Indian J. Pl. Genet. Resources 2(2) : 114–121, 1989

Use of Tissue Culture Technology in *Vanilla* and Possibilities of Germplasm Conservation

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Plantlets were produced from tip cultures of young aerial roots of Vanilla planifolia. *With the breakdown of the root cap, the cells of the quiescent centre divided forming a hemispherical mass of cells which developed either into a single shoot meristem or organized themselves into several meristems bearing leaf primordia. After a few leaves have been formed a root meristem differentiated establishing a well formed plantlet. Since the constituent cells of the root apical meristems are genetically stable, less differentiated and permit plant regeneration in high frequency, they form an ideal material for the long-term preservation of germplasm.*

Renewed interest in recent years in *Vanilla* (*Vanilla planifolia* (Salisb.) Ames (Family Orchidaceae), the natural source of the flavouring and spice, has led to increased interest in improved techniques of cultivation and propagation (Philip & Nainar, 1986, 1988a, b, c). *V. planifolia* produces numerous minute seeds but hardly 1-2 per cent of them germinate under natural conditions. Hence, the crop is commercially propagated by means of long-stem cuttings. Preservation of germplasm in such vegetatively propagated crops is highly taxing on manpower and land resources. Furthermore, a major threat to germplasm collections of *Vanilla* is a root-rot disease caused by *Fusarium batatatis* var. *vanillae* Tucker which often has a devastating effect and completely destroys the plantation.

Meristem culture is a proven technique for the production of disease-free plants and their clonal propagation. While meristem culture ensures short-term storage of germplasm, cryopreservation of meristem can offer an ideal and realistic method for the long-term storage of germplasm in a genetically stable and disease-free condition (Kartha, 1981). However, the current technique of meristem culture and cryopreservation often necessitates the removal of the shoot apex and in monopodial orchids like *Vanilla*, this causes a setback in the growth and development of the mother plant, limiting the availability of shoot meristems. Furthermore, shoot apical meristems are enclosed in many whorls of leaves and require cumbersome aseptic manipulations to dissect out the meristematic domes. Clearly, the development of *in-vitro* techniques of rapid and large scale multiplication of disease-free and genetically stable plants from apical meristems of aerial roots form one of the pre-requisites before an effective method of cryopreservation can be considered in *Vanilla*.

MATERIALS AND METHODS

Aerial root tips (2 cm long) were collected from seven-year old vines of *V. planifolia* grown in the Botanical Gardens of the University of Calicut, Kerala, India. They were surface sterilized with 0.5 per cent $HgCl_2$ for 10 minutes and washed three times with sterile water. The explants were incubated on filter paper bridges in tubes containing liquid MS (Murashige and Skoog, 1962) medium supplemented with IAA (0-10 mg l^{-1}) and 0.2 mg l^{-1} kinetin. The pH of the medium was adjusted to 5.8 prior to autoclaving and the cultures were maintained at 24 $\pm$ 2°C with continuous illumination (7000-8000 lux). After three months in MS medium when the root tips had swelled considerably, the tips (approx. ca 7 mm long) were excised under sterile conditions and planted on the solidified MS medium (0.8 per cent agar) supplemented with IAA (0-10 mg l^{-1}) and 0.2 mg l^{-1} kinetin in 100 ml Erlenmeyer flasks. Each experiment was performed with 40-60 root tips per culture treatment. At weekly intervals, five samples of the cultured root tips were removed from the medium and briefly washed in distilled water. The terminal region of the root tip (approx. 3 mm) was excised and fixed in FAA (formalin, acetic acid, alcohol) or Carnoy's fluid for 24 hours. After dehydration through tert-butyl alcohol series and embedding in paraplast, serial longitudinal sections were prepared at a thickness of 12 μm. Sections were stained with tannic acid and ferric chloride. Starch grains in sections were localized by the periodic acid Schiff reaction. Mitotic indices of cells were estimated from median longitudinal sections of 20 root tip meristems collected from cultures at weekly intervals.

For electron microscopy, 2 mm long root tips were cut and immediately fixed in 2.5 per cent gluteraldehyde (2 h., 0.05 M phosphate buffer, pH 7) at room temperature, followed by over night fixation with 2 per cent osmium tetraoxide at 4°C. After dehydration in graded ethanol series, the samples were pre-stained with 2 per cent alcoholic uranyl acetate, infiltrated with 99.5 per cent propylene oxide and embedded in ERL-4206 (Serva, Heidelberg). Ultrathin sections were post-stained with 0.3 per cent lead citrate (Reynolds, 1963) and examined under a Zeiss EM 109.

RESULTS

Morphology of the aerial root apex

V. planifolia produces two adventitious roots from each node with which it clings to the support (Fig. 1). The apical portion of an aerial root taken from a seven-year old vine in longisection is rather flat with a tightly adhering root cap (Fig. 5). Three promeristem layers, 2 to 5 cells wide, show continuity with (1) the pole of the central cylinder, (2) the cortex, and (3) the root cap and epidermis.

The root cap, at its deepest point, consists of approximately 12 layers of cells. The innermost layer, the cap meristem, consists of small densely cytoplasmic cells devoid of conspicuous starch grains and vacuoles. At about the fifth cell layer, cells increased in size and become highly vacuolated. These cells accumulate large

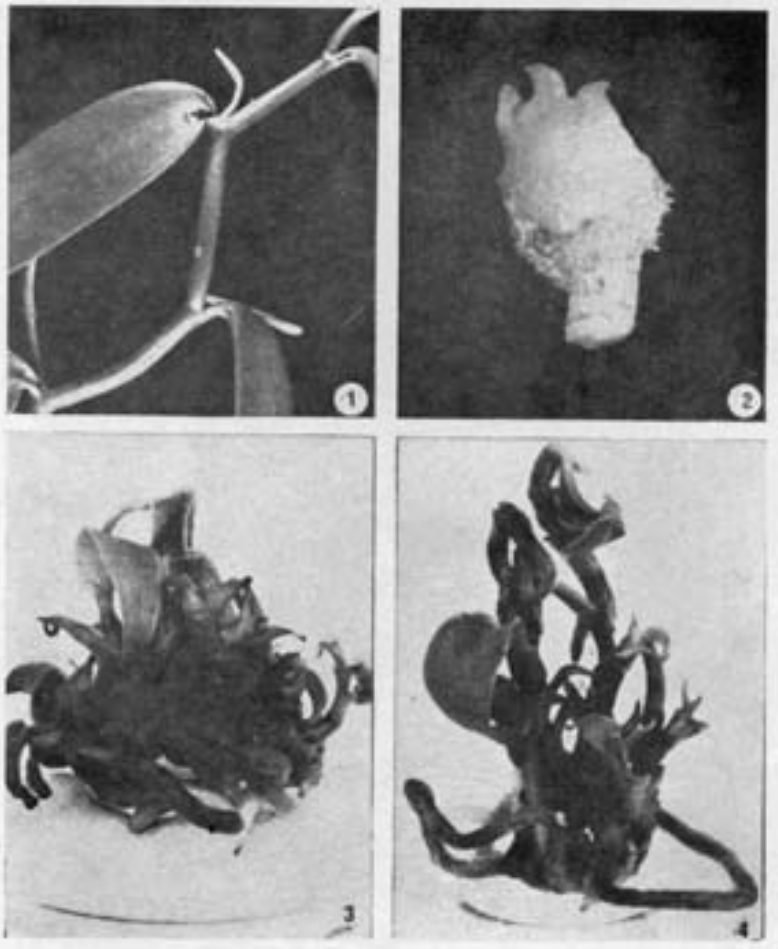

PLATE I

Fig. 1. An apical portion of *V. planifolia* vine with four nodes, young adventitious roots (arrow) and leaves. ×0.75.

2. 2 cm long aerial root tip excised from a young 6 cm long root after eight weeks in MS medium containing 2 mg l^{-1} IAA and 0.2 mg l^{-1} kinetin. ×5

3. Development of multiple shoots from swollen root tips subcultured on solidified MS medium after 12 weeks in culture. ×1.25

4. Multiple plantlets after 14 weeks in culture. ×1.25

amounts of amyloplasts in their plastids (Fig. 6). Abutting on the proximal surface of the root cap meristem is a small hemispherical group of 25-30 cells. The constituent cells of the hemisphere are relatively the smallest at the root apical region, nearly isodiametric, less densely cytoplasmic with no particular thickenings on their walls (Fig. 7). The nuclei in these cells, before transfer to the culture, medium, do not show mitotic figures in contrast to the neighbouring cells. These cells correspond closely to the quiescent centre as shown by Clowes (1971).

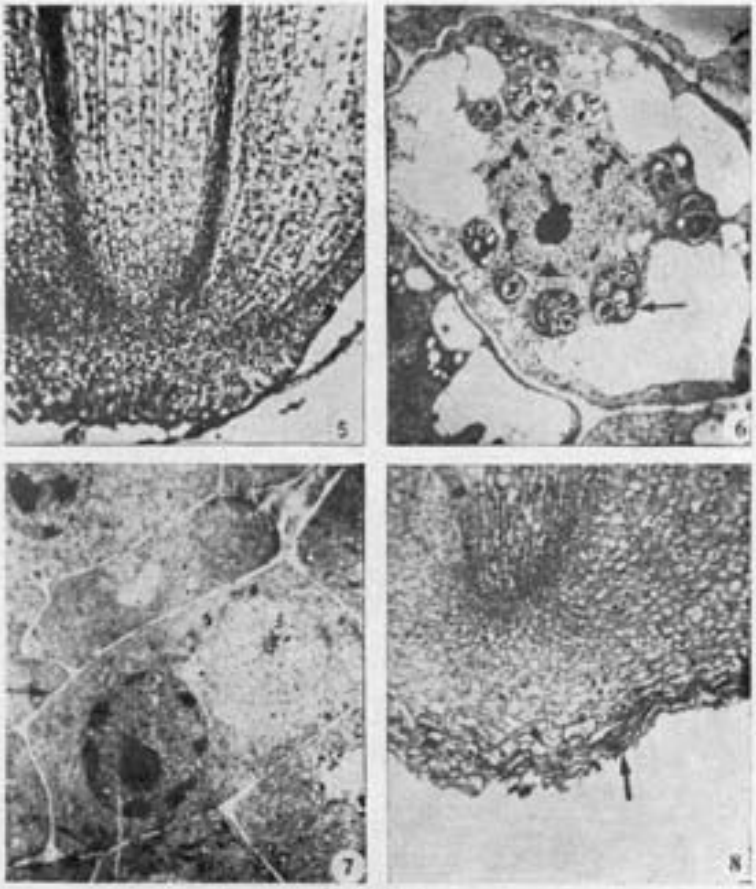

PLATE II

Fig. 5. Median longisection of a young aerial root tip of *V. planifolia* from a seven year-old vine showing the structure of the root meristem. ×126

6. A root cap cell showing multigranular amyloplasts (arrow) and large vacuoles. ×3000.

7. A quiescent centre cell. ×3000

8. Median longisection of a young aerial root tip after four weeks in MS medium containing 2 mg l^{-1} IAA and 0.2 mg l^{-1} kinetin showing remnants of the cap (arrow) and mitoses in the quiescent centre region. ×60

Organogenesis in-vitro

2 cm long root tips excised from aerial roots less than 15 cm long cultured on liquid MS medium supplemented with 2 mg l^{-1} of IAA and 0.2 mg l^{-1} of kinetin, swelled considerably within two months. After three months in culture, the swelled root cap tissue cracked, exposing small spherical bulges with surrounding dark sheets of necrotic material (Fig. 2). Median longisections of the root apices after two weeks in culture indicated that the root cap lysis began at the distal layer and progressed centripetally towards the meristem. Lysis began with the

breakdown of the starch granules in amyloplasts followed by the disintegration of the cytoplasm (Fig. 8).

Concomitant with these changes in the root cap, the cells of the quiescent centre increased in volume and divided at a rapid rate. The activation of the quiescent centre cells commenced with the breakdown of the distal half of the cap. In about four weeks in culture, lysis of the cap was complete and the number of cells in the quiescent centre region had increased considerably resulting in an increase in girth of the apex; the originally pointed apex now assumed a hemispherical outline.

The cells on the periphery of the hemispherical mound were relatively smaller, had dense cytoplasm and prominent nuclei, and accumulated small starch granules in their cytoplasm before dividing periclinally. Subsequent anticlinal and periclinal divisions in these cells resulted in a homogenous mass of densely stained cells which developed either into a single shoot meristem (Fig. 9) or organized themselves into several meristemoids (Fig. 10). The meristemoids thus produced were delimited by a thin layer of cells which were raised above the surface of the hemispherical mound giving it a corrugated appearance (Fig. 10). Each meristemoid enlarged further and their constituent cells continued to divide and form a shoot meristem on the flanks of which leaf primordia differentiated in an acropetal sequence. Within nine weeks in culture, a number of shoot apices each surrounded by a whorl of leaves were formed in the apical region of this mound of meristematic tissue (Fig. 11).

The new root was initiated only after four to five leaves had formed. The root emerged approximately at an angle of 120° with the mid-line of the plumule and the nodal plate (Fig. 12) by piercing through the surrounding tissue. However, none of the plantlets formed in liquid media emerged from the swollen root tip and required excision and transfer to a solid medium. When the swollen root tips were excised and grown in agar solidified media of the same constitution, they enlarged progressively and became further corrugated and covered with various outgrowths. Many new meristems developed, leading directly to shoots (Fig. 3) followed by roots. In about nine months, plantlets with well formed shoots, leaves and roots differentiated (Fig. 4) from each explant.

DISCUSSION

Potentialities of excised root cultures have been explored so far on only a very modest scale largely due to the difficulties encountered with majority of dicrty-ledonous species and with almost all monocotyledons. Root tissues are rarely used for *in-vitro* propagation purposes, because of contamination problems and the substantial disadvantages of the constituent cells of a differentiated root being of varying ploidy, resulting in uneven growth. Consequently, roots of very few species have been maintained in continuous cultures and in even fewer species have root buds been grown *in-vitro*. Shoot initiation from callus tissue derived from cultured roots of dandelion, tomato, *Isatis tinctoria*, *Atropa belladona* and

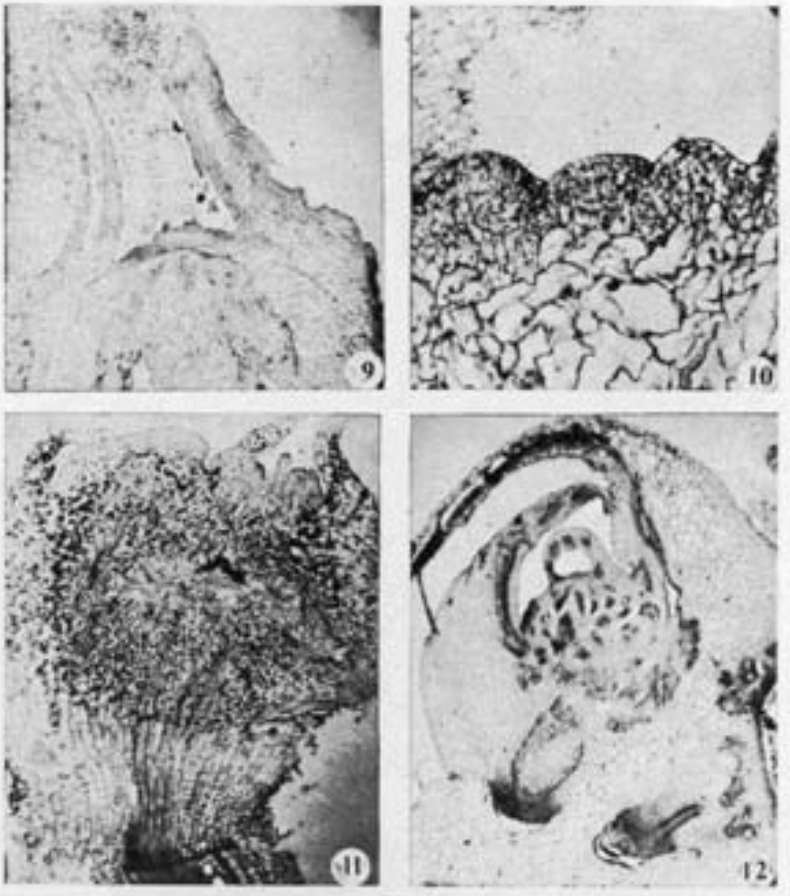

PLATE III

Fig. 9. Median longisection of a young aerial root tip after nine weeks in liquid MS medium showing differentiatton of a shoot meristem and a pair of leaf primordia from the root meristem. ×40

10. Some, showing meristemoids. ×40

11. Differentiation of multiple shoots from meristemoids formed at the distal end of the root tip after nine weeks in liquid MS medium. ×18.

12. Formation of plantlet by differentiating a root three weeks after transfer to solidified MS medium supplemented with 2 mg l^{-1} IAA and 0.2 mg l^{-1} kinetin. ×18

protocorm-like bodies from callus of cultured root tips of the orchids *Epidendrum obrienianum*, *Catasetum trulla* × *Catasetum* berthrand and *Oncidium varicosum* have been obtained. In all these reports, either the root apical meristem is not involved or its precise involvement in organogenesis is obscured due to the callus interphase.

The dual role of the root cap in graviperception and maintenance of the quiescent centre has been well documented (Barlow & Pilet, 1983; Wilkins, 1984).

Removal of the cap from actively growing roots of *Zea mays* have been observed to trigger off a series of changes, especially in the quiescent centre leading to the regeneration of a new cap (Barlow, 1974). In the present study on *Vanilla*, lysis of the cap cells began with the loss of its meristem and subsequent breakdown of starch in amyloplasts. As in decapped roots of *Zea mays*, degeneration of the cap cells of *Vanilla* roots in culture, stimulated growth and division of cells of the quiescent centre. However, in none of the 500 cultured root tips, sectioned at various stages of development and examined from day 3 to 90, was there any indication of a cap regeneration comparable to that of *Zea mays*. In contrast, the descendants of the quiescent centre either developed into a single shoot meristem or some of their peripheral cells organized themselves into meristemoids and then to entire plantlets indicating that a fundamental switch over in the organizational activity occurs so that formerly organized the root meristem now organize the shoot meristem.

Endogenous shoot buds without the callus interphase have been induced in cultured roots of a few taxa: A few such cases have been reported in ferns (Peterson, 1975) but with the exception of *Selaginella*, other examples have turned out to be more properly interpreted as the initiation of lateral buds from the derivatives of the root apical cells. The only reported case of root meristem transformation to shoot meristem in seed plants is for the orchid *Neottia nidus-avis* (Champagnat, 1971). However, there is little conclusive histological data on the fate of root apical meristem during transformation, although, in the case of *Neottia*, it was shown that the root meristem first differentiated into a protocorm from which a shoot primordium arose (Champagnat, 1971). Aerial root tips of *Vanilla* cultured in the present study, however, produced plantlets directly from the meristematic zone without a callus interphase, minimizing the possibility of induced epigenetic changes in the resultant plants. Using the methods reported in this paper, disease-free clonal material of *V. planifolia* could be easily produced for use in plantations. Furthermore, the root tips of *Vanilla* could perhaps be an ideal candidate for cryopreservation because of a small unit size, high regenerative potentiality, the constituent cells being vacuolated, meristematic, genetically uniform and without intercellular spaces.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Department of Science and Technology, Government of India (Grant SP/SO/AO2/85).

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