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In Vitro Studies on Tropical Orchid, *Vanilla planifolia* using different Concentration of Growth Regulators

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Abstract: ***Natural vanilla flavours are presently in high demand in food industry and alternate routes of production of these through secondary metabolic pathways using plant cell and tissue culture could constitute the best technology for immediate future. In vitro production of natural vanillin could be achieved through two different methods. In the first, organised root tissue formed from the explants produces vanilla flavour compounds in precursor feeding experiments. The flavour production proceeds through the phenylpropanoid pathway in which the organised root tissues function as biocatalysts. Ferulic acid has proved to be the best source for the flavour compounds. In the second method, the callus maintained in Murashige and Skoog (MS) medium containing a specific combination of growth regulators is used in precursor feeding experiments as before for production of flavour.***

Keywords: nodal explants, organised root tissues, vanilla flavours, *Vanilla planifolia* Andr.

1. Introduction

Vanilla flavour (*Vanilla planifolia* Andr.) is an important food additive and the present worldwide consumption of vanillin is estimated to be 12,000 tonnes. Approximately 50 tonnes are in the form of natural vanillin extracted from beans of *Vanilla planifolia,* with synthetic vanillin providing the remainder from petrochemical and wood pulping industry. The natural vanillin costs US $ 4,000/kg depending on quality, compared with US $ 15/kg for synthetic vanillin (Lomascolo *et al.,.,* 1999). The synthetic vanillin currently in widespread use as in the process of losing demand as it contains only a single compound made from plant lignin (Nabard, 1998). Strong market demand for natural and environmentally friendly products has spawned efforts to produce vanillins by natural means. But the natural vanilla flavour is far superior due to the presence of more than 250 compounds (Riley and Kleyn, 1989; Adedeji *et al.,.,* 1993). The traditional method of production of natural vanillin is cumbersome as it involves cultivation of plants in restricted climatic regions, hand (manual) pollination with limited success and the tedious processes for curing and extraction of products from the pods. Again, as flowering is seasonal the production of natural vanillin is also seasonal. The tissue culture method envisaged here could be used throughout year in laboratories / factories and for commercial production.

Plant tissue cultures are potentially valuable for studying the biosynthesis of secondary metabolites. In view of the current consumer demands for natural foods and plant-derived medications, *in vitro* production of valuable secondary products has become an industrially promising venture. The production of flavour compounds with cultured plant cells is based upon their unique biochemical and genetic capacity and the totipotency of plant cells (Schleiden, 1838; Schwann, 1839). Thus, every cell of the *Vanilla* plant contains the genetic information necessary to produce the numerous biochemical components (or their precursors) that constitute natural vanilla flavour. The potential advantages of plant tissue culture systems include; independence from environmental factors, geographical indications, uniformity and controlled production in response to demand.

Biosynthesis of natural vanillin and other related compounds follows the phenylpropanoid pathway (Funk and Brodelius, 1994, Knorr et al., 1993). All the enzyme systems required for the biosynthesis of natural flavours from precursor are present in cultured cells. These cells could thus function as biocatalysts if cultures are provided with the precursors. Since the natural vanillin flavour contains over 250 organoleptic compounds, the ideal precursors for their synthesis through phenylpropanoid pathway appear to be phenylalanine - cinnamic acid - coumaric acid - caffeic acid - isoferulic acid - 3,4-dimethoxycinnamic acid - 3,4-dimethoxybenzoic acid - vanillic acid (after Funk and Brodelius, 1992).

Plant cell cultures of *V.planifolia* and other species have been initiated from cells, tissues and plant organs such as leaves and stems (Davidonis and Knoor 1991, Zerihum et al.,., 2009, Geetha and Shetty 2000, Zuraod et al.,., 2013, Foong Jing et al., 2014, Renuga and Saravana Kumar 2014, Jadid et al., 2015, Nurcahyani et al., 2015, Vijaykumar Biradar et al., 2016), growing plant shoot tip, roots and seeds (Knuth *and* Sahai, 1991) or green capsules (Funk and Brodelius 1990; Knuth and Sahai, 1991).

The effect of several plant hormones and their combinations on flavour production and composition of vanilla components are examined by Knuth and Sahai (1991). The Influence of growth regulators on cell growth and formation of phenolic substances has also been explored by Funk and Brodelius (1990). Funk *and* Brodelius (1992) have also studied the activation effect of kinetin on some enzymes involved in the phenylpropanoid pathway. The possibility of a bioprocess for the production of natural vanillin from a ferulic acid precursor with aerial roots as the biocatalysts

and charcoal product reservoir has been carried out by West Cott et al (1994). However in our experiment, the *in vitro* protocol was developed to mass culture vegetative tissues of *V.planifolia* for production of flavour compounds.

2. Material and Methods

Cultivation of cuttings

Cuttings of *V.planifolia* raised in pots, which contains soil and farmyard manure (FYM) 1:1 ratio (Rosman, R; Tasma-IM 1998) in a partially natural shaded place in our centre from where the explants are collected. Previously mother cuttings were obtained from M/s. Parry Agro Industries Ltd., Candura - Sheikalmudi Estate, Coimbatore District, Tamil Nadu.

Explants preparation

Shoot tips and young nodal segments with petioles were excised approximately 2 cm from mature healthy vines. These explants were soaked in liquid detergents (Tween 20) for 3 min. and then rinsed in running tap water for 10 min. The cleaned explants were surface sterilized with mercuric chloride 0.1% (W/V) for 8 minutes followed by 10 rinses with 2 minutes intervals in sterile distilled water aseptically. Surface sterilized explants were trimmed out at their cut ends.

Media and primary culture initiation

Murashige and Skoog (1962) (MS) medium supplemented with various plant growth hormones (HiMedia, Mumbai, India) in the combination of Naphthaleneaectic acid (NAA) 2-6 mg/l with 2,4-dichlorophenoxy acetic acid (2,4-D) 0.5-1mg/l and 6-benzyladenine (BA) 1-2 mg/l were used for primary culture initiation. The pH of the MS medium with 3% sucrose was adjusted 5.6 ±.2 prior to sterilisation and gelled with 0.9% (W/V) agar (HiMedia, Mumbai, India) and sterilized for 20 minutes under 1.05 kg/cm² pressure at 121ºC. The cultures were incubated under cool fluorescent lights with 1500-2000 Lux for 16 hrs at a temperature of 25±2 and 70±10 relative humidity. Axillary bud with base of petiole containing nodes and shoot tips were inoculated vertically by inserting around 5mm of the lower end in to medium aseptically. Each treatment had ten replicates and data were documented after 40 days of culture.

Organised Root Tissues (ORT) and callus establishment

MS media supplemented with NAA 2.5 mg/l and BA 1.0 mg/l were used for organised root tissue formation from nodal explants, after four weeks that the cultures were subcultured in the same medium with increased concentration of NAA up to 8.0 mg/l and BA 3.0 mg/l.

Callus cultures were well established from the base of shoot bud and young leaf petiole in MS media supplemented with low concentration of NAA and BA. Nature of callus was pale yellow in colour. The callus was subcultured for further proliferation and biomass production by altering the concentration and combination of PGRs under same physical parameters.

Extraction and analysis of flavour compound

Organised root biomass were transferred from specific hormonal media (Table 1) to the hormone free medium and treated with ferulic acid as precursor, after incubation for 72 hrs extracted of flavour compounds according to the method followed by Westcott *et al.,.,* 1994. Similar experiments were done with callus tissues.

The various explants were also tried for callus initiation on the MS media containing 2,4-D and other auxins. In vitro callus cultures 1g were harvested extracted using 70% ethanol (Kunth and Shai, 1989) in a soxhlet for 48 hours. The extract was dissolved in HPLC grade water and remaining residue was dissolved in of HPLC grade methanol separately. These extracts were filtered through sartorius RC-membrane syringe filter (0.20µm) and 10 µl was injected. Chromatography was carried out using Shimadzu HPLC with auto sampler and standard C18 column (25 cm x 4.6 mm). The mobile phase consisting of methanol: acidified water was used in ratio of 1: 9. Acidified water was prepared by mixing HPLC grade glacial acetic acid and water in the ratio of 1: 80. Degassing was performed under vacuum. Flow rate was 2.5 ml/min; back pressures 250 psi and compounds read at 254 nm in an UV detector. The total run time was 30 min, but it is preferable if the time is extended to 40 min. The identification and quantification of compounds were achieved by direct comparison with authentic standard (AOAC Official method 990.25).

3. Results and Discussion

Primary culture establishment

Initially the primary culture establishment was from young nodal explants of *V.planifolia* cultured in 3% sucrose containing basal MS medium supplemented with different PGRs at various concentrations in different proportion. The medium supplemented with NAA upto 2.0 mg/l and BA upto 1.0 mg/l showed that only axillary bud was developed into a single plantlet without forming any remarkable initiation. Primary culture could be established in medium supplemented with increased level of NAA (> 2.0 mg/l) and BA 1.0 to 2.0 mg/l (Fig1). The organised yellow tissue was formed at base of axillary bud as well as at cut base of young nodal segment (Table 1). There was a clear indication that the NAA and BA combination resulted in formation of organised tissues from various young explants such as axillary bud, leaf and shoot tips, but not with rootlet. When culture of mature nodal explant was grown in media supplemented with NAA 2.5 mg/l and BA 2.0 mg/l multiple shoots were observed (Fig 1e), but with the same combination the young shoot tips respond to form organised tissues at cut base (Fig 1a). The type of explants also plays a vital role in explant culture morphology.

Table 1: Effect of PGRs on organised tissue development in *V. palnifolia* (Data were reordered after 40 days in culture)

No.	*MS media with PGRs mg/l*	*Explants /Organised Root Tissue (ORT) culture*	*Tissue proliferation*
A	NAA 2.5 + BA 2.0	Matured nodes	Multiple shoot buds
B	NAA 2.5 + BA 2.0	Young shoot tip	Yellow coloured organised tissues at cut end
C	NAA 2.5+ 2,4-D 1.0+ BA 2.0	Axillary bud / young leaf base	Yellow coloured organised tissues at cut end
D	NAA 3.0+ BA 1.0	Axillary bud	Yellow coloured organised tissues at base of bud
E	NAA 4.0+ BA 2.0	Nodal explant/ORT	ORT with pale yellowish green colour projections
F	NAA 5.0+ BA 2.0	Nodal explant/ORT	Globular ORT with root hairs
G	NAA 6.0+ BA 2.0	Nodal explant/ORT	Globular green fused ORT
H	NAA 6.5+ BA 2.0	Nodal explant/ORT	Globular ORT with numerous root hairs

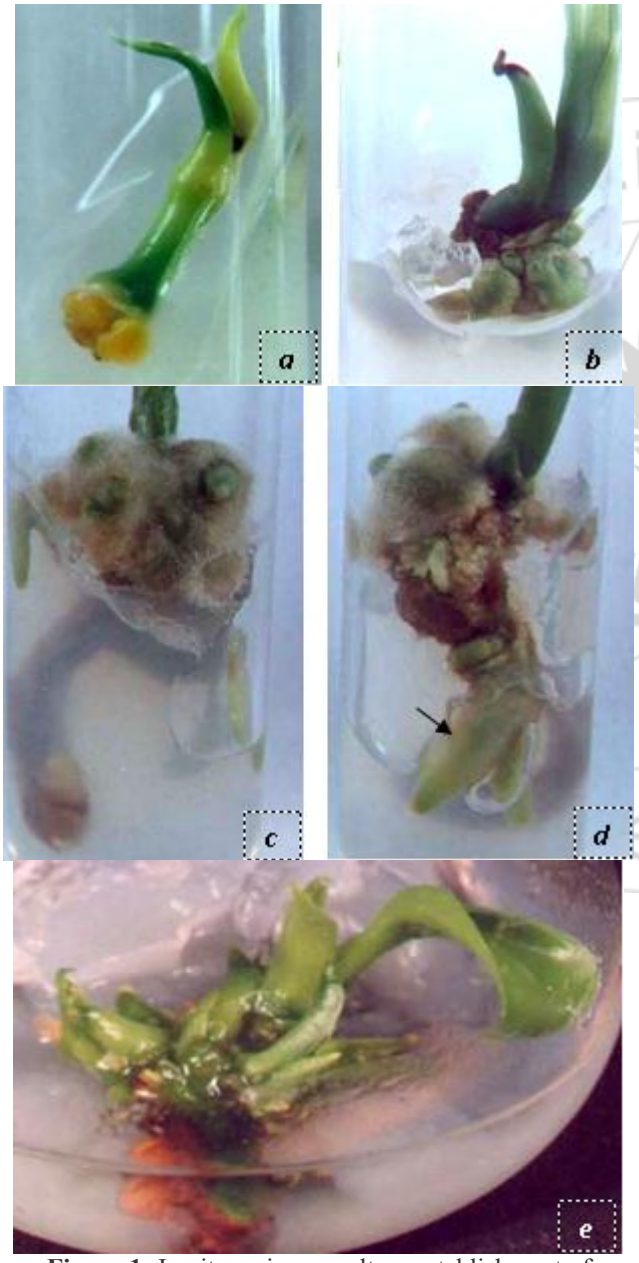

Figure 1: In vitro primary culture establishment of V.planifolia on MS medium with various PGRs. **a-d** organised tissue formations from various explants. **a-**Young shoot tip on medium with NAA 2.5 mg/l and BA 2.0 mg/l. **b-d** Nodal explant **b** on medium with NAA 4.0 mg/l and BA2.0 mg/l. **c** and **d** on medium with NAA 5.0 mg/l and BA2.0 mg/l (observe the development of root let in **d**) **e -** Multiple shoots development from mature node on medium with NAA 2.5 mg/l and BA 2.0 mg/l.

Organised Root Tissues (ORT) and Callus establishment

The organised yellow tissue was developed into organised root tissue when subcultured in increased level of NAA (4-6.5mg/l) and BA (2.0 mg/l) containing MS media (Fig. 2 a-d) and (Table E-H). The maximum biomass could be harvested from these cultures (Fig. 3). In the media containing less than 4 mg/l NAA does not develop into organised root tissue.

The observation also revealed that the nodal explant of axillary bud developed into plantlet. In this plantlet multiple buds developed around its nodes. Those nodal segments were subcultured in same combination of NAA 2.0 mg/l and BA 2.0 mg/l for establishment of multiple buds. Each bud around 1cm length was separated and subcultured after 21 days in different combinations. Callus was formed at the base of shoot bud in NAA 1.0 mg/l and BA 1.5 mg/l in MS medium. (Fig 2f)

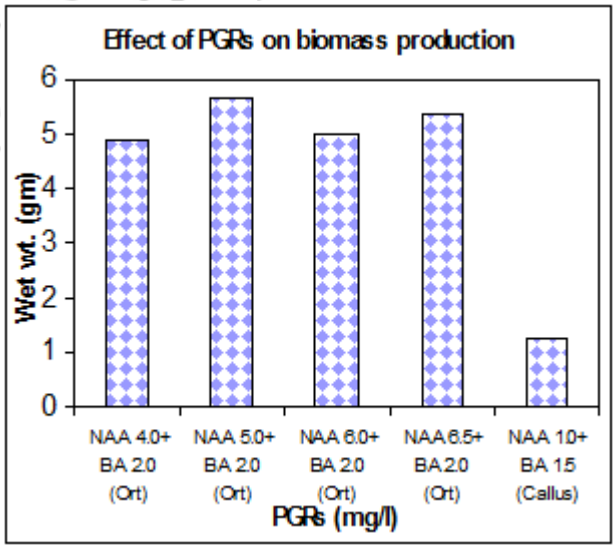

Figure 3: Data were reordered after 40 days in culture

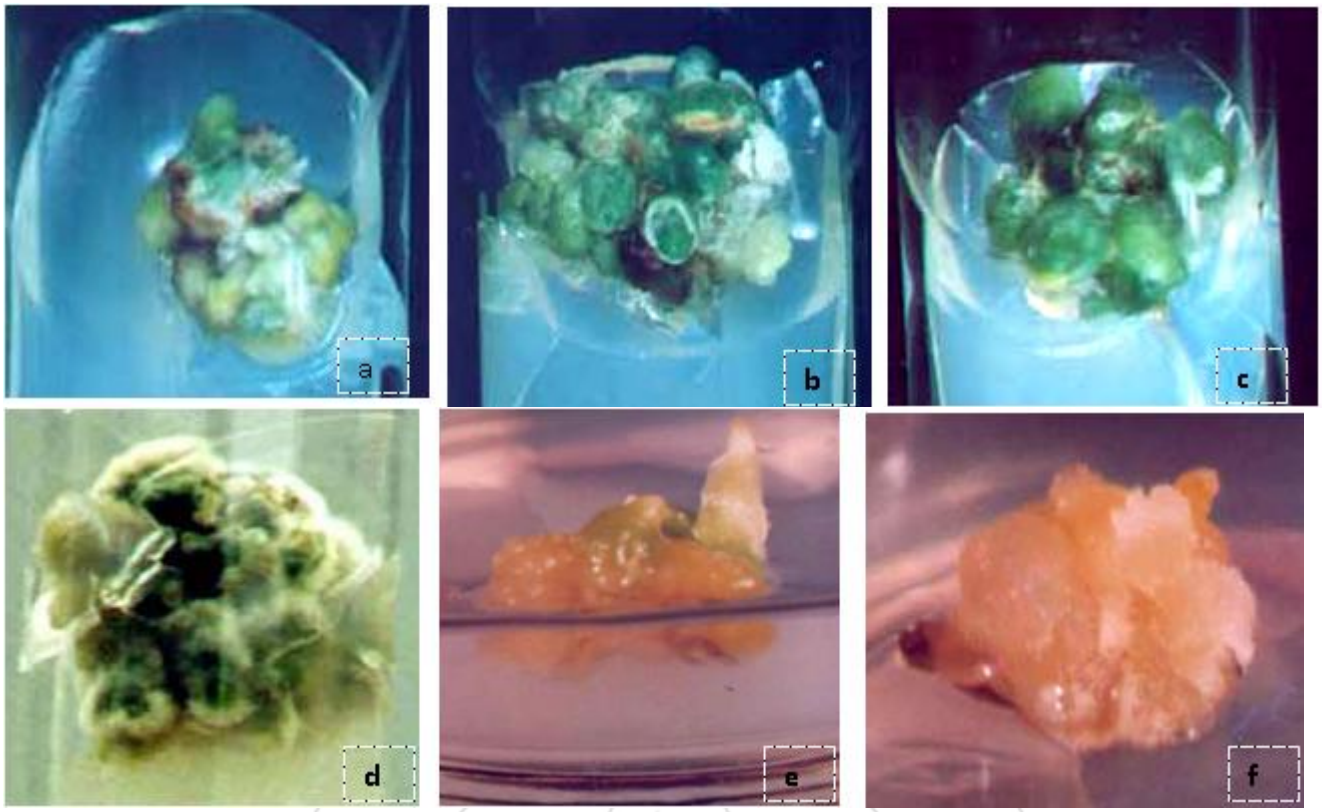

Figure 2: Establishment organised root tissue (ORT) and callus of *V.planifolia* on MS medium with various PGRs. **a-d** Organised root tissue formations. **a-**Pale yellowish green ORT on medium with NAA 4.0 mg/l and BA 2.0 mg/l. **b** medium with NAA 5.0 mg/l and BA2.0 mg/l. **c** medium with NAA 6.0 mg/l and BA2.0 mg/l **d** ORT with numerous hairs on medium with NAA 6.5 mg/l and BA2.0 mg/l **e -** shoots development on medium with NAA 2.0 mg/l and BA 2.0 mg/l. **f** callus development on medium with NAA 1.0 mg/l and BA 1.5 mg/l.

Vanilla plant tissue provides an excellent source for the production of vanillin and other related compounds. Organised vanilla tissue very rarely forms a callus. This is not surprising because vanilla is a monocotyledon and an orchid, a plant group with little tendency towards dedifferentiation in tissue cultures. In our experiments we could develop callus but biomass yield was very low, only 20% of the root biomass (Fig 2 -f). Hence option for organised root tissue biomass development is more apt since the root contains highest concentration of vanillin compared to other somatic tissues (200 µg/g dry wt.). The root tissue also is amenable to precursor treatment as it acts as biocatalyst. The addition of ferulic acid increases the vanillin concentration to 489µg/g after 72-hrs incubation (Westcott *et al.,,* 1994). The flavour production proceeds through the phenylpropanoid pathway in which the organised root tissues function as biocatalysts. In our experiments the yield of vanillin from organised root tissue was more than 30% to that present in vanilla beans. The results are of considerable significance for the development of an efficient biocatalyst process for continuous production of the vanilla flavour from cultured vegetative root tissue. This holds immense potential for the commercial production of natural vanillins using precursors which can be obtained residues from sugar and paper-mill industries in scale up studies. This has dual benefits by using wastes the pollution low is reduced and cheap substrates are used to produce highly valuable flavours.

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