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Advances in Micropropagation of Selected Aromatic Plants: A Review on Vanilla and Strawberry

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ABSTRACT

Aromatic plants have been used commercially as spices, natural flavor, raw material for essential-oil industry and other medicinal purpose. Tropical and sub-tropical Asia are rich in the number of aromatic plant species due to their suitable ecological conditions. Micropropagation has superiority over conventional method of propagation because of high multiplication rate but, field performance of these tissue cultured plants depends on the selection of the initial material, media composition, growth regulators, cultivar and environmental factors. Some well developed *in vitro* techniques are currently available to help growers for meet the demand of the spices and pharmaceutical industry. Identification of somatic clones of plants derived through tissue culture can facilitate commercially viable *in vitro* propagation for medicinal and aromatic plants. An overview of the regeneration of aromatic plants by *in vitro* organogenesis from various types of explants is presented in this review article.

Key words: Acclimatization, aromatic plants, clonal fidelity, field evaluation, *in vitro*, isozymes, molecular markers

INTRODUCTION

Human beings are dependent on plant secondary metabolites for their medicinal and aromatic purpose since the beginning of civilization. Of the 2,50,000 higher plant species on earth, more than 7000 species of plants found in different Indian agro-ecosystems and used by various indigenous systems of medicine and industries (Mathew *et al*., 2005). Aromatic plants possess odorous volatile compounds, most of which are essential oil, gum exudates, balsam and oleoresin various plant parts, namely, root, wood, bark, stem, foliage, flower and fruit. They have been used as raw materials for the extraction of essential oils which are used in the flavor and fragrance industries. These plants are also the sources of spices, herbs, plant based medicines, pharmaceuticals, cosmetics, botanical pesticides, insect repellents and herbal teas/drinks (Chomchalow, 2002). Essential oils constitute about 17% in the world wide flavor and fragrance market, whereas, world production of essential oils varies from 40,000 to 60,000 tones per annum. India is well known throughout the world as the land of aromatic plants or the land of spices, or the land of traditional perfumes because it possesses favorable climatic conditions suitable for the development of aromatic plants. These plants have been used commercially as spices and as sources of raw material for essential-oil industry. The West Asians and Europeans downplayed the Indian

Table 1: List of some major aromatic plants of India*

Scientific name	Common name	Family	Parts used
Amomum subulatum	Small cardamom#	Zingiberaceae	Fruit
Cinnamomum verum	Cinnamon	Lauraceae	Bark
Cinnamomum tamala	Indian cassia	Lauraceae	Bark
Curcuma longa	Turmaric#	Zingiberaceae	Root
Cymbopogon citratus	Lemongrass (W.Indian)	Graminae	Leaf
Cymbopogon flexuosus	Lemongrass (E.Indian)	Graminae	Leaf
Cymbopogon martini var. *motia*	Palmarosa	Graminae	Leaf
Cymbopogon nardus	Citronella (Ceylon)	Graminae	Leaf
Cymbopogon winterianus	Citronella (Java)	Graminae	Leaf
Elettaria cardamomum	Small cardamom#	Zingiberaceae	Fruit
Eucalyptus globulus	Eucalypt	Myrtaceae	Leaf
Jasminum officinale	Jasmine	Oleaceae	Flower
Jasminum sambac	Arabian jasmine	Oleaceae	Flower
Hedychium spicatum	Tahitian flame	Zingiberaceae	Flower
Kaempferia galanga	Kencur	Zingiberaceae	Root
Lavandula angustifolia	Lavender	Lamiaceae	Flower
L. officinale	Lavender	Lamiaceae	Flower
Mentha arvensis	Japanese mint#	Lamiaceae	Aerial parts
Mentha citrata	Bergamot mint#	Lamiaceae	Aerial parts
Mentha piperita	Peppermint#	Lamiaceae	Aerial parts
Mentha spicata	Spearmint#	Lamiaceae	Aerial parts
Ocimum basilicum	Basil#	Lamiaceae	Aerial parts
Ocimum gratissimum	Lemon basil#	Lamiaceae	Aerial parts
O. tenuiflorum	Holy basil#	Lamiaceae	Aerial parts
Pelargonium capitatum	Alta of rose geranium	Geraniaceae	Leaf
P. crispum	Curly-leaved geranium	Geraniaceae	Leaf
P. fragrans	Nutmeg-scented geranium	Geraniaceae	Leaf
P. graveolens	Pot geranium	Geraniaceae	Leaf
P. macrorrhizum	Scented geranium	Geraniaceae	Twig
P. pratense	Scented geranium	Geraniaceae	Twig
Pimpinella anisum	Anise	Umbelliferae	Seed
Piper nigrum	Black pepper#	Piperaceae	Berry
Rosa damascene	Damask rose	Rosaceae	Flowers
Trigonella foenum-graecum	Fenugreek#	Fabaceae	Seed
Vanilla planifolium	Vanilla	Orchidaceae	Pod
Vetiveria zizanioides	Vetiver	Poaceae	Root
Zingiber officinale	Zinger #	Zingiberaceae	Root

*From Country Reports of ASIUMAP, FAO/RAP, Bangkok, 4-9 Nov. 96. #Used both for aromatic and culinary purposes. NB: Some aromatic plants in this partial list have the medicinal properties also

biodiversity and de-emphasized its advanced status and many rare plant species were taken from India by Europeans for further development without given the basic credit to India (Chomchalow, 2002). Due to overexploitation, many species have become extinct or scarce so they now have to be cultivated. Aromatic plants were originally collected from the wild and cultivated within India are shown in Table 1.

A general overview *of in vitro* clonal propagation in aromatic plants: *In vitro* culture is one of the key tools of plant biotechnology that exploits the totipotency nature of plant cells

(Haberlandt, 1902) and unequivocally demonstrated for the first time in plants by Steward *et al.* (1958). Cell tissue and organ culture through *in vitro* condition (Debergh and Zimmerman, 1991) can be employed for large scale propagation of disease free clones and gene pool conservation. Aromatic plant industry has applied immensely *in vitro* propagation approach for large scale plant multiplication of elite superior varieties. As a result, hundreds of plant tissue culture laboratories have been constructed worldwide, especially in the developing countries due to cheap labor costs. However, micropropagation technology is more costly than conventional propagation methods and unit cost per plant becomes unaffordable, thus compels to adopt strategies to cut down the production cost (IAEA-TECDOC-1384, 2004).

Micropropagation of aromatic plants: Micropropagation has superiority over conventional method of propagation because of high multiplication rate and disease free plants. Efficient plant regeneration protocol is essential for genetic manipulation of crop species. Aromatic plants are used as dried roots, buds, seeds, berries and fruits commonly used for their flavor and other properties (Samuel *et al.*, 2001). India is in rich repository of aromatic plants used as spices and accounts for about 47% of the global trade (Peter *et al.*, 2007). The productivity of many of these crops is low due to lack of high yielding, biotic stress resistant varieties and the absence of disease-free planting material of elite genotypes. Though, vegetative propagation is prevalent in many tropical and herbal aromatic plants, it is not adequate to meet the demand (Nirmal Babu and Divakaran, 2003). Mustafa and Hariharan (1998) have developed a new tissue culture method for the large-scale multiplication of Zingiberaceous family which constitutes a vital group of rhizomatous aromatic plants characterized by the presence of volatile oils and oleoresins of export value. Protocols for clonal multiplication of many economically and medicinally important Zingiberaceous species like *Amomum subulatum* (large cardamom), *Curcuma aromatica* (kasturi turmeric), *C. amada* (mango ginger) (Prakash *et al.*, 2004), *C. domestica, C. zedoaria* (Yasuda *et al.*, 1988; Prakash *et al.*, 2004), *C. aeruginosa* (Balachandran *et al.*, 1990). *C caesia* (Bharalee *et al.*, 2005), *Alpinia* sp. (Barthakur and Bordoloi, 1992; Geetha *et al.*,1997) *Kaempferia galangal* (Ajith and Seeni, 1995; Chan and Thong, 2004; Chirangini *et al.*, 2005), ginger (Hosoki and Sagawa, 1977; Nadgauda *et al.*, 1980; Nirmal Babu *et al.*, 1997) and *Hedychium spicatum* (Badoni *et al.*, 2010) were developed. However, regeneration of ginger plantlets through callus phase has been reported from leaf, vegetative bud, ovary, anther explants (Nirmal Babu *et al.*, 1992, 1996, 1997; Kackar *et al.*, 1993) and anther callus from diploid and tetraploid ginger (Nirmal Babu, 1997).

Black pepper micropropagation was reported using various explants from both mature and juvenile tissues (Broome and Zimmerman, 1978; Mathews and Rao, 1984; Philip *et al.*, 1992; Nazeem *et al.*, 1993, 2004; Nirmal Babu *et al.*, 2005). Plant regeneration was reported in black pepper from shoot tip and leaf with or without intervening callus phase (Nirmal Babu *et al.*, 1997; Nazeem *et al.*, 1993; Bhat *et al.*, 1995), whereas, Shaji *et al.* (1998) reported variability among genotypes for callus induction. But cyclic somatic embryogenesis from black pepper zygotic embryos was reported by Joseph *et al.* (1996) and Nair and Gupta (2003, 2005). Successful cardamom regeneration from callus of seedling explants was reported by many scientist (Priyadarshan and Zachariah, 1986; Nirmal Babu *et al.*, 1993), whereas, commercialization of micropropagated turmeric plant was reported by Nadgauda *et al.* (1978), Keshavachandaran and Khader (1989), Nirmal Babu *et al.* (1997) and Meenakshi *et al.* (2001). Organogenesis and plantlet formation were achieved from the callus cultures of turmeric (Nirmal Babu *et al.*, 1997; Salvi *et al.*, 2000, 2001;

Praveen, 2005). Successful plant regeneration and variations among regenerated plants were also reported in some rear plants like, *Alpinia conchigera* and *A. galangal* (Balachandran *et al*., 1990; Borthakur *et al*., 1998). A list plant regeneration protocols are given in Table 2. It is evident that AC eliminates light offers an improved consequence of micro-environment for the rooting in *A. andreanum* and *Dendrobium* (Gantait *et al*., 2008; 2009a).

Vanilla: Though MS basal media is used in almost all experimental combinations, the use of WP media (Ganesh *et al*., 1996) can also be used. Root meristem, node, axillary bud, shoot tip and even pods are used as explant source for multiple shoot proliferation in vanilla. Table 3 describes in details about the Plant Growth Regulators (PGRs) like IBA, NAA or PAA (Giridhar *et al*., 2003) as auxin and BA or BAP can be used as cytokinin (Divakaran *et al*., 1996; Mathew *et al*., 2000) for direct organogenesis. Addition of sucrose 15-20 g L^{-1} (Geetha and Shetty, 2000; Divakaran *et al*., 2006) acts as the carbon source for shoot multiplication medium. Ten percent coconut milk (George and Ravishankar, 2004), or d-Biotin 0.05 mg L^{-1} with folic acid 0.5 mg L^{-1} (Geetha and Shetty, 2000) enhances and elongate multiple shoot. Shoot section of first node is the best explant for callus culture in vanilla. MS or ½ MS basal media including BAP 0.5 mg L^{-1} (Pett and Kembu, 1999) and 0.2% activated charcoal (George and Ravishankar, 1997) as an additive induces maximum percentage of *in vitro* root. It is also reported that AC abolishes light offers a rational physical environment for the rhizosphere and facilitates rooting in vanilla (Gantait *et al*., 2009b).

Strawberry: Wide range of plant parts like apical meristems from stolon bud, runner tip, leaf disc, meristem and even single shoot from rosette or leaf petiole may be used successfully for the *in vitro* shoot multiplication. It is clear from Table 4 that like other above mentioned plants, MS was the only basal media used by almost all research workers. IBA and NAA were the auxin source, combined with BA, BAP or TDZ as cytokinin. Sucrose served as carbon source whereas casein hydrolysat or thiamine enhanced shoot induction in some cases. MS at full or half concentration serves as the basal media for root induction in *in vitro* shoots. Only auxin as IBA or NAA can be used for root formation without applying any cytokinin. Activated charcoal caused an increase in the number and length of the roots with 95-100% success rate.

***Ex vitro* field evaluation of acclimatized plants:** These recent advances in plant tissue culture have resulted in the development of protocols for micropropagation of many aromatic plants. Some of which were scaled up to commercial scale, but the process of acclimatization of micropropagated plants to the soil environment has not fully been studied. The transplantation stage continues to be a major bottleneck in the micropropagation of aromatic plants. Plantlets grown *in vitro* have been continuously exposed to a unique microenvironment and have been selected to provide minimal stress to achieve optimum conditions for rapid multiplication. Acclimatization of a micropropagated plant to a greenhouse or a field environment is essential because anatomical and physiological characteristics of *in vitro* plantlets necessitate that they should be gradually acclimatized to the environment of the greenhouse or field (Hazarika, 2003). Successful acclimatization procedures provide optimal conditions for a high percentage of survival of plants, they minimize the percentage of dead and damaged plants in the micropropagation process and they enhance the plant growth and establishment (Sha Valli Khan, 2003). Efficient acclimatization procedure saves the resources of time, labor, money and reduces the cost of production of qualified and deliverable products (Gantait, 2009). Dynamics of the process as well as the final percentage

of fully acclimatized plants are related to plant species and both *in vitro* and *ex vitro* culture conditions (Pospisilova *et al*., 1999). Some plant species are unable to adapt *in vitro* formed leaves to *ex vitro* conditions, but leaves of many other plant species are fully capable of *ex vitro* acclimatization and they function until new leaves are formed (Van Huylenbroeck and Debergh, 1996).

In order to assess yield potential of *in vitro* generated plants, information about field performance is necessary. To get idea of the *ex vitro* morphogenetic efficiency existing among the micropropagated plants with regard to the quantitative characters of economic importance, it is necessary to study them under an array of distinguishable environments. As yield is the main object of a breeder, it is important to know the relationship between various characters that have direct and indirect effects on yield. Micropropagated plants, obtained through *in vitro* culture to retrieve virus free initial planting material, have been widely accepted on field scale. A few trials for comparing conventionally propagated and micropropagated plants however, have shown a mild to striking difference in morphology, flowering behaviour as well as other quality (Radhakrishnan and Ranjitha Kumari, 2009) and quantity parameters. Smith and Hamill (1996) compared the performance of micropropagated ginger (*Zingiber officinale* Roscoe) with normal ginger plant and they found the first generation micropropagated plants had significantly ($p<0.01$) reduced rhizome yield with smaller knobs and more roots. Field performance of plants obtained via tissue culture depends on the selection of the initial material, media composition and number of transfers in culture, the cultivar and many other factors (Libek and Kikas, 2003). Micropropagated and standard propagated strawberry seedlings of cv. Teresa also demonstrated the significant differences between analyzed characteristics (Zebrowska and Hortynski, 2002).

Acclimatization of micropropagated vanilla plants: After rooting of plantlets is achieved, those plantlets are passed through a hardening process for better establishment in the field. Hardening is done in greenhouse on proper growing substrate (organic substrate) with intermittent water supply. When robust root proliferation occurs, these plantlets are then transferred to the main field. Gantait *et al*. (2009b) successfully acclimatized tissue cultured vanilla plantlets on a mixture of sand, soil, coconut fibre and charcoal (1:1:1:1 v/v).

Acclimatization of micropropagated strawberry plants: After achieving the *in vitro* multiple plantlet regeneration, acclimatization of those plantlets is of utmost importance. Tissue culture derived plants can be directly transferred to small pots and allowed to raise on self system with manual water supply. Though it takes much more time to keep them in rooting medium, but the survival percentage reached up to 95-100% during the months of April-June (Koga *et al*., 1999). Following the ideal hardening procedure, micropropagated plantlets were hardened in polyethylene bags and plastic trays filled with soil:farm yard manure (v/v) at 1:1 ratio. Hardening in February gives best result but planting in early April results in even more than a 95% survival rate (Kaur and Chopra, 2004).

Clonal fidelity: Identification of somatic clones of plants derived through tissue culture, with respect to their trueness to their mother or between themselves can be done in various ways. Use of highly discriminatory methods for the identification and characterization of genotypes in this respect is very much essential. Organ culture (e.g., cotyledon, root, bulb scales), somatic embryogenesis and nodule culture, these three alternative directions in developing plantlets may

be appropriate for commercial scales (George, 1996). A major consideration in using an adventitious system is the potential of recovering unusually high numbers of genetic variants. In a commercial setting, this threat is often serious enough to eliminate any further consideration of micropropagation as a cloning method. This is especially true for suspension or callus-culture which seed to generate the higher incidences of somaclonal variation but, somatic embryogenesis appears to be not as susceptible to such problems (Kuehnle *et al*., 1992). Somaclonal variation can also be an occurrence in shoot cultures that have been maintained by stimulation of axillary bud growth. This situation can often be the case in which cytokinin levels are maximized to maintain maximum axillary shoot proliferation (Veilleux and Johnson, 1998). Identification of somatic clones of aromatic plants derived from tissue culture, with respect to their trueness to their mother or between themselves can be done in different way (Gantait *et al*., 2009b). The use of highly discriminatory methods for the identification and characterization of genotypes is essential for breeding programmes. Several cytological and molecular markers have been used to detect the variation and/or confirm the genetic fidelity in micropropagated plants (Vasil, 1984). There are many reports available for genetic fingerprinting and clonal fidelity of medicinal and aromatic plants using allozymes, RAPD, SSR, ISSR etc. (Divakaran *et al*., 1996; Damiano *et al*., 1997; De Benedetti *et al*., 2001).

Test of clonal fidelity by isozymes analysis: Isozymes arise in nature due to genetic and epigenetic mechanisms. The polyacrylamide gel electrophoresis of isozymes as standardized by Schields *et al*. (1983) is being widely followed by research workers with modifications for specific crops. Excellent reviews of enzyme activity staining by Vallejos (1983) and by Wendel and Weeden (1990) are still being referred to by many workers. However, somaclonal variations mostly occur as a response to the stress imposed on the plant in culture conditions and are manifested in the form of DNA methylations, chromosome rearrangements and point mutations (Phillips *et al*., 1994). This is apparent in studies conducted to screen somaclonal variations produced in tissue cultured aromatic plants such as in turmeric and neem (Salvi *et al*., 2001; Singh *et al*., 2002). Association between isozyme patterns of *in vitro* regenerated plants and different growth regulators has reported in different medicinal plants like *Gentiana lutea* L. (Petrova *et al*., 2006), *Hypericum brasiliense* (Abreu *et al*., 2003), *Gymnema sylvestre* R.Br. (Reddy *et al*., 1998), *Aegle marmelos* L. (Ajith Kumar and Seeni, 1998), *Chlorophytum arundinaceum* Baker (Lattoo *et al*., 2006) etc.

Test of clonal fidelity by molecular markers: Molecular markers have been found to be the most desirable tool for establishing genetic uniformity of the micropropagated plantlets. An extensive study on genetic fidelity and molecular diagnostics in micropropagation systems was carried out in micropropagated clones of three species namely *Populus deltoids, Eucalyptus tereticornis, E. camaldulensis* and *Coffea arabica* (Vasil, 1984). In this study, the authors inferred genetic fidelity in those micropropagated clones where molecular markers failed to detect and polymorphism. However, preliminary results on RAPDs, MP-PCR and AFLPs also showed lack of polymorphism in these genotypes, since other molecular markers (e.g., DAF, STS and STMS) did detect adequate and reproducible polymorphism in the same material (Roy *et al*., 1999; Prasad *et al*., 1999). It is of the opinion that any failure to detect polymorphism should not be used to infer genetic fidelity. It is to be emphasized that each marker system screens only a fraction of the genome and not the whole genome and the different markers may screen different fractions of the genome. The entire genome cannot be studied on the basis of only on type of molecular marker.

Table 2: Micropropagation of some commercially important aromatic plants

Plant species	Explant	Response	Medium (PGR in mg L^{-1})	Reference
Amomum subulatum Roxb.	Rhizome bud	Mult Sht	MS + 0.5 KIN	Sajina *et al*. (1997b)
		Rt	MS + 1 BAP + 0.5 IBA	
Amomum krervanh	Axillary buds	Mult Sht	MS + 2 IMA + 0.5 TDZ	Tefera and
		Rt	MS	Wannakrairoj (2004)
Carcuma amada (Roxb.)	Rhizome	Mult Sht	B5 + 0.5 NAA + 4 BA	Barthakur and
		Rt	MS	Bordoloi (1992)
Carcuma longa L.	Rhizome sets,	Mult Sht	MS + 1 NAA	Nirmal Babu *et al*.
	vegetative buds		MS + 72.64 µM TDZ	(1997)
		Rt	MS	Prathanturarug *et al*.
				(2005)
Cassia sp.	Shoot tip	Mult Sht	WPM + 3 BA + 1 KIN	Nirmal Babu *et al*.
		Rt	WPM + 2 g L^{-1} AC	(1997)
Cinnamommum verum	Shoot tip	Mult Sht	WPM + 3 IBA + 1 KIN	Mathai *et al*.
		Rt	WPM + 2 AC	(1997)
Crocus sativus L.	Corms	Mult Sht	MS + 0.5-3 NAA + 1-3 Zeatin	Milyaeva *et al*. (1995)
Elettaria cardamomum	Vegetative buds	Mult Sht	MS + 1 BA + 0.5 NAA	Nadgauda *et al*.
Maton.		Rt	MS + 0.5 BA + 0.5 KIN + 2 IAA +	(1983)
			0.1nBiotin + 0.2 CP + 5% CW	
	Inner core rhizome	Somatic embryo	MS + 4.4 µM BAP + 0.5 µM NAA	Manohari *et al*.
		Mult Sht	MS + 13.2 µM BAP + 0.5 µM NAA	(2008)
		Rt	MS + 13.2 µM BAP + 0.5 µM NAA	
Eryngium foetidum L.	Shoot tip	Mult Sht	MS + 8 BA + 4 GA_3	Daniel *et al*.
		Rt	MS + 3-4 IAA	(1997)
	Leaf, stem-disc and	Mult Sht	Sucrose free MS	Martin (2004)
	root	Rt		
Kaempferia rotunda,	Vegetative buds,	Mult Sht	MS + 1 BA + 0.5 NAA/IBA	Sajina *et al*.
K. galanga	rhizome bits	Rt	MS	(1997a)
				Geetha *et al*. (1997)
				Chirangini *et al*. (2005)
Languas galanga L.	Rhizome bud	Mult Sht	MS + 2 BA	Ferwerda (1994)
Stuntz				
Lavendula angustifolia	Shoot tip	Mult Sht	MS + 1 BA + 0.5 IBA	Sajina *et al*. (1997a)
Mentha piterita L.	Axillary bud	Mult Sht	MS + 4.44 µM BA + 2.32 µM KIN	Sunandakumari *et al*.
		Rt	MS + 0.49 µM IBA	(2004)
Murraya koenigii (L.)	Shoot tip	Mult Sht	MS + 2 BA + 0.5 NAA	Rao *et al*. (1997)
Spremg.				
Murraya koenigii Spreng.	Shoot tip	Mult Sht	MS + 0.75 BA	Hazarika *et al*. (1995)
Myristica fragrans Houtt.	Single node	Mult Sht	SH + 2 BA + 1 NAA + 5 g L^{-1} AC	Iyer (2007)
Piper nigram L.	Shoot tip, node, buds	Mult Sht	MS + 1 IAA + 1 BA	Rema *et al*. (1995)
		Rt	MS + 1 BA	Nirmal Babu *et al*.
				(1997)
Piper nigram L.	Shoot tip, node, buds	Mult Sht	MS + 1 IAA + 1 BA	Rema *et al*. (1995)
		Rt	MS + 1 BA	Nirmal Babu *et al*.
				(1997)
Syzygium aromaticum	Node	Mult Sht	WPM + 2 2iP	Suparman and
				Blake (1990)
Trachyspermum ammi	Hypocotyl	Ca	MS + 1 NAA	Sehgal and Abbas
L. Spreng		Em	MS + 0.2 NAA	(1994)

Table 2: Continued

Plant species	Explant	Response	Medium (PGR in mg L^{-1})	Reference
Turnera diffusa	Leaf	Mult Sht	MS + 7 BA + 6 IBA	Alcaraz-Melendez *et al.*
		Rt		(1994)
Zingiber officinale	Leaf sheath	Mult Sht	MS + 2 BA + 0.6 NAA	Huang (1995)
		Sht RegRt	MS + 1 BA + 0.6 NAA	
			MS	

Mult Sht: Multiple shoot; Rt: Root; Em: Somatic embryo; Ca: Callus; Sht Reg: Adventitious shoot regeneration; CW: Coconut water; AC: Activated charcoal, TDZ: Thidiazuron

Table 3: *In vitro* clonal propagation in vanilla

Explant	Response	Medium (PGR in mg L^{-1})	Reference
First node	Ca	MS + 500 casein hydrolysate + 1g L^{-1} inosotol + NAA + BA	Davidonis and Knorr (1991)
Root meristem	Mult Sht	MS + 0.5 IBA + 1.0 BA	Divakaran *et al.* (1996)
Node	Mult Sht	WPM + 1 BAP	Ganesh *et al.* (1996)
	Rt	MS	
Axillary bud	Mult Sht	MS + 1 NAA + 2 BA	George and Ravishankar
	Rt	½ MS + 2 g L^{-1} AC	(1997)
Pods	Mult Sht	½ MS + 1 NAA + 1-2 BAP	Mary *et al.* (1999)
Middle part of plant	Mult Sht	MS + 0.5 BAP	Pett and Kembu (1999)
	Mult Sht	MS + 0.1 NAA + 1 BAP	Mathew *et al.* (2000)
Shoot tip, node	Mult Sht	MS + 1 BAP	Geetha and Shetty (2000)
Shoot tip, node	Mult Sht	N69 + 0.5 BAP + 0.05 d-Biotin + 0.5 folic acid	Geetha and Shetty (2000)
Node	Mult Sht	MS + 2 IBA	Giridhar *et al.* (2001)
Axillary bud	Mult Sht	MS + Phenylacetic acid + BA	Giridhar *et al.* (2003)
	Rt	MS + IBA	George and Ravishankar
Axillary bud	Mult Sht	MS + 2.69 µM NAA + 10% CW	(2004)
Shoot tip	Mult Sht	MS + 0.5 IBA + 1 BA	Divakaran *et al.* (2006)
Shoot tip, axillary bud	Mult Sht	MS + 2 BAP + 0.5 NAA	Chitra *et al.* (2007)
Axillary budNode	Mult Sht	MS + 9.55 µM BA + 150 Citric acid + 100 Ascorbic acid	Lee- Epinosa *et al.* (2008)
	Rt	MS + 0.44 µM NAA	
	Mult Sht	MS + 2.0 BAP	Gantait *et al.* (2009b)
	Rt	MS + 0.25 IAA + 2 g L^{-1} AC	

Mult Sht: Multiple shoot; Rt: Root; Em: Somatic embryo; Ca: Callus; Sht Reg:Adventitious shoot regeneration; CW: Coconut water; AC: Activated charcoal

For instance, the oligonucleotide in-gel hybridization is only suitable for studying the repetitive DNA (Bhat *et al.*, 1997); RFLPs are suitable only for the study of variation in restriction sites of a particular restriction enzyme.

However, in some other studies, the lack of polymorphism in micropropagated plants screened through molecular markers was used to suggest genetic fidelity. Similarly, Rout *et al.* (1998) used RAPD markers to evaluate the genetic stability of micropropagated plants of *Zingiber officianales*. Molecular markers like RAPD, AFLP and ISSR polymorphism was used for assessment of genetic variability in black pepper (Pradeepkumar *et al.*, 2001, 2003; Babu *et al.*, 2003; Nirmal Babu, 2003; Ganga *et al.*, 2004; Nazeem *et al.*, 2005; Keshavachandran *et al.*, 2005) and cardamom (Peter *et al.*, 2007) to characterize important cultivars, varieties and related species to develop finger prints for the inter relationships study. Ajith *et al.* (1997) used RAPD markers to estimate genetic fidelity of micropropagated *Piper longum* whereas, Banerjee *et al.* (1999) reported male sex

Table 4: *In vitro* clonal propagation in strawberry

Explant	Response	Medium (PGR in mg L^{-1})	Reference
Leaf lamina, petiole	Ca	MS + 5 µM 2,4-D or NAA + 5 µM BA	Green *et al*. (1990)
	Em	10.05 µM IAA + 105 µM BA	
Apical meristem	Leaf rosette	MS + 1 BA + 0.1 IBA + 0.1 GA_3	Petrovic and Jacimovic (1990)
	Mult Sht	MS + 1 BA + 1 IBA	
	Rt	MS + 0.5 IBA	
Anthur	Ca, Sht Reg	MS + 0.05-1 NAA + 0.5-10 BA	Xilin (1992)
	Rt	½ MS + 0.1 NAA	
Leaf disc	Sht Reg	MS + 0.1 IBA + 3 BA + 600 Casin hydrolysate	Sorvari *et al*. (1993)
	Mult Sht	MS + 1.48-4.44 µM BA	Lopez-Aranda *et al*. (1994)
	Rt	MS + 0.5 g L^{-1} AC	
Axillary bud	Mult Sht	LS + 2 BA + 0.1 NAA	Kang *et al*. (1994)
	Rt	MS	
Shoot tip	Mult Sht	MS + 0.02 NAA + 2 BA	Jeong *et al*. (1996)
	Rt	½ MS	
Single shoot	Mult Sht	MS + 0.1 IBA + 0.5 BA	Maodobry *et al*. (1997)
Leaf disc	Sht Reg	MS + 80 µM TDZ	Sutter *et al*. (1997)
		MS + 2 µM IBA	
Stipule	Ca	MS + 5 2,4-D + 1 BAP + 9% Sucrose	Damiano *et al*. (1997)
	Sht Reg	MS + 1 BAP	
Axillary bud	Mult Sht	MS + 1 BA	Hammaudeh *et al*. (1998)
	Rt	MS + 0.1-0.2 NAA	
Leaf, petiole	Ca	MS + 0.1 NAA + BA 0.25	Infante *et al*. (1998)
Nodal segment	Mult Sht	MS + 0.1 µM NAA + 4.0 µM BA	Bhatt and Dhar (2000)
		½ MS + NAA 1.0 µM	
Meristem	Mult Sht	MS + 1 IAA + 1 BA	Adak *et al*. (2001)
	Rt	MS + 5% AC	
Axillary bud, runner tip	Rt	½ MS + 1 IBA + 0.2 g L^{-1} AC	Mahajan *et al*. (2001)
Leaf	Sht Reg	MS + 3.2 BAP	Zebrowska and Hortynski (2002)
Stipules	Sht Reg	MS + 1 BAP + 0.4 Thiamine	Palombi *et al*. (2003)
Runner tip	Mult Sht	MS + 4 BAP	Lal *et al*. (2003)
	Rt	½ MS + 1 IBA	
Leaf, petiole	Ca	MS + 0.75 NAA + 0.5 BAP	Kaushal *et al*. (2004)
	Em	MS + 0.25 NAA + 2 BAP	
Leaf disc	Ca	MS + 1 BA + 1 2,4-D	Khan and Spoor (2004)
	Sht Reg	MS + 2.25 BA + 0.18 NAA	
Shoot tip	Mult Sht	MS + 1 BA + 1 IAA	Singh *et al*. (2004)
Vegetative bud	Mult Sht	MS + 0.5 KIN + 1 BAP + 2 GA_3	Kaur *et al*. (2005)
Sepal	Mult Sht	MS + 1-2 µM Zeatin	Debnath (2006)
	Rt		

Mult Sht: Multiple shoot; Rt: Root; Em: Somatic embryo; Ca: Callus; Sht Reg: Adventitious shoot regeneration; CW: Coconut water; AC: Activated charcoal

associated RAPD markers. RAPD based genetic stability analysis was reported among micropropagted plants of turmeric (Salvi *et al*., 2000, 2001), *Bacopa monnieri* L. (Ramesh *et al*., 2010) and *Swertia chirata* (Chaudhuri *et al*., 2008).

In comparison to molecular assays such as Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphism (RFLP), ISSR is cost efficient, overcomes the hazards of radioactivity and requires lesser amounts of DNA (25-50 ng). Further ISSR markers

have higher reproducibility than Random Amplification for Polymorphic DNAs (RAPDs) (Meyer *et al*., 1993; Fang and Roose, 1997), are more informative, (Nagaoka and Ogihara, 1997), require no prior sequence information and hence were the choice markers for the present study. Also the mentioned advantage of cost efficiency associated with ISSR assay can help in a regular genetic uniformity check of the micropropagated plantlets without adding much to the cost of tissue culture-raised plants. Inter Simple Sequence Repeat (ISSR) marker assay was employed to validate the genetic fidelity of *Swertia chirayita* plantlets multiplied *in vitro* by axillary multiplication upto forty-two passages. The results confirmed the clonal fidelity of the tissue culture-raised *S. chirayita* plantlets and corroborated the fact that axillary multiplication is the safest mode for multiplication of true to type plants (Joshi and Dhawan, 2007). ISSR markers are considered suitable to detect variations among tissue culture produced plants (Leroy *et al*., 2001; Rahman and Rajora, 2001). Johnson *et al*. (2003) reported ISSR-PCR is a valuable tool for genetic diversity analysis in spices. The competence of ISSR in clonal fidelity assessment on micropropagated *Allium* and Aloe was established successfully by Gantait *et al*. (2010a, b).

Vanilla: To test the genetic variability among progenies, isozyme analysis of leaf tissues can be done by Native PAGE (Divakaran *et al*., 1996). Besse *et al*. (2004) successfully demonstrated that genetic diversity can be detected through RAPD interference in vanilla. Later, Sreedhar *et al*. (2007) assessed the genetic fidelity of vanilla using both RAPD and ISSR primers, but this resulted in no difference in their monomorphic banding pattern. Most recently Verma *et al*. (2009) successfully used RAPD and ISSR markers in vanilla to assess the genetic diversity.

Strawberry: The strawberry clones derived from micropropagation should be true to the type. But there may be any variation due to different physical factors causing spontaneous somaclonal variation. To test the fidelity of the clones PAGE can be used for the analysis of banding patterns of different isozymes extracted from young leaf tissues (Nehra *et al*., 1991). Another way to detect variation is use of DNA fingerprinting with RAPD markers. Samples are randomly chosen from total regenerants and compared to those of mother plants (Palombi *et al*., 2003). Examination of clonal fidelity can be done by both isozyme pattern and RAPD analysis (Damiano *et al*., 1997). Debnath *et al*. (2008) used ISSR assay to discriminate the relatedness of strawberry cultivars.

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