ort. Sci.
1 (1): 1-14, 2006

OCUS

Spices biotechnology

K. V. Peter, K. Nirmal Babu[1] and D. Minoo[1]
Kerala Agricultural University
Thrissur, Kerala, India
E-mail: kvptr@yahoo.com

ABSTRACT

In recent times, biotechnological tools have supplemented various conventional approaches in conservation, characterization, improvement and utilization for increasing production and productivity of spices. In many spices, viable micropropagation technologies are available for commercial production and generation of disease - free planting material. Somaclonal variation is important in crops where natural variability is low and a few useful somaclonal variants have been identified in ginger, turmeric and vanilla. Protoplast technology is also available for capsicum, black pepper, fennel, fenugreek, garlic, saffron and peppermint. *In vitro* cryopreservation, Synseed and Micro-rhizome technologies are available for safe propagation, conservation, movement, and exchange of spices germplasm. Studies are in progress for *in vitro* production of flavour and colouring compounds like capsaicin, vanillin, anethole, crocin, picrocrocin, saffranal, etc. using immobilized and transformed cell cultures. Use of molecular markers for crop profiling, fingerprinting, molecular taxonomy, identification of duplicate hybrids, estimation of genetic fidelity and tagging of genes for marker aided selection (MAS) is gaining importance. Isolation of important and useful genes and development of transgenics is in the preliminary stage.

Key words: Spice crops, micropropagation, somaclonal variation, DNA fingerprinting, secondary metabolites

TRODUCTION

Spices and herbs are aromatic plants, parts of which e used to flavour culinary preparations, in confectionery, d in medicines and perfumery. Spices and herbs are grown oughout the world; different plant species are grown in ferent regions. India is a rich repository of spices with er 100 species of herbs and spices being grown. Black pper, cardamom, ginger, turmeric, vanilla, capsicum, namon, clove, nutmeg, tamarind, pimenta, etc., constitute e major spices. Seed spices like coriander, cumin, fennel, ugreek, dill, caraway, anise and herbal spices like saffron, ender, thyme, oregano, celery, anise, sage and basil are o important. Crop improvement aims to increase ductivity and quality of a target crop to meet increasing man demands. Lack of high yielding, pest and disease istant varieties, and a limited genetic variability in some ps, is a major production constraint in spices. Use of technological tool stands to play a major role in ieving the above through commercial propagation, velopment of novel varieties and new breeding lines *via* naclonal variation, anther culture, protoplast fusion, reactor and recombinant DNA technologies for improving, conserving and utilizing the diversity and increasing the utility of spices.

Micropropagation and Plant regeneration

High and rapid rate of multiplication coupled with additional advantage of obtaining disease-free planting material makes micropropagation an important and viable alternative to conventional propagation.

Black pepper and related species

Methods for micropropagation of black pepper have been reported using various explants from both mature and juvenile tissues (Broome and Zimmerman, 1978; Lissamma Joseph *et al,* 1996). Phenolics and endogenous bacterial contaminants severely hamper establishment in black pepper cultures. Treating explants with fungicides prior to routine sterilization followed by frequent transfer to fresh medium, use of activated charcoal and antibiotics in culture media have been suggested for reducing phenolic interference and systemic contamination. Efficient plant regeneration protocols are essential for genetic manipulation of any crop species. Plants have been successfully regenerated from callus cultures of many *Piper* species. Plant regeneration

ian Institute of Spices Research, Marikunnu P.O., Kozhikode 673 012, Kerala, India

was reported from shoot tip and leaf, with or without an intervening callus phase, (Bhat *et al*, 1995).

Techniques for somatic embryogenesis in black pepper are reported by Nair and Gupta (2003). Cyclic somatic embryogenesis from maternal tissues like integuments has tremendous potential for automated micropropagation. These systems are useful for transgenic experiments for transfer of *Phytophthora* resistance. Methods for micropropagation of medicinally important species of piper viz., *Piper longum P. chaba* and *P. betle* have also been developed (Sarasan *et al*, 1993). Plants were regenerated from leaf and stem explants of related species of black pepper like *Piper longum*, *P. betle*, *P. chaba, P. attenuatum* and *P. colubrinum* through both direct and indirect organogenesis (Bhat *et al,* 1992;1995). Somatic embryogenesis is also reported in betelvine (Johri *et al,* 1996).

Cardamom

Efficient and commercially viable technology for rapid clonal propagation of cardamom is available (Vatsya *et al,*1987). Many commercial laboratories use micropropagation techniques for large-scale production of clonal material.

Successful high-frequency regeneration of plantlets from cardamom has been reported. Attempts on anther and microspore culture were reported to be inconsistent in plant regeneration from anther derived callus on MS medium.

Ginger

Clonal multiplication of ginger has been reported by many workers (Rout *et al,* 2001). Micropropagation helps in production of pathogen-free planting material in ginger where diseases often spread through infected seed rhizomes. Regeneration of plantlets through callus has been reported from leaf, vegetative bud, ovary and anther explants. Ginger fails to set fruit in nature. However, in *vitro* pollination could be effected to overcome prefertilization barriers to develop the 'fruit' and subsequently, plants could be recovered from these fruits (Valsala *et al*, 1997).

Turmeric

Technologies for micropropagation turmeric of for production of disease-free planting material were developed (Nadgauda *et al*, 1978; Yasuda *et al,* 1988, Rahman *et al,* 2004; Prathanturarug *et al*, 2003, 2005). Organogenesis and plant regeneration has been reported in turmeric by various workers (Shetty *et al,* 1982; Praveen *et al,* 2005).

Renjith *et al* (2001) reported *in vitro* pollinati and hybridization using two short duration types VK and VK-76 and reported seed set and seed developme This reduces breeding time and helps in recombinati breeding which had not been attempted in turmeric earl

Other Zingiberaceous taxa

Protocols for micropropagation of ma economically and medicinally important zingiberace species like *Amomum subulatum* (large cardamo *Curcuma aromatica* (kasturi turmeric), *C. domestica* 'Koova', *C. aeruginosa, C. caesia , C. amada* (ma ginger), *Curcuma domestica* [*C. longa], C. zedoa Kaempferia galanga, K. rotunda, Alpinia* spp., *Alp conchigera, Alpinia galanga, etc have been* develop (Barthakur and Bordoli, 1992; Chang and Criley, 19 Yasuda (1988) reported successful callus induction fr rhizomes. Prakash *et al* (2004), Lakshmi and Myth (2003) and Rahman *et al* (2004) reported efficient pl regeneration through somatic embryogenesis from leaf ba derived callus of *Kaempferia galanga* L.

Vanilla

Micropropagation of vanilla has been standardi for large-scale multiplication of disease-free plants (Cerv and Madrigal, 1981; Kononowicz and Janick, 1984; Gee and Shetty, 2000).

In vitro germination of vanilla seeds and selecti of useful genotypes from segregating progenies is a reported. This technique was also used to resc interspecific hybrids between cultivated *V. planifolia* a wild *V. aphylla* through embryo rescue.

In vitro propagation of *Vanilla tahitiensis* (M Mathew *et al*, 2000) and endangered species of *Vanill wightiana* , *V. andamanica, V. aphylla* and *V. pilifera* also reported to save these species from extinction.

Successful plant regeneration from shoot and se derived callus was reported in vanilla (Davidonis and Kn 1991; Nirmal Babu *et al,* 1997). This efficient system c be used for creation and exploitation of somaclonal variati in this crop where the existing variation is limited.

Tree spices

Micropropagation protocols have been report in many tree spices like cinnamon, nutmeg, cassia, clov camphor, curry leaf, pomegranate, camboge and tamari (Zhang and Stoltz, 1981; Mascarenhas, *et al* 1987; Math and Hariharan, 1990; Hazarika *et al,* 1995; Mini *et at,* 19 Mallika, et al, 1997; Bhuyan *et al,* 1997; Huang *et al,* 19 Nirmal Babu *et al,* 2000; Mehta *et al,* 2000).

Plant regeneration through somatic embryogenesis has been reported in *Cinnamomum verum* and *C. camphora.* Induction of somatic embryogenesis from zygotic embryos of *Syzygium cumini* and nutmeg was reported by Iyer *et al,*. (2000).

Seed and herbal spices

Micropropagation protocols for many seed and herbal spices are available. These include coriander, fennel, anise, peppermint, spearmint, celery, thyme, lavender, savory, ocimum, oregano, basil, sage, fennel , parsley, sweet marjoram, dill and garlic (Bhojwani, 1980; Ahuja, 1982; Miura, *et al,* 1987; Cellarova, 1992; Furmanowa and Ozszowska, 1992; Hunault and Du-Manoir, 1992; Panizza and Tognoni, 1992; Toth and Lacy, 1992; Patnaik and Chand, 1996; Vandemoortele *et al,* 1996; Sajina *et al,* 1997; Iyer and Pai, 1998).

Plant regeneration has been successfully induced from callus cultures of peppermint, coriander, celery, cumin, fennel, lavender, anise, parsley, poppy, oregano, dill, caraway and sage (Ratnamba and Chopra, 1974; Sehgal, 1978; Chand and Roy, 1981; Jha *et al,* 1982; Ammirato,1983; Van Eck and Kitt, 1990;1992; Neena Kumari and Sarathy, 1992; Kataeva and Popowich, 1993; Onisei *et al,* 1994; Okamoto *et al,* 1994; Donovan *et al,* 1994; Hunault and Maatar, 1995; Kim *et al,* 1996; Sajina *et al,* 1997; Sastry *et al,* 1997).

Propagation through somatic embryogenesis and *in vitro* flowering and seed set in coriander was reported by Stephan and Jayabalan (2001). *In vitro* flowering and seed formation in cumin has been reported. Bertaccini *et al* (2004) used micropropagation for elimination of mite-brone virus and for establishment of virus-free garlic (*Allium sativum*).

Plant regeneration from anther and microspore cultures has been reported in fennel and celery.

Capsicum

Micropropagation and plant regeneration in chilli was reported using various explants (Agarwal 1988; Anu *et al,* 2004).

Development of haploid capsicum through androgenesis is reported. New approaches for induction of pollen embryogenesis in *Capsicum annuum* were reported by Gonzalez *et al* (1996) and Regner (1996). Occurrence of unreduced gametes and ploidy restoration in haploid peppers (*Capsicum annuum*) was reported.

Saffron

Reports are available on micropropagation and plant regeneration in saffron. *In vitro* proliferation of saffron stigma was also reported (Homes *et al*, 1987; Ilahi *et al*, 1987; Yang *et al,* 1996).

Field evaluation of tissue cultured plants

Black pepper and related species

Large-scale field evaluation of tissue cultured black pepper plants, in over 30 ha in all the pepper growing districts of Kerala, indicated that tissue cultured plants were superior to conventional propagules in field establishment, plant height, internodal length, number of laterals per unit area, number of spikes per unit area, fruit set, mean yield, dry weight, oil content, oleoresin content, etc. Preliminary field performance of micropropagated plantlets of *Piper longum, P. chaba* and *P. betle* indicated that these were on par with conventionally propagated plants (Nirmal Babu *et al,* 2003).

Cardamom

Large-scale field evaluation of tissue cultured plants of cardamom was carried out by the Spices Board of India and the IISR. Results showed that micropropagated plants performed on par with suckers.

Ginger and turmeric

Field evaluation of tissue cultured plants of ginger and turmeric indicate that micropropagated plants require at least two crop seasons to develop rhizomes of normal size that can be used as seed rhizomes for commercial cultivation. Tissue cultured plants of kasturi turmeric, mango ginger, *Kaempferia galanga,* etc. also show a similar pattern.

Salvi *et al* (2002) reported in turmeric that micropropagated plants showed significant increase in shoot length, number of tillers, number and length of leaves, number of gingers and total fresh rhizome weight per plant compared to conventionally propogated plants. Variations among regenerated plants have been reported in *Kaempferia galanga.* Anu *et al,*(2004) reported variation among somaclones and their seedling progeny in *Capsicum annuum.*

Estimation of genetic fidelity in micropropagated pepper using RAPDs

Genetic fidelity of micropropagated plants of black pepper was confirmed by Nirmal Babu *et al* (2003). RAPD (Random Amplification of Polymorphic DNA) profiling and

morphological characterization indicated that the micropropagation protocol can be used for commercial cloning of black pepper. Genetic uniformity of micropropagated *Piper longum* using RAPD profiling was reported by Ajith *et al* (1997) and Parani *et al* (1997).

In ginger, RAPD profiles did not show any polymorphism among micropropagated plants. However, Nirmal Babu *et al* (2003) reported RAPD profile differences in micropropagated ginger.

Salvi *et al* (2001) reported that RAPD analysis of regenerated plants in turmeric showed variation. Genetic stability and uniformity of *Foeniculum vulgare* Mill. plants regenerated through organogenesis and somatic embryogenesis was reported by Bennici *et al* (2004).

Somaclonal variation

Induction and utilization of somaclonal variation was attempted in many spices to develop genotypes resistant to biotic and a biotic stresses.

In black pepper, a few *Phytophthora* foot rot tolerant somaclones were identified through *in vitro* selection of calli using crude culture filtrate and toxic metabolites isolated from *Phytophthora capsici*.

Attempts to induce somaclonal variation in cardamom resulted in identification of a few Katte virus tolerant somaclones (Nirmal babu *et al,* 1997).

In ginger, field evaluation of somaclones indicated variability and resulted in identification of a few promising, high yielding lines with tolerance to rhizome rot (Nirmal Babu *et al*, 1996; Nirmal Babu, 1997). RAPD characterisation of these somaclones also showed profile variations indicating genetic differences Isolation of *Pythium*-tolerant ginger by using culture filtrate as the selecting agent has also been reported.

Variants with high curcumin content were isolated from tissue cultured plantlets of turmeric. Root rot disease tolerant clones of turmeric cv. Suguna were isolated using continuous *in vitro* selection technique against pure culture filtrate of *Pythium graminicolum* (Gayatri *et al*, 2005).

Variation in essential oil composition of plants regenerated from protoplasts of peppermint was reported. Reports are also available on *in vitro* selection for salt tolerance in fenugreek, *Trigonella foenum-graecum; in vitro* selection for resistance to *Alternaria* blight in cumin and drought tolerance in coriander through tissue culture has also been showed. Somaclonal variation and virus elimination for improvement of garlic has been reported. Ghosh *et al* (1997) reported generation of virus free pla by thermotherapy and meristem culture in garlic. MSU SHK 5, a somaclonally derived *Fusarium* yellows resista line in celery has been identified.

Microrhizomes

Microrhizomes form an important source disease-free planting material in rhizomatous crops li ginger and turmeric and are ideally suited for germpla exchange, transportation and conservation.

In vitro induction of microrhizomes in ging turmeric and *Kaempferia* is reported by many worke (Bhat *et al,* 1994; Nirmal Babu, 1997; Raghu Rajan, 199 Sunitibala *et al,* 2001; Nirmal Babu *et al,* 2003).

Microrhizome derived plants had more tillers b the plant height was smaller. They gave fresh rhizome yie ranging from 100- 800 g per plant with an estimated yie of 10 kg per 3m^2 bed. *In vitro* formed rhizomes were fou to be genetically more stable compared to micropropagat plants (Nirmal Babu *et al,* 2003).

Synthetic seeds

Artificial or synthetic seeds can be an ideal syste for low-cost plant movement, propagation, conservation a exchange of germplasm.

Synthetic seeds were developed by encapsulatio *in vitro* developed small shoot buds in 3% calcium algina in black pepper, cardamom, ginger, turmeric, camph cinnamon, celery, lavender and fennel. These synthe seeds could be stored from 7 to 10 months in sterile wa with over 80 % viability (Redenbaugh *et al,* 1986; Pra 1992; Sharma *et al*, 1994).

Protoplast culture

The protoplast is a naked cell and absence of cell wall makes a protoplast suitable for a variety manipulations that are not normally possible with int cells. Hence, protoplast is an important tool for parasexu modification of genetic content of cells.

Successful isolation and culture of protoplasts w reported in *P. nigrum* and *P. colubrinum* (Shaji *et al*, 199 Plant regeneration, however, was observed only in *colubrinum*. Protoplasts could be successfully isolated fro *in vitro* grown leaf mesophyll tissues of cardamom, ging and turmeric. These were cultured upto the microcalli sta (Nirmal Babu,1997; Geetha *et al*, 2000).

Isolation and fusion of protoplasts in vanilla reported. Isolation of protoplasts from leaves of nutm has been reported by Iyer *et al* (2000).

Successful isolation and culture of protoplasts was reported in fennel (Miura and Tabata, 1986), fenugreek (Sen and Gupta, 1979), peppermint and garlic (Ayabe *et al*, 1995) and saffron (Isa *et al*, 1990). Suh Sang Ki and Park (1995) reported protoplast fusion and culture in garlic. Successful production of interspecific hybrids between peppermint and gingermint was reported by Sato *et al* (1996).

Organogenesis and plant regeneration from isolated protoplasts have been demonstrated in chillies (Fari and Czako 1981; Agarwal, 1988; Prakash *et al,* 1997).

Genetic transformation

Preliminary reports are available on *Agrobacterium* - mediated gene transfer in *P. nigrum* (Sasikumar and Veluthambi, 1996). They obtained primary transformants for kanamycin resistance in cotyledons using *Agrobacterium tumefaciens* binary vector strains LBA 4404 and EHA 105. Sim *et al* (1998) reported *Agrobacterium*-mediated transfer of GUS gene to black pepper. Nirmal Babu *et al* (2005) reported *Agrobacterium* - mediated transformation of black pepper with the gene for osmotin, a PR (Pathogenesis related) protein known to induce resistance to *Phytophthora*.

Preliminary experiments to standardize optimum conditions for gene delivery and efficiency of the plasmid vector pAHC25 and promoter Ubi-1 and transformation of cardamom using biolistic process resulted in transient expression of GUS gene in bombarded callus tissue.

A few reports are available on *Agrobacterium*-mediated genetic transformation of capsicum (Liu *et al,* 1990; Shivegowda *et al,* 2002). Regeneration of transgenic pepper plants resistant to TMV and CMV has been reported.

Molecular characterization and development of mapping populations

In recent times, there is increased emphasis on using molecular markers for characterization of genotypes for genetic fingerprinting, to identify and clone important genes, for marker assisted selection and in understanding inter-relationships at the molecular level.

Black pepper

In black pepper, molecular markers like RAPD, AFLP and ISSR were used for assessment of genetic variability to characterize important cultivars, varieties, related species to develop fingerprints and to study inter relationships (Pradeep Kumar *et al,* 2001). A mapping population was developed for preparation of the genetic map in black pepper (Nirmal Babu *et al,* 2003). Male parent-specific RAPD markers were used by Johnson *et al* (2005) to identify hybrids.

Jaramillo and Manos (2001) used phylogenetic analysis of sequences of the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA based on a world wide sample of the genus *Piper.*

In long pepper (*Piper longum*), Banerjee *et al* (1999) reported male sex associated RAPD markers. Genetic diversity among landraces of a dioecious *Piper betle* using molecular markers was reported by Anjali *et al* (2004).

Cardamom

Molecular techniques like RAPD, RFLP and ISSR polymorphism were used to characterize cardamom germplasm collections comprising important cultivars, varieties and related genera to develop fingerprints and to study inter-relationships. The study indicated no duplicates in the 100 lines characterized and that the Kerala and Karnataka populations were divergent as they formed two separate clusters in the phylogram. RAPD and ISSR profiling of 11 species representing 5 major, related tribes of cardamom indicated that *Ammomum* is closest to the cultivated cardamom (Nirmal babu *et al,* 2005). A protocol for isolation and molecular characterization of DNA from market samples of cardamom was standardized and can be used to identify different grades of commercial cardamom and to identify adulttrents if any (IISR Annual Report, 2004).

Ginger

RAPD profiling of various ginger cultivars and related species is in progress at the Indian Institute of Spices Research to study the inter- relationships and to identify core collections in the germplasm. Ninetysix accessions of ginger were analysed and interrelationships studied. Polymorphism detected is moderate to low in ginger. RAPD profiling of ginger somaclones and selected 'variants' among micropropagated, callus regenerated and microrhizome derived plants indicated differences in RAPD profiles.

Phylogenetic analysis of the tribe *Zingibereae* (Zingiberaceae) was performed by Ngamriabsakul *et al* (2003) using nuclear ribosomal DNA (ITS1, 5.8S and ITS2) and chloroplast DNA. The study suggested that the tribe *Zingibereae* and the genus *Curcuma* are monophyletic. Kress *et al* (2002) studied phylogeny of the gingers (Zingiberaceae) using DNA sequences of the nuclear

internal transcribed spacer (ITS) and plastid *matK* regions and proposed a new classification of the Zingiberaceae.

Turmeric

Sasaki *et al* (2004) used single nucleotide polymorphism (SNP) analysis of the trnK gene to identify *Curcuma* plants.

Sasikumar *et al* (unpublished) studied over 96 Indian cultivars and related species of turmeric using RAPD profiling for establishing interrelationship. RAPD analyses showed good polymorphism among the 96 accessions studied. Five species of *Curcuma* were characterized using 12 primers. Intra species polymorphism in (*curcuma* was high compared to the interspecies polymorphism (IISR 2003, 2004).

An efficient protocol for isolation of high molecular weight DNA from dried. Powder samples of turmeric, including market samples, is described by Remya *et al* (2004). This will help in PCR-based detection of adulteration in marketed turmeric powder. Cao *et al* (2001) and Sasaki *et al* (2002) used sequence analysis of Chinese and Japanese *Curcuma* drugs on the 18S rRNA gene and trnK gene and the application of amplification-refractory mutation system analysis for authentication.

Vanilla

In the absence of classical phenotypic markers in perennial crops like vanilla, molecular markers such as RAPD and AFLP were used to establish genetic similarities and interrelationships in cultivars, seed progenies, somaclones and interspecific hybrids. Isoenzyme, RAPD and AFLP polymorphisms, supplemented by morphological characters, have been used to study the existing variability in cultivated vanilla, species interrelationships, identification of interspecific hybrids, and, fingerprinting of important genotypes. The study indicated limited variability among the cultivated collections of *V. planifolia* grown in India. *Vanilla tahitensis* was found to be closest to *V. planifolia.* Significant variations exist among selfed seed progenies of *V. planifolia.* This variation was further magnified when plant regeneration was through callus or when explants were grown in colchicine containing medium. Progeny obtained from crosses between *V. planifolia* and *V. aphylla* is truly hybrid, and thus, *in vitro* technology can be used for generation of variability in crop improvement (Minoo *et al*, 2006).

In tree spices, Shibu *et al* (2000) identified sex - specific DNA markers for identifying female trees in nutmeg. Yapwattanaphun *et al* (2004) used ITS sequen data to elucidate phylogenetic relationship in mangoste (*Garcinia mangostana*) and its wild relatives (*Garci* spp.). Molecular characterization and preparation molecular maps has been done in *Capsicum*. Arnedo-And *et al* (2002) developed RAPD and SCAR markers linked the Pvr4 locus for resistance to PVY in capsicum. Blum *al* (2002) reported mapping of the locus for pungency *Capsicum.* Kang *et al* (2001) developed interspeci (*Capsicum annuum* x *C. chinese*) F2 linkage map in pep using RFLP and RAPD markers. Caranta *et al* (19 developed CAPS marker for the Pvr 4 locus for pyramid potyvirus resistance genes in pepper.

Isolation of candidate genes

Work on isolation of genes responsible agronomically important characters, especially for bi and a biotic stresses, has also been attempted in spices.

In black pepper, programmes on isolation, clon of genes and validation is in progress and a few putati genomic and cDNA fragments associated with resista genes have been isolated (IISR 2004, 2005; Johnson *et* 2005). Molecular cloning of a cDNA fragment encod the defense related protein â-1,3-glucanase in black pep (*P. nigrum* L.) and methyl glutaryl CoA reductase in *Pi colubrinum* has been reported. Bhat *et al* (2005) repor isolation and sequencing of CMV coat protein gene w reference to black pepper.

Chen *et al* (2005) reported cDNA cloning characterization of a mannose-binding lectin from *Zingi officinale* Roscoe (ginger) rhizomes.

Molecular cloning of mannose-6-phosph reductase and its developmental expression in celery w studied by Everard *et al* (1997). Wang and Kumar (20 reported that heterologous expression of *Arabidopsis* ER causes delayed senescence in coriander.

Huh *et al* (2001) utilized the candidate ge approach to identify phytoene synthase as the locus mature fruit color in red pepper (*Capsicum* spp).

Tai and Staskawicz (2000) constructed ye artificial chromosome (YAC) library of hot pep (*Capsicum annuum* L.) and identified clones from the B resistance locus.

Bai *et al* (2004) reported successful cloning expression of *Crocus sativus* phytoene desaturase gene preparation of antiserum. Tsaftaris *et al* (2004) repor isolation of three homologous AP1-like MADS-box genes in *Crocus sativus* L. and characterized their expression.

Conservation of genetic resources

In vitro conservation

Genetic resources of most spices are conserved either in seed gene banks or in field repositories. Storage of germplasm in seed banks is not practical in some crops as these are vegetatively propagated and seeds are either recalcitrant or heterozygous. Conservation of germplasm in *in vitro* gene banks and cryobanks is a viable and safe alternative.

Conservation of pepper, cardamom, ginger, turmeric, vanilla, seed and herbal spice germplasm *in vitro* in gene banks by slow growth was reported (Dekkers *et al*, 1991; Nirmal Babu *et al*, 1996, 1997,1999).

Conserved material of all the species developed into normal plants without any deformities and was morphologically similar to the mother plants. RAPD profiling of the conserved plants too showed genetic integrity. Suspensions of embryogenic cell lines of fennel, conserved at 4 ^{0}C for upto 12 weeks, produced normal plants upon transfer to normal laboratory conditions (Umetsu *et al*, 1995). Conservation of genetic resources in *in vitro* gene banks is now an established convention and gene banks for conservation of spice germplasm functions at the IISR and at the National Bureau of Plant Genetic Resources, New Delhi. About 500 accessions of spice germplasm are currently conserved the *in vitro* repository of IISR.

Cryopreservation

Cryopreservation of black pepper and cardamom seeds in liquid nitrogen (LN_2) has been reported. Plants could be successfully regenerated from cryopreserved seeds of capsicum and anise, and, technologies for cryopreservation of black pepper, cardamom, ginger, turmeric and vanilla germplasm - using vitrification and encapsulation methods is available. Choudhary and Chandel, (1995); eported cryopreservation of vanilla pollen for conservation of the haploid genome and for assisted pollination between species that flower during different seasons and successful fertilization was effected using cryopreserved pollen.

Production of secondary metabolites

Use of biotechnology for biosynthesis of secondary metabolites particularly in plants of pharmaceutical significance holds an interesting alternative to conventional production of plant constituents.

In vitro proliferation of the stigma of saffron, *Crocus sativus* and chemical analysis of metabolites produced through tissue cultures has been reported by Himeno *et al* (1988); Koyama *et al* (1987). *In vitro* metabolite production from saffron tissue cultures has also been demonstrated by Venkataraman *et al* (1989) and Vishwanath *et al* (1990).

Production of flavour components and secondary metabolites *in vitro* using immobilised cells is an ideal system for spice crops. Production of saffron and capsaicin was reported using cell cultures (Johnson *et al,* 1996). Reports on *in vitro* synthesis of crocin, picrocrocin and saffranal from saffron stigma (Himeno and Sano, 1995) and colour components from cells derived from pistils (Hori *et al,* 1988) are available for further scale up. Johnson *et al* (1996) reported biotransformation of ferulic acid vanillamine to capsaicin and vanillin in immobilised cell cultures of *Capsicum frutescens.*

Callus and cell cultures have been established in nutmeg, clove, camphor, ginger, lavender, mint, thyme, celery, etc. Cell immobilization techniques have been standardized in ginger, sage, anise and lavender (Ilahi and Jabeen, 1992). Production of essential oils from cell cultures (Ernst, 1989) and accumulation of essential oils by *Agrobacterium tumefaciens* transformed shoot cultures of *Pimpinella anisum* has been reported (Salem and Charlwood, 1995). Regulation of the shikimate pathway in suspension culture cells of parsley (Conn and McCue, 1994) and production of anethole from cell cultures of *Foeniculum vulgare* (Hunault *et al*, 1989) is also reported. Growth of shoot cultures and production of monoterpene by transformed shoots of *Mentha citrata* and *Mentha piperita* in flasks and fermentors was reported. Chavez et al (1996) reported biosynthesis of the sesqiterpene phytoalexin capsidol in elicited root cultures of chilli pepper. Production of rosmarinic acid in suspension cultures of *Salvia officinalis* has been discussed by Hippolyte *et al* (1992). Phenyl propanoid metabolism in suspension cultures of *Vanilla planifolia* was studied by Funk and Brodelius (1990 a, b). Reports on production of phenolic flavour compounds using cultured cells and tissues of vanilla are also available (Dorenburg and Knorr, 1996). *In vitro* production of petroselinic acid was reported from cell suspension cultures of coriander (Kim *et al*, 1996). Kintzios *et al* (2004) reported scaling up of micropropagation in *Ocimum basilicum* L. in an airlift bioreactor and accumulation of rosmarinic acid thereof.

Though the feasibility of *in vitro* production of spice

principles has been demonstrated, methodology for scaling up and reproducibility needs to be developed before it can reach commercial levels. Once standardized, this technology can have tremendous potential in industrial production of important compounds like capsaicin, vanillin, crocin, picrocrocin, saffranal, myristicin, anethole, menthol and curcumin.

Micropropagation technology is available for rapid cloning of many spices. Technology for conservation of genetic resources in *in vitro* gene banks is another useful development. Molecular characterization of germplasm has made reasonable progress. Identifying markers for important agronomic characters will help in marker assisted selection to shorten breeding time. Application of recombinant DNA technology for production of transgenics resistant to biotic and abiotic stress has a long way to go in spices improvement. Although programmes have been initiated in many laboratories on *in vitro* secondary metabolite production, these techniques need to be refined and scaled up for possible industrial application. Considering commercial possibilities, intensification of biotechnological activities in spices is needed in the coming decades.

REFERENCES

Agarwal, S. 1988. Shoot tip culture of pepper and its micropropagation. *Curr. Sci.,* **57**:1347 - 1348.

Ahuja, A., Verma, M and Grewal S. 1982. Clonal propagation of *Ocimum* species through tissue culture. *Ind. J. Exptl. Biol.*, **20**:455-458.

Ajith, A., Parani, M., Rio, C.S., Latha, R. and Balakrishna, P. 1997. Micropropagation and genetic fidelity studies in *Piper longum* L. p. 94-97. In: Biotechnology of Spices, Medicinal & Aromatic Plants. Edison, S., K.V. Ramana, B. Sasikumar, K.N.Babu, S. J. Eapen. (eds.), Indian Society of Spices, Calicut, Kerala, India.

Ammirato, P.V. 1983. The regulation of somatic embryo development in plant cell cultures : Suspension culture techniques and hormone requirements. *Bio/Tech*, **1**:68-74.

Anjali,V., Nikhil, K and Ranade, S.A. 2004. Genetic diversity amongst landraces of a dioecious vegetatively propagated plant, betelvine (*Piper betle* L.). *J. Biosci.*, **29**:319-328.

Anu, A., Nirmal Babu, K and Peter, K.V. 2004. Variations among somaclones and its seedling progeny in *Capsicum annuum*. *Pl. Cell Tiss. Org. Cult.*, **76**:261-267.

Arnedo-Andres, M.S., Gil Ortega, R., Luis Arteaga,M a Hormaza, J.I. 2002. Development of RAPD a SCAR markers linked to the Pvr4 locus for resistan to PVY in pepper (*Capsicum annuum* L.). *The Appl. Genet.,* **105**:1067-1074.

Ayabe, M., Taniguchi, K and Sumi, S.I. 1995. Regenerati of whole plants from protoplasts isolated from tiss cultured shoot primordia of garlic (*Allium sativu* L.). *Pl. Cell Rep.,* **15**:17-21.

Bai. J., Miao, C., Xu, Y., Tang, L., Wang, Z. T & Chen, 2004. Cloning, expression of *Crocus sativus* phytoe desaturase gene and preparation of antiserum again it. Wuhan University *J. Natural Sci.*, **9**:252–258.

Banerjee, N.S., Manoj, P and Das, M.R. 1999. Male se associated RAPD markers in *Piper longum* L. *Cu Sci.*, **77**:693-695.

Barthakur, M.P and Bordoloi, D.N. 1992. Micropropagati of *Curcuma amada* (Roxb.). *J. Spices and Aroma Crops,* **1**:154-156.

Bennici, A., Anzidei, M & Vendramin, G.G. 2004. Gene stability and uniformity of *Foeniculum vulgare* Mi regenerated plants through organogenesis a somatic embryogenesis. *Pl. Sci.,* **166**:221–227.

Bertaccini, A., Botti, S., Tabanelli, D., Dradi, G., Fogh C., Previati, A., da Re, F., Nicola, S., Nowak, J a Vavrina, C.S. 2004. Micropropagation a establishment of mite-brone virus-free garlic (*Alliu sativum*). *Acta Hort.* **631**:201–206.

Bhat, A.I., Haresh, P.S and Madhubala, R 2005. Sequenci of coat protein gene of an isolate of Cucumber Mos Virus infecting black pepper in *Ind. J. Pl. Biochem Biotech.*, **14**:37- 40.

Bhat, S.R., Chandel, K.P.S and Malik, S.K. 1995. Pla regeneration from various explants of cultivat *Piper* species. *Pl. Cell Rep.,* **14**:398-402.

Bhat, S.R., Chandel, K.S.P and Kacker, A. 1994. *In vit* induction of rhizome in ginger *Zingiber officina* Rosc. *Ind. J. Exptl. Biol.,* **32**:340 - 344

Bhat, S.R., Kackar, A and Chandel, K.P.S. 1992. Pla regeneration from callus cultures of *Piper longu* L. by organogenesis. *Pl. Cell Rep.,* **11**:525-528.

Bhojwani, S.S. 1980. *In vitro* propagation of garlic by sho proliferation. *Sci. Hort.,* **13**:47-52.

Bhuyan, A.K., Pattnaik, S and Chand, P.K. 199 Micropropagation of curry leaf tree (*Murra koenigii* (L.) Spreng.) by axillary proliferation usi intact seedlings *Pl. Cell Rep.,* **16**:779- 782.

Blum, E., Liu, K., Mazourek, M., Yoo, E.Y., Jahn, M a Paran, I. 2002. Molecular mapping of the C loc

for presence of pungency in *Capsicum*. *Genome*, **45**:702-705.

Broome, O.C and Zimmerman, R.N. 1978. *In- vitro* propagation of black pepper. *Hortl. Sci.*, **43**:151-153.

Buyukalaca, S and Mavituna, F. 1996. Somatic embryogenesis and plant regeneration of pepper in liquid media. *Pl. Cell, Tiss. Org. Cult.,* **46**:227-237.

Cao, H., Sasaki, Y., Fushimi, H., Komatsu, K and Cao, H. 2001. Molecular analysis of medicinally used Chinese and Japanese *Curcuma* based on 18S rRNA and trnK gene sequences. *Bio. Pharmaceutical Bull.,* **24**:1389-1394.

Caranta, C., Thabuis, A and Palloix, A. 1999. Development of CAPS marker for the Pvr 4 locus: a tool for pyramiding potyvirus resistance genes in pepper. *Genome,* **42**:1111-1116.

Cellarova, E .1992. Micropropagation of *Mentha*. In *Biotechnology in Agriculture and Forestry*, Vol 19. High Tech and Micropropagation III (Ed. Y P S Bajaj) pp:262-275.

Cervera, E and Madrigal, R. 1981. *In vitro* propagation of *Vanilla (Vanilla planifolia* A.). *Environ. Experi. Botany,* **21**:441.

Chan, L.K and Thong, W.H. 2004. In vitro propagation of Zingiberaceae species with medicinal properties. *J. Pl. Biotech.* **6**:181-188.

Chand, S and Roy, S.C. 1981. Induction of organogenesis in callus cultures of *Nigella sativa* L. *Ann. Bot.*, **48**:1-4.

Chang, B.K.W and Criley, A. 1993. Clonal propagation of pink ginger *in vitro*. *Hortl. Sci.,* **28**:1203.

Chavez Moctezume, M.P and Lozoya Gloria, E. 1996. Bio synthesis of sesquturpenic phytoalexin capsidol in elicited root cultures of chilli pepper. *Pl. Cell Rep.*, **15**:360-367.

Chen, Z.H., Kai, G.Y., Liu, X.J., Lin, J., Sun, X.F and Tang, K.X. 2005. cDNA cloning and characterization of a mannose-binding lectin from Zingiber officinale Roscoe (ginger) rhizomes. *J. Biosci.*, **30**:213-220 .

Conn, E.E and McCue, K.F. 1994. Regulation of the shikimate pathway in suspension cultured cells of parsley (*Petroselinum crispum* L.) In *Advances in Plant Biotechnology* (Ryu D. D. Y. and Furusaki S. eds.) Elsevier Science, Netherlands pp : 95-102.

Davidonis, G and Knorr, D. 1991. Callus formation and shoot regeneration in *Vanilla planifolia*. *Food Biotech.,* **5**:59 66.

Dekkers, A.J., Rio, A.N and Ghosh, C.J. 1991. *In vitro* storage of multiple shoot culture of gingers at ambient temperatures of 24 29°C. *Sci. Hortic.,* **47**:157-167.

Donovan, A., Collin, H.A., Isaac, S and Mortimer, A.M. 1994. Analysis of potential sources of variation in tissue culture derived celery plants. *Ann. Appl. Bot.,* **124**:383-398.

Dornenburg, H and Knorr, D. 1996. Production of phenolic flavour compounds with cultured cells and tissues of vanilla species. *Food Biotech.,* **10**:75-92.

Ernst, D. 1989. *Pimpinella anisum* L. (Anise) : Cell culture, somatic embryogenesis and production of anise oil. In *Biotechnology in Agriculture and Forestry* Vol 7- Medicinal and Aromatic Crops II (Y P S Bajaj Ed.), Springer-Verlag Berlin pp:381-397.

Everard, J.D., Cantini, C., Grumet, R., Plummer, J and Loescher, W.H. 1997. Molecular clonning of mannose-6-phosphate reductase and its developmental expression in celery. *Pl. Physiol.,* **113**:1427.

Fari, M and Czako, M. 1981. Relationships between position and morphogenic responses of pepper hypocotyl explants cultured *in vitro*. *Scientia Horticulturae*. **15**:207.

Funk, C and Brodelius, P.F. 1990a. Phenyl propanoid metabolism in suspension cultures of *Vanilla planifolia* Andr. II. Effects of precursor feeding and metabolic inhibitors. *Plant Physio.,* **94**:95-101.

Funk, C and Brodelius, P.F. 1990b. Phenyl propanoid metabolism in suspension cultures of *Vanilla planifolia* Andr. III Conversion of 4-methoxycinnamic acids into 4 - hydroxybenzoic acids. *Plant Physio.*, **94**:102-108.

Furmanowa, M and Olszowska, O. 1992. Micropropagation of Thyme (*Thymus vulgaris* L.) pp : 230-242 In *Biotechnology in Agriculture and Forestry* Vol.19 High-Tech and Micropropagation III (Y P S Bajaj Ed) Springer Verlag, Heildelberg.

Gayatri, M.C., Roopa, D.V and Kavyashree, R. 2005. Selection of turmeric callus for tolerant to culture filtrate of *Pythium graminicolum* and regeneration of plants, *Pl. Cell, Tiss. Org. Cult.*, **83**:33-40.

Geetha, S and Shetty, S.A. 2000. *In vitro* propagation of *Vanilla planifolia*, a tropical orchid. *Curr. Sci.,* **79**:886-889.

George, P.S and Ravishankar, G.A. 1997. *In vitro* multiplication of *Vanilla planifolia* using axillary bud explants. *Pl. Cell Rep.*, **16**:490-494.

Ghosh, D.K., Ahlawat, Y.S and Dutta, G.M. 1997. Production of virus free garlic plants by thermotherapy and meristem culture. *Ind. J. Agril. Sci.,* **67**:591-594.

Gonzalez-Melendi, P., Testillano, P.S., Ahamadian, P.,

Fadon, B and Risueno, M.C. 1996. New *in situ* approaches to study the induction of pollen embryogenesis in *Capsicum annuum*. *European J. Cell Bio.*, **69**:373- 386.

Hazarika, B.N., Nagaraju, V and Parthasarathy, V.A. 1995. Micropropagation of *Murraya koenigii* Spreng. *Ann. Pl. Physiol.*, **9**:149-151.

Himeno, H and Sano, K. 1995. Synthesis of crocin, picrocrocin and safranal by saffron stigma like structures proliferated *in vitro*. *Agril. Bio. Chem.*,. **51**:2395-2400.

Hippolyte, I., Marin, B., Baccou, J.C and Jonard, R. 1992. Growth and rosmarinic acid production in cell suspension cultures of *Salvia officinalis* L. *Pl. Cell Rep.*, **11**:109-112.

Homes, J., Legros, M and Laziul. 1987. *In vitro* multiplication of *Crocus sativus* L *Acta Hort.*. **212**:675-676

Hori, H., Enomoto, K and Nakaya, H. 1988. Induction of callus from pistils of *Crocus sativus* L. and production of colour components in the callus. *Pl. Tiss. Cult. Lett.*, **5**:72 -77.

Huang, L.C., Huang, B.L and Murashige, T. 1998. A micropropagation protocol for *Cinnamomum camphora*. *In Vitro Cell. Dev. Biol.- Plant* **34**:141-146.

Huh, J.H., Kang, B.C., Nahm, S.H., Kim, S., Lee, M.H and Kim, B.D. 2001. A candidate gene approach identified phytoene synthase as the locus for mature fruit color in red pepper (*Capsicum* spp.). *Theor. Appl. Genet.*, **102**:524-530.

Hunault, C., Desmarest, P and Du Manoir, J. 1989. *Foeniculum vulgare* Miller: Cell cultures, regeneration and the production of anethole. pp. 185 – 209. In *Biotechnology and Forestry* Vol 7. Medicinal and Aromatic Plants II (Y. P. S. Bajaj ed) Springer-Verlag, Heidelberg..

Hunault, G and Du Manoir, J. 1992. Micropropagation of fennel. pp : 199-216. In: *Biotechnology in Agriculture and Forestry* Vol.19 High-Tech and Micropropagation III (Y P S Bajaj Ed). Springer-Verlag, Heidelberg.

Hunault, G and Maatar, A. 1995. Enhancement of somatic embryogenesis frequency by gibberellic acid in fennel. *Pl. Cell Tiss. Org. Cult.*, **41**:171 -176

IISR. 2003. Annual Report 2002-2003, Indian Institute of Spices Research, Calicut

IISR. 2004. Annual Report 2003-2004, Indian Institute of Spices Research, Calicut

IISR. 2005. Annual Report 2004-2005, Indian Institute of Spices Research, Calicut

Ilahi, I. and Jabeen, M. 1992. Tissue culture studies f micropropagation and extraction of essential oi from *Zingiber officinale* Rosc. *Pak. J. Bo* **24**:54-59.

Ilahi, I., Jabeen, M and Firdous, N.C. 1987. Morphogene with saffron tissue culture. *J. Pl. Physiol.*, **128**:22 223.

Isa, T., Ogasawara, T and Kaneko, H. 1990. Regenerati of saffron protoplasts immobilised in Ca - Algina beads. *Jap. J. Breed.*, **40**:153-159.

Iyer, I.R., Jayaraman, C and Gopinath, P.M. 2000. Isolati of protoplasts from leaves of nutmeg *Myristi fragrans* Houtt. *Biomedicine*, **20**:200-202.

Iyer, I.R., Jayaraman, C., Gopinath, P.M and Sita, G.L. 200 Direct somatic embryogenesis in zygotic embryos nutmeg (*Myristica fragrans* Houtt.) *Trop. Agr* **77**:98-105.

Iyer, P.V and Pai, J.S. 1998. Micropropagation of swe marjoram (*Marjorana hortensis* Moench) *J. Spic and Aromatic Crops,* **7**:47-49.

Jaramillo, M.A and Manos, P.S. 2001. Phylogeny a patterns of floral diversity in the genus *Pip* (Piperaceae). *Amer. J. Bot.*, **88**:706-716.

Jha, T.B., Roy, C and Mitra, G.C. 1982. *In vitro* culture *Cuminum cyminum*: regeneration of flowering shoo from calli of hypocotyl and leaf explants. *Pl. C Tiss. Org. Cult.,* **2** :11-14.

Johnson, G.K., Ganga, G., Sandeep Varma, R., Sasikum B and Saji, K.V. 2005. Identification of hybrids black pepper (*Piper nigrum* L.) using male pare specific RAPD markers. *Curr. Sci.*, **88**:1-2.

Johnson, T.S., Ravishanker, G.A and Venkataraman, L 1996. Biotransformation of ferulic acid vanillami to capsacin and vanillin in immobilised cell cultur of *Capsicum frutescens*. *Pl. Cell. Tiss. Org. Cul* **44**:117-123.

Johri, J.K., Aminuddin and Aruna, P. 1996. Regenerati of betelvine (*Piper betle* L.) through somat embryogenesis. *Ind. J. Exptl. Biol.*, **34**:83-85.

Kado, C.I. 1991. Molecular mechanisms of crown g tumorigenesis. *Critical Rev. Pl. Sci.*, **10**:1-33.

Kang, B.C., Nahm, S.H., Huh, J.H., Yoo, H.S., Yu, J.W Lee, M.H and Kim, B.D. 2001. An interspecif (*Capsicum annuum* L. x *C.chinese*) F2 linkage m in pepper using RFLP and RAPD markers. *Theo Appl. Genet.,* **102**:531-539.

Kataeva, N.V and Popowich, E.A. 1993. Maturation a rejuvenation of *Coriandrum sativum* shoot clon during micropropagation. *Plant Cell Tissue & Org Culture*, **34**:141-148.

Kim, S.W., Park, M.K and Liu, J.R. 1996b. High frequency plant regeneration via somatic embryogenesis in cell suspension cultures of coriander. *Pl. Cell Rep.*, **15**:751-754.

Kintzios, S., Kollias, H., Straitouris, E and Makri. 2004. Scale-up micropropagation of sweet basil (*Ocimum basilicum* L.) in an airlift bioreactor and accumulation of rosmarinic acid. *Biotech. Lett.*, **26**:521-523.

Kononowicz, H and Janick, J. 1984. *In vitro* propagation of *Vanilla planifolia*. *HortScience*. **19**:58-59.

Koyama, A., Ohmori, Y., Fujioka, H., Miyagawa, H., Yamasaki, K and Kondaa, H. 1987. Formation of stigma like structures and pigments in cultured tissues of *Crocus sativus*. *Jap. J. Pharmacog*. **41**:226-229.

Kress, W.J., Prince, L.M. and Williams, K.J. (2002) The phylogeny and a new classification of the gingers (Zingiberaceae): evidence from molecular data. *Amer. J. Bot.,* **89**:1682-1696.

Lakshmi, M and Mythili, S. 2003. Somatic embryogenesis and plant regeneration from callus cultures of *Kaempferia galanga* - a medicinal plant. *J. Medicinal Aromatic Pl. Sci.*, **25**:947-951

Lissamma Joseph., Nazeem, P.A., Mini, S.T., Shaji Philip and Mini Balachandran. 1996. *In vitro* techniques for mass multiplication of black pepper (*Piper nigrum* L.) and *ex vitro* performance of the plantlets. *J. Plantation Crops* (Supplt) **24**:511-516.

Liu, W., Parrot, W.A., Hildebrand, D., Collins, G.B and Williams, E.G. 1990. *Agrobacterium* induced gall formation in bell pepper (*C.annuum* L.) and formation of shoot like structures expressing induced genes. *Pl. Cell Rep.*, **9**:360-364.

Madhusudhanan, K and Rahiman, B. A. 1997. *In vitro* response of *Piper* species on activated charcoal supplemented media. p.16-19. In: *Biotechnology of Spices, Medicinal and Aromatic Crops,* Edison, S. Ramana, K. V., Sasikumar, B., Nirmal Babu, K. and Santhosh J. E. (eds.), Indian Society for Spices.

Mallika, V.K., Rekha, K., Marymol, M., Manjula, M and Vikraman Nair, R. 1997. *In vitro* shoot initiation from explants of field grown trees of nutmeg (*Myristica fragrans* Houtt.). p.29-34. In: *Biotechnology of Spices, Medicinal and Aromatic Crops*. Edison S, Ramana K V, Sasikumar B, Nirmal Babu K and Santhosh J E (eds.), Indian Society for Spices.

Mary, M., Rio, Y,S., Kuruvilla, K.M., Lakshmanan, R., Gilu, L.G., Madhusoodanan, K.J and Potty, S.N. 2000. Multiple shoot regeneration in kokum and camboge. *J. Spices and Aromatic Crops*, **9**:151-152.

Mary, M., Rio, Y.S., Gilu, L.G., Lakshmanan, R and Madhusoodanan, K.J. 2000a. *In vitro* propagation of *Vanilla tahitensis* Moore. *J. Spices and Aromatic Crops,* **9**:171-173.

Mascarenhas, A.F., Nair, S., Kulkarni, V.M., Agrawal, D.C., Khuspe, S.S and Mehta, U.J. 1987. Tamarind. p. 316-330. In: Cell and Tissue Culture in Forestry, Vol.3 J M Bonga & D J Durzan, (eds.) Martinus Nijhoff, Dordecht.

Mathew, M.K and Hariharan, M. 1990. *In vitro* multiple shoot formation in *Syzygium aromaticum*. *Ann. Bot.* **65**:277 279.

Mehta, U.J., Krishnamurthy, K.V and Hazra, S. 2000. Regeneration of plants *via* adventitious bud formation from mature zygotic embryo axis of tamarind (*Tamarindus indica* L.). *Curr. Sci.,* **78**:1231-1234.

Mini, P.M., John, C.Z., Samsudeen, K., Rema, J., Nirmal Babu, K and Ravindran, P.N. 1997. Micropropagation of *Cinnamomum verum* (Bercht & Presl), p.35-38. In. *Biotechnology of Spices, Medicinal and Aromatic Crops*. Edison S, Ramana K V, Sasikumar B, Nirmal Babu K and Santhosh J E (eds.), Indian Society for Spices.

Minoo, D., Sajina, A., Nirmal Babu, K and Ravindran, P.N.1997. Ovule culture of vanilla and its potential in crop improvement. , p.112-118. In: *Biotechnology of Spices, Medicinal and Aromatic Plants*, Edison, S., Ramana, K.V., Sasikumar, B., Nirmal Babu, K. and Santhosh, J. Eapen (eds.). Indian Society for Spices, Calicut, India.

Miura, Y and Tabata, M. 1986. Direct somatic embryogenesis from protoplasts of *Foeniculum vulgare*. *Pl. Cell Rep.,* **5**:310 -313.

Miura, Y., Fukui, H and Tabata, M. 1987. Clonal propagation of chemically uniform fennel plants through somatic embryoids. *Planta Medica*, **53**:92-94.

Nadgauda, R.S., Mascarenhas, A.F., Hendre, R.R and Jagannathan, V. 1978. Rapid clonal multiplication of turmeric *Curcuma longa* L. plants by tissue culture. *Ind. J. Exptl. Biol.*, **16**:120 122 .

Nair, R.R and Gupta, S.D. 2003. Somatic embryogenesis and plant regeneration in black pepper (*Piper nigrum* L.): Direct somatic embryogenesis from tissues of germinating seeds and ontogeny of somatic embryos. *J. Hort. Sci. and Biotech.,* **78**:416-421.

Neena Kumari and Saradhi, P.P. 1992. Regeneration of plants from callus cultures of *Oreganum vulgare* L. *Pl. Cell Rep.,* **11**:476-479.

Ngamriabsakul, C., Newman, M.F. and Cronk, Q.C.B.

(2003) The phylogeny of tribe *Zingibereae* (*Zingiberaceae*) based on its (nrDNA) and *trn*l–f (cpDNA) sequences. *Edinburgh J. Bot.*, **60**:483-507.

Nirmal Babu, K., Anu, A., Remasree, A.B and Praveen, K. 2000. Micropropagation of curry leaf tree *Murraya koenigii* L. (Spreng). *Pl. Cell Tiss. Org. Cult.*, **61**:199-203.

Nirmal Babu, K., Geetha, S.P., Minoo, D., Ravindran, P.N and Peter, K.V. 1999. *In vitro* conservation of germplasm. pp :106-129, In: *Biotechnology and its application in Horticulture*. S P Ghosh (ed). Narosa Publishing House, New Delhi.

Nirmal Babu, K., Minoo, D., Geetha, S.P., Samsudeen, K., Rema, J., Ravindran, P.N and Peter, K.V. 1998. Plant biotechnology - it's role in improvement of spices. *Indian J. Agril. Sci.*, **68** (Special Issue.):533-547.

Nirmal Babu, K., Ravindran, P.N and Peter, K.V. 1997. Protocols for micropropagation of spices and aromatic crops. Indian Institute of Spices Research, Calicut, Kerala.

Nirmal Babu, K., Rema, J., Sree Ranjini, D.P., Samsudeen, K and Ravindran, P.N. 1996b. Micropropagation of an endangered species of Piper, *P. barberi* Gamble and its conservation. *J. Pl. Genet. Resources* **9**:179-182.

Nirmal Babu, K., Sajina, A., Minoo, D., John, C.Z., Mini, P.M., Rema, J and Ravindran, P.N. 2003. Micropropagation of camphor tree *Cinnamomum camphora* (Presl.). *Pl. Cell Tiss. Org. Cult.*, **74**:179-183.

Nirmal Babu, K.,. George, J.K., Anandaraj, M., Venugopal, M.N., Nair, R.R 2005. Improvement of selected spices through Biotechnology tools – Black pepper, Cardfamom, Ginger, Vanilla. Final Report, Department of Biotechnology, Government of India. pp. 111.

Nirmal Babu, K. 1997. *In vitro* studies in *Zingiber officinale* Rosc. Ph.D Thesis. Calicut University, Kerala, India.

Okamoto, A., Sakurazawa, H and Arikawa, K. 1994. Regeneration of plantlets from celery (*Apium graveolens* L.) callus using a fermentor. *J. Fermentation and Bioengineering* **77**:208 -211.

Onisei, T., Toth, E.T and Amariei, D. 1994. Somatic embryogenesis in lavender tissue culture I. Isolation and characteristics of an embryogenic callus line. *J. Herbs, Spices and Medicinal Plants* **2**:17-29.

Panizza, M and Tognoni, F. 1992. Micropropagation of lavender (*Lavandula officinalis* Chaix X *Lavandula latifolia* villars cv. Grosso). pp : 295-305. In: *Biotechnology in Agriculture and Forestry.* Vol.19. High-Tech and Micropropagation III. Y. P. S. Baj (ed.) Springer-Verlag, Heidelberg.

Parani, M., Anand, A and Parida, A. 1997. Application RAPD finger printing in selection micropropagated plants of *Piper longum* f conservation . *Curr. Sci.*, **73**:81-83.

Patnaik, S and Chand, P.K. 1996. *In vitro* propagation medicinal herbs *Ocimum americanum* L. syn. *canum* Sims (hoary basil) and *Ocimum santum* (holy basil). *Pl. Cell Rep.*, **15**:846-851.

Pradeep Kumar, T., Karihaloo, J.L and Archak. S. 200 Molecular characterization of *Piper nigrum* cultiva using RAPD markers. *Curr. Sci.*, **8**:246-248.

Prakash, A.H., Rio, K.S and Kumar, M.U. 1997. Pla regeneration from protoplasts of *Capsicum* L. California Wonder. *J. Biosci.*, **22**:339-344.

Prakash, S., Elangomathavan, R., Seshadri, S., Kathirav and Ignacimuthu, S. 2004. Efficient regeneration *Curcuma amada* Roxb. plantlets from rhizome a leaf sheath explants. *Pl. Cell Tiss. Org. Cult.*, **78**:15 165.

Pratap, K.P. (1992) Artificial Seeds. *Vatika*. **1**:27-30.

Prathanturarug, S., Soonthornchareonnon, N., Chuakul, W Phaidee, Y. and Sarakamp, P. 2005. Rap micropropagation of *Curcuma longa* using b explants pre-cultured in thidiazuron-supplement liquid medium. *Pl. Cell Tiss. Org. Cult.*, **80**:347-35

Praveen, K. 2005. Variability in Somaclones of Turme (*Curcuma longa* L.)Ph.D Thesis. Calicut Universi Kerala, India.

Rahman, M.M., Amin, M.N., Jahan, H.S and Ahmed, 2004. *In vitro* regeneration of plantlets of *Curcu longa* Linn: a valuable spice plant in Banglade *Asian J. Pl. Sci.*, **3**:306–309.

Rao, S.R and Ravishankar, G.A. 2000. Vanilla flavo production by conventional and biotechnologi routes. *J. Sci. Food Agri.*, **80**:289-304.

Ratnamba, S.P and Chopra, R.N. 1974. *In vitro* inducti of embryoids from hypocotyls and cotyledons *Anethum graveolens* seedlings. *Z Pflanzenphysi* **73**:452-455.

Redenbaugh, K., Brian, D.P., James, W., Mary, E., Peter and Keith, A.W. 1986. Somatic seeds : Encapsulati of asexual plant embryos. *Biotech.*, **4**:9-83.

Regner, F. 1996. Anther and microspore culture in capsicu In: VitroHaploid Production in Higher Plants. pp. 7 89. Jain S M, Sopory S K and Veilleux R E (ed Vol. 3. Kluwer Academic Publishers, T Netherlands.

Remya, R., Syamkumar, S. and Sasikumar, B. 2004 Isolati and amplification of DNA from turmeric powder . *British Food J.*, **106**:673-678.

Renjith, D., Valsala, P.A. and Nybe, E.V. 2001. Response of turmeric (*Curcuma domestica* Val.) to *in vivo* and *in vitro* pollination. *J. Spices and Aromatic Crops*, **10**:135-139.

Rout, G.R., Palai, S.K., Samantaray, S and Das, P. 2001. Effect of growth regulator and culture conditions on shoot multiplcation and rhizome formation in ginger (*Zingiber officinale* Rosc.) *in vitro*. *In Vitro Cell. Devtl. Biol. - Plant,* **37**:814-819.

Sajina, A., Mini, P.M., John, C.Z., Nirmal Babu, K., Ravindran, P.N and Peter, K.V. 1997. A note on micropropagation of large cardamom. *J. Spices and Aromatic Crops* **6**:145-148

Salem, K.M.S.A and Charlwood, B.V. 1995. Accumulation of essential oils by *Agrobacterium tumefaciens* transformed shoot cultures of *Pimpinella anisum*. *Pl. Cell Tiss. Org. Cult.,* **40**:209-215.

Salvi, N.D., George, L. and Eapen S. 2001 Plant regeneration from leaf base callus of turmeric and random amplification polymorhic DNA analysis of regenerated plants. *Pl. Cell Tiss. Org. Cult.,* **66**:113-119.

Salvi, N.D., George, L. and Eapen, S. 2002 Micropropagation and field evaluation of micropropagated plants of turmeric. *Pl. Cell Tiss. Org. Cult.*, **68**:143-151.

Sano, K and Himeno, H. 1987. *In vitro* proliferation of saffron (*Crocus sativus* L.) stigma. *Pl. Cell Tiss. Org. Cult.,* **11**:159-166.

Sarasan, V., Elizabeth, T., Beena, L and Nair, G.M. 1993. Plant regeneration in *Piper longum* L. (Piperaceae) through direct and indirect adventitious shoot development. *J. Spices and Aromatic Crops* **2**:34 40.

Sasaki, Y., Fushimi, H and Komatsu, K. 2004. Application of single-nucleotide polymorphism analysis of the trnK gene to the identification of Curcuma plants. *Biological and Pharmaceutical Bulletin* **27**:144-146.

Sasaki, Y., Fushimi, H., Cao, H., Cai, S.Q. and Komatsu, K. 2002 Sequence analysis of Chinese and Japanese Curcuma drugs on the 18S rRNA gene and trnK gene and the application of amplification-refractory mutation system analysis for their authentication. *Biol. Pharm. Bull.,* **25**:1593-9.

Sasikumar, B and Veluthambi, K. 1996b. Transformation of black pepper (*Piper nigrum* L.) using *Agrobacterium* Ti plasmid based vectors. *Ind. Perfumer,* **40**:13-16.

Sastry, E.V.D., Sanjeev, A.,Rajendra, K and Sharma, R.K .1997. *In vitro* responses of fennel (*Foeniculum vulgare* Mill.), p. 49-50. In: *Biotechnology of Spices, Medicinal and Aromatic Crops*. Edison S, Ramana K V, Sasikumar B, Nirmal Babu, K. and Santhosh, J.(eds.) Indian Society for Spices.

Sato, H., Yamada, K., Mii, M., Hosomi, K., Okuyama, S., Uzawa, M., Ishikawa, H and Ito, Y. 1996. Production of an interspecific somatic hybrid between peppermint and gingermint. *Pl. Sci.,* (Limerick) **115**:101-107.

Sehgal, C.B. 1978. Differentiation of shoot buds and embryoids from inflorescence of *Anethum graveolens* in cultures. *Phytomor.*, **28**:291-297.

Sen, B and Gupta, S. 1979. Differentiation in callus culture of leaf of two species of *Trigonella. Physiologia Plantarum* **45**:425-428.

Shaji, P., Anandaraj, M and Sharma, Y. R. 1998. Comparative study of protoplast isolation and development in *Piper nigrum* (black pepper) and *P. colubrinum*. pp. 51-53. In: Developments in plantation crops research, NM Mathew and CK Jacob(eds.), Allied Publishers, New Delhi.

Sharma,T.R., Singh, B.M and Chauhan, R.S. 1994. Production of encapsulated buds of *Zingiber officinale* Rosc. *Pl. Cell Rep.,* **13**:300-302.

Shibu, M.P., Ravishankar, K.V., Lalitha, A., Ganeshaiah, K.N and Shanker, R.U. 2000. Identification of sex specific DNA markers in the dioecious tree, nutmeg (*Myristica fragrans* Houtt.). *Pl. Genet. Resources Newslett.*, **121**:59-61.

Shivegowda, S.T., Mythili, J.B., Saiprasad, G.V.S., Gowda, T.K.S., Anand, L and Gowda, R. 2002. *In vitro* regeneration and transformation in chilli pepper (*Capcisum annuum* L.). *J. Hortl. Sci. Biotech.*, **77**:629-634.

Sim, S.L., Jafar, R., Power, J.B and Davey, M.R. 1998. Development of an *Agrobacterium*-mediated transformation system for black pepper (*Piper nigrum* L.). *Acta Hort*., **461**:349-354.

Stephan, R and Jayabalan, N. 2001. Propagation of *Coriandrum sativum* L. through somatic embryogenesis. *Ind. J. Exptl. Biol.,* **39**:387-389.

Suh Sang Ki and Park, H. 1995. Protoplast isolation, fusion and culture of garlic (*Allium sativum* L.). *J. Korean Soc. Hort. Sci.*, **36**:614-619.

Sunitibala, H., Damayanti, M and Sharma, G.J. 2001. *In vitro* propagation and rhizome formation in *Curcuma longa* Linn. *Cytobios*, **105**:71-82.

Tai, T and Staskawicz, B.J. 2000. Construction of yeast

artificial chromosome library of pepper (*Capsicum annuum* L.). and identification of clones from the Bs2 resistance locus. *Theor. Appl. Genet.*, **100**:112-117.

Toth, K.F and Lacy, M.L. 1992. Micropropagation of celery (*Apium graveolens* var. dulce). pp:218-228. In: *Biotechnology in Agriculture and Forestry* Vol.19 High-Tech and Micropropagation III.Y. P. S.Bajaj(ed.) Springer - Verlag, Heidelberg.

Tsaftaris, A.S., Pasenti, K., Iliopoulos, I and Polidoros, A.N. 2004. Isolation of three homologous AP1-like MADS-box genes in Crocus (Crocus sativus L.). and characterization of their expression. *Pl. Sci.*, **166**:1235–1243.

Umetsu, H., Wake, H., Saitoh, M., Yamaguchi, H and Shimomura, K. 1995. Characteristics of cold preserved embryogenic suspension cells in fennel *Foeniculum vulgare* Miller. *J. Plant Physiol.*, **146**:337-342.

Valsala, P.A., Nair, S.G and Nazeem, P.A. 1997. *In vitro* seed set and seed development in ginger, *Zingiber officinale* Rosc. p. 106-108. In: *Biotechnology of Spices, Medicinal and Aromatic Plants*, Edison, S., Ramana, K.V., Sasikumar, B., Nirmal Babu K and Santhosh J. Eapen (eds.). Indian Society for Spices, Calicut, India,

Van Eck, J.M and Kitto, S.L. 1990. Callus initiation and regeneration in *Mentha*. *HortScience*. **25**:804-806.

Van Eck, J.M and Kitto, S.L. 1992. Regeneration of peppermint and orangemint from leaf disc. *Pl. Cell Tiss. Org. Cult.*, **30**:41-49.

Vandemoortele, J.L., Billard, J.P., Boucaud, J and Gaspar, T. 1996. Micropropagation of parsley through axillary shoot proliferation . *Pl. Cell Tiss. Org. C* **44**:25-31.

Vatsya, B., Dinesh, K., Kundapurkar, A.R and Bhaska S. 1987. Large scale plant formation of cardam (*Elettaria cardamomum*) by shoot bud cultures. *Physiol. Biochem.*, **14**:14 -19.

Venkataraman, L.V., Ravishanker, G.A., Sarma, K.S Rajasekaran, T. 1989. *In vitro* metabolite product from saffron and capsicum by plant tissue and cultures. p. 147-151. In: *Tissue Culture Biotechnology of Medicinal and Aromatic pla* Kukreja *et al* (eds.), CIMAP, Lucknow, India.

Vishwanath, S., Ravishankar, G.A and Venkataraman, 1990. Induction of crocin, crocetin, picrocrocin safranal synthesis in callus cultures of saffr *Crocus sativus* L. *Biotech. Appl. Biochem.*, **12**:3 340.

Wang, Y and Kumar, P.P. 2004. Heterologous expres of Arabidopsis ERS1 causes delayed senescenc coriander. *Pl. Cell Rep.*, **22**:678-683.

Yapwattanaphun, C., Subhadrabandhu, S., Honsho, Yonemori, K., Chinawat, Y and Suranant, S. 20 Phylogenetic relationship of mangosteen (*Garc mangostana*) and several wild relatives (*Garc* spp.) revealed by ITS sequence data. *J. Amer. Hortl. Sci.*, **129**:368-373.

Yasuda, K., Tsuda, T., Shimiju, H and Sugaya, A. 19 Multiplication of *Curcuma* sp. by tissue cult *Planta Medica* **54**:75-79.

Zhang, B and Stoltz, L.P. 1981. *In vitro* shoot forma and elongation of dwarf pomegrante. *Hort.* **26**:1084.