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EFFECT OF DIFFERENT GROWTH ADDITIVES ON SEED GERMINATION OF *VANDA TESSELLATA* (ROXB.) HOOK. EX G. DON- A MEDICINAL ORCHID

M Madhavi and P Chandramati Shankar

Department of Biotechnology, Yogi Vemana University, Vemana Puram Kadapa- 516 005, Andhra Pradesh, India

Abstract

Vanda tessellata (Roxb.) Hook. ex G. Don is a wild epiphytic orchid with high medicinal values. The seeds of this orchid can be germinated asymbiotically *in vitro* for rapid propagation. In this study, different growth additives such as banana powder (1-3% w/v), tomato powder (1-3% w/v), and coconut water (5, 10, 15, and 20% v/V) in half strength Murashige and Skoog medium showed various responses, either independently or in combinations. Amongst these three additives used, (15% v/V) coconut water gave the maximum percentage response (78±1.15) followed by banana powder (46.6±1.2) and tomato powder (19±1.3), when used alone. A combination of banana powder (10 gml^{-1}) and tomato powder (10 gml^{-1}) with coconut water (15% v/V) showed best response (96±1.9) in half strength Murashige and Skoog media. Amongst 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 seen in half strength MS medium with IAA (0.1 mgl^{-1}), KN (1.0 mgl^{-1}), and coconut water (15% v/V).

Introduction

ORCHIDACEAE IS one of the most diverse and largest families of flowering plants. Orchids comprised of 28,237 species (Willis, 2017) distributed widely in the humid tropical forests of India, Sri Lanka, South Asia, South and Central America, and Mexico. In India, 1,256 species of orchids in 155 genera are found in different habitats (Singh *et al.*, 2019). Himalayas is their main habitat followed by Eastern and Western Ghats. The Orchidaceae family is all listed in International Union of Conservation of Nature (IUCN) Red Data Book. Orchid species represent the most highly evolved family among monocotyledons with approximately 850 genera (Guitierrez, 2010; Singh *et al*., 2007; Stewart and Griffith, 1995). In peninsular India about 371 species have been reported (Saranya *et al*., 2012).

The name *Vanda* originated from Sanskrit (William Jones, 1795); it is known throughout the Indian subcontinent as Rasna (Sushruta Samhita, 1907-1911, Chakra Samhita 1888-1903, Hoernle 1893-1912). Orchids are being used as medicine due to the presence of alkaloids, flavonoids, glycosides, and other phytochemicals (Gutierrez, 2010). Kumari *et al*. (2012) studied the beneficial and medicinal values of 58 orchids. The juice of some orchid plants is given as a liver tonic and to temper the bile (Dasari *et al*., 2013). *Vanda* is listed as threatened plant in the International union for conservation of nature IUCN, Red list (2014). The population has decreased from 2009 to 2013 (Indian Biodiversity Portal, 2013). Over exploitation of these species has led to great genetic and ecological erosion; many orchids have been listed as endangered species (Machaka-Houri *et al*., 2012). In nature, orchid seeds do not germinate easily, these germinate only if infected by a suitable mycorrhizal fungus (Kauth *et al*., 2008). Multiplication of orchids using tissue culture methods have been preferred, as there exists great opportunity in improving the quality and increasing the numbers of plantlets through mass propagation of several important orchids, hybrids or a new variety within a short time period (Bhattacharjee and Hossain, 2015; Bhatti *et al.,* 2017; Borah *et al.,* 2015; Chen and Chang, 2000; Chen *et al.*, 2004; Decruse and Gangaprasad, 2018; Goh and Wong, 1990; Mohanty and Salam, 2017; Pathak *et al.,* 2001; Sibin and Gangaprasad, 2016). In the present study, rapid and efficient method for mass propagation through asymbiotic seed germination using different growth additives is reported for *Vanda tessellata*. Asymbiotic germination is an excellent technique to study the biotic and abiotic factors of orchid seed biology, while symbiotic germination provides a way to investigate the physiological mechanism of orchid seed germination. Although great advances are being made, orchid seed physiology, ecology, and whole plant ecology still needs to be understood.

Material and Methods

Source and Collection of Materials

Immature seeds from undehisced green capsules of *Vanda tessellata* were collected from Seshachalam forest of Chittoor district and Rapur Ghat which belongs to Kadapa district of Andhra Pradesh, during month of November (2018).

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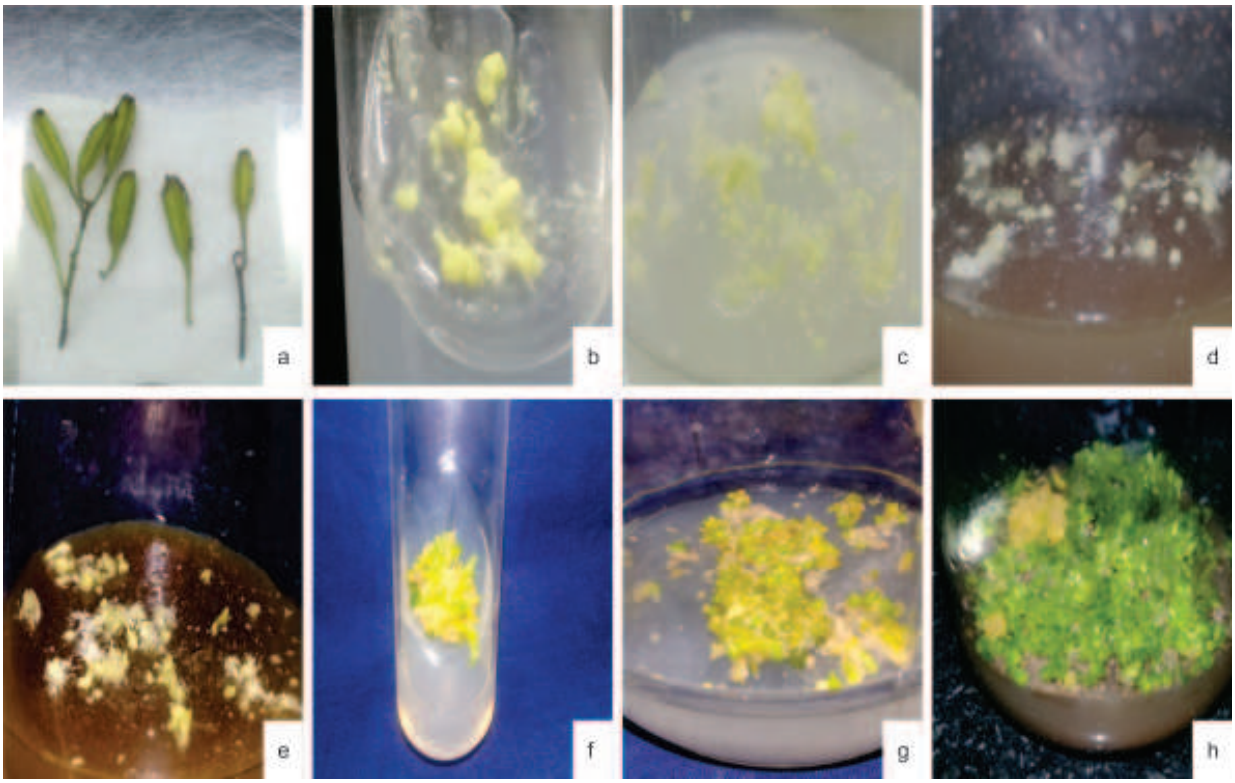

Fig. 1. a-h. Different stages of seed germination on half strength MS medium with growth additives in *Vanda tessellata*: a, Undehisced capsule; b, Freshly inoculated *Vanda* seeds (Control); c, Germinating seeds [(½ MS+ CW (15% v/V)]; d, Germinating seeds [(½ MS+ BP (1%)]; e, Protocorms [(½ MS+ TP (1%)]; f, Protocorm multiplication and leaf differentiation (Control); g, Protocorm multiplication and leaf differentiation [½ MS+CW (15%)]; h, Protocorm differentiation [½ MS+BP (1%)].

Sterilization and Culture Conditions

The collected capsules were washed with running tap water for 5-10 min. The capsules were transferred to laminar air flow; where they were surface sterilized with 3% sodium hypochlorite solution for 10 min followed by rinsing with sterile distilled water, subsequently they were treated with $HgCl_2$ (0.5% w/v) solution for 5 min followed by rinsing with sterile distilled water for three to four times. Then, the capsules were dipped in 80% ethanol for 1 min and flamed aseptically. Subsequently, these were cut open longitudinally with a sterile surgical blade and the seeds were scooped out and inoculated on the nutrient media.

Media Preparation and Inoculation

Murashige and Skoog (1962) medium (half strength) was prepared using standard stock solutions of macronutrients, micronutrients, iron, and vitamins. The pH was adjusted at 5.7±0.1. Sucrose (3%) and Agar

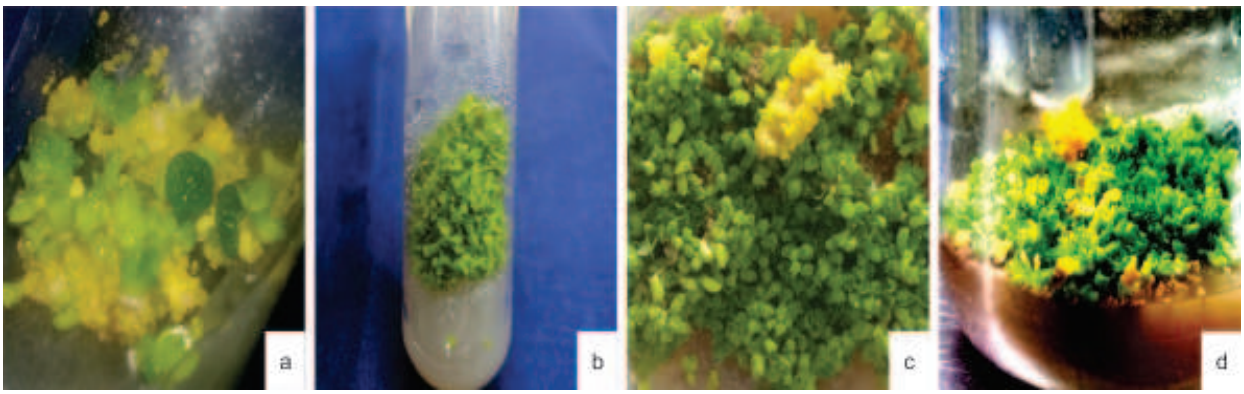

Fig. 2. a-d. Protocorm formation on half MS medium with growth additives at different time intervals in *Vanda tessellata*: a, Protocorm multiplication and differentiation (control) after 90 days; b, Leaf and root differentiation [(½ MS+CW (15% v/V)] after 70 days; c, Leaf and root differentiation [(½ MS+BP (1%)] after 45 days; d, Seedling formation [(½ MS+BP (1%) + TP (1%) +CW (15% v/v)].

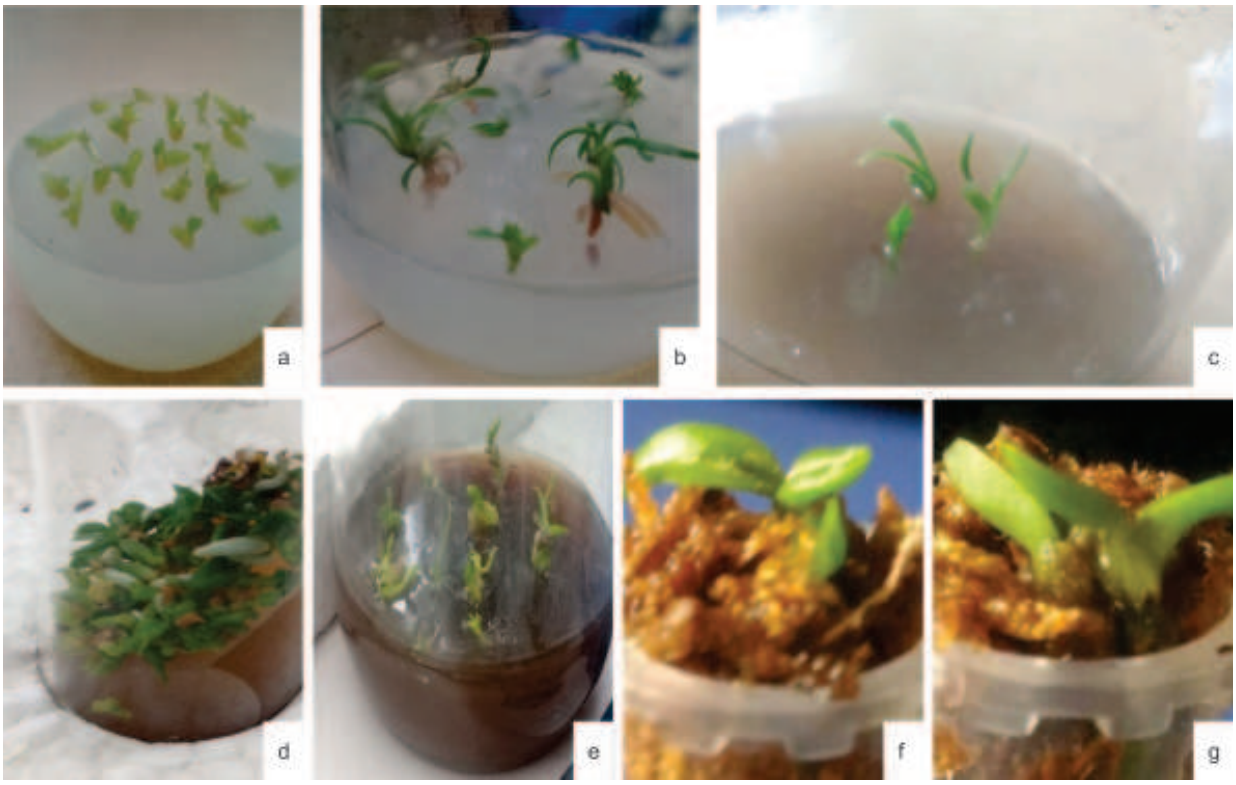

Fig. 3. a-g. Root induction on different nutritional combinations in *Vanda tessellata*: a, Initiation of rooting [½ MS + IAA (0.1 mgl^{-1}) + BAP (1.0 mgl^{-1})]; b, Root development [½ MS + CW (15%) + IAA (0.1 mgl^{-1}) + KN (1.0 mgl^{-1})]; c, Root development [½ MS + CW (15%) + NAA (0.1 mgl^{-1}) + BAP (1.0 mgl^{-1})]; d, Seedling development [½ MS + CW (15%) + NAA (0.1 mgl^{-1}) + KN (1.0 mgl^{-1})]; e, Seedling development [½ MS + CW (15%) + IAA (0.1 mgl^{-1}) + TDZ (1.0 mgl^{-1})]; f-g, Hardened seedlings in a pot with *Sphagnum* moss.

(0.8%) was added to the medium. Different concentrations of banana powder (BP: 1%, 2%, 3% w/v), tomato powder (TP: 1%, 2%, 3% w/v) (Himedia) and coconut water (CW: 5, 10, 15, 20 % v/v) were also incorporated in the medium. MS medium with different plant growth regulators such as IAA (0.1, 0.5, 1.0 mgl^{-1}), NAA (0.1, 0.5, 1.0 mgl^{-1}), KN (1.0, 2.0, 3.0 mgl^{-1}), BAP (1.0, 2.0, 3.0 mgl^{-1}), TDZ (1.0, 2.0, 3.0 mgl^{-1}) individually, and in combination with natural additives were prepared. The medium was sterilized at 121°C, 15 psi for 15 min in an autoclave.

Culture Conditions

All the cultures were maintained at 25±1°C continuous dark and 60-70% relative humidity. Observations were recorded every wk for the production of protocorms as a successful sign of *in vitro* germination. They were subcultured every 2 wks into a fresh media containing the same composition. For each treatment, 5 sets were taken. After 2 to 3 wks, the cultures were transferred to light conditions, 16 hrs photoperiod. The observation on the seed germination and protocorm developmental stages were observed and the data was documented. The germinated seeds and protocorms were subcultured on to the fresh medium containing growth additives like banana powder, tomato powder, and coconut water, and plant growth regulators like IAA, NAA, KN, BAP, and TDZ. The parameters in all the cultures were taken and recorded.

Growth Additives

After sterilization process, the seeds of *Vanda tessellata* were transferred on to half strength MS medium with plant hormones like IAA, NAA (0.1, 0.5, 1.0 mgl^{-1}), KN, BAP, TDZ (1.0, 2.0, 3.0 mgl^{-1}) individually or in combination and sucrose (3%) was added to the medium with the gelling agent agar (0.8%) and the pH was adjusted at 5.7.

Subculturing

Subcultures were maintained in the same conditions as for germination. Regular subculturing was done at every 3-4 wks of time period till the protocorms grew and formed complete seedlings.

Rooting and Hardening

The green and healthy seedlings obtained were subcultured on half strength MS mediun containing auxins IAA, NAA with (0.1, 0.5, 1.0 mgl^{-1}) along with banana powder and tomato powder (1-3%) individually

or in combination so as to assess the effect of these additives on the rooting of *Vanda tessellata* seedlings. Then, the complete seedlings were thoroughly washed with water to remove the chemical media. They were transferred to sterile plastic cups containing coco bricks and *Sphagnum* moss. The hardened seedlings were covered with a sterile plastic cover in order to maintain humidity for a long period and placed in culture room at 25±2^{0}C under photoperiod of 16 hr in cool fluorescent white light for few a wks to observe the growth.

Data Recording and Statistical Analysis

The experiments were conducted thrice using 5 replicates per treatment. The first subculture was performed in 10 wks after which subculturing to freshly prepared medium was done every 3 wks. The culture responses with regard to callus formation, shoot, and root development were recorded at regular intervals. Seed germination response was also examined by recording the seed germination percentage on different combinations of growth hormones.

The seed germination percentage was derived using the following formula:

Seed germination percentage = Number of seeds successfully germinated by swelling /Total number of seeds inoculated × 100.

The data obtained from the present investigation were subjected to analysis of variance (ANOVA) and significant differences were determined by employing Tukey's test at $p \geq 0.05$. The statistical data analysis was performed using graph pad prism version 5.03 program.

Results and Discussion

In the present study, seeds successfully germinated with varied germination frequency in different nutritional combinations. The time taken for onset of germination, protocorm development and differentiation thereof was also different depending upon the growth additive(s) used in the medium (Tables 1-4). It was observed that additional presence of banana extract and tomato extract at low concentration in MS medium was effective in enhancing the growth and development of seedlings. The protocorms obtained up to two leaf stage were dark green and healthy. Incorporation of coconut water (15%) in the medium favoured growth and multiplication of the protocorms and differentiation thereof. Gnanasekaran *et al.* (2010) had reported that the banana extract is rich in minerals, vitamins, and amino acids that play an important role in the growth of protocorm like bodies. The combination of banana powder, tomato powder, and coconut water with plant hormones such as IAA (0.1 mgl^{-1}) and KN (1.0 mgl^{-1}) in the nutrient medium showed the best response (46.6±1.20%), followed by IAA (0.5 mgl^{-1}) and KN (1.0 mgl^{-1}), NAA (0.1 mgl^{-1}) and BAP (1.0 mgl^{-1}), and IAA (0.1 mgl^{-1}) and TDZ (1.0 mgl^{-1}) (Table 4).

Table1. Effect of growth additives on seed germination of *Vanda tessellata* on half strength MS medium.

Growth additives			Germination percentage±S.E
Banana powder (g/l)	Tomato powder (g/l)	Coconut water (%)	
10	-	-	46.6±1.20
20	-	-	13.3±0.33
30	-	-	11.6±0.6
40	-	-	9.9±0.0
	10	-	19±1.30
	20	-	0±0.00
	30	-	0±0.00
	40	-	0±0.00
		5	9.6±0.0
		10	15.2±0.8
		15	78±1.15
		20	0±0.00

The values represent Mean ± standard error with in column followed by the significant difference by tukeys test ($p \geq 0.05$).

Exogenous cytokinin treatments showed increase in asymbiotic germination of many orchid species. De Pauw *et al*. (1995), Miyoshi and Mii (1995, 1998), and Stewart and Kane (2006) also reported increased levels of germination of several terrestrial orchids. The exact role of cytokinins in orchid seed germination is not well understood. Cytokinins, in general promote cell division, as well as RNA and protein synthesis (Bewley and Black, 1994). Certain mycorrhizal fungi are known to produce cytokinins (Crafts and Miller, 1974).

Table 2. Effect of combination of growth additives on the development of *Vanda tessellata* protocorms on half MS medium.

CW (%)	BP+TP (1%)	BP+TP (2%)	BP+TP (3%)	BP+TP (4%)
Control	10±0.03	6.6±0.1	0±0.0	0±0.0
5	20±0.04	15±0.00	10±0.8	9±0.6
10	16±0.33	4±0.3	11±0.57	4±0.33
15	96±1.9	11±1.00	15±0.8	0±0.00
20	12±0.4	0±00	0±0.00	0±0.00

The values represent Mean ± standard error with in column followed by the significant difference by tukeys test ($p \geq 0.05$). BP, Banana powder; TP, Tomato powder; CW, Coconut water.

Table 3. Response of *Vanda tessellata* seed germination on different nutritional combinations.

Nutrient combination	Time taken for onset of seed germination	Number of days taken for formation for protocorms	Per cent seed germination
½ MS (control)	60	80-90	50
½ MS+ CW (15%)	15-18	60-70	83.3
½ MS +CW (15%) + BP (1%)	30	45	78
½ MS + CW (15%) + TP (1%)	30-45	45	70
½ MS + CW (15%) + BP (1%) + TP (1%)	20-25	30-35	96

Time taken for germination: n = 120, df = 7, F = 18.3, $p \geq 0.001$ ($p = 0.00$); Time taken for protocorms: n = 120, df = 7, F = 3.7, $p \geq 0.005$; Result: n = 120, df = 7, F = 18.3, $p \geq 0.001$ ($p = 0.00$).

Table 4. Effect of combination of plant growth regulators along with BP and TP on the protocorm development in *Vanda tessellata.*

IAA (mgl^{-1})	NAA (mgl^{-1})	KN (mgl^{-1})	BAP (mgl^{-1})	TDZ (mgl^{-1})	%±S.E
0.1	-	1.0	-	-	46.6±1.20
0.1	-	2.0	-	-	33.3±1.9
0.1	-	3.0	-	-	23.3±0.67
0.5	-	1.0	-	-	40.3±2.5
0.5	-	2.0	-	-	19.9±0.66
0.5	-	3.0	-	-	13.3±0.00
1.0	-	1.0	-	-	9.9±0.3
1.0	-	2.0	-	-	7±0.3
1.0	-	3.0	-	-	0±0.00
-	0.1	-	1.0	-	26.6±3.83
-	0.1	-	2.0	-	23.3±1.90
-	0.1	-	3.0	-	19.9±0.66
-	0.5	-	1.0	-	16.6±0.03
-	0.5	-	2.0	-	11±0.57
-	0.5	-	3.0	-	9.96±1.93
-	1.0	-	1.0	-	0±0.0
-	1.0	-	2.0	-	4±0.3
-	1.0	-	3.0	-	7±0.00

Table 4. Effect of combination of plant growth regulators along with BP and TP on the protocorm development in *Vanda tessellata* (contd.).

IAA (mgl^{-1})	NAA (mgl^{-1})	KN (mgl^{-1})	BAP (mgl^{-1})	TDZ (mgl^{-1})	%±S.E
-	0.1	1.0	-	-	19.9±0.6
-	0.1	2.0	-	-	15.5±0.88
-	0.1	3.0	-	-	9.9±1.93
-	0.5	1.0	-	-	13.3±0.01
-	0.5	2.0	-	-	11±0.57
-	0.5	3.0	-	-	7±0.66
-	1.0	1.0	-	-	7±0.66
-	1.0	2.0	-	-	0±0.00
-	1.0	3.0	-	-	0±0.00
0.1	-	-	-	1.0	26.6±0.88
0.1	-	-	-	2.0	19.9±1.9
0.1	-	-	-	3.0	11.6±2.2
0.5	-	-	-	1.0	13.3±3.8
0.5	-	-	-	2.0	9.9±0.3
0.5	-	-	-	3.0	7±0.3
1.0	-	-	-	1.0	7±0.3
1.0	-	-	-	2.0	11±0.00
1.0	-	-	-	3.0	9.6±1.93

The values represent Mean ± standard error with in column followed by the significant difference by tukeys test (p=0.05). -, Nil; IAA, Indole-3-acetic acid; NAA, Napthalene acetic acid; BAP, 6-Benzyl Amino purine; KN, Kinetin; TDZ, Thiadiazuron.

Exogenous cytokinins supplied *in vitro* may substitute for naturally occurring compounds released during mycorrhizal infection. Only two species of fungi screened produced cytokinins in appreciable amounts for detection (Crafts and Miller, 1974). Cytokinins may also aid in lipid mobilization within orchid embryos (De Pauw *et al.,* 1995). Dimalla and Van Staden (1977) found that storage lipids in pecan nuts (seeds with high levels of lipids) were mobilized when treated with exogenous cytokinins. Auxins stimulate ethylene evolution especially under stress conditions, which in turn stimulate seed germination in many plant species (Liberman, 1979; Taiz and Zeiger, 1998). Although not investigated in orchid seeds, auxins may lead to low levels of ethylene evolution. The role of PGRs in asymbiotic orchid germination is uncertain, and responses of growth regulators are often species specific. A major obstacle in understanding the role of exogenous and endogenous PGRs in promoting/ inhibiting germination of orchid seeds may be the small size of the seeds and the possible low levels of PGRs in the embryos. Investigations on the concentrations of endogenous PGRs in orchid seeds as well as when PGRs are active in germination, would greatly enhance the information on how PGRs affect germination of orchid seeds.

Amongst the different concentrations of growth additives used, individually in half strength MS medium for germination, coconut water (15% v/V) gave the best results for frequency of germination (78%), followed by 46% in banana powder (1%) (Table 1). Table 3 shows the effect of combinations of growth additives with 15% coconut water on the frequency, onset of germination and time taken for protocorm development. Highest response of seed germination (96%) was achieved in half strength MS medium supplemented with CW (15% v/V)+BP(1%)+TP(1%). The chlorophyllous protocorms obtained showed better growth and development and differentiation thereof and healthy seedlings were developed.

Conclusion

Asymbiotic germination is an excellent technique to study biotic and abiotic factors of orchid seed biology. The populations of these orchid plants in nature is

depleting due to various reasons, one amongst these is their over collections due to their use in ethno medicine. During the present study, the growth additives showed a stimulating effect on seed germination, growth and multiplication of protocorms and differentiation thereof. Presently, highest response of seed germination (96%) was achieved in half strength MS medium supplemented with CW(15% v/V)+BP(1%)+TP(1%) in presently studied *Vanda tessellata*. The chlorophyllous protocorms obtained showed better growth and development and differentiation thereof. Although great advances are being made, orchid seed physiology still needs to be completely understood.

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