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# An efficient nutrient medium for asymbiotic seed germination and *in vitro* plant generation of *Vanda tessellata* (Roxb.) Hook. ex G. Don

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*Key words:* Banana, charcoal, genetic stability, micropropagation, protocorm, RAPD analysis, seedling development.

**Abstract: *Vanda tessellata* (Roxb.) Hook. ex G. Don is an epiphytically grown orchid well-known for its excellent floral value and therapeutic qualities. The present investigation deals with a study of asymbiotic seed germination and large-scale *in vitro* plant generation of *Vanda tessellata* by using three different basal media (MS, KC, and VW) and two supplements, charcoal and banana. Of these three media used for seed germination, MS (Murashige and Skoog) gave the best response, followed by KC (Knudson C) and VW (Vacin and Went). MS medium took less time to germinate seeds and maximum protocorm formation was also observed. MS medium with banana powder (15,000 mg/l) showed the best result for developing seedlings from protocorm and maximum growth of leaves and roots of the seedlings. Propagation through secondary protocorm formation was highest in MS media with charcoal (1000 mg/g). *In vitro*-grown plants were successfully acclimatized with an 89.4% survival rate. According to a random amplified polymorphic DNA (RAPD) analysis, the *in vitro* generated plants were clone copies of their parent plant and did not exhibit variations. These findings validated the most trustworthy techniques, which can also be applied for to large-scale medicinal *Vanda tessellata* plant production at the commercial level.**



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All relevant data are within the paper and its Supporting Information files.

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## 1. Introduction

Orchids are a unique group of flowering plants belonging to the family Orchidaceae. Orchids are popularly known for their beautiful, attractive

flower, long shelf life, and high purchase prices. Among monocotyledons, the Orchidaceae is a highly evolved family comprising nearly 850 genera (Stewart and Griffith, 1995; Singh *et al*., 2007; Gutierrez, 2010; Madhavi and Shankar, 2019). It has been reported that 28,237 species are distributed in the tropical forests of India, South Asia, Sri Lanka, South and Central America, and Mexico (Willis, 2017). In India, 155 recognized genera with 1256 orchid species are found in different habitats (Singh *et al*., 2019). According to available records, 466 taxa of orchids are found In West Bengal (Mitra, 2021). There are 12 orchid genera recorded in the Purulia district (Paramanik *et al.,* 2020).

*Vanda tessellata* (Roxb.) Hook. ex G.Don is a medicinally important epiphytic orchid of the family Orchidaceae. The pollination mechanism of orchids is highly specialized and seeds are small, thin, and non-endospermic. It is reported that approximately 1,300 to 4 million seeds are present per capsule (Pierik, 1987). Due to the lack of endosperm, a symbiotic association with mycorrhizal fungi is required to provide nutrients to embryos that are required for the seed germination of orchids in their natural habitat (Paramanik *et al.,* 2021). The epiphytic orchid's 30- 60 cm-tall stem is furnished with thickly coriaceous, recurved, plicate, and obtuse-keeled leaves. Flowers are greenish-yellow, with brown specks on the lip's middle lobe (Chauhan, 1999). The petals are shorter than the sepals, yellow with brown lines and white borders. The lip measures 16 mm in length and is bluish with purple specks. Capsules are with acute ribs that are 7.5-9.0 cm long and narrowly clavate-oblong (Fig. 1).

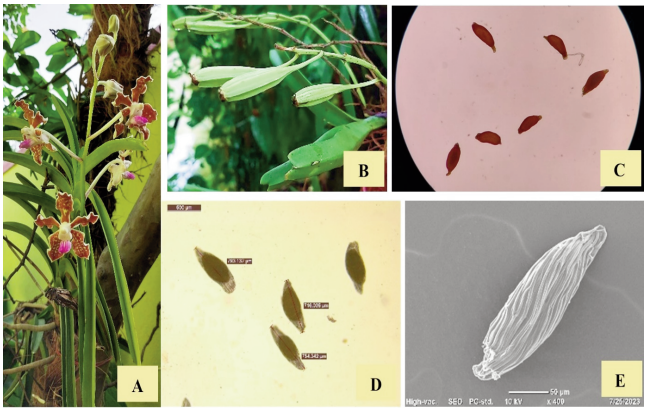


Fig. 1 - Morphological image of *Vanda tessellata*. (A) Plant with flower spike, (B) Mature capsule, (C) Light microscopic image of seeds (10x), (D) Stereo microscope image of seeds, (E) Scanning Electron Microscopy image of seed (400x).

In traditional medicine, *V. tessellata* has been commonly used to treat a variety of ailments, including fever, rheumatism, dysentery, and dyspepsia. The juice of the leaves is applied topically to treat otitis media. The root is used as a bronchitis cure and as an antidote to scorpion stings (Chauhan, 1999).

The value of orchids as a commodity has grown daily. Due to demands from massive collections in the past, the habitat of this medicinally significant orchid is being destroyed, which has caused the species to become rare and limited to minimal areas within its native habitats (Kaur and Bhutani, 2009). In nature, orchids have been propagated vegetatively to solve this problem, but it is a prolonged process. Therefore, plant tissue culture and micropropagation can be extremely effective in preventing the extinction of this orchid and increasing its population (Wochok, 1981). As a result, orchids must depend on external sources of nutrients for germination and large-scale production. The asymbiotic seed germination culture method, which was first commenced by Knudson (1946), is commonly used for seed germination of orchids. Another method for micropropagating orchids is to employ aseptically produced seedlings (Bhadra, 1999).

Molecular markers are crucial for determining the genetic diversity, variation, and resemblance of various plants and their population structure. Genetic variation is responsible for various factors related to *in vitro* culture settings (Pradhan *et al*., 2023). During *in vitro* culture, sometimes somaclonal variation changes the genetic composition of the regenerants (Rawat *et al*., 2013). Several molecular markers have been used to evaluate the genetic fidelity of clones generated *in vitro*. One effective and affordable method for identifying plant genetic variability is random amplified polymorphic DNA (RAPD)(Hussain *et al*., 2008).

This study aimed to develop an efficient nutrient medium for asymbiotic seed germination and large-scale *in vitro* plant generation of *V. tessellata* (Roxb.) Hook. ex G.Don. and also assess the genetic fidelity of *in vitro* regenerants with mother plants through RAPD analysis.

## 2. Materials and Methods

*Establishment of culture*

Seeds from 7-month-old, undehisced green pods

were used to establish cultures. Undehisced green pods of *V. tessellata* (Roxb.) Hook. ex G. Don were collected from the trunks of different trees in the Ajodhya hills of Purulia district, West Bengal.

The freshly harvested capsules were first given a five to ten-minute rinse under running tap water. After that, pods were rinsed in 90% ethanol for 20-30 seconds, treated with 0.1% (w/v) mercuric chloride solution for 10 minutes, and then the surface sterilization procedure was completed by washing the material three times in sterile distilled water. After excising the sterilized pods lengthwise, the seeds were scooped out and put in a conical flask with 100 ml of autoclaved distilled water. The mixture was then slowly shaken for five minutes. Culture tubes (25 x 150 mm) with 10 ml of nutrient media were inoculated with 100 µl of the seed suspension. Conical flasks (250 ml, 500 ml) and culture bottles (500 ml) contained 50 to 100 ml of nutrient media were used for plantlet development.

Three basal media, KC (Knudson, 1946), MS (Murashige and Skoog, 1962), and VW (Vacin and Went, 1949), hormone-free, were used for asymbiotic seed germination. Sucrose (3% w/v) was used as the carbon source. The pH of the medium was adjusted to 5.6 before autoclaving. After adding 0.8% (w/v) agar to solidify the media, the media were autoclaved at 125°C (15 psi) for 20 min. The cultures were kept at 24±2°C with a 10-hour photoperiod supplied by 3000-lux white fluorescent Philips lights.

*Multiplication and rooting*

MS media containing different concentrations of banana powder (15000 mg/l, 30000 mg/l, 60000 mg/l) and activated charcoal (1000 mg/l, 2000 mg/l, 3000 mg/l) added to the medium singly, were used to obtained well-developed seedlings from healthy protocorms. A combination of banana and charcoal in three different concentrations was also used in MS medium for plantlet development. Rooting occurs in the same medium. The cultures were kept at 24±2°C with a 10-hour photoperiod supplied by 3000-lux white fluorescent Philips lights.

*Acclimatization of seedlings*

Only seedlings with fully grown roots were chosen for the acclimatization phase. The nutrient medium was then completely removed from the entire seedlings by giving them a thorough water wash. The rooted seedlings were transferred to containers filled with potting mix containing small charcoal pieces, coconut husk, sphagnum moss, broken breaks, and dead tree bark (mango). Subsequently, they were kept in the growth chamber to maintain humidity (80%) and temperature (24±2°C) for a few weeks. After that, the plantlets were moved to a moist, shady place in the departmental garden, and water was applied to the plants twice a day.

*RAPD fingerprinting analysis*

The genetic stability of wild and *in vitro* propagated plantlets was assessed in the current study through the RAPD fingerprinting technique. Genomic DNA was isolated from the leaf tissue of both the control mother plant and five consecutive generations of *in-vitro*-grown plants. Leaf tissue was subjected to whole genomic DNA extraction following the supplied protocol of the DNA extraction kit (DNeasy® Plant Mini Kit-Qiagen, part no. 69104). The quality and quantity of the DNA samples were determined by recording the ratio of absorbance at A260/A280 in a UV-VIS spectrophotometer (UV-1800 SHIMADZU). The integrity of the genomic DNA was confirmed by electrophoresis on a 0.8% agarose gel. PCR amplification was done in 25 µl reaction volume containing 50 ng of genomic DNA, 8 µM primer (RAPD), molecular biology grade water, and 12.5 µl Hi-Chrome PCR Master Mix containing Taq DNA polymerase, dNTPs, $MgCl_2$.

A total of 10 primers from the OPA and OPB series (Integrated DNA Technologies) were used for PCR. Amplification was executed in DNA Thermal Cycler (Eppendorf Mastercycler Nexus X2 Thermal Cyclers). The initial denaturation temperature for the PCR was 94°C, which was succeeded by 35 denaturation cycles for 45 s at 94°C, annealing for 45 s at 27-38°C, and extension at 72°C for 30 s. Following the last cycle, a final extension step was included, lasting 7 minutes at 72°C. The amplified products were electrophoresed in a horizontal gel apparatus (Power PackTM Basic, Bio-Rad) with a "100 bp" DNA ladder (BioLitTM ProxiB) used to visualize and take pictures of the gels. Finally, the Gel Documentation system (Gel DocTM XR+, Bio-Rad, USA) was used to examine and evaluate the stained (0.5 µg/l) gel with ethidium bromide. To verify the reproducibility of each PCR, it was performed three times.

*Data collection and statistical analysis*

All parameters (germination percentage, protocorm formation percentage, survival, leaf formation, root formation, callus formation,

secondary protocorm formation percentage) were evaluated and analyzed using SPSS and expressed as mean ± standard error (SE). Three replicate cultures were set up for each treatment. One-way analysis of variance (ANOVA) was used to identify significant differences in data of all treatments, and Duncan's multiple range test (p=0.05) was performed to separate the means.

## 3. Results and Discussion

*Asymbiotic seed germination*

The shape and colour change of the seed was used to observe the response to seed germination. Most of the seeds were embryonated, and the testa of the seed was ruptured to form a swollen globular structure (Fig. 2 A). The beginning of seed germination and the development of protocorm on three basal media following, MS, KnC, and VW media were periodically recorded from the first inoculation day (Table 1). Among the three basal media, the highest percentage of germination (83.50±0.31%) was recorded in the MS medium in shorter time (45 days). Second-stage protocorm with a slightly elongated apical region was also (70.00±0.06) observed in MS medium after 20 days. In the other media assessed the germination occurred beyond 100 days with lower germination and protocorm formation percentages (Table 1).

Fig. 2 - Asymbiotic seed germination and seedling development of *Vanda tessellata*. (A-B) Early globular stage, (C) Protocorm showing initiation of leaf primordia, (D-E) Protocorm with distinct leaf and developed seedling with leaf and root, (F) Multiple protocorms with many leaf primordia, (G) Axillary shoot formation from multiple protocorms (H) Leaf formation from secondary protocorm in MS with charcoal (1000 mg/l), (I-J) Close view of leaf primordia from secondary protocorm, (K) Protocorm with callus, (L-O) Sequential stage of seedling growth after subculture on medium containing MS with 15000 mg/l banana powder, (P-Q) Stepwise acclimatization.

*Seedling development: multiplication and rooting*

Healthy protocorms were transferred to MS media supplemented with charcoal or banana powder. The highest percentage of leaf formation (82.40±2.74) and healthy root formation (83.50± 2.00) were found in MS medium-containing banana powder (15000 mg/l). Leaf formation and root formation both were observed in the same medium composition. A little callus development in this medium was also noticed (Fig. 2 K). Secondary protocorms originated from protocorms, were observed and the highest percentage was formed in MS with charcoal (2000 mg/l) (Fig. 2 I and J). A combination of banana and charcoal at different concentrations showed no significant result (Table 2). Only a combination with low concentration

Table 1 - Effect of different media on the seed germination of *V. tessellate*

| Media | Time duration required for germination (days) | Germination (%) | Protocorm formation (%) |
|---|---|---|---|
| Murashige and Skoog (MS) | 46 | 83.50±0.31 c | 70.00±0.06 c |
| Knudson C (KnC) | 103 | 28.00±0.05 b | 35.00±0.08 b |
| Vacin and Went (VW) | 190 | 10.00±0.14 a | 2.00±0.14 a |

The mean of three replicates ± SE (standard error) is displayed in each column. Mean values followed by the same letter do not differ significantly at the 0.05 level (DMRT).

Table 2 - Effect of charcoal and banana and their combination on the morphogenetic responses and growth of *V. tessellata* seedlings after 3 months of *in vitro* culture

| Treatments | Survival (%) | Leaf formation (%) | Root formation (%) | Callus formation (%) | Secondary protocorm formation (%) |
|---|---|---|---|---|---|
| MS | 64.33±2.14 e | 37.08±0.93 e | 30.30±1.38 b | 18.97±1.36 c | 10.71±0.82 c |
| MSC1 | 63.50±1.79 e | 26.70±1.13 d | 0.00±0.00 a | 0.00±0.00 a | 35.50±1.75 e |
| MSC2 | 42.00±1.32 d | 18.70±0.66 b | 0.00±0.00 a | 0.00±0.00 a | 23.50±1.80 d |
| MSC3 | 12.20±1.43 b | 10.20±0.70 b | 0.00±0.00 a | 0.00±0.00 a | 0.00±0.00 a |
| MSB1 | 99.50±0.28 f | 82.40±2.74 g | 83.50±2.00 d | 11.40±0.77 b | 5.90±0.94 b |
| MSB2 | 42.50±2.34 d | 42.50±1.89 f | 40.20±1.74 c | 0.00±0.00 a | 0.00±0.00 a |
| MSB3 | 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 |
| MSC1B1 | 24.40±1.13 c | 24.40±1.92 d | 0.00±0.00 a | 0.00±0.00 a | 0.00±0.00 a |
| MSC2B2 | 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 |
| MSC3B3 | 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 |

The mean of three replicates ± SE (standard error) is displayed in each column. Mean values followed by the same letter do not differ significantly at the 0.05 level (DMRT). C1= Charcoal 1000 mg/l, C2= Charcoal 2000 mg/l, C3= Charcoal 3000 mg/l, B1= Banana 15000 mg/l, B2= Banana 30000 mg/l, B3= Banana 60000 mg/l.

(MSC1B1) showed a slight survival response (24.40±1.13) along with leaf formation as compared to the other two combinations (Table 2). Previously, Aktar *et al*. (2008) and Islam *et al*. (2015) reported that banana homogenate (BH) had beneficial and boosting effects on the regeneration of new PLBs and a healthy shoot system established from PLBs in *Dendrobium* orchids. Mature bananas are fairly rich in vitamin B6 or pyridoxine. However, they have comparatively high concentrations of vitamins A (carotene), C (ascorbic acid), and B-complex (niacin, thiamine, and riboflavin)(Qamar and Shaikh, 2018). Minerals including sodium (Na), iron (Fe), copper (Cu), phosphorus (P), manganese (Mn), zinc (Zn), copper (Fe), and especially potassium (K) can also be found in bananas (Sarma *et al.,* 2021).

Potassium can help to provide resistance to drought, helping orchids to transport water from the roots to the apices, and preventing the orchids from wilting (Xu *et al.,* 2021). According to Minea *et al*. (2004), 10% banana homogenate increased the size of the leaves on *Spathoglottis kimballiana* Hook. f. In *Dendrobium nobile* Lindl. cultures, banana homogenate considerably boosted the formation of leaves (Sudeep *et al*., 1997). Activated Charcoal (AC) can be used in media to reduce phenolic browning. Browning of explant of several plant species has been controlled using the AC (Meziani *et al*., 2016; Mittal *et al*., 2016; Rani and Dantu, 2016; Magrini and Devitis, 2017; Irshad *et al*., 2018). Kim *et al.* (2019) reported that MS medium supplemented with AC had prevented browning in seedling development of *Pecteilis radiata*. The adsorptive qualities of AC are principally noted for both its beneficial and detrimental effects. By adsorbing phenolic molecules and inactivating peroxidase and polyphenol oxidase, the AC stopped browning (Pan and van Staden, 1998), but large concentrations of AC can absorb the PGRs and mineral nutrients in the culture medium, reducing the frequency of seedling conversion. All plants lost their viability after subculturing on the media containing MSB3, MSC2B2, and MSC3B3 (Table 2). Indeed, high concentrations of AC and banana powder drastically affected the survival.

*Acclimatization*

A vital stage in the micropropagation process is acclimatization. Plantlets (about 5-6 cm) were moved to pots containing charcoal, coconut husk, brick, mango bark, and sphagnum moss in a 2:2:2:1:1 ratio. With a 92% survival rate, the *in vitro*-raised seedlings were acclimated in a plant growth chamber in the laboratory for 2 months. After that, an 89.4% survival rate with more or less similar healthy plants was observed after 10 months of transfer in the poly-house.

*Genetic fidelity and assessment of in vivo and in vitro plants by RAPD fingerprinting analysis*

According to earlier studies, RAPD is a widely used marker to assess the genetic fidelity of different micropropagated plants (Kawiak and Lojkowska,

2004; Tikendra *et al*., 2019). The genetic make-up of *in vivo* and *in vitro* generated plants was compared using ten randomly selected Random amplified polymorphic DNA (RAPD), a dominant marker. The leaves of the mother plant and the leaves of five successive generations of *in vitro*-grown plants were collected, and the leaf tissues were used to extract the DNA. Seven of the ten randomly chosen RAPD primers produced unique band patterns in the current investigation. These are OPA-03, OPA-10, OPA-11, OPA-15, OPA-18, OPA-19 and OPB-01 (Table 3). *In vitro,* regenerated plants and the mother plant growing in the garden (the plant from which the explants were collected) were both genetically homogeneous, as evidenced by the lack of variance in the banding pattern displayed by any of the primers. The annealing temperature of the seven primers that exhibited scorable monomorphic band patterns is shown in a tabular format. The temperature at which all seven primers were annealed was 27°C. According to Pradhan *et al*. (2023), RAPD is the successful marker to assess the genetic fidelity of *in vitro* grown plants with the mother plant. The current investigation validates the earlier reports.

Six unique monomorphic bands having a size range of 320 bp to 900 bp were generated by OPA 03 (Fig. 3). OPA 18 yielded four unique monomorphic bands having a size range of 450 bp to 1000 bp. Two distinct monomorphic bands were produced from OPA 10, and the size range of the bands was from 800 to 920 bp (Fig. 3). The fact that all of the bands were monomorphic demonstrated the genetic stability of *in vitro* regenerants and the similarity of the genetic makeup of the micropropagated plants to the mother plant, which was the actual objective of the current investigation.

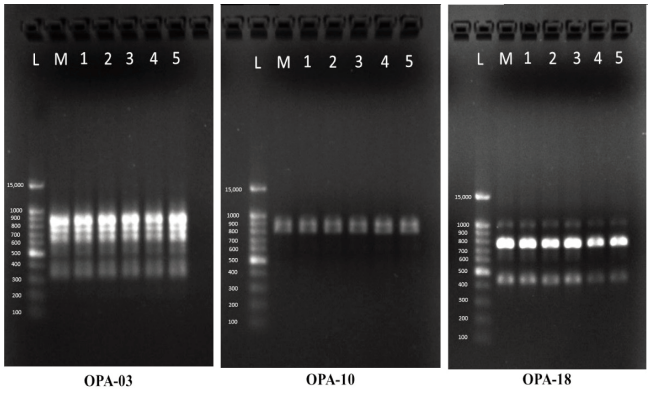


Fig. 3 - Gel electrophoresis of RAPD fragments of *Vanda tessellata* obtained with primer OPA-03, OPA-10, and OPA-18. Lane L 100 bp DNA ladder; lane M mother plant, lanes 1-5 are i*n vitro* regenerated plants of five successive generations.

## 4. Conclusions

The present research reports a successful nutrient culture medium for asymbiotic seed germination of *V. tessellata*. An efficient, cost-effective nutrient

Table 3 - Effects of sowing date and mulching on marketable root yield (MRY), unmarketable root yield (URY), and weed density (WD)

| Primer | Sequence (5'-3') | Tm (°C) | Total bands |
|---|---|---|---|
| OPA-03 | AGTCAGCCAC | 27 | 6 |
| OPA-10 | GTGATCGCAG | 27 | 2 |
| OPA-11 | CAATCGCCGT | 27 | 1 |
| OPA-12 | TCGGCGATAG | 27 | 0 |
| OPA-15 | TTCCGAACCC | 27 | 2 |
| OPA-18 | AGGTGACCGT | 27 | 4 |
| OPA-19 | CAAACGTCGG | 27 | 2 |
| OPA-20 | GTTGCGATCC | 27 | 0 |
| OPB-01 | GTTTCGCTCC | 27 | 2 |
| OPB-12 | CCTTGACGCA | 27 | 0 |
| Total | | | 18 |

Mean values within rows and columns followed by a different letter(s) are significantly different at a 5% probability level.
CV = coefficient of variation. LSD = least significant difference.

media for large-scale *in vitro* plant generation was also achieved by using banana powder supplementation. From the above findings, it may be concluded that MS medium with banana powder is the best medium for overall seedling growth and multiple protocorm formation of *V. tessellata*. Through RAPD analysis, it has been successfully proved that all regenerants were genetically similar to the parent plant. The media reported in the current study does not include the use of plant growth regulators (PGRs) for plant development and multiplication, this condition minimizes the possibility of occurrence of genetic alterations. This study would help the pharmaceutical and floriculture industry and conserve wild populations of orchids in the near future.

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