ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 6, Issue, 4, pp.3488-3496, April, 2015

International Journal of Recent Scientific Research

RESEARCH ARTICLE

EX SITU CONSERVATION OF TRICHOGLOTTIS TENERA (LINDL.) A THREATENED, AND ENDANGERED ORCHID OF WESTERN GHATS USING ASYMBIOTIC SEED GERMINATION TECHNIQUE

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ARTICLE INFO

Article History:

Received 5th, March, 2015
Received in revised form 12th, March, 2015
Accepted 6th, April, 2015
Published online 28th, April, 2015

Key words:

Trichoglottis tenera, Seed germination, Asymbiotic seed culture, BAP, NAA TDZ.

ABSTRACT

Orchids have been used as traditional healing system of medicine as well as in the treatment of a number of diseases in many parts of the country and in the world. Orchids are a group of extremely interesting plants now recognized as economically important crops having floricultural, commercial and therapeutic values. They are well represented in India, distributed in Himalayan, NE Indian, Western Ghats (WGs) and Eastern Ghats regions. *Trichoglottis tenera* is an endangered epiphytic orchid of Western Ghats. Orchid seeds are dust-like structures and difficult to use directly in the field for mass propagation of orchid seed germination. We have used ten different media for orchid seed germination namly; we investigated seed germination in the ten basic inorganic media which were a). full and half strength Knudson-C medium6-benzylaminopurine (BAP- 0.2 – 2.0 mg/l), α-Naphthalene acetic acid (NAA-0.1 -1.0 mg/l), *Thidiazuron (TDZ 0.2 – 2.0 mg/l)* coconut water (15% ml/l) and banana powder (0.4 % mg/l). b). Full and half strength Murashige & Skoog (MS) medium (1962) full strength and half strength of MS medium has been fortified with different combinations of plant growth regulators i.e., 6-Benzylaminopurine (BAP 0.2 – 2.0 mg/l and 0.1-1.0 mg/l), α-Naphthalene acetic acid (NAA 2.0 - 0.2 mg/l and 0.2-2.5mg/l), Kinetin (1.0 – 1.0 mg/l), *Thidiazuron (0.2 – 2.0 mg/l),* coconut water (15% ml/l) and tomato powder (4.0% and 0.4%). c). Full and half strength Oat meal agar medium (OMA) full strength of banana agar medium (BAM) was supplemented with different types of plant growth regulators (PGRs) i.e., 6-Benzylaminopurine (BAP 2.0 – 0.2 mg/l and 0.2-1.0 mg/l), α-Naphthalene acetic acid (NAA 0.2 – 2.5 mg/l and 2.0 -0.2 mg/l), Kinetin (1.0 – 0.1 mg/l), Gibberellic acid (GA_3 0.2 – 2.0 mg/l) and different concentrations of hormonal additives like Tryptone powder (0.1 – 1.0 mg/l) banana powder (0.4%) coconut water (15% ml/l).

d). Full and half strength Banana agar medium (BAM) which were contains 6-Benzylaminopurine (BAP 0.1 – 1.0 mg/l and 0.2 – 2.0 mg/l), α-Naphthalene acetic acid (NAA 2.0 – 0.2 mg/l), Kinetin (1.0 – 0.1 mg/l) and *Thidiazuron (0.1 – 1.0 mg/l)* different strength of hormonal additives like Tryptone powder (0.1 – 1.0 mg/l) banana powder (0.1 – 1.0 mg/l and coconut water (15% ml/l). e). Full and half strength R medium which were contain 6-Benzylaminopurine (BAP 0.2 – 2.0 mg/l and 0.1 – 1.0 mg/l) α-Naphthalene acetic acid (NAA 2.0 – 0.2 mg/l and 2.0 -0.2 mg/l), *Thidiazuron (0.1 – 1.0 mg/l and 0.2 – 2.0 mg/l), and different concentrations of additives of t*omato powder (0.4% mg/l) and coconut water (15% ml/l). The rooting media were prepared and supplemented with plant growth regulators (IBA -0.2 mg/l), and IAA (0.3 mg/l). A maximum seed germination of 90% percentage was observed after 60-70 days on half strength MS medium with banana powder. 90% of seed germination was observed on half strength MS medium with commercially purchased oats. Significant seed germination was observed on half strength MS medium with corn flour. Optimum seed germination was observed on half strength MS medium and banana powder. The well developed shoots were removed from their conical flask and transferred in to subculture medium, and supplemented with BAP (0.2 – 0.5 mg/l), NAA (0.3 – 0.7 mg/l). The rooting media also media prepared and supplemented with plant growth regulators (IBA -0.2 mg/l) and IAA (0.3 mg/l). The results of these experiments showed that the studied species can be effectively propagated by *in vitro* seed germination of orchids with the aim of *ex situ* biodiversity conservation.

INTRODUCTION

Trichoglottis tenera is a rare, endangered epiphytic orchid largely restricted to the reserved forests of Western Ghats of India. In this study the endangered epiphytic orchid may play a key role for confirmation of mycorrhizal status and requires isolation of the fungi and restoration of functional mycorrhiza. Orchids are popular horticultural and ornamental plants mostly because of their exquisite flowers. Asymbiotic orchid seed germination is one of the most prevalent methods of orchid plant production in commercial settings and has been highly

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favored by scientific researchers as a tool to study orchid seed germination physiology and early life stages (Kauth *et al*., 2008).

Many epiphytic orchid species are horticulturally desirable and few have resisted cultivation from seed using artificial media. In nature, both epiphytic and terrestrial orchids use fungi as a carbon source as well as vitamins, hormones, and amino acids, all of which contribute to the growth and development of orchid seedlings. Epiphytic orchid seedlings may also use fungi as a critical source of free water to resist desiccation resulting from their small size and arboreal habit (Yoder *et al*., 2000). The relative ease by which epiphytic orchids have been cultivated artificially without fungal assistance has led to a prevailing attitude that these plants can be conserved as long as seed banks are maintained. Given the importance of fungi to orchids in situ, the preservation of seeds alone has raised conservation concerns (Johansen and Rasmussen, 1992; Zettler *et al*., 2003). All orchids need fungi to provide inorganic and organic nutrients for seed germination and/or early protocorm development (Smith and Read, 1997). In adult photosynthetic orchids N, P, and water continue to flow from the fungal partner but carbon exchange is essentially reversed with photosynthate providing incentive for continued fungal colonization. Orchids, like all other flowering plants, produce flowers as a means of achieving pollination to produce seed. The seed is necessary to produce new plants to continue the species. Orchid seed, in comparison, is tiny, like dust. It contains virtually no nutrition to grow the new plant, so orchid seed relies on a mycorrhizal fungus to provide the nutrition required to grow. Until the young orchid grows leaves and roots large enough to support the orchid, the fungus must provide all the nutrition for the growing plant. Without this fungus, there is no possibility of the seed developing. Orchid seeds are among the smallest in the plant world (hence their nickname of “dust seeds”), so special care is needed while handling them. A little flask probably holds a few millions of orchid seeds. The majority of orchids are photosynthetic at maturity. However more than 100 species of orchids are completely achlorophyllous (Leake, 2005) and are nutritionally dependent on their fungal partners throughout their lifetime. The mycorrhizal fungi continued to provide some carbon to adult photosynthetic plants (Alexander *et al*., 1985). Mycorrhizal fungi may also be a key source of water for orchids. P and N (as glycine) transfer from fungus to plant was confirmed in radiolabelling experiments (Cameron *et al*., 2006), (2007). Epiphytic and lithophytic orchids have provided opportunities to investigate aspects of orchid-fungal ecology (Bayman *et al*., 2002). In certain orchids, self pollination is not possible and even if possible as in the case of various species of *Vanda*, one has to wait for 4 to 6 months for pod development (Fitch, 1981).

A single pod contains millions of orchid seeds which germinate all the seeds depending upon the suitable culture medium and lab condition. The reliable protocol is needed for propagation of *in vitro* orchid seed germination technique. This technique would develop *in vitro* seed germination and production of protocorm like bodies (PLBs) which is very useful for re-establishment of plant in the wild and for commercial production. A consequence of this lifestyle is that it is very difficult or impossible to germinate orchid seeds by typical gardening methods (http: //www.Orchidmeadow. co.uk/about_OM.html). The reliable protocol of this technique would be very useful and commercially important for orchid growers and for re-establishment in the wild.

MATERIALS AND METHODS

Procurement of orchid pods

Mature green pods and dry pods were collected during the month of November to February in 2012, from Western Ghats of India. The collected pods were stored in (7*5) polythene bags and transferred to our lab.

Sterilization of glassware and metal instruments

To begin with the following necessary items must be treated to dry heat sterilization process for one hour in a hot air oven (65°C). Glassware such as beaker, conical flask, culture tubes, pipettes, and petri dish are to be soaked in soap oil (Teepol) solution for 24 hours. The following items are such as petri dish with inside plotting paper, scalpels, forceps (small and medium size), double distilled water (500 ml), and cotton wool should be wrapped in aluminium foil and subjected to auto clave treatment at 121°C for 15 lb pressure for 20 min. After this, the articles are gently kept inside the laminar airflow chamber and then wiped with 100% alcohol and placed in a sterilization chamber.

Seed storage and inoculation

Two types of pods were separated and stored in a cooling refrigerator at 4°C to make the seed mature until continue studies (Batty. A. L. *et al*., 2001).

Sterilization of orchid pod

The collected pods were first washed in running tap water for 30 Secs to detach unwanted soil particles from the pods. Then they were washed with Teepol solution for 3min,and then three times washed with sterilized double distilled water in the laminar air flow hood. The pods were opened out a with help of sterile surgical blade (Kehr surgical (Pvt. Ltd) and orchid seeds were collected by Centrifuge tube (50ml). Further sterilized by 0.1 % NaClO (HiMedia, Mumbai) solution was poured to Centrifuge tube (50 ml) was subjected to constantly vortex for 2 min. After the vortex solution has been discarded to sterilized beaker (250 ml) under the laminar air flow hood. Again wash with sterilized double distilled water for two times. Further they were treated with 0.1% $HgCl_2$ (HiMedia, Mumbai) solution was poured to centrifuge tube (50 ml) and handled to routinely up and right shaking for 30 sec and the solution was discarded. Now it’s for ready to introduce culture medium.

Culture medium and culture conditions

The ten different media have been used for orchid seed germination i.e. the full strength of Knudson-C medium (KCM)

was supplemented with auxins i.e., 6-benzylaminopurine (BAP 0.2 – 2.0 mg/l), cytokinins i.e., α-Naphthalene acetic acid (NAA 1.0 – 0.1 mg/l), *Thidiazuron (TDZ 0.2 – 2.0 mg/l) the* additives of coconut water (15% ml/l) and banana powder (0.4 % mg/l) and the half strength of Knudson-C medium (KCM) was supplemented with different concentrations of 6-benzylaminopurine (BAP 2.0 – 0.2 mg/l), α-Naphthalene acetic acid (NAA 0.1 – 1.0mg/l), *Thidiazuron (TDZ 0.2 – 2.0 mg/l) the* additives of coconut water (15% ml/l) and tomato powder (3.5 % mg/l) were also added. The contaminations were also marked and have been removed from the cultures. The full strength and half strength of MS medium has been fortified with different combinations of plant growth regulators i.e., 6-Benzylaminopurine (BAP 0.2 – 2.0 mg/l), α-Naphthalene acetic acid (NAA 2.0 - 0.2 mg/l), Kinetin (1.0 – 1.0 mg/l), and different strength additives of coconut water (15% ml/l) and tomato powder (0.4% mg/l). The half strength of MS medium have also been supplemented with different combinations of plant growth regulators i.e., 6-Benzylaminopurine (BAP 0.1 – 1.0 mg/l), α-Naphthalene acetic acid (NAA 0.2 – 2.5 mg/l), *Thidiazuron (0.2 – 2.0 mg/l)* and different strength additives of coconut water (15% ml/l) and banana powder (4.0 % mg/l). The contaminated cultures was observed instantly and removed from the cultures. The full strength of banana agar medium (BAM) was supplemented with different types of plant growth regulators (PGRs) i.e., 6-Benzylaminopurine (BAP 2.0 – 0.2 mg/l), α-Naphthalene acetic acid (NAA 0.2 – 2.5 mg/l), Kinetin (1.0 – 0.1 mg/l), and different combinations of additives like banana powder (4.0 mg/l). The half strength of banana agar medium (BAM) was supplemented with different types of plant growth regulators (PGRs) i.e., 6-Benzylaminopurine (BAP 0.2 – 1.0 mg/l), α-Naphthalene acetic acid (NAA 2.0 – 0.2 mg/l), Gibberellic acid (GA_3 0.2 – 2.0 mg/l) and different concentrations of hormonal additives like tomato powder (4.0 mg/l).

The full strength of Oat meal agar medium (OMA) was supplemented with different combinations of plant growth regulators i.e., 6-Benzylaminopurine (BAP 0.1 – 1.0 mg/l), α-Naphthalene acetic acid (NAA 2.0 – 0.2 mg/l), Kinetin (1.0 – 0.1 mg/l) and different strength of hormonal additives like Tryptone powder (0.1 – 1.0 mg/l) and coconut water (15% ml/l). The half strength of Oat meal agar medium (OMA) was also supplemented with different combinations of plant growth regulators i.e., 6-Benzylaminopurine (BAP 0.2 – 2.0 mg/l), α-Naphthalene acetic acid (NAA 2.0 – 0.2 mg/l), *Thidiazuron (0.1 – 1.0 mg/l)* and different concentrations of additives like banana powder (0.1 – 1.0 mg/l) and coconut water (15% ml/l). The contaminations were also marked and removed from the cultures. The full strength of R medium was supplemented with 6-Benzylaminopurine (BAP 0.2 – 2.0 mg/l) α-Naphthalene acetic acid (NAA 2.0 – 0.2 mg/l), *Thidiazuron (0.1 – 1.0 mg/l), and different concentrations of additives of t*omato powder (0.4% mg/l) and coconut water (15% ml/l). The half strength of R medium was supplemented with 6-Benzylaminopurine (BAP 0.1 – 1.0 mg/l) α-Naphthalene acetic acid (NAA 2.0 – 0.2 mg/l), *Thidiazuron (0.2 – 2.0 mg/l), and different concentrations of additives of t*omato powder (0.4% mg/l) and coconut water (15% ml/l). The contaminated cultures was marked and removed from the cultures.The above medium were added with sucrose 0.25% gm/l and set pH was adjusting at 5.7 using 1N of NaOH prior to add the gelling agent with 0.8% gm/l agar (PTC grade, Hi Media). Each flask contains hundred milliliters medium and was dispensed autoclaving at 121°C for 20 min at 15lb/psi pressure maintaining at tissue culture room for 8/16 hours in continuous light (60 µmolm2s-1) conditions and dark conditions at 25 ± 2°C.

Tetrazolium seed viability test

Freshly isolated orchid seeds from capsules were stained with 1% (v/v) 2, 3, 5-triphenyl tetrazolium chloride (TTC) and placed in a shaker at 5 rpm for 30 min and kept in darkness overnight under static condition at 30 °C for 24 hrs. The seeds were then observed under a stereomicroscope. Percentage of seed viability was calculated by the number of red color staining embryos divided by the total number of seeds examined and multiplied by 100 (Sainiya Samalaa.2014). The embryos could germinate in an appropriate culture medium and lab condition (Zeng. S. *et al*., 2013).

Seed germination and seedling development

All the flasks were transferred into laminar air flow chamber for inoculation of orchid seed. The sterilized orchid seeds were spread over on the ten different medium, approximately 300 seeds per medium containing vessels. After 65 days the seed coat (testa) the inoculated seeds were ruptured and the embryos were formed. My research has clearly showed that orchid seed was first developed by protocorm and then the leaf grew inside the culture medium before emerging in the culture vessel. After the inoculation of orchid seeds the principal factors of the light intensity was good for induction of leaf primordial and darkness in nature it could be better for protocorm like bodies (PLBs). The cyclic photometer was installed in our lab and to maintain the photoperiod conditions of the temperature at 16/8 hrs under light from fluorescent lamps with the intensity 25 µmol m–2s–1.The protocorm subsequently differentiated into first leaf and then root primordial which were formed from the complete seedlings within 95 days.

Germination and growth index

The seed cultures were observed every day and the number of germinated seeds was recorded. After 60 days of inoculated seed in conical flask, few of the seeds were shown to swell. This is observed within 60 days after seed setting in conical flask with contain maximum number of seeds were germinated. A seed was considered to be germinated as seed coat ruptured, plumule and radicle came out and were >2mm long. Germination count was expressed in percentage (Islam *et al.* 2012). The germination percentage was calculated using the following formula (GI). Growth index (GI) was used to measure the seedling development. The GI calculation was based on the formula given by Arditti (3).

$$\text{Germination (\%)} = \frac{\text{Number of seed germinated}}{\text{Total number of seeds set for test}} \times 100$$

Statistical analysis

The experiment was designed in a completely Randomized Design (CRD). The results are expressed as mean ± SD of two

experiments. All data were analyzed by one-way ANOVA using Statistical Package for the Social Sciences (SPSS) Software version 16.0 (SPSS Inc., Chicago, IL, USA). Comparisons of the mean and standard deviations were determined by t- tests at $p<0.05$ level of significance.

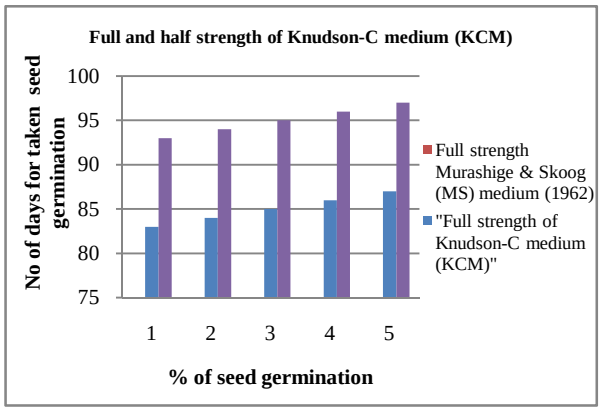

Figure 1

*After 60 days ruptured by orchid seed coat.
**After 75 days development of Protocorm Like bodies (PLBs) on the medium.
***After 90 days culture of seedlings on the medium.

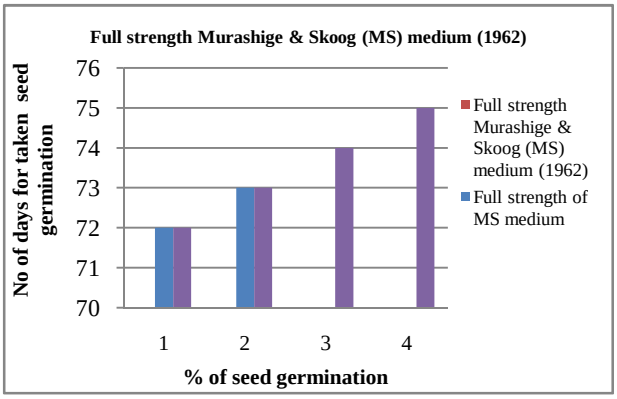

Figure 2

*After 60 days ruptured by orchid seed coat.
**After 75 days development of Protocorm Like bodies (PLBs) on the medium.
***After 80 days plantlets developed from the orchid seedlings on the culture medium.

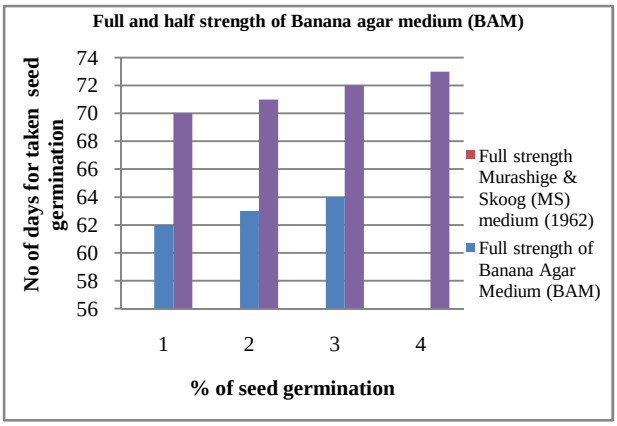

Figure 3

*After 45 -60 days ruptured by orchid seed coat.
**After 75 days development of Protocorm Like bodies (PLBs) on the medium.
***After 95 days complete plant developed from orchid seedlings on culture medium.

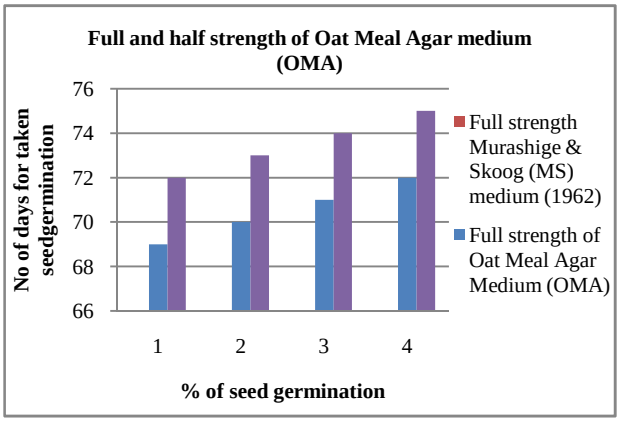

Figure 4

*After 45 days ruptured by orchid seed coat.
**After 60 days development of Protocorm Like bodies (PLBs) on the medium.
***After 75 days complete plantlet raised from orchid seedlings on culture medium.

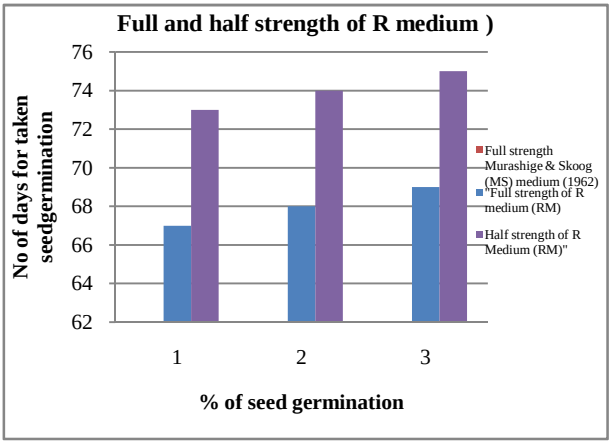

Figure 5

*In both medium after 45-60 days ruptured by orchid seed coat.
**After 70 days development of Protocorm Like bodies (PLBs) on the medium.
***After 90 days complete plantlet developed from orchid seedlings on culture medium.

RESULTS AND DISCUSSION

The ten different culture media (including full strength and half strength) – KC medium (KCM), MS (Murashige and Skoog medium 1962), Banana agar medium (BAM), Oat meal agar medium (OMA), R medium (RM) have been used for orchid seeds culture and observed regularly for germinating process. Germination of seedling growth and development were scored on a scale of 0 –5 (Stewart *et al.,* 2003). MES (2-(N-morpholino) ethanesulfonic acid) is used as a buffering agent and added with ten different media. This function of a buffering agent is to prevent a rapid change in pH when acids or bases are added to the solution. Seed germination percentages were calculated based on viable seeds determined by visual inspection with the aid of a dissecting stereomicroscope (Nikon, Japan) examining the seeds after 60 days of culture. The orchid seedlings were subsequent assessed every week by germination of seedlings growth and development of protocorm on all the above media and supplemented with 6 Benzylaminopurine (BAP), α-Naphthalene acetic acid (NAA), Thidiazuron (TDZ) and Kinetin. These ten different media were also enriched with

additives like Coconut water (CW), Banana powder (BP). All cultures were maintained under suitable environmental conditions (25±2) °C under cool white fluorescent tubes (Philips, India) 25 µmol m^{-2} s^{-1} with photosynthetic photon flux density (16/8 hrs) daily. Comparisons of the mean and standard deviations were determined by t- tests at $p<0.05$ level of significance. The experiments were established in a completely randomized design. ANOVA and t- test were used for comparison among the treatment means.

Effect of full strength Knudson-C medium (KCM)

The effectiveness of asymbiotic seed germination was examined in full strength of Knudson-C medium to promote seed germination and subsequent protocorm development of *Trichoglottis tenera* seeds. These medium was fortified with coconut water (CW; 15%, v/v) and sucrose (0.25% w/v) and different concentrations of plant growth hormones were added in the order of 6 Benzylaminopurine (BAP 0.2 – 2.0 mg/l), α-Naphthalene acetic acid (NAA 1.0 – 0.1 mg/l), Thidiazuron (TDZ 0.2 – 2.0 mg/l) and in additives of banana powder (0.4% mg/l) Table-1. The viable orchid seeds were ruptured by seed coat within three weeks of inoculation of seeds and immediately protocorm was formed in the appearance of a protomeristem. Seedlings cultured in full strength of Knudson-C (KC) medium in the initial 4 weeks, 16-hr photoperiod significantly had longer leaves. The best response of seedlings development in the concentrations of (NAA- 0.5, 0.7, 1.0), (BAP-0.9, 0.8, 0.7), (TDZ 0.4, 0.6, 0.8) and banana powder (0.4%) exhibited good response within 60 days of seed culture. Initially, light had a significant effect on promoting seed swelling and subsequently leaf developed in full strength KC medium**.** Since cultures were maintained under dark light conditions (25±2°) in 75 days, the protocorm had reached chlorophyllous with rhizoids and chlorophyllous leaves were formed. They had two leaves primordial and were also possessed in protocorm with rhizoids. The 87 % germination was achieved.

Effect of half strength Knudson-C medium (KCM)

The commercially produced orchid plants were handled by asymbiotic media for seed germination (Campos 2004). Several techniques are investigated in asymbiotic media to germinate seeds of terrestrial and endangered species of orchids (Roy *et al*., 2011; Zeng *et al*., 2012). The effect of the present study is to determine using half strength of Knudson-C medium supplemented with 6 Benzylaminopurine (BAP 2.0 – 0.2 mg/l), α-Naphthalene acetic acid (NAA 0.1 – 1.0 mg/l), Thidiazuron (TDZ 0.2 – 2.0 mg/l) were added with artificial additives of Coconut water (CW; 15%, v/v) tomato powder (3.5% ml/l) and sucrose (0.25% w/v) (Table-1). Best results were detectable in the concentrations of BAP – 0.2, 0.4, 0.8, NAA- 0.7, 0.9, 1.0 and TDZ – 1.4, 1.8, 2.0 and hormonal additives (tomato powder – 3.5%) were also added. The results found after 45 days, seed swelling were formed and followed by protocorm on 60 days of seed inoculation (Bhattacharjee *et al*., 1999). The embryos were grown transparently after which was clearly visible with the aid of stereomicroscope. The results obtained from half strength Knudson-C medium supplemented with different concentration of growth regulators. The protocorms were observed after 60 days, and it multiplied with chlorophyll containing leaves. The protocorms like bodies (PLBs) were also produced with the first leaf within 75 to 90 days of inoculation. To obtain the germinated seedlings were regenerated from seedlings and reintroduced to their natural habitats with a survival rate reaching 70% of the seedlings after two years in the field (Zeng *et al*., 2012).

Effect of full strength MS medium (MS) (BAP , NAA and Tomato powder)

The *in vitro* orchid seed germination was assessed by full strength of MS media were found to be effective for germination of immature orchid seeds only after the rupturing the seed coat to initiate protocorms within 60 days on seed inoculation. The first shows seed swelling and initiation of plantlets developed from protocorms like bodies (PLBs 3-5) were cultured on full strength of MS media are fortified with 6 Benzylaminopurine (BAP - 0.2 – 2.0 mg/l), α-Naphthalene acetic acid (NAA – 2.0 – 0.2 mg/l), Kinetin (Kin - 0.1 – 1.0 mg/l), and tomato powder (0.4 % mg/l). The great number (75%) of germinated orchid seeds were observed (0.73 ± 0.01) and (0.73 ± 0.01) in the concentrations of BAP -0.2 and 0.4mg/l, NAA – 2.0 and 1.8 mg/l, Kinetin – 0.1 and 0.2 mg/l) and tomato powder (0.4 mg/l) (Table-2**).** This is considered to be the banana powder (0.4%) has been shown to very effective response for the germination of orchid seeds. Therefore the present study was showed the better result for orchid seed germination on full strength of MS media supplemented with hormones after 60 and 70 days of primary culture respectively.

Effect of half strength MS medium (MS)

The half strength of MS salt gives impression of being a high response for *in vitro* orchid seed germination. Therefore, in the present study; half of the strength of MS medium was appeared to be a positive response. The orchid seeds were inoculated on half strength MS medium supplemented with different concentrations of auxins and cytokinins viz. 6 Benzylaminopurine BAP (0.1 – 1.0 mg/l), α-Naphthalene acetic acid (NAA 0.2 - 2.5 mg/l), Thidiazuron (TDZ – 0.2 – 2.0 mg/l) and organic additives of banana powder (4.0 % mg/l). The organic additives have been commonly used to have a stimulation effect in seed germination and protocorm growth and development. In this study, addition of organic additives into half strength MS media at different concentrations of auxins and cytokinins showed a significant stimulation in germination of orchid seeds within 75 days. The fastest germination was observed in the concentrations of BAP- 0.7 and 0.8 mg/l, NAA- 1.8 and 2.0 mg/l, TDZ – 1.4 and 1.6 mg/l and organic additives (4.0 % mg/l). The highest germination, protocorm formation and plant growth ratio were obtained from half strength MS medium (0.75 ± 0.02), (0.75 ± 0.0) and (0.74 ± 0.01) within 90 days completely. Banana powder, peptone and Coconut water is reported to contain carbohydrate, vitamin, acid amino and organic acid, organic ion and enzyme which are usually important to plant cell development (Nitsch, J. P. 1951). Hence, the present study has shown the more desirable for orchid seed germination on half strength of MS medium and fortified with plant growth regulators and added with organic additives of banana powder.

Effect of full strength Banana agar medium (BAM)

In this experiment, orchid seeds were cultured by using full strength of banana agar medium which promoted orchid seed germination. The germination of orchid seed shows a better result in the concentrations of banana powder (Hi Media, Mumbai) with fortified plant growth regulators in the culture media (Table-3). The different combinations of plant growth regulators are 6 Benzylaminopurine (BAP-2.0 – 0.2 mg/l), α-Naphthalene acetic acid (NAA-0.2 – 2.5 mg/l), Kinetin (Kin-1.0 – 0.1mg/l) with banana powder (0.4% mg/l). The banana powder has been added to the culture medium which showed highest responses of orchid seeds swelling and subsequently germination within 45 to 60 days of seed culture. In contrast, MES (0.5 g/l) buffer added to pH of the medium has not been changed. In this occurrence, the pH-7 has been maintained for germination of orchid seed. Combination of BAP (1.6, 1.2 mg/l) NAA (0.7 and 1.2 mg/l), and with Kin (0.8 and 0.6 mg/l) showed effective germination of orchid seeds which formed protocorm like bodies (PLBs) within 75 days, when added to banana powder. The maximum number (73 %) of germinated orchid seeds were observed (0.64 ± 0.01) and (0.64 ± 0.01) (Table- 3).

Effect of half strength Banana agar medium (BAM)

The half strength banana agar medium was used to test orchid seeds germination only after 75 days, germination of orchid seed initially embryos formed and swelled with white colored PLBs were formed. The half strength banana agar medium supplemented with different concentrations of plant growth regulators i.e., 6 Benzylaminopurine (BAP-0.2 – 1.0 mg/l), α-Naphthalene acetic acid (NAA-2.0 – 0.2 mg/l), Gibberellic acid (GA_3- 0.2 – 2.0 mg/l) and Banana powder (4.0% mg/l) (Hi Media, Mumbai).The germination of orchid seeds, which means as rupture of seed coats, swelling of embryos became first leaf only after 70 days (Arditti, 1967). The immature orchid seeds were first turned into smooth walled globular structures called spherules with sticky hair after 70 days of seed culture due to the swelling of embryos (Shreeti. P. 2013). A great numbers of complex additives have been used to study their effects on orchid seed germination like coconut water, banana pulp, peptone, tomato juice, salep, honey and beef extract (Mitra, 1971; Vij, 1993; Hua and Zhiguo, 1998**;** Mohammad *et al*., 2013). Based on this report, it appears that addition of plant growth regulators to the culture medium may increase the rate of seedlings germination and development of orchid plantlets. Seed germination frequency was lower in half strength culture medium when compared to their full strength of banana agar medium. In addition of PGRs are BAP- 0.5 and 0.8 mg/l, NAA-1.0 and 0.6 mg/l and Gibberellic acid (GA_3 1.0 and 1.6mg/l) were proved (0.73 ± 0.01), (0.72 ± 0.01) to be formation of shoot buds and followed by protocorm like bodies (PLBs). However the addition of banana powder has been added to their culture media to promote to better response for their protocorm like bodies (PLBs) (Mohammad *et al*., 2013). Hence, the aim of this study is to identify the effect of banana agar medium which better resulted in visible sign of orchid seed germination.

Effect of full strength Oat meal agar (OMA) medium

Full strength of oat meal agar medium proved to be effective for orchid seedling germination. The first stage of orchid seedling germination was observed within 45 days and all seedlings were in viable condition followed by swelling of embryos by nutrition. The swelled embryos increased number of cells and led to cell division resulting in the formation of rupturing of the seed coat. The ruptured seed coats produced germinated mass seedlings forming green spherule. This occurrence was observed within 60 days of primary inoculation of orchid seeds in full strength of oat meal agar medium with fortifying with 6-benzyladeninepurine (BAP-0.1 – 1.0 mg/l), α-Naphthalene acetic acid (NAA2.0 – 0.2 mg/l), and Kinetin (Kin- 1.0 – 0.1mg/l) and organic additives of tryptone Powder (1.0% mg/l) (Table-4). The seeds were swelled on full strength Oat meal agar medium after 60 days of culture. The initial seed germination took place after 75 days of culture and protocorm like bodies (PLBs) were formed 80 days of culture. The complete green spherule was modified into protocorm like bodies (PLBs) within 85 days of seed culture. The best results were (0.72 ± 0.00) and (0.71 ± 0.01) in the concentrations of plant growth regulators namely; BAP (0.3, 0.7 1.0 mg/l), NAA (1.6, 0.8, 0.2), Kin (0.8, 0.4, 0.1) and organic additives of Tryptone powder (1.0 % mg/l). The similar mode of differentiation was also reported from *Platanthera clavellata* (Zettler and Hofer 1998), *Cypripedium acaule* (Leroux *et al*. 1997). *Habenaria macroceratitis* (Stewart and Kane 2006), *Cymbidium aloifolium* (Hossain *et al*., 2009), *C. giganteum* (Hossain *et al*., 2010) roots emerged from the basal part of the protocorms and gradually differentiated into young seedlings. The well developed seedlings were produced first leaf and root primordial within 12 to 15 weeks of culture. The study the oat meal agar medium was better to identify the effect of orchid seedlings.

Effect of half strength Oat meal agar (OMA) medium

In vitro orchid seed germination is scrutinized to be an appropriate method for *in* vitro propagation and conservation of orchids (Arditti. J, 1982; Buyun *et al*., 2004; Rahman, A.R.M.M. *et al*., 2004; Lo, S.F. *et al*., 2004; Kannonont, N. *et al*., 2010; Park, S.Y. *et al*., 2000). The *in vitro* asymbiotic orchid seed germination has been used for production of many orchid species (Arditti, J. 1992). After two months, the inoculation of germinating orchid seeds were produced different protocorm. It was observed that seed germination and protocorm production was enhanced by the addition of Banana Powder (mg/l) and the effect was identified in the concentration of 6-benzyladeninepurine (BAP 0.2 – 2.0 mg/l), α-Naphthalene acetic acid (NAA 2.0 – 0.2 mg/l), Thidiazuron (TDZ 0.1 – 0.1 mg/l), and organic additives of banana powder (1.0% mg/l) within the fifth week of seed culture. Several orchids hobbyist were reported and orchid seed germination increased within short period of light illumination (Rasmussen *et al*., 1990a). In our results, 75% of seed germination occurred in the half strength of oat meal agar when seeds were subjected to dark incubation with short periods. This was a remarkable increase at an alarming rate from 25% of seed germination under frequent darkness. The exact illumination of light (16/8)

is not well understood for the swelling of seed germination. The protocorm production was good (0.75 ± 0.01), (0.74 ± 0.01) with green shoot in the concentration of (BAP 0.8, 1.2 mg/l), (NAA 1.4, 1.0 mg/l), (TDZ 0.4, 0.6 mg/l), and Banana powder (0.4, 0.6 mg/l) (Table 4). Significantly, the yellow protocorms like bodies (PLBs) with green primordial was also produced by addition of banana powder (0.4 and 0.6% mg/l). This experiment was conducted to study the effect of oat meal agar produced by cumulative percentage of plantlets with root primordial, complete leaflets and development of number of PLBs after 95 days of seed culture.

Effect of full strength r medium (RM)

Asymbiotic seed germination of orchid seeds is an efficient propagation method for large-scale production of orchid plants. The effect of full strength R media on the seed germination and seedling development was investigated in asymbiotic seed cultures. The observation on orchid seed germination and seedling development was made after 40 days of inoculating the seedlings. The average number of orchid seeds sown for germination in a conical flask (250 ml) was about 500 in this case. Germination of seedlings, growth and development were also noted (Table 5; Stewart *et al*., 2003). The first stage was only viable seeds swelled and ruptured by seed coats, and it was occurred undifferentiated embryos formed an irregular shaped cell mass as spherules within 60 days after inoculation of seed culture (Kiyoshi Tsutsui and Masanori Tomita, 1986**).** The seeds developed into protocorms in 90 days and well developed into plantlets. The individual plantlets were transferred to separate conical flask (250 ml) with supplemented plant growth regulators. This observation was enhanced to identify in the concentration of 6-benzyladeninepurine (BAP0.4, 1.0 mg/l), α-Naphthalene acetic acid (NAA1.8, 1.2 mg/l), Thidiazuron (TDZ 0.2, 0.5 mg/l), and Tomato powder (0.4% mg/l). The percentage of seed germinations (0.68 ± 0.01), (0.69 ± 0.02) were determined based on viable seeds by visual inspection with the help of a dissecting stereomicroscope (Nikon, Japan). The observations of the level of contamination and the number of germinated seeds were evaluated periodically, starting from the first day of inoculation of seeds. The results of this work are extremely important for the success of in vitro asymbiotic seed germination of orchids, which varies according to species in analysis. Maximizing the success of asymbiotic seed germination depends on identifying abiotic conditions and finding the most appropriate medium for each species (Pericles B.L.de Sousa, *et al*., 1999).

Effect of half strength R medium (RM)

The present study was mainly aimed at understanding the mode of seed germination and protocorm development from seedlings. Several researchers have suggested many nutrient media for *in vitro* asymbiotic seed germination of terrestrial and endangered species of orchids (Zeng *et al*., 2012; Roy *et al*., 2011). *In vitro* orchid seed germination plantlet development is considered to be a suitable method for ex-situ conservation of orchids (Van Waes and Debergh 1986). The immature seeds were sown on half strength R media. The highest percentage (0.74 ± 0.02), (0.75 ± 0.01) of germinating seed on these media was intensely green embryos when grown on medium (Table-5) fortified with different concentration of 6-benzyladeninepurine (BAP-0.5, 0.7, 0.9, 1.0 mg/l), Naphthalene acetic acid (NAA-1.2, 0.8, 0.4, 0.2 mg/l), Thidiazuron (TDZ-1.0, 1.4, 1.8, 2.0 mg/l) and Tomato powder (0.3, 0.4, 0.5, 0.7 mg/l). After inoculate orchid seeds, seeds coat were ruptured to produce embryos which was observed using stereo light microscopic (Nikon, Japan) within 45 days. The evaluations were made simultaneously for the efficiency of proliferation, number of seed germination, and number of contamination on media. The germination process has been not been initiated when shown on light. The growth regulators of auxins and cytokinins are extensively used in orchid tissue culture (Arditti and Ernst 1993), their effect on seed germination may vary among orchid genera. The half strength R media was beneficial to the production of protocorm like bodies (PLBs) with higher frequency, vigorous growth, and less differentiation (Table-5).When the orchid seeds was maintained on the culture media, the protocorm like bodies differentiated into buds. So it was necessary to instantly subculture for further development of the protocorm-like bodies (PLBs) before their differentiation to keep their complete plantlets raised on after 90 days of seed culture without further delaying growth. So, it has been successfully raising the plantlets from orchid seed germination and has been considered as a promising isolate for the achievement of orchid seedlings. This is considered to be the best for tomato powder and plant growth regulators have showed effective response for the germination of orchid seeds. Therefore, the present study was most suitable for the germination of orchid seedlings.

CONCLUSIONS

In vitro seed germination protocol has been developed for asymbiotic seed germination of *Trichoglottis tenera* using matured orchid seeds cultured on different types of culture medium supplemented with different concentrations of plant growth regulators like cytokinins, auxins and additives of banana powder, coconut water and tomato powder. This study exhibited asymbiotic seed germination as a favorable method for the production of orchid plants. However, the potential of PLB production is increased when asymbiotic medium containing hormones and hormone containing additive compounds (e.g., coconut water, banana powder, and tomato powder) are used. The mature seed is largely virus free therefore; results would also be virus-free plants. Many tropical epiphytic orchids are produced in this way (Arditti *et al*., 1990; Arditti *et al*., 1982). However, attempts to germinate terrestrial orchid's asymbiotically have not been as successful as species have been germinated asymbiotically (Arditti and Oliva, 1981; Oliva and Arditti, 1984). Micropropagation is successfully employed for disappearing from its natural habitats due to extensive collections by the orchid enthusiasts. The plants produce very minute seeds, which lack endosperm. Seeds are wind dispersed. Naturally, they require mycorrhizal association for their germination. Hence, a fast method of growing and conserving them in the green houses is an urgent need.

Therefore, in this technique conservation of species is achieved through asymbiotic seed germination technique. It is most effective method through the management of wild populations and natural habitats *(in situ* and *ex situ* conservation).

Techniques can be used to complement *in situ* methods, and in some instances, may be the only option for some species (Maunder *et al*., 1998; Ramsay *et al*., 2000). Number of species faces the risk of extinction; *in vitro* techniques have been increasingly used in the conservation of threatened plants in recent years also (Fay, 1992; Arditti and Krikorian, 1996; Benson *et al*., 2000). Sarasan *et al*. (2006) considered that, future conservation biotechnology research and its applications must be aimed at conserving highly threatened, mainly endemic plants from conservation hotspots.

Acknowledgement

The authors are gratefully acknowledging University Grants Commission, New Delhi for financial support, and they also thank the management of St. Joseph's College (Autonomous), Tiruchirappalli -2 for having provided laboratory facilities to carry out this research work.

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