 0.1cm) (D) Seed germination in Mitra medium (after 60 days; bar = 0.1cm) (E) Seed germination in KC medium (after 60 days; bar = 0.1cm) (F) Development of protocorm in KC medium (bar = 0.5 cm) (G) PLBs with leaves (bar = 1cm) (H) Well developed plantlets at 15 µM NAA (I) Well developed plantlets with root at 15 µM of NAA (J) Well developed plantlets in KC medium supplemented with 10 µM NAA and 20 µM SA (K) Rooting in KC medium with15 µM NAA and 30 µM PG (L) Hardened plantlets in net house

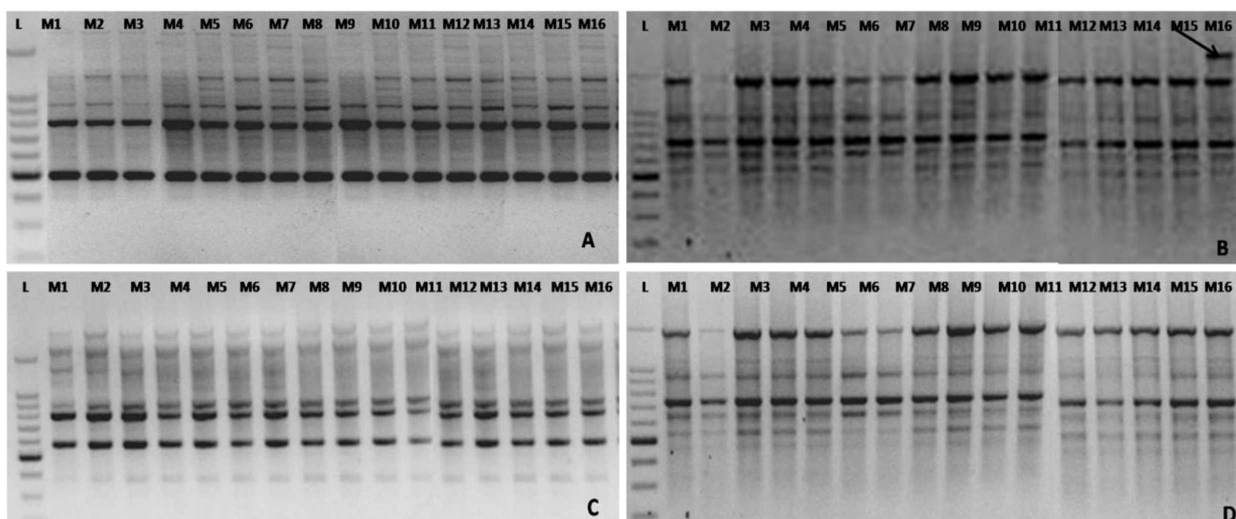

Fig. 2 - Banding profile in *C. ovalis* using SCoT and ISSR markers, A) S2 primer B) S5 C) CH7 D) CH8 primer; Lanes (M1- M16) micropropagated plantlets prior to hardening, L - 100bp ladder, arrow in figure B showing the polymorphic band

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Table 1 - Effect of BAP and 2, 4-D on seed germination of *C. ovalis* in different media

Medium	PGRs (µM)	Time required for germination (weeks)		Seed germination (%)
	BAP 2,4-D	Spherule	Protocorm	
KC	0	9 - 10	11 - 12	75.08 ± 0.59c
	1	6 - 7	8 - 9	70.80 ± 0.71d
	3	6 - 7	8 - 9	88.74 ± 0.71a
	6	8 - 9	9 - 10	62.68 ± 0.75f
	1	7 - 8	9 - 10	64.12 ± 1.21f
	3	8 - 9	9 - 10	66.02 ± 1.6e
	6	6 - 7	8 - 9	62.55 ± 0.77f
B5	0	9 - 10	10 -11	65.91 ± 0.67f
	1	8 - 9	9 - 10	69.90 ± 0.21e
	3	6 - 7	7 - 8	79.75 ± 0.71b
	6	6 - 7	7 - 8	70.93 ± 0.71d
	1	7 - 8	9 - 10	59.47 ± 1.13g
	3	7 - 8	9 - 10	65.99 ± 0.64f
	6	7 - 8	9 - 10	57.91 ± 0.98g
Mitra	0	10 - 11	12 - 13	47.31 ± 0.65i
	1	9 - 10	11 - 12	59.18 ± 0.45g
	3	9 - 10	11 - 12	53.48 ± 0.63h
	6	8 - 9	10 - 11	43.82 ± 0.75j
	1	9 - 10	11 - 12	46.52 ± 0.51i
	3	9 - 10	11 - 12	33.88 ± 0.46k
	6	9 - 10	11 - 12	31.01 ± 0.27k

Above values represent mean ± SE of 5 replicates per concentration. Means values followed by the same letter in the column are not significantly different as given by Tukey-Kramer HSD ($P \leq 0.05$) and all the experiments were repeated three times with five replicates

Table 2 - Effect of BAP and NAA in KC medium on growth and development of seedlings of *C. ovalis* (after 5 weeks of transfer)

PGRs	Concentration (µM)	Regeneration frequency (%)	Shoot no.	Shoot length (cm)
Control	-	-	-	-
BAP	5	65.00	3.06 ± 0.24g	2.10 ± 0.04d
	10	70.00	5.20 ± 0.20d	3.06 ± 0.15b
	15	80.00	6.20 ± 0.28c	2.86 ± 0.19c
	20	60.00	3.86 ± 0.48f	1.26 ± 0.07g
NAA	5	55.00	2.06 ± 0.20h	1.20 ± 0.15g
	10	75.00	3.26 ± 0.18g	1.42 ± 0.02f
	15	60.00	2.26± 0.05h	0.93 ± 0.02h
	20	50.00	2.00 ± 0.29h	0.63 ± 0.01h
BAP+NAA	5 + 5	66.66	4.26 ± 0.15e	1.89 ± 0.04e
	10 + 5	73.33	6.33 ± 0.28c	2.43 ± 0.14d
	15 + 5	86.66	8.46 ± 0.19a	4.70 ± 0.18a
	20 + 5	80.00	7.80 ± 0.17b	2.73 ± 0.15c

Values represent mean ± SE of 5 replicates per concentration. Means values followed by the same letter in the column are not significantly different as given by Tukey-Kramer HSD ($P \leq 0.05$) and all the experiments were repeated three times with five replicates

Table 3 - Effect of NAA, SA and PG supplemented in KC medium on root induction and proliferation of *C. ovalis*

Phenolic compounds with PGRs (µM)			Regeneration frequency (%)	No. of roots	Root length (cm)
NAA	PG	SA			
0	0	0	-	-	-
5			55.00	2.33 ± 0.18h	1.30 ± 0.11d
10			65.00	4.86 ± 0.23f	1.9 ± 0.13d
15			75.00	5.53 ± 0.21e	3.33 ± 0.26b
20			50.00	3.53 ± 0.35g	2.41 ± 0.09c
5	10		58.06	4.56 ± 0.16f	2.36 ± 0.12c
10	20		74.19	12.13 ± 0.49c	3.87 ± 1.29b
15	30		80.64	16. 6 ± 0.94a	5.3 ± 0.15a
20	40		67.74	14.4 ± 0.4b	3.73 ± 0.34b
5		10	54.83	4.8 ± 0.37f	1.93 0.29d
10		20	70.96	8.53 ± 0.33d	3.54 ± 0.33b
15		30	61.29	8.2 ± 0.24d	3.33 ± 1.49b
20		40	51.61	5.93 ± 0.26e	2.66 ± 0.20c

Table 4 - Data of SCoT and ISSR marker used in the genetic fidelity analysis

Sl no.	Primer name	Primer sequence (5' - 3')	Total no. of bands	No. of mon-morphic bands	No. of poly-morphic bands	% of poly-morphism
Start codon targeted polymorphism(SCoT)						
1	S1	CAACAATGGCTACCAGCA	5	5		
2	S2	ACGACATGGCGACCGCGA	5	5		
3	S3	CCATGGCTACCACCGCCA	3	3		
4	S4	ACCATGGCTACCACCGTG	3	3		
5	S5	ACCATGGCTACCACCGGC	5	4	1	20
6	S6	CCATGGCTACCACCGCAC	6	6		
7	S7	ACCATGGCTACCACCGTC	2	2		
8	S8	ACGACATGGCGACCAACG	5	5		
9	S9	ACCATGGCTACCACCGAG	5	5		
10	S10	CAACAATGGCTACCAGCC	2	2		
Inter Simple Sequence Repeat (ISSR)						
11	CH1	GTGTGTGTGTGTGG	3	3		
12	CH2	TGTGTGTGTGTGTGTGA	4	4		
13	CH3	ACACACACACACACACGA	4	4		
14	CH4	GAGGAGGAGGC	3	3		
15	CH5	CACACACACACAAC	4	4		
16	CH6	CTTCACTTCACTTCA	3	3		
17	CH7	ACACACACACACACACT	6	6		
18	CH8	GAGAGAGAGAGAGAGAYT	5	5		
19	CH9	CACCACCACGC	3	3		
20	CH10	ACGACGACGACGACGACG	5	5		
Total			81	80	1	1.23

IV. CONCLUSION

An efficient protocol has been developed for *ex-situ* multiplication of *C. ovalis*, a medicinal as well as horticultural important epiphytic orchid. Among the various media tested, KC medium proved to be effective for seed germination. Best plantlet growth and development was achieved in KC medium incorporated with BAP (15 µM) and NAA (5µM). Further, multiplication of the clonally stable plantlets has also been accomplished. The developed protocol can be utilized for large scale production of plantlets to be re-introduced in natural habitats not only to reduce the threat from extinction but also for sustainable commercial utilization.

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