CHAPTER I

INTRODUCTION

1.1 Background:

Orchids are the most fascinating, varied and beautiful of all flowers that belong to the family Orchidaceae, one of the largest and most diverse plant families (Singh, B. and S. R. Voletri, 1995). This family is also known to be most successful family among plants representing the peak in evolutionary history of monocots. This is clear with its wide distribution and large number of species (Just and Beardsell, 2015). The beauty of the flowers, variety of fragrance, brilliance in color and attractive habit of orchids has aroused highest admiration among the people throughout the world with 26,567 accepted species in the world (Kew World Checklist of Orchids).

Orchids are one of the major families of angiosperms, are having immense importance to ornamental and medicinal potential. Nowadays, many orchids' species are threatened and enlisted in Red Data Book of IUCN (Chugh et al., 2009 and IUCN, 2011). The entire family is now included in Appendix II of convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) where the international trade is strictly controlled and monitored (Chugh et al., 2009). Orchid species are under serious threat with an uncertain future due to unscrupulous collection for commercial uses (Swarts and Dixon, 2009).

In Nepal, orchid species are distributed from 60-5200 m (a.s.l.). Nepal is gifted with 450 species from 107 genera of which 18 endemic species (Rajbhandari, K. R., 2014).

Eurasia, America and Canada are known as the homes of orchids but today they are found almost every part of the world except for the arid zones that support orchid growth with few native species (source- orchid.origin/Google). Orchids are known for most beautiful range of flowers, not only for their aesthetic value but also for medicinal value (Hrashel and Thangjan, 2015). Medicinal orchids are primarily belong to genera Anoectochilus, Bletilla, *Calanthe, Coelogyne, Cymbidium, Ephemerantha, Eria, Galeola, Gastrodia, Gymnadenia, Habenaria, Ludisia, Luisia, Nervilia and Thunia* (Pant and Raskoti, 2013).

Orchids are threatened species. These beautiful flower plants are important for aesthetic value and also work as ecological indicators. The disappearance of orchids indicates the change in soil and air of the particular region (Joshi *et al*, 2009). Usually orchids are grown for ornamental purpose but they are used in herbal medicine, as food and in cultural purpose by different tribes in different parts of the world. Orchids are used worldwide as traditional healing and also used to cure number of many diseases (Pant B., 2013). Several species of *Vanda, Cymbidium, Ascocentrum, Paphiopedum, Phalaenopsis, Calanthe, Coelogyne, Dendrobium, etc* have huge demand in international market for breeding materials (De and Medhi, 2014).

Orchid is a vegetative propagated plant. It produces a huge number of seeds but the seeds cannot germinate easily in nature due to the absence of endosperm in the seeds is an incompatibility barrier that limits germination of orchid seed in nature. Seeds usually germinate in symbiotic association with some species specific mycorrhiza (a kind of symbiotic fungus) which supplies nutrient to the germinating undifferentiated orchid embryos. Therefore, the presence of species specific mycorrhiza is as important factor for the distribution of orchids in nature (Bhadra, 1999, Bhadra and Hossain, 2003). Moreover, orchid seeds have poor germination capacity that may be ruined very quickly in nature of favorable environment. The most interesting feature of orchids is that they grow in different types of habitat. They need specific microhabitat which varies with habitats they grow in.

1.1.1Tissue culture practice and its importance in orchids

Tissue culture is the scientific method for propagation of plant through any parts. This technique requires trained personals with sophisticated lab. After the totipotency theory by Schwann and Scheilden in 1838, there has been continuous exercise that resulted in many valuable discoveries by various workers in tissue culture. The first attempt of tissue culture was made by Haberlandt in 1902. Noel Bernard from 1900 to 2011 was the first to introduce the symbiotic germination of orchid seeds *in vitro*. Micro propagation of orchid successfully carved its way only after the discovery of Knudson C medium by Lewis Knudson. This medium is now used mostly for orchid seed germination (Arditti, 1990, 2010). These discoveries lead to the possible *in vitro* germination of orchid seeds. Another famous discovery of Murashige and Skooge (MS) medium (1962) and other discoveries as well made tissue culture of plants possible. This MS medium (1962) is now the most used nutrient medium for tissue culture.

Different researches have been carried out of many valuable orchids up to date. About 22 genera of orchids have been propagated using different explants like root tip, shoot tip, young embryos and leaf (Murashige and Skoog, 1962, 1974). Successful plant regeneration of different orchid species, *Geodorum densiflorum* through seed (Bhadra and Hossain, 2003), *Vanda teres* using protocorm obtained from seed culture (Sinha and Roy, 2004), *Dendrobium* hybrid through protocorm (Aktar *et al*., 2007), *Vanda tessellata* through shoot tip culture (Rahman *et al*., 2009) *Vanda tesatacea* (Kaur and Bhutani, 2009) has been carried out using different media and phytohormones and some organic additives like charcoal, coconut water and sucrose.

Tissue culture for preservation of germplasm only began after 1988 in Nepal (Teixeira da Silva and Acharya, 2014). Some medicinally important and endangered orchids like *Aerides odorata, Coelogyne cristata, C. fuscescensm C. ovalis, Cymbidium devonianum, C. elegans, C. irridioides, Dendrobium densiflorum, D. primulinum, Phaius tankervilleae, Pholidata articulate, Thunia alba* and *Vanda tessellata* have been successfully studied *in vitro* through immature seeds culture, synthetic seed germination, etc. in CDB, TU Nepal. Department of Plant

Resources has also successfully developed protocol for more than 40 orchid species in National Herbarium and Plant Laboratory (NHPL) of Government of Nepal.

Different researchers have given various conclusions and views in tissue culture of orchids for conservation and growth as they are important to many aspects. As the orchids are sold as pot flowers and cut flowers in international markets in developed countries (Aktar *et al*.,2007) increased popularity has positive effect on production as well as sale. For breeding materials some species of orchids like *Vanda, Cymbidium, Ascocentrum, Paphiopedilum, Phalaenopsis, Calanthe, Coelogyne, Dendrobium,* etc. have great demands in international market. Tissue culture techniques as well as strong breeding technique, seed storage and cryo preservation technique can be used to overcome the loss of orchids (De and Medhi., 2014) and to fulfill today's demand.

Comparatively, micropropagation technique is significant to reproduce and introduce the orchid plants into environment than traditional way which is really difficult one (Long *et al*.,2010; Asghar *et al*.,2011; Mohanthy *et al*., 2012; Habiba *et al*., 2014). Propagation through PLBs is really a rapid method to obtain large number of plantlets in short period of time because PLBs can be obtained in short time (Long *et al*., 2010; Mohanthy *et al*., 2012). For tissue culture, plant growth regulators play really important role in differentiation of shoot, roots and leaves. Growth regulators have a low importance for germination but are important for the subsequent development of protocorms (Habiba *et al*., 2014). Rooting hormones are used to induce roots as shooting hormones do not form roots (Aktar *et al*., 2007).

1.1.2 Description Taxonomic

Vanda tessellata (Roxb.) Hook. ex G. Don

Synonyms: *Vanda roxburghii*. R.Br, *Epidendrum tessellatum* Roxb.,

Figure 1: Image of *Vanda tessellata*

Common name: Tessellated Vanda, Vanda

Ayurvedic name: Atirasa & Rasna (Singh and Duggal, 2009) (Kasera and Sukla, 2001)

Distribution: Eastern and Central Nepal, Sri Lanka, Burma, India and Myanmar (Chauhan *et al.*, 1999, Raskoti, 2009)

Botany: *Vanda tessellata* is an epiphytic orchid, 30-60 cm high, with leafy stem. Leaves are thickly cariaceous, recurved, plicate, obtuse and keeled. Flowers are greenish, yellow mottled with brown in the mid lobe of lip with purple caruncles (Chauhan *et al*., 1999).

Photochemistry: Alkaloid, glucoside, bitter principle, tannins, resin, saponins, sirosterol and colouring matter (Nayak *et al*., 2005), Ahmad *et al*., 2001)

Habitat: The growth habit is monopodial. Because of their relatively thin stem, they do not withstand drying as well as orchids with pseudobulbs. Their natural range is from the Philippines through the Asian tropics to Australia and Africa. They occur in regions that are warm and humid. Some are found in areas with heavy rains throughout the year but others are from areas with moderate rains throughout the year (McConnell and Cruz, 1996)

1.1.3 Medicinal and other uses

Traditionally the root of *Vanda tessellata* (Roxb.) Hook. ex G. Don (syn *Vanda roxburghii* R. Br.) is used for antidote for scorpion stings, and remedy of bronchitis and rheumatism and paste of leaves is used to treat fevers (Raskoti, 2009, Subedi *et al*., 2012). *Vanda tessellata* have been also used in the indigenous medicine such as Ayurvedic and local traditional medicinal practices. The leaf juice is used for treatment of inflammatory conditions. It is also instilled in ear as remedy of otitis. The leaves in the form of paste are applied to the body to bring down fever. The leaves are used in rheumatism, nervous problems, bronchitis, dyspepsia and fever. Unani practices hold it to be laxative and tonic to the liver. It is also used to treat ccough, piles, boils on the scalp, etc. (Rao *et al*., 2012). *Vanda tessellata* is used to care 13 different diseases with several properties (Shanavaskhan *et al*., 2012) and also have antiepileptic property of rat (Prasad and Achari, 1966, Rajalakshimi *et al*., 2012). It is an ingredient of *Rasna Panchaka Quatha,* an Ayurvedic formulation used in the treatment of arthritis and rheumatism (Singh and Duggal, 2009, Chauhan *et al*., 1999).

Pre-clinical Studies: - Aphrodisiac (Kumar *et al*., 2000), anti-inflammatory (Basu *et al*., 1971), Anti-arthritic (Prasad *et al*., 1966) antimicrobial (Ahmed *et al*., 2001) and wound healing (Ahmed *et al*., 2002)

1.2 Hypothesis

Orchids germinate poorly in its natural habitat. This is due to the lack of endosperm or very small endosperm which is inefficient for the seed germination. These seeds need a very special kind of fungal association for germination which has very rare probability. Along with this they need microclimate of favorable temperature, humus, humidity, etc in nature. As few seeds make germination too but do not make it up to mature plant. As we can now fulfill such needs artificially *in vitro* culture techniques by tissue culture method can fulfill such needs and propagate through any parts and tissues of the plant, Orchids do not need symbiotic association for *in vitro* culture. So the hypothesis of this research is, "it is possible to propagate *Vanda tessellata* (Roxb.) Hook.ex G. Don through protocorm as explants grown *in vitro*."

1.3 Objectives

1.3.1 General objective

- To develop an efficient protocol for *in vitro* multiplication and mass propagation of *Vanda tessellata* through protocorms culture method.

1.3.2 Specific objectives

- To determine the effect of different concentration of phytohormones and organic additives in growth and development of shoots from protocorms of *Vanda tessellata*.
- To develop an efficient protocol for rapid propagation from *in vitro* grown protocorms of *Vanda tessellata* Roxb.

1.4 Rationale:

Orchids are well known for their beautiful flowers; beside this ornamental value they possess high medicinal value as well. But now days these beautiful herbal plants are in considerable threat due to the envasion by man. They are in real threat because they face usually infrequently encountered in their natural habitat and grow only in distributed forest areas with high light intensity. They are also highly vulnerable to habitat destruction. Habitat loss, deterioration and fragmentation, over exploitation, pollution, global warming, etc are other reasons as well.

Vanda tessellata is a monopodial orchids, with endowed with beautiful fragrant flowers and high medicinal properties which is used to cure several diseases by ethnic groups as well as in Ayurvedic formulations. There is a high demand for breeding material for breeding technique so some species of genus *Vanda* and *Vanda tessellata* is one of them. They produce large number of seeds but due to the lack of endosperm they can't germinate. They need special kind of fungal association for germination. Only 2-5% of orchid seeds germinate in nature with mycorrhizal association. Out of which very few survive in nature. To save these valuable plants for future perspective and coming generation possible steps must be taken in action. Tissue culture is the

most efficient method of few to make this possible for today's demand for conservation and commercial purpose. So this present research study has been carried out to develop efficient protocol for propagation of *Vanda tessellata* (Roxb.) Hook. ex G. Don through *in vitro* grown protocorm fro *ex situ* as well as germplasm conservation.

CHAPTER II

LITERATURE REVIEW

After Scheilden (1838) and Schwann (1839) gave the totipotency theory that cells are autonomous, and in principle, are capable of regenerating to give a complete plant; tissue culture have been practiced by different plant biologists. The first attempt of tissue culture was carried out by Haberlandt (1902) in practice.

The famous discovery of Knudson that seeds of orchids can be germinated in simple sugar containing medium without any fungal association, the tissue culture in orchid was revolutionized. He named this new modified medium as 'Knudson C' (1946).

In 1960, Morel carried out the vegetative propagation of orchids by meristem culture in *Cymbidium*. It was the first clonal propagation in orchids that succeeded.

Murashige and Skoog (1962) developed the famous Murashige and Skoog medium. This MS formulation is now the most widely used nutrient medium in plant tissue culture.

Hadly and Harvais (1968) concluded that gibberellic acid increased the survival period of protocorms of seeds of *Dactylorchis purpurella* but was neutral to growth and size of protocorms.

Bhadra and Hossain (2003) studied *in vitro* germination and micropropagation of *Geodorum densiflorum* through seed culture in agar solidified MS and PM media supplemented with different concentrations of PGRS (NAA, IAA, BAP) and sucrose. They recorded maximum elongation of shootbuds on 0.8% (w/v) agar solidified MS medium supplemented 3% (w/v) sucrose, 2 mgl^{-1} BAP and 2 mgl^{-1} NAA. They also found highest number of shootbuds per segment in 0.8% (w/v) agar solidified MS medium supplemented 3% (w/v) sucrose plus BAP (3.0 mgl^{-1}).

Sinha and Roy (2004) developed protocol for successful regeneration of *Vanda teres (Roxb.)* using protocorm obtained from germinated seeds achieved from Vacin and Went medium supplemented with Auxin and cytokinin. The best shoot proliferation was found on same medium containing 10% coconut water.

Akter *et al*. (2007) conducted organogenesis of *Dendrobium* orchid using traditional media and organic extracts using *in vitro* multiple protocorm like bodies in Knudson C (Kn C), Vacin and Went (WC), half strength of Mourashige and Skooge (½MS) and New *Phalaenopsis* (NP) media supplemented with sabribanana pulp (Sb), Charcoal (C) and coconut water (CW). Among the media ½ MS showed the better performance on fresh weight, number of protocorm like bodies (PLBs), shoot and leaves of plantlets.

Zhao *et al*. (2008) induced callus from *Dendrobium candidum* Wall. ex Lindl. from longitudinal bisected segments of protocorms and sub cultured two times every 40 days on MS medium with micronutrients at half strength, micronutrients at full strength, 2% sucrose with 8.8 μm 6-Benzyl aminopurine they achieved PLBs when calluses was transferred onto same basal medium without any plant growth regulators.

Rahman *et al*. (2009) studied *in vitro* micropropagation of *Vanda tessellata* L. from shoot tip explants and found that MS medium supplemented with NAA (1.5mgl^{-1}) and BAP (1.0 mgl^{-1}) as best medium for promoting shoot formation and elongation. And MS medium supplemented with NAA (0.5 mgl^{-1}) and IBA (1.0 mgl^{-1}) was found to be best medium for root formation.

Kaur and Bhutani (2009) studied *in vitro* propagation of *Vanda testacea* in three different media *viz.* VW, MS and NAA supplemented various concentrations of BAP and NAA and concluded that VW medium supplemented with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA superior to the MS and KC medium with same

Pant and Pradhan (2010) conducted micropropagation of *Cymbidium elegans* in MS medium supplemented BAP and NAA with different combinations through immature seeds. They found MS supplemented BAP (1.0 mgl^{-1}) and NAA (0.5 mgl^{-1}) as the most suitable medium for multiplication of protocorm and healthy shoots.

Jawan *et al*. (2010) studied *in vitro* culture of Borneo's endemic orchid *Vanda dearei* and concluded that protocorm growth in half MS was superior to KC and VW along with maximum percentage of protocorm with leaf and mean number of leaf and roots produced.

Pant and Shrestha (2010) used shoot tip explants derived from *in vitro* grown seedlings of *Phaius tankervilleae* for proliferation of shoots in MS medium supplemented BAP and NAA of various combinations. They found MS medium supplemented BAP (1.0 mgl^{-1}) to be best medium for shooting and MS with NAA (0.5 mgl^{-1}) for rooting.

Islam *et al*. (2011) studied effect of potato extract (PE) on *in vitro* seed germination and seedling growth of local *Vanda roxburghii* orchid and concluded that PE at optimum concentration can be supplemented in mediums for high percentage of seed and seedling germinations. They also found production of protocorm to be affected significantly by PE.

Pant and Swar (2011) cultured *Cymbidium irridioides* and found that protocorms germinated rapidly from seeds in MS supplemented BAP (1 mgl^{-1}) and NAA (1 mgl^{-1}).

Poudel *et al*. (2012) studied *in vitro* seed culture of *Esmeralda clarkei* in MS medium with various combinations of BAP and NAA or alone. They found MS basal medium to be best for protocorm formation.

Bindiya *et al*. (2012) found that MS medium was best medium for seed germination and protocorm formation in comparison to Knudson C (1946), Vacin and Went (1949) and Raghavan and Torrey (1964) without using any growth hormone.

Bindiya *et al*. (2013) concluded that MS medium adjusted at pH 5.5 is the best for seed germination of *Vanda tessellata* orchid in comparison to the pH adjusted at 3.5, 4.5, 5.5 and 6.5.

Lee, Y., Hsu S. and Yeung, E.C. (2013) found that protocorm like bodies are important in orchid micropropagation and outwardly resemble somatic embryos in form and development.

Baker *et al*. (2014) studied micropropagation of *Orchis catasetum* using protocorms as explants cultured in MS medium fortified with different concentrations of N^6- Benzyl adenine, alpha-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) either individually or in combination of 0.5 mgl^{-1} BA and 0.5 mgl^{-1} NAA as most suitable medium for maximum PLBs regeneration (20.40 per plantlet).

Habiba *et al*. (2014) studied effect of different types of Cytokinin Kinetin (Kn), 6-benzylaminopurine (BA) and 2-isopentyladenine (2ip) with variable concentration of 0, 0.1, 1 and 10mg/L in modified Mourashige and Skooge (MS) medium on *in vitro* organogenesis of *Epidendrum* 'Rouge Star No. 8'. They found that highest number of protocorm like bodies (PLBs) in 0.1 mg/L of Kinetin but 100% PLBs induction was formed in 0.1 mg/L of BA.

Habiba *et al*. (2014) used different concentrations of 0, 0.1, 1 and 10 mg/L of plant growth regulators BA and Hyaluronic acid (HA9) on organogenesis of PLBs in *Dendrobium kingianum* under white light emitting diodes (LEDS) and found that 1mg/L of BA to be favourable condition for increase in average number of PLBs, shoot and fresh weight. They found lower concentration of BA to be potential PGR and HA9 act as a PGR like BA for organogenesis in PLBs of *D. kingianum*.

Bhattacharjee and Islam (2014) studied development of an efficient protocol for *in vitro* germination and enhancing protocorm like bodies (PLBs) development in three indigenous orchid species *viz. Acampe premorsa, Agrostophylum khasianum* and *Phalaenopsis kornorreris* and found that half MS supplemented 1.5% (w/v) sucrose to be most efficient medium for plantlets and root formation.

Bhattacharjee and Islam (2014) studied effects of PGRS on multiple shoot induction in *Vanda tessellata* in four culture media *viz.* PM, MS, ½ MS and B_5. Out of 20 different combinations of PGRS tested for multiple shoot induction, MS medium fortified 1 mgl^{-1} NAA and 1.0 mgl^{-1} BAP was proved to be best medium formulation for multiple shoot development and elongation.

Sebastinraj *et al*. (2014) studied rapid propagation of *Vanda testacea* in half strength MS, KC and VW medium and concluded the MS medium as most suitable medium for seed germination than KC and VW media.

Shekarriz *et al*. (2014) studied the effect of CW and peptone on seed germination and PLBs formation on hybrid orchid *Phalaenopsis* using five media *viz.* modified FAST, modified KC, modified MS, modified half-strength MS and VW. The best medium for PLBs formation was modified half-strength MS medium containing CW and peptone.

Dwiyani *et al*. (2015) studied *in vitro* germination and its subsequent growth of an orchid *Vanda tessellata* in NP medium supplemented CW and tomato juice. They concluded that NP medium supplemented tomato juice (100 gl^{-1} and 200 gl^{-1}) showed better growth result than NP medium supplemented CW.

Hrahsel and Thangjam (2015) studied asymbiotic *in vitro* seed germination and regeration of *Vanda coerulea* and found full strength of MS basal medium superior over half strength of MS medium with high rate of seed germination and protocorm like bodies (PLBs) formation.

David D *et al*. (2015) studied *in vitro* growth of native orchid *Vanda helvola* Blume adding organic additives such as tomato juice, coconut water, peptone and yeast extracts taking three basal media such as MS, KC and VW. KC was found to be best medium for seed germination. They concluded that these organic additives significantly enhanced the development of seedlings from protocorms obtained from seed germination in KC medium.

Sachin (2015) studied impact of temperature and pH variation on *in vitro* protocorm formation of *Vanda tessellata* in MS without various combinations of growth hormones. Among culture maintained at different temperature (15^0C, 20^0C, 25^0C, 30^0C) and pH (4.5, 5.5, 6.5, 7.5), Sachin found highest protocorm formation at temperature 20^0C with pH 5.5 than other culture conditions.

CHAPTER III

MATERIALS AND METHOD

3.1 Plant materials

The plant materials used were protocorm of *Vanda tessellata* (Roxb.) Hook. ex G. Don which were maintained on *in vitro* biotechnology lab of CDB, T.U. Kirtipur at room temperature 25 ± 2^0C (at an altitude of 1300m).

3.2 Methodology

3.2.1 Preparation of stock solutions

The solidified medium with different strengths (full-, half-, quarter strengths) of MS medium (1962) and with or without hormones (BAP and NAA) were used for the multiplication of protocorm. Quarter strength of MS medium with different hormone concentration was used for shooting and rooting of the germinated plants. For the preparation of nutrient mediums, first their stock solutions with higher concentration were made and stored. From these stock solutions, the required volume was diluted to make the required concentration during the time of media preparation.

3.2.1.1 Preparation of stock solutions for MS medium

The MS medium (1962) consists of macronutrients (stock A), micronutrients (stock B), iron source (stock C), vitamins (stock D), sucrose and agar (as gelling agent). The composition of the MS medium is as follows:

Table 1: Composition of Murashige and Skoog (1962) medium

Stock A (macronutrients)

Components	Composition of MS (final conc.) mg/l	(10X) mg/100ml stock concentration	Volume to be taken for liter
Potassium nitrate (KNO_3)	1900	19.00	100ml

Ammonium Nitrate(NH_4NO_3)	1650	16.5	100ml
Calcium Chloride ($CaCl_2.2H_2O$)	440	4.4	100ml
Magnisium Sulphate ($MgSO_4.7H_2O$)	370	3.7	100ml
Potassium dihydrogen phosphate (KH_2PO_4)	170	1.7	100ml

Stock B (micro-nutrients)

Components	Composition of MS (final conc.) mg/l	(100X) mg/100ml stock concentration	Volume to be taken for liter
Boric acid (H_3BO_4)	6.2	620	1ml
Manganese sulphate (MnSO4.4H2O)	22.3	2230	1ml
Zinc sulphate (ZnSO4.7H2O)	8.6	860	1ml
Sodium molybdate (Na2MoO4.2H2O)	0.25	25	1ml
Cobalt chloride (CoCl2.6H2O)	0.025	2.5	1ml
Copper sulphate (CuSO4.5H2O)	0.025	2.5	1ml
Potassium iodide *(KI)	0.83	83	1ml

*100 ml stock solution for KI was made separately and used as 1ml/l in MS medium

Stock C (Iron source)

Components	Composition of MS (final conc.) mg/l	(10X) mg/100ml stock concentration	Volume to be taken for liter
Sodium ethylene diamine tetra acetate (Na_2.EDTA)	37.3	373	10ml
Ferrous sulphate ($FeSO_4.7H_2O$)	27.8	278	10ml

Stock D (Vitamins)

Components	Composition of MS (final conc.) mg/l	(100X) mg/100ml stock concentration	Volume to be taken for liter
Glycine	2.0	200	1ml
Nicotinic acid	0.5	50	1ml
Pyridoxine HCl	0.5	50	1ml
Thiamine HCl	0.1	10	1ml
Myo-inositol*	100	10000	1ml

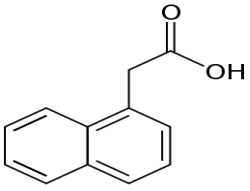
*Myo-inositol was made freshly and used at the time of media preparation (100mg/l)

Carbon source
Sucrose $30gL^{-1}$

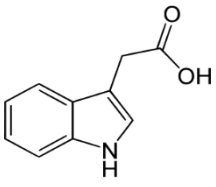
Gelling Agent
Agar $8gL^{-1}$

The above mentioned chemicals were weighed accurately using the digital balance and dissolved completely one by one in sterile distilled water with the help of magnetic stirrer. For MS medium, the final volume for stock solution for stock A was raised to 1 liter and 100ml each for stock 'B','C' and 'D' adding sterile water. The stock solution for KI was made separately because it causes precipitation for stock solution B. After preparation of all the stock solutions they were kept in the dark bottles and preserved in refrigerator due to the light sensitivity of the chemicals.

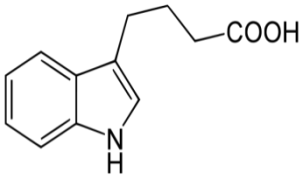
3.2.2 Hormones used for the experiments



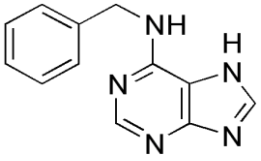
α-Napthaleneacetic acid



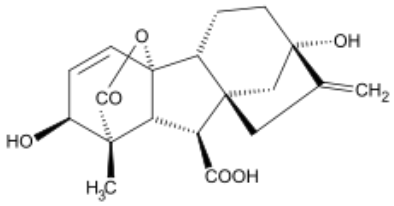
Indole-3-acetic acid



Indole-3-butyric acid



6-Benzylaminopurine



Gibberlic Acid

3.2.3 Preparation of Hormones stock solution

For the preparation of stock solutions of Auxin- Naphthalene acetic acid (NAA), 10 mg was weighed accurately and dissolved completely in 2.5 ml of 1N NaOH in a beaker and 70 ml of sterile water was added. The pH solution was maintained 5.8 using 0.1N NaOH and 0.1N HCl. Final volume was made up to 100 ml by adding sterile distilled water.

For the Auxin- Indole-3-acetic acid (IAA) and Indole-3-butyric acid (IBA), 10 mg of them were weighed accurately and dissolved in 2.5 ml of 95% ethyl alcohol in separate beakers and 70 ml of sterile distilled water was added and the pH was maintained 5.8 using 0.1N NaOH and 0.1N HCl. The final volume was made up to 100 ml by adding sterile distilled water.

For the preparation of cytokinin i.e. 6-Benzylaminopurine (BAP), 10 mg of it was weighed accurately and dissolved in 2.5 ml of 0.5N NaOH with gentle heating in a beaker. Then 70 ml of sterile distilled water was added and the pH was maintained 5.8 using 0.1N NaOH and 0.1N HCl. The final volume was mesh up to 100 ml by adding sterile distilled water.

For Gibberlic acid (GA_3), 10 mg of it was weighed accurately and dissolved in few drops of 95% ethyl alcohol and the final volume was mesh up to 100 ml by adding sterile distilled water. All the stock solutions of plant hormones were of 100 ppm. All the stock solutions of plant hormones were then kept in clean sterile dark brown bottles due to light sensitivity and preserved in the refrigerator at 4°C.

3.2.4 Preparation of nutrient medium

For the preparation of MS nutrient medium, required amount of each stock solutions- macronutrients, 100ml/l; micronutrients, 1ml/l; iron source, 10ml/l and vitamins, 1ml/l were poured one by one in one-liter sterile conical flask. 1 ml/l of KI was also added and mixed homogenously using magnetic stirrer. Then, 100 mg/l of Myo-inositol was added to the

solution. Sucrose (3% and 6%) was added to the mixture for carbon sources and dissolved. Then, 700 ml of sterile distilled water was added and the pH of the medium was adjusted to 5.8 with 0.1N NaOH and 0.1N HCl. The final volume of medium was made up to 1000 ml by adding required amount of distilled water. Further addition of required amount of sterile distilled water was done for making the half strength and $1/4^{th}$ strength. The required volume of medium was then poured in different conical flasks to make 100 ml of the required concentration and different hormones in combination or in single are added according to the need. For the solidification of the medium, 0.8% agar was added and boiled on heater till the agar got completely dissolved.

About 15 to 17 ml of prepared medium supplemented with or without hormones was poured in each clean, autoclaved culture tubes and these culture tubes were capped by aluminium foil and labeled with permanent marker. Six replicates were made for each treatment. All culture tubes were noted with the glass marker with the concentration of the medium used in each. The culture tubes containing medium were sterilized by autoclaving at pressure of 15 lb /in^2 (psi) and temperature of 121^0C for 20 minutes. After cooling, the culture tubes were kept in culture room in slanting position (25±2°C).

For 5% and 10% coconut water supplemented medium, the required volume of coconut water was added to the MS medium. For 5% CW supplemented MS medium, 50 ml/l and for 10% CW supplemented ¼ MS medium 100 ml/l coconut water was added as needed.

[Note: - for the hormone supplemented media the hormone stock solutions were added using the volumetric analytical formula $S_1V_1=S_2V_2$, where

S_1= Initial strength of hormone stock (mg/l)

V_1= Initial volume of hormone stock to be taken

S_2= Required strength of the media (mg/l)

V_2= Final volume of media to be made**]**

3.2.5 Method of sterilization

3.2.5.1 Sterilization of glasswares and metallic instruments (dry heat or wet heat sterilization)

During the experiment all the necessary equipments like glasswares and metallic instruments were sterilized using the dry heat or wet heat sterilization technique before their use. Glasswares such as beakers, conical flasks, measuring cylinder, glass rods, petridish, pipettes, culture tubes, etc and metallic instruments like forceps, scalpels, surgical blade handles, needles etc were dipped in detergent water for about 24 hours and washed first thoroughly in tap water and finally with distilled water. Those cleaned glasswares were sterilized by moist heat in an autoclave at 15psi and 121°C for 20 minutes. Cotton, filter papers and the metallic instruments

like forceps, spatula and surgical blade handle were wrapped with aluminum foil before keeping inside the hot air oven for sterilization at 180°C for 2-3 hours.

3.2.5.2 Sterilization of inoculation chamber (with 70% ethyl alcohol)

Before inoculation of explants, the laminar air flow chamber was sterilized by cleaning with the help of cotton soaked with 70% ethyl alcohol. All the requirements for inoculation i.e. 70% ethyl alcohol, sterile water, petriplates, beaker, forceps, spatula, surgical blade with its handle, culture tubes with media (except the plant material) were exposed under ultraviolet (UV) radiation for 45 minutes inside the laminar air flow cabinet for removing the possible contaminants present in the working area. Then UV light was turned off and air blower switched on. After 20 minutes, the laminar air flow chamber was ready for inoculation.

3.2.5.3 Sterilization of media and distilled water

The prepared media and distilled water were sterilized by moist heat in an autoclave at 15psi and 121°C for 20 minutes.

3.2.6 Inoculation of protocorm for germination

Protocorm and shoot buds like bodies obtained from *in vitro* culture were sub cultured in the full half and quarter strength of the MS medium supplemented with or without hormones with the help of forceps inside the Laminar Air Flow cabinet around the burning Bunsen burner. The inoculated culture tubes were then tightly sealed with the help of aluminum foil and rubber band and labeled. The culture tubes were transferred to the culture room and exposed to artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25 ± 2^0C.

3.2.7 Inoculation of protocorms for shooting

Protocorms and PLBs obtained from the *in vitro* multiplication of protocorms were sub cultured in quarter strength of MS medium in different concentration of NAA and BAP alone or in combination, different concentration of GA_3, coconut water and BAP plus 6% sucrose. The culture tubes were transferred to the culture room and exposed to artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25 ± 2^0C.

3.2.8 Rooting of shoots

In vitro grown shoots of *Vanda tessellata* (Roxb.) Hook.ex G. Don of about 0.8 cm in length were transferred to quarter strength of MS media containing different concentrations of various rooting phytohormones like NAA, IAA and IBA for development of roots. The culture tubes were transferred to the culture room and exposed to artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25 ± 2^0C.

3.2.9 Data recording

After the culture tubes were transferred to the culture room after inoculation, they were regularly observed for initiation of multiplication of protocorm, first shoot, and leaf and root formation. The data was taken at regular intervals of one week. The data for multiplication protocorm formation, first shoot formation, first leaf formation and first root formation was recorded and the data for shoot proliferation was taken for 32 weeks of protocorm inoculation. The shoot number, shoot lengths, leaf number, leaf length, root number and root length of plantlets were recorded up to 32^{th} week of subculture.

3.2.10 Statistical Analysis

Average value of week was taken for germination. Statistical analysis was done using SPSS 16.0. Normality test (Shapiro-Wilk test as $n < 100$) was done of the data of shooting and rooting in different concentration and combinations of hormones in half strength MS medium. Multivariate analysis of variance was done for shooting and rooting data. Data of shoot multiplication was log transformed for the analysis.

CHAPTER IV

RESULTS

In vitro multiplied protocorms of *Vanda tessellata* Roxb.ex G.Don were inoculated on different strength of MS medium (MS, ½ MS and ¼ MS) without hormone. The protocorms showed positive response in all tested medium. Later the protocorms were transferred again for shooting along with various parameters in quarter strength of MS medium supplemented various concentrations of NAA and BAP alone or in combinations, coconut water, GA_3 and BAP plus 6% sucrose. The *in vitro* grown plantlets were then transferred to quarter strength of MS supplemented with various concentrations of rooting hormones.

Table 2: Effect of different MS medium on *in vitro* growth of protocorm of *Vanda tessellata* Roxb. (Observation taken in weeks)

Nutrient Medium	PLBs formation	PLBs multiplication	Shoot initiation	Leaf initiation	Shoot proliferation
MS free	3	5	5	8	13
1/2 MS	4	5	6	9	14
1/4 MS	3	5	5	8	11

Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25 ± 2^0C.

4.1 **Shoot Multiplication**

Quarter strength of different concentration of BAP, NAA, GA_3 and Coconut water were used for the further experiment for growth and multiplication of shoots because quarter strength of MS medium showed better growth result than the full and half strength of MS medium. *In vitro* germinated shoot buds of length 2-4 mm were used as explants. These micro shoots were cultured in quarter strength of MS media supplemented with different concentration(0.5, 1.0, 1.5 and 2.0 mg/l) of GA_3, ¼ MS supplemented with different concentration of coconut water(5% and 10%), ¼ MS supplemented with different concentration of NAA(0.5, 1.0, 1.5 and 2.0mg/l), ¼ MS supplemented with different concentration of BAP(0.5, 1.0, 1.5 and 2.0mg/l), ¼ MS supplemented with different concentration of BAP(0.5, 1.0 and 1.5mg/l) plus 6% sucrose and ¼ MS supplemented with different concentration of NAA(0.5, 1.0, 1.5 and 2.0mg/l) plus 0.5 BAP. The shoot buds showed varied response to the above mentioned types of media in the number of shoot buds, number of shoots, length of shoots, number of leaver and length of leaves. The first shoot was developed in 0.5 BAP supplemented medium along with small leaf primordial. The protocorm like bodies and shoot buds were observed to be developed in early 4 weeks in all mediums.

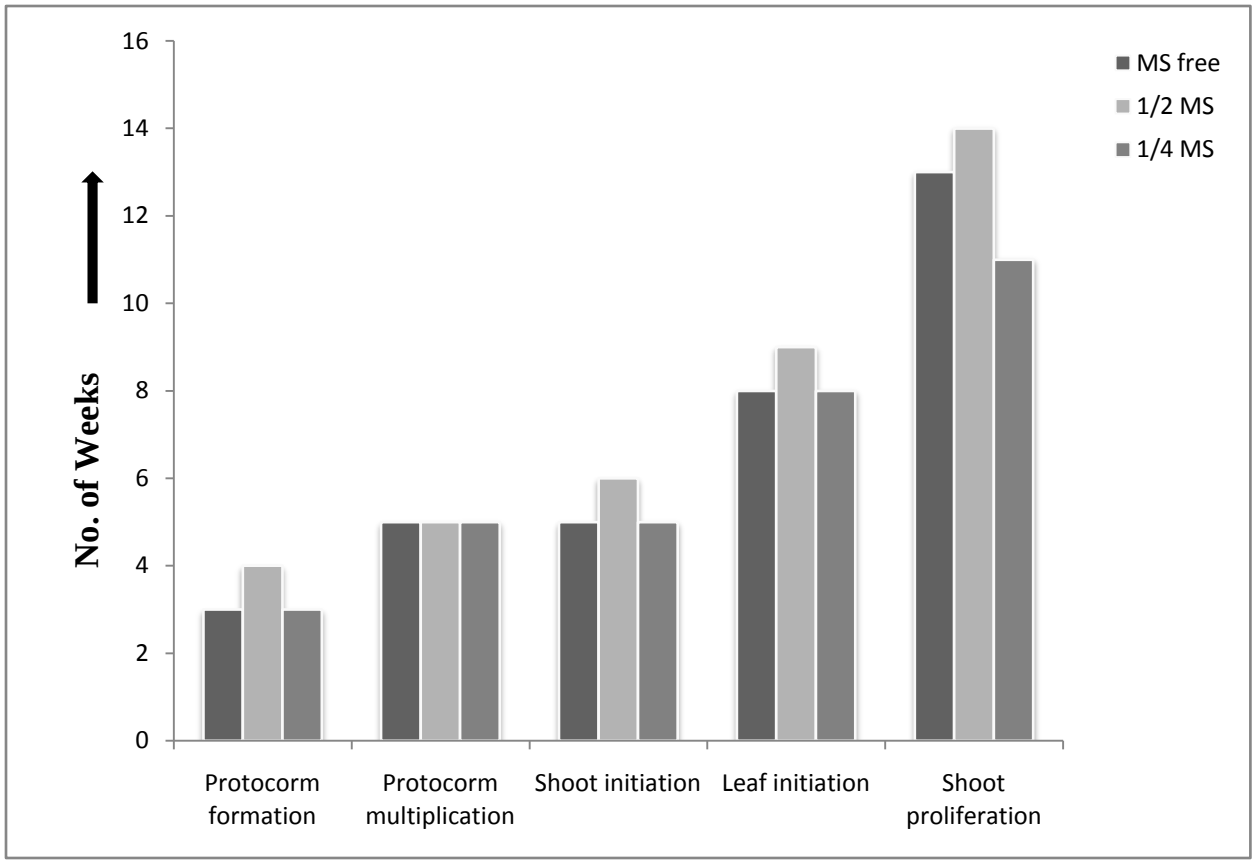


Figure 2: Effect of different strength of MS media on initiation of protocorm formation and multiplication, shoot initiation, leaf initiation and shoot proliferation observed in weeks. (Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25±2^{0}C)

4.1.1 Shoot multiplication in different concentration of GA_3

In the various concentration of GA_3 (0.5, 1.0, 1.5 and 2.0mg/l) supplemented ¼ MS media were used for shoot multiplication. All the tested media gave the positive result in different parameters observed (shoot bud number, shoot number, shoot length, leaf number and leaf length). Shoot multiplication was observed after 4 weeks of culture. GA_3 (1.5mg/l) supplemented medium showed highest number of shoots (more than 12 shoots per culture) (FIG 11 'F') but the length of shoot was found to be highest in GA_3 (0.5) supplemented medium with 1.78cm (FIG 11 'D'). The numbers of leaves were found increased with increased number of shoots. The root formation was absence even after 32 weeks of primary culture.

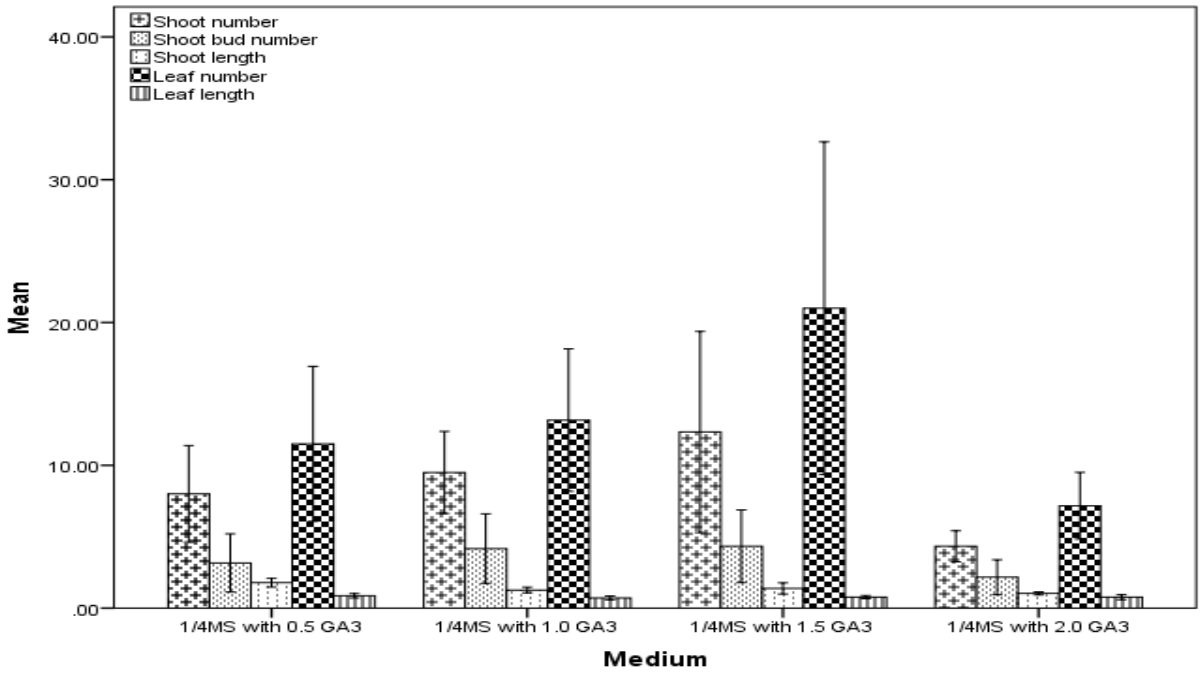


Figure 3: Average number of shoot, shoot bud number, shoot length, leaf number and leaf length produced per responsive protocorm culture in ¼ MS medium supplemented GA_3 in different concentrations. (Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25±2^{0}C)

4.1.2 Shoot multiplication in different concentration of coconut water

In the ¼ MS medium supplemented with different concentrations (5% and 10% of coconut water) there was positive response in different parameters of growth recorded(shoot bud number, shoot number, shoot length, leaf number and leaf length) was observed. The number of shoot multiplication was found higher in 10% coconut water supplemented ¼ MS than 5% coconut water supplemented ¼ MS medium. The numbers of leaves were found higher with increased number of shoots.

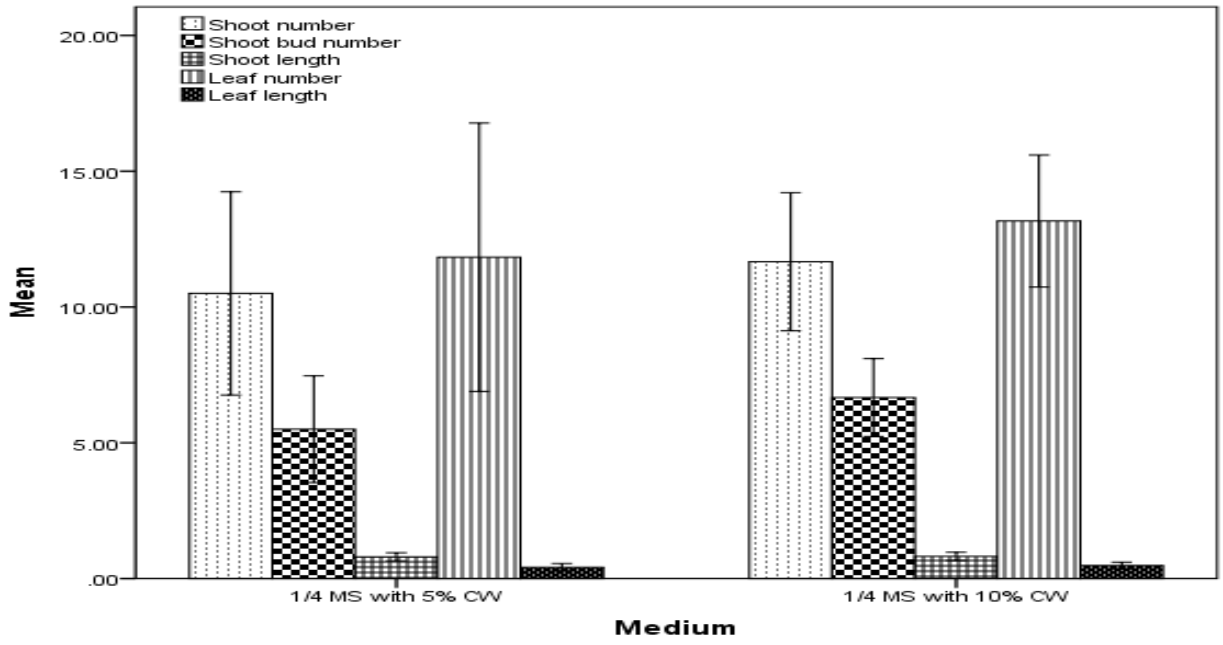


Figure 4: Average number of shoot, shoot bud number, shoot length, leaf number and leaf length produced per responsive protocorm culture in ¼ MS medium supplemented 5% and 10% coconut water. (Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25±2^{0}C)

4.1.3 Shoot multiplication in different concentration of BAP+6% Sucrose

In the various concentration of BAP (0.5, 1.0, 1.5 and 2.0mg/l) plus 6% sucrose supplemented ¼ MS medium, positive response in the different parameters of growth recorded (shoot bud number, shoot number, shoot length, leaf number and leaf length) was observed. Shoot multiplication was observed in the 4th week of primary culture. BAP (1.5mg/l) plus 6% sucrose supplemented 1/4MS medium developed more shoots (more than 13 shoots per culture) but the highest shoot length was developed in the 0.5mg/l plus 6% sucrose supplemented ¼ MS medium used. The number of leaves and leaf length were found increased with increased number of shoots.

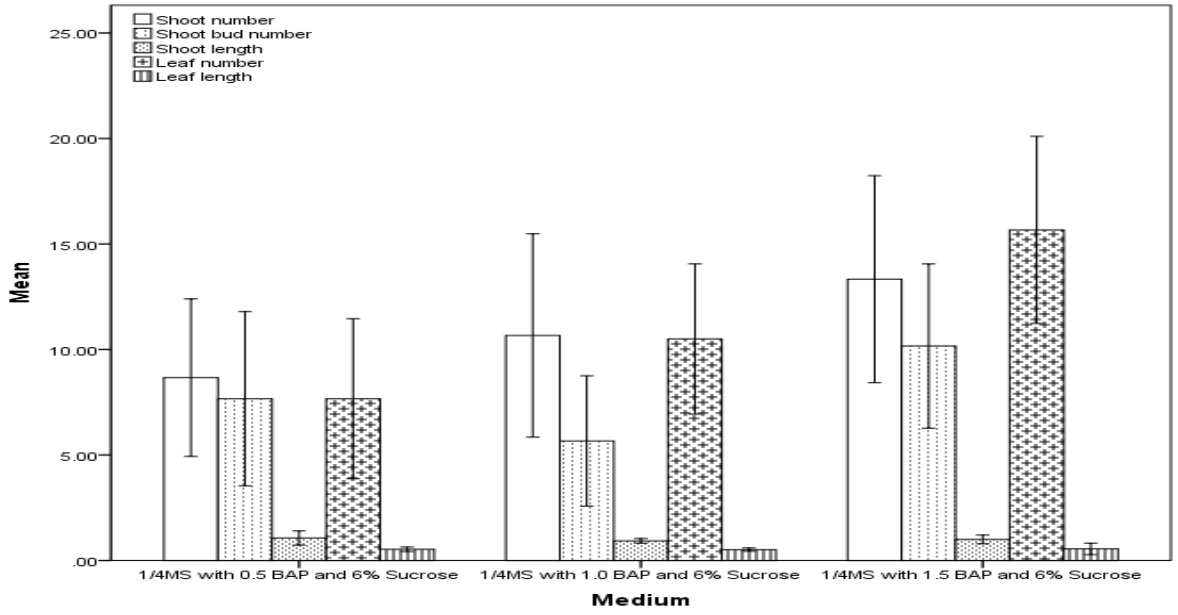


Figure 5: Average number of shoot, shoot bud number, shoot length, leaf number and leaf length produced per responsive protocorm culture in ¼ MS medium supplemented BAP plus 6% sucrose in different combinations. (Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25±2^{0}C)

4.1.4 Shoot multiplication in different concentration of NAA

In NAA supplemented medium shoot multiplication was observed in 5th week of culture. Various concentration of NAA (0.5, 1.0, 1.5 and 2.0mg/l) supplemented with ¼ MS medium, positive response in the different parameters (shoot bud number, shoot number, shoot length, leaf number and leaf length) were recorded. 0.1mg/l NAA supplemented 1/4MS medium developed more number of shoots (more than 4 shoots per culture) than other medium used but the length of shoot was found highest in 2.0mg/l (1.15cm) supplemented ¼ MS medium. More leaf numbers were developed also in 1.0mg/l NAA supplemented ¼ MS medium than other medium used

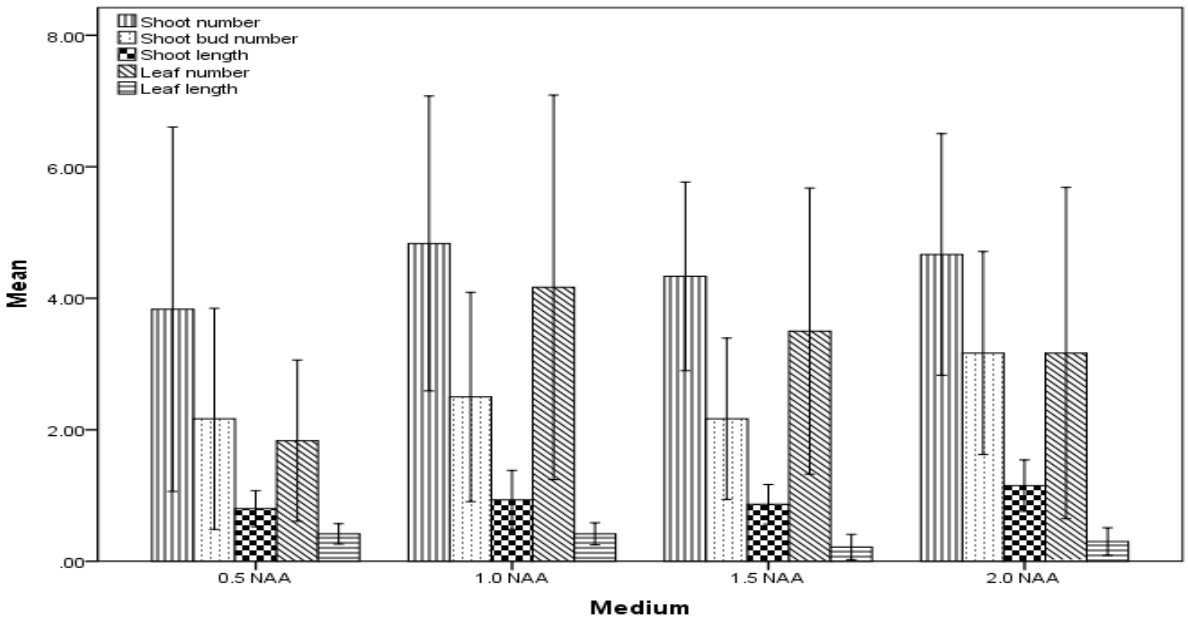


Figure 6: Average number of shoot, shoot bud number, shoot length, leaf number and leaf length produced per responsive protocorm culture in ¼ MS medium supplemented NAA with different concentrations. (Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25±2^{0}C)

4.1.5 Shoot multiplication in different concentration of BAP

In various concentration of BAP (0.5, 1.0, 1.5 and 2.0mg/l) supplemented ¼ MS medium there was positive response in the different parameters (shoot bud number, shoot number, shoot length, leaf number and leaf length) recorded. Shoot multiplication was observed in 5th week of primary culture. 0.5mg/l BAP supplemented MS medium developed more shoots but the length of shoot was found to be highest in 2.0mg/l supplemented ¼ MS. The numbers of shoot buds were found similarly developed in 0.5mg/l BAP and 1.0mg/l BAP supplemented ¼ MS medium. Leaf number were developed more with increased number of shoots. Leaf length was found highest in 2.0mg/l BAP (0.51cm per culture) supplemented ¼ MS medium.

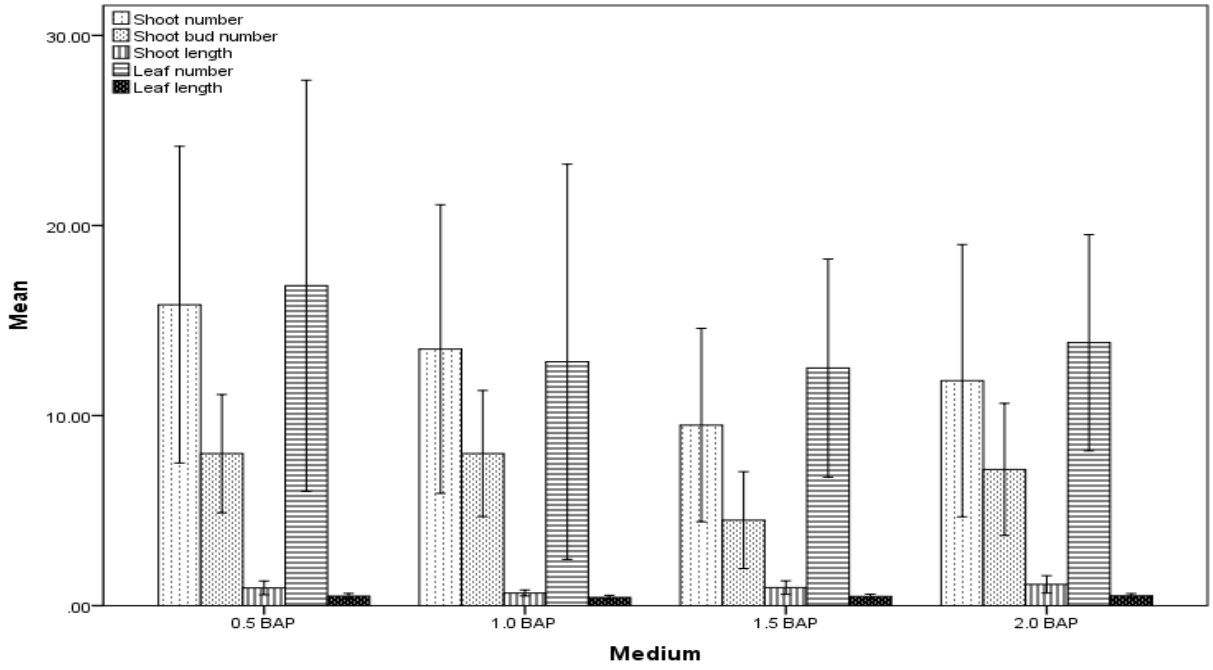


Figure 7: Average number of shoot, shoot bud number, shoot length, leaf number and leaf length produced per responsive protocorm culture in ¼ MS medium supplemented BAP with different concentrations. (Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25±2^{0}C)

4.1.6 Shoot multiplication in different concentration of NAA plus 0.5 BAP

In ¼ MS supplemented various concentration of NAA (0.5, 1.0, 1.5 and 2.0mg/l) plus 0.5mg/l BAP there was positive response in the different parameters recorded. Shoot multiplication was found in 5th week of primary culture. 1.0mg/l NAA plus 0.5mg/l and 2.0mg/l NAA plus 0.5mg/l BAP supplemented ¼ MS medium developed more number of shoots along with highest shoot length (1.05cm) than other medium used. Shoot bud and leaf numbers recorded increased with the increased number of shoots.

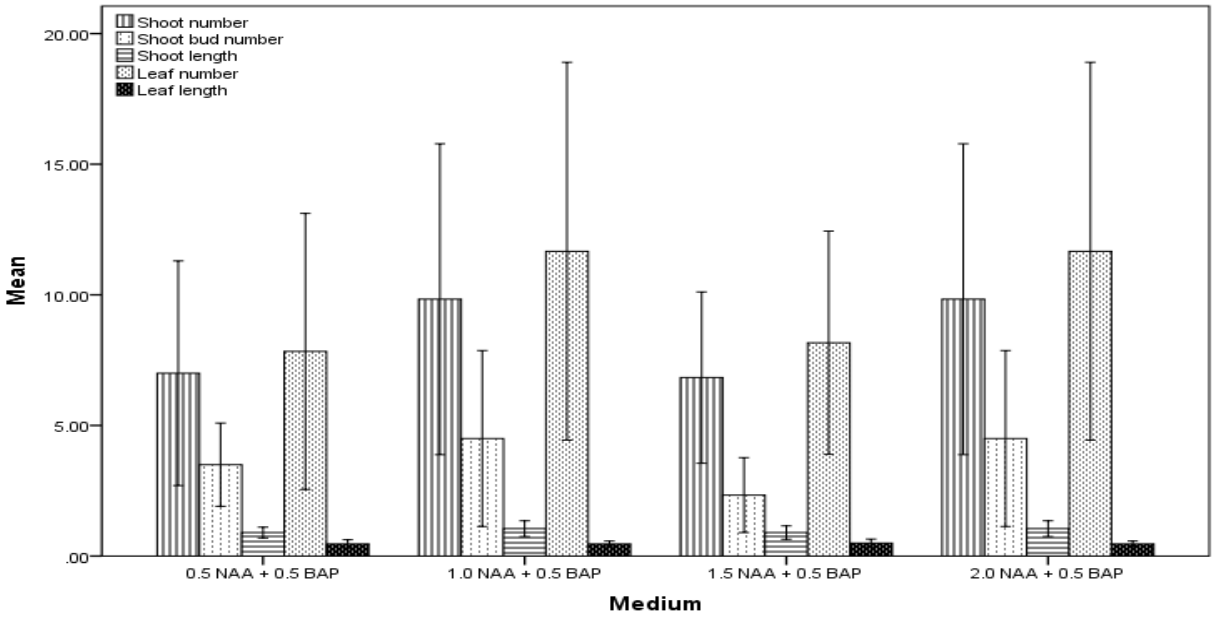


Figure 8: Average number of shoot, shoot bud number, shoot length, leaf number and leaf length produced per responsive protocorm culture in ¼ MS medium supplemented NAA and BAP with different combinations. (Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25±2^{0}C)

4.2 Rooting of Shoots

The shoots of about 1cm in length were used as explants for rooting. Rooting was done in ¼ MS supplemented with different concentrations (0.25, 0.5 and 1.0mg/l) of Auxin (NAA, IAA and IBA). Single shoot was used for inoculation in the rooting medium. The first root was developed in 1.0 mgl^{-1} NAA supplemented medium. In IBA and IAA supplemented medium some callus were seen after 4 weeks of shoot inoculation.

4.2.1 Root formation in IAA

On ¼ MS supplemented various concentrations of IAA 0.25, 0.5 and 1.0mg/l) shoot multiplication was observed in the 6^{th} week of culture. Highest number of shoot formation was observed in 1.0mg/l IAA with more than 8 shoot per culture along with shoot length 1.02cm. Root formation was observed after 8 weeks of primary culture. Highest number of root was developed in 0.25mg/l IAA supplemented ¼ MS medium with highest root length than other medium used. ¼ MS with 1.0mg/l IAA supplemented medium developed more number of leaves (14 per culture) with highest leaf length (0.62cm per culture).

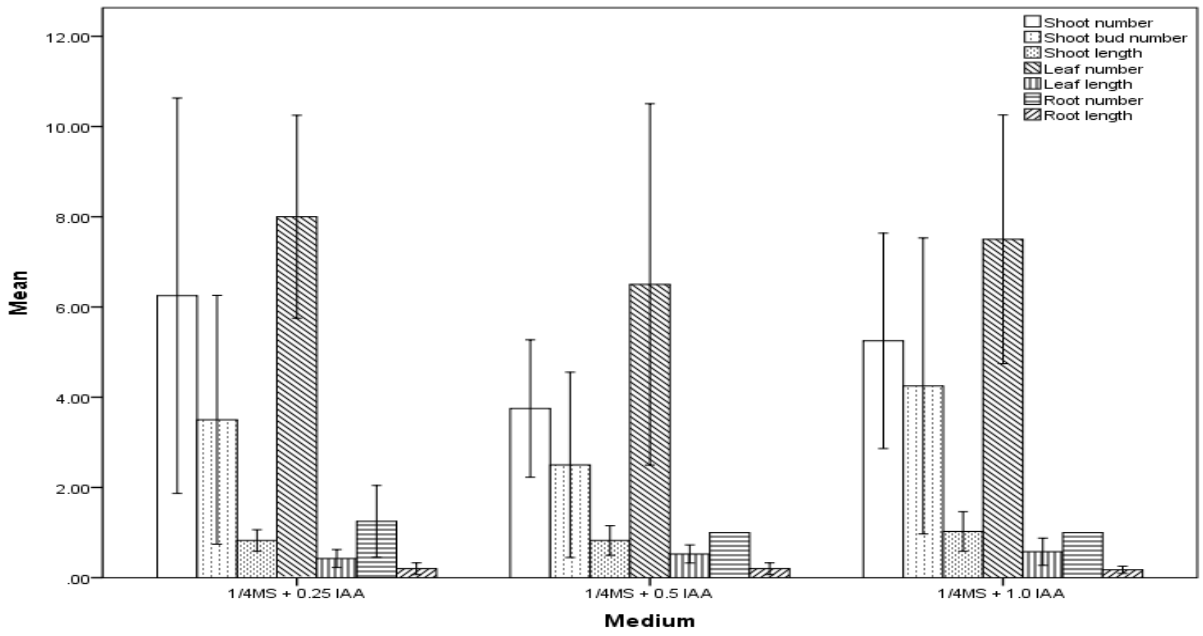


Figure 9: Average number of shoot, shoot bud number, shoot length, leaf number, leaf length, root number and root length produced per responsive protocorm culture in ¼ MS medium supplemented IAA with different concentrations. (Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25 ± 2^0C)

4.2.2 Root formation in IBA

On ¼ MS supplemented various concentration of IBA (0.25, 0.5 and 1.0mg/l) shoot multiplication was seen in 6th week of primary culture. 0.5mg/l IBA supplemented medium developed more shoot than other medium used but number of shoot buds was recorded in 1.0mg/l IBA supplemented ¼ MS medium. The length of shoot along with root length was developed highest in 0.25mg/l supplemented ¼ MS medium than other medium used. ¼ MS supplemented with 1.0mg/l IBA developed more roots (0.75 per culture) and highest leaf length (0.55 per culture). The number of leaf was found highest in 0.5mg/l IBA supplemented ¼ MS medium than other.

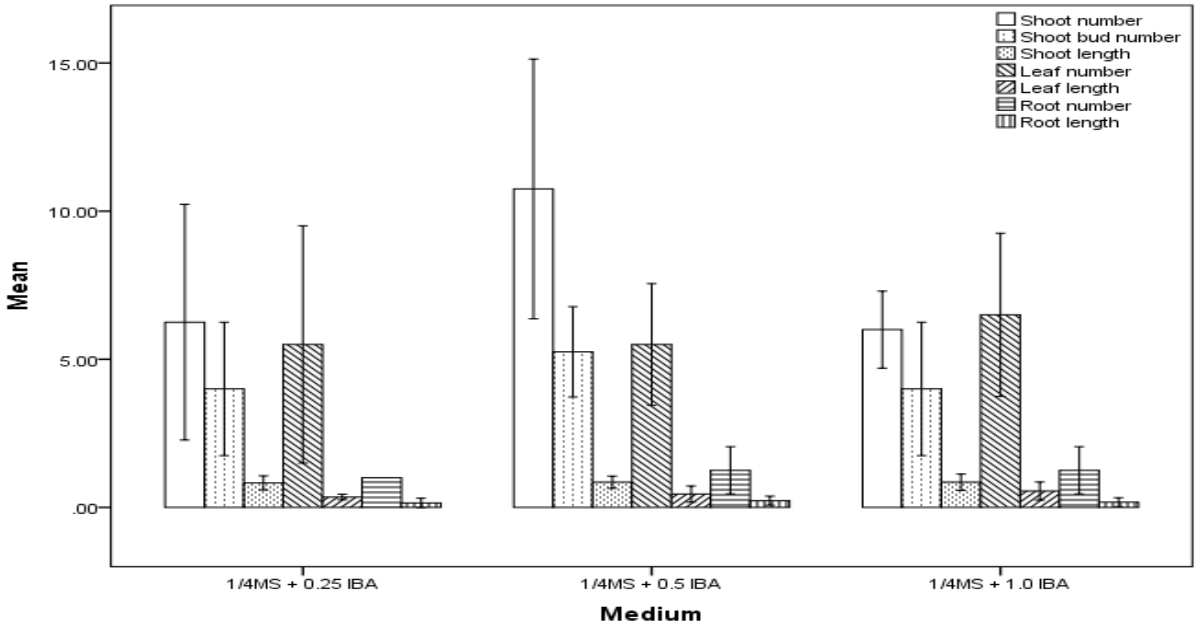


Figure 10: Average number of shoot, shoot bud number, shoot length, leaf number, leaf length, root number and root length produced per responsive protocorm culture in ¼ MS medium supplemented IBA with different concentrations. (Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25±2^{0}C)

4.2.3 Root formation in NAA

On ¼ MS supplemented with various concentration of NAA (0.25, 0.5 and 1.0mg/l) there was positive response in all parameters observed. Shoot multiplication was observed in 6th week of primary culture. Maximum numbers of shoots (more than 11 shoots per culture) were developed in 1/4MS supplemented with 0.5mg/l NAA. The length of shoot was also highest in the same medium along with leaf number and leaf length than other medium used. The root number was developed equally in 0.5mg/l and 1.0mg/l NAA supplemented ¼ MS with same length of root.

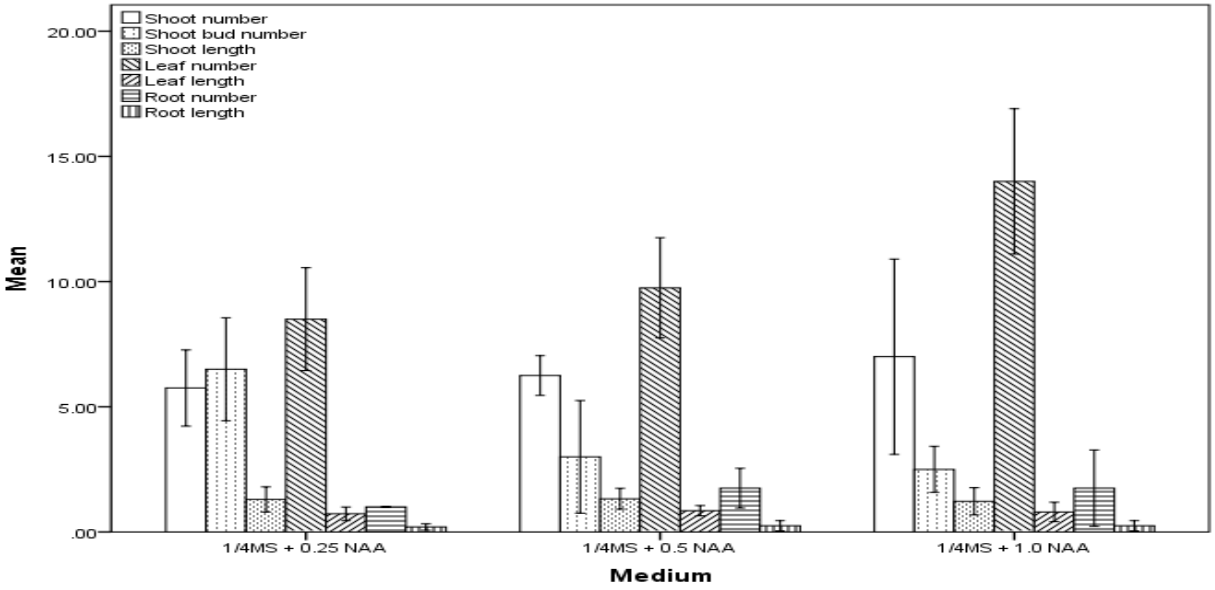
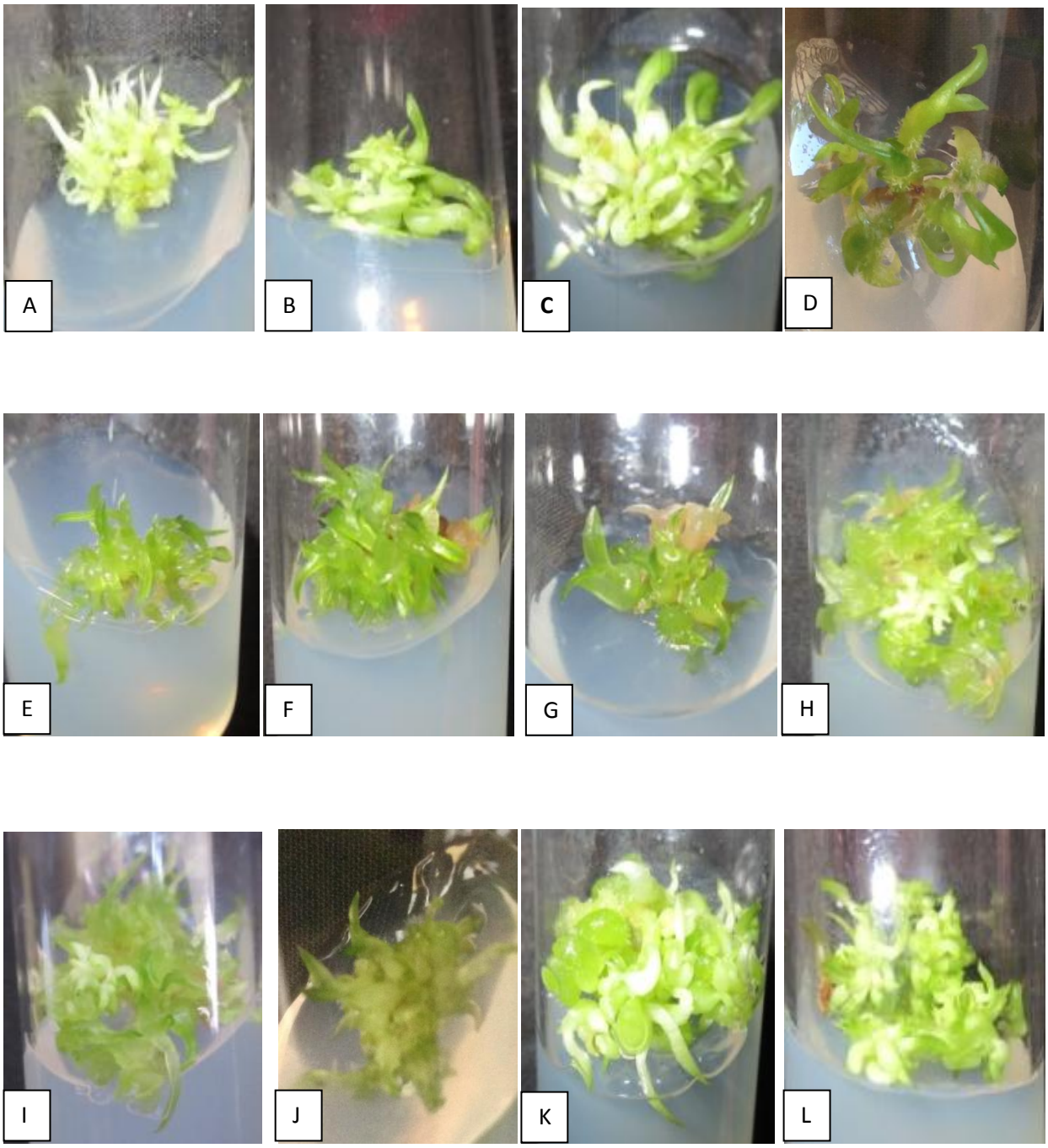
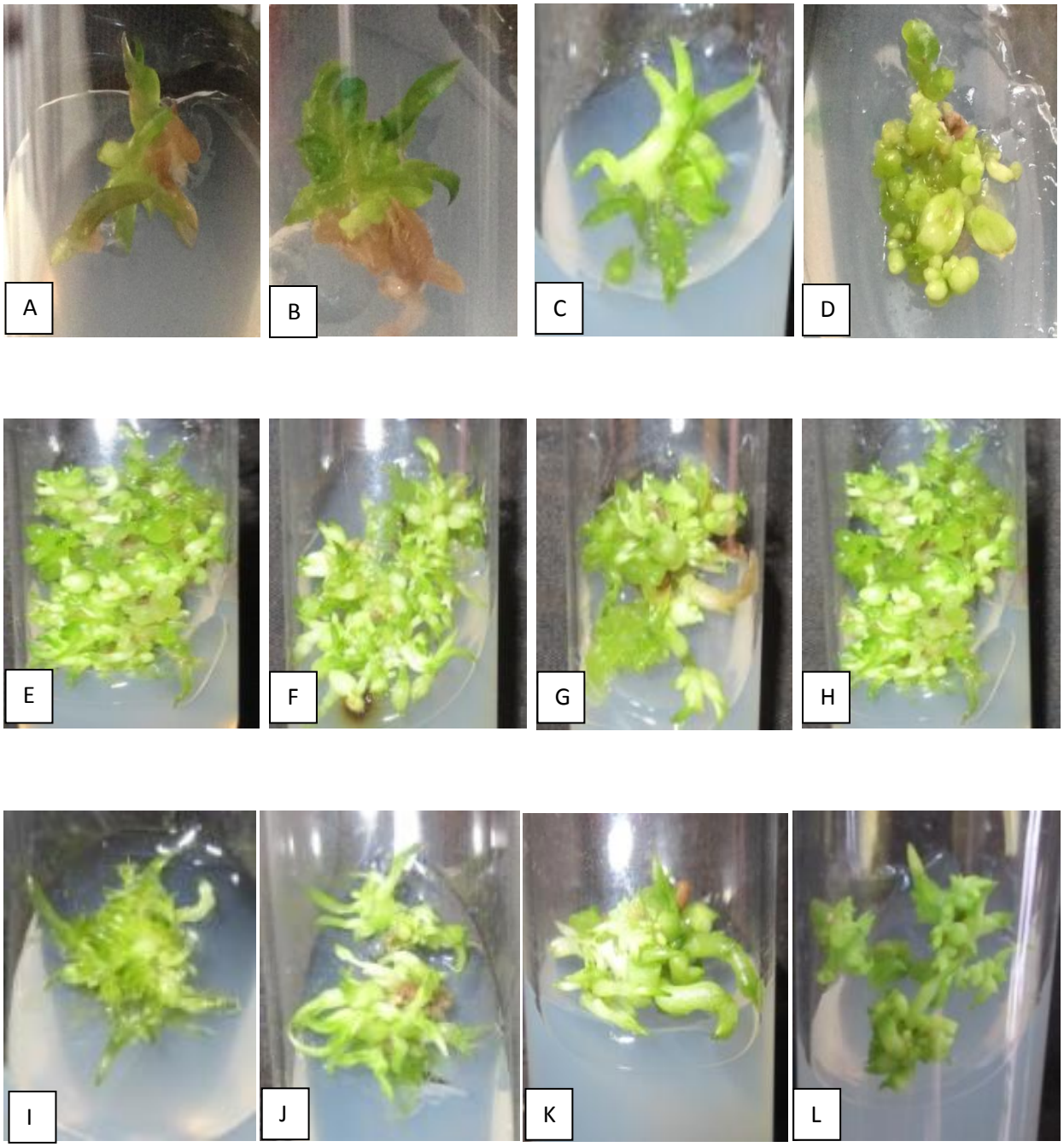


Figure 11: Average number of shoot, shoot bud number, shoot length, leaf number, leaf length, root number and root length produced per responsive protocorm culture in ¼ MS medium supplemented NAA with different concentrations. (Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25±2^{0}C)



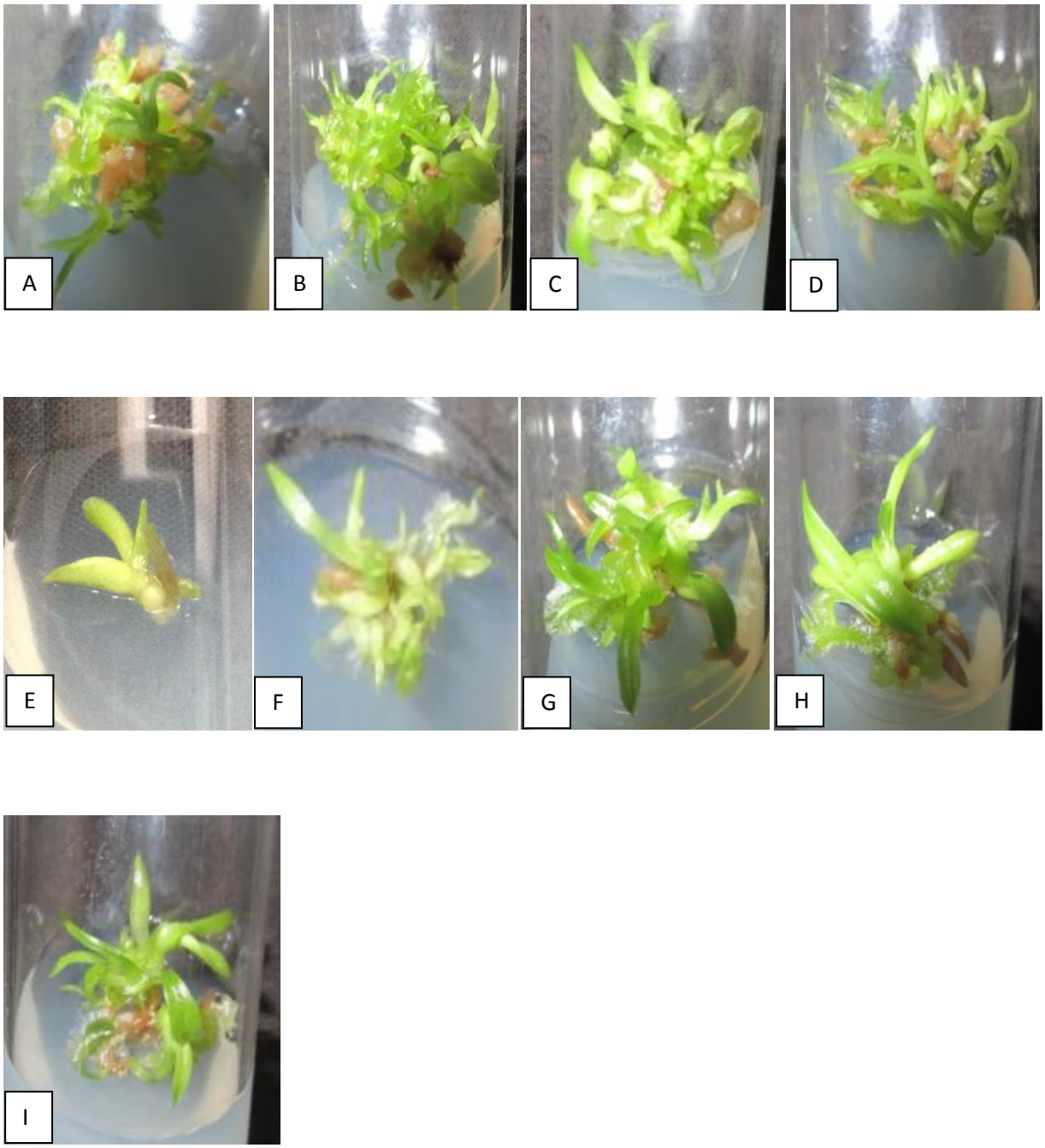
Figures 12 (A-L): Germination from single protocorm A-C in quarter strength of MS medium of A) full strength MS B) ½ MS C) ¼ MS Shooting from single protocorm in quarter strength of MS medium D-L supplemented with D) 0.5 mgl^{-1} GA_3 E) 1.0 mgl^{-1} GA_3 F) 1.5 mgl^{-1} GA_3 G)2.0 mgl^{-1} GA_3 H) 5% CW I) 10% CW J) 0.5 mgl^{-1} BAP plus 6% sucrose K) 1.0 mgl^{-1} BAP plus 6% sucrose L) 1.5 mgl^{-1} BAP plus 6% sucrose

(Pictures taken after 14 weeks of culture (A-C); 32 weeks of culture (D-L)



Figures 13 (A-L): Shooting from single protocorm in quarter strength of MS medium supplemented with A) 0.5 mgl^{-1} NAA B) 1.0 mgl^{-1} NAA C) 1.5 mgl^{-1} NAA D) 2.0 mgl^{-1} NAA E) 0.5 mgl^{-1} BAP F) 1.0 mgl^{-1} BAP G) 1.5 mgl^{-1} BAP H) 2.0 mgl^{-1} BAP I) 0.5 mgl^{-1} NAA and 0.5 mgl^{-1} BAP J) 1.0 $mgl^{-1}$and 0.5 mgl^{-1} BAP K) 1.5 mgl^{-1} NAA and 0.5 mgl^{-1} BAP L) 2.0 mgl^{-1} NAA and 0.5 mgl^{-1}

(Pictures taken after 32 weeks of culture)



Figures 14 (A-J): Rooting in Quarter strength of MS medium supplemented A) 0.25 mgl^{-1} IAA B)0.5 mgl^{-1} IAA C) 1.0 mgl^{-1} IAA D) 0.25 mgl^{-1} IBA E) 0.5 mgl^{-1} IBA F) 1.0 mgl^{-1} IBA G) 0.25 mgl^{-1} NAA H) 0.5 mgl^{-1} NAA J) 1.0 mgl^{-1} NAA (Pictures taken after 32 weeks of culture)

CHAPTER V

DISCUSSION

5.1 Protocorm germination in different strength of MS medium

In the present study, MS medium with different strength showed varied response on development of shoot buds and shoots from *in vitro* grown protocorms of *Vanda tessellata* (Roxb.) Hook. ex G. Don. *In vitro* grown protocorms were cultured in quarter strength MS media supplemented with different hormones and organic additives in different concentrations. These *in vitro* grown protocorms responded positively in quarter strength of MS medium supplemented with different concentrations of plant hormones and organic additives. The multiplication and development of shoot buds and PLBs started after 3 weeks of primary culture. The shoot bud and protocorm like bodies along with some callus was observed in all strength of MS basal medium. The shoots were observed after 5-6 weeks of culture. The organic additives increased the growth and development of protocorm and shootbuds.

5.2 Shoot Multiplication

In vitro grown protocorms were used as explants for shooting. The protocorm of similar size were inoculated on quarter strength of MS medium supplemented with different concentration of GA_3 (0.5, 1.0, 1.5 and 2.0 mgl^{-1}), CW (5% and 10%), BAP (0.5, 1.0 and 1.5 mgl^{-1}) plus 6% sucrose, NAA (0.5, 1.0, 1.5 and 2.0 mgl^{-1}), BAP (0.5, 1.0, 1.5 and 2.0 mgl^{-1}) and NAA (0.5, 1.0, 1.5 and 2.0 mgl^{-1}) with 0.5 mgl^{-1} BAP. MS medium is best for protocorm formation (Prakash et al., 2012). The multiplication of protocorm and differentiation of protocorm was promoted in basal MS basal media supplemented with combination of BAP and Kinetin in comparison to BAP and Kn alone in *Vanda coerulea* (Hrashel and Thangjam, 2015). The multiplication of protocorms and growth of healthy shoots was promoted on MS supplemented BAP and NAA in *Cymbidium elegans* (Pant and Pradhan, 2010). The effect of different combinations with hormones showed the significant variation for shooting. There was significant variation in all parameter observed in shoot multiplication, shoot bud number, shoot length, leaf number and leaf length ($P< 0.05$).

The explants cultured in quarter strength of MS medium supplemented 1.5 mgl^{-1} GA_3 developed more number of shoots and leaves (21 leaves) as compared to other concentration of GA_3 used. But the length of the shoot was found highest (1.76cm) with longest leaf length (0.86cm) in same strength of MS supplemented 0.5 mgl^{-1} GA_3. The shoots and leafs were long and green and many but protocorm were fewer.

The ¼ MS medium supplemented with 10% CW was found to be superior over ¼ MS supplemented 5% CW in all parameters observed. This may be due to this species favors organic additives rich media for its growth and multiplication. Shoot proliferation was best in 10% CW supplemented medium in *Vanda teres* (Sinha and Roy, 2004), organic additives with half MS

media showed better growth in shoot development of *Dendrobium* orchid (Akter *et al*., 2007) and in hybrid orchid *Phalaenopsis* (Shekarriz et al., 2014) and organic additives in KC media promoted better growth of seedlings from protocorm in *Vanda helvola* (David D. *et al*., 2015).

Since the BAP plus 3% sucrose supplemented ¼ MS medium didn't gave better result (data not included), the higher concentration of BAP plus 6% sucrose supplemented ¼ MS medium was used. The most interesting observation was found in BAP plus 6% sucrose supplemented ¼ MS medium. The number of PLBs and shoot buds developed vigorously in these media along with shoot formation. In ¼ MS medium supplemented BAP (1.5 mgl^{-1}) produced more shoot and shoot buds by addition of 6% sucrose than without sucrose. Similar result was obtained by Bhattacharjee and Islam (2014) in *Acampe premorsa, Agrostophylum Khasianum* and *Phalaenopsis kornorerries* and PLBs formation in *Dendrobium candidum* (Zhao *et al*., 2008).

In the ¼ MS supplemented different concentration of NAA, the highest shoot number multiplication was observed in ¼ MS supplemented with 1.0 mgl^{-1} NAA along with higher leaf number. Very few shoots and leaf numbers were observed in same concentrations supplemented NAA as compared to all medium used.

In BAP supplemented medium, the highest number of shoot was observed in ¼ MS supplemented BAP (0.5 mgl^{-1}) among all the culture conditions. This may be due to this species need low concentration of cytokinin for shoot multiplication. The shoot bud number was found more (8 Shoot buds per culture) in BAP (0.5 mgl^{-1} and 1.0 mgl^{-1}) supplemented medium. Shoot length (1.11cm) and leaf length (0.51cm) was found higher in 2.0 mgl^{-1} BAP supplemented medium. But the leaf number were developed more in 0.5 mgl^{-1} BAP supplemented medium. Similar findings were observed in *Geodorum densiflorum* (Bhadra and Hussain, 2003), *Vanda teres* (Sinha and Roy, 2004), *Phaius tankervilleae* (Pant and Shrestha, 2011) along with other combinations. The use of BAP supplemented medium gave better result in shooting in *Vanda tessellata* (Rahman *et al*, 2009).

In 0.5 mgl^{-1} BAP and different concentration of NAA supplemented medium, highest number of shoot was developed in 2.0 mgl^{-1} NAA plus 0.5 mgl^{-1} BAP (9.83cm). Similar findings were observed by Nongdam and Tikendra (2014) in *Dendrobium chrysotoxum*,

5.3 Rooting of Shoots

For rooting, *in vitro* grown shoots were used as explants obtained from the shooting of protocorms. The plantlets responded variously for the root development in different concentration of Auxin.

In IAA supplemented medium highest number of root was found in 0.25 mgl^{-1} IAA supplemented medium (1 root per culture) but Bhattacharjee and Islam (2014) recorded highest number of root formation in 1mgl^{-1} IAA in half strength of MS. This may be due to the difference in media or the shoot explants used. The longest root length (0.15cm) was found in

0.25 mgl^{-1} IAA supplemented medium. But the shoots were more (more than 8) in 1.0 mgl^{-1} IAA supplemented medium with longest shoot length (1.02cm). Shoot buds formation was also found in the 1.0 mgl^{-1} (more than 5) supplemented medium.

In IBA supplemented medium, 0.5 mgl^{-1} IBA supplemented medium produced more shoot (more than 9 shoot per culture) as compared to other medium. The more numbers of shoot buds (more than 8 per culture) and roots (0.75 per culture) were developed in 1.0 mgl^{-1} IBA supplemented medium along with leaf length (0.55cm). The highest root length (1cm) was developed in 0.25 mgl^{-1} IBA supplemented medium. Same result was seen in *Vanda tessellata* (Rahman *et al.*, 2009),

In NAA supplemented medium, highest number of shoot (more than 11 per culture) were developed in 0.5 NAA supplemented medium along with shoot length (1.32cm), leaf number (more than 14 per culture) and leaf length (0.95cm). More number of shoot buds (more than 6) was developed in 0.25 NAA supplemented medium. Highest number of roots (1.75 per culture) with highest root length (0.25cm) was developed by both NAA (0.5 mgl^{-1} and 1.0 $mgl^{-}1$) supplemented medium. This result of rooting in 0.5 mgl^{-1} supplemented medium was similar to the findings of in *Phaius tankervilleae* (Pant and Shrestha, 2010) and in *Vanda tessellata* (Rahman *et al.*, 2009) and in *Esmeralda clarkei* (Paudel and Pant, 2012. MS medium supplemented NAA promotes rooting (Pant and Shrestha, 2011).

All the quarter MS medium supplemented auxin gave positive result for rooting. Among them quarter MS supplemented 0.5 mgl^{-1} NAA and 1.0 mgl^{-1} NAA favored growth of more roots and highest root length as compared to other concentrations used.

CHAPTER VI

CONCLUSION

Vanda tessellata (Roxb.) Hook. ex G. Don is one of the most popular orchid that is known for its high medicinal and horticultural value. In the present research study, quarter MS was found to be most effective medium for initiation of different parts (protocorm, shoot and leaf) compared to MS and half MS without any supplement of plant hormones. Quarter MS supplemented BAP (0.5 mgl^{-1}) was found to be best medium to develop the highest number of shoot among all the culture conditions. Shoot buds development was found to be best in quarter strength of MS medium supplemented BAP (1.5 mgl^{-1}) plus 6% sucrose as compared to all other combinations of MS medium. Root development was found to be best in both quarter strength MS medium supplemented NAA (0.5 mgl^{-1}) and NAA (1.0 mgl^{-1}). This result shows that this species favors BAP for multiplication compared to other medium used. This protocol might be useful for rapid growth of shoot buds and multiplication of slow growing orchid *Vanda tessellata* for its conservation and for its commercial utilization.

CHAPTER IV

RECOMMENDATIONS

Some recommendations can be outlined from this present study

- Some other organic additives can be used for testing mass propagation.
- Acclimatization of this plant can be the further research work as the plants are not acclimatized in this research work.
- Phytochemical analysis can be carried out *in vitro* as this plant is well known for its high medicinal value.
- The plant can be mass propagated for horticulture commercially as it has beautiful fragrant flower.

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ANNEXES

1. **Shoot multiplication of *Vanda tessellata* in quarter strength of MS medium supplemented with different hormone concentrations.** (Observations of 32nd week)

Mean					
Concentrations	No. of Shoot buds	No. of shoots	Length of shoots (cm)	No. of leaves	Length of leaves (cm)
0.5 GA_3	3.16	8.00	1.78	11.50	0.86
1.0 GA_3	4.16	9.50	1.26	13.16	0.71
1.5 GA_3	4.33	12.33	1.38	21.00	0.76
2.0 GA_3	2.16	4.33	1.03	7.16	0.76
5% CW	5.50	10.50	0.80	11.83	0.41
10% CW	6.66	11.66	0.81	13.16	0.48
0.5BAP + 6% Sucrose	7.66	8.66	1.06	7.66	0.53
1.0BAP + 6% Sucrose	5.66	10.66	0.93	10.50	0.51
1.5BAP + 6% Sucrose	10.16	13.33	1.00	15.66	0.55
0.5NAA	2.16	3.83	0.80	3.50	0.41
1.0NAA	2.50	4.83	0.93	5.83	0.41
1.5NAA	2.16	4.33	0.86	2.66	0.21
2.0NAA	3.16	4.66	1.15	2.66	0.30
0.5BAP	8.00	15.83	0.93	16.83	0.50
1.0BAP	8.00	13.50	0.66	12.83	0.43
1.5BAP	4.00	9.50	0.95	12.50	0.48
2.0BAP	7.16	11.83	1.11	13.83	0.51
0.5NAA + 0.5BAP	3.50	7.00	0.90	7.83	0.46
1.0NAA + 0.5BAP	4.50	3.83	1.05	11.66	0.46
1.5NAA + 0.5BAP	2.33	6.83	0.90	8.16	0.50
2.0NAA + 0.5BAP	4.50	9.83	1.05	11.66	0.46

2. **Effect of different concentrations of Auxin (IAA, IBA and NAA) on rooting of shoots.**

Mean							
Concentration of Auxin (mg\l)	No of shoots	No of shoot buds	Length of shoots(cm)	No of leaves	Length of leaves (cm)	No. of Roots	Length of Roots (cm)
0.25 IAA	7.50	3.75	0.82	11.0	0.45	1.0	0.15
0.5 IAA	5.25	2.50	0.82	8.25	0.52	0.50	0.05
1.0 IAA	8.75	5.25	1.02	14.00	0.62	0.50	0.02
0.25IBA	6.25	4.00	0.92	8.00	0.35	0.50	0.10
0.5IBA	9.75	5.25	0.85	10.50	0.45	0.50	0.07
1.0IBA	7.25	8.25	0.87	9.00	0.55	0.75	0.05
0.25NAA	8.25	6.75	1.30	11.50	0.75	1.00	0.20
0.5NAA	11.25	5.50	1.32	14.75	0.97	1.75	0.25
1.0NAA	6.00	2.00	1.22	9.00	0.85	1.75	0.25

Conditions: Artificial light (fluorescent light) with a light/dark cycle of 16/8 hours at 25 ± 2^0C. (Observation at 32nd week)

3. Normality Test of Data: Shoot Multiplication

	Hormone concentration	Shapiro-Wilk		
		Statistics	df	Sig.
Log No. of Shoots	0.5 GA_3	.804	6	.064
	1.0 GA_3	.897	6	.357
	1.5 GA_3	.871	6	.230
	2.0 GA_3	.915	6	.473
	5% CW	.874	6	.241
	10% CW	.904	6	.400
	0.5BAP + 6% Sucrose	.902	6	.387
	1.0BAP + 6% Sucrose	.907	6	.418
	1.5BAP + 6% Sucrose	.902	6	.387
	0.5NAA	.908	6	.423
	1.0NAA	.818	6	.085
	1.5NAA	.927	6	.554
	2.0NAA	.974	6	.918
	0.5BAP	.943	6	.684
	1.0BAP	.824	6	.095
	1.5BAP	.921	6	.512
	2.0BAP	.746	6	.018
	0.5NAA + 0.5BAP	.784	6	.042
	1.0NAA + 0.5BAP	.904	6	.398
	1.5NAA + 0.5BAP	.878	6	.259
	2.0NAA + 0.5BAP	.904	6	.398
Shoot-bud Number	0.5 GA_3	.912	6	.452
	1.0 GA_3	.823	6	.094
	1.5 GA_3	.782	6	.040
	2.0 GA_3	.908	6	.421
	5% CW	.982	6	.961
	10% CW	.927	6	.554
	0.5BAP + 6% Sucrose	.967	6	.869
	1.0BAP + 6% Sucrose	.908	6	.425
	1.5BAP + 6% Sucrose	.889	6	.315
	0.5NAA	.809	6	.070
	1.0NAA	.902	6	.389
	1.5NAA	.908	6	.421
	2.0NAA	.958	6	.804

	0.5BAP	.893	6	.332
	1.0BAP	.928	6	.565
	1.5BAP	1.000	6	1.000
	2.0BAP	.966	6	.866
	0.5NAA + 0.5BAP	.902	6	.389
	1.0NAA + 0.5BAP	.828	6	.104
	1.5NAA + 0.5BAP	.823	6	.093
	2.0NAA + 0.5BAP	.828	6	.104
Shoot Length	0.5 GA_3	.838	6	.126
	1.0 GA_3	.862	6	.195
	1.5 GA_3	.783	6	.041
	2.0 GA_3	.915	6	.473
	5% CW	.982	6	.960
	10% CW	.958	6	.804
	0.5BAP + 6% Sucrose	.954	6	.772
	1.0BAP + 6% Sucrose	.915	6	.473
	1.5BAP + 6% Sucrose	.823	6	.094
	0.5NAA	.780	6	.039
	1.0NAA	.964	6	.851
	1.5NAA	.950	6	.741
	2.0NAA	.884	6	.290
	0.5BAP	.871	6	.232
	1.0BAP	.866	6	.212
	1.5BAP	.921	6	.515
	2.0BAP	.912	6	.453
	0.5NAA + 0.5BAP	.823	6	.094
	1.0NAA + 0.5BAP	.865	6	.208
	1.5NAA + 0.5BAP	.827	6	.101
	2.0NAA + 0.5BAP	.865	6	.208
Leaf Number	0.5 GA_3	.963	6	.839
	1.0 GA_3	.860	6	.187
	1.5 GA_3	.859	6	.185
	2.0 GA_3	.890	6	.316
	5% CW	.779	6	.037
	10% CW	.823	6	.094
	0.5BAP + 6% Sucrose	.920	6	.503
	1.0BAP + 6% Sucrose	.921	6	.515
	1.5BAP + 6% Sucrose	.874	6	.245
	0.5NAA	.685	6	.004

	1.0NAA	.876	6	.249
	1.5NAA	.831	6	.111
	2.0NAA	.828	6	.102
	0.5BAP	.930	6	.581
	1.0BAP	.815	6	.079
	1.5BAP	.831	6	.109
	2.0BAP	.766	6	.028
	0.5NAA + 0.5BAP	.828	6	.104
	1.0NAA + 0.5BAP	.961	6	.828
	1.5NAA + 0.5BAP	.962	6	.835
	2.0NAA + 0.5BAP	.961	6	.828
Leaf Length	0.5 GA_3	.866	6	.212
	1.0 GA_3	.805	6	.065
	1.5 GA_3	.915	6	.473
	2.0 GA_3	.920	6	.505
	5% CW	.805	6	.065
	10% CW	.908	6	.421
	0.5BAP + 6% Sucrose	.915	6	.473
	1.0BAP + 6% Sucrose	.866	6	.212
	1.5BAP + 6% Sucrose	.881	6	.272
	0.5NAA	.958	6	.804
	1.0NAA	.809	6	.070
	1.5NAA	.850	6	.158
	2.0NAA	.976	6	.933
	0.5BAP	.982	6	.960
	1.0BAP	.915	6	.473
	1.5BAP	.908	6	.421
	2.0BAP	.908	6	.421
	0.5NAA + 0.5BAP	.866	6	.212
	1.0NAA + 0.5BAP	.915	6	.473
	1.5NAA + 0.5BAP	.982	6	.960
	2.0NAA + 0.5BAP	.915	6	.473

4. Normality Test for Data: Rooting of Shoots

	Hormone concentration	Shapiro-Wilk		
		Statistics	df	Sig.
Shoot Number	0.25 IAA	.929	4	.589
	0.5 IAA	.717	4	.018
	1.0 IAA	.862	4	.268
	0.25IBA	.982	4	.911
	0.5IBA	.895	4	.408
	1.0IBA	.753	4	.041
	0.25NAA	.790	4	.085
	0.5NAA	.666	4	.004
	1.0NAA	.838	4	.189
Shoot-bud Number	0.25 IAA	.801	4	.103
	0.5 IAA	.863	4	.272
	1.0 IAA	.799	4	.100
	0.25IBA	.827	4	.161
	0.5IBA	.863	4	.272
	1.0IBA	.806	4	.113
	0.25NAA	.939	4	.650
	0.5NAA	.684	4	.008
	1.0NAA	.827	4	.161
Shoot Length	0.25 IAA	.630	4	.001
	0.5 IAA	.926	4	.572
	1.0 IAA	.939	4	.650
	0.25IBA	.808	4	.117
	0.5IBA	.993	4	.972
	1.0IBA	.926	4	.572
	0.25NAA	.940	4	.653
	0.5NAA	.887	4	.369
	1.0NAA	.863	4	.271
Leaf Number	0.25 IAA	.984	4	.925
	0.5 IAA	.880	4	.337
	1.0 IAA	.935	4	.623
	0.25IBA	.946	4	.689
	0.5IBA	.831	4	.171
	1.0IBA	.827	4	.161
	0.25NAA	.893	4	.395

	0.5NAA	.742	4	.033
	1.0NAA	.950	4	.714
Leaf Length	0.25 IAA	.840	4	.195
	0.5 IAA	.801	4	.103
	1.0 IAA	.801	4	.103
	0.25IBA	.729	4	.024
	0.5IBA	.840	4	.195
	1.0IBA	.863	4	.272
	0.25NAA	.998	4	.995
	0.5NAA	.828	4	.163
	1.0NAA	.849	4	.224
Root Number	0.25 IAA	.945	4	.683
	0.5 IAA	.729	4	.024
	1.0 IAA	.630	4	.001
	0.25IBA	.729	4	.024
	0.5IBA	.630	4	.001
	1.0IBA	.863	4	.272
	0.25NAA	.630	4	.001
	0.5NAA	.863	4	.272
Root Length	0.25 IAA	.993	4	.972
	0.5 IAA	.729	4	.024
	1.0 IAA	.630	4	.001
	0.25IBA	.827	4	.161
	0.5IBA	.630	4	.001
	1.0IBA	.729	4	.024
	0.25NAA	.945	4	.683
	0.5NAA	.993	4	.972
	1.0NAA	.993	4	.972

5. Multivariate analysis: for Shooting

Source	Df	F	Sig.
Shoot Number	20	3.435	.000
Shoot-bud Number	20	5.289	.000
Shoot Length	20	4.382	.000
Leaf Number	20	3.970	.000
Leaf Length	20	6.844	.000

6. Multivariate analysis: for rooting:

Source	df	F	Sig.
Shoot Number	8	.577	.787
Shoot-bud Number	8	.821	.591
Shoot Length	8	2.639	.028
Leaf Number	8	.574	.790
Leaf Length	8	3.256	.010
Root Number	8	1.765	.129
Root Length	8	2.567	.032