IN VITRO PROPAGATION STUDIES ON VANILLA
(Vanilla planifolia Andr.)

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By

V.S. NEELANNAVAR

DEPARTMENT OF HORTICULTURE
COLLEGE OF AGRICULTURE, DHARWAD
UNIVERSITY OF AGRICULTURAL SCIENCES
DHARWAD – 580005

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(P.U. KRISHNARAJ)

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I. INTRODUCTION

India, 'the land of spices' is the largest producer, consumer and exporter of spice in the world. The major share (90%) of spices produced in the country is used to meet the domestic demand and only 10 per cent is exported. India's share in the world spice market is estimated at 46 per cent by volume and 27 per cent by value. The area under various spice crops in India is 24 lakh ha with a production of 25 lakh tonnes. Indian export of spices is 1.98 lakh tonnes valued at Rs. 1,185.15 crores (Singhal, 2003a).

Vanilla (*Vanilla planifolia* Andr. or *Vanilla fragrans* Salisb.) is a herbaceous, perennial, climbing orchid belonging to the family Orchidaceae and order Orchidales. Vanilla is indigenous to Mexico and systematic cultivation of vanilla on a commercial basis began with the introduction of this crop to Madagascar, Java, Mauritius, Tahiti, Seychelles, Zanzibar, Brazil, Indonesia, Comoro islands and India.

Vanilla was introduced into India during 1835 by British rulers along with clove and nutmeg. In India, it is grown in an area of 2545 ha with an average production of 92 tonnes. In India, vanilla is mainly grown in Karnataka, Kerala and Tamil Nadu. In Karnataka, it is grown in an area of 1465 ha with an average production of 54 tonnes. In Karnataka, it is mainly grown in the districts of Chikkamagalur, Shimoga, Uttara Kannada, Dakshina Kannada, Kodagu, Hassan, Mysore, Belgaum and Dharwad (Chandrashekar and Shankar, 2004).

Vanilla is explored and domesticated as a value based export crop. It is cultivated for its beans containing sweet scent, aroma and pleasant flavour, which is mainly due to the presence of vanillin. It is used as flavouring substance in several items such as chocolates, ice-creams, soft drinks, liquors, egg-nogs, candies, baked foods, cakes, biscuits etc. It is also used in scenting tobacco, perfumery and pharmaceuticals.

Vanilla is the second most expensive spice after saffron and is still big money spinner. It is often referred to as green gold and princess of spices. In the world market, India occupies the sixth position and exports 1.86 per cent of the world total. In the year 2001-02, India earned Rs. 1436.09 Lakhs by exporting 23.7 metric tonnes of vanillin. In 1994-95 it earned Rs. 0.81 lakh by exporting 0.6 metric tonnes of vanilla. During the period 1994-2002 the quantity of vanillin exported showed a growth rate of 50.29 per cent and the value from export showed 247.09 per cent growth rate. This tremendous increase is due to high demand for natural vanillin (Singhal, 2003b).

Though vanilla was introduced into India as early as 1835 not much headway has been made in extending the area under the crop. One of the major limitations in the cultivation of vanilla in India is the shortage of quality planting material. The availability of planting material from the existing plantation is insufficient to meet the ever-increasing demand. The need of the hour is to produce large quantum of planting material from a limited plant material economically. The exploitation of the concept of totipotency (Haberlandt, 1902) has progressed from remote possibility to a rapidly expanding reality as evident from the number of species that is now being successfully propagated through tissue culture. Recent progress in the field of plant cells and tissue culture has made this area of research one of the most dynamic and promising in the disciplines of biotechnology, agriculture, horticulture and forestry. Besides this *in vitro* culture technique is becoming increasingly popular as alternative and feasible means of vegetative propagation in some commercially important plants (Murashige, 1974). Morel's (1960) discovery that explained the orchid meristems could be induced to develop multiple shoots laid a firm foundation for *in vitro* propagation of other horticultural crops.

Although the conventional methods of vegetative propagation have reached commercial acceptability for want of better alternatives, tissue culture techniques have been shown to have definite and indispensable advantages over the former, as it ensures an extremely rapid rate of multiplication. Tissue culture technique is not season dependent and requires only a limited quantity of plant tissue as a source of initial explants. It can also aid in the production of disease free plants.

The technique has great potential for rapid and large-scale multiplication of true to type planting material (Pierik, 1990). Attempts have been made in vanilla to exploit the

possibility of micro-propagation technique for rapid multiplication. Keeping these aspects in view the present investigation was conducted on vanilla at the College of Agriculture, Dharwad, with the following objectives.

1. Identification of suitable source of explants for culture.
2. Evaluation of different gelling agents as an alternate to agar.
3. Standardization of use of growth regulators for shoot proliferation and root initiation
4. Identification of suitable hardening medium for better establishment.

II. REVIEW OF LITERATURE

The present experiment was conducted to find out the best explant, growth regulator, gelling agent and hardening medium for *in vitro* multiplication of vanilla. The relevant literature pertaining to these aspects has been reviewed and presented below.

The concept of totipotency, which is inherent in the cell theory of Schleiden (1838) and Schwann (1839) is the basis for plant tissue culture. Haberlandt (1902) suggested the use of embryosac fluids for inducing divisions in vegetative cells and the culture of isolated plant cells. He also predicted that "artificial embryos" could be grown from isolated mature plant tissue.

When Haberlandt (1902) attempted the first plant cell culture studies, his intentions were to develop a versatile tool to explore morphogenesis and to demonstrate totipotency of plant cells. With the Passage of time most of these ideas were confirmed experimentally proving his broad vision and foresight. Recent progress in the field of plant cell and tissue culture has made this area of research into one of the most dynamic and promising tools in experimental biology *in vitro* cultures are now also being used as tools for the study of various basic problems in plant physiology, plant pathology, cell biology and genetics in addition to agriculture, forestry and horticulture which subsequently have turned the "dreams" of Haberlandt, White and Gautheret into realities.

Major breakthroughs in plant tissue culture were achieved after the discovery of auxins and cytokinins. It was followed by the revelation that the organogenesis of shoots and roots in cultured cells could be manipulated by changing the concentrations of these growth regulators in the nutrient media by Skoog and Miller (1957). The pioneering experiments of White (1934, 1939), Gautheret (1939), Morel and Martin (1952), Skoog and Miller (1957), Reinert (1958), Steward *et al.* (1958), Morel (1960), Cocking (1960, 1966), Murashige *et al.* (1972), Carlson *et al.* (1972), Murashige (1974, 1978), Navarro *et al.* (1975), Hu and Wang (1983) and Litz (1984, 1985) are often cite as the landmarks in the developmental phases of plant tissue culture.

Commercial application of plant tissue culture techniques has been in the production of clonal plants at a very rapid rate. The plantlets (ramets) developed through tissue culture are known to have by passed juvenility, grow faster and mature earlier than those propagated through seeds. While applying the technique of apical meristem culture for raising virus-free plants of an orchid, Morel (1960) also realized the potential of this method for the rapid multiplication of these plants. Realizing this tremendous advantage in the technique, the commercial orchid growers soon adopted this novel technique as a standard method for propagation. Murashige (1974) was instrumental in giving the technique of *in vitro* culture, a status of a viable practical approach for rapid and mass propagation of horticultural species.

According to Murashige (1974) there are three possible methods available for *in vitro* micro propagation *viz,* (a) release of axillary buds from the influence of apical dominance, (b) production of adventitious shoots through direct or indirect organogenesis and (c) somatic embryogenesis. Callus mediated organogenesis and somatic embryogenesis are not recommended for clonal propagaion since there is a possibility of producing aberrants. In shoot tips and axillary bud cultures, genetic fidelity is maintained to a large extent. *In vitro* somatic embryogenesis is limited to a few species but still, acts as the most rapid method of plant regeneration (Evans *et al.* 1981).

Currently, *in vitro* clonal propagation strategies have been developed for a number of economically important plant species. More and more species are becoming amenable for *in vitro* studies. Excellent reviews on the subject have been published by Murashige (1974, 1978), Hu and Wang (1983) Styler and Chin (1983), Sharp *et al.* (1984) and Litz (1985).

Propagation by tissue culture

For a long time, the major means for clonally propagation of orchid were the division, splitting and use of shoots. This is very slow method and yields only a small number of plants. The earliest report of using plant tissue culture technique in orchid multiplication goes back to 1947 when it was demonstrated that phalaenopsis plantlets could be developed from the

buds of inflorescence stalks (Rotor, 1949). However the credit for achieving mass clonal propagation of orchids goes to Morel (1960) who was successful in culturing Cybidium shoot apices on nutrient media. He found that within a few months hundreds of plantlets could be obtained from a single meristem.

Plant material (Explant)

Various plant parts like shoot tip or meristem, leaf segments, floral parts, aerial roots, *etc.*, have been successfully used for *in vitro* propagation. Apical meristem of a young shoot is the most commonly used explant both in monopodial and sympodial orchids. However, terminal and axillary buds from shoots tips containing meristem have also been used in many cases (Stewart, 1989).

Reproducible protocols were developed by Mathew *et al.* (1999) for micropropagation, regenerative callus production, root tip cultures, seed cultures, synthetic seeds and their germination in cultivated *Vanilla planifolia* and *in vitro* propagation in *Vanilla wightiana*, a wild species reported to be abundant in eastern Andhra Pradesh. In *Vanilla planifolia*, the average multiplication rate was 1:3 at each sub-culture cycle and a total of 3560 plantlets were produced from 50 nodal buds within a span of 6 months. Plantlets established well in the field.

Seeds of *Vanilla planifolia* obtained from green pods (4-5 months old), collected from elite plants growing in a field in Ramamangalam, India, were cultured on different media to determine the best conditions for germination. The best germination (111.5 seedlings/bottle) was observed on half-strength MS (Murashige and Skoog) medium supplemented with NAA (Naphthalene acetic acid) and BAP [Benzyl Amino Purin] (both at 1 mg/litre), within 4-5 weeks. Seedling proliferation rate was influenced by BAP concentration. BAP at 0.2 mg/litre favoured shoot elongation and rooting. Seedlings could be planted out without any difficulty in small pots containing potting mixture in a net house provided with mist. After hardening for a period of 6 months, the seedlings were established in the field (Mary *et al.*, 1999).

Explants taken from 5-7.5 mm long shoots growing on pseudobulbs were reported to be the best for propagation of cymbidium *in vitro* (Sagawa *et al.,* 1966). Axillary and terminal explants of dendrobium taken from new growth produces better protocorms than stem internodal segments. The growing points excised from dormant buds on old pseudobulbs of Cattleya produced protocorn like bodies, which could be propagated like protocorms grown from meristem of young shoots (Vajrbhaya, 1978). The best results in the propagation of Brassocattleya were obtained by using the third bud from 15 cm long newly growing shoots (Arditti, 1977). According to Sagawa and Kunisaki (1982), the apical shoots or axillary buds were the best source of explants for the successful clonal propagation of orchids.

Pett *et al*., (1999) reported that explants obtained from the middle part of donor plants exhibited the best growth in terms of length and number of nodes, although explants from the base and tip produced more shoots. Genetic variation in shoot production and rooting was observed in *Vanilla tahitensis* lines. Shoot production, growth and rooting was promoted by BAP [Benzyl Amino Purin] at 0.5 mg/litre.

Stem segments with a bud, were successfully cultured for the propagation of Phalaenopsis (Sagawa, 1962). Buds enlarged in two weeks followed by leaf and root development in 6-20 weeks and plantlets were ready for transfer within 30 weeks. Development of plantlets in Vanda 'Miss Joaquim' in 2-3 months was also possible by using similar technique (Sagawa and Sehgal, 1967). With stem and flower stalk explants of Epidendrum Hybrids, dormant bud at the nodes developed into plantlets. Up to 20 plantlets were obtained from a single cane (Stewart and Butten, 1976).

Young leaves and leaf tips of Aranda, Epidendrum, Rhynchostylis, Cattleya and Phalaenopsis have been successfully cultured *in vitro* on Vasin and Went or Murashige and Skoog medium (Goh, 1989; Vij and Pathak, 1990).

Inflorescence stalks of orchids like Dendrobium, Vanda, Phalaenopsis, Oncidium, and Epidendrum are the best explants for tissue culture. (Intowong and Sagawa 1973; Singh and Prakash, 1984).

Clonal propagation of *Vanilla planifolia* was made easy with explant taken from nodal stem segments (Konowicz and Janick, 1984; Jarret and Fernandez 1984; Agarwal *et al.*, 1992).

According to Beechey (1970), aerial roots of orchids could also be used for *in vitro* propagation. Young root tips excised from aerial roots, less than 15 cm long of *Vanilla planifolia* produced number of meristemoids each of which differentiated into a shoot meristem with leaf primordia (Phillip and Nainar, 1986).

Culture media

The success of plant tissue culture as means of plant propagation is greatly influenced by the nature of culture medium used. Nutritional requirements for optimal growth of a tissue may vary with species. Even tissue from different parts of a plant may have different requirement for satisfactory growth (Murashige and Skoog, 1962).

Various nutrient media have been reported. The choice depends on the plant species and the intended use of the culture. Murashige and Skoog (1962) medium characterized by high concentration of mineral salts has been widely used for general plant tissue culture (Murashige, 1974).

The effects of culture media type (Murashige and Skoog (MS), Woody Plant (WP) or Gresshoff and Doy media) and BAP (benzyladenine; 0 or 1 mg/litre), GA (0.1 mg/litre) or AgNO3 (20 mg/litre) on shoot proliferation *in vitro* of *Vanilla planifolia* were investigated by Ganesh *et al.* (1996). Good shoot proliferation was only observed in the presence of BAP. The longest shoots were produced on WP. BAP + GA promoted shoot multiplication and growth. Culture of shoots on MS resulted in rooting. Plantlets were successfully transferred to the soil.

Growth regulators

For obtaining desired response in tissue culture, the role of growth regulators and their concentrations will have to be carefully chosen. The most important developments in the tissue culture of the plants were made with the discovery of growth regulators, auxins, gibberellins, cytokinin and abscicins and other organic compounds like inosityl and B-vitamins.

Wickson and Thimann (1958) discovered that cytokinins could release the lateral buds from apical dominance. In the presence of cytokinins, the dormat buds of vegetative apex are stimulated to grow and elongate. Skoog and Miller (1967) reported that the cell division or cell differentiation was also associated with auxins and cytokinins.

Majority of culture media containing cytokinin 6-benzyl aminopurine, have been most effective for meristem, shoot tip and bud cultures followed by kinetin. For axillary bud proliferation, cytokinins have been utilized to overcome the apical dominance of shoots and to enhance the branching of lateral buds from leaf axils (Murashige, 1974).

Morel (1964) reported that the explants from shoot apices of Cymbidium orchid when cultured *in vitro* became green and then enlarged slowly as a small bulblet similar to a protocorm developed as bulblet from an embryo. The protocorm like body further proliferated into clumps of protocorms each in turn developing into a new plantlet. The portocorm like bodies when cut into small pieces and subcultured, regenerated into more protocorms. He estimated that it could be possible to produce more than 4 million plants in a year from a single explant.

Rao *et al.*, (1993) studied the efficiency of axillary bud culture for rapid propagation in vanilla. Axillary buds were excised from two year old field grown vines, surface sterilized and inoculated on nine different media combinations with MS as the basal medium supplemented with IAA, NAA, Kn, and BAP as growth regulators and d-Biotin and calcium pantothenate as adjuvants. The number of multiple shoots obtained from each of the axillary bud ranged from 4-8 with average of six. The growth of newly formed shoots was more vigorous on MS medium comprising of NAA and Kn as growth harmones.

An efficient micropropagation protocol was developed by Geetha and Shetty (2000) for vanilla using shoot tip and nodal segments as explants. The explants were cultured in MS basal medium with 1mg/l BAP for 10 weeks and subcultured onto fresh medium every 4 weeks. The proliferating clusters were cultured in N59 basal medium with BAP (0.5mg/l)+ d-Biotin (0.05mg/l)+ folic acid (0.5mg/l) and 2% sucrose for elongation of shoots, formation of shoot initials and further proliferation of axillary shoots. Separation and clustering of elongated shoots in fresh medium for 2 to 3 weeks yielded 7-8 cm long plantlets, which were acclimatized in polytunnels.

Giridhar *et al*., (2001) studied the influence of silver nitrate ($AgNO_3$) on *In vitro* shoot multiplication and root formation in vanilla. Incorporation of silver nitrate into the medium induced positive response not only on shoot initiation, number and growth but also increased root number and length. The explants were inoculated onto MS medium salts, supplemented with 2 mg/l BAP and 1 mg/l NAA for shoot initiation and IBA 2 mg/l for rooting along with silver nitrate.

According to Reinert and Mohr (1967), cattleya hybrids, which were propagated to the extent of 10 to 15 folds normally, could be propagated up to 3000 times by tissue culture method with kinetin in the medium.

The effects of auxins, cytokinins and gibberellins alone or in combination of growth of cymbidium protocorms has been studied. IAA alone had no effect, NAA resulted in optimal fresh weight and the protocorms were vigorous. 2,4-D caused increase in fresh weight but protocorms were abnormal. Kinetin resulted in growth of many small shoots and also promoted callus formation, GA alone promoted shoot and leaf growth, NAA and Kinetin resulted in maximum fresh weight increase (Fonnesbech, 1972).

In vitro shoot proliferation of vanilla by axillary branching was achieved on medium consisting of Murashige and Skoog salts supplemented with 0.1 $mg1^{-1}$ nicotinic acid, 2.0 $mg1^{-1}$ inositol, 0.5 $mg1^{-1}$ nicotinic acid, 2.0 $mg1^{-1}$ glycine, 1g 1^{-1} casein hydrolysate, 30 gl^{-1} sucrose, 8 $g1^{-1}$ agar and 0.5 mg 6 benzylamino purine (BA) (Knonowicz and Janick, 1984). Establishment and proliferation were achieved at 1 mg 1^{-1} BA. Proliferated shoots from this treatment were subcultured on MS medium with 0; 0.5, 1.0, 5 mg 1^{-1} BA and shoot proliferation was the highest at 0.5 mg 1^{-1} and 1.0 mg 1^{-1} BA in 8-10 weeks and larger shoot were obtained in 0.5 mg 1^{-1} BA. Jarret and Fernandez (1984) reported that *in vitro* propagated vanilla plantlets transferred to screw cap jars containing MS only produced large size plants.

Mathew *et al.* (2000) reported a commercially viable protocol for mass propagation of *Vanilla tahitensis*, a cultivated species of vanilla. A multiplication ratio of 1:4.7 was observed over a culture period of 60-70 days on benzylaminopurine [benzyladenine] (1 mg litre$^{-1)}$ and alpha-naphthaleneacetic acid (NAA) (0.1 mg litre^{-1}).

Philiph and Padikkala (1989) studied the role of indole acetic acid in the conversion of root meristems to shoot meristems in *Vanilla planifolia*. Aerial root tip explants cultured in MS media containing more than 5 mg/litre IAA continued to grow as roots, but the root meristem of young tips grown in media containing 1-5 mg/litre IAA developed into shoots and plantlets. Scanning the root tip extracts for IAA using UV, TLC, GLC and GC-MS showed higher levels of auxin in root tips from old aerial roots and also in young cultured tips in which the root meristem had transformed to shoots.

A method of clonal propagation was devised by Agarwal (1992) for *Vanilla walkeriae,* which is restricted to the tropical forests of Tamil Nadu, India. MS basal medium supplemented with 0.5 mg kinetin, 1.0 mg BA and 1000 mg casein hydrolysate/litre supported rapid proliferation of multiple shoots from stem node segments. Rooting of shoots could be induced readily on a half strength semi-solid MS medium without hormones. Plantlets were transferred to soil successfully.

George and Ravishankar (1997) developed the efficient multiplication of *Vanilla planifolia*. Multiple shoots were produced by axillary bud explants (excised from 1-year-old, field-grown plants) using semi-solid Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA) at 2 mg/litre + NAA at 1 mg/litre. Multiple shoots were transferred to agitated liquid MS medium with BA at 1 mg/litre + NAA at 0.5 mg/litre for 2-3 weeks, and subsequently cultured on semi-solid medium. *In vitro* produced shoots were placed to root on

MS, half-strength MS or Knudson media supplemented with activated charcoal (0.2%) or coconut milk (15%). Activated charcoal promoted root induction. Best rooting was observed in half-strength MS media containing activated charcoal.

GELLING AGENTS

Gelling or solidifying agents are commonly used for preparing semi-solid or solid media. In static liquid culture, the tissue or cells become submerged and die due to lack of oxygen. Gels provide a surface support to tissue for growing in static condition.

White (1934) used agar as a gelling agent in the medium. Since, its introduction, agar could not be satisfactorily replaced and remains the gelling agent of choice for culture media employed for higher plants as well as micro organisms. However, agar contains some inhibitors, which result in pre-mature abortion of embryoids in anther culture of tobacco (Jain *et al.*, 1997).

The gel must be firm enough to support explant or shoot clusters. But, if it is too hard, it may prevent adequate contact between the medium and the tissue especially after some drying out (Debergh, 1983).

Singh (1984) cultured shoot tips of crab apple and pear on MS medium containing either TC agar or Bacto agar at concentrations ranging from 0 to 1.2 per cent. Optimum shoot proliferation in crab apple was obtained on media containing 0.3 per cent of either agar. Increasing agar reduced the shoot proliferation and shoot growth, but the reduction especially was severe with Bacto agar. Shoot proliferation in pear was found to be best on medium containing 0.6 per cent Bacto agar. Higher concentrations decreased the shoot proliferation and shoot growth. TC agar did not influence fresh weight of pear culture, but with increasing concentrations, increased shoot proliferation.

Sorvari (1986 a, b, c) investigated starches from barley, corn, potato, rice and wheat as alternate gelling agents to agar. Embryo formation from barley anther was greater on media gelled with starch than agar solidified medium. Of the starches examined, barley starch was the most effective. Frequency of plantlet production from anther cultured on media with barley starch was five times higher than on agar solidified media. Subsequently, he also showed that shoot differentiation from potato tuber discs could be obtained within three weeks after inoculation on media gelled with barley starch.

Henderson and Kinnersley (1988) reported that tobacco and carrot cell cultures grown on corn starch (12%) gelled medium produced more than triple the dry weight of culture grown on agar gelled medium.

Tiwari and Rahimbaev (1992) tested five types of different starch sources *viz.*, corn, barley, rice, wheat and potato as substitute for agar. The basal medium taken for anther culture of *Hordeum vulgare* was N6. To this three gel forming components and their combination *viz.*, agar 'Difco' 7.0 g l^{-1}, agarose 'Sorva' 5 g l^{-1}, barley starch 80 g l^{-1} and agar 7 g l^{-1} + barley starch 230 g l^{-1} were added separately. The results showed that N6 medium with barley starch as gelling agent was most suitable for anther culture as it produced 64.3 androgenic calli from 100 anthers which was nearly two times greater than in the medium with agar. Addition of barley starch to agar or agarose did not significantly increase the number of calli from anther. Medium with agarose and starch combination produced fewer calli as compared to starch alone. Lydon *et al.* (1993) reported that growth of shoot cultures *In vitro* could be obtained if the medium were gelled with a mixture of corn starch and gelrite.

Zimmerman *et al.* (1995) reported that medium gelled with corn starch at 50 g l^{-1} plus gelrite at 0.5 g l^{-1} was just as effective as medium gelled with agar for shoot proliferation of apple and raspberry. It also indicated that the gelling agent costs are only 10 to 15 per cent of agar.

Explants from young leaves of one month old greenhouse grown tobacco plants were placed on MS medium containing agar (7 g l^{-1}) or tapioca pearls (100 g l^{-1}). Callus was induced in seven days on agar medium and ten days on tapioca medium. Shoot differentiation occurred in 21 days on agar media and 24 days in tapioca media. Extensive

shoot proliferation occurred in tapioca and agar media in 4 weeks. The percentage of shoot, producing roots on agar was 79.2, but in tapioca gel it was slightly lesser or almost on par with agar. *In vivo* establishment of rooted shoot was found to be good in both the cases when they were transferred to pots (Nene *et al*., 1996).

Micropropagation of chickpea on tapioca based medium using shoot tips was reported by Nene *et al.* (1996). More number of shoots were produced on agar medium (3.6 shoots per explant) than on tapioca. (2.7 shoots/explant). Rooting of shoots was better on tapioca medium (66.7%) than agar (40.0 per cent). Average shoot length was also greater on tapioca medium (4.4 cm) than that on agar (3.8 cm). Establishment of plants raised on tapioca medium was high (83%) as compared to the plants raised from agar medium (78%) in greenhouse.

Hamidah *et al.* (1997) reported that gelrite was better gelling agent as compared to agar for micropropagation of anthurium by using leaf pieces as explant.

Jain *et al.* (1997) used isabgol at 3 per cent for both bacterial and fungal culture. It was found that growth of bacterial cell colonies on isabgol gelled medium subsequently appeared as denser than those on corresponding agar gelled medium. This was because of the presence of some protective substances in isabgol or alternatively due to absence of some inhibitory substances which might be present in agar.

According to Razdan (1996), gelatin at high concentration (10%) can be used as gelling agent but, because of its melting habit at low temperature (25°C) it is limited in use.

Hardening medium

The micropropagated plantlets should be hardened before transferring to the open conditions. The medium used for hardening should have good water holding capacity, drainage and aeration.

Parthasarathy and Nagaraju (1999) studied the effect of different carriers on plantlets of Gerbera. The rooted plantlets when transferred to jars containing soil + sand survived better than sand alone. Plant growth was better with more number of leaves (8.5) in jars containing leaf mould. Considerable mortality was observed with sand (37.3%) and vermiculite (33.3%). The use of jars as containers was found to be better than pots because high humidity was maintained around the plants and reduced the desiccation effect as well as the water loss from the plant and the medium and established better in field.

Qianzhong *et al.* (1998) investigated the effects of rice chaff, perlite, coal cinder or a 1:1 mixture of rice chaff and perlite on seedling survival and root growth of gerbera plantlets. Good root growth and plant survival (93.8%) was observed in rice chaff.

The survival rate of plantlets was almost 100 per cent when plantlets after root development were planted in plastic pots filled with coco peat, red soil, and sand in a 3:1:1 ratio (Aswath and Choudhary, 2002). Greenhouse acclimatization of plantlets was achieved in a 1:1 peat:perlite (volume basis) substrate by Conti *et al.* (1991).

Eapen and Rao (1985) transferred the regenerated anthurium plants to plastic cups with vermiculite and irrigated with a nutrient solution containing MS salts at half strength. Plants were covered with a bell jar to maintain a humid condition. Forty plants were transferred of which 75% survived, flowered and were similar to the parental material.

Martyr (1981) observed that cuttings uptake more water from peat:perlite (1:1, v/v) than from either peat: grit (1:1, v/v) or from peat alone. Water uptake by cuttings was not directly related to the water content of the medium per unit volume, which was greatest in the peat. This higher rate of uptake was reflected in the quicker rooting of the cuttings in the peat: perlite.

Hardening of the *in vitro* plants of *Adhathoda beddomie* was done by Sudha and Seeni (1994). The rooted plants were removed from the culture vessels and washed well. They were transferred on to a potting mixture of sand, topsoil and cattle manure (1:1:1).

These plants were hardened for 4 weeks in a humidity chamber. On transferring to the field 95 percent of the plants survived.

In *Woodfordia fruticosa*, the rooted plantlets were transferred to a mixture of sand. Soil and manure (1:1:1) in 5 cm diameter pots. The plants were watered and maintained under green house conditions for 8 weeks. The establishment rate was 89 per cent (Krishnan and Seeni, 1994).

In vitro plants of *Hemidesmus indicus* were transferred onto a mixture of garden soil, sand and compost (2:1:1). Polythene tents were used to cover the plants to prevent desiccation. The plants were transferred to field after two weeks. Establishment rate was 70 per cent (Patnaik and Debata, 1996).

A potting mixture of sand and soil (1:1) was used for the hardening of *in vitro* plants of *Withania somnifera*. The plants were maintained at 70 per cent humidity under greenhouse conditions for two weeks. The success rate of plant establishment in the field was 100 per cent (Kulkarni *et al.*, 1996).

III. MATERIAL AND METHODS

The present experiment on *In vitro* propagation on vanilla (*Vanilla planifolia* Andr.)" were conducted in the Tissue Culture Laboratory of the Department of Horticulture, College of Agriculture, University of Agricultural Sciences, Dharwad during the year 2003-2004. The details of materials used and methods followed are presented below.

3.1 PLANT MATERIAL

The plant material required for the study was collected from the Shaila Farm, Theerthahalli, Shimoga. This was maintained at the Department of Horticulture, College of Agriculture, Dharwad.

3.2 EXPLANTS AND THEIR PREPARATION

3.2.1 Leaf

The leaf was cut into 2 to 3 mm square pieces and introduced into distilled water. This was inoculated such that the basal portion of the leaf was in contact with the medium.

3.2.2 Shoot tip

Vanilla plants were deheaded and the apical tip was removed with help of razor blade, added into distilled water and used for inoculation.

3.2.3 Internode

The internodes were cut into 2 to 4 mm size and were used for inoculation.

3.2.4 Axillary bud

The shoots were cut into pieces of 2-3cm size along with the bud and the main leaf attached to bud was removed into distilled water. This was used for inoculation.

3.2.5 Aerial roots

The aerial roots are collected, cut into pieces of 2-3 cm and used for inoculation.

3.3 NUTRIENT MEDIA

The nutrient media used in the present study was the Murashige and Skoog media. The composition of these media is furnished in Appendix I.

3.3.1 Preparation of media

For preparing the nutrient media, different stock solutions were prepared along with the stocks of growth regulators.

Stock A: Macro salts

Stock B: Micro salts

Stock E: Vitamins

During the preparation of individual stock solution all the ingradients listed in the Appendix I were measured and the required quantity of each was added one at a time to the distilled water. Each ingradient was dissolved completely before adding the next ingredient. All the stocks were made upto the volume by using double distilled water and were stored in refrigerator at 4°C.

Stock solution of 6-benzyl amino purine was prepared by dissolving it first in few drops of 1N.NaOH and volume was made upto the required concentration with distilled water. Stock of 3-indole butyric acid was prepared by dissolving in few drops of ethanol and volume was made up mixing distilled water.

Leaf segment

Internode

Shoot tip

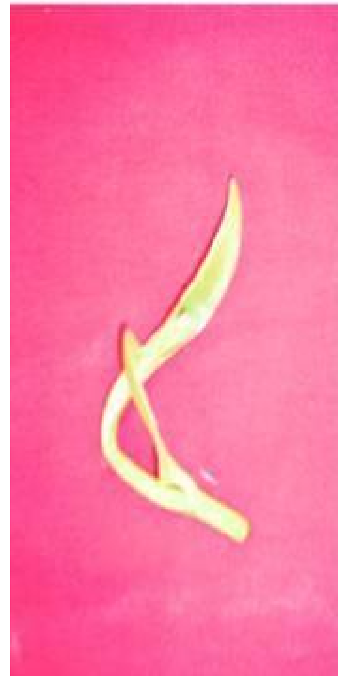
Aerial root

Axillary bud

Plate 1. Explants used

Vanilla plant

Leaf



Shoot tip

Vanilla plant

Plate 4. Explant preparation in vanilla

While preparing individual medium, using stocks, required amount of stock solutions were added along with sucrose and by adding distilled water the volume was made up. The pH was checked and adjusted to 5.6 to 5.8 with the help of 0.1 N HCl or 0.1 N NaOH. The volume was finally made up and the required amount of agar was added into the medium. Agar in the medium was completely melted by gentle heating upto 90°C. 15-20 ml of medium was poured into 25x150 mm pre-sterilized glass culture tubes and plugged with non-absorbent cotton. These tubes were then autoclaved.

3.3.2 Autoclaving

The test tubes with nutrient media were autoclaved at a pressure of 1.06 kg per cm^2 (121°C) for 20 min. The tubes were then removed from the autoclave and allowed to cool. They were kept in the culture room at 25±2°C. The inoculation was done after 4-5 days ensuring that the tubes were free from contaminants.

3.4 GELLING AGENTS

Gelling agents are commonly used for preparing semi-solid or solid tissue culture media. Gels provide a support to growing tissue in static conditions. Four different gelling agents *viz.,* Agar, Isabgol, Arrowroot and Sago were used for the present study

3.4.1 Agar

Agar is a phycocolloid, derived from red algae such as *Gelidium* and *Gracilaria*. It is a polysaccharide mixture of agarose and agaropectin. Agar is a thin translucent, membranous pieces or pale buff powder, strong hydrophillic. It absorbs water 20 times of its weight on swelling and forms strong gel at 40°C (Glessner, 1984).

3.4.2 Isabgol

Isabgol is derived from the seeds of *Plantago ovata* a stemless herb belonging to the family *Plantaginaceae*. Isabgol is a polysaccharide in its nature (Jain *et al*., 1997). Dried seeds of the plant contain over 30 per cent mucilage. The husk that contains all the mucilaginous matter is separated from seeds by crushing and winnowing.

3.4.3 Arrow root

It is also termed as West Indian Arrow root. In the market, it is available as a fine starch powder. The powder is obtained from the dried rhizomes of Maranta. It is tasteless and odourless when dry. When wet or cooked a faint odour develops and contains 64 per cent starch (Anonymous, 1962).

3.4.4 Sago

Sago is derived from tuber of cassava (*Manihot esculenta*) as small grained tapioca pearls. On dry weight basis tapioca pearls contain about 95 per cent starch. On heating, the starch gets converted into a complex polysaccharide, dextrin (Nene *et al*., 1996).

3.5 EXPERIMENTAL CONDITIONS

All the experiments were conducted under well defined conditions of the culture room maintained at 25 ± 2°C. Uniform light was provided by using fluorescent tubes (7200°K lux) over a light/dark cycle of 16/8 hours.

All culture work was carried out in a laminar airflow chamber. For all the experiments, analytical grade chemicals were used. The explants were cultured in 25 x 150 mm tubes.

3.6 CULTURE ESTABLISHMENT

3.6.1 Surface sterilization of explants

The explants were washed serially in Teepol (2-3 drops) for 10 minutes, Bavistin for 10 minutes, water 3 to 4 times thoroughly and later immersed in 0.1 per cent $HgCl_2$ for 3

minutes. This was then mixed with sterile distilled water for 3-4 times in the laminar air flow cabinet.

3.6.2 Inoculation

The sterilized explants were inoculated in test tubes containing the media. Cut ends of explants were kept in such a way so as to have maximum contact with the medium.

3.6.3 Transfer area and maintenance of aseptic conditions

All the aseptic manipulations such as surface disinfection of explants, preparation and inoculation of explants and subsequent subculturing were carried out in the laminar air flow cabinet. The working table of laminar airflow cabinet and spirit lamp were sterilized by swabbing with absolute alcohol. All the required materials like media, spirit lamp, matchbox, glassware etc., were transferred on to the clean laminar airflow. The UV light was switched on for half an hour to achieve aseptic environment inside the cabinet.

3.6.4 Subculture

Microshoots formed in the test tubes were taken out 5-6 weeks after inoculation. The shoots were separated by dissecting them in the sterile environment of laminar airflow cabinet with sterile dissecting needle and forceps. They were placed in the test tubes containing fresh media.

3.6.5 Rooting

The microshoots which were more than 2 to 3 cm in height were taken out and were placed in the tubes containing media with different concentrations of IBA for rooting.

3.6.6 Hardening

Young rooted plantlets were taken out of the test tubes, washed with distilled water and planted in net pots containing different hardening media. These plants were maintained in a prototype polytunnel. The plants were watered twice in a day initially, then once in a day after eight to ten days. Later, they were transferred to greenhouse after 15 days for further acclimatization.

3.7 HARDENING MEDIA

3.7.1 Peat

Four different media used as treatments are;

1) Sand
2) Soil
3) Soil + Cococoir pith (1:1)
4) Sand + Soil + Cococoir pith (1:1:1)

The media were first autoclaved at 121° C for 20 minutes to make it sterile. They were filled into small plastic containers with holes at the bottom to ensure the drainage of excess water.

3.8 EXPERIMENTAL DETAILS

3.8.1 Identification of suitable explant for culture

Design : CRD

Replications : 4

Number of explants used per treatment : 10

Treatments : T_1 – Leaf segments

T_2 – Shoot tip

T_3 – Axillary bud

T_4 – Internodes

T_5 – Aerial roots

3.8.2 Evaluation of different gelling agents as an alternate to purified agar

Design : CRD

Replications : 3

Number of explants used per treatment : 10

Treatments :

T_1 – Agar 06 g/l

T_2 – Sago 80 g/l

T_3 – Sago 50 g/l

T_4 – Sago 50 g/l + Agar 1g/l

T_5 – Isabgol 50 g/l

T_6 – Isabgol 40 g/l

T_7 – Isabgol 30 g/l + Agar 1g/l

T_8 – Arrow root 120 g/l

T_9 – Arrow root 100 g/l

T_{10} – Arrow root 80 g/l + Agar 1 g/l

3.8.3 Standardization of growth regulators for shoot proliferation.

Design : CRD

Replications : 3

Number of explants used per treatment : 10

Treatments :

T_1 – MS

T_2 – MS + BAP 0.5 mg/1

T_3 – MS + BAP 1.0 mg/1

T_4 – MS + BAP 1.5 mg/1

T_5 – MS + BAP 2.0 mg/1

T_6 – MS + BAP 2.5 mg/1

T_7 – MS + BAP 3.0 mg/1

3.8.4 Standardization of growth regulators for root initiation

Design : CRD

Replications : 3

Number of explants used per treatment : 10

Treatments :

T_1 – MS

T_2 – MS + IBA 0.2 mg/1

T_3 – MS + IBA 0.5 mg/1

T_4 – MS + IBA 1.0 mg/1

T_5 – MS + IBA 1.5 mg/1

T_6 – MS + IBA 2.0 mg/1

T_7 – MS + IBA 2.5 mg/1

3.8.5 Identification of suitable hardening medium.

Design : CRD

Replications : 5

Number of explants used per treatment : 10

Treatments : T_1 – Sand

T_2 – Soil

T_3 – Soil + Cococoir pith (1:1)

T_4 – Sand + Soil + Cococoir pith (1:1:1)

3.9 COLLECTION OF DATA

3.9. 1 Number of days taken for emergence of shoot.

The number of days taken to show initial differentiation of shoot from the date of inoculation of different explants was recorded and were expressed as mean number of days.

3.9.2 Number of shoots produced per explant

The number of shoots produced from single explant was noted after 45 days of inoculation.

3.9.3 Shoot length

The shoot length was measured from base to the top of the plantlet and the average length was expressed in centimetres.

3.9.4 Number of leaves per shoot

The number of leaves produced from single shoot was counted and recorded.

3.9.5 Girth of the shoot

The girth of the shoots was measured and expressed in millimeters (mm).

3.9.6 Percent cultures rooted

The number of shoots rooted out of total inoculated was counted and were converted into percentage. Also the number of roots produced per shoot after 45 days of inoculation and number of days taken for initiation of root primodia counted.

3.9.7 Root length

The length of the root from the collar region to the highest root tip was measured as root length in terms of centimeters.

3.9.8 Per cent survival of plantlets

The number of plantlets survived out of total plantlets subjected to hardening was counted at different interval and percentage was calculated.

3.10 STATISTICAL ANALYSIS OF DATA

The experimental data relating to rooting percentage, percent survival of plantlets was transformed to arcsine values and analysed under completely randomized design. The data were subjected to analysis of variance test as suggested by Panse and Sukhatme (1967). Critical difference values were tabulated at one per cent probability wherever F test was found significant.

IV. EXPERIMENTAL RESULTS

The results obtained in the present investigation "*In vitro* propagation studies in vanilla (*Vanilla planifolia* Andr.)" are presented herein.

4.1 IDENTIFICATION OF SUITABLE SOURCE OF EXPLANTS FOR CULTURE

The results obtained on the use of different explants for vanilla multiplication are presented in Table 1.

4.1.1 Time taken for emergence of primordia

The time taken for emergence of primordia by the explants varied significantly. Out of different explants *viz*., leaf segment, shoot tip, axillary bud, internode, aerial root, only shoot tip and axillary bud gave positive results. The first shoot primordia were formed by axillary bud 14 days after inoculation of explants. However, the shoot tip took 21 days for regeneration (Table 1). In other explants, regeneration was not noticed.

4.1.2 Number of shoots produced.

In the explants, axillary bud and shoot tip one shoot was produced in the initial period and there after number increased. Axillary bud produced more number of shoots.

4.1.3 Callus response

Callus production was observed in shoot tip explant and was not in the axillary bud explant.

4.2 EVALUATION OF DIFFERENT GELLING AGENTS AS AN ALTERNATE TO PURIFIED AGAR.

The data on the effect of gels and their concentrations on multiple shoot production and vigour of the shoots, which varied significantly are presented in Table 2.

4.2.1 Number of shoots produced per explant

The multiple shoot production was maximum (3.83 shoots/explant) on medium gelled with 50 g l^{-1} sago + 1 g l^{-1} agar, while it was minimum (2.06 shoots/explant) on isabgol at 50 g l^{-1} gelled medium.

4.2.2. Shoot length

Shoot length was maximum (4.56 cm) on medium gelled with arrow root at 120 g l^{-1} and was minimum (3.10 cm) on medium gelled with isabgol at 30 g l^{-1} + 1 g l^{-1} agar.

4.2.3. Shoot girth

Average shoot girth was maximum (0.43 cm) in medium gelled with arrow root at 120 g l^{-1}, while it was minimum sago 80 g l^{-1} (0.31) gelled medium and (0.32 mm) on isabgol at 50 g l^{-1}.

4.3 STANDARDIZATION OF GROWTH REGULATOR FOR SHOOT PROLIFERATION

4.3.1 Influence of BAP on *in vitro* shoot multiplication

The shoot regeneration at different concentrations of Benzyl amino purine (BAP) was determined on full strength MS medium. Significant differences were observed with respect to number of shoots, shoot length and number of leaves among different concentrations of BAP. The results pertaining to this are presented in the Table 3a, 3b, 4a and 4b.

Table 1. Effect of explant types on the number of days taken for initiation of growth, number of shoots produced, callus response and height

Sl. No.	Explant type	No. of days taken for initiation of growth	Number of shoots produced	Callus response	Increase in growth (cm)
1.	Leaf segment	-	-	-	-
2.	Shoot tip	21.00	1.00	+	1.42
3.	Axillary bud	14.00	1.00	-	4.60
4.	Internode	-	-	-	-
5.	Aerial root	-	-	-	-

+ : Callus development

- : No callus development

Table 2. Influence of gelling agents on shoot multiplication and vigour of the shoots

Sl. No.	Treatment	No. of shoots/explant	Shoot length (cm)	Shoot girth (cm)
1.	Agar 8 g l^{-1}	2.36	3.22	0.33
2.	Sago 80 g l^{-1}	2.56	3.50	0.31
3.	Sago 60 g l^{-1}	2.50	3.53	0.34
4.	Sago 50 g l^{-1} + Agar 1 g l^{-1}	3.83	4.07	0.40
5.	Isabgol 50 g l^{-1}	2.06	3.52	0.32
6.	Isabgol 40 g l^{-1}	2.23	3.46	0.34
7.	Isabgol 30 g l^{-1} + Agar 1 g l^{-1}	2.21	3.10	0.35
8.	Arrow root 120 g l^{-1}	2.73	4.56	0.43
9.	Arrow root 100 g l^{-1}	3.30	3.56	0.36
10.	Arrow root 80 g l^{-1} + Agar 1 g l^{-1}	2.96	3.58	0.37
	S.Em±	0.07	0.07	0.01
	CD at 1%	0.16	0.18	0.02

Table 3a. Growth parameters of shoots as influenced by growth regulators in vanilla (axillary bud explant 25 days after inoculation)

Sl. No.	**Treatment (mg l^{-1})**	**No. of days taken for initiation of shoot**	**No. of shoots/ explant**	**No. of leaves/shoot**	**Mean length of shoots (cm)**
1.	MS	0.00	0.00	0.00	0.00
2.	MS + 0.5 BAP	12.73	1.50	1.49	5.80
3.	MS + 3.0 BAP	11.63	2.70	2.46	4.63
4.	MS + 1.5 BAP	14.80	3.16	2.76	4.20
5.	MS + 2.0 BAP	15.80	1.76	1.83	4.13
6.	MS + 2.5 BAP	16.20	1.51	1.50	3.96
7.	MS + 3.0 BAP	17.01	1.23	1.41	3.40
	S.Em±	0.17	0.04	0.44	0.08
	CD at 1%	0.41	0.10	0.11	0.19

Table 3b. Growth parameters of shoots as influenced by growth regulators in vanilla (shoot tip explant 25 days after inoculation)

Sl. No.	Treatment (mg l^{-1})	No. of days taken for initiation of shoot	No. of shoots/ explant	No. of leaves/shoot	Mean length of shoots (cm)
1.	MS	0.00	0.00	0.00	0.00
2.	MS + 0.5 BAP	22.00	1.00	1.00	1.00
3.	MS + 3.0 BAP	20.00	1.00	1.00	1.00
4.	MS + 1.5 BAP	20.00	1.00	1.00	1.00
5.	MS + 2.0 BAP	18.00	1.00	1.00	1.00
6.	MS + 2.5 BAP	20.00	1.00	1.00	1.00
7.	MS + 3.0 BAP	23.00	1.00	1.00	1.00
	S.Em±	0.27	NS	NS	0.04
	CD at 1%	0.68	-	-	0.10

Table 4a. Growth parameters of shoots as influenced by growth regulators in vanilla (axillary bud explant 45 days after inoculation)

Sl. No.	Treatment (mg l^{-1})	No. of shoots/ explant	No. of leaves/shoot	Mean length of shoots (cm)
1.	MS	0.00	0.00	0.00
2.	MS + 0.5 BAP	2.66	3.26	10.73
3.	MS + 3.0 BAP	3.23	3.73	9.30
4.	MS + 1.5 BAP	4.70	4.50	8.60
5.	MS + 2.0 BAP	2.43	3.33	6.70
6.	MS + 2.5 BAP	2.10	2.53	5.46
7.	MS + 3.0 BAP	1.40	2.33	4.41
	S.Em±	0.07	0.06	0.09
	CD at 1%	0.16	0.16	0.22

Table 4b. Growth parameters of shoots as influenced by growth regulators in vanilla (Shoot tip explant 45 days after inoculation)

Sl. No.	Treatment (mg l^{-1})	No. of shoots/ explant	No. of leaves/shoot	Mean length of shoots (cm)
1.	MS	0.00	0.00	0.00
2.	MS + 0.5 BAP	2.60	3.10	3.08
3.	MS + 3.0 BAP	3.40	3.33	3.51
4.	MS + 1.5 BAP	2.80	4.40	4.46
5.	MS + 2.0 BAP	2.33	2.60	3.28
6.	MS + 2.5 BAP	1.80	2.20	2.50
7.	MS + 3.0 BAP	1.30	2.10	2.19
	S.Em±	0.06	0.05	0.03
	CD at 1%	0.14	0.12	0.08

4.3.2 Number of shoots produced per explant

There was significant difference between axillary bud and shoot tip explants with respect to the number of shoots produced. The maximum number of shoots were produced in axillary bud.

After 45 DAS, in axillary bud maximum number of shoots (4.7 shoots/explant) were produced in media supplemented with 1.5 mg/l BAP and minimum (1.40 shoots/explant) number of shoots were produced in 3.0 mg/l BAP supplemented media (Table 4a). In shoot tip explants maximum (3.40 shoots/explant) were produced in media supplemented with 3.00 mg/l BAP and minimum (1.30 shoots/explant) number of shoots were produced in 3.0 mg/l BAP supplemented media (Table 4b). In control there was no initiation of shoot growth in both the cases.

4.3.3 Shoot length

After 45 DAS, there was significant difference with respect to shoot length among axillry bud and shoot tip. Axillary bud produced highest shoot length compared to shoot tip. In axillary bud explant, the highest (10.73cm) length was observed, at 0.5 mg/l BAP supplemented media and minimum (4.41 cm) was observed in media supplemented with 3.0mg/l BAP. In shoot tip the maximum (4.46 cm) was recorded in medium supplemented with 1.5 mg/l BAP and minimum (2.19 cm) was recorded in media supplemented with 3.0 mg/l BAP.

4.3.4 Number of leaves per shoot

Maximum (4.50 leaves/shoot) number of leaves were produced in media supplemented with 1.5 mg/l BAP and lowest (2.33 leaves/shoot) was observed in 3.0 mg/l BAP supplemented media. In shoot tip maximum (4.40 leaves/shoot) number of leaves were produced in media supplemented with 1.5 mg/l BAP and lowest (2.10 leaves/shoot) was observed in 3.0 mg/l BAP supplemented media.

4.4 STANDARDIZATION OF GROWTH REGULATOR FOR ROOT INITIATION.

In this experiment, influence of IBA at different concentrations on *in vitro* rooting of shoots was studied. The results on number of days taken for emergence of root primordia, per cent rooting, length of roots are presented in Table 5.

4.4.1 Per cent rooting of microshoots

The highest rooting percentage (100%) was registered in media supplemented with 0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l and 2.5 mg/l, IBA, while the lowest was in control (46.66%).

4.4.2 Number of days taken for emergence of root primordia

There was significant difference with respect to time taken for emergence of root primordia among the auxins used. Root initiation was early (8.8 days) in media supplemented with 0.5mg/l IBA. However, it was maximum (13.50 days) in control.

4.4.3 Root length

Maximum root growth (4.29 cm) was recorded in 1.0mg/l IBA supplemented media and minimum root growth (0.56 cm) was recorded in control.

4.5 IDENTIFICATION OF SUITABLE HARDENING MEDIUM FOR BETTER ESTABLISHMENT

The results of the experiment on hardening of rooted plantlets are presented in Table 6.

Table 5. Influence of different concentrations of auxin (IBA) on *in vitro* rooting

Sl. No.	Treatment (mg l^{-1})	Per cent rooting	No. of days for emergence of primordia	Length of root (cm)
1.	MS	46.66	13.50	0.56
2.	MS + 0.2 IBA	76.66	9.94	1.85
3.	MS + 0.5 IBA	100.00	8.80	3.87
4.	MS + 1.0 IBA	100.00	10.36	4.29
5.	MS + 1.5 IBA	100.00	10.93	3.61
6.	MS + 2.0 IBA	100.00	11.20	3.56
7.	MS + 2.5 IBA	100.00	12.70	3.59
	S.Em±	1.78	0.18	0.05
	CD at 1%	4.43	0.45	0.12

Table 6. Effect of media on survival percentage, plant height and number of leaves during hardening

Sl. No.	Medium	15 DAT			30 DAT		
		Survival percentage	Plant height (cm)	Number of leaves	Survival percentage	Plant height (cm)	Number of leaves
1.	Sand	100	13.20	4.20	100	15.54	6.24
2.	Soil	80	12.50	3.26	80	14.22	5.76
3.	Soil + Coco coirpith (1:1)	100	14.40	5.08	100	16.32	6.72
4.	Sand + Soil + Coco coirpith (1:1:1)	100	15.60	5.28	100	16.74	7.56
	S.Em±	1.23	0.06	0.05	1.23	0.06	0.07
	CD at 1%	3.02	0.15	0.11	3.02	0.14	0.17

DAT – Days after transfer to hardening medium

4.5.1 Survival percentage of plantlets

The data regarding the survival percentage of plantlets during hardening at different intervals are presented in the Table 6. The maximum (100%) survival was noticed on sand, soil+cococoir pith (1:1) and sand + soil + cococoir pith (1:1:1) at 15 and 45 days after transfer to hardening medium. While minimum (80%) was recorded in soil at 15 and 45 days after transfer to hardening medium.

4.5.2 Height of the plantlets

Significant differences were observed among the treatments. The maximum height (15.60 cm) was recorded in sand + soil + cococoir pith (1:1:1) this was followed by soil+cococoir pith (1:1) with 14.40cm height.

4.5.3 Number of leaves per plant

Highest number of leaves was recorded in soil+cococoir pith (1:1) and sand+soil+cococoir pith (1:1:1) with 5.08 and 5.28 leaves respectively. Minimum (3.26) leaves were observed in soil.

V. DISCUSSION

The present studies were conducted to know the efficacy of different explants for *in vitro* propagation, to standardize growth regulators for vanilla multiple shoot production and *in vitro* rooting. The effect of different gelling agents on multiple shoot production and suitable hardening media for micropropagated plantlets were also assessed. The results of the experiment are discussed in this chapter.

5.1 IDENTIFICATION OF SUITABLE SOURCE OF EXPLANT FOR CULTURE

In the present investigation, regeneration of plantlets from the cultured explants occurred by direct organogenesis in axillary bud and shoot tip. However, callus growth was also noticed at the cut end of shoot tip explant.

Axillary bud gave quickest response for the regeneration of shoots. On the other hand, shoot tip took more time for the regeneration of shoots. This difference in response, among the different explants might be due to the difference in physiological state of the explants (Sreelatha *et al.*, 1998). Axillary bud has a potential bud, which has organized tissue, meristematic in nature. Hence, it will take less time for shoot initiation. Whereas in shoot tip, callus growth was observed, for which, it has to undergo process of dedifferentiation that is to return to an undifferentiated mass state. In the first stage of dedifferentiation, the cells organize themselves in a meristematic mass and in the second stage reorientation of meristematic cells take place, which will be the origin of new individual and the whole process take more time for emergence of primordia. Physiological state may also account for the changes in the content of indigenous phytoharmones, nutrients and metabolites.

Generally, shoot tips are highly regenerative since they have meristematic cells. However in the present experiment, regeneration was observed maximum in axillary bud. Similar report was made by Geetha and Shetty (2000) where in they found that axillary bud produced more number of shoots with broader leaves than in shoot tip explants. This result was in accordance with Sagawa *et al.*, (1966) who also reported that axillary bud of dendrobium taken from new growth produced better protocorms than other explants. Therefore, axillary bud was found to be a suitable source of explant followed by shoot tip for micropropagation in vanilla.

5.2 EVALUATION OF DIFFERENT GELLING AGENTS AS AN ALTERNATE TO PURIFIED AGAR

The highest number of multiple shoots were in media gelled with sago at 50 g l^{-1} + 1 g l^{-1} agar followed by arrow root at 100 g l^{-1}. The shoot proliferation in sago at 50 g l^{-1} + 1 g l^{-1} agar gelled medium was maximum. The rich nutrients present in sago (Appendix II) probably have supplemented the growth and development.

Isabgol at all concentrations produced less number of multiple shoots per explant compared to sago and arrow root. The results were in accordance with Prabhakara (1999) and Shailaja and Patil (2002).

Isabgol showed decreased shoot proliferation with its increasing concentrations. The same results were also found in arrow root and sago. Similar observation was made by Prabhakara (1999) in anthurium. He observed that, as the concentration of gelling agents *viz.*, sago, arrow root and isabgol increased, the consistency also increased and as the surface hardness increased, the per cent seed germination decreased. He reported that it is due to poor contact between the surface of the medium and the explant, resulting in poor absorption of nutrients. Debergh (1983) opined that gel must be firm enough to support explant or shoot cluster. If it is too hard, it may prevent adequate contact between the medium and tissue. Due to solidification, water stress is created, which affects severely the growth and development in tissue culture (Bouniolus, 1974).

Isabgol

Arrow root

Sago

Agar

Plate 2. Different gelling agents

Axillary bud

Shoot tip

Plate 3. Multiple shoot formation in vanilla

Sago Isabgol Arrow root

Plate 5. Comparison of shoot formation in different gelling agents

a b c d

Plate 6. Multiple shoot production in MS medium

a. 2.0 mg/l BAP
b. 2.5 mg/l BAP
c. 3.0 mg/l BAP
d. 3.5 mg/l BAP

Table 7: Production cost of vanilla plants (per plant)

Sl. No.	Particulars	Rate (Rs./plant)
1.	Media cost	0.70
2.	Inoculation cost	0.45
3.	Preparation cost	0.55
4.	Washing cost	0.20
5.	Electricity cost	0.45
6.	Others	0.40
	Total	**2.75**
	Primary hardening cost	
1.	Hardening medium cost	0.20
2.	Filling cost	0.15
3.	Mortality (%)	0.25
4.	Net pots cost	0.30
5.	Other costs	0.15
	Total	**1.05**
	Grand total	**3.80**

Table 8. Cost for one litre media using different gelling agents at different concentrations

Product	Price (Rs. kg^{-1})	Quantity (g l^{-1})	Price (Rs. l^{-1})
Agar	1880	008	15.04
Isabgol	50	050	2.50
Isabgol	50	040	2.00
Isabgol	50	030	1.50
Sago	56	080	4.48
Sago	56	060	3.36
Sago	56	050	2.80
Arrow root	40	120	4.80
Arrow root	40	100	4.00
Arrow root	40	080	3.20

*Lobo chemicals price list 2004-05

The highest shoot length, girth and shoot weight were recorded in medium gelled with arrow root at 120 g l^{-1}, but the number of shoots produced per explant was less in this medium.

Vigour of the shoots was poor in isabgol gelled medium at all concentrations used. This might be due to poor absorption of nutrients because of poor contact with the surface of media as a result of hardiness in its surface (Prabhakara, 1999). Similarly, Jain *et al.* (1997) reported the hardiness imparted by isabgol during the culture period.

More number of vigorous shoots were produced in sago gelled medium at 50 g l^{-1} + 1 g l^{-1} agar. This is probably because of its richness in nutrients like carbohydrates, aminoacids and minerals.

The medium gelled with sago produced more number of shoots than agar. Agar contains some inhibitory substances because of which Jain *et al.* (1997) observed pre-mature abortion of embryoids in tobacco anther culture. On the other hand, Nene *et al.* (1996) reported that on dry basis tapioca pearls (sago) contain about 95 per cent starch. Starch helps in absorbing the inhibitory substances, hence increases the regeneration of plants (Tiwari and Rahimbaev, 1992).

Isabgol and arrow root gave poor results with respect to multiple shoot production. Arrow root was found to be much better than isabgol.

The production cost of one plant is estimated Rs. 3.80 (Table 7) (excluding secondary hardening), whereas in commercial units, it is selling at the rate of Rs. 15 – 25 per plant. Further, the cost of the plantlets (Table 8) was reduced by 4-5 times in sago gelled media when compared with agar.

Considering all aspects of multiple shoot production, quality of plants and cost, sago at 50 g l^{-1} + 1 g l^{-1} agar found to be a suitable replacement for agar. With regard to multiple shoot production sago was superior to agar, but quality wise agar and sago were almost equal. With regard to cost (Table 8), the best alternative was sago in combination with agar. However, though it reduced the price to some extent their practical handling is a problem during media pouring and removal of plantlets as it is laborious and chance of damaging the plantlets is also there. Similar problems were also noticed in isabgol and arrow root.

5.3 STANDARDIZATION OF GROWTH REGULATORS FOR SHOOT PROLIFERATION

The results revealed that, BAP at lower concentrations produced more vigorous shoots. The highest shoot length observed in media supplemented with 0.5 mg/l, but number of shoot produced per explant was less. The similar results were recorded by Rao *et al.* (1993). However, BAP at 1.5 mg/l produced maximum number of shoots but their size (both hight and girth) was significantly reduced. This may be due to the fact that suppression apical dominance leads to production of more number of multiple shoots and reduced shoot length. This was in accordance with the results of Murashige (1974) where in for axillary bud proliferation, cytokinins have been utilized to over come apical dominance of shoot and enhances branching of lateral buds from leaf axils.

The mean number of leaves per shoot was maximum in media supplemented with 1.5 mg/l BAP. This is because of maximum number of shoots produced. In case of shoot tip explant, mean shoot length was highest in media supplemented with BAP 1.5 mg/l, but number of shoots reduced considerably. This is because of apical dominance which leads to production of more number of multiple shoots and reduced shoot length.

As the concentration of BAP increased, the shoot length, number of shoots, number of leaves reduced. Similar result was recorded by Bhatt (1994). Wherein, they found that high level of cytokinins have deleterious effect on shoot growth and protocorm production in vanilla.

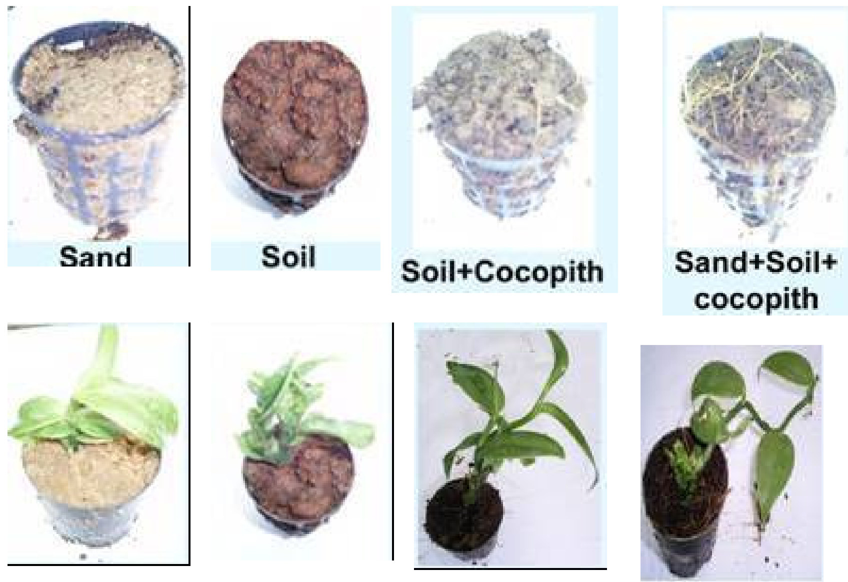


Plate 7. Establishment of plantlets on different hardening media

Plate 8. Roots induced in vitro on MS medium

Plate 9. Fully developed plant

5.4 STANDARDIZATION OF GROWTH REGULATOR FOR ROOT INITIATION

In the present investigation the root growth was present in the absense of growth regulators. Similar results were obtained by Agarwal *et al.* (1992); Ganesh *et al.* (1996). However, the per cent rooting and length of roots were less compared to media supplemented with IBA at 1.0mg/l, where in rooting was 100% with better roots. This may be due to fact that IBA enhances root production and its growth.

5.5 IDENTIFICATION OF SUITABLE HARDENING MEDIUM FOR BETTER ESTABLISHMENT

The *in vitro* grown plantlets were used for hardening on four different media *viz*., sand, soil, soil +coco coirpith(1:1), sand + soil + coco coirpith(1:1:1). The highest survival percentage and better vigour of plantlets were observed in sand + soil + coco coirpith(1:1:1) media followed by soil + coco coirpith(1:1). However, soil comparatively poor results. This could be because of poor aeration and compactness of soil when compared to other media. The medium sand + soil + coco coirpith(1:1:1) proved its superiority as hardening medium for establishment of vanilla plantlets due to the optimum conditions like good aeration and high water holding capacity.

5.6 PROTOCOL FOR MICROPROPAGATION OF VANILLA

Based on the results, a protocol for micropropagation of vanilla is given below.

1. Shoots should be cut into pieces of 2-3 cm size along with a bud. In shoot tip explant the terminal portion should be retained and the matured leaves removed.
2. The isolated explants need to be treated with a bactericide and a fungicide for 10 minutes and washed with distilled water. They are to be treated with 0.1 per cent $HgCl_2$ for 10 minutes and washed 3-4 times with sterile distilled water in laminar air flow cabinet.
3. After sterilization, explants need to be cultured on MS basal medium supplemented with 1.0mg/l BAP for 40-50 days.
4. The shoots may be subcultured on MS medium supplemented with 1.0 mg l^{-1} BAP at regular interval.
5. The shoots of more than 3 cm in length are to be cultured on MS medium containing 0.5 mg l^{-1} IBA for 30-40 days for rooting.
6. These rooted plantlets should be hardened on soil + coco pith (1:1) or sand + soil + coco pith (1:1:1) media for 45 days.
7. To reduce the cost of micropropagated plantlets, sago at 50 g l^{-1} + 1 g l^{-1} agar can be used as an alternate gelling agent to agar.

5.7 FUTURE LINE OF WORK

1. Different explants for shoot initiation may be tried for higher concentration of growth regulators.
2. Rooting of *in vitro* produced shoots may be tried *ex vitro* in the polytunnel.
3. Hardening of *in vitro* rooted plantlets on different media may be tried.

VI. SUMMARY

The present investigations on *In vitro* propagation studies on vanilla (*Vanilla planifolia* Andr.) was conducted in the Tissue Culture Laboratory in the Department of Horticulture, University of Agricultural Sciences, Dharwad during 2003-2005.

Vanilla is an important spice crop, and offers excellent scope for cultivation in the tropical high rainfall regions in southern India. There is a growing demand for natural vanilla flavour in the global trade. Vanilla essence, vanillin extracted from the cured beans is widely used for flavouring cakes, sweets, chocolates, ice creams, beverages, in cosmetics and perfumery industries. Vanilla is generally propagated by stem cuttings. However, this method of propagation is rather slow, labour intensive and time consuming. Obtaining the stem cuttings from the mother plant causes set back to their growth and yield. To overcome this micropropagation plays an important role in rapid mass propagation of vanilla. Generally, the cost of micropropagated plant is very high. For this one of the major contributing factor is the cost of agar. Keeping this in view, the present investigation was conducted to find out suitable explant, gelling agent, and growth regulator for shoot multiplication and rooting for culture establishment and to screen suitable hardening media.

Among the explants *viz*., leaf segment, shoot tip, axillary bud, internode and aerial roots, axillary bud and shoot tip emerged as suitable explants for vanilla culture establishment.

Sago at 50 g l^{-1} + agar at 1 g l^{-1} was found as a suitable replacement for agar. Sago produced more number of vigorous shoots. Regarding other gelling agents, arrow root was found promising, but its practical handling problems restricts its use. The studies are therefore needed to overcome its handling problem.

Among different concentrations of BAP, 1.0 mg/l BAP gave more number of better sized shoots. Among different concentrations of IBA used, 0.5 mg/l IBA resulted in 100 per cent rooting with better plantlet growth.

Four different medium *viz*., sand, soil, soil+cococoir pith (1:1), sand+soil+cococoir pith (1:1:1) were tried for hardening of plantlets. Soil+cococoir pith (1:1) and sand+soil+cococoir pith (1:1:1) gave the maximum (100%) survival percentage with better plant vigour resulting as a suitable medium for hardening.

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Appendix I : Media composition

Contents (mg 1^{-1})	**MS**
Ammonium nitrate	1650
Potassium nitrate	1900
Magnesium sulphate	370
Manganese sulphate	-
Zinc sulphate	8.6
Ferrous sulphate	27.8
Copper sulphate	0.025
Potassium phosphate	170
Potassium iodate	0.83
Calcium chloride	440
Coblt chloride	0.025
Boric acid	6.2
Sodium molybdate	0.25
Sodium EDTA	37.3
Nickel chloride	-
Ferric chloride	-
Aluminum chloride	-

George *et al.* (1988)

Appendix II : Composition of sago and arrow root

Nutrient (g/100g)			Minerals (mg/100g)		
Component	**Sago**	**Arrow root**	**Component**	**Sago**	**Arrow root**
Moisure	59.4	16.5	Calcium	50	10
Protein	0.7	0.2	Phosphrous	40	20
Fat	0.2	0.1	Iron	0.9	1.0
Mineral	1.0	0.1	Magnesium	66	-
Fibre	0.6	-	Sodium	7.5	-
Carbohydrates	38.1	83.1	Potassium	70.4	30
			Copper	0.15	20
			Sulphur	58	-
			chlorine	10	-

Vitamins (mg/100g)			Amino acids (mg/100g)		
Component	**Sago**	**Arrow root**	**Component**	**Sago**	**Arrow root**
Thiamine	0.05	-	Argenine	5800	-
Riboflavin	0.10	-	Histidine	110	-
Niacin	0.3	-	Lysine	290	-
Vitamin C	25	-	Tryptophan	080	-
			Phenylalanine	108	-
			Tyrosine	100	-
			Methionine	050	-
			Cystine	090	-
			Threonine	200	-
			Leucine	300	-
			Isoleucin	250	-
			valine	240	-

(Gopalan *et al.*, 1994)

Appendix III : Impurities present in different gels (%)

Impurities	Agar	Sago	Arrow root	Isabgol
Ash	1.75	6.25	0.3	2.9
Calcium	0.27	10.64	0.1	-
Barium	0.01	-	-	-
Silica	0.09	0.94	-	-
Chloride	0.13	2.75	-	-
Sulphate	1.32	-	-	-
nitrogen	0.14	0.02	-	-

(Perik, 1987 and Gopalan *et al.,* 1994)

APPENDIX IV : Abbreviations used in the text

MS	-	Murashige And Skoog Medium
CGS	-	Common Grade Sugar
BAP	-	6-Benzyl amino purine
BA	–	6-Benzyl adenine
KIN	–	Kinetin
NAA	-	Napthyl acetic acid
IAA	-	Indole-3-acetic acid
IBA	-	Indole-3-butyyric acid

IN VITRO PROPAGATION STUDIES ON VANILLA (*Vanilla planifolia* Andr.)

V.S. NEELANNAVAR 2005 Dr. SATISH S. PATIL
Major Advisor

ABSTRACT

An investigation on "in vitro propagation studies on vanilla (*vanilla planifolia* Andr.)." was carried out during 2003-2005 at the tissue culture laboratory of department of horticulture, college of agriculture, UAS, Dharwad.

In the present investigation five sub experiment were carried out by following CRD design in order to find out best explant, gelling agent, growth regulators for shoot and root initiation and hardening media.

Regarding the suitability of explants, axillary bud and shot tip were the best for culture establishment, by producing more number of adventitious shoots with early emergence of primordia. Among the gels used for standardization, sago at 50 g/l + agar 1g/l emerged as the sole replacement of agar, which gave best result in terms of shoot proliferation and subsequent growth. With respect to cost, sago at 50 g/l + agar 1g/l was best replacement for agar which minimized the cost by four times.

Among BAP concentration, 1.0 mg/l produced more number of better sized shoots. Among the IBA concentration used, the maximum number of roots with good length in short time were observed on 0.5 mg/l IBA supplemented media.

Soil + coco coirpith (1:1) and sand + soil + coco coirpith (1:1:1) medium gave 100% survival percentage at 15 and 45 days after transfer to hardening media and plantlets so hardened were more vigorous.