SEED CHARACTERIZATION AND STANDARDIZATION OF *IN VITRO* GERMINATION PROTOCOL IN *Vanda tessellata* (Roxb.) Hook. ex G. Don.

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by

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(2020-11-014)

THESIS

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KERALA, INDIA

2023

DECLARATION

I, hereby declare that this thesis entitled **"Seed characterization and standardization of *in vitro* germination protocol in *Vanda tessellata* (Roxb.) Hook. ex G. Don."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **"Seed characterization and standardization of *in vitro* germination protocol in *Vanda tessellata* (Roxb.) Hook. ex G. Don."** is a record of research work done independently by **Ms Athira Chandranath** (2020-11-014) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Introduction

1. INTRODUCTION

Orchidaceae, which is one of the largest and most highly evolved families among monocotyledons with about 900 genera and 30,000–35,000 species, is distributed worldwide (Seeja and Sreekumar, 2022). Orchids are perennial plants with the most diverse, fascinating, varied, and beautiful flowers. They have outnumbered other families of flowering plants by evolving higher levels of specialisation in their vegetative and reproductive traits. It can grow in a variety of habitats and is a model plant for plant breeders because over 1,000,000 hybrids have already been released; no other crop has produced this many hybrids to date. Orchids are commercially traded as ornamentals, medicinal products, and food (Hinsley *et al.,* 2018). However, it ranks in the top position among the floricultural crops in the global market. The global orchid trade value was $51521 million in 2020 and is expected to grow at a compound annual growth rate (CAGR) of 4.6% from 2021 to 2027. The North American orchid industry is expected to be valued at US $ 1617.3 million in 2020 (https://www.alltheresearch.com/report/735/orchid-market). India has a rich diversity of native orchid species with commercial value and is blessed with a wide range of agroclimatic conditions, but its contribution to the global market is meagre, and 80% of the orchids sold in Indian markets are imported from Thailand.

Vanda is the third most important orchid genus in terms of global market share. It is a monopodial and mostly epiphytic orchid that is widely cultivated in Southeast Asia and the Indian subcontinent (Islam *et al.,* 2014). The genus Vanda consists of about 87 species, the majority of which are commercially used as cut flowers, potted plants, herbal products in cosmetics and medicine, and some of which are used as food additives. Most Indian species of Vanda have been recognised in breeding programs, especially to produce primary hybrids, due to their inherent attractiveness coupled with their ability to transmit these characters to hybrids. They are cross compatible with other genera like Ascocentrum, Aerides, Rhynchostylis, Neofinetia, Renanthera, and Phalaenopsis (De *et al*., 2014).

Vanda tessellata (Roxb.) Hook. ex G. Don. commonly known as ‘rasna’ (Sanskrit) and ‘Grey orchid" or "checkered Vanda" (English), is distributed in certain regions of India and Indo-China since it requires specific agro-climatic conditions. It is a monopodial epiphytic orchid with

a wide array of medicinal and ornamental values. It has attractive and fragrant flowers all year and is diverse in the wild, with nearly 50 different colours of tessellate markings on the petal. It was one of the parents of the Vanda 'Mimi Palmer', an international award-winning orchid for its strong sweet fragrance (Osman, 2008), and several other commercially leading orchid hybrids have been developed from this wild species. Its population in its natural habitat is rapidly diminishing due to overexploitation and habitat destruction, and it has become an endangered species in India. The International Union for Conservation of Nature (IUCN) ranked this species as a conservation priority and recommended it for ex situ conservation (Khela and Chadburn, 2014). Its multifaceted uses and scarcity necessitate the development of appropriate techniques for large-scale multiplication, as seed germination in orchids is generally very low (2-5%). Hence, an alternative propagation method is a pre-requisite for sustainable utilisation and conservation of this species.

Plant tissue culture is an established method for effective propagation, offers the ability for large-scale production, and ensures clonal stability, irrespective of season and weather (Singh and Duggal, 2009; Silva *et al*., 2015). Orchids' seeds are microscopic and unpretentious (Dressler, 1993). They differ from those of most angiosperms and resemble the so-called 'dust seeds' of other plants (Fleischer, 1929; 1930; Ziegenspeck, 1936; Rauh *et al*., 1975; Rasmussen, 1995). The orchid seeds generally lack a well-defined endosperm and contain a globular-shaped embryo covered by thin layers of seed coats (Arditti, 1992). Although the macroscopic appearances of various orchid seeds are similar, they are highly diverse owing to their seed coat embellishment (Clements and Molvray, 1999). Micro-morphological seed traits are species-specific, reflect various modes of seed dispersal, and can be used to prioritise species for conservation and select appropriate *in situ* and *ex situ* conservation strategies.

Generally, an orchid capsule consists of enumerable numbers of non-endospermic seeds that require symbiotic association with certain fungi for germination. Hence, in natural conditions, the percentage of seed germination is very low. *In vitro* seed germination is the best option to overcome this problem. Seed propagation and multiplication response mainly depend on the seed traits. Hence, seed characterization is inevitable for the development of an efficient propagation technique. Moreover, understanding morphological, morphometric, and reproductive characteristic features is inevitable for practical conservation and sustainable utilisation of any crops, particularly endangered species like *Vanda tessellata*.

In these backdrops the present investigation was aimed at studying the morphological and morphometrical seed characterization and standardisation of the *in vitro* seed germination protocol for *Vanda tessellata*.

Review of Literature

2. REVIEW OF LITERATURE

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

The taxonomic description of the accepted plant name *Vanda tessellata* was first made by W. Roxburgh (1795), who coined the name *Epidendrum tessellatum*. W.J. Hooker proposed the current name *V. tessellata,* and George Don proposed the same name following the International Code of Botanical Nomenclature in 1830 (Roxb.) Hook. ex. Done 1830. Many flora include descriptions of the plant (Gamble, 1928). It is a monopodial epiphytic orchid, also known as the grey orchid, that can reach a height of 60 cm and has a leafy stem. The leaves are thickly coriaceous, recurved, plicate, obtuse, and keeled (https://en.wikipedia.org/wiki/Vanda_tessellata; Singh and Duggal, 2009). Kirankumar *et al.* (2021) reviewed the taxonomic and medicinal properties of this species. According to Bhattacharjee and Islam (2014), the demand for this orchid is increasing day by day due to its medicinal and floricultural importance. The whole plant body is used in Ayurvedic medicines. Fever is treated with a leaf paste. It is an ingredient in 'Rasna Panchaka Quatha," the Ayurvedic formulation used in the treatment of arthritis and rheumatism (Singh and Duggal, 2009). A novel aphrodisiac compound was also isolated from the flower of *V. tessellata* which activates neuronal (nNOS) and endothelial nitric oxide synthase (eNOS) and influences the levels of nitric oxide (NO) in tissue and blood (Subramonium *et al*., 2013).

2.1. EVALUATION OF FLORAL CHARACTERS

2.1.1. Floral characters (qualitative)

2.1.1.1. Number of inflorescences per plant/year

The number of inflorescences produced per year in orchids is a crucial characteristic feature for determining whether it will be profitable to use as a cut flower or a pot plant (Garay, 1972).

2.1.1.2. Length of inflorescence

Inflorescence length has been identified as being one of the significant characters in any orchid breeding programme (McDonald, 1991). It is one of the biometric characteristics that determines the size and shape of the inflorescence. Variation in spike length among varieties is a genetic characteristic (Thomas, 2008). In *V. tessellata*, the inflorescence is a raceme (Sahu and Chaudhary, 2021). The average length of inflorescence was 19.02 cm (Seeja, 2018). The inflorescence of *V. tessellata* is short (<15 cm) (De *et al*., 2015).

2.1.1.3. Number of flowers per inflorescence

In orchid breeding, the number of flowers per inflorescence is one of the important traits (McConnel and Kamemoto, 1983; McDonald, 1991). According to Islam *et al*. (2016), *V. tessellata* has peduncles that are 15-20 cm long, with 6–10 flowering racemes and 6–10 blooms per inflorescence (Sahu and Chaudhary, 2021).

2.1.1.4. Flower inter-nodal length (cm)

The internodal length of the inflorescence should be ideal for better display of the inflorescence; more clearance results in an ungainly, prominent exposure of the inflorescence axis, while sufficient clearance is necessary to avoid blossom overcrowding (Thomas, 2008). Flower internodal length in *V. tessellata* was reported to be 2.02 cm (Seeja, 2018), which is an ideal distance to expose the flower.

2.1.1.5. Length of the pedicel (cm)

Pedicel size differed significantly among orchid species, and ranged from 1.2 to 10.3 cm (Anand *et al.,* 2013). The length of the pedicel is a highly heritable trait. To increase yield in orchids, direct selection for pedicel length would be useful. Pedicel length ranges from a minimum of 3.50 cm to a maximum of 54.67 cm in orchids (Vanlalruati *et al.,* 2016).

2.1.1.6. Flower colour and pattern of tessellate marking

Schiestl *et al.* (1997) reported that after pollination, flower colour and aroma decreased. The sepals and petals are brownish green with darker tessellation, and the lip is indigo blue (De, 2021). Flowers have yellow sepals, tessellated with brown lines and white margins. Petals are yellow with brown lines and white margins (Islam *et al*., 2016). Flowers are greenish yellow with brown in the lip's midlobe and purple caruncles (Singh and Duggal, 2009).

2.1.1.7. Flower longevity (days)

Generally, Vanda blooms once every month, and flowers last for two to three weeks (Garay, 1972). Pollen removal from stamens and pollination reduce floral longevity (Evanhoe and Galloway, 2002), and floral scent and colour diminish after pollination (Schiestl *et al*., 1997; Theis and Raguso, 2005). The longevity of an individual flower in *V. tessellata* is around one month (Sahu and Chaudhary, 2021).

2.1.1.8. Nature of flowering—free or seasonal

Monopodial orchids bloom all year or only occasionally. Since seasonal flowering is confined to specific months or seasons, it is not as desirable as free-flowering. The demand for flowers is related to their constant availability on the market, which is possible in the case of free-flowering commercial hybrid varieties (Thomas, 2008). The nature of flowering in *V. tessellata* is noted as "free" (Seeja, 2018).

2.1.1.9. Season of flowering

The rate of flower opening and the number of flowers produced per inflorescence determine the flowering time of a species (Thomas, 2008). In India, its flowering has been reported in various climatic seasons from January to December (Matthew, 1995; Seidenfaden, 1999; Seeja, 2018). The grey orchid has a wide tolerance for temperature (26–31ºC) and low relative humidity (31%–44%) (Jayaweera, 1981).

2.1.1.10. Anthesis.

In orchids, anthesis is a genetically controlled trait. (Sanford, 1971). Anthesis in *V. tessellata* is reported to be from 6 a.m. to 6 p.m. (Seeja, 2018).

2.1.1.11. Time of stigma receptivity

The receptivity of the stigma decreases with flower aging. (Dongarwar and Thakur, 2014). Pollinating young, fully open flowers is recommended in cases of artificial pollination in orchids in general since pollen and stigma are most receptive 1–8 days after flowers are open (Proctor, 1998; Shiau *et al.*, 2002). Stigma receptivity remained throughout the day after anthesis and up to six days after anthesis in *V. tessellata* (Seeja, 2018).

2.1.2. Pollen characters

2.1.2.1. Pollen morphology

Pollen forms different types of aggregated entities called pollen dispersal units, or PDUs (Pacini, 1997). In angiosperms, Orchidaceae plants have the greatest number of PDU types, and pollen is often packed in PDUs called pollinia (Pacini, 2009; Pacini and Hesse, 2002). In orchids, pollen grains mostly existed as tetrads (Abraham and Vatsala, 1981; Das and Goshal, 1988; Varghese, 1995; and Sobhana, 2000). Pollinium has two pollinia, which are waxy and globular (Mulgaonkar, 2005). The Vandeae consistently exhibit tectate-granular and atectate wall types, no footlayer, in-aperturate pollen, and compact pollinia, suggesting this is the most palynologically derived group of the family (Zavada, 1990).

2.1.2.2. Pollen production per pollinium

Pollen production per flower per anther varied from variety to variety within an orchid species (Nair *et al.*, 1964). The numbers of pollen grains per pollen dispersal unit have both genetic and ecological consequences. The most important is that the more grains per pollinia, the more likely seeds in a pod will have the same male parent; the more pollen adhering to the stigma and fertilising it, the more male competition inspiring greater female competition, leading to

reproductive fitness of the species in its ecology (Pacini and Franchi, 1999). *Vanda tessellata*, produces an average of 900000 pollen grains per pollinium (Seeja, 2018).

2.1.2.3. Pollen fertility percentage

The carbohydrate and water content of pollen grains at the beginning of anthesis, the environment during exposure and dispersal, and the location of pollen presentation (whether pollen is exposed or protected)—all these factors together appear to affect pollen longevity (Pacini, 1997; Dafni and Firmage, 2000; Nepi *et al*., 2001). Long-term viability requires a significant amount of sucrose and cytoplasmic polysaccharides. Pollen that has a high water content (more than 30%), along with a high percentage of sucrose and cytoplasmic polysaccharides, is more resistant to desiccation than pollen with a lower carbohydrate content (Dafni and Firmage, 2000). Pollen longevity is also influenced by environmental conditions such as temperature, light intensity, and relative humidity (Pacini, 1997). The average pollen fertility percentage is 91.35 percent (Seeja, 2018). Pollen is most receptive in orchids, one to eight days after flowers open (Shiau *et al*., 2002). Pollen longevity in orchids lasts from a few hours to several weeks (Endress, 1994).

2.1.2.4. Pollen germination percentage

The germination requirements of pollen vary appreciably from species to species. Apart from moisture, they generally require a carbohydrate source, boron, and calcium for satisfactory germination and tube growth (Patel and Mankad, 2014). In addition to serving as a pollen metabolic substrate, externally provided sucrose maintains the osmotic pressure (Shivanna and Johri, 1989). Evidence is abundant that sucrose in the medium is metabolised by germinating pollen (Linskens and Stanley, 1974).

2.1.3. Capsule characters

2.1.3.1. Days taken for pod setting after anthesis

The duration between pollination and fertilisation is highly variable in the Orchidaceae (Swamy, 1943), but it is usually short in terrestrial orchids (Swamy, 1949). In orchids, after the completion of pollination, the development of capsules starts, and flowers start to senesce within 1–5 days. (Dongarwar and Thakur, 2014).

2.1.3.2. Days to mature capsule harvest

The point of mass seed maturity achieved at the end of capsule maturation is associated with a reduction in moisture content (Schwallier *et al.,* 2011). The development of brownish and yellowish colours on the capsule's surface was a sign of capsule maturity (Slater, 1991). During the development stages of some orchids, capsules turned light green, yellow, or brown (Dongarwar and Thakur, 2014). Vanda fruit capsules took 6–9 months or even up to 20 months to attain maturity (PhytoTech Labs, 2019). The maturation of the capsule took three months in *V. tessellata* (Sahu and Chaudhary, 2021).

2.1.3.3. Length of capsule (cm)

The size of the fruit also depends on the amount of pollen applied to the stigma (Rasmussen and Johansen, 2006). Capsules are 7.5–9.0 cm long, narrowly clavate-oblong, and have acute ribs (Seeja, 2018).

2.1.3.4. Width of capsule (cm)

Capsule width is approximately 1.5 cm (Thakur *et al*., 2012).

2.1.3.5. Percentage of filled seeds

There is no effect of pollen age on the percentage of non-embryonated seeds (Proctor, 1998). Female reproductive success in orchids can be highlighted by the proportion of seeds with embryos (Lock and Profita, 1975; Nilsson, 1980; Patt, *et al.,* 1989; Proctor and Harder, 1994).

2.1.3.6. Seed viability percentage

Seed viability tests, can be used to effectively solve conservation problems (Cooke *et al*., 2013). A seed viability test is defined as any technique used to determine whether individual seeds appear to be dead or alive within a sample, which enables the proportion of live seeds in a population to be estimated (Gosling, 2003). In order to retain the seed viability in storage, it is important to collect mature seeds of orchids, since orchid seed viability is highest at the time of physiological maturity and gradually declines thereafter (Copeland and McDonald, 2001).

Some chemical methods of seed viability testing rely on the presence or absence of enzymatic activity connected to the live or dead status of a seed or the differential uptake of a stain by living versus dead tissue, commonly known as enzymatic and nonenzymatic tests, respectively (Copeland and McDonald, 2001; Wood *et al.*, 2003). These staining tests are based on the premise that viability is an inherent characteristic of a seed's potential to germinate, hence, a resting seed is a potential seedling. This pioneering view of characterizing a seed's viability was developed by Lakon (1949) through the establishment of the Tetrazolium test (TTC) for seeds.

The 2,3,5-triphenyl tetrazolium chloride changes from its oxidized, colourless form to the reduced, red or pink 2,3,5-triphenyl formazan within hydrated living tissues containing respiratory enzymes or dehydrogenases. The TTC test is one of the most commonly used seed viability tests to date and has the advantage of being a rapid test (Copeland and McDonald, 2001).

The Evan's blue test (EB) is a less commonly used nonenzymatic seed viability test (Hooi *et al*., 2010; Pouzi *et al*., 2011). Instead of enzymatic activity within the cells, it relies on membrane integrity, with non-viable seeds detected as having a damaged or leaky membrane, which allows the Evan's blue stain to penetrate the cytosol of the embryonic cells. The absence of a blue stain within cells indicates that cell membrane pumps are active, and hence that seeds are potentially viable. Therefore, seeds with completely unstained embryos are considered viable, and seeds with embryos stained blue are considered non-viable. The EB test is fast and is considered more accurate.

2.2. MORPHOLOGICAL AND MORPHOMETRIC CHARACTERIZATION OF SEEDS

The taxonomic importance of seed characteristics was first pointed out by Clifford and Smith (1969).

2.2.1. Morphological characters

The morphological characteristics of the seeds not only serve as taxonomical markings but also aid in deducing phylogenetic relationships (Barthlott, 1976). The seeds of orchids vary in size, morphology, structure, colour, and other finer details. Molvray and Kores (1995) have brought to light that the seeds vary in shape from filiform to fusiform, clavate to ellipsoidal, and sometimes

prominently winged. In some cases, the seed is covered by a hard coat, but in most cases, the seed coat is papery in texture and loosely surrounds the embryo. The walls of the testa can be smoother or reticulated. If reticulation is present, the patterns may be diverse.

The fusiform testa and prolate-spheroid embryo are the most common shapes in the Orchidaceae family (Diantina *et al.,* 2020).

Fresh orchid seeds appear in many different colours. Most often, it is whitish, brownish, or dark brown, but it can also be beige, yellow, reddish, orange, greenish, yellowish brown, or black. The colour is determined by the testa and embryo (Barthlott *et al*., 2014).

2.1.2. Morphometric characters

The morphometric characteristics of seeds are constantly challenging to taxonomic and phylogenetic issues, which would be extremely beneficial in both academic and applied ventures (Rani *et al.,* 1993; Augustine *et al.,* 2001).

The seed size varies from 150 to 6000 µm and in the majority of taxa, the range is from 300 to 800 µm (Molvray and Kores, 1995).

Seeds were categorised by size (seed length) as follows: very small (100–200 µm), small (200–500 µm), medium (500–900 µm), large (900–2000 µm), and very large (2000–6000 µm). Seed pods were classified according to size as follows: very small (10–20 mm), small (20–30 mm), medium (30–40 mm), large (40–50 mm), and very large (>50 mm) (Barthlott *et al*., 2014).

According to Arditti *et al*. (1980) and Augustine *et al*. (2001) length/width ratio provides some very important data on the relative degree of truncation of the seeds in orchids. Elongated seeds have a SL/SW ratio greater than 5, whereas truncated seeds have a SL/SW ratio less than 5 (Diantina *et al.,* 2020).

EL/EW ratios above 1 show that they have prolate-spheroid shaped embryos. (Diantina *et al.*, 2020). According to Arditti *et al*. (1979), the seed volume in orchids is a reflection of the size of the seeds.

The volume of the embryo changes during the development of the seed. Young seeds have small, undifferentiated embryos, whereas the mature seeds from the dehisced capsules have embryos of a larger volume (Swamy *et al.*, 2004).

There is evidence that larger embryo volume is positively correlated with seed weight (Dangat and Guray, 2016), influencing the seed's ability to float in air and its distribution (Arditti and Ghani, 2000).

The orchid seeds are small in weight; they are adapted for wind dispersal and belong to the category of fliers. They consist of an undifferentiated embryo contained within a loose testa and an air-filled space between the testa. Consequently, seeds with a higher ratio of seed volume/embryo volume are expected to be more buoyant than those with a lower ratio of seed volume/embryo volume. The fact that the seed volume to embryo ratio is greater than two indicates that they are a widely distributed species. While orchid taxa whose ratio of seed volume/embryo volume is less than two shows that they are restricted or localised in distribution.

Similarly, the percentage of air space has an impact on the phytogeographical distribution of the taxa. Very light and buoyant seeds with a greater percentage of air space may get dispersed over wide geographical areas more quickly than the seeds with minimum air space (Augustine *et al*., 2001). Therefore, if such species become endangered, conservational measures become more difficult.

According to Verma *et al*. (2014), terrestrial species have bigger air-spaces because of their larger seed volumes, resulting in a bigger SV/EV ratio than epiphytic species. Moreover, seeds with SV/EV ratios above 2.2 were suggested to be more buoyant than those with lower ratios, thus enabling wider plant distribution ranges. Plant adaptations to specific or limited geographic distribution ranges stimulate the evolution of a wide variety of morphological characters, including seed dispersal mechanisms.

2.3. *IN VITRO* SEED GERMINATION STANDARDIZATION

There are difficulties in propagating orchids through conventional methods as it possess very complex and unique characteristics in their flowers, which slow the rate of successful pollination by natural means (Mukhopadhyay and Roy, 1994).

Pierik (1987) reported that an orchid plant produces 1,300 to 4 million seeds per capsule and only 0.2–0.3 percent germinate in nature (Singh, 1992) due to the lack of endosperm or very small endosperm, which is inefficient for seed germination. Seeds require a symbiotic association with some species-specific mycorrhiza (a kind of symbiotic fungus) for nutrient supply, successful germination, and establishment of the plant at an early stage of development. Therefore, the presence of species-specific mycorrhiza is an important factor for the distribution of orchids in nature (Bhadra, 1999; Bhadra and Hossain, 2003). Only 2–5 percent of orchid seeds germinate in nature with mycorrhizal associations. out of which very few survive in nature. It also needs a microclimate of favourable temperature, humidity, etc. Few seeds germinate but do not develop into mature plants.

This necessitates the application of *in vitro* seed propagation techniques for orchid conservation. As a result, *in vitro* cultural techniques are now being used for rapid propagation of commercially important orchid species in order to develop rapid propagation of economically important and critically endangered orchids for proper conservation (Singh, 1992; Bhattacharjee *et al.*, 2015 a, b).

Tissue culture, an established method for effective propagation, offers the ability for large-scale production and ensures clonal stability, irrespective of season and weather (Singh and Duggal, 2009; Silva *et al*., 2015). The ambient *in vitro* culture environment allows plants to grow in aseptic conditions with reduced light intensity, higher humidity, and rich carbohydrates, nutrients, and growth regulators (Hazarika, 2003; Teixeira da Silva *et al*., 2015).

Seed culture is probably the most effective technique so far to get lots of new seedlings, despite the long maturity time of Vanda capsules that could reach 6–9 months or even up to 20 months (PhytoTech Labs, 2019).

Orchids do not need symbiotic associations for *in vitro* culture. Therefore, asymbiotic germination of orchid seeds could be a perfect system to ensure maximum germination because the heterotrophic *in vitro* conditions provide the required nutrient supply for seed germination (Otero Ospina and Bayman, 2009; Valadares *et al*., 2012).

Seeds could provide large quantities of explants where adult orchid plants are limited. Seeds could evenly germinate using basal medium without the addition of hormones or complex organic matter. Seeds will grow into protocorms and become seedlings later (Yildiz, 2012).

In orchids, seeds directly produce single, vigorous seedlings after about 3–5 months in culture. Yet, once they reach the protocorm stage (3–6 weeks), there is an indirect production of multiple shoots with structures resembling protocorm-like bodies (PLBs) (Rubluo *et al*., 1989).

Protocorms have the flexibility to induce shoots, roots, and/or secondary protocorms (PLBs) (Sujjaritthurakarn and Kanchanapoom, 2011; Setiaji *et al*., 2018). The protocorm phase usually begins when the bipolar structure cannot be distinguished between basal and apical (Setiari *et al.,* 2016). By definition, protocorms are produced by seeds, whereas protocorm like bodies (PLBs) are produced by explants. PLBs are important in orchid micropropagation and outwardly resemble somatic embryos in form and development (Lee *et al.,* 2013).

Knudson (1946) developed a protocol for asymbiotic *in vitro* orchid seed germination on a medium containing minerals, nutrients, and sugar. The first experiments on Vanda *in vitro* culture were carried out at the University of Singapore with calluses derived from seedlings in an undefined medium containing tomato juice and 2,4-dichlorophenoxy acetic acid (2,4-D) (Rao, 1963; Rao, 1967).

In general, the most commonly used basal medium for Vanda cultures is MS (35.7%) and VW (Vacin and Went, 1949) (21.4%). MS medium is widely used in a variety of plants, including orchids. This medium contains high concentrations of ammonia, potassium, and nitrates; and is relatively cheaper compared to other media such as the White medium (Stewart Jr, 2016).

Bindiya *et al*. (2012) found that MS medium without hormones was best for seed germination and protocorm formation in comparison to Knudson C (1946), Vacin and Went (1949), and Raghvan and Torrey (1964) media in *V. tessellata*.

MS medium with pH 5.5 is the best for seed germination of *V. tessellata* in comparison to the pH adjusted at 3.5, 4.5, 5.5, and 6.5 (Bindiya *et al.,* 2012).

Seeds of *V. tessellata* germinated in absence of any exogenous plant growth regulators. This might be due to the presence of adequate amounts of endogenous growth regulators in the seed tissue, which are needed at the initial stage of germination (Hajong *et al*., 2010; Islam *et al*., 2014).

The ideal formulation for seed germination may not always be the same as that required for protocorm development and plantlet formation (Kauth *et al*., 2008; Koene *et al.,* 2019).

Bhattacharjee and Islam (2014) investigated the effects of PGR(s) on *V. tessellata* multiple shoot induction in four culture media: PM, MS, 12 MS, and B5. The MS medium fortified with 1 mgL^{-1}NAA and 1.0 mgL^{-1} BAP was the most effective for multiple shoot development and elongation.

According to Bhattacharjee *et al*. (2015b), MS medium is the best for seed germination and protocorm formation. Similarly, MS supplemented with BAP (1.0 mgL^{-1}) and NAA (1.5 mgL^{-1}) was found to be the most suitable for PLBs development and plantlet formation. On the other hand, the combined effect of BAP and IAA was a suitable hormonal combination for root development.

Dwiyani *et al.* (2015) studied *in vitro* germination and the subsequent growth of *V. tessellata* in NP medium supplemented with CW and tomato juice. They concluded that NP medium supplemented with tomato juice (100 gL^{-1} and 200 gL^{-1}) showed better growth than NP medium supplemented with CW.

Sachin (2015) studied the impact of temperature and pH variation on the *in vitro* protocorm formation of *V. tessellata* in MS without various combinations of growth hormones. Among cultures maintained at different temperatures (15 °C, 20 °C, 25 °C, and 30 °C) and pH (4.5, 5.5, 6.5, and 7.5), temperature $20^{o}C$ with pH 5.5 produced the most protocorms.

The pH set for Vanda culture varies from 4.7–5.9. The better preference was at pH 5.8 (16.3%), followed by 5.6 and 5.2 (Setiaji *et al*., 2021).

Madhavi and Shankar (2019) reported that the highest response of seed germination (96%) in *V. tessellata* was achieved in half strength MS medium supplemented with coconut water (15% v/v) + banana powder (1%) + tomato powder (1%). Among the combinations of auxins (IAA and NAA) used along with cytokinins (BAP, Kn, and TDZ) and coconut water (15% v/v) in half strength MS medium, the best response of seed germination (46.6±1.2) was observed in half strength MS medium with IAA (0.1 mgL^{-1}), Kn (1.0 mgL^{-1}), and coconut water (15% v/v).

Aditya *et al.* (2020) recommended that in general, MS +VIT (vitamin), MKC (Modified Knudson C) + VIT, MKC + 0.1% P (peptone), VW + VIT and VW + VIT media could be used to produce maximum number of healthy seedlings in *V. tessellata* instead of a single medium. However, of all these, MS + VIT medium might be the optimal one since all the developmental stages were recorded in this particular treatment.

Plant growth regulators (PGRs) can be used simultaneously to match Vanda growth stages. The most commonly used PGRs in Vanda cultures, either in combination or as a single dose, are the cytokinins 6-benzylaminopurine (BAP), kinetin (Kin), N6-isopentenyladenine (2-iP), and thidiazuron (TDZ); and auxin (indole-3-acetic acid (IAA)), indole-3-butyric acid (IBA), 2,4-Dichlorophenoxyacetic acid (2,4-D), and a-naphthaleneacetic acid (NAA). In combination, 15.47% used higher concentrations of cytokinins such as BAP (4.44-66.6 µM), while 10.71% used a higher concentration of auxin such as NAA (0.27-8.06 µM). Single auxin (15.47%) is generally used to induce roots or germination, with optimum concentrations ranging at 0.54–22.80 µM for NAA, while single cytokinin (10.71%) is generally used to induce shoots, with optimum concentrations ranging at 0.91–11.35 µM for TDZ. The rest, 41.66% do not use any PGRs, and generally prefer to add complex organic materials for germination or seedling maintenance (Setiaji *et al*., 2021).

The combination of 1.5 mgL^{-1} NAA and 1.0 mgL^{-1} BAP was proved to be the best medium formulation for multiple shoot formation as well as maximum shoot elongation. The single shoots were isolated from the multiple shoots and subcultured in MS medium having NAA and IBA

individually and in combinations for root induction. Maximum root induction was obtained in MS agarified medium having 0.5 mgL^{-1} NAA and 1.0 mgL^{-1} IBA (Rahman *et al*., 2009).

The maximum frequency of seed germination (100%) was achieved on MS medium fortified with 1.5 mgL^{-1} indole-3 butyric acid (IBA). After the fourth subculture, the protocorm seedlings were used for further proliferation and the development of multiple shoots on MS medium supplemented with 0.5 mgL^{-1} each of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA) + 100 mgL^{-1} activated charcoal (AC). The optimal rooting of individual shoots was achieved on MS medium with 1.0 mgL^{-1} IBA (Manokari *et al*., 2021).

The germination of *V. tessellata* seeds cultured on KC (72.1%), VW (70.7%), and ½MS media (70.4%) did not show a significant difference (Roy and Banerjee, 2002).

Seeds cultured on full strength MS medium containing 2.0 mgL^{-1} 6-benzylaminopurine (BAP) had the highest frequency of asymbiotic seed germination (94%) as compared to half- and one-fourth strength MS media supplemented with various concentrations and combinations of 6-furfurylaminopurine (kinetin) and BAP. The PLBs gained dedifferentiation and proliferated into embryogenic calli on MS medium containing 2.0 mgL^{-1} BAP and 0.5 mgL^{-1} indole-3 acetic acid (IAA). Somatic embryos (SEs) were differentiated from the callus when cultured on MS medium in combination with 1.0 mgL^{-1} BAP and 0.5 mgL^{-1} IAA (Manokari *et al.*, 2020).

Materials and Methods

3. MATERIALS AND METHODS

The experiment entitled “Seed characterization and standardization of *in vitro* germination protocol in *Vanda tessellata* (Roxb.) Hook. ex G. Don.” was conducted at College of Agriculture, Vellayani and Saraswathy Thangavelu Extension Centre of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (STEC-JNTBGRI), Puthenthope, Thiruvananthapuram during the period 2021-2022.

3.1. Materials

Vanda tessellata plants maintained in the Orchid Germplasm Conservatory at Saraswathy Thangavelu Extension Centre of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (STEC-JNTBGRI), Puthenthope, Thiruvananthapuram were used for the experiment (Plate 1).

3.2. EXPERIMENT I - EVALUATION OF FLORAL CHARACTERS

Observations with respect to various floral characters as detailed below were taken from fifteen plants in three replications and its average was calculated.

Statistical analysis were done using the software GRAPES of KAU.

3.2.1. Floral characters (qualitative)

3.2.1.1. Number of inflorescences per plant/year

Total number of inflorescences produced per plant per year was counted from 15 plants and mean was worked out.

3.2.1.2. Length of inflorescence (cm)

Length of spike from the base of inflorescence to its tip was measured from 15 plants and average was worked out.

3.2.1.3. Number of flowers per inflorescence

Total number of flowers present in a spike was counted from 15 plants and mean was worked out.

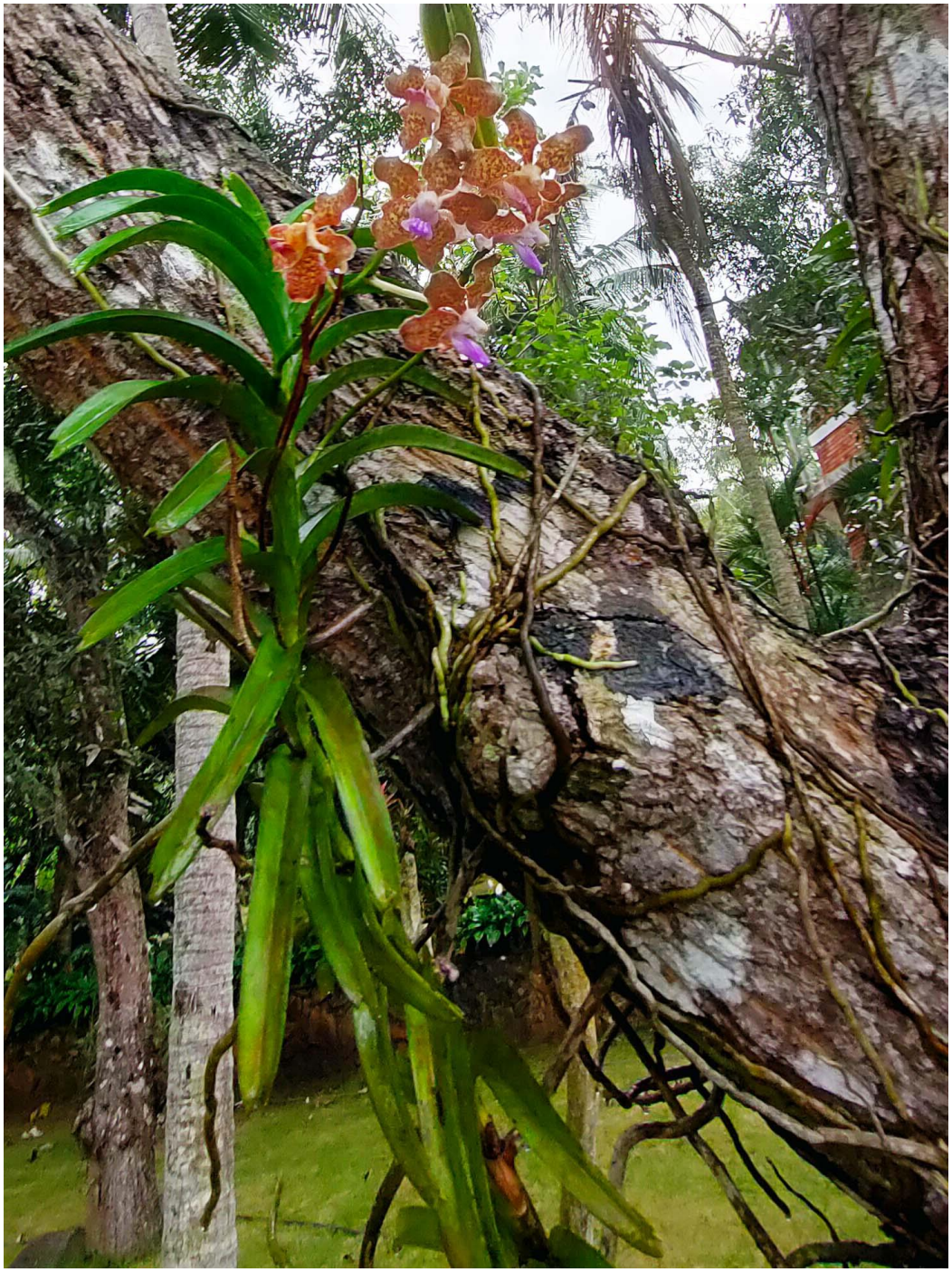


Plate 1. *Vanda tessellata* – A flowering plant on its natural habitat

3.2.1.4. Flower inter-nodal length (cm)

Distance between two adjacent flowering nodes from the base to tip of a spike was measured from fifteen plants and mean was worked out.

3.2.1.5. Length of the pedicel (cm)

Distance between the points of attachment of the pedicel from the stem to the base of the flower was measured from different flowers and mean was worked out.

3.2.1.6. Flower colour and pattern of tessellate marking

Flower colour was visually observed and compared with the report in the reference book entitled “Introduction to Orchids” (Abraham and Vatsala,1981).

3.2.1.7. Flower longevity (days)

Total number of days of flower retentiveness in a spike was counted.

3.2.1.8. Nature of flowering – free or seasonal

Appearance of inflorescence and flowering time in a year was observed and recorded.

3.2.1.9. Season of flowering

Season of inflorescence emergence and flowering was observed and recorded.

3.2.1.10. Time of anthesis

Time of opening of the flowers in an inflorescence was perceived.

3.2.1.11. Time of stigma receptivity

Total number of days of the stigma receptivity for operative pollination in a flower was taken.

3.2.2. Pollen characters

3.2.2.1. Pollen morphology

Pollinium was collected from freshly opened flowers and was put in a slide holding acetocarmine stain and crushed well with a needle base. Nature of pollen grains were observed under microscope for shape, ornamentation etc.

3.2.2.2. Pollen production per pollinium

The pollen production per pollinium was estimated using haemocytometer. Pollinium was collected in an Eppendorf tube and crushed well. The prepared pollen suspension was gently filled into the two chambers underneath the coverslip of the haemocytometer and the pollen grains were observed under microscope and counted in four sets of sixteen corner squares and centre square of 25 sub squares and calculated the average number of pollen grains present in a pollinium.

3.2.2.3. Pollen fertility percentage

Pollen fertility was estimated using acetocarmine staining technique. Pollinia were crushed with needle and pollen grains liberated were transferred into acetocarmine stain for one minute. Later the pollen grains were observed under the microscope. Counted stained pollen grains were viable and unstained ones were non-viable. The viable pollen grains were counted randomly from five different microscopic fields and mean was worked out and expressed it in percentage.

$$\text{Pollen fertility percentage} = \frac{\text{Number of stained pollen grains}}{\text{Total number of pollen grains}} \times 100$$

3.2.2.4. Pollen germination percentage

Pollen grains were kept for germination in sucrose solution and observed under microscope to count the total number of germinated pollen grains from the total number of pollen grains in the microscopic field and percentage was calculated using the formula:

$$\text{Pollen germination percentage} = \frac{\text{Number of germinated pollen grains}}{\text{Total number of pollen grains}} \times 100$$

3.2.3. Capsule characters

3.2.3.1. Days taken for pod setting after anthesis

Number of days taken from the day of pollination to greening and swelling of gynostegium (mainly ovary) present between the pedicel and sepals was logged.

3.2.3.2. Days to mature capsule harvest

Number of days from pollination to harvesting of green indehiscent mature capsule was recorded.

3.2.3.3. Length of capsule (cm)

Distance from the base of pedicel to the base of calyx of the capsule from fifteen plants was measured with a ruler and mean was calculated.

3.2.3.4. Width of capsule (cm)

Diameter of capsule was measured from fifteen plants using a ruler and mean was worked out.

3.2.3.5. Percentage of filled seeds

Seeds were scooped out from mature capsules and mixed with water. Samples were taken on a slide and from each sample five microscopic fields were observed for seeds with well filled embryo inside seed coat and calculated percentage of filled seeds.

$$\text{Percentage of filled seeds} = \frac{\text{Number of well filled embryo inside seed coat}}{\text{Total number ofseeds}} \times 100$$

3.3. EXPERIMENT II - MORPHOLOGICAL AND MORPHOMETRIC CHARACTERIZATION OF SEEDS

3.3.1. Morphological characters

3.3.1.1. Seed colour

The colour of seed mass inside capsule was observed after opening the capsule. Seed colour was visually assessed, recorded and compared based on the general orchid seed colour proposed by Barthlott *et al.* (2014). The colour of embryo was observed under light compound microscope.

3.3.1.2. Seed shape

Seed shape was visually assessed under a light compound microscope and recorded and compared based on the general orchid seed shape proposed by Barthlott *et al.* (2014).

3.3.1.3. Seed testa ornamentation

Seed testa ornamentation was visually assessed under a light compound microscope and recorded.

3.3.2. Morphometric characters

3.3.2.1. Seed length (*µ*m)

Seed length was measured under light compound microscope using LAS EZ software and mean was calculated. Length was measured at the longest axis of the seed and average was expressed in *µ*m.

3.3.2.2. Seed width (*µ*m)

Seed width was measured under light compound microscope using LAS EZ software and the mean was calculated. Width was measured at the widest axis of the seed and average was expressed in *µ*m.

3.3.2.3. Seed length/width ratio

From the observed seed length and seed width measurements, seed length/width ratio was calculated.

3.3.2.4. Seed volume (μm^3)

Based on seed length and width measurements seed volume was calculated following Prasongsom *et al.* (2017) as below:

SV= 4.19 (SL/2) $(SW/2)^2$, where SV is seed volume, SL is seed length, SW is seed width.

3.3.2.5. Embryo volume (μm^3)

Based on embryo length and width measurements embryo volume was calculated for prolate spheroid embryos following Prasongsom *et al.* (2017) as below:

EV= 4.19 (EL/2) $(EW/2)^2$, where EV is embryo volume, EL is embryo length, EW is embryo width.

3.3.2.6. Percentage of air space between seed testa and embryo

From seed volume and embryo volume percentage of air space is calculated as

$$\text{Percentage air space } = \frac{\text{SV} - \text{EV}}{\text{SV}} \times 100$$

Where SV is seed volume, EV is embryo volume.

3.3.2.7. Seed viability percentage

Seeds were extracted from mature capsules and were dipped in 1% tetrazolium solution for 24 hours. Seeds were mounted on slides and observed through microscope. Counted total number of seeds, total number of seeds stained and total number of seeds unstained present in each microscopic fields and its average was worked out. From this estimated seed viability percentage by the following formula.

$$\text{Seed viability percentage } = \frac{\text{Total number of stained seeds}}{\text{Total number of seeds}} \times 100$$

3.4. EXPERIMENT III - PREPARATION OF *IN VITRO* SEED GERMINATION MEDIA

Four different basal nutrient media (solid and liquid) supplemented with various additives were prepared for asymbiotic seed germination

Full-strength MS medium (Murashige and Skoog,1962) - solid

Full-strength MS medium - liquid

Half-strength MS medium - solid

Half-strength MS medium - liquid

Knudson C medium (Knudson, 1946) - solid

Knudson C medium - liquid

Mitra medium (Mitra *et al*., 1976) - solid

Mitra medium - liquid

Growth regulators prepared and added were BAP (6 - bezylaminopurine), IAA (Indole-3-acetic acid) and NAA (1-naphthelene acetic acid) at concentrations ranged from 0.1 to 2 $mgL^{-1.}$

pH of the medium was adjusted to 5.8 with either 1.0 N sodium hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl) before being solidified with 8 gL^{-1} agar in case of solid media. Thereafter, 10 ml /50 ml of the medium was dispensed into culture tubes/ bottles and autoclaved.

3.5. EXPERIMENT IV - STANDARDISATION OF *IN VITRO* SEED GERMINATION

Mature capsules/pods were collected from the plants after 4-4.5 months of pollination. They were soaked in aqueous solution of detergent for 10 minutes, followed by rinsing with running tap water. Capsules were then surface sterilized using 0.1% (w/v) mercuric chloride solution for 5 min. and washed systematically for 4-5 times with sterile distilled water under laminar airflow cabinet. Then the pod was dipped in absolute ethanol and the alcohol is burnt off. This was to burn off any bacteria or fungi on its surface. Pod was then placed on a sterilized surface and was cut opened longitudinally using a sterile surgical blade and the seeds were scooped out of the capsules using sterilized spatula and small mass of the aggregated seeds was inoculated on different culture media for *in vitro* asymbiotic seed germination. Observations were made by using five replicates for each medium.

3.5.1. Number of days taken for seed with chlorophyll embryo in different media after inoculation

Number of days taken for seed greening from the day of seed inoculation was observed and average was worked out in days.

3.5.2. Number of days taken for protocorm development

Number of days taken for protocorm development from the day of seed inoculation was observed and average was worked out in days.

3.5.3. Number of days taken for first leaf

Number of days taken for formation of first leaf from the day of seed inoculation was observed and average was worked out in days.

3.5.4. Number of days taken for root initiation

Number of days taken for root initiation from the day of seed inoculation was observed and average was worked out in days.

3.5.5. Number of seeds with chlorophyll embryo

Number of seeds with chlorophyll embryo was counted from different media and average was worked out.

3.5.6. Number of protocorm with shoot apex

Number of protocorm with shoot apex was counted from different media and average was worked out.

3.5.7. Number of protocorm with 1-2 leaves

Number of protocorm with 1-2 leaves was counted from different media and average was worked out.

3.5.8. Number of protocorm with two or more leaves and root (seedling)

The number of protocorm with two or more leaves and root was counted from different media and the average was worked out.

Results

4. RESULTS

4.1. EXPERIMENT I - EVALUATION OF FLORAL CHARACTERS

4.1.1. Floral characters (qualitative)

The mean performance of different traits was observed and depicted in Table 1.

The average number of inflorescences produced per plant per year was 4.6, with a mean length of 22.3 cm (Plate 2B). The average number of flowers produced per inflorescence was 7.25 with an inter-nodal length of 1.77 cm and a green pedicel length of 3.97 cm.

Vanda tessellata flowered throughout the year (from January to December). Flowers are bilaterally symmetric (zygomorphic) and resupinate. Calyx consists of three sepals, one dorsal and two laterals. The inside of the sepals is whitish at the base, greyish brown at the apex, and greenish white along the margins. Outside, the sepals are greenish-white at the base and apex and grey-brown along the margin. Corolla consists of three petals, with one of the petals modified into a labellum. Petals are white inside the base, grey brown at the apex, and greenish white along the margin, while outside the base, apex, and margin; they are greenish white and greyish brown. The sepals and petals were dark brown tessellated, the labellum was white speckled with a violet tinge inside, and the mid-lobe was lilac-blue (Plate 2D, E); they lasted about 22 days.

The anthesis period lasted from 6 a.m. to 6 p.m. Since artificial pollination was successful from morning to evening in these days, stigma receptivity remained throughout the day after anthesis and continued until the withering of the flower. The correlation analysis of the floral characters is depicted in Table 2. None of the floral characters showed significant positive or negative correlation.

Table 1: Floral characters of *Vanda tessellata*

Sl. No.	**Traits**	**Description**
1.	Number of inflorescences per plant/year	4.6
2.	Length of inflorescence (cm)	22.3
3.	Number of flowers per inflorescence	7.25
4.	Flower inter-nodal length (cm)	1.768
5.	Length of the pedicel (cm)	3.971
6.	Flower colour and pattern of tessellate marking	Green pedicel, greyish green calyx and corolla with dark brown tessellation, labellum white speckled with violet tinge inside and mid lobe lilac- blue.
7.	Flower longevity (days)	Around 22 days
8.	Nature of flowering- free or seasonal	Free
9.	Season of flowering	January - December
10.	Time of anthesis	6am - 6pm
11.	Time of stigma receptivity	Remain receptive till withering of flower.

Table 2: Correlation analysis of floral characters

Sl. No.	Characters	No. of Inflorescence / plant/year	Length of inflorescence (cm)	No. of flowers/ inflorescence	Flower internodal length (cm)	Length of pedicel (cm)
1.	Inflorescence/plant/ year	1	-0.296	-0.372	-0.184	0.565
2.	Length of inflorescence (cm)		1	0.813	0.869	0.411
3.	No. of flowers/ inflorescence			1	0.519	0.006
4.	Flower internodal length (cm)				1	0.393
5.	Length of pedicel (cm)					1

** Correlation is significant at 0.01 level.

* Correlation is significant at 0.05 level.

4.1.2. Pollen characters

Pollen grains are packed together in a pollinium (Plate 2F, G). It was smooth and ovate to obovoid; the exine was smooth and thick; and the intine was not as prominent or absent. Pollen grains are mostly in the tetrad stage (Plate 2I, J). Average pollen production per pollinium was 7,30,000. The mean pollen fertility percentage and pollen germination percentage (Plate 2K) were 98.85% and 78.2%, respectively. The pollen characters were depicted in Table 3.

Table 3: Pollen characters

Sl. No.	**Trait**	**Description**
1.	Pollen morphology	Ovate to circular, smooth, granular, exine thick and intine thin and inconspicuous, mostly tetrad form.
2.	Pollen production per pollinium	7,30,000
3.	Pollen production per pollinium	98.85%
4.	Pollen germination percentage	78.2%

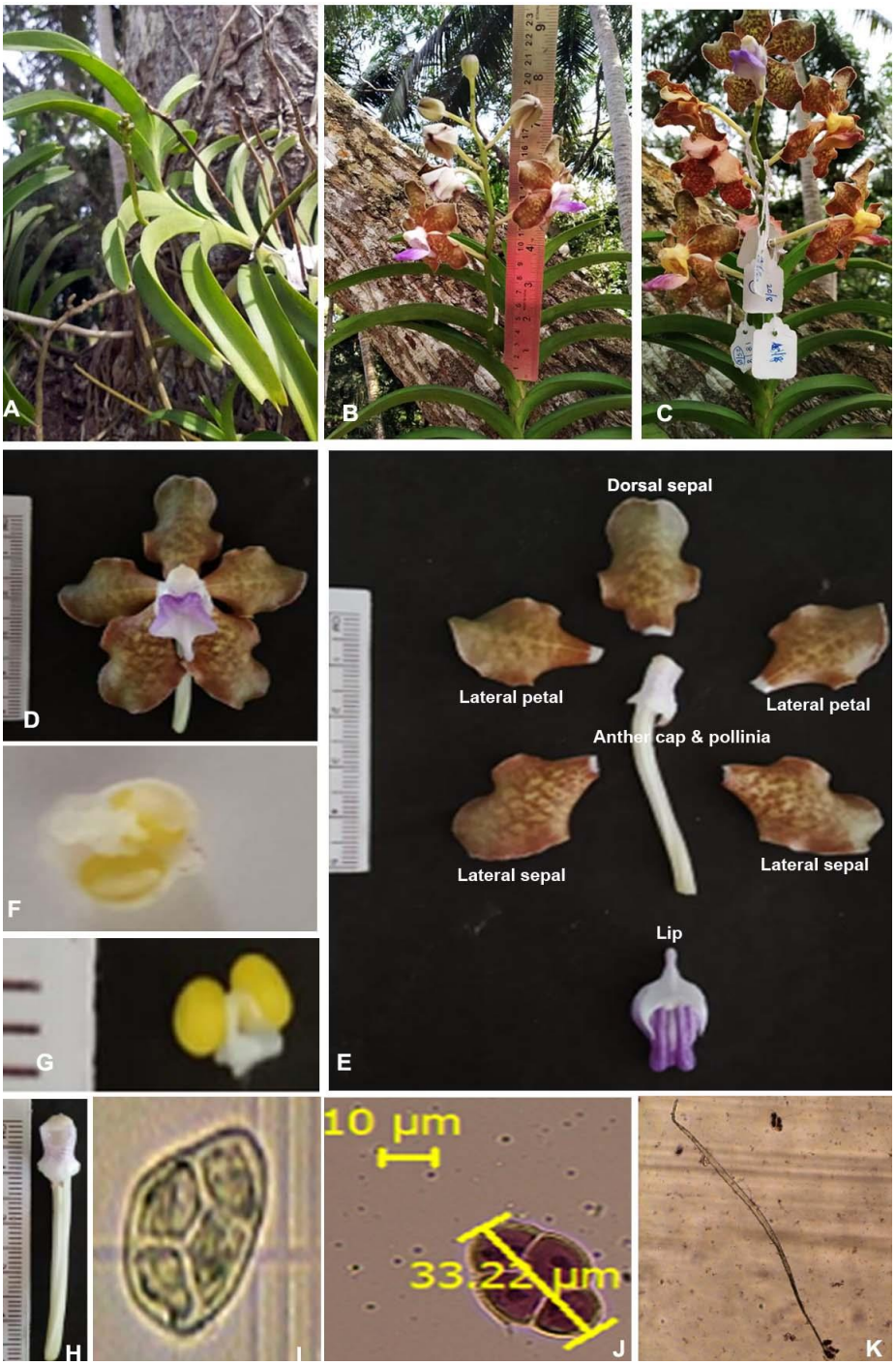


Plate 2. Floral characters: (A, B &C) - Different stages of flower opening and selfing, (D) - Single flower, (E) - A dissected out flower parts, (F) - Anther cap with pollinia, (G) - Pollinia, (H) – Gynostemium, (I) – Pollen grain in tetrad stage, (J) – Pollen size, (K) – Pollen germination.

4.1.3. Capsule characters

After pollination, flowers started to wilt, greening and swelling of the ovary occurred, and it took one week for pods to set. (Plate 3 A-F) The fertilised ovary developed into a capsule. The ripening of a capsule took 120–130 days (4–4.5 months) (Plate 3H). Capsules turned green, yellow, and brown in accordance with maturity. A mature capsule had an average length and width of 10.39 cm and 1.48 cm, respectively, with a maximum diameter towards the distal end. 76% of the seeds in the capsule have fully filled embryos. All pods were dehisced after attaining maturity, and mature seeds were dispersed. The capsule characters were shown in Table 4. The correlation analysis of capsule and pollen characters revealed that none of these characters showed significant positive or negative correlation (Table 5).

Table 4: Capsule characters

Sl. No.	**Trait**	**Description**
1.	Days taken for pod setting after anthesis	7 days
2.	Days to mature capsule harvest	120 - 135 days
3.	Length of capsule (cm)	10.396
4.	Width of capsule (cm)	1.4798
5.	Percentage of filled seeds	76%

Table 5: Correlation analysis of capsule and pollen characters

Sl. No.	Characters	Length of capsule (cm)	Width of capsule(cm)	Percentage of filled seeds	Pollen fertility percentage	Pollen germination percentage
1.	Length of capsule(cm)	1	0.605	-0.257	-0.478	0.186
2.	Width of capsule(cm)		1	0.358	-0.097	0.105
3.	Percentage of filled seeds			1	0.834	0.639
4.	Pollen fertility Percentage				1	0.719
5.	Pollen germination Percentage					1

** Correlation is significant at 0.01 level.

* Correlation is significant at 0.05 level.

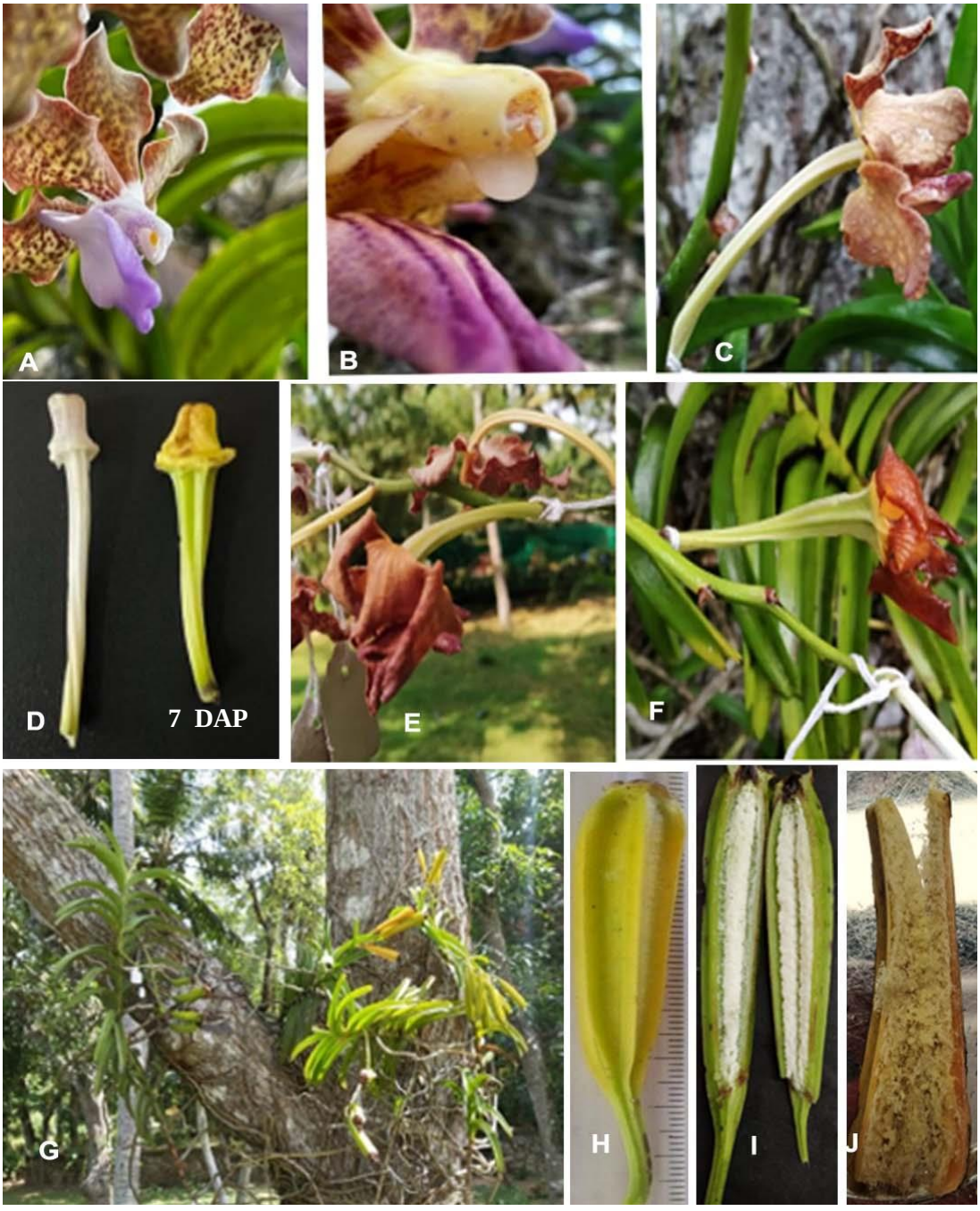


Plate 3. Capsule characters: (A- F) Fertilisation and stages of pod development, G – Plants with mature capsules, H – A mature capsule, I – An immature capsule of 60 days old, J – Yellow seed mass in a mature capsule.

4.2. EXPERIMENT II - MORPHOLOGICAL AND MORPHOMETRIC CHARACTERIZATION OF SEEDS

Mean performance of different traits was recorded in Table 6.

4.2.1. Morphological characters

Capsules, when split open, had a seed mass that appeared yellow (Plate 3J) and had a light violet embryo when seen under a compound microscope. A unique characteristic of seeds is that instead of an endosperm, there is an air space surrounding a small globular embryo within a membranous seed testa. Seeds were spathulate in shape, containing a prolate-spheroid embryo covered by a reticulated testa, giving them a characteristic rope-like appearance (Plate 4A, B).

4.2.2. Morphometric characters

The morphometric characters of seeds were depicted in Table 6. The average seed length, seed width, seed length/width ratio, embryo length, embryo width, and embryo length/width ratio of *V. tessellata* were 192.28 µm, 52.66 µm, 3.75, 126.24 µm, 38.98 µm and 3.32 µm respectively.

Seed volume was 167930.56 µm, embryo volume was 112066.344 µm, seed volume/embryo volume ratio was 1.5 with a percentage of air space 31.69%.

Seed viability was tested using 1% (w/v) 2,3,5-triphenyl tetrazolium chloride (TTC) solution and Evan's blue staining technique. Under the TTC test, seeds were scored as viable if the embryo was stained golden yellow and as non-viable if the embryo was unstained (Plate 5A). Under Evan's blue test, seeds with unstained embryos were considered viable, and those with embryos stained blue were considered non-viable (Plate 5B). The seed viability percentage observed was 70.6%.

Table 6: Morphological and morphometric characterization of seeds

Sl. No.	***Morphological characters***	**Description**
1.	1. Seed colour	Mature seeds have yellow colour
2.	2. Seed shape	Spathulate seed with prolate spheroid embryo
3.	3. Seed testa ornamentation	Reticulated testa
Morphometric characters		
4.	4. Seed length µm	192.28
5.	5. Seed width µm	52.66
6.	6. Embryo length µm	126.24
7.	7. Embryo width µm	38.98
8.	8. Seed length/width ratio	3.75
9.	9. Embryo length/width ratio	3.32
10.	10. Seed volume µm	167930.56
11.	11. Embryo volume μm^3	112066.34
12.	12. Seed volume/embryo volume ratio	1.5
13.	13. Percentage of air space	31.69%
14.	14. Seed viability percentage	70.6%

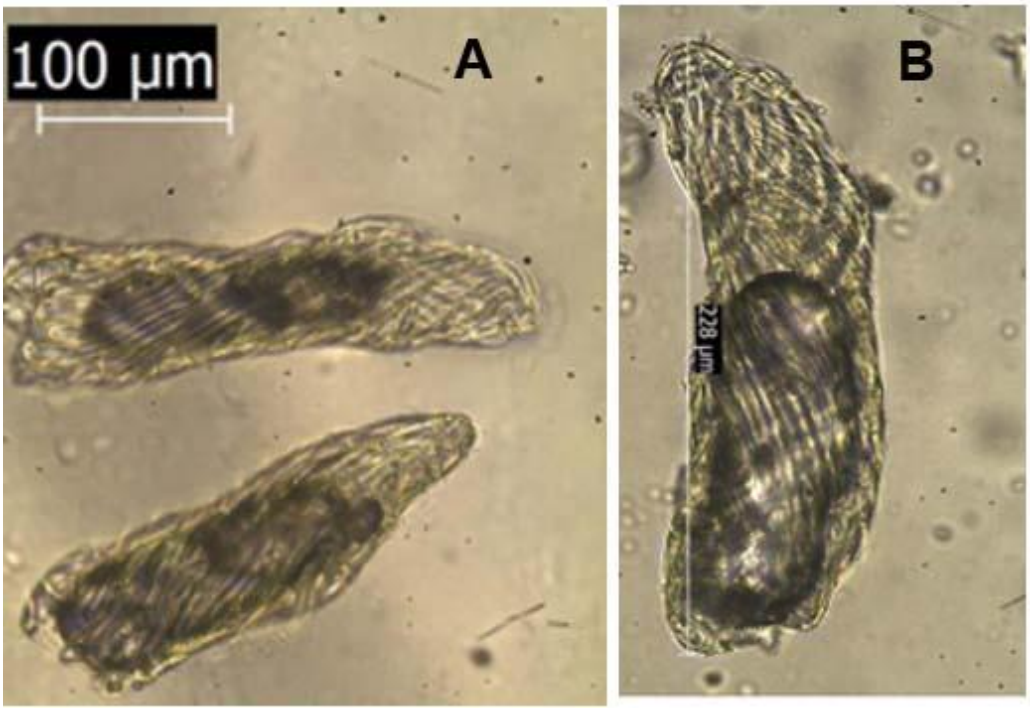


Plate 4. (A,B) Microscopic view of *Vanda tessellata* mature seeds showing reticulate testa and light violet coloured viable embryo.

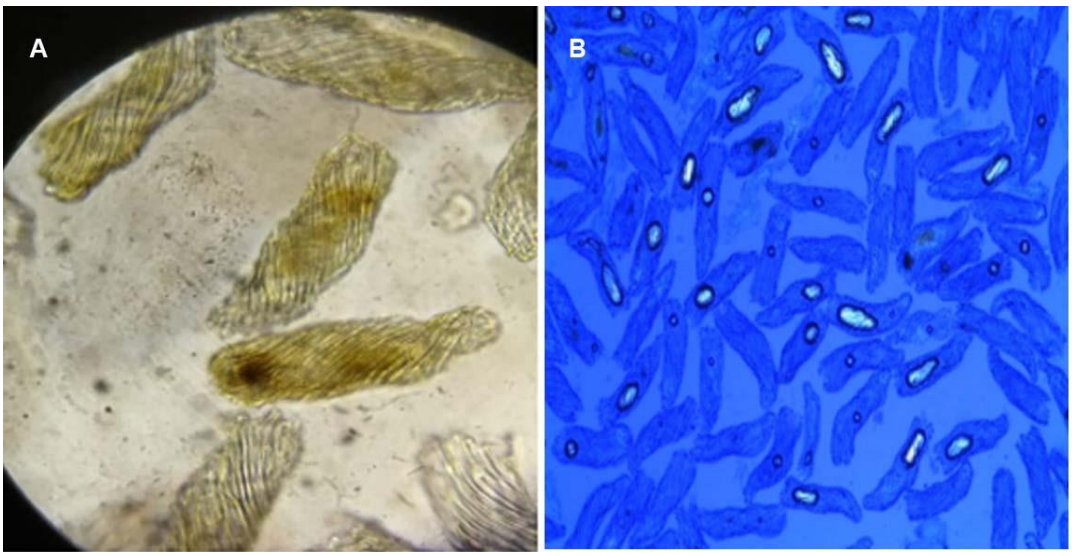


Plate 5. Seed viability staining tests, (A) - 2,3,5-triphenyl tetrazolium chloride, (B)- Evan's blue staining

Table 7: Correlation analysis of seed characters

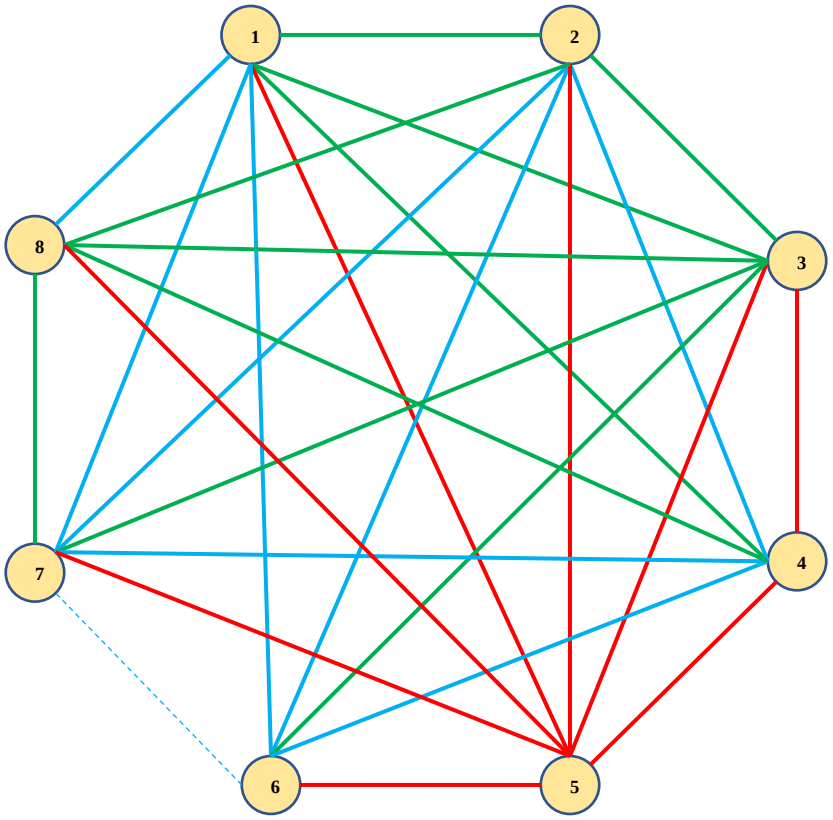
	Seed length (µm)	Seed width (µm)	Embryo length (µm)	Embryo width (µm)	Seed length / width ratio	Seed volume ($µm^3$)	Embryo volume ($µm^3$)	Air space
Seed length (µm)	1							
Seed width (µm)	0.831	1						
Embryo length (µm)	0.134	0.285	1					
Embryo width (µm)	0.753	0.914*	-0.077	1				
Seed length /width ratio	-0.487	-0.861	-0.065	-0.867	1			
Seed volume ($µm^3$)	0.906*	0.953*	0.144	0.94*	-0.735	1		
Embryo volume ($µm^3$)	0.879*	0.922*	0.052	0.957*	-0.726	0.993***	1	
Air space	0.944*	0.699	0.341	0.514	-0.27	0.757	0.702	1

*** Correlation is significant at 0.001 level.

** Correlation is significant at 0.01 level.

* Correlation is significant at 0.05 level.

Embryo width shows a positive and significant correlation with seed width. Seed volume shows a positive and significant correlation with seed length, seed width, and embryo width. Embryo volume has a strong positive and significant relationship with seed length, seed width, and embryo width, as well as a very strong positive and significant relationship with seed volume. The percentage of airspace has a positive and significant correlation with seed length.



1. Seed length
2. Seed width
3. Embryo length
4. Embryo width
5. Seed length/width ratio
6. Seed volume
7. Embryo volume
8. Air space

Positive

Negative

Positive significant

Highly positive significant

Fig. 1. Diagrammatic representation showing correlation of seed characters

4.3. EXPERIMENT III - PREPARATION OF *IN VITRO* SEED GERMINATION MEDIA

Four different basal nutrient media, *viz.*, full-strength MS, half-strength MS, Knudson C, and Mitra media (solid and liquid), supplemented with various growth regulators (BAP, IAA, and NAA), were prepared for asymbiotic seed germination. In the case of solid media, the pH of the medium was adjusted to 5.8 with either 1.0 N sodium hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl) before solidification with 8 g^{-1} agar. Thereafter, 10 ml /50 ml of the medium was dispensed into culture tubes/ bottles and autoclaved.

4.4. EXPERIMENT IV - STANDARDISATION OF *IN VITRO* SEED GERMINATION

Flow chart showing different stages of seed germination and plantlet formation in *in vitro* is depicted in Plate 8.

4.4.1. Number of days taken for seeds with chlorophyll embryo

The seeds in the mature capsule were minute, spathulately shaped, and embryonate but non-endospermic. The seeds were enlarged in size within two weeks in all the media tested, but their further development was dependent on the media composition and supplemented hormonal concentrations. In the basal media, most of the enlarged embryos became browned and disintegrated after 6-7 weeks, and no further growth was observed even after 10 weeks of inoculation. Among the solid and liquid media tested, initial embryonic development was more prominent in the liquid medium. However, it was discovered that keeping developing embryos in liquid medium had a negative impact on their future growth. Most of those embryos were browned and died when they were kept in the same medium for a prolonged period, *i.e.*, more than one month. Among the basal media tested seed germination was noticed in Knudson C, Mitra, and MS while in ½ MS sign of seed germination was not observed even after two months. Among the different media supplemented with various concentrations of BAP (0.1 to 1.0 mgL^{-1}), half MS and full strength MS showed better performance than others. The cultures were kept under alternate 12-hour light and 12-hour dark and 24-hour dark conditions, but only the 12-hour alternate light and dark conditions resulted in embryonic development and greening. The embryo's development with greening was observed after 45 days of inoculation in MS liquid medium supplemented with 0.5 mgL^{-1} BAP, while in the same medium without BAP the same observation was noticed only after 50 days. In half MS both liquid and solid medium supplemented with 0.5 mgL^{-1} BAP embryo

development and greening was noticed after 60 days. All other media, both liquid and solid, didn't show any significant seed germination. The number of days required for the development of green embryos in different nutrient media is depicted in Table 8.

Microscopic observation of the germinating embryos at different period of germination in progress revealed that after 15 days the embryo were enlarged in size (Plate 6 A-C). After a month a beak-like structure was formed at terminal region of the embryo and subsequently the seed coat ruptured and exposed the beak-like projection (Plate 6 D,E). After two months of inoculation these embryos were completely emerged out of the seed coat forming into a protocorm which appeared more or less round (Plate 6F). Subsequently, after 3 months of inoculation, an apical notch was formed on the protocorms which was later developed in to small leaf primordium at the apex. Apart from apical leaf primordia, root primodia was also evoked at the basal region (Plate 6 G-J). Then, leaf primordia expanded to develop leafy shoots (Plate 6 K, L) which was followed by root induction at the basal region of the protocorm to form complete plantlet and it take place for a period of 6-7 month of inoculation of seeds into the medium for germination. Apart from these, during the developmental stages, from embryos multiple protocorms were emerged (Plate 6M) which on subculturing produced multiple shoots (Plate 7 I).

4.4.2. Number of days taken for protocorm formation

In MS basal liquid and MS liquid supplemented with 0.5 mgL^{-1} BAP, protocorm formation was noticed after 75 days. While in half-strength MS liquid and solid media supplemented with with 0.5 mgL^{-1} BAP the foregoing observation was only noticed after 90 days (Plate 7C). There was no protocorm development in Knudson C media and Mitra media after seed greening (Table 8), as germinated embryos turned brown and disintegrated into these media.

4.4.3. Number of days taken for first leaf

The germinated embryos with a mass of multiple shoots (20-30) within 120 days. Subculture of these shoots on fresh agar gelled medium resulted to the emergence of first leaves (Plate 7 D, E) and these leaves were more prominent after 150 days of culture. In half-strength MS medium supplemented with 0.5 mgL^{-1} BAP required 180 days for the same observation.

4.4.4. Number of days taken for root initiation.

The first root appeared after the formation of the first leaf when it was subcultured onto MS medium supplemented 0.5 mgL^{-1} BAP and 0.5 mgL^{-1} NAA (Plate 6 F,G). To reach this phase total 210 days culture period was required.

4.4.5. Number of seeds with chlorophyll embryo

The numbers of seeds with chlorophyll embryos obtained in each 250 ml culture bottle were counted after 75 days of culture. The average number of developing embryos in MS liquid medium was 9.5, 86.33 in Knudson C liquid, 72.5 in Mitra liquid, 36 in half MS liquid supplemented with 0.5 mgL^{-1} BAP, 2 in half MS solid supplemented with 0.5 mgL^{-1} BAP, and 39.75 in MS liquid supplemented with 0.5 mgL^{-1} BAP.

Analysis of variance revealed that Knudson C (liquid) was the best medium for the formation of seeds with chlorophyll embryos. Mitra medium (liquid) was on par with Knudson C medium for the formation of seeds with chlorophyll embryos (Table 8.).

4.4.6. Number of protocorm with shoot apex

The average number of protocorm with shoot apex in different media were observed as follows: MS media liquid 7.5, Half MS liquid supplemented with 0.5 mgL-1BAP - 37, Half MS solid with 0.5 mgL-1BAP – 2 and MS liquid with 0.5 mgL-1BAP - 41.75.

Analysis of variance revealed that the best medium for protocorm formation and development was MS supplemented with 0.5 mgL-1BAP (liquid) followed by half MS medium supplemented with 0.5 mgL-1BAP (liquid).

4.4.7. Number of protocorm with 1-2 leaves in different media

The average number of protocorm with leaves differentiation was as follows:

MS medium liquid - 7, half MS liquid augmented with 0.5 mgL^{-1} BAP - 36.3, half MS solid with 0.5 mgL^{-1} BAP - 2 and MS liquid with 0.5 mgL^{-1} BAP - 38.75 respectively.

Analysis of variance revealed that the best media for leaf formation was MS medium supplemented with 0.5 mgL^{-1} BAP (liquid) and half MS media with 0.5 mgL^{-1} BAP (liquid).

From all these observations made, it is concluded that the best medium for seed germination and greening was liquid medium of Knudson C followed by Mitra and MS supplemented with 0.5 mgL^{-1} BAP. However, further growth was not significant in Knudson C medium. The best medium

for further protocorm development and shoot initiation was MS supplemented with mgL^{-1} BAP (liquid). For leaf and root development the best medium was MS solid supplemented with 0.5 mgL^{-1} BAP and 0.5 mgL^{-1} NAA. Some protocorms that were inoculated into MS medium (solid) supplemented with 1 mgL^{-1} BAP and mgL^{-1} NAA produced multiple shoots. Therefore, the best media for multiple shoot production was MS 1 mgL^{-1} BAP + 1 mgL^{-1} NAA (solid).

4.4.8. Number of protocorm with two or more leaves and root (seedling)

All the protocorms with 1-2 leaves differentiated were further developed into more number of leaves.

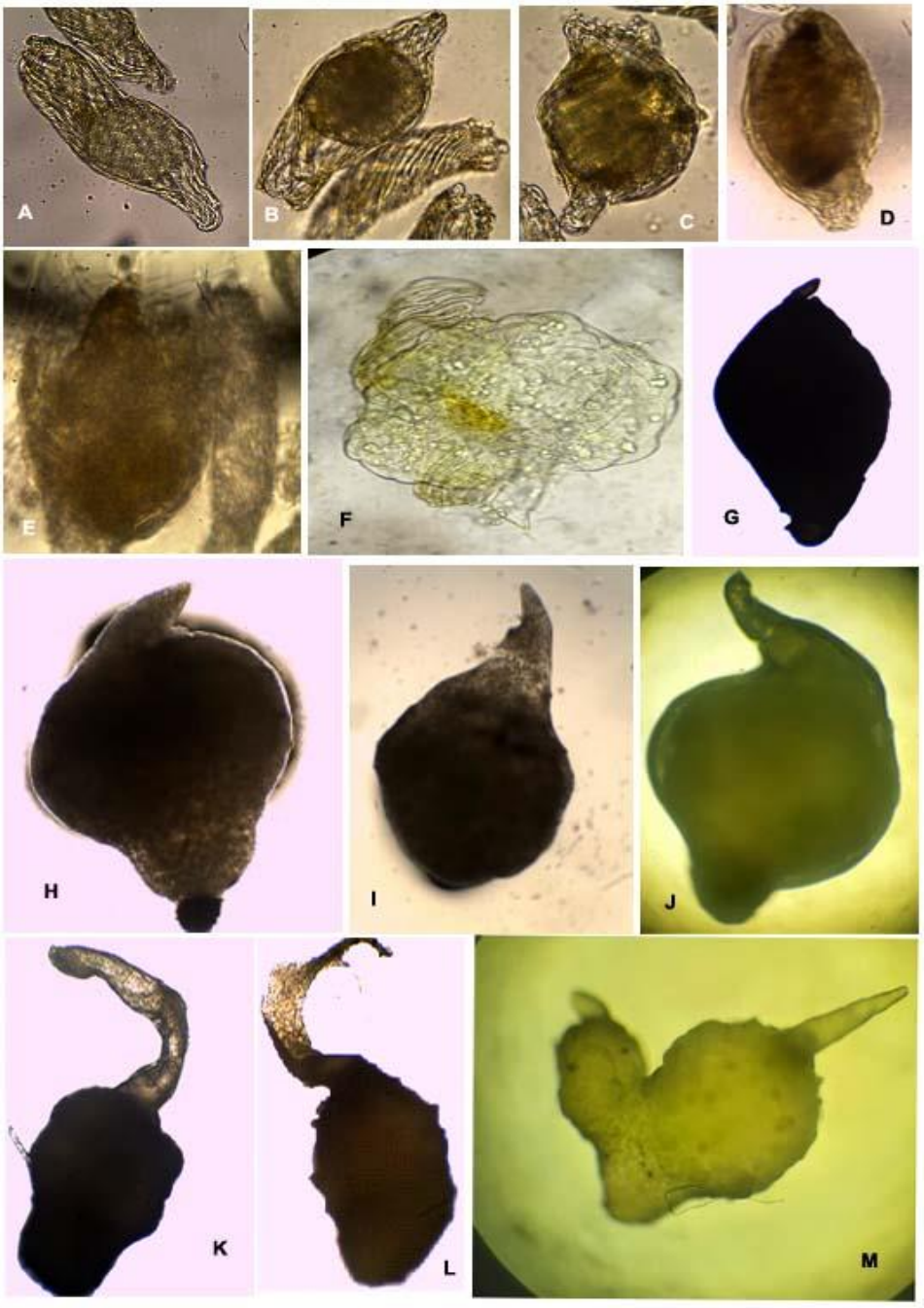


Plate 6. Microscopic view of different stages of embryo development in *in vitro*: (A -C) Bulging of embryo after inoculation, (D,E) Embryo forming into a beak like structure, (F) Complete rupturing of seed coat, (G-J) Formation of protocorm with leaf and root primordia, (K,L) Expansion of shoot, (M) Multiple protocorms

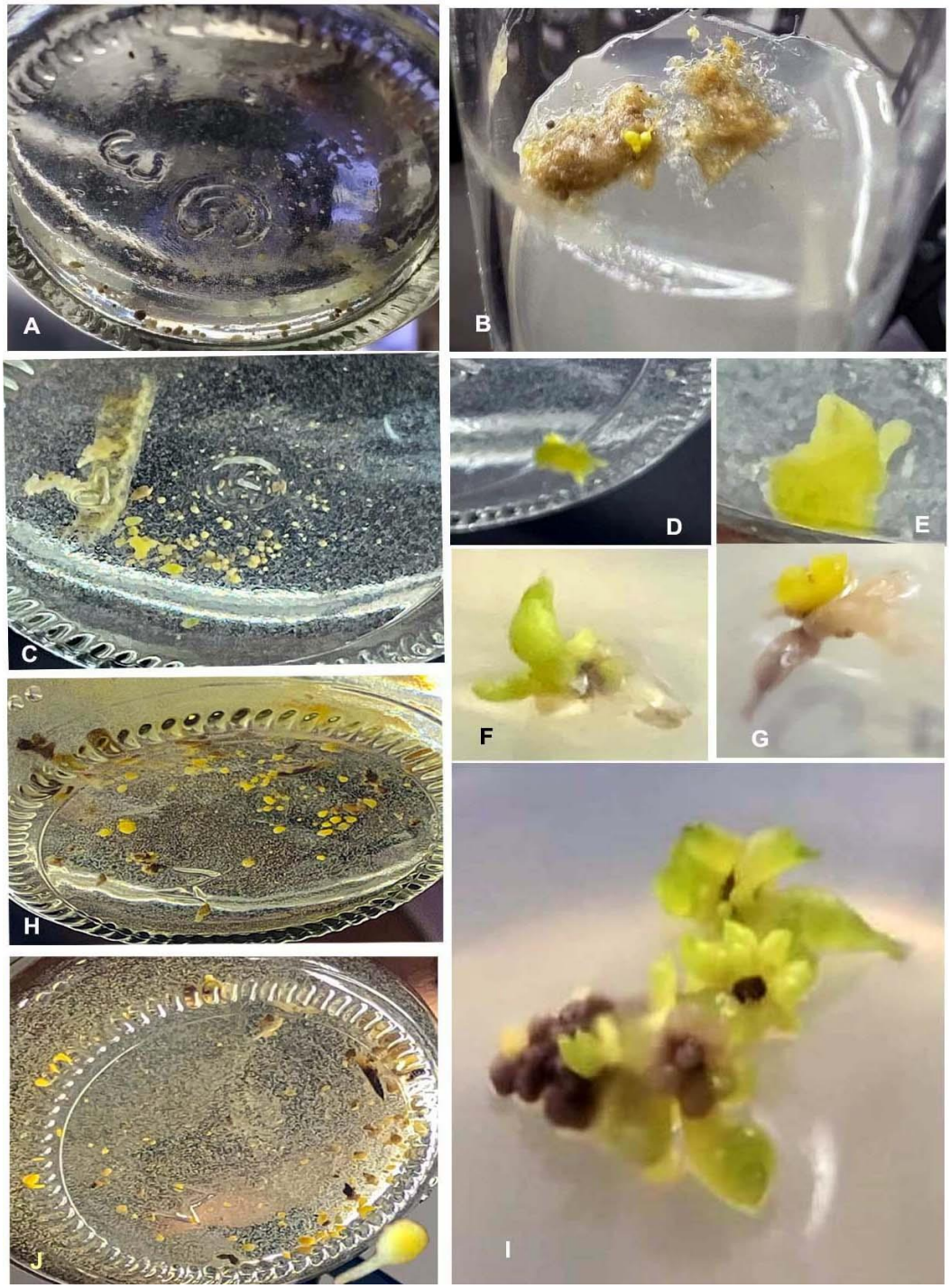


Plate 7. Stages of *in vitro* seed germination: Seed germination in (A) liquid medium, (B) solid medium, (C) Protocorm formation, (H, J) Enlargement of protocorms with leaf and root primordia (D, E) Leaf formation, (F, G) Leaf and root development (I) Multiple shoot formation

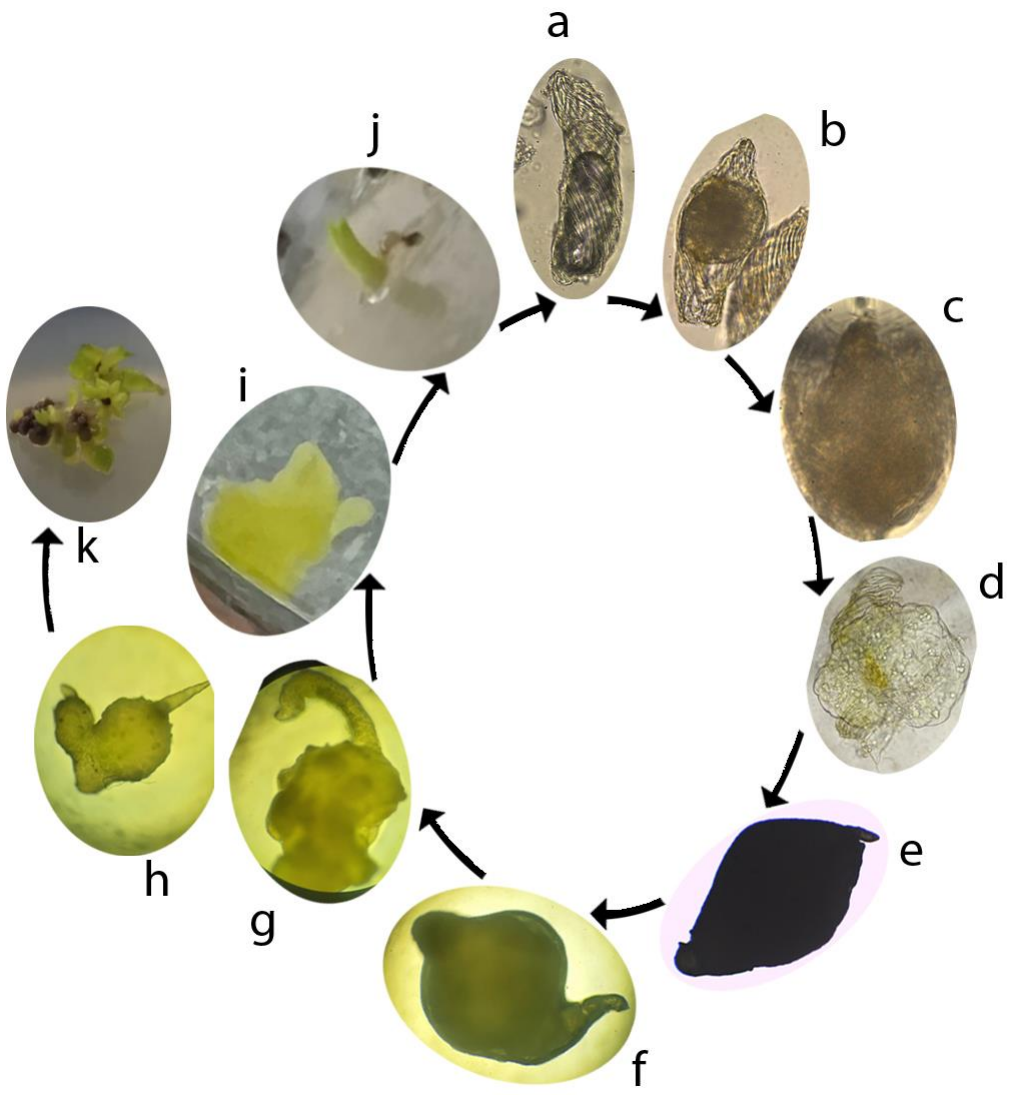


Fig.2. Flow chart showing different stages of seed germination and plantlet formation in *in vitro*. (a) Mature seed, (b) 15 days after inoculation, (c) 30 days after inoculation – embryo formation into a beak like structure, (d) 45-60 days after inoculation – complete rupturing of seed coat, (e) 3 months after inoculation – protocorm formation, (f) 4 months after inoculation – embryo with leaf and root primordia, (g) Enlargement of shoot, (h) multiple protocorm, (i) 5 months after inoculation – first leaf formation, (j) 7 month after inoculation – leaf and root development, (k) multiple shoot formation.

Table 8. Standardisation of *in vitro* seed germination

Sl. No.		MS (liquid)	KC (liquid)	Mitra (liquid)	Half MS 0.5BAP (liquid)	Half MS 0.5BAP (solid)	MS 0.5BAP (liquid)
1.	Number of days taken for seed with chlorophyll embryo in different media after inoculation	52.5	45	45	60	60	45
2.	Number of days taken for protocorm development	77.5	-	-	90	90	75
3.	Number of days taken for first leaf	150	-	-	180	180	150
4.	Number of seeds with chlorophyll embryo	9.5	86.3	72.5	36	2	39.75
5.	Number of protocorm with shoot apex	7.5	-	-	37	2	41.75
6.	Number of protocorm with 1-2 leaves	7	-	-	36.3	2	38.75

Table 9. LSD test Matrix of CD values of number of seeds with chlorophyll embryo

	HMS 0.5BAP (l)	HMS 0.5BAP(s)	KC (l)	M (l)	MS (l)	MS 0.5BAP (l)
HMS 0.5BAP (l)	0					
HMS 0.5BAP(s)	36.139	0				
KC (l)	36.139	40.405	0			
M (l)	40.405	33.805	36.139	0		
MS (l)	40.405	40.405	33.805	40.405	0	
MS 0.5BAP (l)	40.405	33.805	44.261	38.331	38.331	0

Table 10. LSD test Matrix of CD values of number of protocorm with shoot apex

	HMS 0.5BAP (l)	HMS 0.5BAP(s)	MS (l)	MS 0.5BAP (l)
HMS 0.5BAP (l)	0			
HMS 0.5BAP(s)	23.828	0		
MS (l)	26.640	22.289	0	
MS 0.5BAP (l)	26.640	22.289	25.273	0

Table 11. LSD test Matrix of CD values of number of protocorm with 1-2 leaves

	HMS 0.5BAP (l)	HMS 0.5BAP(s)	MS (l)	MS 0.5BAP (l)
HMS 0.5BAP (l)	0			
HMS 0.5BAP(s)	21.462	0		
MS (l)	23.995	20.076	0	
MS 0.5BAP (l)	23.995	20.076	22.764	0

Discussion

5. DISCUSSION

Vanda tessellata, one of the native orchids in India, is vulnerable to extinction because of the unscrupulous overexploitation and destruction of its native habitat. This wild species has many desirable characters for commercial exploitation in the floriculture industry, and the plant breeders have already developed commercial hybrids using it as one of the parents. Furthermore, this species has unique medicinal properties that have all been thoroughly researched. Given its population loss and commercial utility in a variety of fields such as floriculture, medicine, and food formulation, strategies and technologies for the conservation and sustainable use of this crop are critical. In order to achieve the foregoing goal, an in-depth inquest on the morphological, morphometric, and reproductive aspects of the species and its large-scale multiplication technique through the application of biotechnological intervention is a prerequisite. In these circumstances, the present study is the most relevant one.

5.1. EVALUATION OF FLORAL CHARACTERS

5.1.1. Floral characters

Monopodial orchids either have year-round flowering or seasonal blossoming. *V. tessellata* is a monopodial orchid having year-round flowering from January to December, which is in agreement with the findings of Matthew (1995), Seidenfaden (1999), and Seeja (2018). The plants have a wide tolerance for temperature (26-31ºC) and low relative humidity (31–44%) (Jayaweera, 1981). while some other Vanda species, like Vanda John Club, Vanda Popoe Diana, Vanda Ruby Prince, and *Vanda spathulata*, have seasonal flowering (Thomas, 2008).

It is a free-flowering species; the average number of inflorescences produced per plant per year was 4.6, as observed in *Vanda spathulata.* The inflorescence of *V. tessellata* lasts for 2–3 months, and generally, new inflorescence occurs after the complete wilting of the previous spike; if pod set occurs, then new inflorescence emergence occurs only after the dehiscence of all the capsules on the plant. Therefore, in natural conditions, a plant can produce an average of 4.6 inflorescences per year. As reported earlier (Seeja, 2018), the average inflorescence length was 22.3 cm, and the number of flowers produced per spike was 7.25, with a maximum of 8 flowers in some inflorescences and a minimum of 5 flowers in others. These observations were in agreement

with the findings of Islam *et al.* (2016) and Sahu and Chaudhary (2021). The reported floral characters, such as a green pedicel, a greyish green calyx and corolla with dark brown tessellation, a white speckled labellum with a violet tinge inside, and a lilac-blue mid-lobe, were corroborated by the observation of De *et al.* (2021).

In most species of orchids, anthesis was observed from early morning to noon. But in *V. tessellata,* anthesis was from morning to evening in a day (6 a.m. to 6 p.m.), and the same observation was recorded by Seeja (2018). Stigma receptivity remained throughout the day after anthesis and continued until the withering of the flower. However, Seeja (2018) has recorded stigma receptivity up to 6 days only.

Under natural conditions, the longevity of an unfertilized flower was found to be around 22 days, and this observation is in agreement with the earlier reports of Garay (1972) and Sahu and Chaudhary (2021). After pollen is removed from the stamens and stigmas, pollination-supporting features such as flower scent and floral colour diminish, and the flower begins to enter senescence and gradually withers within 4 days. The same findings were reported by Schiestl *et al.* (1997), Evanhoe and Galloway (2002), and Theis and Raguso (2005).

In *Aerides crispum* and *Cattleya* sp., flower longevity was only six days (Anand *et al.*, 2013). The genetic makeup of the species determines the longevity of flowers on the plant (Sakai *et al*., 1995; Nagare and Pal, 2008).

The mean pedicel length in this species was 3.97 cm, which is in agreement with the general orchid pedicel length ranges reported by Vanlalruati *et al.* (2016) and Anand *et al.* (2013).

The average flower internodal length in *V. tessellata* was 1.76 cm. The flower intermodal length may vary from species to species (Thomas, 2008).

5.1.2. Pollen characters

Pollen is ovate to obovoid and smooth; exine is smooth and thick; and intine is not so prominent or absent. Microscopic observations showed that pollen grains mostly existed as tetrads. This is in agreement with the reports of Abraham and Vatsala (1981); Das and Goshal (1988); Varghese (1995); and Sobhana (2000).

Pollen production per pollinium averages 7,30,000. Given the fact that there are millions of ovules in an orchid flower, to obtain a sustainably good number of fertile seeds, sufficient pollen loading has to be ensured. In orchids, pollen-to-ovule number ratios are several orders of magnitude lower than in other angiosperms with powdery pollen due to the lack of wastage during transport to the stigmatic surface (Lukasiewicz, 1999).

The mean pollen fertility percentage was observed by staining with acetocarmine. Almost all the pollen grains were viable, with a pollen fertility percentage of 98.85%, the same observation was made by Seeja (2018).

In vitro pollen germination showed a percentage of 78.2. The 100% success rate of artificial pollination was also an indication of the ability of pollen grains to form well-developed pollen tubes leading to the ovary.

5.1.3. Capsule characters

As reported by Dongarwar and Thakur (2014), after pollination, flowers started to wilt, greening and swelling of the ovary occurred, and it took one week for pods to set. It is a general observation in orchids that indicates the effectiveness of pollination and fertilization. The ripening of a capsule took 4–4.5 months. This is not in agreement with the report of PhytoTech Labs (2019), where it took 6–9 months or even up to 20 months to attain maturity.

According to Slater (1991), capsules matured to a yellowish green colour. A mature capsule had an average length and width of about 10.39 cm and 1.48 cm, respectively, with a maximum diameter towards the distal end.

The capsules dry and shrivel, and millions of seeds escape through the grooves running longitudinally. The seeds are generally almost microscopic and very numerous—over a million per capsule. 76% of these were filled seeds, meaning they had well-filled embryos within, and this may be due to the production of an immense quantity of seeds in a capsule. Generally, orchids produce innumerable viable seeds since the seeds are non endospermic and the chance of germination is very low.

Generally, orchids have dust-like seeds that are minute in size and weight; thus, they are ideal resources for seed banking, enabling large volume storage without the need for large facilities (Magrini *et al*., 2019). According to Kauth *et al*. (2008), epiphytic orchids commonly have dry cracks in the seed testa and usually exhibit higher viability, especially with TTC. The high seed viability percentage (70.6) was observed when staining with tetrazolium, which means it ensures germination under appropriate conditions. The viability test aids in the reduction of errors in the early stages of research, thus speeding up the research process, and improves the efficiency of species conservation measures like seed banking (Pradhan *et al*., 2022).

5.2. MORPHOLOGICAL AND MORPHOMETRIC CHARACTERIZATION OF SEEDS

5.2.1. *Morphological characters*

According to Barthlott (1976), the colour of the seed is the colour of the testa, and here the seed mass possessed a yellow and a light violet embryo.

Fusiform-shaped seeds and prolate-spheroid embryos are the most common shapes in the Orchidaceae family (Diantina *et al.*, 2020). *V. tessellata* seeds were spathulate in shape, containing a prolate-spheroid embryo covered by a reticulated testa, giving them a characteristic rope-like appearance. This may help with the dispersal of seeds.

5.2.2. *Morphometric characters*

Seeds are very small since the size was 192.28 µm which is consistent with the result of Molvray and Kores (1995), where the orchid seed size varies from 150 to 6000 µm.

According to Arditti *et al*. (1980) and Augustine *et al*. (2001), the length/width ratio provides an idea of the relative degree of truncation of the seeds in orchids. In *V. tessellata* seeds were truncated as they had a SL/SW ratio 5 (3.75). It has possessed a prolate spheroid embryo, which is in agreement with the result of Diantina *et al*. (2020). They have also reported that seeds with EL/EW ratios above 1 have prolate-spheroid-shaped embryos.

The volume of the embryo changes during the development of the seed. In an immature capsule of rwo months' duration, seeds were in the developing stage, and embryos were

undifferentiated. While 5-month-old mature capsules had fully developed seeds with fully filled embryos of a larger volume, this was corroborated with the findings of Swamy *et al*. (2004).

According to Augustine *et al.* (2001), seeds with a greater percentage of air space may get dispersed over wider geographical areas than the seeds with the minimum air space. Terrestrial species have a bigger SV/EV ratio than epiphytic species. Thus they are more buoyant than those with lower ratios, enabling wider plant distribution ranges (Verma *et al*., 2014). This statement was supported by the findings of Diantina *et al.* (2020) in temperate terrestrial species *D. cunninghamii*, *G. cunninghamii*, and *P. banksii*, compared to the tropical epiphyte orchid *D. lineale*. *V. tessellata* seeds have a lower percentage of air space (31.69%), and their SV/EV ratio of 1.5 shows that they are adapted to restricted or localised distribution.

5.3. STANDARDISATION OF *IN VITRO* SEED GERMINATION

The orchids produce an enumerable number of microscopic seeds, but they are non-endospermous, and therefore mycorrhizal association is inevitable for their germination. In this circumstance, the percentage of seed germination in orchids is very low. In order to overcome the foregoing problem, *in vitro* seed germination is the best option, and this technique has been widely used for the commercial multiplication of orchids. The nutrients and physical conditions required for orchid seed germination differ between species. Hence, four different nutrient media, such as MS, ½MS, Knudson C, and Mitra were tried. For seed germination, both liquid and agar-gelled media were tried by various authors, and the response depended on the orchid species. In certain orchids, liquid medium enhanced the initial germination percentage, and subsequent transfer of the germinated seeds into the agar gelled medium promoted shoot and root development (Seeja 2018). Plant growth regulators play an important role in the development of shoots and roots. Although many orchid seeds are germinated in hormone-free nutrient media, for the rapid, high-percentage germination, the development of shoots and roots, and the eventual formation of multiple plants from a single embryo, nutrient media supplemented with growth hormone are inevitable. Therefore, both basal and hormone supplemented media were tried for seed germination.

As reported earlier (Paul *et al*., 2012), the seeds were enlarged within 1-2 weeks, but all those enlarged seeds were not further developed into a plant, this indicated the requirement of growth hormone for further development. It was noted that the seeds of *V. tessellata* germinated in

the absence of any exogenous PGRs. This is in agreement with the findings of Aditya *et al. (*2020) and Yildiz (2012). This might be due to the presence of adequate amounts of endogenous growth regulators in the seed tissue, which are needed at the initial stage of germination (Hajong *et al*., 2010; Islam *et al*., 2014). Specific endogenous growth-promoting and growth-inhibiting compounds are directly involved in controlling seed development, dormancy, and seed germination (Hartman *et al*., 1997). However, for the germinated embryos' further development, growth hormone is found to be essential. Germination can be dependent on the choice of media due to their differences in the balance and supply of organic and inorganic salts.

The greening of the embryo may be due to the formation of chlorophyll in the presence of light. When compared to solid medium, the sign of seed germination was more prominent in liquid medium, but solid medium favoured further embryonic development. It is a well-established fact that the seeds were imbibed easily in the nutrient liquid medium that evoked the embryos' germination, but in static liquid, proper gaseous exchange (supply of O_2) will not take place, and as a result, the germinating embryos will die and brown, subsequently disintegrating into the medium.

The microscopic observations of the germinating embryos at different stages were in agreement with the earlier report in Dendrobium (Gurudeva, 2019). The enlarged embryo then emerged from the seed coat and developed into a protocorm, which appeared more or less round as reported by Setiari *et al*. (2016) since the protocorm phase usually begins when the bipolar structure that cannot be distinguished between rhizoids and shoots forms the apical portion. Subsequently, those quickly turned green, and an apical notch was formed, which was later developed into a small leaf primordium at the apex. Apart from apical leaf primordia, the protocorms developed root primodia at the basal region. Later, leaf primordia expanded to develop leafy shoots, which were followed by root induction at the basal region of the protocorm to form a complete plantlet. These developmental stages are common for most of the orchid species, especially epiphytic orchids (Gurudeva, 2019). Apart from these developmental stages, some seeds developed into multiple protocorm-like bodies, which, on subculturing, produced multiple shoots in MS medium (solid) supplemented with 1 mgL^{-1} BAP and 1 mgL^{-1} NAA. This is in agreement with the findings of Bhattacharjee and Islam (2014) and Rahman *et al*. (2009).

The frequency of seed germination was the highest in Knudson C (86.33), followed by Mitra (72.5), and MS liquid with 0.5 mgL^{-1} BAP (39.75). This is in agreement with the report of Roy and Banerjee (2002). And this finding is against the observation made by Bindiya *et al.* (2012) and Bhattacharjee *et al*. (2015), who found MS basal medium to be the best medium for germination.

Though seed germination frequency was the highest in Knudson C and Mitra media, the rate of survival of the germinated seeds was remarkably low in these media. After germination, the protocorms did not survive, which might be due to the lack of an adequate amount of nutrients and/or essential growth-stimulating substances (Stoutamire, 1974).

Out of all the seed germination media, the highest survival rate and lowest necrosis were noted in MS liquid with 0.5 mgL^{-1} BAP, which could be due to the presence of an adequate amount of nitrogen in the form of ammonium nitrate in the MS medium, whereas in Knudson C and Mitra, nitrogen was present in the form of ammonium sulphate (Paul *et al.,* 2012).

In MS (liquid) and MS liquid with 0.5 mgL^{-1} BAP, protocorm formation took about 75 days. And for half MS liquid with 0.5 mgL^{-1} BAP and half MS solid with 0.5 mgL^{-1} BAP, it took around 90 days. The maximum number of protocorms with shoot apex was observed in MS liquid with 0.5 mgL^{-1} BAP (41.75), followed by half MS liquid with 0. 5 mgL^{-1} BAP (37), MS media (7.5), and half MS solid supplemented with 0. 5 mgL^{-1} BAP (2).

The first leaf appeared after approximately 150 days of inoculation in MS liquid with 0.5% BAP followed by the same agar gelled medium, with the highest frequency of protocorms having 1-2 leaves.

The first root appeared after the formation of the first leaf in the seedling when it was subcultured onto solid MS media with 0.5 mgL^{-1} BAP and 0.5 mgL^{-1} NAA in about 7 months. This indicates that the formation of the shoot and root required an equal amount of cytokinin and auxin.

The best medium for initiation of seed germination was not ideal for further seed development. The best medium for seed germination was Knudson C, but for further development of seedlings, MS medium supplemented with 0.5 mgL^{-1} BAP was the best. This observation is in

agreement with the reports of Kauth *et al.* (2008) and Koene *et al*. (2019). NH^{4+} and NO^{3-} ions (individually or in combination) have a pivotal role in the *in vitro* germination of orchid seeds as well as the growth and development of the germinated propagules (Dohling *et al*., 2008; Poothong and Reed, 2016; and Zhang *et al*., 2019). The enhanced protocorm development in MS medium may be due to the presence of a rich source of macro- and microelements (Hossain *et al.*, 2009; Paul *et al*., 2012; and Bhowmik and Rahman, 2017). The addition of BAP to the basal medium also stimulated the survival rate of germinated propagules and had an effect on the morphogenesis of the protocorms.

Summary

6. SUMMARY

Vanda tessellata (Roxb.) Hook. ex G. Don. is a monopodial epiphytic orchid with a wide array of medicinal and ornamental values. Hence, the species has been overcollected from its natural habitat, coupled with unscrupulous habitat destruction for urbanization, leading to the verge of extinction of this species in the wild, and the IUCN has identified this species as a conservation priority. Moreover, the unique characteristic features of this species, such as attractive flowers with tessellate marking, fragrance, compatibility with intra- and inter-generic wild and commercial hybrids, as well as medicinal properties, invited the breeders, who produced several commercial hybrids using this species as one of the parents. The possibility of more elegant and superior-quality hybrid production using this species is a rewarding one. It can be achieved only through the formulation of pragmatic strategies for its conservation and the development of appropriate technologies for its sustainable utilisation. To achieve the foregoing goals, in-depth analysis of morphology, morphometry, reproductive biology, and the development of an *in vitro* seed germination protocol are required. In these backdrops, the present investigation, entitled "Seed characterization and standardisation of *in vitro* germination protocol in Vanda tessellata (Roxb.) Hook. ex G. Don," was carried out for the standardisation of *in vitro* seed germination protocol in *V. tessellata* along with morphological and morphometrical characterization of seeds.

The experiment was conducted at the Saraswathy Thangavelu Extension Centre of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Puthenthope, Thiruvananthapuram, and at the College of Agriculture, Vellayani, Thiruvananthapuram, during 2021–22. The *V. tessellata* germplasm repository maintained in STEC-JNTBGRI was utilised for morphological characterization and in vitro seed germination studies. The present investigation included four experiments: (i) evaluation of floral characters; (ii) morphological and morphometric characterization of seeds; (iii) preparation of *in vitro* seed germination media; and (iv) *in vitro* seed germination in culture media and evaluation.

Vanda tessellata is a monopodial epiphytic orchid that blooms from January to December. Inflorescence is an axillary, erect, and simple raceme of medium length (22.7 cm) bearing an average of seven flowers with an average internodal length of 1.77 cm. It takes around one month from inflorescence emergence to first flower opening, and 4-5 days from first flower opening to

last flower opening. Flowers are bilaterally symmetric (zygomorphic), resupinate, and have two petals, one dorsal sepal, two lateral sepals, and a labellum. They had a green pedicel with a mean length of 3.97 cm, a greyish green calyx and corolla with dark brown tessellation, a white speckled labellum with a violet tinge inside, and a lilac-blue mid-lobe. The time of anthesis was from morning to evening in a day (6 a.m. to 6 p.m.), and under natural conditions, the longevity of an unfertilized flower was found to be around 22 days. But after pollen removal from the stamens and pollination of the stigma, flower scent and colour reduce, and the flower starts to senesce and will wither in about 4 days. Stigma receptivity remained throughout the day after anthesis and continued until the withering of the flower.

The male and female reproductive parts are fused together and become the gynostemium, or column. Pollen grains are packed together as a pollinium with two waxy, globular pollinia, with an average of 7,30,000 pollen grains per pollinium. Pollen is ovate to obovoid and smooth; exine is smooth and thick; and pollinia are mostly in the tetrad stage. Intine is not so prominent or absent. Almost all pollen grains are viable, with a fertility percentage of 98.85% and a pollen germination rate of 78.2%.

After pollination, flowers started to wilt, greening and swelling of the ovary occurred, and it took one week for pods to set. The ripening of a capsule can take 4–4.5 months. A mature capsule can have an average length and width of about 10.39 cm and 1.48 cm, respectively, with a maximum diameter towards the distal end.

The capsules dry and shrivel, and millions of seeds escape through the grooves running longitudinally. The seeds are generally almost microscopic and very numerous—over a million per capsule—where 76% of them have well-filled embryos and 70.6% of them are viable. After ripening, they blow off like dust particles or spores. Mature seeds have a spathulate shape, and the seed mass has a yellow colour with a prolate-spheroid, a light violet-colored embryo, and a reticulated testa. The average seed length, width, embryo length, and width of V. tessellata are 192.28, 52.66, 126.24, and 38.98, respectively. Seed length/width ratio is 3.75, showing they are truncated seeds. A unique characteristic of orchid seeds is that instead of an endosperm, there is an "air pocket" surrounding a small globular embryo within a membranous testa. The airspace volume in orchid seeds varies depending on the species. There is a relationship between seed size and

embryo volume that determines the proportion of air space. Here, the seed volume is 167930.56µm^3, the embryo volume is 112066.344 µm^3, with a percentage of air space is 31.69. The amount of trapped air influences seed dispersal by affecting floatability (in air and water) and buoyancy, reflecting ecological adaptations to different distribution ranges (which is relevant to in situ conservation). For these reasons, studies investigating the airspace within the seed and its relation to taxonomy and living habitat are important to identify the best conservation strategy. *V. tessellata* seeds had a lower percentage of air space (31.69), and their SV/EV ratio of 1.5 shows that they are adapted to restricted or localised distribution.

Matured seeds were cultured on four different media, all solid or liquid, with or without plant growth regulators: MS, half MS, Knudson C, and Mitra. The enlargement of the embryos was the first sign of germination, and the embryos turned green because of the production of chlorophyll. Except for half MS, all of the basal media responded in 45 days. MS and half-MS supplemented with 0.5 mgL^{-1} BAP showed seed greening and germination with 45 and 60 days of inoculation, respectively. The only solid medium that showed germination was half MS with 0.5 mgL^{-1} BAP after 60 days of inoculation. The frequency of seed germination was the highest in Knudson C (86.33), followed by Mitra (72.5), and MS liquid with 0.5 mgL^{-1} BAP (39.75). It was noted that the seeds of *V. tessellata* germinated in the basal medium itself.

Though seed germination frequency was highest in Knudson C and Mitra media, the rate of survival of the germinated seeds was remarkably low in these media. Out of all the seed germination media, the maximum survival rate and lowest necrosis were noted in MS liquid with 0.5 mgL^{-1} BAP, which could be due to the presence of an adequate amount of nitrogen in the form of ammonium nitrate in the MS medium, whereas in Knudson C and Mitra, nitrogen was present in the form of ammonium sulphate.

Germinated seeds later developed into protocorms and differentiated into shoot meristems and rhizoids in opposite directions. Leaves were produced from green protocorm. Real roots were formed later. In MS (liquid) basal and MS liquid fortified with 0.5 mgL^{-1} BAP, protocorm formation was noticed within 75 days of inoculation of the seeds. For half-MS liquid augmented with 0.5 mgL^{-1} BAP and half-MS solid augmented with 0.5 mgL^{-1} BAP, it took around 90 days. The maximum number of protocorms with shoot apex was observed in MS liquid supplemented

with 0.5 mgL^{-1} BAP (41.75), followed by half MS liquid supplemented with 0. 5 mgL^{-1} BAP (37), MS medium (7.5), and half MS solid supplemented with 0. 5 mgL^{-1} BAP (3) respectively.

The first leaf appeared after 150 days of inoculation in MS liquid medium supplemented with 0.5 mgL^{-1} BAP, with the highest frequency of protocorms having 1-2 leaves. The first root appeared after the formation of the first leaf in the seedling, and it took about 7 months in MS solid supplemented with 0.5 mgL^{-1} BAP and 0.5 mgL^{-1} NAA.

Apart from these developmental stages, some seeds developed into multiple protocorm-like bodies, which, on subculturing, produced multiple shoots in MS medium (solid) supplemented with 1 mgL^{-1} BAP and 1 mgL^{-1} NAA.

The above study revealed that the best media for seed germination was Knudson C, for further protocorm development and shoot initiation it was MS media supplemented with 0.5 mgL^{-1} BAP (liquid), for leaf and root development it was MS media supplemented with 0.5 mgL^{-1} BAP and 0.5 mgL^{-1} NAA (solid), and the best media for multiple shoot production was MS supplemented with 1 mgL^{-1} BAP + 1 mgL^{-1} NAA (solid).

Vanda tessellata is an endangered and valuable wild orchid species, so its conservation and utilisation for genetic improvement is worthwhile, and therefore it can be multiplied on a large scale by adopting a proper *in vitro* propagation method.

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SEED CHARACTERIZATION AND STANDARDIZATION OF *IN VITRO* GERMINATION PROTOCOL IN *Vanda tessellata* (Roxb.) Hook. ex G. Don.

by

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ABSTRACT OF THE THESIS

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ABSTRACT

The present investigation entitled "Seed characterization and standardization of *in vitro* germination protocol in *Vanda tessellata* (Roxb.) Hook. ex G. Don" was carried out with the objective of standardization of *in vitro* seed germination protocol in *Vanda tessellata* along with morphological and morphometrical characterization of seeds. The study was conducted at Saraswathy Thangavelu Extension Centre of JNTBGRI, Puthenthope, Thiruvananthapuram and at College of Agriculture, Vellayani, Thiruvananthapuram during 2021-22.

The research work included four experiments. Experiment I - evaluation of floral characters, experiment II - morphological and morphometric characterization of seeds, experiment III - preparation of *in vitro* seed germination media and experiment IV - *in vitro* seed germination in culture media and its evaluation. All experiments were carried out in five replicates and mean was calculated.

For experiment I & II, *V. tessellata* germplasm repository maintained at Saraswathy Thangavelu Extension Centre of JNTBGRI, Puthenthope was used for morphological characterization. The study revealed that *V. tessellata* is an epiphytic, monopodial orchid with year-round flowering. Inflorescence is axillary, erect, and simple raceme, bearing an average of seven flowers. Flowers are bilaterally symmetric, resupinate, having green pedicel, greyish green calyx and corolla with dark brown tessellation, labellum white speckled with violet tinge inside and mid lobe lilac- blue. Time of anthesis was from morning to evening in a day (6am to 6pm) and under natural conditions, longevity for an unfertilized flower was around 22 days. Stigma receptivity remained throughout the day after anthesis and continue till the withering of flower.

The male and female reproductive parts are fused together and become the gynostemium or column. Pollen grains are packed together as a pollinium with 2 waxy, globular pollinia with an average 7,30,000 pollen production per pollinium. Pollen was ovate to obovoid, smooth, exine was smooth and thick and pollinia were mostly in tetrad stage. Intine was not so prominent or absent. Almost all pollen were viable with a fertility percentage of 98.85 and a pollen germination percentage of 78.2.

After pollination, flowers started to senesce, greening and swelling of ovary occurred and it took one week for pod setting. The ripening of a capsule took 120-135 days. The capsule

contained millions of seeds, where 76% of them had well filled embryo and 70.6% among them were viable. Mature seeds have spathulate shape and seed mass have yellow colour with a prolate-spheroid, light violet coloured embryo and a reticulated testa. *V. tessellata* seeds had a lower percentage of air space (31.69%) and SV/EV ratio of 1.5 shown that they are adapted to restricted or localized distribution.

In experiment III, four different basal nutrient media (solid and liquid) *viz*. MS, Half MS, Knudson C and Mitra, supplemented with various growth regulators were examined for asymbiotic seed germination. pH of the medium was adjusted to 5.8 with either 1.0 N sodium hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl) before being solidified with 8 gL^{-1} agar in case of solid media. Thereafter, 10 ml or 50 ml of the medium was dispensed into culture tubes/bottles and autoclaved.

Under experiment IV, matured seeds of 120-135 days old were cultured on four different media *viz.* MS, Half MS, Knudson C and Mitra, both solid and liquid with and without plant growth regulators. The enlargement of the embryo and rupturing of seed coat was the first sign of seed germination followed by greening and emergence of embryo out of the seed coat and later developed into protocorm and differentiated into shoot meristem and rhizoids in opposite directions. Leaves were produced from green protocorm. Real roots were formed later. Apart from these developmental stages, some seeds developed into multiple protocorm like bodies which on sub-culturing produced multiple shoots with small leaves and roots.

From the present investigation it can be concluded that the best media for seed germination was Knudson C (liquid) media followed by Mitra (liquid) media, for further protocorm development and leaf formation was MS media with 0.5 mgL^{-1} BAP (liquid), for leaf and root development was MS media with 0.5 mgL^{-1} BAP and 0.5 mgL^{-1} NAA (solid) and for multiple shoot production with leaves and roots was MS media 1 mgL^{-1} BAP + 1 mgL^{-1} NAA (solid).

Vanda tessellata is an endangered and valuable wild orchid species so its conservation and utilization for genetic improvement is worthful and therefore it can be multiplied on large scale by adopting proper *in vitro* propagation method

സംഗ്രഹം

"ആരാത്തവാഴ" (വന്യ ഓർക്കിഡ്) സസ്യവിത്ത് സ്വഭാവപഠനവും അതിനോടൊപ്പം അവയുടെ ഇൻ-വിട്രോ വിത്ത് മുളപ്പിക്കൽ പ്രോട്ടോക്കോൾ തയ്യാറാക്കൽ" എന്ന വിഷയം സംബന്ധിച്ചുള്ള ഗവേഷണ പഠനം തിരുവനന്തപുരം വെള്ളായണി കാർഷിക കോളേജിലും തിരുവനന്തപുരം പുത്തൻതോപ്പുള്ള ജവഹർലാൽ നെഹ്റു ട്രോപ്പിക്കൽ ബൊട്ടാണിക്കൽ ഗാർഡൻ ആൻഡ് റീസർച്ച് ഇൻസ്റ്റിറ്റ്യൂട്ടിലുമായി 2021-22 കാലയാളവിൽ നടത്തുകയുണ്ടായി. ആരാത്തവാഴയുടെ വിത്തിന്റെ സ്വഭാവം പഠിക്കുക എന്നതും ഇൻ-വിട്രോ വഴി വിത്ത് മുളപ്പിക്കാൻ പ്രോട്ടോക്കോൾ വികസിപ്പിക്കുക എന്നതുമായിരുന്നു പരീക്ഷണത്തിന്റെ ലക്ഷ്യം.

ജവഹർലാൽ നെഹ്റു ട്രോപ്പിക്കൽ ബൊട്ടാണിക്കൽ ഗാർഡൻ ആൻഡ് റീസർച്ച് ഇൻസ്റ്റിറ്റ്യൂട്ട് പുത്തൻതോപ്പ് -ൽ (JNTBGRI, Puthenthope) പരിപാലിച്ചുവരുന്ന സസ്യവൈവിധ്യ ശേഖരത്തിൽ നിന്നു പരീക്ഷണ വസ്തുക്കൾ ശേഖരിച്ചു.

ഗവേഷണ പ്രവർത്തനങ്ങൾ നാല് പരീക്ഷണങ്ങളായി തിരിച്ചിരിക്കുന്നു. പരീക്ഷണം I - പുഷ്പത്തിന്റെ സ്വഭാവവിലയിരുത്തൽ, പരീക്ഷണം II- വിത്തുകളുടെ രൂപഘടനയും മോർഫോമെട്രിക് സ്വഭാവവും വിലയിരുത്തൽ, പരീക്ഷണം III- ഇൻ വിട്രോ വിത്ത് മുളയ്ക്കൽ മാധ്യമം തയ്യാറാക്കൽ, പരീക്ഷണം IV- ഇൻ വിട്രോ വിത്ത് മുളപ്പിക്കലിന്റെ വിവിധ പരീക്ഷണങ്ങളും മൂല്യനിർണ്ണയവും. എല്ലാ പരീക്ഷണങ്ങളും അഞ്ച് പകർപ്പുകളിലായി നടത്തുകയും ശരാശരി കണക്കാക്കുകയും ചെയ്തു.

ആദ്യ പരീക്ഷണത്തിൽ വിവിധ പുഷ്പങ്ങൾ, പൂമ്പൊടി, കായ്കൾ എന്നിവ യുടെ പ്രതീകങ്ങൾ രേഖപ്പെടുത്തുകയും സസ്യങ്ങളുടെ സ്വഭാവം സൂക്ഷ്മമായി നിരീക്ഷിച്ച് ഗുണപരമായ പ്രതീകങ്ങൾ വിവരിക്കുകയും അളവിലുള്ള പ്രതീകങ്ങൾ വിലയിരുത്തുകയും ശരാശരി രൂപപ്പെടുത്തുകയും ചെയ്തു. വർഷം മുഴുവനും പൂക്കുന്ന ഒരു എപ്പിഫൈറ്റിക്, മോണോപോഡിയൽ ഓർക്കിഡാണ് വാണ്ട ടെസ്സെല്ലാറ്റ പൂങ്കുലകൾ ശരാശരി ഏഴ് പൂക്കൾ വഹിക്കുന്ന റസീം ആണ്. പൂക്കൾക്ക് ഉഭയകക്ഷി സമമിതി, ചാരനിറത്തിലുള്ള പച്ച ദളങ്ങൾക്കും വിദളങ്ങൾക്കും കടും തവിട്ട് നിറത്തിലുള്ള ടെസ്സലേഷൻസ്, ഉള്ളിൽ വയലറ്റ് നിറത്തോടുകൂടിയ വെള്ള ലേബല്ലത്തിന്റെ മധ്യ ലോബ് ലൈലാക് ബ്ലൂവുമാണ്. പൂവിടരുന്ന സമയം ഒരു ദിവസം രാവിലെ മുതൽ വൈകുന്നേരം വരെ ആയിരുന്നു (രാവിലെ 6 മുതൽ വൈകുന്നേരം 6 വരെ). സ്വാഭാവിക സാഹചര്യങ്ങളിൽ, ബീജസങ്കലനം ചെയ്യാത്ത പുഷ്പത്തിന്റെ ദീർഘായുസ്സ് ഏകദേശം 22 ദിവസം നിലനിന്നു. പൂവിടർന്നു കഴിഞ്ഞ് വാടുന്നതുവരെ പൂക്കളിൽ ജനിതലം പൂമ്പൊടിയോടു സ്വീകാര്യമായിരുന്നു.

പരാഗരേണുക്കൾ കൂടിച്ചേർന്ന് പൊളീനിയ ആകുന്നു. പോളീനിയയുടെ രൂപഘടന ദൃശ്യപരമായി നിരീക്ഷിച്ചു. വ്യക്തിഗത പൂമ്പൊടിയുടെ ഘടന, സംഖ്യ, ക്രമീകരണം,

പ്രവർത്തനക്ഷമത മുതലായവ മനസ്സിലാക്കാൻ അവയെ ചതച്ചശേശം അസറ്റോകാർമൈൻ സ്റ്റെൻ ഉപയോഗിച്ച് നിറം നൽകി. ഹീമോസൈറ്റോമീറ്റർ കണക്കാക്കിയ പോളീനിയത്തിലെ പൂമ്പൊടിയുടെ അളവ് ശരാശരി 7,30,000 ആണ്. കൂടാതെ, മിക്കവാറും എല്ലാ പൂമ്പൊടിയും ടെട്രാഡ് ഘട്ടത്തിലുമായിരുന്നു, 98.85 ശതമാനം പാരാഗരേണുവും പ്രവർത്തനക്ഷമമാണെന്ന് കണ്ടെത്തി, അവയിൽ 78.2 ശതമാനം 10 ശതമാനം വീര്യമുള്ള പഞ്ചസാര ലായനിയിൽ മുളച്ചു.

പരാഗണത്തിനു ശേഷം, പൂക്കൾ വാർദ്ധക്യം പ്രാപിക്കാൻ തുടങ്ങി, അണ്ഡാശയത്തിന് പച്ചനിറവും വീക്കവും സംഭവിക്കുകയും കായ്കൾ സജ്ജീകരിക്കാൻ ഒരാഴ്ചയെടുക്കുകയും ചെയ്തു. ഒരു കായപക്വമാകാൻ ഏകദേശം 120-135 ദിവസമെടുത്തു.

പരീക്ഷണം II-ൽ, വാണ്ട ടെസ്സെല്ലാറ്റയുടെ വിത്തുകൾ സൂക്ഷ്മദർശിനി-കോമ്പൗണ്ട് ലൈറ്റ് മൈക്രോസ്കോപ്പിന് കീഴിൽ രൂപഘടനാപരമായി ചിത്രീകരിക്കുകയും മോർഫോമെട്രിക് വിവരശേഖരണത്തിനു മൈക്രോസ്കോപ്പിക് സോഫ്റ്റ്വെയർ പാക്കേജ് ലെയ്ക ലാസ് 500 ഉപയോഗിച്ച് വിശകലനം ചെയ്യുകയും ചെയ്തു. പക്വമായ ഒരു കായിൽ ദശലക്ഷക്കണക്കിന് വിത്തുകൾ അടങ്ങിയിരുന്നു. 76 ശതമാനം വിത്തുകളും സാധാരണ ആകൃതിയിലുള്ള വലിയ ഭ്രൂണത്താൽ നന്നായി നിറഞ്ഞിരിക്കുന്നതായി കണ്ടെത്തി. വിത്തുകൾ സ്പാത്തുലേറ്റ് ആകൃതിയിലുള്ളതും വയലറ്റ് നിറത്തിലുള്ള പ്രോലേറ്റ് സ്ഫെറോയിഡ് ഭ്രൂണത്തോടുകൂടിയതുമാണ്. ടെസ്റ്റ കോശങ്ങൾ ജാലിതങ്ങളുമായിരുന്നു. പാകമാകുമ്പോൾ വിത്തിന്റെ 31.69 ശതമാനവും വായുവിൽ നിറഞ്ഞു.

പരീക്ഷണം III- ഇൻ -വിട്രോ വിത്ത് മുളയ്ക്കൽ പഠനത്തിനായി നാല് വ്യത്യസ്ത മാധ്യമങ്ങൾ അതായത് എംഎസ് (MS), ഹാഫ് എംഎസ് (half MS), നഡ്സൺ സി (Knudson C), മിത്ര എന്നിവ വളർച്ചാ ഹോർമോണുകളുള്ളതും അല്ലാത്തതുമായ ദ്രാവകവും ഖരവും ഉൾപ്പെടെയുള്ള മാധ്യമങ്ങൾ തയ്യാറാക്കി ഉപയോഗിച്ചു. 1.0 N സോഡിയം ഹൈഡ്രോക്സൈഡ് (NaOH) അല്ലെങ്കിൽ 1.0 N ഹൈഡ്രോക്ലോറിക് ആസിഡ് (HCl) ഉപയോഗിച്ച് മാധ്യമത്തിന്റെ pH 5.8 ആയി ക്രമീകരിച്ചു, ഖരമീഡിയയുടെ കാര്യത്തിൽ 8 g L−1 അഗർ ഉപയോഗിച്ച് ദൃഢമാക്കും. അതിനുശേഷം, 10 മില്ലി അല്ലെങ്കിൽ 50 മില്ലി മീഡിയം കൾച്ചർ ട്യൂബുകളിലേക്കും കുപ്പികളിലേക്കും വിതരണം ചെയ്യുകയും ഓട്ടോക്ലേവ് ചെയ്യുകയും ചെയ്തു.

പരീക്ഷണം IV- ൽ 120-135 ദിവസം പ്രായമുള്ള വിത്തുകൾ അണുവിമുക്തമായ അവസ്ഥയിൽ പുറത്തെടുക്കുകയും മാധ്യമത്തിൽ വയ്ക്കുകയും ചെയ്തു. ഓരോ മാധ്യമത്തിന്റെയും അഞ്ച് പകർപ്പുകൾ വിത്തുകൾ ഉപയോഗിച്ച് നിറയ്ക്കപ്പെട്ടു. വിത്ത് ഹരിതവൽക്കരണവും ക്ലോറോഫിൽ ഭ്രൂണവും കാണിക്കുന്നതിനുള്ള ദിവസങ്ങളുടെ എണ്ണം, പ്രോട്ടോകോർം വികസനം, ഉൽപ്പാദിപ്പിക്കുന്ന പ്രോട്ടോകോമുകളുടെ എണ്ണം, കാണ്ഡഉത്പാദനവും ഒന്നിലധികം ചിനപ്പുപൊട്ടലുകളുമുള്ള പ്രോട്ടോകോർമുകളുടെ എണ്ണം, വേരുകളും ഇലകളും മുളക്കുന്നതിനുള്ള ദിവസങ്ങളുടെ എണ്ണം എന്നിവയിൽ നിരീക്ഷണങ്ങൾ

രേഖപ്പെടുത്തി. ഭ്രൂണത്തിന്റെ വികാസവും വിത്ത് കോട്ട് പൊട്ടുന്നതുമാണ് വിത്ത് മുളകലിന്റെ ആദ്യ ലക്ഷണം. തുടർന്ന് പച്ചനിറവും വിത്ത് കോട്ടിൽ നിന്ന് ഭ്രൂണം പ്രത്യക്ഷപ്പെടുകയും പിന്നീട് പ്രോട്ടോകോം ആയി വികസിക്കുകയും എതിർദിശകൾ ഷൂട്ട് മെറിസ്റ്റം, റൈസോയ്ഡുകൾ എന്നിങ്ങനെ വേർതിരിക്കപ്പെടുകയും ചെയ്തു. പിന്നീടു ഇലകളും യഥാർത്ഥ വേരുകളും രൂപപ്പെട്ടു. ഈ വളർച്ചാ ഘട്ടങ്ങൾ കൂടാതെ, ചില വിത്തുകളിൽ നിന്നും ഒന്നിലധികം പ്രോട്ടോകോർമുകൾ വികസിച്ചു, അവയിൽ സബ് കൽച്ചർ ചെയ്തപ്പോൾ ഒന്നിലധികം ചിനപ്പുപൊട്ടൽ ഉണ്ടാക്കി.

വാണ്ട ടെസ്സെല്ലാറ്റയുടെ വിത്ത് മുളയ്ക്കുന്നതിനുള്ള ഏറ്റവും നല്ല മാധ്യമം ക്നഡ്സൺ സി (ദ്രാവകം) മാധ്യമവും തുടർന്ന് മിത്ര (ദ്രാവകം) മാധ്യമവും ആണെന്ന് ഇപ്പോഴത്തെ അന്വേഷണത്തിൽ നിന്ന് നിഗമനം ചെയ്യാം, കൂടുതൽ പ്രോട്ടോകോം വികസനത്തിനും ഇല രൂപീകരണത്തിനും 0.5mg/L BAP ഉള്ള MS മീഡിയ (ദ്രാവകം) ഉത്തമം. ഇലയുടെയും വേരിന്റെയും വികാസത്തിന് 0.5mg/L ബാപ്പ യും 0.5mg/L നാ യും (ഖരം) ഉള്ള MS മീഡിയയും ഒന്നിലധികം ചിനപ്പുപൊട്ടൽ ഉത്പാദനത്തിന് MS മീഡിയ 1mg/L BAP + 1mg/L NAA (ഖരം) ഉത്തമം.

വംശനാശഭീഷണി നേരിടുന്നതും വിലപ്പെട്ടതുമായ ഒരു വന്യഓർക്കിഡ് ഇനമാണ് വാണ്ട ടെസ്സെല്ലാറ്റ. അതിന്റെ സംരക്ഷണവും ജനിതക മെച്ചപ്പെടുത്തലിനുള്ള ഉപയോഗവും മൂല്യവത്താണ്, അതിനാൽ ശരിയായ ഇൻ വിട്രോ വിഭജന രീതി അവലംബിച്ച് വലിയ തോതിൽ ഇത് വർദ്ധിപ്പിക്കാൻ കഴിയും.