Biotechnological Approaches in Improvement of Spices: A Review

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Abstract

Biotechnological approaches like micropropagation, somaclonal variation, *in vitro* conservation, synseed technology, protoplast fusion, production of flavour and colouring components and development of novel transgenics have great potential in conservation, utilization and increasing the production of spices. Efficient micropropagation systems are available for many spices which are being used for propagation, conservation, safe movement and exchange of germplasm, crop improvement through somaclonal variation and transgenic pathways. Studies on the production of metabolites, flavour and colouring compounds using immobilized and transformed cell cultures are being attempted. Molecular markers and maps are being generated for crop profiling, fingerprinting, identification of duplicates, and marker-assisted breeding. Transcriptome sequencing is becoming an important tool for identification, isolation and cloning of useful genes. Bio-technological approaches involving microbials (antogonists/hyper parasites and PGPRs) with broad spectrum of disease suppression and growth promotion have been found effective in crop health management in spice crops.

Keywords

Fingerprinting • Ginger • *In vitro* conservation • Micropropagation • Molecular markers • Somaclonal variation • Spices • Synseed technology • Transgenics • Tree spices • Turmeric • Vanilla

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25.1 Introduction

Spices and herbs are aromatic plants – fresh or dried plant parts of which are mainly used to flavour our food and confectionery and also in medicine and perfumery industry. Spices and herbs are grown throughout the world, and it has been estimated that these crops are grown on an area of 8 million ha globally contributing to 31.6 million tons of spices annually. India's share to world spices production is 6 million tons. The global spice industry amounts to 1.1 million metric tons, accounting to US$3.475 billion in value. India's share at the global level is 0.575 million metric tons, accounting to US$2.037 billion, i.e., 52 % in volume and 58.6 % in value (Source: Spices Board of India 2014).

Black pepper, cardamom, ginger, turmeric, vanilla, cinnamon, clove, nutmeg, tamarind, etc. constitute the major spices, while coriander, cumin, fennel and fenugreek are important seed spices followed by saffron, lavender, thyme, oregano, celery, anise and sage are important herbal spices. The productivity of many of these crops is low due to the lack of high-yielding, pest and disease-resistant varieties. The past few years have witnessed a quantum jump in utilization of biotechnological tools to achieve the above through commercial propagation, development of novel varieties and marker-assisted breeding (Nirmal Babu et al. 2011d).

25.2 Black Pepper

25.2.1 Micropropagation and Plant Regeneration

Micropropagation has been employed for large-scale production of disease-free planting materials and germplasm conservation. High rate of multiplication coupled with the additional advantage of obtaining disease-free planting material makes micropropagation a viable alternative to conventional propagation (Nirmal Babu and Minoo 2003). Black pepper, *Piper nigrum* L., is native to India and is the most important spice in the world. Conserving the genetic diversity and development of *Phytophthora* foot rot resistance are the immediate priorities for all breeding programmes. Technologies for micropropagation of black pepper using various explants were reported (Nirmal Babu 1997; Nirmal Babu et al. 2012b). Multiple shoots can be induced using BA in the culture medium (MS or SH Medium) either alone or in combination with auxins (Fig. 25.1a). Endogenous contamination severely hampers establishment of black pepper cultures to over come this constrains. A commercially viable protocol for large-scale *in vitro* multiplication of black pepper these problems was reported by Nazeem et al. (2004). Protocols were standardized for micropropagation of other endangered and medicinally important species of *Piper* like *P. longum* and *P. chaba*, *P. betle*, *P. barberi* and *P. colubrinum* (Nirmal Babu 1997; Nirmal Babu et al. 2012b). Joseph et al. (1996) and Yamuna (2007) reported the somatic embryogenesis from zygotic embryos, while Nair and Gupta (2003, 2006) reported the cyclic somatic embryogenesis from the maternal tissues, which have tremendous potential for automated micropropagation. Nirmal Babu et al. (2005a) reported the somatic embryogenesis from mature leaf tissues (Fig. 25.1b, c). These protocols are useful in transgenic experiments. Plant regeneration was reported in other *Piper* species like *P. longum*, *P. betle, P. chaba, P. attenuatum* and *P. colubrinum* through direct and indirect organogenesis (Nirmal Babu et al. 2012b). Attempts on induction of variability on somaclones for tolerance to *Phytophthora* foot rot resistance by Shylala et al. (1996) resulted in identification of tolerant somaclones through *in vitro* selection of calli as well as somaclones using crude culture filtrate and toxic metabolite isolated from *Phytophthora capsici.*

25.2.2 Molecular Characterization

Recent advances in molecular biology led to more emphasis on molecular markers for characterization of the genotypes, genetic fingerprinting, identification and cloning of important genes and marker-assisted selection and in understanding interrelationships at the molecular level. Menezes et al. (2009) and Joy et al. (2007, 2011) developed and characterized a total of 16 microsatellite markers from black pepper and used them to

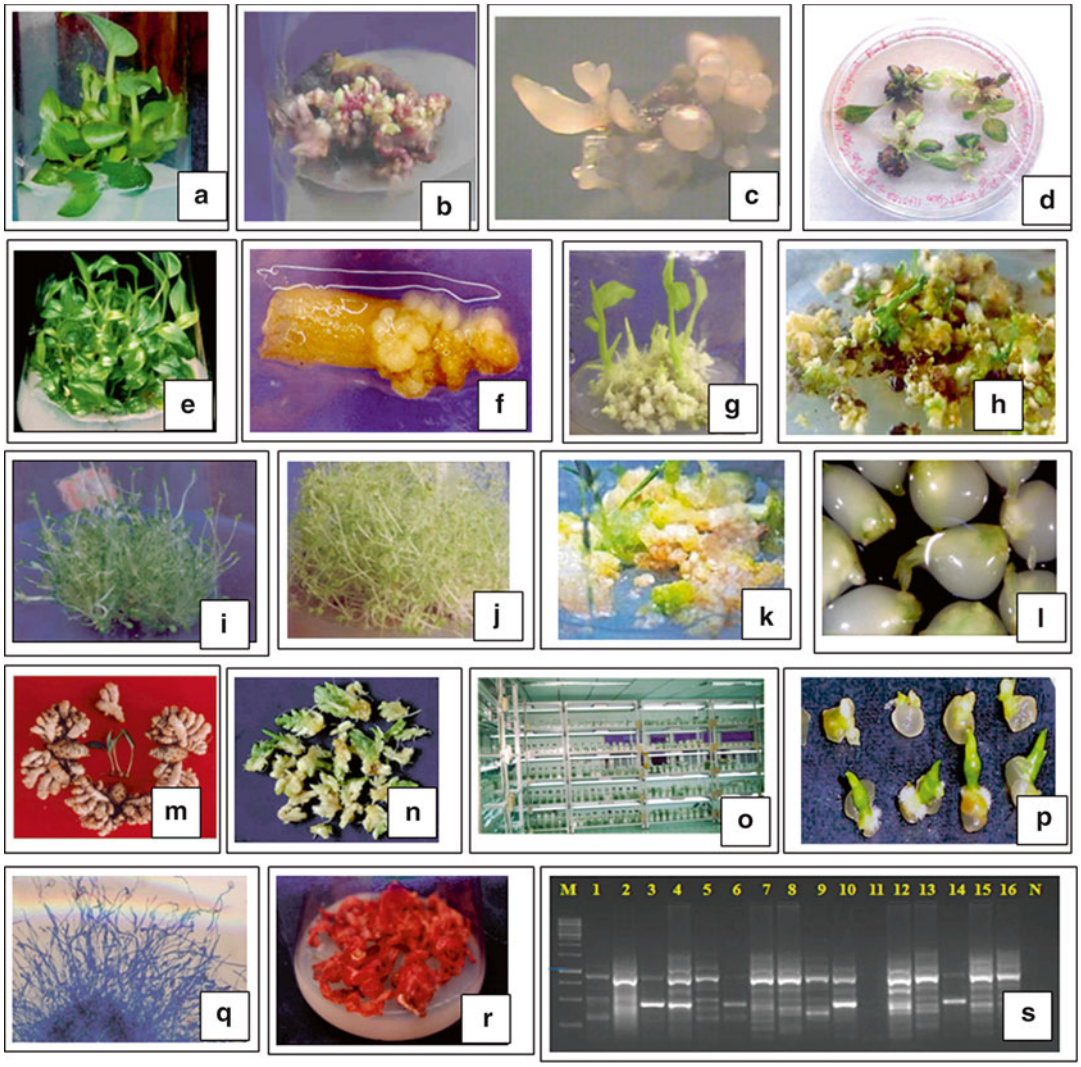

Fig. 25.1 (**a**) Biotechnological approaches for spices. Micropropagation in black pepper; (**b**) plant regeneration in black pepper; (**c**) somatic embryogenesis in black pepper; (**d**) regeneration of transformants in black pepper; (**e**) micropropagation in cardamom; (**f**) regeneration of embryoids from cardamom anthers; (**g**) plant regeneration in ginger; (**h**) somatic embryogenesis in cinnamon; (**i**) micropropagation in thyme; (**j**) micropropagation in mint; (**k**) plant regeneration in coriander; (**l**) synthetic seeds in cardamom; (**m**) microrhizomes in turmeric; (**n**) microrhizomes in ginger; (**o**) *in vitro* gene bank of spices; (**p**) germinating cryopreserved encapsulated buds of vanilla; (**q**) germinating cryopreserved pollen of vanilla; (**r**) *in vitro* multiplication of nutmeg mace; (**s**) ISSR profiles of black pepper varieties

study the genetic diversity of 20 varieties from Brazilian collections and 40 popular genotypes and 4 different species of black pepper from India, respectively. Liao et al. (2009) reported the isolation and characterization of 11 and 9 polymorphic microsatellites loci from a *Piper polysyphonum* and *Piper solmsianum*, respectively. These microsatellite markers provide a reliable means to understand the population structure and interrelationships in the genus *Piper*. Jaramillo and Manos (2001) used phylogenetic analysis of sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA based on a worldwide sample of the genus *Piper.* Nirmal Babu et al. (2011a) reported the molecular interrelationships between 24 *Piper* species using RAPD profiles. Chaveerach

et al. (2002) studied interrelationships between three *Piper* species, viz., *P. kadsura, Piper retrofractum* and *P. chaba* using morphological characters and RAPD profiles to demonstrate a closer relation between *P. retrofractum* and *P. kadsura* than between *P. chaba* and *P. retrofractum*. Genetic diversity of *Piper* species using RAPD and ISSR (Fig. 25.1s) was also reported recently by many workers (Sen et al. 2010; Nirmal Babu et al. 2012b). Jiang and Liu (2011) applied RAPD and SRAP markers to analyze genetic diversity in 74 individual plants of *Piper* spp. in Hainan Island. The SRAP technique clearly distinguished all *Piper* spp. from each other.

Molecular markers like RAPD, AFLP, ISSR, SSR and ITS polymorphism were used for assessment of genetic variability in black pepper and to characterize important cultivars, varieties and related species of black pepper to develop fingerprints and to study the interrelationships (Pradeep Kumar et al. 2003; Nazeem et al. 2005; Nirmal Babu et al. 2011a). A mapping population was developed for the preparation of genetic map of black pepper (Nirmal Babu et al. 2011a), and DNA markers were also used to study the genetic fidelity among micropropagated black pepper and long pepper plants (Nirmal Babu et al. 2011a).

Johnson et al. (2005) used male parent specific RAPD markers for the identification of hybrids in black pepper (*Piper nigrum* L.). Sex-specific markers were developed in *Piper longum* L. using RAPD (Banerjee et al. 1999) and differential display (Manoj et al. 2008) *Piper betle* L. using ISSR (Khadke et al. 2012) and RAPD markers (Samantaray et al. 2012b). Sheji et al. (2006) developed a SCAR marker for identifying *Phytophthora*-resistant lines of black pepper. RAPD technique was also employed for the authentication of dried black pepper from its adulterant *Carica papaya* L (Dhanya et al. 2009).

25.2.3 Protoplast Culture

The 'protoplast' is devoid of cell wall and this makes the protoplast technology suitable for genetic transformation by introduction of transgene DNA and somatic hybridization by protoplast fusion of species or subspecies resistant to traditional cross-breeding, or isolation of subcellular organelles etc. Reliable procedures are available for isolation, culture and fusion of protoplasts from a range of spices. Successful isolation and culture of protoplasts were reported in leaf tissues in black pepper (Shaji et al. 1998), and these protoplasts could be successfully developed up to microcalli stage.

25.2.4 Genetic Transformation

Reports are available on *Agrobacterium*-mediated gene transfer system (Fig. 25.1d) in *P. nigrum* (Nirmal Babu et al. 2005a, 2011d, 2013). Maju and Soniya (2012) reported an efficient protocol for genetic transformation using *Agrobacterium tumefaciens* strain EHA 105 and multiple shoot production in *Piper nigrum* var. Panniyur-1 to understand the mechanisms that control the regeneration process in the species. Mani and Manjula (2011) reported the *Agrobacterium-mediated* transformation and endogenous silencing in *Piper colubrinum* by vacuum infiltration. The *in planta* transformation via pollen tube pathway was done by Asha and Rajendran (2010) in black pepper variety Panniyur-2, using the total exogenous DNA of *Piper colubrinum*, a species resistant to *Phytophthora capsici*.

25.2.5 Cloning and Isolation of Candidate Genes

Spices are sources of many important genes with antibiotic and pharmaceutical properties. Efforts are being made to identify and isolate genes of interest both for crop improvement and of industrial value. Molecular cloning of a cDNA fragment encoding the defence-related protein β-1,3-glucanase in black pepper (*P. nigrum* L.) and methyl-glutaryl-CoA reductase in *Piper colubrinum* was reported (Girija et al. 2005a, b). PCR-based SSH technique was used to generate a leaf-specific subtracted cDNA library for *Piper nigrum* L. for the identification of more number

of tissue-specific genes (Alex et al. 2008). Bhat et al. (2005) reported the isolation and sequencing of CMV coat protein gene infecting black pepper and its possible deployment in black pepper to induce virus resistance. Dicto and Manjusha (2005) used PCR-based SSH to identify *P. colubrinum* resistance genes which are differentially expressed in response to salicylic acid. Mani and Manjula (2011) reported the cloning and characterization of two isoforms of osmotin, an antifungal PR-5 gene homologue, from a salicylic acid-induced subtracted cDNA library in *P. colubrinum*. Cloned isoforms of osmotin from resistant species could be used for molecular breeding of black pepper. Bioprospecting of novel genes from black pepper was attempted by Sujatha et al. (2005). Heterologous probes were used to identify the presence of pea lectin genes and tomato protease inhibitor genes in black pepper. Resistance gene analogues (RGAs) have been isolated in *Piper nigrum* and *Piper colubrinum* by Tiing et al. (2012).

25.3 Cardamom

25.3.1 Micropropagation and Plant Regeneration

Cardamom, considered the 'Queen of Spices', is also native to India. The productivity of cardamom is hampered by various diseases of viral aetiology. Utilization of virus-free planting material is an important input into disease management strategy. Cardamom is one of the first crops where commercialization of micropropagation has been achieved. Efficient *in vitro* methods for rapid clonal propagation of cardamom (Fig. 25.1e) are available (Nadgauda et al. 1983; Nirmal Babu et al. 1997; Nirmal Babu and Minoo 2003, 2011b, 2011e). Successful regeneration of plantlets from callus of seedling explants and anthers (Fig. 25.1f) of cardamom was reported (Nirmal Babu 1997; Nirmal Babu et al. 2011b, Rao et al. 1982). Manohari et al. (2008) also reported an efficient protocol for the induction of somatic embryogenesis and plant regeneration in small cardamom. Identification of a few Katte tolerant somaclones was reported by Peter et al. (2001).

25.3.2 Molecular Characterization

Molecular profiling of 11 species representing 5 major tribes, viz., *Amomum, Aframomum, Alpinia, Hedychium* and *Elettaria*, and 96 collections of cardamom germplasm using RAPD, PCR, RFLP and ISSR markers to elucidate their interrelationships, identification of duplicates and geographical origins of Indian cardamom was reported by Nirmal Babu et al. (2011a). Molecular characterization of selected cardamom genotypes using AFLP markers was also done in Columbia by Tamayo 2007. Molecular profiling of Indian, Sri Lankan and Guatemalan exported cardamoms using RAPD/ISSR primers indicated that though there is a lack of genetic polymorphism among them, they show the variation in quality parameters (Thomas et al. 2006).

25.3.3 Protoplast Culture

Successful isolation and culture of protoplasts were reported from cell suspensions and leaf tissues in cardamom (Geetha et al. 2000), and these protoplasts could be successfully developed up to microcalli stage.

25.3.4 Genetic Transformation

A preliminary study on the transformation of cardamom was attempted using the biolistic process to study the optimum conditions for gene delivery and the efficiency of the plasmid vector pAHC 25 and promoter Ubi-1 (maize ubiquitin) for transformation and gene expression in cardamom embryogenic callus. Transient expression of GUS gene was noticed in the bombarded callus tissue (Nirmal Babu et al. 1998).

25.4 Ginger

25.4.1 Micropropagation and Plant Regeneration

Ginger is the third most important spice that originated in South Asia. There is no seed set in ginger

leading to limited variability, and this hampers crop improvement programme. Rhizome rot caused by *Pythium aphanidermatum* and bacterial wilt caused by *Ralstonia solanacearum* are the major diseases affecting ginger. Diseases of ginger are often spread through infected seed rhizomes. Tissue culture will help in the production of pathogen-free planting material. Clonal multiplication of ginger from vegetative buds (Nadgauda et al. 1980; Nirmal Babu et al. 1997, 1998; Sharma and Singh 1997); optimization of media composition for micropropagation of ginger (Nirmal Babu et al. 2011c); regeneration of plantlets *via* callus phase from leaf (Nirmal Babu et al. 1992a), vegetative bud (Fig. 25.1g), ovary and anther explants (Kacker et al. 1993; Nirmal Babu 1997; Nirmal Babu et al. 2005a, b; Lincy et al. 2009; Ramachandran and Chandrashekaran 1992) and plant regeneration from ginger immature anthers (Nirmal Babu et al. 1992b) and anther have been reported (Samsudeen et al. 2000). This system was used for inducing somaclonal variability in ginger where lack of seed set hampers conventional breeding. A few promising high-yielding rhizome rot-tolerant somaclones also have been identified (Nirmal Babu et al. 1996; Sumathi 2007).

In nature, ginger fails to set fruit. However, by supplying required nutrients to young flowers and by *in vitro* pollination, 'fruit' development and subsequently plants could be recovered. *In vitro* pollination attempts were successfully made by Nazeem et al. (1996) to overcome the pre-fertilization barriers like spiny stigma, long style and coiling of pollen tube that interfered with natural seed set in ginger successful seed set was obtained. Induction of tetraploid ginger through *in vitro* colchicine treatment and tetraploid somaclone with extra bold rhizomes was also reported (Adaniya and Shirai 2001; Smith et al. 2004; Nirmal Babu et al. 1996, 2005b; Wang et al. 2010).

25.4.2 Molecular Characterization

RAPD profiling of 90 accessions of ginger indicated moderate to low level of polymorphism (Sasikumar and Zachariah 2003; Nirmal Babu et al. 2005a, b; Parthasarathy and Nirmal Babu et al. 2011c). Various markers like AFLP (Wahyuni et al. 2003; Sajeev et al. 2011) and ISSR (Kizhakkayil and Sasikumar 2010) were used to study the genetic diversity in ginger. Jiang et al. (2006) used metabolic profiling and phylogenetic analysis to investigate the diversity of plant material within the ginger species and between ginger and closely related species in the genus *Zingiber* and also for authentication of ginger. Chavan et al. (2008) developed SCAR markers as complementary tools for distinguishing *Z. officinale* from the other *Zingiber* species.

25.4.3 Protoplast Culture

Successful isolation and culture of protoplasts were reported from cell suspensions and leaf tissues in ginger (Nirmal Babu 1997; Geetha et al. 2000), and these protoplasts could be successfully developed up to microcalli stage. Somatic hybridization of ginger through chemical fusion (PEG mediated) and its regeneration was reported by Guan et al. (2010). RAPD technique was used for the identification of hybrids, and flow cytometry analysis revealed the diploid nature of all regenerated progenies.

25.4.4 Genetic Transformation

Transient expression of GUS was successfully induced in ginger embryogenic callus bombarded with plasmid vector pAHC 25 and promoter Ubi-1 (maize ubiquitin) callus tissue (Nirmal Babu 1997). *Agrobacterium tumefaciens* strain EHA105/p35SGUSInt, effective in expressing β-glucuronidase activity, was used to transform ginger by Suma et al. (2008).

25.4.5 Cloning and Isolation of Candidate Genes

Nair and Thomas (2007) identified the three primer pairs designed from the conserved motifs of NBS domain of NBS-LRR gene class as most successful in isolating RGCs in ginger, and these

provide a base for isolation of RGC mining in ginger. They also reported the efficiency and sensitivity of SSCP (single-strand conformation polymorphism) analysis in discriminating *Pythium* susceptible and resistant *Zingiber* accessions (Nair and Thomas 2012; Nair et al. 2010) and the isolation of resistant gene designated ZzR1 from *Z. zerumbet* and its correlation between ZzR1 expression and resistance of the wild taxa to *Pythium aphanidermatum* infection (Nair and Thomas 2013). Swetha Priya and Subramanian (2008) reported the presence of R gene of CC-NBS-LRR class of plant resistant gene in ginger varieties against *Fusarium oxysporum* f. sp. *zingiberi.* Fujisawa et al. (2010) cloned a novel gene that could encode s (beta) bisabolene synthetase from ginger. Prasath et al. (2011, 2013) reported the isolation of PR5 protein genes CaPR5 and ZoPR5 which code for the precursor proteins of 227 and 224 amino acids. They used a PCR-based suppression subtractive hybridization (SSH) method to identify *C. amada* (a potential donor for bacterial wilt resistance to *Zingiber officinale*) genes that are differentially and early expressed in response to the *R. solanacearum* infection compared to *Z. officinale*. The study highlighted the expression of LRR, GST and XTG much higher in resistant species (*C. amada*) than in susceptible species (*Z. officinale*).

25.5 Turmeric

25.5.1 Micropropagation and Plant Regeneration

Turmeric of commerce is the dried rhizomes of *Curcuma longa* L. which belongs to the family Zingiberaceae. India is the major producer and exporter of this spice. Curcumin is the important colouring material from turmeric, and development of varieties with high recovery of curcumin is the need of the hour. Successful micropropagation of turmeric has been reported (Nadgauda et al. 1978; Nirmal Babu et al. 1997; Sunitibala et al. 2001; Salvi et al. 2002; Panda et al. 2007; Ghosh et al. 2013). This technique is used for the production of disease-free planting material. Organogenesis and plantlet formation were achieved via callus cultures of turmeric (Nirmal Babu et al. 1997; Sunitibala et al. 2001; Salvi et al. 2000). Variants with high curcumin content were isolated from tissue-cultured plantlets (Nadgauda et al. 1982). 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).

Renjith et al. (2001) reported the *in vitro* pollination and hybridization between two short duration types VK-70 and VK-76 and reported the seed set and seed development. This reduces the breeding time and helps in recombination breeding which was so far not attempted in turmeric. Protocols for micropropagation of many economically and medicinally important zingiberaceous species like *Amomum subulatum* (large cardamom), *Curcuma aromatica* (kasturi turmeric), *C. amada* (mango ginger), *C. zedoaria, Kaempferia galanga, K. rotunda* and *Alpinia* spp. were developed (Vincent et al. 1992; Chang and Criley 1993; Ravindran et al. 1996; Geetha et al. 1997; Chan and Thong 2004; Chithra et al. 2005; Raju and Anita-D 2005. Islam et al. (2004) described an efficient protocol for microrhizome production, *in vitro*, in turmeric. Rahman et al. (2004) reported the efficient plant regeneration through somatic embryogenesis from leaf base-derived callus of *Kaempferia galanga* L.

25.5.2 Molecular Characterization

Kress et al. (2001) proposed a new phylogenetic analysis of Zingiberaceae based on DNA sequences of the nuclear internal transcribed spacer (ITS) and plastid *mat K* regions. The results suggest that *Curcuma* is paraphyletic with *Hitchenia, Stahlianthus* and *Smithatris.* Sasaki et al. (2004) applied single-nucleotide polymorphism analysis of the trnK gene for the identification of *Curcuma* plants. Genetic diversity evaluation among *C. longa* and other 14 *Curcuma* species was done using ISSR and RAPD markers (Syamkumar and Sasikumar 2007) placing them into seven groups which is somewhat congruent with classification based on morphological

characters proposed by the earlier workers. Genetic diversity in turmeric was assessed by using molecular markers like RAPD (Jan et al. 2011), both RAPD and ISSR (Singh et al. 2012) and PCR-based markers, viz., RAPD, ISSR and AFLP (Das et al. 2011; Nirmal Babu et al. 2007, 2011d). Development and characterization of EST-derived and genomic microsatellites in *Curcuma longa* were reported by Joshi et al. (2010), Siju et al. (2010) and Senan et al. (2013) which could be used for diversity analysis. Nayak and Naik (2006) carried out four C nuclear DNA content and RAPD analysis of 17 promising cultivars of turmeric from India.

Salvi et al. (2003) and Praveen (2005) using RAPD analyzed turmeric somaclones and concluded that plants regenerated using shoot tips showed genetic stability, while the callus-derived and inflorescence-derived plants showed variations. Tyagi et al. (2007) confirmed the genetic stability of 12-month-old *in vitro* conserved turmeric by RAPD profiling. Sinus et al. (2010) developed and amplified microsatellite markers from ESTs of turmeric. Sasaki et al. (2002) used sequence analysis of Chinese and Japanese *Curcuma* drugs on the 18S rRNA gene and *trnK* gene and its application based on amplification-refractory mutation system analysis for their identification and authentication. Sasaki et al. (2004) used SNP analysis based on the differences in the *nucleotide* positions in the 177, 645, 724 and a 4 base indel on the *trnK* gene obtained using three different lengths of 26 mer, 30 mer and 34 mer reverse primers for the identification of four *Curcuma* sp. studied by Xia et al. (2005) used 5S rRNA spacer and chemical fingerprints for quality control and authentication of Rhizoma *Curcumae*, a Chinese medicine used for the removal of blood stasis and alleviating pain.

25.5.3 Protoplast Culture

Successful isolation and culture of protoplasts were reported from cell suspensions and leaf tissues in turmeric (Geetha et al. 2000), and these protoplasts could be successfully developed up to microcalli stage.

25.5.4 Genetic Transformation

An efficient method for stable transformation was developed in turmeric using particle bombardment on callus cultures (Shirgurkar et al. 2006). Transgenic shoots regenerated were multiplied, and stably transformed plantlets were produced. Polymerase chain reaction (PCR) and histochemical GUS assay confirmed the stable transformation. Transformed plantlets were resistant to glufosinate. A protocol for genetic transformation and regeneration was established in *C. alismatifolia* using shoot explants through *Agrobacterium* strain AGLO harbouring binary vector pBI121 or pBI121-Ca-ACSI. Transformation was confirmed by PCR, GUS assay and Southern blotting of regenerated plants (Mahadtanapuk et al. 2006).

25.5.5 Cloning and Isolation of Candidate Genes

Isolation, cloning and characterization of a mannose-binding lectin from cDNA (Chen et al. 2005), a stress-responsive CDPK gene (ZoCDPK1) from ginger rhizome, using rapid amplification of cDNA end (RLM-RACE) technique (Vivek et al. 2013) and violaxanthin deepoxidase (GVDE) (Huang et al. 2007) have been reported. Over-expression of ginger CDPK1 gene in tobacco conferred tolerance to salinity and drought stress. ZoCDPK1 functions in the positive regulation of the signalling pathways that are involved in the response to salinity and drought stress in ginger, and it is likely operating in a DRE/CRT independent manner. Joshi et al. (2010) used degenerate primers designed based on known R gene in combination to elucidate resistance gene analogues from *Curcuma longa* cultivar Suvarna. Kar et al. (2013) used a previously isolated resistance gene candidate Czp11 from *C. zedoaria* resistant to *Pythium aphanidermatum* as a template to characterize a major resistance gene CzR1 through candidate gene approach in combination with RACE-PCR strategy.

25.6 Vanilla

25.6.1 Micropropagation and Plant Regeneration

Vanilla planifolia, native to Mexico and Central America, now cultivated in other parts of the tropics, is the source of natural vanillin. Micropropagation of vanilla using apical meristem was standardized for large-scale multiplication of disease-free and genetically stable plants (Kononowicz and Janick 1984; Minoo 2002; Minoo et al. 2006; Minoo and Nirmal Babu 2009). Successful plant regeneration from shoot- and seed-derived callus was reported in vanilla (Nirmal Babu et al. 1997; Minoo 2002).

25.6.2 Molecular Characterization

Markers like RAPD and AFLP coupled with morphological characters were utilized to assess the variability and hybrid nature of genotypes and of successful interspecific hybridization and production of hybrids between *V. planifolia* and *V. aphylla* (Minoo et al. 2006). RAPD marker was used to estimate the level of genetic diversity and interrelationships among different clones of *V. planifolia* and related species (Minoo et al. 2010). The data showed very limited variation within accessions of *V. planifolia* indicative of its narrow genetic base and its close relationship with *V. tahitensis* (Besse et al. 2004; Schluter et al. 2007; Minoo et al. 2008b; Minoo and Nirmal Babu 2009, Minoo et al. 2010). A comparative study of RAPD and ISSR was reported to analyze the interrelationships among nine cultivated, wild and hybrid *Vanilla* species (Verma et al. 2009). Fourteen microsatellite loci developed from *V. planifolia* shown to be monomorphic within the cultivated accessions and 11 markers out of 14 were polymorphic when transferable to *V. tahitensis* (Bory et al. 2008).

25.6.3 Protoplast Culture

Minoo et al (2008a) reported the isolation of viable protoplasts in *Vanilla* species, i.e. in *V. andamanica*, and that of PEG-mediated protoplast fusion between *V. planifolia* and *V. andamanica.* The protoplast fusion technology can be useful in gene transfer of useful traits to *V. planifolia* especially the natural seed set and disease tolerance observed in *V. andamanica.*

25.6.4 Genetic Transformation

Protocols are available for genetic transformation of *Vanilla planifolia* using indirect procedure, viz*., Agrobacterium tumefaciens* using shoot tip sections (Malabadi and Nataraj 2007) and protocorm-like bodies from shoot tips as explants (Ratheesh and Ishwara Bhat 2011), providing a very useful basis for further genetic improvement of the orchid.

25.6.5 Cloning and Isolation of Candidate Genes

Sequencing of neutral genes has been used for reconstructing the evolutionary history of vanilloid orchids, including a few vanilla species (Cameron et al. 1999; Cameron and Chase 2000; Cameron 2004, 2009; Cameron and Molina 2006). Nuclear and plastid sequences were also used for unravelling the origin of the Tahitian vanilla (Lubinsky et al. 2008). Recently, the length polymorphism of the nonneutral caffeic acid O-methyl transferase gene was also used to analyze 20 vanilla species and confirmed the strong differentiation of Old World versus New World species in the genus (Besse et al. 2009).

25.7 Tree Spices

25.7.1 Micropropagation and Plant Regeneration

Cinnamon, clove, nutmeg, curry leaf, pomegranate, tamarind, allspice and garcinia are some of the important tree spices. In these perennial tree crops, identification and clonal multiplication of high-yielding 'elite' genotypes become a priority due to long pre-bearing period. Micropropagation

of cinnamon, Chinese cassia and camphor was reported from seedlings and mature tree explants (Mini et al. 1997; Nirmal Babu et al. 1997; Huang et al. 1998). Multiple shoots were induced from shoot tips and nodal segments of *Cinnamomum camphora* on Woody Plant Medium (WPM) (Huang et al. 1998 and from a cotyledonary node on MS medium (Azad et al. 2005). Successful micropropagation of Chinese cassia was reported by Inomoto and Kitani (1989) using nodal explants from seedlings on MS medium. Micropropagation protocols for *C. camphora* were developed by Nirmal Babu et al. 2003.

In vitro multiple shoot induction was worked out in *G. indica* (Kulkarni and Deodhar 2002). Murashige and Skoog's medium supplemented with BAP gave optimal response in different genotypes investigated. Micropropagation of three species of garcinia was reported by Huang et al. (2000), Malik et al. (2005) and Mohan et al. (2012). *In vitro* shoot initiation from explants of field-grown trees of nutmeg was reported by Mallika et al. (1997). Micropropagation of clove from seedling explants have been reported (Mathew and Hariharan 1990; Suparman and Blake 1990). MS medium supplemented with IBA or activated charcoal induced root formation. However, there are no reports on successful micropropagation of clove from mature shoot explants.

Reports on micropropagation of curry leaf, pomegranate, camboge and tamarind are also available (Mascarenhas et al. 1987; Hazarika et al. 1995; Rao et al. 1997; Bhuyan et al. 1997; Mathew et al. 1999; Nirmal Babu et al. 2000; Mehta et al. 2000). High-frequency direct shoot proliferation was induced in intact seedlings of *M. koenigii* (Bhuyan et al. 1997). Shoot proliferation is also reported from different explants like nodal cuttings (Nirmal Babu et al. 2000), leaves (Mathew and Prasad 2007) and immature seeds (Rani et al. 2012). Efficient micropropagation protocols for Pomegranate were reported (Bin and Jiang 2003; El-Agamy et al. 2009; Patil et al. 2011). The plantlets grown on WPM were found to be significantly better in average survival, plantlet height and average leaf number per shoot when compared to MS and NN media (El-Agamy et al. 2009; Kaji et al. 2013). *In vitro* regeneration and high-frequency regeneration of tamarind were achieved in different media compositions (Hussain et al. 2004; Pattepur et al. 2010). Thidiazuron can play a major role to induce germination in tamarind seedlings Mehta et al. (2004). Reports on successful callus induction and plant regeneration in nutmeg, cinnamon (Fig. 25.1h), camphor, pomegranate and curry leaf are available (Bhansali 1990; Iyer et al. 2000; Kong et al. 2009; Shi et.al. 2009, 2010; Paul et al. 2011).

25.7.2 Molecular Characterization

Genetic identification among four cinnamon species (*Cinnamomum cassia*, *C. zeylanicum*, *C. burmannii* and *C. sieboldii*) using nucleotide sequences of chloroplast DNA was studied by Kojoma et al. (2002). The nucleotide variation at one site in the *trnL-trnF* IGS and at three sites in the *trnL* intron was used for the correct identification of *Cinnamomum* species. Furthermore, single-strand conformation polymorphism (SSCP) analysis of PCR products from the *trnL-trnF* IGS and the *trnL* intron resulted in different SSCP band patterns among *C. cassia*, *C. zeylanicum* and *C. burmannii*. RAPD technology was applied for the analysis of normal and excellent types of *Cinnamomum camphora* L. and genetic interrelationship of nine *Cinnamomum* species (Hui and Linshui 2003 and Joy and Maridass 2008), respectively. Sheeja et al. (2013) reported the diversity analysis of nutmeg (Myristica) and related genera using both RAPD and ISSR markers. Species-specific bands could be identified from all the accessions, which can be converted into SCAR markers for genotype identification and authentication.

Yapwattanaphun et al. (2004) used ITS sequence data to elucidate phylogenetic relationship of mangosteen (*Garcinia mangostana*) and several wild relatives (*Garcinia* spp.). The ITS sequence analysis showed that *G. atroviridis, G. cowa, G. dulcis, G. malaccensis, G. mangostana, G. rostrata* and *G. vilersiana* have nucleotide additivity (two different nucleotides at the same nucleotide position) at several sites in the ITS region. The occurrence of these species might be related to hybridization with ancestors, but the

genomic compositions, even chromosome numbers, of these species are still unknown. Sulassih and Santosa (2013) worked on the diversity analysis of mangosteen and its relatives based on morphological and ISSR markers, revealing a close relationship between *G. celebica, G. malaccensis* and *G. mangostana*. It was determined that *G. malaccensis* and *G. celebica* were ancestors based on morphological and ISSR markers. Thatte and Deodhar (2012) identified male- and female-specific molecular markers in *Garcinia indica*.

Genetic variability and relationship studies of curry leaf were done using RAPD, DAMD and ISSR by Verma and Rana (2013). Genetic diversity studies of pomegranate using RAPD, SSR, AFLP, RAMP, nuclear rRNA and internal transcribed spacer were reported by many workers (Durgaç et al. 2008; Jbir et al. 2008; Awamleh et al. 2009; Zamani et al. 2010; Hasnaoui et al. 2010; Pirseyedi et al. 2010; Ebrahimi et al. 2010; Soriano et al. 2011; Zhao et al. 2013; Singh et al. 2013). Genetic diversity studies of tamarind using RAPD and AFLP markers were done by Diallo et al. (2007) and Algabal et al. (2011) and in allspice by Wadt et al. (2004).

25.7.3 Cloning and Isolation of Candidate Genes

Genes encoding cinnamomin (a type II RIP), which has three isoforms, were isolated from camphor seeds by Yang et al. (2002). A geraniol-synthase gene is also isolated from *Cinnamomum tenuipilum* by Yang et al. 2005. Expression of CtGES was exclusively observed in the geraniol chemotype of *C. tenuipilum*. Furthermore, *in situ* hybridization analysis demonstrated that CtGES mRNA was localized in the oil cells of the leaves.

25.8 Seed and Herbal Spices

25.8.1 Micropropagation and Plant Regeneration

Seed spices and herbs constitute a large group of widely different aromatic plants which are used as spices, culinary herbs and medicinal herbs and those which are used in aroma therapy. Micropropagation protocols for many seed and herbal spices are available. They include coriander, anise, thyme (Fig. 25.1i), peppermint (Fig. 25.1j), spearmint, celery, lavender, savory, *Ocimum*, oregano, basil, sage, fennel, parsley, dill and garlic, saffron and *Eryngium foetidum* capsicum (Bhojwani 1980; Venkataraman and Ravishankar 1987; Cellarova 1992; Furmanowa and Olszowska 1992; Panizza and Tognoni 1992; Patnaik and Chand 1996; Vandemoortele et al. 1996; Sajina et al. 1997a; Ochoa-Alejo and Ramirez-Malagon 2001; Gupta and Bhargava 2001; Sharma et al. 2004; Aflatuni et al. 2005; Karaoglu et al. 2006; Majourhat et al. 2007; Minas 2009; Song et al. 2009; Ascough et al. 2009; Falk et al. 2009; Kothari et al. 2010; Fadel et al. 2010; Irikova et al. 2011; Samantaray et al. 2012a; Nhung and Quynh 2012; Kara and Baydar 2012; Navroski et al. 2012; Zeybek et al. 2012; Rodeva et al. 2013; Santoro et al. 2013; Dixit and Chaudhary 2013; Keller and Senula 2013). Clonal propagation of chemically uniform fennel plants through somatic embryoids was reported by Miura et al. (1987). Shoot regeneration protocols for fenugreek, cumin and coriander (Fig. 25.1k) were reported (Nirmal Babu et al. 1997; Tawfik and Noga 2001; Ebrahimie et al. 2003; Aasim et al. 2009). Jakhar et al. (2003) reported the *in vitro* flowering and seed formation in cumin.

Somatic embryogenesis has been established in saffron, garlic (Keles et al. 2010) and chilli (Blazquez et al. 2003; Sheibani et al. 2006; Munyon et al. 1989). An efficient protocol for organogenesis from root protoplasts (Xu et al. 1982) and adventitious shoot formation in fennel was developed by investigating the effect of plant growth regulators by Jakhar and Choudhary (2012). Profuse callus differentiation was observed when medium was supplemented with 1.0 mg/l BAP followed by 1.0 mg/l BAP + 0.5 mg/l IBA. The shoot morphogenesis was observed in callus proliferated from the shoot apex explants incubated at 1.0 mg/l BAP + 0.5 mg/l IBA, upon subculture on the same levels of plant growth regulator. In order to create variability, organogenesis followed by mutagenesis

has also been identified as a potential *in vitro* technique. In this process, stock organogenetic callus is treated with physical or chemical mutagens. The studies have shown positive indications to isolate promising mutants in cumin (Raje et al. 2004). Similar type of efforts can be made for the creation of variability in cumin for resistance to *Alternaria* blight, root rot in fenugreek, and for many other stresses.

In vitro methods of screening could prove highly useful in screening a large germplasm collections or cell lines for resistance to prevalent fungal diseases and tolerance to drought and salt stress. The reports are available on *in vitro* selection for salt tolerance in fenugreek on media containing 0.025–1.5 % NaCl (Settu et al. 1997) and drought-tolerant cell lines cultured on media containing 0.25–1.50 % PEG in coriander (Stephen and Jayabalan 2000) through tissue culture. Selection of somaclonal variants resistant to *Septoria apiicola* by callus culturing in the presence of fungal culture filtrate in celery (Evenor et al. 1994), *Fusarium* yellow-resistant celery line, a somaclonal variant (Lacy et al. 1996), and resistant to *Alternaria* blight in cumin (Shukla et al. 1997b) has been reported by different workers.

25.8.2 Molecular Characterization

Genetic diversity study of fennel, fenugreek, cumin, coriander, thyme, dill, garlic and saffron using RAPD, ISSR, AFLP and SSR was reported by many workers (Paran et al. 1998; Lopez et al. 2008; Rubio-Moraga et al. 2009; Imran et al. 2010; Zahid et al. 2009; Abdoli et al. 2009; Singh et al. 2011; Jana and Shekhawat 2012; Jo et al. 2012; Keify and Beiki 2012; Khalil et al. 2012; Suresh et al. 2013; Siracusa et al. 2013; Singh et al. 2013). Molecular marker systems based on SCAR, CAPS, RAPD, STS, SSR. TRAP and SRAP are available for identification and fingerprinting of fennel varieties by Agbiotech (2009). Genetic diversity and identification of variety-specific AFLP markers were developed in fenugreek by Vinay Kumar et al. (2012). Twelve novel polymorphic microsatellite loci were developed and characterized from a repeat-enriched genomic library of *Crocus sativus* to study population and conservation genetics of this economically and medicinally important species Nemati et al. (2012). Fenwick and Ward (2001) studied the RAPD-based cultivar identification in mint. RAPD- and AFLP-based hybrid identification in Mentha was reported by Shasany et al. (2005).

25.8.3 Protoplast Culture

Shekhawat and Galston (1983) reported the isolation, culture and shoot regeneration from mesophyll protoplasts of fenugreek. Successful regeneration of whole plants from tissue-cultured shoot primordial of garlic was reported by Ayabe et al. (1995).

25.8.4 Genetic Transformation

Wang and Kumar (2004) reported the heterologous expression of *Arabidopsis* ERS1 which causes delayed senescence in coriander. *Agrobacterium rhizogenes* which mediate transformed root cultures were analyzed for the production of essential oils in dill (Santos et al. 2002). The development of efficient genetic transformation protocol using glucuronidase gene in caraway was studied, and gene transfer was more efficient when cotyledonary node explants were used (Krens et al. 1997). Preliminary experiments on *Agrobacterium*-mediated transformation of uidA (GUS) gene into cotyledon and hypocotyls explants in fenugreek were reported by Khawar et al. (2004). Stable transformation in cumin through particle bombardment was reported by Singh et al. (2010) and through *Agrobacterium*-mediated transformation by Pandey et al. (2013).

25.8.5 Cloning and Isolation of Candidate Genes

Molecular cloning of mannose-6-phosphate reductase and its developmental expression in celery was studied by Everard et al. (1997). Wang and Kumar (2004) reported the heterologous

expression of *Arabidopsis* ERS1 that causes delayed senescence in coriander.

25.9 Development of Synthetic Seeds

Synthetic seeds or artificial seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates or any other tissue which can be used for sowing as a seed and those that posses the ability to convert into a plant under *in vitro* or ex vitro conditions and that retain its potential after storage also. Artificial or synthetic seeds can be an ideal system for low-cost plant movement, propagation, conservation and exchange of germplasm. Synthetic seeds were developed by encapsulation of *in vitro* developed small shoot buds in 3–5 % calcium alginate in black pepper, shoot buds in cardamom (Fig. 25.1i), somatic embryos and *in vitro* regenerated shoot buds in ginger and turmeric, *in vitro* regenerated shoot buds, protocorms in vanilla (Sharma et al. 1994; Sajina et al. 1997b), somatic embryos in cinnamon and curry leaf and nodal segments in pomegranate leaf (Sundararaj et al. 2010; Minoo 2002; Gayatri et al. 2005; Naik and Chand 2006; Nikhil and Shukla 2013). Synseeds have been reported in cumin (Tawfik and Noga 2002a, b), coriander (Kim et al. 1996; Stephen and Jayabalan 2000), fennel (Sajina et al. 1997b), celery (Pratap 1992), dill (Ratnamba and Chopra 1974; Sehgal 1978) and nigella (Hamid Elhag and Olemy 2004), and regeneration of these plants has been successfully obtained.

25.10 Microrhizome

Microrhizome technology is useful for developing disease-free planting material and hence is an ideal source of planting material suitable for germplasm exchange, transportation and conservation. *In vitro* induction of microrhizomes in ginger (Fig. 25.1m) was reported by many workers (Bhat et al. 1994; Sharma and Singh 1995; Nirmal Babu 1997; Nirmal Babu et al. 2003, 2005b; Tyagi et al. 2007; Sumathi 2007; Zheng et al. 2008). The microrhizome-derived plants have more tillers but were shorter. They gave fresh rhizome yield ranging from 100 to 800 g per plant with an estimated yield of 10 kg per 3 m^2 bed. Many reports are available on *in vitro* microrhizome formation in turmeric (Fig. 25.1n) (Nirmal Babu et al. 2003; Cousins and Alderberg 2008). Low sucrose is reported to decrease the size of microrhizome, but optimum microrhizome production at 6–9 % sucrose was also reported. Sucrose (6–9 %) was most effective in rhizome formation.

25.11 Conservation of Genetic Resources

25.11.1 *In Vitro* Conservation of Germplasm

The genetic resources of spices are conserved either in seed gene banks and/or in field repositories. Conservation of the germplasm in *in vitro* (Fig. 25.1o) and cryobank is a viable and a safe augment to conventional conservation strategies (Nirmal Babu et al. 2012c). Conservation of pepper, cardamom, herbal spices, vanilla and ginger germplasm in *in vitro* gene bank by slow growth was reported (Nirmal Babu et al. 1999; Tyagi et al. 2007). Protocols for *in vitro* conservation by slow growth of black pepper and its related species, viz., *P. barberi*, *P. colubrinum, P. betle* and *P. longum*, were standardized by maintaining cultures at reduced temperatures, in the presence of osmotic inhibitors and at reduced nutrient levels or by minimizing evaporation loss by using closed containers. The technology for *in vitro* conservation of zingiberaceous crops like ginger, turmeric, *Kaempferia*, cardamom and their related species was standardized by Geetha (2002) and in vanilla by Minoo (2002), Minoo and Nirmal Babu (2009). Slow growth techniques are being used for medium-term conservation of spices in *in vitro* repository at NBPGR (Mandal et al. 2000). Suspensions of embryogenic cell lines of fennel, conserved at 4 °C for up to 12 weeks, produced normal plants upon transfer to laboratory conditions (Umetsu et al. 1995).

25.11.2 Cryopreservation

Cryopreservation of black pepper and cardamom seeds in liquid nitrogen (LN_2) was reported by Choudhary and Chandel 1994, 1995. The technology for cryopreservation of black pepper, cardamom, ginger, turmeric and vanilla germplasm (Fig. 25.1p) using vitrification, encapsulation and encapsulation-vitrification methods is available (Minoo 2002; Yamuna 2007; Minoo et al. 2012; Nirmal Babu et al. 2012). Cryopreservation of encapsulated shoot buds of endangered *Piper barberi* has achieved (Nirmal Babu et al. 2012c). Efficient cryopreservation technique for *in vitro* grown shoots of ginger based on encapsulation-dehydration, encapsulation-vitrification and vitrification procedures was reported by Yamuna (2007). Minoo (2002) reported the cryopreservation of vanilla pollen (Fig. 25.1q) for the conservation of haploid genome as well as assisted pollination between species that flower at different seasons and successful fertilization using cryopreserved pollen. Cryopreservation of coriander (*Coriandrum sativum* L.) somatic embryos using sucrose preculture and air desiccation was reported by Popova et al. 2010. Cryopreservation of celery using LN_2 was reported by González-Benito and Iriondo 2002.

25.12 Production of Secondary Metabolites

Biotechnology can be utilized to exploit the potential of spices for bio-production of useful plant metabolites. The use of tissue culture for the biosynthesis of secondary metabolites particularly in plants of pharmaceutical significance holds an interesting alternative to control production of plant constituents. This technique is all the more relevant in recent years due to the ruthless exploitation of plants in the field leading to reduced availability. *In vitro* proliferation of nutmeg mace (Fig. 25.1r) and synthesis of flavour components in culture were reported by Nirmal Babu et al. (1992a). Since mace is the source of anticarcinogenic compound myristicin, this technique with improvement can be used for the production of myristicin. Most of the reports in saffron were on the *in vitro* proliferation of stigma and *in vitro* synthesis of colour components and metabolites. The proliferation of stigma of saffron *in vitro* and chemical analysis of metabolites produced through tissue cultures of *Crocus sativus* were reported (Sano and Himeno 1987; Himeno et al. 1988; Sarma et al. 1991).

Plant cells cultured *in vitro* produce wide range of primary and secondary metabolites of economic value. Production of phytochemicals from plant cell cultures has been presently used for pharmaceutical products. Production of flavour components and secondary metabolites *in vitro* using immobilized cells is an ideal system for spices crops. Ahmad et al. (2013) concluded that regenerated tissues of *P. nigrum* are a good source of biologically active metabolites for antimicrobial activities, and callus culture presented itself as a good source for such activities. Production of saffron and capsaicin was reported using such system (Ravishankar et al. 1993, 1995; Johnson et al. 1996; Venkataraman and Ravishankar 1997). Johnson et al. (1996) reported the biotransformation of ferulic acid vanillylamide to capsaicin and vanillin in immobilized cell cultures of *Capsicum frutescens.* Reports on the *in vitro* synthesis of crocin, picrocrocin and safranal from saffron stigma (Himeno and Sano 1995) and colour components from cells derived from pistils (Hori et al. 1988) are available for further scaling up. Callus and cell cultures were established in nutmeg, clove, camphor, ginger, lavender, mint, thyme (Furmanowa and Olszowska 1992), celery, etc. Cell immobilization techniques have been standardized in ginger, sage, anise and lavender (Ilahi and Jabeen 1992). The production of essential oils from cell cultures (Ernst 1989) and accumulation of essential oils by *Agrobacterium tumefaciens-transformed* shoot cultures of *Pimpinella anisum* were reported (Salem and Charlwood 1995). The 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 and Du Manoir 1992) were reported. Growth and production of monoterpene by transformed shoot

cultures of *Mentha citrata* and *Mentha piperita* in flasks and fermenters was reported by Hilton et al. 1995. The production of rosmarinic acid in suspension cultures of *Salvia officinalis* has been discussed by Hippolyte et al. (1992). Reports on the 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. 1996a). Kintzios et al. (2004) reported the scaling up of micropropagation of *Ocimum basilicum* L. in an airlift bioreactor and accumulation of rosmarinic acid. Though the feasibility of *in vitro* production of spice principles has been demonstrated, methodology for scaling up and reproducibility need to be developed before it can reach commercial levels. Once standardized, this technology has tremendous potential in industrial production of important compounds like capsaicin, vanillin, crocin, picrocrocin, safranal, myristicin, anethole, menthol and curcumin.

25.13 Biotechnological Approaches on Disease Management in Spice Crops

Crop losses in spice crops due to biotic stress are serious. Particularly, soilborne nature of the plant pathogens, poor amenability for effective disease management and lack of durable host resistance are the real constraints of production (Sarma et al. 2014). Foot rot and slow decline of black pepper (Sarma 2010), clump and capsule rot of small cardamom rhizome rot, *Fusarium* yellows and bacterial wilt in ginger and turmeric and root rots and wilts in cumin, coriander and fenugreek still remained as major threats in these crops. Though high degree of host resistance was identified for *Phytophthora capsici*, *Radopholus similis* and *Meloidogyne incognita* (root/foot rot and slow decline diseases), in *Piper colubrinum*, a native of Brazil, efforts to develop resistant transgenics are yet unsuccessful. Pending successful incorporation of R genes in susceptible cultivars, it is imperative to explore the available natural resources to combat these biotic stresses in these crops. Microbial biocontrol technology as a part of integrated disease management (IPM) is an ideal option. This technology is ecofriendly and would reduce pesticide load into the ecosystem. Hence, it received considerable attention during the last four decades. In this well-proven microbial biotechnology, biological control (Cook 1985) was found effective in reducing the crop losses in soilborne diseases of several crops, including spice crops (Sarma et al. 2014). Antagonism/antibiosis, hyperparasitism, growth promotion and induced systemic resistance (ISR) are the basic principles that govern this technology. Hyperparasitic fungi like *Trichoderma harzianum* and *T. viride*, *Pochonia chlamydosporia* and *Paecilomyces lilacinus* and PGPRs (plant growth-promoting rhizobacteria) like *Pseudomonas fluorescens, Pseudomonas cepacia* (Fridlender et al. 1999), *Bacillus subtilis* and Arbuscular mycorrhiza (AM) were found effective in reducing crop losses caused by soilborne plant pathogens in the spice crops. The large-scale production of these microbes adopting both solid and liquid fermentation technologies is in place. The delivery systems/formulations of these microbials both in solid and liquid (Chet 1987; Batta 2004) are now available in the market for the farming community to utilize them for the seed treatment and soil application. Application of these along with organic inputs that would serve as food base for their multiplication is suggested. The seed treatment is particularly important for seed spices, ginger and turmeric, where contaminated seed is the primary source of infection (Singh et al. 1972; Sarma and Anandaraj 1998).

Either fungus or bacterium to be successful biocontrol agents in a given ecosystem, rhizosphere competence (Ahmed and Baker 1987; Harman 1992) and competitive saprophytic ability (CSA) are the important traits that are of paramount importance (Sarma 2006).

25.13.1 *Trichoderma*

Intensive investigations carried out during the last four decades clearly established the potential

of *Trichoderma* as an effective biocontrol agent in suppression of several soilborne plant pathogens (Papvizas 1985; Papvizas and Chet 1987). These biocontrol agents received considerable attention in the management of spice crop diseases, viz., black pepper (Rajan et al. 2002; Saju 2004; Sarma and Saju 2004; Sarma 2010; Sarma et al. 2013), small cardamom (Suseela Bhai 1998; Suseela Bhai et al. 1993), ginger (Sharma and Jain 1979; Usman et al. 1996; Usman 1997), turmeric, cumin (Vyas and Mathur 2002; Hagag and Abosedera 2005), coriander (Gopal Lal et al. 2010) and fenugreek (Kakani et al. 2009). *In vitro* selection based on the inhibition of the target pathogen, the inhibitory effects of both volatile and nonvolatile antibiotics on suppression of the target pathogen, disease suppression and growth promotion of the target crop both in pot culture and field evaluation are the criteria adopted to evaluate their bio-efficacy. The mode of action of this hyperparasite includes production of the lytic enzyme like cellulases, glucanases and chitinase, and antibiotics that are necessary for the degradation of the cell wall of pathogen were studied in these pathogens of spice crops. The disease suppressive effect of *T. harzianum* was established through soil application that reduced the capsule rot of cardamom and was on par with treatment with copper fungicides and potassium phosphonate (Suseela Bhai 1998). Suppression of root rot in cardamom nurseries was demonstrated with soil application of *T. harzianum* (Sarma 2006). Similar results were obtained in rhizome rot of ginger caused by *Pythium aphanidermatum* both as seed treatment and soil application along with neem cake (Usman et al. 1996). In cumin, seed contamination with *F. oxysporum f. sp. cumini* and also wilt suppression were accomplished with seed treatment and soil application (Vyas and Mathur 2002). Root rot caused by *Rhizoctonia solani* in fenugreek was successfully controlled with *T. viride* application both as soil treatment and seed coating (Kakani et al. 2009). Economic viability of *Trichoderma* technology in black pepper was reported by Madan et al. (2006).

25.13.2 Plant Growth-Promoting Rhizobacteria (PGPRs)

Rhizosphere of any crop plant is associated with abundant microbial load that would impact the crop health and productivity. This is an established fact and has been researched extensively. Microbes associated with rhizoplane, rhizosphere and endophytes (Hallmann 2001) received greater attention in plant microbe interaction to focus on growth promotion and disease suppression in crop plants (Kloepper et al. 1980, 1993). In general, the parameters for selection and studies on mode of action remained the same as that were adopted in fungal biocontrol agents. There is a spurt of research activity on this group of organisms taking advantage of their potential both for growth promotion and disease suppression in several plant pathosystem (Lopper 1988). Kumar et al. (2013) have characterized *Pseudomonas aeruginosa* for their genetic and functional properties. Extensive studies carried out on black pepper – *P. capsici* – pathosystem revealed that *Pseudomonas fluorescens* increased growth in black pepper through production of growth promoters (Diby et al. 2001). Similarly, the induced systemic resistance (ISR) was achieved through the enhancement of defence-related enzymes like PAL, peroxidase, polyphenol oxidase (Diby and Sarma 2005; Diby et al. 2001) and mycolytic enzymes in black pepper (Diby et al. 2005). The enhancement of nutrient uptake in black pepper through this microbial association was found promising (Diby et al. 2001). In the case of ginger, the bio-efficacy of a bacterial strain mixture (*Bacillus subtilis -S2BC1* and *Burkholderia cepacia)* in suppressing rhizome rot and *Fusarium* yellows (Shanmugam et al. 2012, 2013) has been reported. The efficacy of microbial mixtures in root disease suppression has been reported (Raupach and Kloepper 1978). Similar results were reported in *P. capsici*-Black pepper pathosystem (Jisha et al. 2002). There is a need for field evaluation of these microbial mixtures to exploit their commercial potential. Harman and Stasz (1991) have utilized protoplast

fusion technique, for the production of superior biocontrol fungi.

25.13.3 Arbuscular/Vesicular Arbuscular Mycorrhiza (AM/VAM)

The importance of AM/VAM was extensively studied for their role in enhanced nutrient uptake and the protection of root system from soilborne plant pathogens (Atkinson et al. 1994; Schenk 1981). Increased nutrient uptake, altered host metabolism through enhanced production of phenolics, growth regulators and defence mechanisms are the factors implicated in root protection from soilborne plant pathogens. *Glomus fasciculatum* inoculum incorporated in black pepper nursery mixture was found effective in reducing root infection caused by *P. capsici, R. similis* and *M. incognita* in black pepper (Anandaraj et al. 1996).

25.13.4 Production of Healthy Nursery Stock of Spice Crop

The production of root rot-free rooted cuttings of black pepper received considerable attention. Root rot caused by *Phytophthora* and plant parasitic nematodes at nursery stage go unnoticed without any visible foliar symptoms. Hence, fortifying nursery mixture with biocontrol agents (AM, fungi and PGPRs) is an accepted technology which is becoming popular in all pepper-growing countries (Sarma 2010; Sarma et al. 2013, 2014). Similar methodology is also practiced in cardamom nurseries.

25.13.5 Biosafety Regulations

The formulations of these microbials both solid and liquid (Batta 2004) with ideal population of these biocontrol agents are now available in the market for seed treatment and soil application. In general, the use of these formulations along with organic inputs is suggested to ensure their multiplication and colonization. However, biosafety of microbials, to be used for crop protection, is an essential prerequisite. The regulatory norms for many of these are now in place (Kulakshetra 2004) and are further being improved. Alarmingly *Pseudomonas aeruginosa* an endophyte in black pepper highly effective in suppressing all the three major pathogens of the crop could not be used in crop protection, since *P. aeruginosa* is a human and animal pathogen (Kumar et al. 2012). The use of *P. aeruginosa* has been banned in Europe for crop protection as a policy. Similar biosafety regulatory mechanisms are needed in India and elsewhere to make this technology farmer friendly. However, large-scale field demonstrations and correct appraisal among the farming community is called for to popularize this technology. Critical ecological studies of these microbes, their quantification in relation to protection through molecular techniques (Aravind et al. 2011), identification of organisms with multiple mode of action to address the disease complexes, strainal improvement and the field demonstrations are needed to popularize this microbial biotechnology for crop protection in spice crops (Sarma et al. 2014).

25.14 Conclusions

Biotechnology has the potential to be a key tool to achieve sustainable agriculture and agri-based industry, through improvement of food production in terms of quantity, quality and safety while preserving the environment. Significant progress has been made in the field of biotechnology for micropropagation, conservation and management of genetic resources, disease and pest management and molecular characterization. Identifying markers linked to important agronomic characters will help in marker-assisted selection to shorten breeding time. Application of recombinant DNA technology for the production of resistant types to biotic and abiotic stress has to go a long way before they can be effectively

used in spices improvement. Though programmes have been initiated in many laboratories for *in vitro* secondary metabolite production, these techniques are to be refined and scaled up for possible industrial production of the products. Owing to their commercial potential, intensification and application of biotechnology in spices is important and indispensable in the coming decade. Microbial intervention through *T.harzianum, T.viride, P.florescens* and AM fungi has been found effective in disease suppression and growth promotion. Induced systemic resistance (ISR) as reflected in defense response appears to be one of the modes of action in disease suppression in black pepper and ginger. This microbial biocontrol technology at present is being practiced by the farming community for effective disease management in spice crops.

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