

Effects of Media composition on Asymbiotic Seed Culture of *Vanda tessellata* : An Approach to *In vitro* Conservation

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Abstract

For initiation of seed germination and protocorm growth in *Vanda tessellata* (Roxb.) Hook. ex G. Don different media, namely Knudson's C basal medium, modified Knudson's C basal medium, Murashige and Skoog basal medium, Vacin and Went basal medium and Lindeman basal medium either alone or supplemented with vitamin or 0.1% peptone (w/v) were used. Vitamin mixture consisted of nicotinic acid, pyridoxine and thiamine HCl at 1 : 1 : 10, respectively. For the initiation of germination, all the treatments exhibited promising results without showing significant variation. But the rate of survival of the germinated seedlings was remarkably low in all the basal media. Maximum survival rate of germinated seedlings was recorded in MS + 0.1% peptone. Necrosis of protocorms was a common phenomenon in all the treatments and 100% necrosis was recorded in LM basal medium and LM + vitamin. Addition of vitamin mixture and peptone in the basal media increased the rate of survival as well as differentiation of the germinated protocorms. Maximum rooted plantlets were recorded in VW + 0.1% peptone.

Introduction

Orchidaceae is comprised of one of the most popular ornamental crops for the mesmerizing beauty of the flowers with long shelf lives and occasionally for the fragrance (Roy and Banerjee 2002). Depending upon the current demand, they are commercially utilized as cut flowers as well as potted plants.

However, there are some difficulties in propagation of orchids through conventional methods as it possesses very complex and unique characteristics in its flowers which

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slows the rate of successful pollination by natural means. Even after successful pollination, germination of seeds depends on its specific mycorrhizal partner. Due to this constraint, hardly 5% seeds germinate in nature at a slow rate. Due to these reasons, nowadays the horticultural industry mostly depends on *in vitro* techniques for commercial production of ornamental orchids (Mukhopadhyay and Roy 1994, Suttle 1996). Apart from its horticultural values, many orchids are medicinally important and as such orchids are indiscriminately exploited in nature. In addition unauthorized trade and collection of orchids by orchid lovers, the rare and precious orchid species are disappearing day by day. This is an indication of extinction of these orchid species (Abraham et al. 2012). To save the valuable orchid germplasm from extinction, at present *in vitro* clonal propagation particularly the asymbiotic seed germination technique is being practiced (Arditti 1967, 1979, 1982, Arditti et al. 1981, Thompson et al. 2006, Kauth et al. 2006, Steward and Kane 2006).

Vanda tessellata (Roxb.) Hook. ex G. Don. is an epiphytic orchid which is widely distributed throughout the sub Himalayan tract to peninsular India and Sri Lanka (Roy and Banerjee 2002). This species is highly important for its medicinal properties. The whole plant body is used in Ayurvedic medicines. Paste of leaves is used in fever. It is the ingredient of 'Rasna Panchaka Quatha', the Ayurvedic formulation used in treatment of arthritis and rheumatism (Singh and Duggal 2009). Apart from this, a novel aphrodisiac compound has been isolated from the flower of *V. tessellata* which activates neuronal (nNOS) and endothelial nitric oxide synthase (eNOS) that influences the levels of nitric oxide (NO) in tissue and blood (Subramonium et al. 2013). The wild population of *V. tessellata* has been reduced drastically due to its high economic importance, over-exploitation, habitat deterioration and heavy deforestation of host trees (Pandey et al. 2003). Therefore, this orchid has been designated as an endangered species in India (Prakash et al. 2013a,b; Bhattacharjee and Islam 2014). International Union for Conservation of Nature (IUCN) ranked *V. tessellata* as conservation prioritized orchid species with decreasing population and has recommended for its *ex situ* conservation (Khela and Chadburn 2014).

In this study, suitable culture media were standardized using five basal media for *in vitro* asymbiotic seed germination, protocorm growth and their morphogenesis in the presence of vitamins and organic supplements. Asymbiotic seed culture under aseptic condition is the ideal method for conservation and propagation of orchids in general (Rasmussen 1995). Therefore, a fruitful initiative was undertaken to optimize the asymbiotic seed culture of *V. tessellata* using vitamin and organic supplements followed by the detailed study of the growth and morphogenesis of seedlings and protocorms.

Materials and Methods

Aseptic culture was initiated from the seeds of *Vanda tessellata* (Roxb.) Hook. ex G. Don collected from 9-month-old undehisced mature capsules raised through manual self-

pollination method. The freshly collected capsules were first thoroughly washed with liquid detergent and then were kept under running tap water for about 45 min. After that the capsules were rinsed in 0.1% (w/v) mercuric chloride solution for 10 - 12 min for surface disinfection followed by washing thrice with sterile distilled water. Finally, the capsules were dipped in 95% (v/v) ethanol for 60 sec and then kept on sterile Petri dishes for a few minutes to evaporate the last drop of ethanol from the surface of the capsule. After surface sterilization, the capsules were dissected carefully in a laminar air flow cabinet using sterile scalpel and a pair of forceps. The microscopic seeds were taken out aseptically and carefully spread on the surface of the culture medium. For each treatment, three replicate cultures were maintained. The culture vessels with inoculated seeds were incubated at $25 \pm 2^\circ\text{C}$ and 10 hrs of photoperiod was provided by Phillips white fluorescent lights of 3000 lux intensity for their germination.

The seed germination media were based on inorganic salts of Knudson C basal medium (KC; Knudson 1946), Modified Knudson C medium (MKC), Murashige and Skoog medium (MS; Murashige and Skoog 1962), Vacin and Went medium (VW; Vacin and Went 1949) and Lindemann medium (LM; Lindemann et al. 1970). In MKC, original iron and micronutrient sources were replaced with those of MS in half strength. All the five media were supplemented with 2% (w/v) sucrose. 0.1% (w/v) peptone was added separately to all the basal media to observe the effect of organic supplement on seed germination. Vitamin mixture (VIT) was prepared using three vitamins (Nicotinic acid: pyridoxine : thiamine HCl) at 1 : 1 : 10 (1 : 1 : 10 mg/l, respectively). pH was adjusted at 5.6 and 0.9% (w/v) agar (Merck) was added.

After maintaining the cultures for three months, raw data of growth and morphogenesis were collected. Only the healthy seedlings were counted and transferred to fresh medium, while the remaining propagules were scooped out from the medium and every stage of development was counted and recorded carefully. The non-germinated seeds were also counted under the microscope to determine the exact amount of seed inocula. Necrotic propagules were also counted. The emergence of embryo through the ruptured seed testa was considered as the early stage of germination (De Pauw et al. 1995). Germination as well as survival rates of seedlings were expressed through quantitative assessment. Different developmental stages of seedlings such as globular stage and with leaf primordial, seedlings with leafy shoot, with root and expanded leaves were also recorded.

Data were analyzed by one-way ANOVA, followed by DMRT at 5% significance level. The statistical analysis was performed on arcsine transformed values of the recorded data using SPSS (version16) software package.

Results and Discussions

Five types of asymbiotic media were tested with different mineral salts at different concentrations, organic supplements and vitamin mixture. No significant difference in

the rate of seed germination was observed in various media compositions. Previous studies on asymbiotic seed germination of orchids reported the influence of various culture media on seed germination (Sinha and Roy 2000, Mohanty et al. 2012, Pyati 2019, Kang et al. 2020). After establishment of aseptic cultures, it was observed that seed germination was initiated approximately after three weeks in each treatment. The effect of different basal media either singly or along with peptone and vitamin mixture on seed germination and survival of propagules are shown in Fig. 1A,B. Frequency of seed germination was very high in all the treatments without showing any significant differences which ranged from 80 to 89%. The highest rate of seed germination has been noted in LM basal medium (Fig. 1A). It was noted that the seeds of *V. tessellate* germinated in absence of any exogenous PGRs. This might be due to the presence of adequate amount 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 inhibiting compounds are directly involved in controlling seed development, dormancy and seed germination (Hartman et al. 1997). It has also been reported that depending upon the choice of culture medium, germination has been affected due to their differences in the balance and supply of organic and inorganic salts (Arditi 1982, Arditi and Ernst 1984, Van Waes and Debergh 1986, Paul et al. 2012).

Though the seed germination frequency was very high in all the treatments, a huge difference was noticed at the same time, when the question of the survival rate of those germinated propagules arose. Although LM basal medium gave maximum frequency of germination, but after germination, the protocorms did not survive, which might be due to the lack of adequate amount of nutrient and/or essential growth stimulating substances (Stoutamire 1974). Maximum frequency of necrosis and death of protocorms was noticed at stage 1 (globular stage). However, fewer cases of protocorm death were noticed at later stages too. This phenomenon is very common in case of seed culture of orchids (Stoutamire 1974, De Pauw et al. 1995, Roy and Banerjee 2002). Of the five seed germination media, maximum survival rate and lowest necrosis were noted in MS with 0.1% P (Fig. 1B). Maximum seedling survival was recorded in MS + 0.1% P which could be due to the presence of nitrogen in the form of ammonium nitrate in the MS medium, whereas in rest of the basal media, nitrogen was present in the form of ammonium sulphate (Paul et al. 2012). In earlier studies, MS was reported as most suitable for seed germination in a number of orchid species like *Geodorum densiflorum* (Bhadra and Hossain 2003), *Vanda teres* (Sinha and Roy 2004), *Cymbidium findlaysonianum* (Tawaro et al. 2008), *Dendrobium hookerianum* (Paul et al. 2012), *Vanda tessellata* (Bhattacharjee and Islam 2014, Manokari et al. 2020).

Immediately after germination, the protocorms remained white, without any shoot apex and appeared more or less round (Stage 1: globular stage; Fig. 2B). Subsequently, those quickly turned green and an apical notch was formed which was later developed in to small leaf primordium at the apex (Stage 2: leaf primordial stage; Fig. 2C). Apart from

apical leaf primordia, the protocorms developed shiny rhizoids at the basal region. Later, leaf primordia expanded to develop leafy shoots (Stage 3: leafy stage; Fig. 2D) which was followed by root induction at the basal region of the protocorm to form complete seedlings (Stage 4: rooted stage; Fig. 2E). These developmental stages are common for most of the orchid species especially for epiphytic orchids (Arditi 1967). Apart from these four developmental stages, two more stages were also recorded during the process of morphogenesis, which are multiple protocorm like bodies and the callus stage. After three months of culture, the seedlings exhibited a number of morphogenetic stages which were eventually been considered as growth parameters (Table 1).

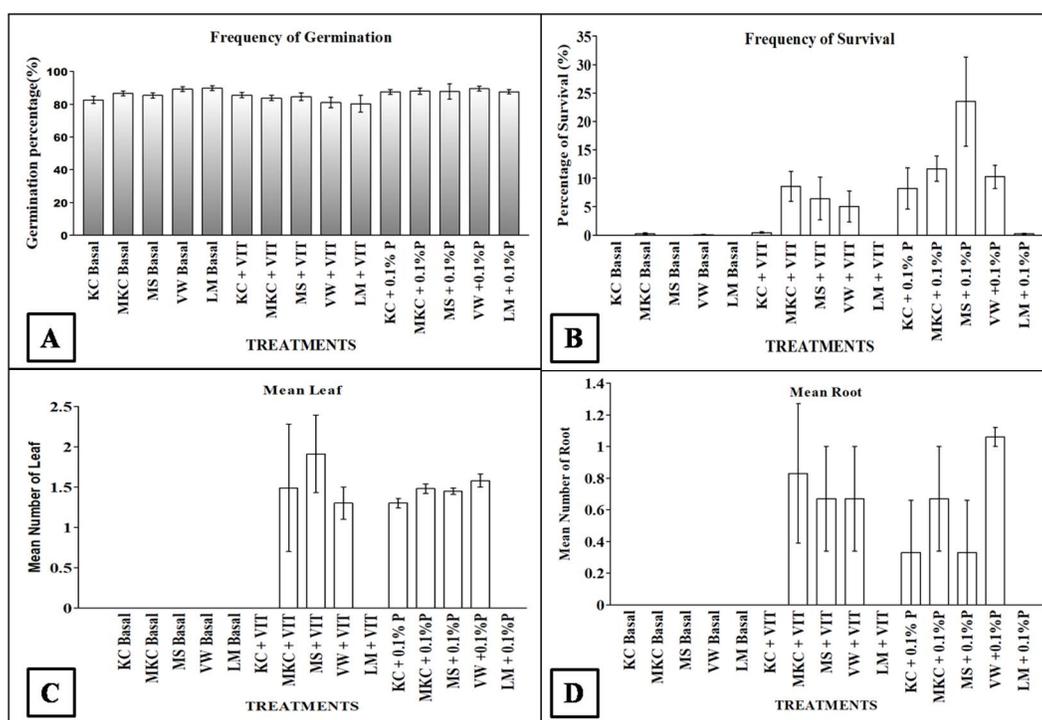


Fig. 1. Different *in vitro* responses during seed germination of *Vanda tessellata*: (A) Frequency (%) of seed germination. (B) Frequency (%) of survival of germinated protocorms. (C) Mean number of leaves. (D) Mean number of roots in each treatment. [Bar diagram shows the mean value of three replicates of each treatment and the error bar shows standard error occurred during experiment and were plotted using KY plot software].

Although, in the present study germination rate was not optimum in MS, various post germination developmental stages were recorded in the MS + 0.1% P medium. In previous studies it was revealed that 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). In case of media supplementation with peptone, majority of the propagules were restricted at leaf primordial stage in LM + 0.1% P medium. Globular stage protocorms (Stage 1) were only recorded in MS + 0.1% P medium as maximum necrosis in all other treatments occurred at globular stage. Leafy stage (Stage 3) and rooted stage (Stage 4) were recorded in all the four media combinations except in LM + 0.1% P. Leaf primordial stage (Stage 2) was found to be most abundant and was found in all the media combinations (Fig. 2C, Table 1). Apart from these four stages, multiple protocorm like bodies was only recorded in MKC + 0.1% P medium. Maximum number of roots was developed in VW + 0.1% P medium (Fig. 1D).

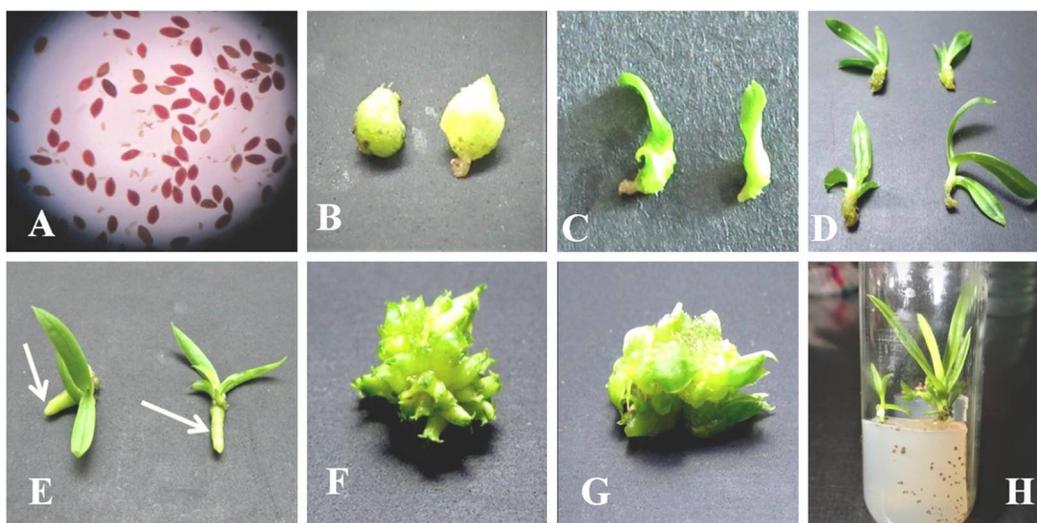


Fig. 2. The seeds and different developmental stages of seedlings of *Vanda tessellata*: A. TTC treated seeds of *V. tessellata*. B. Globular stage (Stage 1). C. Leaf primordial stage (Stage 2). D. Leafy stage (Stage 3). E. Rooted stage (Stage 4). F. Multiple protocorm like bodies. G. Callus differentiating into PLBs. H. Rooted plantlets during culture condition.

It is well-established that NH_4^+ and NO_3^- ions (individually or in combination) are very crucial for *in vitro* germination of orchid seeds as well as for growth and development of the germinated propagules (Dohling et al. 2008, Poothong and Reed 2016, Zhang et al. 2019). The high rate of germination followed by further protocorm development in MS could be due to the presence of rich source of macro and micro elements (Hossain et al. 2009, Paul et al. 2012, Bhowmik and Rahman 2017). In MS + 0.1% P medium, where peptone was added, the survival rate of germinated seedlings was accelerated and the propagules subsequently passed through sequential developmental stages. Similar response was also recorded in case of MKC + 0.1% P, VW + 0.1% P and KC + 0.1% P media except LM + 0.1% P (Fig. 1B, Table 1). These results suggest that the nitrogen content of the medium is an important factor for healthy growth and

development of protocorms. It has been previously reported that, peptone could act as a good source of organic nitrogen and contains about 16.16% total nitrogen comprising primarily of amino nitrogen as amino acids (Olivia and Arditi 1984, Arditi and Ernst 1993). Thus, addition of 0.1% peptone would provide additional 161.6 mg/l nitrogen into the culture medium (Roy and Banerjee 2002). Therefore, it seems likely that the addition of peptone stimulated the growth of germinated propagules in KC+0.1%P, MKC+0.1%P, VW+0.1%P, and MS+0.1%P media. Similar response was recorded in case of *Cymbidium giganteum*, where peptone was found to be beneficial for increasing seed germination as well as the size of protocorm (Hossain et al. 2010).

Table 1. Effect of different media composition on various developmental stages of germinated protocorms.

Treatment	Globular stage % (Stage 1) ± SE	Leaf primordial stage % (Stage 2) ± SE	Leafy stage % (Stage 3) ± SE	Routed stage % (Stage 4) ± SE	MPB% ± SE	Callus stage % ± SE
KC basal	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
MKC basal	0.00 ± 0.00 ^a	100.00 ± 0.00 ^c	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
MS basal	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
VW basal	0.00 ± 0.00 ^a	33.33 ± 33.33 ^{ab}	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
LM basal	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
KC + VIT	66.67 ± 33.33 ^b	33.33 ± 33.33 ^{ab}	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
MKC + VIT	0.00 ± 0.00 ^a	66.67 ± 9.99 ^{bc}	13.55 ± 11.75 ^b	6.09 ± 3.21 ^a	13.68 ± 4.67 ^c	0.00 ± 0.00 ^a
MS + VIT	2.22 ± 2.22 ^a	19.20 ± 6.71 ^{ab}	66.25 ± 14.92 ^d	5.66 ± 3.21 ^a	2.96 ± 2.96 ^{ab}	3.70 ± 3.70 ^b
VW + VIT	5.56 ± 5.56 ^a	62.28 ± 2.00 ^{bc}	26.43 ± 0.72 ^c	5.73 ± 2.88 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
LM + VIT	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
KC + 0.1% P	0.00 ± 0.00 ^a	60.07 ± 10.11 ^{bc}	38.59 ± 8.79 ^c	1.33 ± 1.33 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
MKC + 0.1% P	0.00 ± 0.00 ^a	46.66 ± 6.76 ^{ab}	43.30 ± 9.13 ^c	7.31 ± 5.47 ^a	2.72 ± 1.75 ^b	0.00 ± 0.00 ^a
MS + 0.1% P	44.67 ± 18.74 ^b	24.52 ± 6.77 ^{ab}	26.81 ± 10.74 ^c	4.00 ± 4.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
VW + 0.1% P	0.00 ± 0.00 ^a	40.60 ± 0.22 ^{ab}	41.95 ± 0.90 ^c	17.45 ± 1.08 ^b	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
LM + 0.1% P	0.00 ± 0.00 ^a	66.67 ± 33.33 ^{bc}	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a

Data shown are the mean of three replicates ± SE. In each column, mean values followed by the same letter are not significantly different at the 5 % level (DMRT).

Although basal media gave high frequency of germination, it failed to induce further development of germinated propagules. Seeds became necrotic after swelling in the KC, MS and LM basal media. However, in case of MKC and VW basal media, the germinated propagules remained restricted to Stage 2 (leaf primordial stage). The highest frequency of leaf primordial stage was found in MKC basal medium.

Addition of vitamin mixture to the basal media also stimulated the survival rate of germinated propagules and its effect on morphogenesis of the protocorms is clearly

shown in Table 1. In the presence of vitamin mixture, all the four morphogenetic developmental stages were recorded in VW + VIT and MS + VIT media (Fig. 2 B-E; Table 1). Maximum frequency of globular stage (Stage 1) was observed in KC + VIT medium. On the other hand, frequency of mature leafy stage (Stage 3) was found highest in MS + VIT medium. Apart from these four stages two more types of morphological features were also observed in these set of media. Maximum frequency of multiple protocorm like bodies (MPB) (Fig. 2F) was recorded in MKC + VIT medium, while callus stage (Fig. 2G) was recorded only in MS + VIT medium. Maximum number of leaves was observed in MS + VIT medium (Fig. 1C). Sharma et al. (1991) reported that during seed germination and seedling growth of *Coelogyne punctulata* and *Cymbidium elegans*, addition of various vitamins exhibited synergistic effect. On the other hand, thiamine, nicotinic acid and biotin were found most effective in germination and differentiation of *Cattleya* hybrids (Mariat 1949). In *Orchis laxiflora*, it was reported that pyridoxine with nicotinic acid and biotin enhanced seed germination process (Mead and Bulard 1979).

All the developmental stages of seedlings were recorded in MS + VIT medium. Maximum frequency of MPB was recorded in MKC + VIT medium. On the other hand, VW + 0.1% P medium was able to produce maximum number of rooted plantlets. Again, MKC + 0.1% P medium gave satisfactory result in this regard. So, it is not an easy task to recommend a single medium which would be exclusively suitable for seed germination and seedling survival of this particular orchid species. Thus, the primary objective of this study was to produce maximum number of healthy seedlings which in turn could be utilized for *in vitro* storage and conservation of this species. Therefore, in general MS + VIT, MKC + VIT, MKC + 0.1% P, VW + VIT and VW + VIT media could be recommended for this purpose instead of a single one. However, of all these, MS + VIT medium might be the optimal one since all the developmental stages were recorded in this particular treatment.

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