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In vitro propagation of *Vanda* orchid: a review

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Abstract

Vanda for the past decade has been the primadonna of ornamental orchids in the south and southeast Asia, along with *Phalaenopsis* and *Dendrobium*. Along with the increase in demand for Vanda, this genus has faced several threats from illegal collection to habitat loss. Mass propagation through in vitro culture is a promising strategy to make ensure sustainable business in horticulture, as well as for conservation purposes. This review provides an overview and synthesizes of various *Vanda* in vitro culture literature. We showed the researchers' preferences on several aspects for growing Vanda, including species, basal medium, plant growth regulators, explant, and culture conditions. The most commonly used as explants are seeds or protocorms, growing on Murashige & Skoog or Vacin & Went medium. This medium can be added banana homogenate to increase its nutritional value. *Vanda* seedlings can be incubated at 25 ± 1-3°C, with a lighting intensity of 50 µmol m^{-2} s^{-1} at 12/16 h PP. Choosing a medium that is cheaper but still rich in nutrients and its additives especially during the subculture phase; selection of explants that are responsive and minimizing the possibility of contamination; as well as seeing the target market in particular, can make Vanda's propagation efforts more effective, efficient, and profitable.

Keywords: efficient protocols, in vitro culture, Orchidaceae, *Vanda*x

Introduction

Orchidaceae is the second largest family of the angiosperms after Asteraceae. There are many approximate versions of orchid species numbers because almost every expedition in biodiversity hotspots is reported to discover new species. A provisional checklist suggests 28,000 species of orchids including 736 recognized genera, represent 8% species of the angiosperms (Chase et al., 2015; Christenhusz & Byng, 2016; Willis, 2017). It has exceeded the estimated maximum number (25,000) (Atwood, 1986).

The fascinating charisma of orchids defines them as an ornamental plant not only in terms of color but also in the uniqueness of the flower shape. Orchids flower could be kept indoors in fresh conditions for a long time as a symbol of beauty (Rahman et al., 2008). Cut flowers of the hybrids of *Mokara*, *Dendrobium*, and *Vanda* remain fresh from 7-30 days, while *Phalaenopsis* and *Cattleya* remain fresh for 1-4 weeks, and 18-28 days for *Aranda* (De et al., 2014). Orchid flowers have persistent perianth characters, unlike other cut flowers that easily fall off (Rahman et al., 2009). Today, orchid cultivation is an international business that has great potential to take part in the economic growth of countries. In the world floriculture trade, around 8% of sales are covered by orchids (Martin & Madassery, 2006), while in the ornamental plant industry, they are second favorites cut flowers and potted plants (Hossain, 2008).

One of the orchids that are widely cultivated in Southeast Asia and the Indian subcontinent is the genus *Vanda*, which was established by Sir W. Jones in 1795. His type species of this genus is *Vanda roxburghi*. *Vanda* is a monopodial orchid and mostly epiphytic (Islam et al., 2014). There are about 184 species plants, native to China, Himalayas, Bangladesh, Indonesia, and northern Australia, which 62 are accepted names, 122

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are synonyms, and 5 names remaining unresolved (The Plant List, 2019).

The name of *Vanda* came from an Indian language called Sanskrit (Garay, 1974), means that people like these plants by their fragrance, color, and the shape of the flower. Many *Vanda* hybrids have characteristics that preferred to mass consumption such as variable of color pallets, produce fragrant flowers, free-blooming, long-lasting flowers, multiple inflorescences, compact growth habits, and cold tolerance. These superior traits make *Vanda* become a great potential to dominate the American and European markets. In the 1950s, Hawaii, United States became the center of *Vanda* orchid development, where they produced primary and secondary hybrids of *Vanda,* with round and large-sized flowers. Later in the 1960s, Hawaii was replaced by Thailand. *Vanda* orchid breeders in Thailand produce more complex *Vanda* hybrids due to the segregation of progeny genes with new flower colors and shapes (Motes, 2004).

One of the obstacles of *Vanda* cultivation is they required three or more years of maintenance to reach flowering-size since deflasking (compared to *Phalaenopsis* that required only 18–24 months). Small-scale production and duration of *Vanda* culture causing the relatively high per-unit cost of production, which causes high selling prices at the farm level (Johnson & Kane, 2007). *Vanda*, like some other orchids genus, was also threatened by habitat destruction, climate change, and unsustainable harvest (often illegal) for horticulture, food, or medicine (Fay, 2018).

To meet the demand of the orchid consumers and innovation for the world floriculture industry, mass production of orchids is important. There are many ways to propagates orchids. The conventional propagation was the separation of pseudobulb clumps and keiki, but these methods are not suitable for mass production because it is inefficient in time and space, and has high risks of parent plant's death. Tissue culture is now an established method for the effective propagation method, offers the ability of large scale productions and ensure clonal stability, and irrespective cultured of season and weather (Singh & Duggal, 2009; Silva et al., 2015). Protocol for asymbiotic in vitro orchid seed germination on a medium containing mineral nutrients and sugar was developed by Knudson's (1922). The first experiments on *Vanda* in vitro culture were carried out at the University of Singapore with callus derived from seedlings in undefined media containing tomato juice and 2,4-Dichlorophenoxy acetic acid (2,4-D) (Rao, 1963; Rao, 1967). This technique continues to be developed, including applied along with genetic engineering, becomes an important method for mass scale propagation and conservation of orchid species. The main objective of this review is to provide a thorough understanding of *Vanda's* germplasm response to in vitro conditions by compiling what is known from various published literature and research. Detailed information is shown in the Supplementary File.

Micropropagation for conservation and sustainable utilization

Research publications about *Vanda* have not been as much as *Dendrobium*, *Phalaenopsis*, *Oncidium*, *Cattleya,* and another popular genus of orchids that may have reached hundreds, but this information could be used as initial information to develop in vitro *Vanda* cultivation.

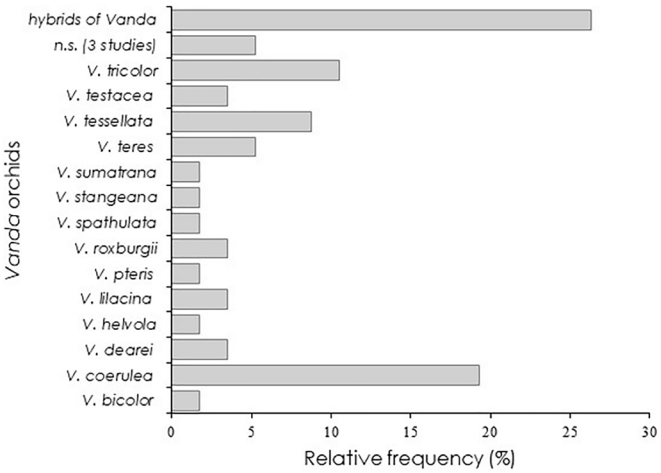

Figure 1. Relative frequency (%) of micropropagation studies showing various *Vanda* species and hybrids. Some of the studies use more than one type of orchids. n.s. not specified.

Based on the number of papers that have been published, species of *V. coerulea* (19.3 %), *V. tricolor* (10.6 %), and *V. tesselata* (8.8 %) are the most widely studied (Figure 1). *V. coerulea* is one of the most popular native orchids found in the northeastern region of India with a range of distribution is extending to China (southern Yunnan). Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) listed this species in appendix 2 (removed from the appendix 1 due to the discovery of new populations, especially in the Himalaya region (Christophe, 2012) and the global conservation status is vulnerable (Walter & Gillet, 1998). Habitat loss and degradation, mainly from human activities, and illegal hunting were the major cause of the decline in the population of these orchids. In other regions, *V. tricolor* (appendix 2) also face the same threats. It is widespread and highly cultivated in

South East Asia, while the wild populations are small and highly fragmented especially on Java and Bali (Gardiner, 2007). Gardiner (2007) reported that this species has been rare in nature due to over-collecting and natural disasters such as Mount Merapi eruption, one of the most active volcanoes in Indonesia with a 4-year eruption cycle. Anticipating a similar threat, the researchers developed an in vitro propagation technique for *V. tesselata* earlier to maintain a population whose trend tended to decline even though it still had the least concern status (Khela & Chadburn, 2014). Distribution of *V. tesselata* broad enough covering Bangladesh, India, Myanmar, Nepal, Sri Lanka, and Thailand.

In addition to horticultural uses, *Vanda* is also for medicinal uses, especially in India, Nepal, China, and Bangladesh. Khan et al. (2019) have summarized the use of *Vanda* in the field of traditional medicine and the bioactive compounds detected in it. The class of compounds detected included eucomic acid and its derivatives from *V. teres* (Simmler et al., 2011); Phenanthrene derivatives from *V. tessellata*, *V. parviflora*, and *V. coerulea* (Anuradha et al., 2008; Anuradha & Rao, 1998; Simmler et al., 2010); Bibenzyl derivatives from *V. coerulea* and *V. roxhburghii* (Simmler et al., 2010; Uddin et al., 2015); Phenolic compounds from *V. roxburghii*, *V. parishii*, and *V. tessellata* (Chawla et al., 1992; Dahmén & Leander, 1976; Prakash & Bais, 2016); Anthocyanins from *V.* hybrid (*V. teres* x *V. hookeriana*) (Junka et al., 2012); Alkaloids from *V. hindsii* (Brandange & Granelli, 1973); Steroids and triterpenoids from *V. roxburghii* (Mohammed-Usman et al., 2012). Based on the examination of *Vanda* extracts of various species, these orchids known to have pharmacological activity like anti-inflammatory, antioxidant neuroprotective, membrane stabilizing, anti-aging, hepatoprotective, antimicrobial, and wound healing activities (Khan et al., 2019). Traditional uses are usually for treating rheumatism, dyspepsia, indigestion, piles, wounds, bronchitis, and hepatitis (Khan et al., 2019).

Vanda breeding both with genetic transformation and interspecific hybrids being consumers favorites since they have a variety of choices in flower colors and other superior properties. On the other hand, wild *Vanda* tends to have some disadvantages such as difficult to adjust the growth to local climate, usually does not meet the dosage of commercials fertilizers and hormones (maintainers need to determine the optimal dose for the orchid themselves), and more expensive. So far, the relative frequency of research conducting on *Vanda* hybrid micropropagation is 26.3%.

Seed culture is probably the most effective technique so far to get lots of new seedlings, despite long maturity time of *Vanda* fruit capsule that could reach 6-9 months or even up to 20 months (PhytoTech Labs, 2019). This problem could be overcome by applying 6-Benzylaminopurine (BAP) and gibberellic acid (GA) hormones to stimulate flowering in plants, continued by spraying 6-30-30 sodium-phosphate-potassium fertilizer after pollination for fruit ripening. However, unripe seeds are still able to be planted and show a better result in some cases. When the seeds are riped, the inner coat surrounding the embryo may be thickened, makes it difficult for water and nutrients to reach the seeds. Some seeds may carry some poor traits that lead to nonuniformity clones. This could be avoided by choosing the superior breeds and maintain the stability of their genetic content. The optimum preference for explants, medium, and incubation conditions will be explained later.

Explant, culture medium and its constituents

Explant selections are an important factor to consider before initiating a culture method. Explant taken from potted plants in the greenhouse (ex vitro) may carry fungal and bacterial infections due to exposure to open environments. In monopodial orchids, such as *Vanda*, choosing shoot tip as explant could be caused death to the mother plant, since monopodial orchid relies their growth on its apical dominance. Flower stalk could only be obtained during the flowering season (the flowering could be induced but cannot be continuously carried out). Indeed, flower stalk of *Vanda* has limited plantable parts, for comparison they have a shorter length than the stalk of *Phalaenopsis*, which is commonly used as explant for *Phalaenopsis* micropropagation. The flower stalks of *Vanda* also mature rapidly, where young flower stalks are known better to used as explants. In general, choosing juveniles and other young tissues over mature parts needs to be considered. Other than that, flowering plants of *Vanda* are 2-3 times more expensive than their vegetative plants.

Seeds are the most commonly used explant for *Vanda* propagation (25.6%) (Figure 2). The next preference explants used are protocorm (23.2%), shoot/axillary tip (13.4%), shoot (12.2%), and others below 10% (seedling, nodal segment, callus, leaf, root, and flower stalk). Seeds could provide large quantities of explants where adult orchids plant are limited. Seeds could germinate even only using basal medium without the addition of hormones or complex organic matter. Seeds will grow into protocorms and become seedlings later (Yildiz, 2012). Protocorms have the flexibility to induce

shoots, roots, and/ or reproduce secondary protocorms/ PLBs (Sujjaritthurakarn & Kanchanapoom, 2011; Setiaji et al., 2018). The protocorm phase usually begins when the bipolar structure that cannot be distinguished between basal and apical (Setiari et al., 2016). By definition, protocorms are produced by seeds whereas protocorm like bodies (PLB) are produced by explants (Lee et al., 2013).

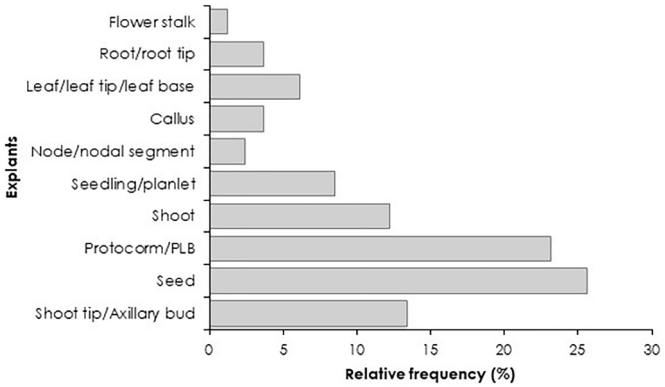

Figure 2. Relative frequency (%) of micropropagation substudies showing different explant used in *Vanda* in vitro culture. Some of studies used more than one explants.

In general, the most commonly used basal medium for *Vanda* cultures is MS (35.7%) and VW (Vacin & Went, 1949) (21.4%) (Figure 3). 28.5% of the sub-studies using seeds were planted on Murashige & Skoog medium (1962) (Figure 4). MS is widely used in a variety of plants, including orchids. This medium contain high concentrations of ammonia, potassium, and nitrates; and relatively cheaper compared to other mediums such as the White medium (Stewart Jr, 2016). On the other hand, VW media was specifically intended for orchid species at the beginning of the formulation, especially for *Cymbidium*. In this medium, $Ca_3(PO_4)_2$ is added in abundant quantities, providing phosphate to increase the formation of PLB (protocorm like bodies) (Silva, 2012). The seeds of 18 different orchid genera, planted on the VW medium, produce a chance of more than 70% of protocorms formation (Kartikaningrum et al., 2017).

The pH set for *Vanda* culture varies from 4.7-5.9 (Figure 5). The greatest preference was at pH 5.8 (16.3%), thereafter 5.6 and 5.2. The pH of the MS medium is usually set between 5.6-6.3. Adjusting the pH of the culture medium is important to make sure the physiological processes of the plant are not disturbed. Acidic medium prevents the uptake of phosphoric acid, Ca^{2+}, and Mg^{2+}; while alkalic medium prevents the uptake of iron, Cu^{2+}, Zn^{2+}, Mn^{2+}, and boron (Bell et al, 2020; Jakobsone & Osvalde, 2019; Ichinose et al., 2018). pH affects the solubility and absorption of nutrients through the activation of certain enzymes; solidification gelling agents; and prevent absorption of toxic substances (Sahu et al., 2017; Lager et al., 2010). A slightly acid medium seems to be preferred by most of the orchids, and also important for auxin action. Sachin (2015) reported that the highest protocorm formation on *V. tesselata* was observed at temperature 20°C and pH 5.5. It is difficult to determine whether the pH of the medium could affect the orchid seedlings because it is related to other components of the culture media.

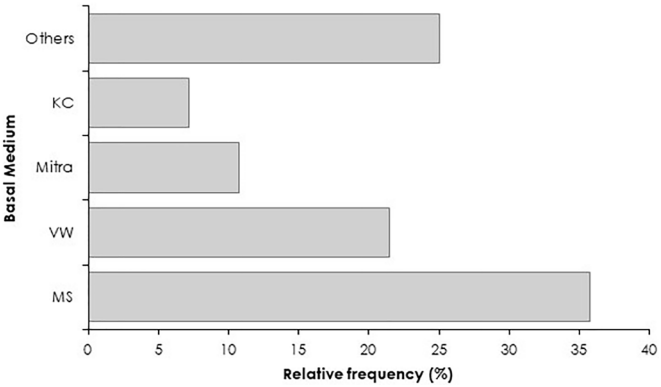

Figure 3. Relative frequency (%) of micropropagation substudies showing basal medium used in *Vanda* in vitro culture. Some of studies used more than one basal medium.

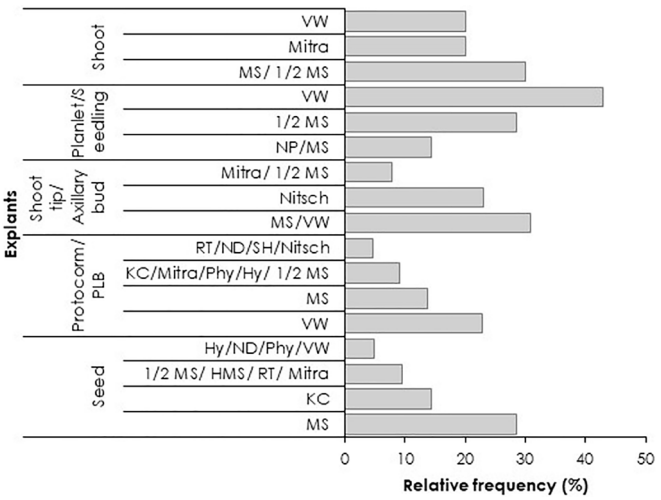

Figure 4. Relative frequency (%) of micropropagation studies showing basal medium used based on explant types in *Vanda* in vitro culture. A study may had one or more types of explant.

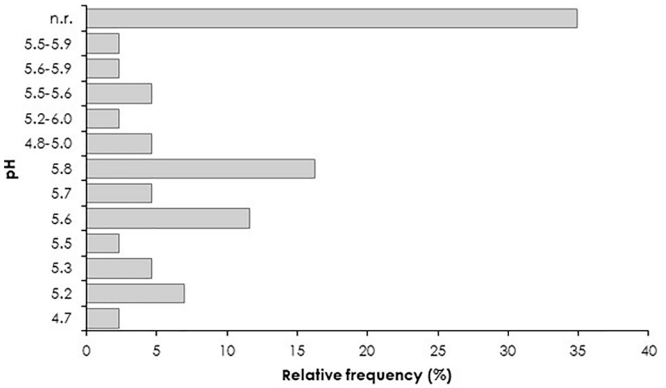

Figure 5. Relative frequency (%) of micropropagation studies showing different pHs used of basal medium in *Vanda* in vitro culture. n.r. not reported.

Plant growth regulators (PGRs) can be used simultaneously to match with *Vanda* growth stages. The most commonly used PGRs in *Vanda* cultures, either as combined or 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) (Figure 6). 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 range at 0.54-22.80 µM for NAA, while single cytokinin (10.71%) is generally used to induce shoots with optimum concentrations to range 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.

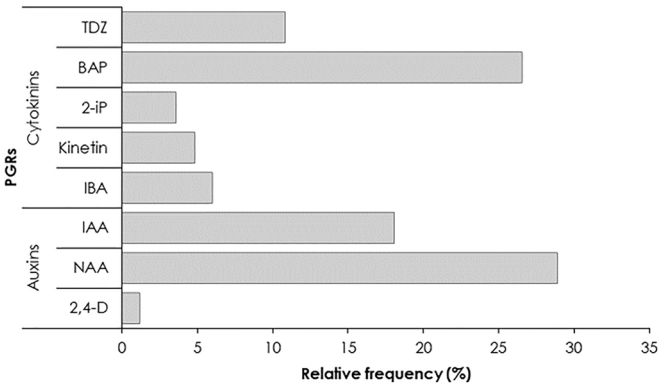

Figure 6. Relative frequency (%) of micropropagation substudies showing different plant growth regulators (PGRs) used in *Vanda* in vitro culture. Some of studies may used more than one substudies.

Organic complex materials contained different levels of sucrose, fructose, agar, peptone, nicotinic acid, biotin, folic acid, auxin, glutamic acid, glycine, adenine, niacin, nitrogen (Park & Yeung, 2018; Acemi & Ozen, 2019). Any of these components are responsible for promoting the growth and development of the cultures (Islam et al., 2015). The most commonly used organic complex materials in *Vanda* cultures are banana homogenate (18.8%), peptone (16.7%), activated charcoal (16.7%), coconut water (16.7%), tomato juice (8.3%), and potato homogenate (8.3%) (Figure 7). In *Vanda*, few papers explain the function of adding these additives because they may have complex effects and more focus on the effects of PGRs. Beneficial effects of organic complex materials (BH, CW, peptone) on growth and differentiation of protocorms and seedling have been carried out by Arditti (1979).

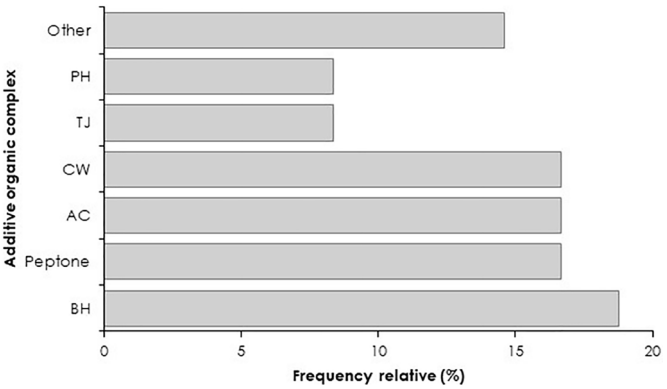

Figure 7. Relative frequency (%) of micropropagation substudies showing various additives used in *Vanda* in vitro culture. A study may had one or more substudy. PP potato pulp, TJ tomato juice, CW coconut water, AC activated charcoal, BP banana pulp.

Banana is known to be rich in carbohydrates, certain vitamins, minerals, carotenoids, and polyphenols. Studies that employed BH usually used a concentration of 3.5-15 % (v/v), combining with auxin and/or auxin-cytokinin and other additives. BH might help to stabilize the pH of the medium which may change due to the addition of activated charcoal. The pH of the medium could drops due to the acid residues of HCl in AC, since AC needs to be washed by HCl solutions in its production (George et al., 2008). Peptone generally consists of high tryptophan, a low molecular weight protein, vitamins, and plant growth factors. These factors may induce changes in *Vanda*, which can provide plant cells with an easily absorbed source of nitrogen (George et al., 2008). CW can induce cell division thus promoting early protocorm differentiation a wide spectrum of growth factors, and has been successfully used in some orchid production (Intuwong & Sagawa, 1973; Pyati et al., 2002). In epiphytic orchids the addition of 15% CW to the basal medium able

to increase growth performance in various parameters: shoot length, number of roots, leaf width, leaf area, fresh and dry weight of shoots and roots, and stimulating new shoots (Baque et al., 2011; Yong et al., 2009; Paris et al., 2019). The main hormone contained in CW is IAA, while cytokinin, gibberellin, abscisic acid are also detected (Yong et al., 2009; Tan et al., 2014).

The addition of activated charcoal improves the growth of *Vanda*. Some of the positive effects of AC are improved aeration, established polarity of microelements, stabilizes substrate temperature, and adsorbs toxic substances (phenolic compounds), all because of the nature of AC which has small pores and a large surface area (Thomas, 2008; Zeng et al., 2015). AC is suitable for root induction because it creates dark conditions of the medium in accordance to the original environment of the underground root. AC together with BAP can increase flowering frequency from 65% to 100%, increase in vitro germination and plantlet development, effective in increasing rhizome production and fresh weight gain during micropropagation, increasing the formation of orchid buds and promoting bud induction of orchid seeds (Thomas, 2008). However, in some cases, the addition of 1% activated charcoal to culture media caused acidification, which was largely due to an increase in the hydrolysis of sucrose during sterilization (Saad & Elshahed, 2012). Another disadvantage of AC is the adsorption is not selective, some beneficial substances may also be adsorbed.

Incubation condition

Lighting, temperature, and humidity are important aspects for the incubation chamber to be maintained to support plant growth, aimed for their adaption to in vivo environments. However, this review does not explain the humidity conditions because too few papers have mentioned it, since it may be difficult to measure humidity inside culture bottles.

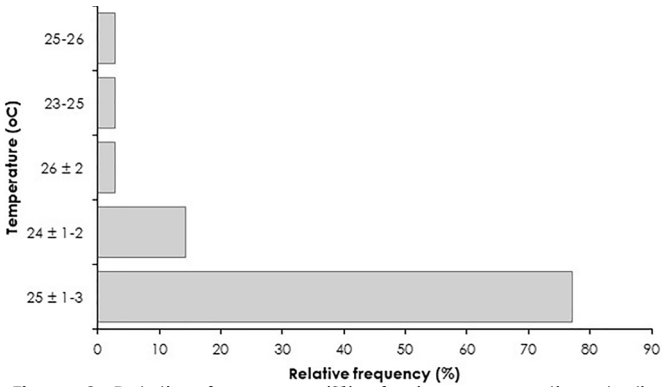

Figure 8. Relative frequency (%) of micropropagation studies showing different temperature conditions in *Vanda* in vitro culture

Table 1. Number of micropropagation studies showing different lighting conditions during *Vanda* in vitro phase.

Light intensity (µmol m^{-2} s^{-1})	Photoperiod (h)				
	0	12	16	24	Nr
25	-	-	1	-	
30	-	-	2	-	1
35	-	1	1	-	-
40	-	1	1	-	-
45	-	-	2	-	-
50	-	4	3	-	-
NR	1	-	2	1	-
28-35	-	-	1	-	-
30-50	-	1	-	-	-
NR	4	-	-	-	-
20-50	-	1	-	3	-
37	-	-	1	-	-
10	-	1	-	1	1
9	-	-	-	2	-
20-30	-	-	1	-	-
56	-	-	1	-	-
15	-	-	1	-	-
100	-	-	-	-	1

The studies on *Vanda* in vitro culture used the temperature ranged from 23-26°C, but 25 ± 1-3°C (77.1 %) was most commonly used. The intensity of the light used varies, from 25 to 100 µmol m^{-2} s^{-1}, but the most widely used is 50 µmol m^{-2} s^{-1} at 12/16 h PP (7 studies). Only 5 studies of reports using dark culture, the light intensity is not reported (Table 1). All studies employing light-emitting diodes.

Conclusions

The growth of *Vanda* orchids during the in vitro phase requires optimal controlled conditions. It is make sure the viability of seedlings during acclimatization and uniformity during flowering induction in the greenhouse. This review attempts to infer the basic needs for in vitro culture in *Vanda* based on the preferences of previous studies. For the source of explants, the most commonly used are seeds or protocorms, planted on MS or VW medium with a pH of 5.8. Banana homogenate 3.5-15% is the most used additives. *Vanda* seedlings mostly incubated at 25 ± 1-3°C, with a lighting intensity of 50 µmol m^{-2} s^{-1} at 12/16 h PP. *Vanda*'s in vitro culture technique still needs to be developed and expanded with the application of molecular biotechnology. The potential and uniqueness in ornamental, horticultural, and medicinal values are also slightly mentioned. This review can temporarily serve as a basis for *Vanda* producers to avoid confusion in choosing culture procedures from the various studies that have been conducted.

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