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In Vitro Seed Germination Studies and Flowering in Micropropagated Plantlets of Dendrobium Ovatum Lindl

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Abstract: *The genus Dendrobium is one of the most important genera with distribution of eleven species in the Western Ghats, belonging to the family Orchidaceae. It is known for its long lasting, showy flowers which has significance in floriculture industry. Dendrobium ovatum Lindl. is an epiphytic orchid found in the Western Ghats. A rapid in vitro seed germination technique is described here. MS, VW, B5 and KC media supplemented with various concentrations of auxins and cytokinins were used in combination for asymbiotic seed germination and plantlet formation. In the evaluation of the media VW medium supplemented with 2 mg BAP/L^{-1} and NAA 5mg/L^{-1} is found to be suitable. Further hormonal concentrations of auxins and cytokinins were evaluated for a minimal and optimal level in the medium. VW basal medium fortified with 10 mg NAA/L^{-1} and 2 mg BAP/L^{-1} and 50 ml. CM induced formation of PLBs which further differentiated into plantlets. In the optimization process for phytohormones, 0.5 mg BAP/L^{-1} and 5 mg NAA/L^{-1} favoured maximum number of plantlet formation. However, rhizogenesis was found to be minimal in the above medium. In vitro rooting was successful with VW medium supplemented with IAA 2mg/L^{-1} , 0.5 mg BAP/L^{-1}, CM 50 ml, and 500mg of activated charcoal. 90 days old in vitro plantlets inside the tissue culture bottles were seen with inflorescence production with 10-12 flowers per axis. Hardened plants were transferred to green house after ex vitro rooting technique. Significance of the present work is discussed here.*

Keywords: *Dendrobium ovatum, PLB's, VW, KC, BAP, NAA, IAA, CM, Ex vitro rooting, rhizogenesis.*

I. Introduction

Orchids belong to the largest and most diverse group among the angiosperms. The genus Dendrobium is the third largest in the family Orchidaceae comprising of about 1184 species worldwide[9]. Dendrobium ovatum[1,2] is an epiphytic species which grows on angiospermic trees at 300-900 m altitude along the Western Ghats. It has tufted stems of deep muddy brown colour and ivory white flowers with a creamy centre. [4,10]. Dendrobium ovatum has medicinal significance and has been widely used as a tonic [12,14]. The juice of Dendrobium ovatum is helpful in all kinds of stomachache, bile secretion and is used as a laxatives [7].

The minute orchid seeds are produced profusely but are non-endospermous and contain almost no nutrients[15].In nature, germination and early development are therefore reliant upon a highly specialized fungal association [5,13]. Asymbiotic seed germination by in vitro culture is essential[8].Since then, in vitro seed germination protocols have been established for many orchid species, and a number of media and salts have been used for germination and propagation[3]. It is fast disappearing from its natural habitats due to excessive habitat destruction and extensive collections by the orchid enthusiasts. Hence tissue culture is a solution which can help in large scale multiplication [11]. Dendrobium ovatum Lindl. in vitro seed germination studies and flowering in micropropagated plantlets is selected here for the present investigation which deals with the selection of a suitable medium for the asymbiotic culture.

II. Material And Methods

Dendrobium ovatum was collected from Sagar, Shimoga district and were grown in Green house at St. Joseph's College Post Graduate and Research Centre. The fruit capsules approximately 90 days old were collected for culture. Two protocols were used for surface sterilization of capsules. In the first protocol, healthy mature fresh capsules were collected from the green house. Dried up tepals and dead tissues at the region of gynostegium were carefully removed using a sterile scalpel. The capsules were washed in 5 % teepol (liquid detergent) to remove the surface dirt and further rinsed in double distilled water to remove the traces of detergent. Inside LAF, fruits were dipped in 100% alcohol and flamed for 3-5 seconds and repeated 2 to 3 times. The capsule was cut open and the seeds were dusted on the surface of the nutrient medium Tissue culture bottles and test tubes were kept under controlled conditions of growth room.

In the second protocol, healthy mature fresh capsules were collected from the green house using a scalpel, carefully trim the dried up tepals and dead tissues of the capsule. Then the capsule is washed in 5 % teepol liquid detergent to remove the surface dirt, rinsed in double distilled water to remove the traces of

detergent. Inside LAF fruits are treated with 0.1 % of $HgCl_2$ for 4-5 minutes and rinsed with autoclaved double distilled water for 2 or 3 times. The capsule was cut open and the seeds were dusted on the surface of the nutrient medium and TC bottles and test tubes were kept under controlled conditions of growth room.

Inoculations of disinfected explants and sub-culturing were carried out under aseptic environment, in a horizontal Laminar Air Flow Unit. Explants were placedon the nutrient medium in culture bottles/tubes with a sterilized forceps .Various basal media like MS, B5, KC and VW were used supplemented with various combinations of Auxins and Cytokinins. Natural fruit extract of Pineapple and tomato were used as additives to test the healthy growth and multiplication of plantlets. pH of the medium was maintained at 5.6 -5.8.

Culture conditions:

- The cultures were incubated at 25± 2^0C temperature
- Photoperiod 16/8 h with 4- 5000 lux illumination from cool white fluorescent tubes ("Philips", India).
- Humidity level with air condition was between 50-60%.

Maintenance of Cultures:

- Cultures were regularly sub-cultured based on the type of cultures, designed in an experiment.
- Each experiment was repeated twice and consisted of 3 replicates of 10 explants for each treatment.
- Observations were made regularly once in a week.

Media used for sub culturing with pineapple and tomato juice

Pineapple juice

- Basal VW Media + 0.5 mg BAP + 0.5 mg NAA + 25 ml pineapple juice
- **Basal VW Media + 1 mg BAP + 1 mg NAA + 50 ml pineapple juice**
- Basal VW Media + 2 mg BAP + 2 mg NAA + 75 ml pineapple juice

Tomato juice

- **Basal VW Media + 1 mg BAP + 0.5 mg NAA+ 50 ml tomato juice**
- Basal VW Media + 1 mg BAP + 5 mg NAA + 50 ml tomato juice

In vitro rooting

The in vitro rooted regenerants were carefully taken out from the culture bottles and washed thoroughly with distilled water to remove the traces of agar. The plantlets were dipped in 1-2 % bavistin for 5 minutes, then transferred to thumb pots containing various media such as solrite. The potted plants were covered with polythene perforated bags to maintain humidity. These plantlets were maintained at temperature 25 + 20 C and 90-95% relative humidity.

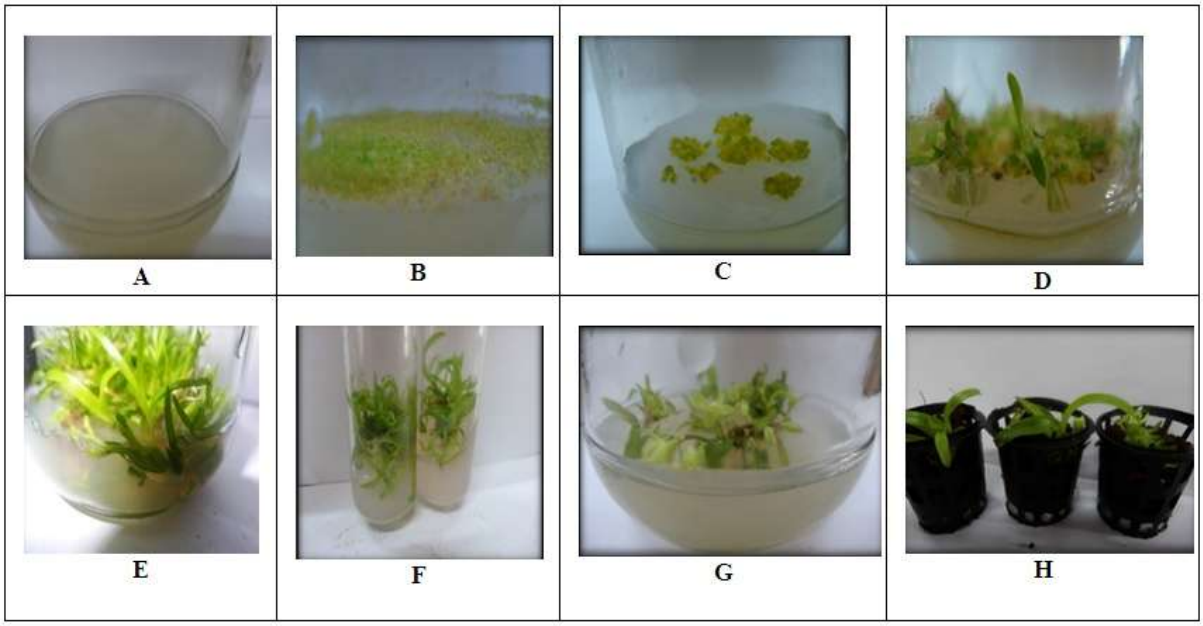
A protocol was developed for in vitro rooting of Dendrobium ovatum Lindl. VW medium with the composition of BAP and NAA and IAA were evaluated in the presence of activated charcoal. A combination of VW medium supplemented with IAA 2mg/L^{-1}, 0.5 mg BAP/L^{-1}, CM 50 ml, and 500mg of activated charcoal were proved excellent for root induction and elongation. 90 days old in vitro plantlet inside the tissue culture bottles were seen with the influence productions in 10 – 12 flowers per axis.

Technique of hardening process

90 days old plantlets with good in vitro rooting and with 3-4 leaf conditions were selected for hardening. Tissue cultured bottles with plantlets were shifted from growth room conditions and were exposed to natural light conditions inside the laboratory area for 4 days. Further Plantlets were carefully removed from the nutrient medium and were subjected to gentle washing of the root system with double distilled water to remove the adhering agar agar. Plants were treated with Auxins to induce ex vitro rooting. Roots were treated with 2% Bevistin, a contact fungicide And transferred to thumb pots containing solrite(a mixure of pearlite and peatmoss). Plants were covered with perforated plastic cup with optimum humidity conditions. Plants were shifted to green house after 10 days.

Observation:

Various stages of growth of plantlets in 90 Days **(Fig. No: 5)**



A - 10 Days Old culture bottles
B - 20 Days Slight swelling of seeds
C - 30 Days Greenish and yellowish swelling of the seeds
D- 45 Days Tiny round PLB's were found (protocorm like bodies)
E - 60 Days - Small plantlets were observed
F - 80 Days – Sub-Culturing, Transferring of the plantlets to test tube and bottles
G - Health plantlets were grown. The flowers were observed in the bottles, after the 3rd sub-culturing
H - Healthy plants grown with 3-4 leaved condition were transferred to the small thumb pots.

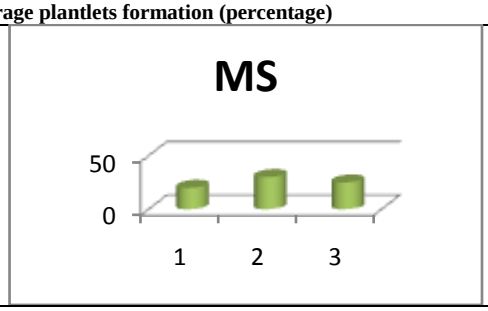
III. Results And Discussion

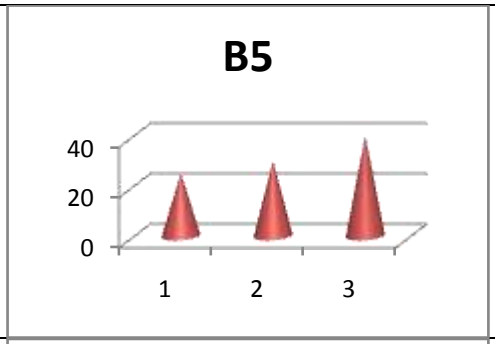
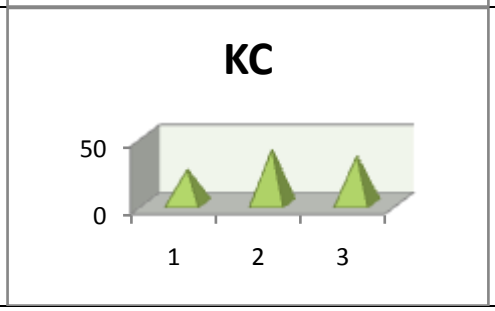
Four different types of media were used MS, VW, B5 and KC media supplemented with various concentrations of auxins and cytokinins were used in combination for asymbiotic seed germination and plantlet formation. After 15 days no results were shown and no contaminants. After 30 days yellow with Greenish colour swelling of seeds were shown with in 40 days small PLB's like bodies, became clear, 60 days small plantlets were observed and 80 days plantlets were sub-cultured. VW media was found to be more suitable with the combination of 0.5 mg BAP/L^{-1} and 5 mg NAA/L^{-1} favoured maximum number of plantlet formation.

The explants were cultured on VW media with the composition of 0.5 mg BAP + 2 mg IAA was used for sub culturing and this composition was found to be more suitable for in vitro flowering. Organic additives viz, coconut water, pineapple juice and tomato fruit extract was supplemented with the medium for better results and rapid proliferation. The inhibitory phenolic and carboxylic compounds produced by the tissues in culture were absorbed by activated charcoal. After the sub culturing the plantlets for 2 weeks the plantlets started flowering inside the culture. The production of plantlets are much higher in number of the same clone in a short time. Those the development of tissue culture protocol is the base requirement for further biotechnological studies viz, meristem culture, embryo culture, molecular breeding and in vitro flowering studies. They is a lot of differences between the flowers bloomed in green house and in vitro flowering.

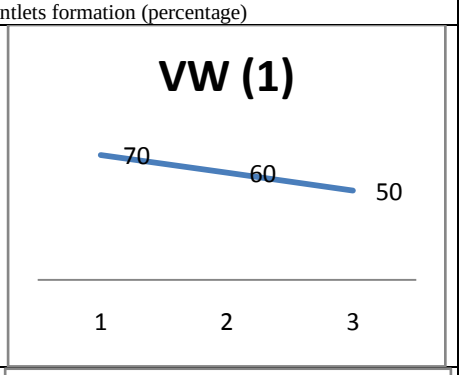
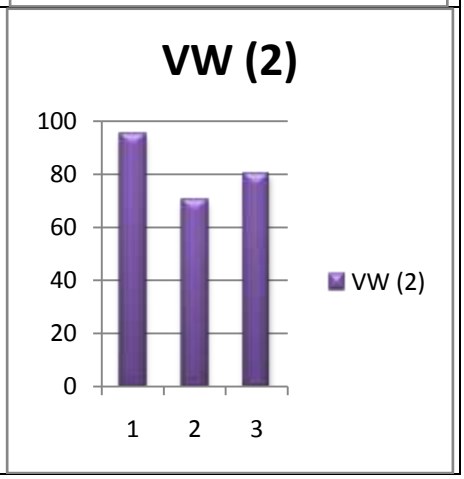
MS, B_5, KC Media Used (Fig. No: 1)

Media Used	Media Composition	Results The average plantlets formation (percentage)
MS	Basal MS Media + 1 mg BAP + 1 mg IAA Basal MS Media + 2 mg BAP + 1 mg IAA Basal MS Media + 3 mg BAP + 2 mg IAA	20% 30% 25%



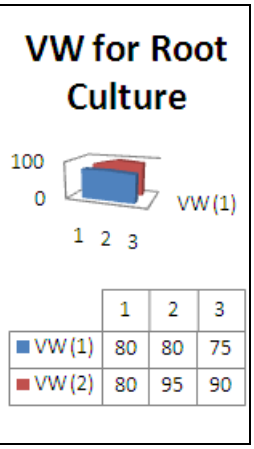
B5,	Basal B5 Media + 1 mg BAP + 1 mg IAA Basal B5 Media + 2 mg BAP + 1 mg IAA Basal B5 Media + 3 mg BAP + 2 mg IAA	25% 30% 40%	
KC	Basal KC Media+ 1 mg BAP + 3 mg NAA Basal KC Media + 2 mg BAP + 5 mg NAA Basal KC Media + 3 mg BAP +10 mg NAA	25% 40% 35%	

Vacin and Went Media (VW) (Fig. No: 2)

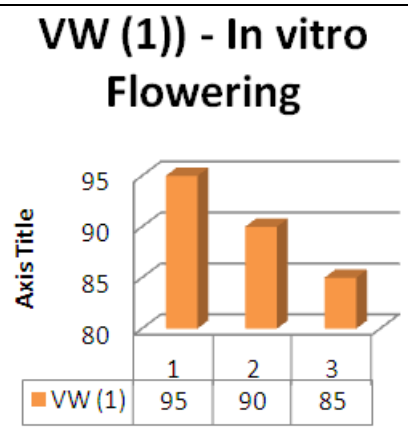
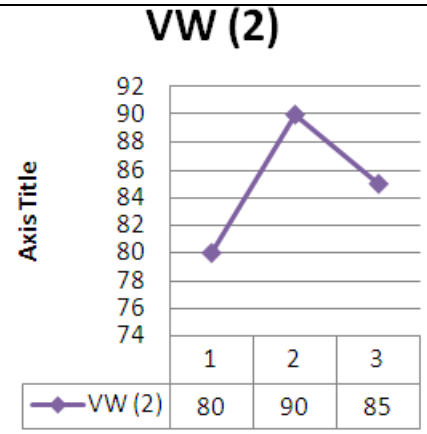
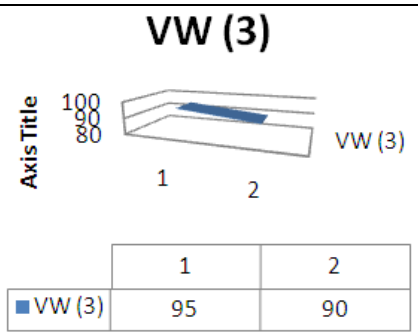
Media Used	Media Composition	Results Average plantlets formation (percentage)	
VW	Basal VW Media + 0.5 mg BAP + 0.5 mg NAA Basal VW Media + 1.0 mg BAP + 1 mg NAA Basal VW Media + 2.0 mg BAP + 2 mg NAA	70% 60% 50%	
VW	**Basal VW Media + 0.5 mg BAP + 5 mg NAA** Basal VW Media + 1 mg BAP + 1 mg NAA Basal VW Media + 2 mg BAP + 2 mg NAA	**95%** 70% 80%	

VW for root culture **(Fig. No: 3)**

VW	Basal VW Media + 0.5 mg BAP + 0.5 mg IAA + 250 mg activated charcoal. Basal VW Media + 1 mg BAP + 1 mg IAA+ 500 mg activated charcoal. Basal VW Media + 2 mg BAP + 2 mg IAA+ 750 mg activated charcoal.	80 % 80 % 75 %	This media was used for rooting along with activated charcoal good growth of roots	

VW	Basal VW Media + 0.5 mg BAP + 0.5 mg IAA + 250 mg activated charcoal. **Basal VW Media + 0.5 mg BAP + 2 mg IAA+ 500 mg activated charcoal.** Basal VW Media + 2 mg BAP + 2 mg IAA+ 750 mg activated charcoal.	80 % **95 %** 90 %	This media used for rooting along with activated charcoal was suitable for rooting	

VW for in vitro flowering (Fig. No: 4)

VW	**Basal VW Media + 0.5 mg BAP + 2 mg IAA+ 50 ml coconut milk + activated charcoal 500 mg** Basal VW Media + 1 mg BAP + 1 mg IAA+ 100 ml coconut milk Basal VW Media + 2 mg BAP + 2 mg IAA + 150 ml coconut milk	**95 %** 90 % 85 %	This media was standardized for in vitro flowering	
VW	Basal VW Media + 0.5 mg BAP + 0.5 mg NAA + 25 ml pineapple juice **Basal VW Media + 1 mg BAP + 1 mg NAA + 50 ml pineapple juice** Basal VW Media + 2 mg BAP + 2 mg NAA + 75 ml pineapple juice	80 % **90 %** 85 %	Media used for sub culturing with pineapple juice	
VW	**Basal VW Media + 1 mg BAP + 0.5 mg NAA+ 50 ml tomato juice** Basal VW Media + 1 mg BAP + 5 mg NAA + 50 ml tomato juice	**95 %** 90 %	Media used for sub culturing with tomato juice	

The speed and extent of seed germination and protocorm development varied with the medium used (fig no 1). Maximum germination percentage and the appearance of protocorms were achieved on VW medium then other mediums (fig no 1). The development of protocorms into seedlings was also superior on VW

medium. On VW medium, the first sign of germination (greening of the embryos) was seen after ~ 2 weeks. The protocorms began to form 2-3 weeks later. VW 0.5 mg BAP/L^{-1} and 5 mg NAA/L^{-1} favoured maximum number of plantlet formation. (fig no 2). The plantlets grown were sub cultured ever 2 weeks using VW media with various composition. Basal VW Media + 0.5 mg BAP + 2 mg IAA+ 500 mg activated charcoal was suitable for good rooting (fig no.3). In vitro flowering was successful in VW medium with (0.5 mg BAP + 2 mg IAA + 50 ml coconut milk) (fig no 4). The production of flowers inside the tissue culture bottles were seen with inflorescence production with 10-12 flowers per axis. In vitro flowering plants were above 2 to 10 cm height excluding the inflorescence, where a normal flowering green house grown plant measured about 11-13 cm height.

Of the various compost mixtures used, the compost made up of broken brick and charcoal with an upper layer of moss was found to be the most suitable for the survival of transferred plantlets. This approach supported 90% survival after 90 days of hardening under glasshouse conditions.

Orchids were grown mainly for their exquisite flowers (Sim et al. 2007) In thin paper, we have demonstrated the possibility of in vitro flowering of Dendrobium ovatum was earlier than time required for conventional breeding method. Not only flowers could be induced early in culture when compared to conventional orchid growing methods. There is a possibility of flowering in vitro throughout the year than seasonal flower between November to January. The auxin and cytokine used have also enhanced the in vitro flowering. Most important of all, there will be tremendous saving of time effort space, man power and cost in orchid breeding by adapting in orchid breeding programmes. These methods will undoubtedly contribute to and benefit the orchid industry as whole.

SCOPE

1. Use of nano biotechnology in control of bacterial and fungal contaminations.
2. In vitro micropropagated plants can be shifted to natural habitats of Western ghats to facilitate In situ conservation of Dendrobium ovatum.
3. Using elicitors (From biological origin) for enhanced plantlet formations.
4. In vitro flowering and Induction of mutations in flowering, using chemical mutagens in the nutrient medium to produce new varieties.
5. Analysis of the chemical content of leaf and stem for Ayurvedic uses: (eg Juice of fresh plant—stomachic, carminative, antispasmodic, laxative, liver tonic. (excites the bile) investigated in a related species, Dendrobium crumenatum Sw., occurs in Andaman Islands. Pounded leaves are used in Malaya for poulticing boils and pimples. Traces of alkaloids have been reported to be present in the pseudobulbs and leaves.

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