



Research review paper

Biotechnological advances in mango (*Mangifera indica* L.) and their future implication in crop improvement — A review

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Abstract

Biotechnology can complement conventional breeding and expedite the mango improvement programmes. Studies involving in vitro culture and selection, micropropagation, embryo rescue, genetic transformation, marker-assisted characterization and DNA fingerprinting, etc. are underway at different centers worldwide. In vitro culture and somatic embryogenesis of several different genotypes have been achieved. The nucellus excised from immature fruitlets is the appropriate explant for induction of embryogenic cultures. High frequency somatic embryogenesis has been achieved in some genotypes; however, some abnormalities can occur during somatic embryo germination. Embryo rescue from young and dropped fruitlets can improve the hybridization success in a limited flowering season. Protocols for protoplast culture and regeneration have also been developed. In vitro selections for antibiotic tolerance and fungal toxin resistance have been very promising for germplasm screening. Genetic transformation using *Agrobacterium tumefaciens* has been reported. Genes that are involved with fruit ripening have been cloned and there have been attempts to deliver these genes into plants. DNA fingerprinting and studies on genetic diversity of mango cultivars and *Mangifera* species are also being conducted at several research stations. The purpose of this review is to focus upon contemporary information on biotechnological advances made in mango. It also describes some ways of overcoming the problems encountered during in vitro propagation of mango.

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Keywords: Mango; Biotechnology; In vitro; Recalcitrance; Somatic embryogenesis; Genetic transformation; Somaclonal variations

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1. Introduction

The mango (*Mangifera indica* L.) is one of the choicest fruit crops of tropical and sub-tropical regions of the world, especially in Asia. Its popularity and importance can easily be realized by the fact that it is often referred as ‘King of fruits’ in the tropical world (Singh, 1996).

Globally, there are a number of problems that affect mango production. Being highly cross-pollinated and owing to the fact that most of superior clones of mango including ‘Indian’ and ‘Floridan’ cultivars are mono-embryonic, propagation through sexual means does not ensure true-to-the type plant reproduction. This leads to long juvenile phase of such plants. Production problems are associated with both scion and rootstock. Scion cultivar problems include biennial bearing habit, large tree size, susceptibility to major diseases and pests, short-post-harvest life and disorders like, malformation, spongy tissue, etc. (Iyer and Degani, 1997). For rootstock breeding, Iyer and Degani (1997) suggested that the priority should include tolerance of various soil related stresses, induction of dwarfing and high degree of polyembryony to ease rapid multiplication, i.e. clonal plants etc. Barring some hybrids, almost all the popular cultivated varieties are chance seedlings resulting from natural cross-pollination. Some hybrids are gaining ground owing to their novel characteristic features; however, there is an acute shortage of this material. Most of the important varieties are not amenable to hi-tech cultivation practices and do not meet the requirements of modern horticultural production systems like precocity

in bearing, dwarfing, regularity in bearing with high yield, resistant to diseases, pests and physiological disorders and good keeping quality. In addition, the world mango trade is narrowed to a great extent owing to the rapid perishable nature of fruits (Lizada, 1993). Mango is a climacteric fruit and long distance transport is sometimes a problem. Anthracnose caused by *Colletotrichum* sp. is regarded as one of the single most significant production and post-harvest problem (Dodd et al., 1998). There is a long-felt need to develop a variety possessing most of the desirable horticultural attributes. Earlier, Singh (1996) suggested that an ideal mango variety should be dwarf, regular bearer with medium size fruit (250–300 g). Additionally, it should be highly tolerant of various fungal and bacterial diseases. The fruits should have stable pleasant flavour combined with good keeping quality. The need for such an ideal phenotype can not be met by conventional breeding. Conventional breeding of woody perennial fruit crops like mango is difficult owing to long juvenile phase, self incompatibility, low fruit set, high fruit drop, single seed per fruit, high degree of cross-pollination, polyembryony, polyploidy and heterozygous nature, meager information on inheritance of important quantitative traits, etc. Inclusion of biotechnology in a breeding programme would expedite the development of desired cultivar(s). Employing biotechnology to correct genetic flaws of existing varieties has great potential importance.

Micropropagation of mango has not met with the commercial success as obtained in other fruit crops like pineapple, banana and strawberry. This is due to many problems associated with it, viz., latent microbial

infection, excessive polyphenol exudation, early explant necrosis, etc. Biotechnology could resolve some of the most serious problems of the mango industry. Moreover, molecular methods are useful for taxonomical characterization, to understand the regulation and expression of important traits/genes, etc. The attempted and potential biotechnological interventions are depicted in Fig. 1.

2. Problems associated with mango micropropagation

A successful *in vitro* culture initiation is a prerequisite for micropropagation, since failure at this very stage leaves a tissue culturist in a predicament, where no prospect of redeeming is in sight.

Preliminary studies has vividly indicated various inherent problems associated with *in vitro* culture of mango such as phenol exudation, medium discoloration, explant browning, deep seated systemic contamination and *in vitro* recalcitrance of tissues, which alone or together jeopardize the whole tissue culture efforts.

2.1. Phenol exudation, media browning and explant necrosis

Mango crops improvement by biotechnological methods such as plants transformation, *in vitro* mutagenesis followed by selection, the recovery of superior somaclonal variations from cell and protoplast culture, etc. requires the development of an efficient regeneration protocol. Mango has been a hard-to-deal crop compared to most other horticultural crops. Several attempts have been made to regenerate mango using leaf (Raghuvanshi and Srivastava, 1995; Singh et al., 1991) and shoots explants (Thomas and Ravindra, 1997; Sharma and Singh, 2002). Though these methods were found inefficient, yet they ensure the availability of explants all

round the year unlike the explants provided by the immature fruitlets only during certain parts of the year.

But high phenolic exudation owing to activation of oxidative enzyme system during the excision of plants, explants browning, deep seated contamination, medium discoloration and slow *in vitro* growth response have made it an ordeal for workers dealing with this crop. In order to reduce phenolic exudation during *in vitro* culture, Raghuvanshi and Srivastava (1995) suggested pretreatment of leaf explants using liquid shaker culture. Explants kept in liquid MS medium supplemented with 1% PVP followed by inoculation on gelled MS medium. Later, Chavan et al. (2000) also suggested dipping apical and axillary buds in 0.5% polyvinylpyrrolidone (PVP)+% sucrose for 30 s to reduce browning of explants. Sharma and Singh (2002) reported that etiolated shoots of mango when cultured *in vitro* registered a marked decline in polyphenol oxidase (PPO) activity and phenol content. There was a negative correlation between PPO activity and phenol content and explants survival. Hare Krishna (2006) also observed a marked decline in total phenols, peroxidase and PPO activities in etiolated mango shoots as compared to non-etiolated control. The total *in vitro* phenol exudation upon culturing was also lower in etiolated shoots and as a result etiolated shoots registered significantly higher survival and reduced necrosis over control.

2.2. Latent systemic contamination

One of the most important limiting factors for culture initiation using vegetative plant parts is the presence of deep seated contamination, which frequently leads to failure of cultures. They may become troublesome later for the culture, or may be transferred to the plantlets, which are produced as latent endophytic contaminants do not immediately reveal their presence of visible growth on the plant material or culture medium. The infection may need to adopt *in vitro*, or may not be able to multiply until the culture is transferred to a medium more favourable to its growth.

Latent fungal infections reported in mango are *Alternaria alternata* (Prusky et al., 1983), *Colletotrichum* spp. (Peterson, 1986), *Dothiorella* spp. (Johnson et al., 1991) and *Fusarium subglutinans* (Ploetz, 1994), which are present in the shoot tips and in different parts of the panicle even in the healthy ones; though, in lower density (Kumar, 1983). These latent contaminants may be distributed intra- and inter-cellular parts of plant tissue such as cortex, phloem, xylem and parenchymatous pith cells. They affect the culture's survival by decreasing the membrane permeability and increasing the release of

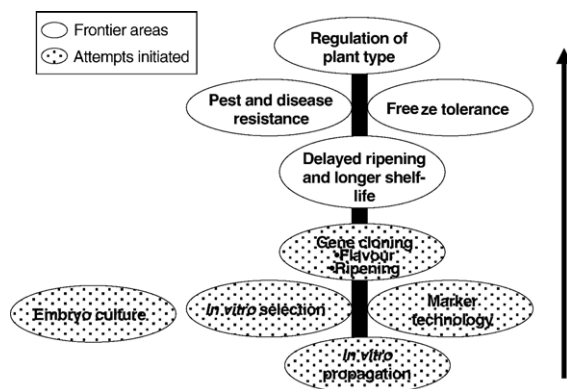


Fig. 1. Biotechnological interventions in mango.

soluble nutrients; thereby, stimulating appressorial germination or the rapid growth and development of subcuticular hyphae or other infection structures within host tissues.

Thomas and Ravindra (1997) and Chandra et al. (2004) tried to remove deep seated endogenous contamination by frequent sterilization but were not able to check these completely. The isolation and identification of endophytic organism(s) would enable to use specific antibiotics against contaminants for maintaining aseptic cultures of mango (Reuveni and Golubowicz, 1997). Hare Krishna (2006) suggested application of imidazole sprays thrice at the interval of three d to field-grown stock plants to reduce the contamination load. Spray with imidazole alone was superior to sprays of bavistin and streptomycin used alternatively at the interval of two d. Besides reducing the infection, imidazole spray delayed the expression of necrosis in shoot tip cultures of monoembryonic mango cvs. Amrapali and Pusa Arunima.

2.3. *In vitro* recalcitrance

Recalcitrance is the inability of plant cells, tissues and organs to respond to tissue culture. Tissue culture responses are greatly influenced by three main factors viz., ‘whole plant’ physiology of donor, *in vitro* manipulation and *in vitro* stress physiology (Benson, 2000).

It would be worthy to collect explants from healthy and well characterized donor plants. However, the choice of donor plants may be limited particularly in mango where successful regeneration was only observed in immature zygotic nucellar embryos/(juvenile tissue), while other explant such as shoot tips, nodal segments, leaf (mature tissues), etc. showed limited success. The reason being juvenility of embryo and nucellar tissues. Though, the success is greatly influenced by genotype (Thomas, 1999); nevertheless, growth status of donor plants; and developmental stage of the embryo on isolation (Laxmi et al., 1999) are also of immense importance. Sometimes, it becomes inevitable to use mature tissues as explanting in former case is dependent on a narrow window of opportunity during the year in which immature fruits are at the appropriate stage for explanting the nucellus.

Recalcitrance of mature explants is exhibited at all stages of culture. For these systems, the selection of explants at a specific responsive stage of a mature tree’s life cycle is of utmost importance to overcome recalcitrance (Benson, 2000). From their results, Thomas and Ravindra (1997) suggested the use of nodal segments from current season’s shoots for better survival, least phenolic

exudation and growth response. Their findings were in line with those reported by Shahin et al. (2003). On the contrary, Yang and Ludders (1993) were able to regenerate shoots from shoot tips. The stock plants were maintained under glasshouse conditions and best results were obtained by explanting in May–June than other months.

In vitro manipulation also exerts a great impact on modification of recalcitrance. Maneuvering different components, such as inorganic, organics, amino acids, enzymes, phytohormones, carbon source, gelling agents and other media additives helps alleviating recalcitrance. One of the most important approaches for overcoming this problem is to optimize the plant growth regulators regime (Gaspar et al., 1996). Raghuvanshi and Srivastava (1995) used different combinations of auxin and cytokinin for instance NAA, IAA, BA and kinetin in MS medium to obtain multiple mango shoots from callus derived from mature leaf explants.

Other important factors which affect the recalcitrance of mango tissues include light regime and *in vitro* plant stress physiology. Maintaining mango culture in dark has been suggested by several workers as discussed elsewhere. The most important *in vitro* stress factor is oxidation of explants on culture owing to high phenol content. Compared to many other crops, it is more prevalent in mango, which has high level of phenols associated with secondary thickening and lignification. The oxidation of phenolic compounds in cut explants is a wound response and culture techniques, such as surface sterilization, may enormously exacerbate this.

3. *In vitro* propagation

Mango genetic engineering requires an efficient *in vitro* regeneration system. Furthermore, *in vitro* propagation also facilitates rapid multiplication of superior clones within a short span. Polyembryonic mango genotypes particularly those which are exploited as rootstocks for their desirable attributes are exclusively propagated by seed that give rise to limited number of clonal seedlings; though identical to mother plant. This is another area, where potentialities of micropropagation can be explored to resolve the problem of clonal rootstocks. Mango tissues darken very quickly *in vitro* as a result of action of enzyme polyphenol oxidase activity (Litz and Vijayakumar, 1988). Work on *in vitro* propagation of mono- and polyembryonic mango cultivars is summarized in Table 1.

3.1. *Somatic embryogenesis*

In comparison to other horticultural crops, mango has been a relatively “hard to deal crop” for tissue culture.

Table 1
Summary of work on somatic embryogenesis and organogenesis in mango

Cultivar (s)	Explant	Response	PGRs	Media	Reference
Cambodiana, Carabao, M20222, Turpentine N2-1-4-3, Earlibush, Chino, Turpentine N2-1-7-2, Sabre and Ono	Ovules	Somatic embryogenesis (SE) and precocious germination	With or Without 1–2 mg l ⁻¹ ; 20% coconut water	Modified MS medium+ 400 mg l ⁻¹ glutamine+ 100 mg l ⁻¹ ascorbic acid	Litz et al. (1982)
Brooks, Golden Brooks, Irwin, Keitt, Kent, Ruby, Sensation, Tommy Atkins and Van Dyke Parris and Saigon	Nucellus Ovular halves	SE and plantlet regeneration SE	1.0 mg l ⁻¹ 2,4-D 4.5 μM 2,4-D	Modified MS medium+ 400 mg l ⁻¹ glutamine+ 100 mg l ⁻¹ ascorbic acid Modified B ₅	Litz (1984) Dewald et al. (1989a)
Hindi	Ovular halves	Inhibition of precocious germination and normal somatic embryo maturation	500 μM ABA	Modified medium (B ₅ major salts+MS organics and micronutrients)	Monsalud et al. (1995)
Amrapali	Leaves	Indirect organogenesis and plantlet regeneration	1.1 μM IAA+ 13.0 μM Kinetin+ 1.0% PVP	MS medium	Raghuvansi and Srivastava (1995)
Hindi, Nam Doc Mai, Lippens, and Tommy Atkins	Nucellus	Induction of somatic embryogenic competence	4.52 μM 2,4-D	Modified B ₅	Litz et al. (1998)
Amrapali	Microshoots from nucellar somatic embryos	Rooting of microshoots	5.0 mg l ⁻¹ ABA	Root initiation medium (Full B ₅ major salts+MS minor and organics)	Ara et al. (1998)
Amrapali	Nucellus	SE and plantlet regeneration from encapsulated somatic embryos	4.5 μM 2,4-D	Modified MS	Ara et al. (1999)
Amrapali	Nucellus	SE and germination	5–20 μM BAP	MS and B ₅ media	Laxmi et al. (1999)
Amrapali and Chausa	Nucellus	SE and plantlet regeneration	1.0 mg l ⁻¹	Modified medium (B ₅ major salts+MS organics and micronutrients)	Ara et al. (2000a,b)
Carabao and Manila Super	Nucellus	SE	–	Modified Medium	Patena et al. (2002)
–	Immature zygotic embryos	Direct SE	5.0 mg l ⁻¹ IBA; 5.0 mg l ⁻¹ kinetin	Modified MS medium	Xiao et al. (2004)
Ambalavi	Nucellus	SE and plantlet regeneration	0.25 mg l ⁻¹ BAP+ 1.0 mg l ⁻¹ NAA; 0.5 mg l ⁻¹ 2ip	Modified medium (BM)	Chaturvedi et al. (2004a)

Nucellus has been utilized as the primary explants. Nucellus derived plants are generally free from viruses and other endophytic disease causing organisms, due to the absence of vascular connection between the surrounding maternal tissue and the nucellus. Therefore, efficient recovery of somatic embryos particularly in monoembryonic mango cultivars would eliminate systemic pathogens and avoid catastrophic loss frequently occurring in clonally propagated genotypes owing to diseases and environmental stresses under tropical environment (Litz, 1984).

This approach has been described in detail both in polyembryonic (Litz et al., 1982, 1998; Dewald et al., 1989a; Patena et al., 2002) and monoembryonic

genotypes (Litz, 1984; Jana et al., 1994; Pliego-Alfaro et al., 1996a; Laxmi et al., 1999; Thomas, 1999; Ara et al., 2000b; Deore et al., 2000; Chaturvedi et al., 2004a,b; Rivera-Dominguez et al., 2004). Though, complaisance has been observed more in the former group. Somatic embryogenesis is a rapid regeneration procedure and is essential for genetic transformation (Mathews et al., 1993).

The induction of embryogenic cultures in mango is primarily dependent upon the morphogenetic potential of the nucellus, a maternal tissue that encompasses the embryo sac. In polyembryonic genotypes, adventitious embryos differentiate from competent cells, which are present within the nucellus (Litz, 2003). Litz et al.

(1982) were first to report induction of embryogenic mango cultures from nucellar tissues of some polyembryonic cultivars after one to two months from ovule cultures excised from 40 to 60 d old fruitlets. Subsequently, the suitable conditions for induction of embryogenic cultures from nucellus of monoembryonic mango cultivars were defined (Litz, 1984). Induction of embryogenic cultures from the excised nucellus of polyembryonic mangoes was described on a medium supplemented with 1–2 mg l⁻¹ 2,4-D, and large numbers of somatic embryos could be produced in liquid medium (Litz et al., 1984).

Relatively low plant recovery from embryogenic cultures was reported in these initial attempts. Numerous developmental anomalies, such as polycotyledony, fasciation, the absence of bipolarity and formation of secondary embryos from hypocotyls were described, which contribute to the failure of embryos to develop to maturity in a normal fashion (Dewald et al., 1989b). In addition, the problems of precocious germination and progressive necrosis have also prevented the efficient recovery of mango plantlets (Dewald et al., 1989a). In an attempt to mitigate these problems, Dewald et al. (1989a) investigated the suitable conditions for optimizing the induction and maintenance of embryogenic cultures and for maturation of somatic embryos in detail. Currently, the protocols employed broadly have four stages, viz., induction of embryogenic culture from nucellus, maintenance of embryogenic culture, maturation and the development of morphologically normal embryos and germination of somatic embryos into well-developed plantlets. Precocious germination of somatic embryos could be minimized by the addition of 3.0 μM ABA and 6.0% (w/v) sucrose. Dewald et al. (1989b) thereafter proposed the suitability of liquid shake culture for the maintenance of embryogenic culture in mango. They observed that modified B5 medium to be optimum for induction with 5 or 6% sucrose and 20% (v/v) coconut water (CW). Also their importance for developing somatic embryos was quantified. Despite the higher rate of embryogenesis from nucellar tissues, it has not given success in cvs. Chaunsa and Anwar Rataul. This was attributed to excessive phenolic exudation from nucellus explant in the medium (Usman et al., 2005).

Different stages of culture initiation and somatic embryo production are presented in Fig. 2.

3.1.1. Induction of embryogenic cultures

Thirty to sixty-day-old fruits, harvested after pollination are suitable for induction of somatic embryogenic culture from the nucellus (Litz et al., 1982; Dewald

et al., 1989a; Pliego-Alfaro et al., 1996a; Ara et al., 1999; Singh et al., 2002; Sulekha and Rajmohan, 2004).

Ara et al. (2004) transferred aseptically dissected monoembryonic ovules to an MS medium consisting half-strength major salts and chelated iron+ full strength microsals and vitamins, 6% sucrose, 400 mg l⁻¹ L-glutamine, 100 mg l⁻¹ ascorbic acid, 0.8% agar and with 1 mg l⁻¹ 2,4-D. The nucellus was removed from the cultured ovules between 1 to 3 weeks after culture, and transferred onto the same medium to prevent browning. The cultures were maintained in dark at 25±2°.

In order to further optimize the somatic embryo production from nucellar tissue in mango, the nucellus was transferred onto a sterile embryo induction medium consisting of B₅ major salts (without ammonium sulphate), MS minor salts and organic components, L-glutamine, sucrose, ascorbic acid, 4.52 to 9.04 μM 2,4-D and 2.0 g l⁻¹ gellan gum (Dewald et al., 1989a; Litz et al., 1993, 1995). Ara et al. (2000b) employed the same medium except the use of half-strength Fe-EDTA in lieu of full-strength and the absence of ascorbic acid in medium. Recently, Chaturvedi et al. (2004a) observed that nucellar tissue under different developmental stages responded differently to the same gelled nutrient medium used for induction of embryogenesis. The youngest fruits (2.5 cm) required 0.25 mg l⁻¹ BAP/1 mg l⁻¹ NAA, while for the oldest fruits (5.0 cm), 0.5 mg l⁻¹ 2iP was sufficient for embryogenesis. Nucellar cultures are incubated in darkness at 25 °C and regularly transferred onto a fresh medium until darkening due to phenol exudation from explant has ceased completely (Litz, 2003). On the other hand, Patena et al. (2002) shifted the basal medium from B5 medium to BP medium (Barba and Patena's formulation) in eight strains of Carabao and two unidentified varieties to effectively control the browning.

Lad et al. (1997) demonstrated that embryogenic competence in 'Carabao' was acquired following exposure to 2,4-D for at least 28 days. Embryogenic nucellar tissue was apparent following culture for approximately 30 d onto induction medium.

The embryogenic response is strongly cultivar dependent. On the basis of their embryogenic response, Litz et al. (1998) classified some varieties as highly embryogenic (polyembryonic Hindi and Parris), moderately embryogenic (monoembryonic Lippens and Tommy Atkins) and difficult-to-regenerate (polyembryonic Nam Doc Mai). They also demonstrated that nurse culture derived from Parris can improve the embryogenic response of non-embryogenic cultures. Likewise, Manzanilla Ramirez et al. (2000) compared the induction responses of three cultivars and observed that 'Ataulfo' (polyembryonic) more embryogenic than

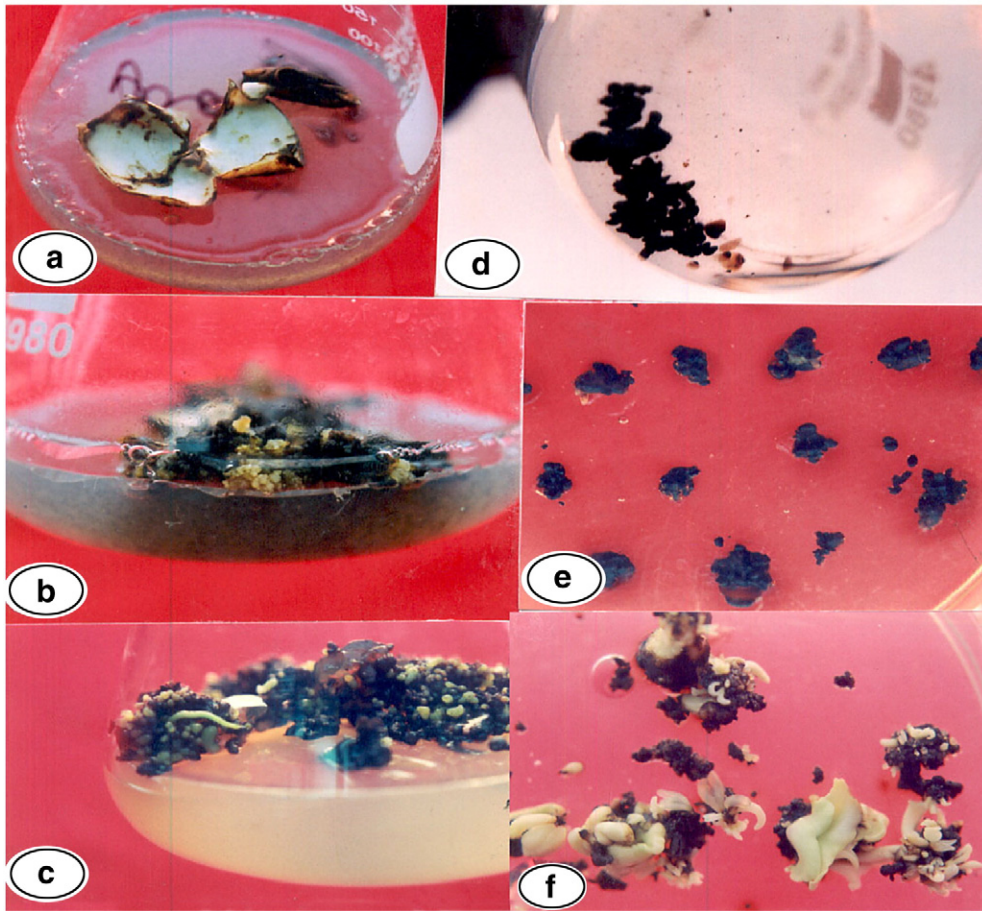


Fig. 2. Culture initiation and callus induction on nucellus in Amrapali (a) In vitro inoculated nucellus explants (b) Callus initiated from nucellus (c) Induction of pro-embryogenic masses on callus (d) Maintenance of embryogenic masses in suspension culture (e) Somatic embryogenesis on growth regulator-free medium (f) Development of heart shaped embryos.

either ‘Tommy Atkins’ (monoembryonic) or ‘Haden’ (Monoembryonic) in that order. [Litz and Yurgalevitch \(1997\)](#) suggested that differential regulation of ethylene biosynthesis and the enzyme spermidine synthase in mango may be major contributing factors in controlling induction of somatic embryos in vitro and confirmed the effects of spermidine in induction of embryogenic competence in mango cultures.

3.1.2. Maintenance of embryogenic cultures

Embryogenic mango culture consists entirely of pro-embryogenic masses (PEMs). Sustained proliferation in embryogenic cultures in most cultivars is possible on induction medium ([Litz, 2003](#)); however, regular subculture at 3 to 4 week intervals is essential to prevent darkening of tissues. Synchronization of embryogenic cultures is difficult to achieve on semi-solid medium due to establishment of polarity within the culture relative to the accessibility of 2,4-D.

Embryogenic cultures of many cultivars exhibit significantly higher proliferation in suspension. Culture darkening was slower in liquid than on solid medium ([Dewald et al., 1989a](#)). In addition, embryogenic suspension cultures can be synchronized according to size of PEMs by passage through different grades of filtration fabrics ([Litz and Gomez-Lim, 2005](#)).

Both cytokinin and auxin are important for proliferation and maintenance of mango globular embryogenic masses ([Litz, 2003](#)). Though, cytokinin is not essential for induction, but is important for stimulating organization of the apical meristem during maturation. [Deore et al. \(2000\)](#) reported good callus induction in medium supplemented with 0.1 mg l^{-1} kinetin plus 2.0 mg l^{-1} 2,4-D. Embryogenic cultures of ‘Paris’ grown in liquid maintenance medium containing 4.0 to $8.0 \text{ }\mu\text{M}$ 2,4-D and $4.6 \text{ }\mu\text{M}$ kinetin produced significantly early and higher number of heart shaped somatic embryos following subculture onto solid medium ([Dewald et al.,](#)

1989a). Ara et al. (2004) suggested the use of 1.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} NAA, either alone or with 1.0 mg l^{-1} kinetin for enhancement of proliferation of the pro-embryogenic cultures (PEC) in liquid as well as solid media; however, proliferation was around five times more profuse in liquid medium. Nevertheless, the dedifferentiation followed by callusing of globular somatic embryos was observed in liquid medium, while on semi-solid medium, the globular somatic embryos successfully developed into cotyledonary stage.

Moreover, developmental abnormalities, e.g. poly-cotyledony, the absence of polarity, vitrification etc. are encountered in somatic embryos that developed in liquid medium in comparison with solid maintenance medium (Dewald et al., 1989b). Gross developmental abnormalities in mango somatic embryos can be prevented up to some extent by careful manipulation of the culture conditions during PEM maintenance and somatic embryo development.

Maintenance as suspension culture as well is cultivar dependent (Litz et al., 2000). Subsequent proliferation of embryogenic cultures has been achieved by production of secondary embryos from PEMs (Dewald et al., 1989a; Litz et al., 1993). Over the time, gradual loss of morphogenetic potential of embryogenic culture occurs.

3.1.3. Maturation of somatic embryos

Important obstacles to normal development of somatic embryos include precocious germination, secondary embryogenesis, etc. from hypocotyl of germinating embryos and the absence of biopolarity. For subsequent germination of somatic embryos into normal seedlings the control of developmental changes is indispensable.

The inductive phase mediated by 2,4-D is necessary for establishment of an embryogenic culture in mono-embryonic mango genotypes. Though, persisted presence of 2,4-D in maintenance medium exerts a considerable negative influence on the somatic embryos production by hindering its development beyond globular stage. Zimmerman (1993) was of the opinion that new gene products are required for the progression from the globular to heart shape stage and synthesis of these products are triggered only in the absence of auxin.

Initiation of maturation including the development of bipolarity in globular embryos followed by the differentiation of cotyledons can be stimulated by transfer of embryogenic cultures from maintenance to maturation medium. Litz and Gomez-Lim (2005) suggested the use of filtration fabric with $1000 \mu\text{m}$ opening size to decant suspension culture for separation of smaller fractions, which are to be transferred either into liquid or onto semi-solid media without 2,4-D in order to arrest re-

petitive somatic embryogenesis and to initiate somatic embryo development. After subculture on medium without 2,4-D, the advanced development stages of somatic embryos were observed. Laxmi et al. (1999) as well observed maximum embryo production on half-strength MS medium supplemented with $20.0 \mu\text{M}$ BAP devoid of 2,4-D.

Maturation of somatic embryo often has been accompanied by gradual necrosis of cotyledon and hypocotyl. Addition of malt extract, casein hydrolysate and reducing agent is not effective (Litz, 1984), while yeast extract was inhibitory (Dewald et al., 1989a). Medium supplemented with coconut water (20% v/v) delayed the necrosis and enhanced the production of somatic embryos (Litz, 1984; Dewald et al., 1989a). According to Dewald et al. (1989a), the maturation medium consisted of modified B₅ major salts, MS minor salts and organics, L-glutamine, casein hydrolysate and 20% (v/v) coconut water.

Among the different concentrations of sucrose tried, a concentration of 6.0% was generally most effective, although ABA in combination with coconut water stimulated a higher production frequency of normal somatic embryos in medium supplemented with 3.0% sucrose (Dewald et al., 1989b). Singh et al. (2001) suggested addition of $100 \mu\text{M}$ ABA to hormone free regeneration medium to improve frequency of embryogenesis as well as percentage of normal bicotyledonary embryo induction.

Maturation of somatic embryos transformation is usually controlled either by embryogenic cultures on semi-solid or liquid medium. Application of growth regulators particularly cytokinin further aids in this process. Somatic embryo development in 'Hindi' occurred in a suspension culture, whereas 'Carabao' showed the response onto semi-solid medium. The apical meristem of globular embryo organizes faster when the maturation medium is supplemented with either kinetin or 6-benzylamino purine at 1 to 3 mg l^{-1} or when incubated in darkness at $25 \text{ }^\circ\text{C}$ (Litz et al., 1993; Lad et al., 1997). Addition of kinetin at $4.65 \mu\text{M}$ or benzyl adenine (BA) at $4.44 \mu\text{M}$ to the maturation medium stimulated the development of cotyledons and helped hastening maturation period (Litz and Gomez-Lim, 2005).

Maturation of nucellar embryos excised from poly-embryonic ovules and raised in vitro was reported to be arrested by pulses of 750 to $1750 \mu\text{M}$ ABA, 7.5 to 12.5% mannitol and combination of both (Pliego-Alfaro et al., 1996a). The absence of developmental arrest of embryos is necessary as lack of this characteristic in recalcitrant seeds has created the need to establish large

living germplasm repositories since these cannot be stored.

When *in vitro* systems for highly embryogenic cultivars are optimized as suspension cultures, the early cotyledonary somatic embryos, which develop in suspension, are hyperhydric. Somatic embryos that demonstrate this physiological disorder are unable to reach maturity (Mathews et al., 1992; Monsalud et al., 1995) and ultimately become necrotic. This has also been a limiting factor for the development of highly embryogenic suspension cultures.

Monsalud et al. (1995) achieved reversion of hyperhydricity of mango somatic embryos in two ways; partial dehydration of heart shaped somatic embryos under controlled conditions at high relative humidity for 24 to 48 h and by increasing the concentration of gelling gum from 2 to 6%.

Precocious germination, another snag in mango somatic embryogenesis can be inhibited by addition of 100 μM ABA by Pliego-Alfaro et al. (1996b). The control of precocious root formation by the use of ABA was also described by Monsalud et al. (1995). Transfer of somatic embryos from medium containing ABA onto medium devoid of it stimulates highly synchronized germination, irrespective of the embryo developmental stages. Thomas (1999) also obtained embryo maturation in the presence of ABA at 1.0 mg l^{-1} . Ara et al. (1999) observed that ABA at 0.004 and 0.02 μM had no significant effect on germination percentage and plantlet development but delayed the germination by three weeks.

Thomas (1999) suggested reduced application of sugar with 1.0 mg l^{-1} ABA for the maturation of early or late heart stage and early cotyledonary embryo. Litz and Gomez-Lim (2005) also advocated reduction in concentration of sucrose in maturation medium from 6 to 4%. The sucrose concentration is gradually reduced to 1.0% during sequential subculture to fresh medium owing to different requirements of developing embryos from heart stage to complete maturity stage. Furthermore, cessation of mango somatic embryo elongation at maturity has also been observed in the presence of high sugar concentration.

3.1.4. Germination and conversion into normal plantlets

The germination medium contains no filter sterilized coconut water and has reduced sugar concentration, i.e. 2.0% (Litz, 2003; Thomas, 1999). Laxmi et al. (1999) also advocated the lowering of sucrose concentration and addition of GA_3 and N^6 -benzylamino purine for improved somatic embryo germination. Germination was achieved on a medium with B5 major salts, MS

minor salts and organics. The germination of a bi-cotyl embryo of mango cv. Amrapali is presented in Fig. 3.

Growing conditions consist of a 16 h photoperiod provided by cool white fluorescent light (40–60 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 25 °C. The conversion efficiency could be enhanced by high light intensity, i.e. 160 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in a CO_2 enriched atmosphere (20,000 ppm) (Litz, 2003).

Converting somatic embryos to ‘Synthetic seed’ or ‘Synseeds’ by encapsulation could possibly be utilized as a means for germplasm storage and transportation of elite germplasm. The desiccated somatic embryos can be stored for several months without any significant decline in their germinability e.g. in alfalfa, somatic embryos desiccated to 10–15% were stored at room temperature for 1 year (McKersie and Bowley, 1993). In contrast, being a recalcitrant crop, mango somatic embryos cannot be desiccated to the extent that alfalfa were dried. In mango, the partially dehydrated immature somatic embryos (4–7 mm long) remained viable for 32 d in the absence of maturation medium under high RH (Monsalud et al., 1995). The viability (%) of stored somatic embryos was assessed by calculating percentage embryos germinated on inoculation/germination medium after a certain period. However, by diligent manipulation of different factors such as somatic embryo induction medium (Gill et al., 1994), dehydration, ABA concentration (Monsalud et al., 1995), gelling agents, encapsulation medium, addition of fungicides/preservatives (Datta et al., 1999) etc., it might be possible to store partially dehydrated immature, recalcitrant somatic embryos for medium- to long-term storage. Ara et al. (1999) attempted encapsulation of cotyledonary stage somatic embryos regenerated from

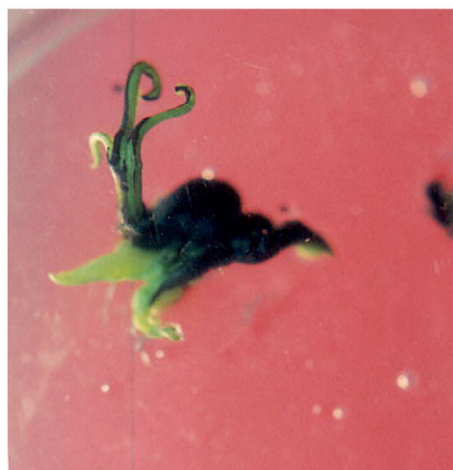


Fig. 3. A germinating bi-cotyl embryo.

nucellar tissue individually in 2.0% alginate gel. Successful plants regeneration occurred when encapsulated somatic embryos were germinated on agar gelled medium containing B5 macro salts (half strength), MS microsalts (full strength), 3% sucrose and 2.9 μM gibberellic acid.

The physical state of the germination medium exerts a great influence on percent germination and subsequent conversion of somatic embryos. Ara et al. (1999) reported successful germination of encapsulated somatic embryos on 0.6% agar gelled semi-solid medium supplemented with full-strength B5 macro salts. Simultaneous encapsulation with sodium alginate helped in increasing the conversion rate of mango somatic embryos. The beads when plated on standard mango basal medium supplemented with 2.0 to 9.0 μM gibberellic acid. Of the different gelling agents, gelrite (Dewald et al., 1989a,b) and phytagel (Laxmi et al., 1999) were superior to agar–agar with regards to germination of somatic embryos. However, Chaturvedi et al. (2004b) stressed upon the need to use liquid state of nutrient medium for near-synchronized development, maturation, germination (visible plumule with developed root) and convertibility (plantlet formation) of cotyledonary embryos. The presence of 0.1 mg abscisic acid/litre together with 100 mg polyethylene glycol and 0.1 mg IAA/litre in the basal BM2 medium promoted development and maturation of embryos followed by their germination. The maximum percentage of germination obtained was 94% with embryos of 1.5 and 2 cm in length. Though, only 80% of the germinated embryos converted into plantlets, with well-developed green leaves, on subculture in the same medium in the former case, while 76% in the latter. The in vitro-raised plantlets showed approximately 50% success in hardening.

Recently, Xiao et al. (2004) reported direct somatic embryogenesis and plantlet regeneration from cotyledon of immature zygotic embryos. PEM-like structures were directly induced on modified MS medium with 25 μM IBA. Later, conversion of somatic embryos was accomplished on a medium containing 23 μM kinetin. Besides, secondary somatic embryogenesis could also be achieved on hypocotyls of mature primary somatic embryos cultured on the conversion medium.

Earlier, Ara et al. (2000a) described a protocol for regeneration of mango plantlets isolated from PEMs in a suspension culture derived from the nucellar callus of mango cv. Amrapali. The dividing protoplasts were transferred to a medium with growth regulators for development of microcalli, which later produced somatic embryos. The mature somatic embryos were ger-

minated into well-developed seedlings and subsequently transferred to soil.

3.2. Organogenesis

As for rooting from field-grown shoots, it is very hard to regenerate rooting on explants, which is attributed to high phenolic exudation and systemic contamination during culture establishment. The first report on organogenesis was by Rao et al. (1981), who induced roots from callus that was initiated from mango cotyledons on MS medium supplemented with kinetin and NAA; however, development of shoots was not observed. Later, Singh et al. (1991) reported induction of callus on different explants such as epicotyl segment, leaf petiole and shoot tip excised from aseptically germinated embryo. Maximum callus was recorded on epicotyl segment, while direct root organogenesis was noted in epicotyl and shoot tip culture with low level of 2,4-D alone.

Thomas and Ravindra (1997) attempted to establish shoot tip culture in some mango genotypes. Their study indicated that several problems such as phenolic exudation, medium discolouration and explant browning are interrelated and are influenced by different factors like medium, genotype, explant, season and decontamination treatment. Browning was overcome by the use of different media additives but deep seated, systemic contamination could not be checked completely. Furthermore, frequent decontamination treatment to explant often stimulated the phenolic exudation in the medium.

The problems associated with field-grown shoots can be obviated by utilizing in vitro grown shoots, which are more responsive to culture conditions/more amenable to in vitro culture.

Ara et al. (1998) devised a two step protocol for in vitro rooting of microshoots obtained from nucellar somatic embryos. Of the three auxins tried, IBA was found most effective with respect to rooting and root growth. Good rooting was accomplished on an auxin-free medium following a 24 h pulse treatment with 5.0 mg l^{-1} IBA.

Resorting initially to somatic embryogenesis preceding rooting of microshoots obtained from somatic embryos compromises the chance of this approach to be used for commercial purpose. The balance between the use of explant derived from field-grown trees and in vitro grown shoots can be struck by employing greenhouse grown shoots.

Reuveni and Golubowicz (1997) tried the small internodes of greenhouse grown mango for culture initiation but were not able to promote shoot formation in established cultures. Hare Krishna (2006) also tried

glasshouse raised shoot segments of cv. Amrapali but was able to achieve only callusing on cut end and sprouting of axillary bud. Further shoot proliferation could not be achieved and cultures survived for more than six months. When, cotyledonary nodes of in vitro germinated seedlings of Kurukkan were used initiation of shoots along with callusing on cut end was observed. These cultures also survived for more than six months.

Yang and Ludders (1993) employed the shoot tips of greenhouse rootstocks for in vitro culture. Shoot proliferation was found to be superior in G medium containing a combination of BA, zeatin, 2iP, IAA and IBA than B5 or Woody Plant Medium (WPM). On the contrary, Shahin et al. (2003) reported that stem node explant was better than shoot tips with respect to shoot proliferation. The highest proliferation was noted on modified WPM supplemented with 20 g l^{-1} sucrose, 30 mg l^{-1} adenine + 2 mg l^{-1} iso-pentyladenine + 0.5 mg l^{-1} IBA + casein hydrolysate or 30 mg l^{-1} adenine + 0.2 or 0.5 mg l^{-1} IBA. Modified WPM medium supplemented with 30 mg l^{-1} adenine + 1.0 mg l^{-1} BA + 4.0 mg l^{-1} IAA registered highest rooting percentage and root number.

Earlier, Raghuvanshi and Srivastava (1995) explored the morphogenic potential of mature leaf explants. Caulogenic callus was initiated and later subcultured onto MS medium for induction of multiple shoot formation. Liquid shake culture was used to reduce phenolic exudation and subsequent necrosis of explants. Multiple shoots were separated and transferred to rooting medium. Only 20% of the cultured explants developed roots on medium supplemented with $9.8 \mu\text{M}$ IBA.

3.3. Embryo culture

Mango suffers from heavy fruit drop and hence embryo culture can improve breeding efforts (Iyer and Subramanyam, 1971). Several factors associated with fruit drop include competition among developing fruitlets, deficient nutrition, moisture stress, hormone imbalance, climatological factors like high temperature, rains during flowering, hailstorms, high wind velocity, varietal factor, lack of fertilization and attack of diseases and pests (Sawke et al., 1990). Research done in this aspect shows that the region of abscission layer formation is pre-determined. Cells are detached in abscission zone by dissolution of middle lamella (Chadha, 1959) and abscission zone undergoes cell wall breakdown because of an increase in cell wall-hydrolyzing enzymes such as cellulose, polygalacturonase, uronic acid oxidase etc. (Sexton et al., 1984; Huberman and Goren, 1982). Later, the xylem tissues are torn by fruit weight. Apart from these, the involve-

ment of ethylene in promoting abscission has been proved unequivocally (Morgan, 1984). The changes in the levels of different hydrolytic enzymes and ethylene could be accompanied by changes in m-RNA governed by abscission specific gene(s); though no abscission specific gene(s) have been identified in mango. A very useful genetic approach that could provide direct evidence for the involvement of abscission specific gene(s) is based on transgene technology. Transgenic plants expressing antisense version of various gene(s) that encodes enzymes involved in degradation of cell wall and middle lamella and synthesis of ethylene. Such plants will produce a very low level of hydrolytic enzymes and enzymes associated with ethylene biosynthetic pathway; therefore, resulting in reduced abscission of fruitlets. Another approach could be transformation of plants with Arabidopsis mutants *etr1* (ethylene resistant) and *ein 2* (ethylene insensitive), which are insensitive to ethylene (Bleecker et al., 1988; Guzman and Ecker, 1990). These mutants have expression of mutated genes under the transcriptional control of their own promoters. This could facilitate the significant reduction in fruit drop of mango. In mango, the technique using immature fruitlets (35–45 day-old) for in vitro regeneration was shown to significantly improve the breeding efficiency in mango (Nathhang, 1999). Chandra et al. (2003a,b) were successful in regenerating immature mango embryo into complete plantlet on MS basal medium supplemented with 9 mg l^{-1} BA + 3 mg l^{-1} kinetin + 400 mg l^{-1} glutamine + 500 mg l^{-1} activated charcoal + 60 g l^{-1} sucrose. Complete plantlet formation was observed in 72% of the cultures. Sahijram et al. (2005) suggested collection of mango fruitlets at 6–8 weeks post-pollination for embryo culture. Hybrid embryos were aseptically excised from immature ovules and thereafter, inoculated in vitro onto semi-solid half-strength modified MS medium containing casein hydrolysate (1.25 g l^{-1}) and sucrose (4.5%). After 12–14 weeks, well-developed seedlings were transferred to non-sterile conditions (tap water in parent culture vessels) under identical environmental conditions for initial hardening-off.

4. Somaclonal variation/in vitro selection

Somaclonal variations could be of great value to the breeders as it is an efficient tool to create variations. Single gene mutations may result in the alteration of a significant horticultural trait and therefore, may give rise to the best available variety in vitro with improved targeted character. Despite the advantages somaclonal

variations proffer, it has been unable to leave a significant impression on mango breeding via development of advantageous off-types of existing selection, which lack in some useful attributes. In order to recover useful off-types of existing cultivars, Litz (2001) subjected embryogenic cultures of three mango cultivars viz., Hindi, Keitt and Tommy Atkins to 0–200 Gy irradiation provided by ^{60}Co . The median lethal doses (LD_{50}) were approximately 125 and 100 Gy for Keitt and Tommy Atkins; however, the LD_{50} for Hindi could not be determined within the given dosage range.

The technique of in vitro selection (fungal toxins) using phytotoxic metabolite(s) has been demonstrated in mango (Litz et al., 1991). Similarly, Jayasankar (1995) challenged the embryogenic cultures of cvs. Hindi and Carabao with purified culture filtrates of *Colletotrichum gloeosporioides* and after several subcultures under stress, tolerant lines could be developed but they lost the further regenerability. In a later study, Jayasankar and Litz (1998) attempted to characterize embryogenic mango cultures selected for resistance to *C. gloeosporioides* culture filtrate and phytotoxin. The resistant embryogenic cultures of cvs. Hindi and Carabao were recurrently selected either with progressively increasing concentrations of culture filtrate or by continuous challenge with the same concentration of either culture filtrate phytotoxin. Mycelium growth was inhibited when the pathogen was co-cultured with the selected, resistant embryogenic mango culture. Conditioned plant growth medium containing macerated resistant embryogenic cultures did not inhibit mycelial growth, confirming that extracellular antifungal compounds were involved in the defense response.

5. Molecular biology

Molecular approaches offer an efficient alternative tool to conventional breeding. It is very useful for characterizing the genetic diversity/relatedness among different cvs. or species of mango, for identifying genes of commercial interest, improvement through gene transfer technology, creation of variations in existing cvs. in vitro, overcoming reproductive isolation barrier via protoplast fusion etc. Some of the important achievements made in mango breeding employing biotechnological tools are presented in Table 2.

5.1. Identification of cultivars

Allopolyploidy, out breeding, wide range of agro-climatic conditions prevailing in different mango

growing areas, widespread hybridization and recombination of characters have contributed immensely to the existing diversity in mango (Ravishankar et al., 2000). In addition, there has been a great degree of confusion in the nomenclature of mango varieties due to the use of synonyms for a single cultivar, which add difficulties in identifying varieties. Moreover, for efficient and effective utilization of plant genetic resources, the characterization of germplasm is inevitable. Though, morphological markers have been in use to assess the genetic diversity, they had limited application in breeding as they are few in numbers as well as season and developmental stage specific/dependent. The use of molecular markers, which comprise isozyme and DNA markers can be used for cultivar identification. Another promising application could be marker aided selection (MAS) to expedite the breeding programme. Fang et al. (2000) studied polymorphism and segregation pattern of AFLP markers in the F_1 progenies derived from cross of mango cultivars viz., Keitt and Tommy. They observed high polymorphism in F_1 progenies, while the average frequency of segregation was 37.16%.

Gan et al. (1981) reported for the first time the use of isozymes to note the genetic variation in mango cultivars. Later, Degani et al. (1990) working on different enzymes viz., GPI (EC 5.3.1.9), TPI (triosephosphate isomerase) (EC 5.3.1.1), LAP (leucine aminopeptidase) (EC 3.4.11.1), IDH (isocitrate dehydrogenase) (EC 1.1.1.42), PGM (phosphogluco mutase) (EC 2.7.5.1) and ACO (1-aminocyclopropane-1-carboxylic acid oxidase) (EC 4.2.1.3) identified six loci with 17 allelomorphs in 41 mango cultivars derived from self-pollinated and open-pollinated trees. It was also possible to find the disparity in the parentage of many cultivars. Schnell and Knight (1992) used five isozymes viz., IDH (isocitrate dehydrogenase), LAP (leucine aminopeptidase), GPI (glucose-6-phosphate isomerase), PGM (phosphogluco mutase) and TPI (triosephosphate isomerase) to differentiate the seedlings derived from zygotic embryos from polyembryonic cultivars. Degani et al. (1992) later demonstrated two separate zones of PGI (phosphogluco isomerase) activity, i.e. PGI-1 and PGI-2 and suggested that PGI-2 is controlled by four alleles. Later, Jintanawongse and Changtragoon (2000) used several enzyme systems to identify mango hybrids and true hybrids resulting from hybridization using eleven isozyme systems.

In addition to identification and characterization of diversity in mango, the modern biotechnological tools can be utilized for determining etiology of dreaded diseases, which may sabotage mango cultivation if not

Table 2
Achievements made in mango breeding through molecular approaches

Biotechnological tool	Achievement	Reference
Molecular markers		
Isozyme markers	Assessment of genetic variation in mango using isozyme markers Identification of different loci with 17 allelomorphs in 41 cultivars Identification of mango hybrids resulting from hybridization	Gan et al. (1981) Degani et al. (1990) Jintanawongse and Changtragoon (2000)
DNA marker		
RAPD	Characterization of fungal toxin tolerant mutants Examination of genetic diversity in mango cv. Kensington Assessment of genetic diversity in land races and advanced cultivars Grouping of varieties based on their similarities and divergence	Jayasankar et al. (1998) Bally et al. (1996) Karihaloo et al. (2003) Souza and Lima (2004)
RFLP	Confirmation of phylogenetic relationship and geographic distribution of <i>Mangifera</i> sp. from Thailand Estimation of genetic relatedness among some poly- and mono-embryonic cultivars	Eiadthong et al. (1999b) Kumar et al. (2001)
VNTR	DNA finger printing and genetic analysis of some mango genotypes and hybrids	Adato et al. (1995)
ISSR	Identification of primer sequences showing clear polymorphism between cvs. grown in Australia	Gonzalez et al. (2002)
SSR	Identification and genetic variation in mango cvs. of Thailand Assessment of diversity in mango germplasm	Eiadthong et al. (1999c) Duval et al. (2005)
AFLP	Estimation of genetic relationship and diversity of mango species	Yamanaka et al. (2006)
Gene cloning	Isolation of peroxisomal thiolase cDNA Isolation of cDNA, which codes for alternate oxidase Isolation of novel ripening specific cDNA clone from mango fruits Construction of cDNA using purified m-RNA of endo-polygalacturonase from mango fruits	Bojorquez and Gomez-Lim (1995) Cruz-Hernandez and Gomez-Lim (1996) Lycett et al. (1997) Suntornwat et al. (2000)
Genetic transformation	<i>Agrobacterium</i> mediated genetic transformation of mango embryogenic cultures Transformation of cv. Hindi with <i>Agrobacterium tumefaciens</i> containing ACO, ACS and alternate oxidase in antisense fashion Optimization of a system for transient and stable transformation studies	Litz et al. (1990), Mathews et al. (1992, 1993) Cruz-Hernandez et al. (1997) Cruz-Hernandez et al. (2000)

controlled in time. One of such disease is mango malformation, which appears both at vegetative and flowering stages. It is inevitable to confirm etiology of disease before working out for its control. Although, confusion exists regarding the etiology of the disease; Freeman et al. (1999) provided unequivocal evidence that the *F. subglutinans* is indeed a causal agent of mango malformation by transforming wild isolates of pathogen with GUS (beta-glucuronidase) reporter and hygromycin resistant gene followed by their inoculation into healthy mango (cv. Kent) vegetative and reproductive buds. After 6–8 weeks after inoculation, typical symptoms of malformation were observed. The presence of GUS-stained mycelium of pathogen within plant organs further corroborated the evidences. Later, genetic diversity among 74 *F. subglutinans*-like isolates from malformed mango in Brazil, Egypt, Florida (USA), India, Israel and South Africa was examined by Zheng and Ploetz (2002). Elsewhere, in Pakistan Iqbal et al.

(2006) estimated the genetic diversity in 20 isolates of *Fusarium* collected from 14 locations using RAPD technique. The isolates were grouped into two main clusters, comprising 13 and 7 isolates, respectively. Within the clusters, the isolates were not essentially related either by geographic origin or by the mango cultivars from which they were isolated. This suggests that some of the infection arose from geographically distant areas, which indicates that infections may have occurred in nurseries prior to plants being transported to various places for subsequent cultivation. Earlier, aiming to characterize *G. fujikuroi* var. *subglutinans* isolates associated with mango malformation using histone H3 gene sequencing and to compare them with other isolates in the *G. fujikuroi* complex, Steenkamp et al. (2000) analysed histone sequence data, which revealed the presence of two phylogenetically distinct groups of *G. fujikuroi* var. *subglutinans* isolates associated with mango malformation. Further, they considered the

identity of the two groups of isolates associated with mango malformation and determined their relatedness to other *Fusarium* spp. For this purpose, portions of the beta-tubulin gene were sequenced and compared with the beta-tubulin sequences. Based on beta-tubulin and histone H3 sequencing, the second group of isolates is conspecific with the *G. fujikuroi* var. *subglutinans* strains previously reported to be the causal agent of mango malformation. With nitrate-nonutilizing (nit) auxotrophic mutants, seven vegetative compatibility groups (VCGs) were identified. RAPD profiles generated with arbitrary decamer primers were variable among isolates in different VCGs, but were generally uniform for isolates within a VCG. In PCR assays, a 20-mer primer pair amplified a specific 448 bp fragment for isolates of *F. sacchari* from sugarcane (MP-B) and what was probably *F. circinatum* (pine, MP-H). A second pair of 20-mer primers was developed from a unique fragment in the RAPD assays, which amplified a specific 608 bp fragment for 51 of 54 isolates from mango (all but the three Brazilian isolates). It also amplified a smaller, 550 bp fragment from isolates of *F. nygamai* (MP-G), but did not amplify DNA of isolates of any other taxon of *Fusarium* that was tested. Besides assessment of genetic diversity in *F. subglutinans*, other plant pathogens for instance *Colletotrichum gloeosporioides*, which causes mango anthracnose have also been examined for genetic diversity employing RAPD analysis (Davis, 2000).

Apart from characterization and assessment of genetic diversity among pathogens in mango, work has also been carried out on characterization of toxins produced by pathogenic organisms such as *Pseudomonas* (Arrebola et al., 2003). Cazorla et al. (2003) reported the production of an antimetabolite toxin (named mangotoxin), primarily by strains of *Pseudomonas syringae* pv. *syringae* pathogenic to mango. Mutants impaired in the production of mangotoxin were constructed followed by sequencing of Tn5 flanking regions of 9 non/low-producers mutants, which revealed that several genes codifying for putative acetyltransferase, non-ribosomal peptide synthetase and the global regulators proteins GacA and LemA could be involved in mangotoxin production.

5.2. DNA markers

The presence of a low level of polymorphism prompted workers to rely more on DNA markers. Different DNA markers have been employed in mango to study monogenic and polygenic traits. As it is a tropical fruit tree, the isolation of a sufficient quality of genomic DNA for use in PCR-based DNA marker technology very

often poses severe problems due to the presence of inhibitors such as polysaccharides, which inhibit the enzymatic DNA processing or phenolics as inhibitors of PCR reactions (Ramirez et al., 2004). The well-established CTAB protocol of Doyle and Doyle yielded excellent DNA templates for PCR amplification for mango (Ramirez et al., 2004). Recently, Gomathi et al. (2005) reported a simple and efficient method for isolation of DNA from of healthy and malformed floral tissues of mango (*M. indica* L.).

5.2.1. Variable number tandem repeat sequence (VNTRS)

Adato et al. (1995) analysed DNA fingerprint patterns of some mango genotypes using minisatellite multilocus probes. The human Jeffery's minisatellite probe resulted in most polymorphism with DraI. They also did genetic analysis of the progeny of the cross 'Tommy Atkins × Keitt' and the six bands of the parents showed polymorphism and the average transmission frequency was 65 and 81% for maternal and paternal specific bands.

5.2.2. Single sequence repeats (SSRs)

Simple sequence repeats or SSRs, also known as microsatellites as DNA markers are advantageous over many other markers as they are highly polymorphic, highly abundant, co-dominant inheritance, analytical simple and readily transferable. Microsatellites are reported to be more variable than RFLPs or RAPDs, and have been widely utilized in plant genomic studies (Weber, 1990; He et al., 2003).

Microsatellite markers to study genetic diversity in mango (*M. indica* L.) were developed using a genomic library enriched for (GA)_n and (GT)_n dinucleotide repeats. Nineteen simple sequence repeat (SSR) loci with clear scorable patterns were chosen to assess diversity in the mango germplasm bank of Guadalupe (FWI). The number of alleles ranged from three to 13 with observed levels of heterozygosity ranging from 0.059 to 0.857 (Duval et al., 2005). Eiadthong et al. (1999c) examined twenty two mango cultivars for 40 SSR anchored primers of 15 to 18 oligo-nucleotides. Seven primers produced reproducible polymorphic DNA patterns. The cultivars could be separated based on their geographic distribution but a distinction between monoembryonic and polyembryonic genotypes could not be established.

In mango, the microsatellite markers are of particular importance to study on identification, variability, germplasm conservation, domestication and movement of germplasm (Viruell et al., 2005). The true potential advantages of microsatellite over other types of genetic markers would be felt more obviously, when they will

be employed to track desirable traits in large-scale breeding programmes and as anchor points for map-based gene cloning strategies (Brown et al., 1996).

5.2.3. Amplified fragment length polymorphism (AFLP)

This PCR-based technique allows inspection of polymorphism at fairly a large number of loci within a very short span of time and at the same time requires a very small amount of DNA. AFLP markers are quite suitable for cultivar identification, estimation of genetic relationship and mapping of QTLs in mango (Kashkush et al., 2001). And the information generated by AFLP analysis regarding genetic relatedness and diversity existing in mango genepool are useful for breeding of improved mango varieties (Yamanaka et al., 2006). Eiadthong et al. (1999a) studied the AFLP analysis of genetic relationship among some mango cultivars in Thailand. Ravishankar et al. (2000) investigated the genetic relatedness of 18 mango cultivars grown in different parts of India through RAPD markers. From their results they concluded that majority of cultivars were evolved from a local mango genepool and were domesticated later.

Apart from the identification of mango cultivars and assessment of genetic relationship, Kashkush et al. (2001) used AFLP information to construct a genetic linkage map, which consisted of 13 linkage groups and covered 161.5 cm defined by 34 AFLP markers.

Fang et al. (1999) constructed a fingerprinting of two mango cultivars viz., Keitt and Tommy employing AFLP. Eiadthong et al. (1999b) studied the AFLP analysis of genetic relationship among some mango cultivars of Thailand.

5.2.4. RAPD and RFLP markers

Schnell and Knight (1992) and Schnell et al. (1995) using RAPD markers tried to determine phylogenetic relationship in 25 cultivars. Ten short primers showed reproducible polymorphic DNA amplification. Jayasankar et al. (1998) used RAPD to characterize fungal toxin (*Colletotrichum gloeosporoides*) tolerant mutations from embryogenic culture of 'Carabao' and 'Hindi' mango.

Recently, Souza and Lima (2004) examined forty mango genotypes of the Embrapa Meio-Norte mango collection for random amplified polymorphic DNA markers with 32 random primers. Thirteen of these 32 initially screened primers that indicated more polymorphic DNA amplification patterns were selected for the RAPD reactions. Based on their initial results, the genotypes were grouped into two distinct groups: one formed by 'Mallika' and 'Amrapali' and another comprised of the other genotypes, which was subdivided into other two groups,

one formed by 'Manzanilo', 'Van Dyke' 'Palmer' and 'Keitt', and the other including the remaining genotypes. This latter group was further divided in two other groups, one including 'Edward', 'Winter', 'Alfa Emprapa 142', 'Kensington' and the advanced breeding selection CPAC 98/86 ('Beta') and the other, including 'Santa Alexandrina', 'Sensation', 'Glenn', 'Irwin' and all 25 Rosa's genotypes. Bootstrap consistency test, however, indicated that only three groups ('Mallika' and 'Amrapali', 'Van Dyke', 'Palmer' and 'Keitt', and 'Irwin' and 'Glenn') really diverged. Likewise, Srivastava et al. (2004) examined the application of molecular markers for parentage analysis of some commercial mango hybrids of India such as Amrapali (Dasher \times Neelum), Mallika (Neelum \times Dasher) and Ratna (Neelum \times Alphonso). Primarily, three different Single Primer Amplification Reaction (SPAR) methods, Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeat (ISSR) and Directed Amplification of Minisatellite DNA (DAMD), have been used for establishing parent-hybrid relationship in case of above mentioned mango hybrids, which were developed using Neelum as one of the parents, and their respective parents. We show that hybrid Ratna (Neelum \times Alphonso) is genetically closer to its male parent Alphonso. While, reciprocal hybrids Amrapali (Dasher \times Neelum) and Mallika (Neelum \times Dasher) are closer to Neelum. Further, one RAPD and two DAMD primers have revealed Neelum-specific bands present in all three hybrids and Neelum exclusively. Further, they suggested that such bands will be useful in breeding programmes by tagging genes as well as by enabling a more efficient early selection of progeny with desirable qualities. Karihaloo et al. (2003) used RAPD analysis for 29 Indian mango cultivars comprising popular landraces and some advanced cultivars. Analysis of molecular variance revealed that 94.7% of the genetic diversity in mango existed within regions. However, differences among regions were significant; northern and eastern regions formed one zone and western and southern regions formed another zone of mango diversity in India. Kumar et al. (2001) estimated genetic diversity in fifty commercial mango cultivars using RAPD markers. Chance seedling hybrids and selections were found to be closely associated, while the genotypes differing morphologically and geographically showed the distinct trends. Earlier, Chunwongse et al. (2000) constructed a molecular map of mango cultivars Alphonso and Palmer employing RFLP and AFLP markers.

Eiadthong et al. (1999b) confirmed the phylogenetic relationship in *Mangifera* by RFLP and amplification of cpDNA. They confirmed the geographic distribution of several *Mangifera* species in Thailand. However, five species viz., *M. gracilipe*, *f.*, *M. lagenifera*, *M. longipes*

and *M. longipetiolata* and *M. quadrifida* were failed to be detected while, of four new species viz., *M. griffithi*, *M. oblongifolia*, *M. gallina* and *M. macrocarpa* identified through cpDNA and RFLP. It was confirmed that *M. indica* and *M. sylvatica* were closely related, while 11 species formed a different cluster. Further, they suggested that the genus *Mangifera* should be reclassified on the basis of molecular analysis, unlike, those classified on the basis of morphological markers by **Kostermans and Bompard (1993)**. Similarly, **Ravishankar et al. (2004)** investigated genetic relatedness among 10 polyembryonic and monoembryonic cultivars each traditionally grown in the west coast of southern India by genomic DNA and chloroplast DNA RFLP analyses. Eight mango cultivars from each of these groups were used for chloroplast DNA RFLP analysis. The primers ORF 106-rbcL and GIF-GI460 were used. All the products were restriction-digested with enzymes. Data from RAPD markers and chloroplast DNA RFLP markers were used for cluster analysis and principal component analysis (PCA), separately. Dendrogram analysis of RAPD and chloroplast DNA RFLP data clearly grouped the cultivars into 2 based on embryo types, i.e. monoembryonic and polyembryonic. In both genomic DNA and chloroplast DNA RFLP analyses, the grouping of cultivars based on their embryo types indicates that monoembryonic and polyembryonic types of Indian mango cultivars have a different genetic base. These results suggest that the polyembryonic types might have been introduced from other parts of south-east Asia and are unlikely to have originated from India. **Deng et al. (1999)** examined 3 mango cultivars using RAPD. Of the various primers used, primers S273, S281 and S286 were found most suitable for RAPD amplification and genomic DNA analysis.

Alternatively, the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) can be used to analyse phylogenetic relationship among different species. **Yonemori et al. (2002)** compared sequences of ITS region of nrDNA to reveal phylogenetic relationship of *Mangifera* sp. Parsimony and neighbor joining (NJ) analyses divulged the close association of common mango (*M. indica* L.) with *M. laurina* Bl., *M. sylvatica* Roxb., and *M. oblongifolia* Hook. f. *Mangifera foetida* Lour. and *M. odorata* Griff. were also related to *M. indica* in both parsimonious and NJ trees, although these two species belong to subgenus *Limus*. ITS sequence analysis revealed that several species have nucleotide additivity (two different nucleotides at the same locus) at several sites in the ITS region. Also, *M. indica* had several polymorphisms among cultivars. On the basis of the findings of this investigation, the authors

suggested the possibility of hybrid origin of *Mangifera* species, although *Mangifera* species are all assumed to be diploid having chromosome number of $2n=2x=40$.

5.2.5. Inter-Simple-Sequence-Repeats (ISSR)

Amplification of inter-simple-sequence-repeats (ISSRs) is a relatively novel technique and has proven to be a powerful, rapid, simple, reproducible and inexpensive way to assess genetic diversity or to identify closely related cultivars in many species, including fruit trees. **Gonzalez et al. (2002)** tested a range of ISSR primer sequences in mango and identified those that show clear polymorphisms between cultivars grown in Australia. Further, they suggested the potential use of DNA markers for mango improvement in the areas of variety identification, validation of parentages, estimation of genetic variation in existing populations, and characterization of rootstocks.

6. Cloning of useful gene(s)

In nature, gene transfer is pretty ambiguous which makes the percentage recovery of desired gene combination subject to efficient screening and selection. Additionally, their range in terms of species involved is dependent on sexual compatibility. These delimit the movement of gene across different taxa. On the other hand, advances made in the field of biotechnology have made gene transfer a reality. This entails targeted manipulation of the genetic material towards a desired end through a pre-determined way. In mango; however, gene cloning technique is mostly confined to fruit ripening.

Bojorquez and Gomez-Lim (1995) isolated peroxisomal thiolase cDNA (1305 bp) that coded for protein for mol wt. 455 kDa, which had high degree of homology with other plant thiolases. **Cruz-Hernandez and Gomez-Lim (1996)** isolated cDNA that codes for alternate oxidase (36.6 kDa) from mesocarp of ripening mango fruit. The gene had the size of peptide equal to *Arabidopsis thaliana* with 68% homology. Likewise, **Lycett et al. (1997)** identified a clone (pNY601) during screening of γ -ZAP cDNA library constructed from mango cv. Tommy Atkins, which was induced to ripen with ethylene, with tomato ACC oxidase 1 (ACO) cDNA. Differential screening resulted in identification of two abundant clones (pNY608 and pNY642) and four less common clones (pNY602, pNY627, pNY633, pNY634). Sequencing and translation of cDNA inserts revealed that pNY608, pNY627, pNY633 and pNY642 had no significant homology to protein sequence in the SWISSPROT database. On the other hand, pNY634 and pNY633 showed strong

homology to a plastid chromatin and Ypt/Rab11 class of small GTPase, respectively. However, no clones related to known fruit ripening specific genes of tomatoes or other species were found during the differential screen.

Chaimanee et al. (1999) observed changes in gene expression during mango fruit ripening. A substantial increase in amount of total RNA, particularly, from unripe to ripe stage was noted. The total RNA and poly (A)+mRNA were isolated and later subjected to in vitro translation. Analysis of translated product by SDS gel electrophoresis and autoradiography revealed accumulation of 67, 61 and 48 kDa polypeptides. These results indicated that the ripening in mango is certainly associated with the expression of specific genes. Later, Chaimanee et al. (2000) isolated exopolysaccharuronase from ripe mango fruit and proposed that exo-PG was highly correlated with ripening in mango. Similarly, Suntornwat et al. (2000) confirmed that endo-polygalacturonase (PG) increases considerably during ripening; cDNA was made using purified mRNA from ripening fruits and one of the cDNAs isolated had the size of 811 bp and encoded 161 amino acids open reading frames and had a match with endo PG of kiwi fruit.

Saiprasad et al. (2004) isolated five ripening related cDNAs from two mango cultivars viz., Alphonso and Totapuri, using RT-PCR technique. The predicted polypeptides of five of these clones showed similarity to database protein sequences of PRI-1 protein, transcription initiation factor, CCR-4 protein, 18S ribosomal RNA gene and 23S ribosomal RNA gene. However, none of these proteins appeared to be directly related to events generally associated with ripening. Further, the authors speculated that these proteins are the regulatory elements/signals known to be involved during fruit ripening and may, therefore, be involved in regulating the expression of other genes directly associated with fruit ripening.

7. Genetic transformation

Genetic transformation provides the means for modifying single horticultural traits in perennial plant cultivars without altering their phenotype. This capability is particularly valuable for tree species in which development of new cultivars is often hampered by their long generation time, high levels of heterozygosity and nucellar embryony. Targeting specific gene traits is predicated upon the ability to regenerate elite selections of what are generally trees from cell and tissue cultures. The integrity of the clone would thereby remain unchanged except for the altered trait (Gomez-Lim and Litz, 2004). Success has been achieved in obtaining genetically

transformed mango embryogenic cultures using *Agrobacterium tumefaciens* (Litz et al., 1990; Mathews et al., 1992, 1993). Engineered *Agrobacterium* strains, viz., (i) C 58CI with plasmid pG3850::1103 with selectable marker NPT II and (ii) A208 contained pTit37-SE::pMON9749, a co-integrate vector with genes encoding for NPT-II and GUS were used for transformation of 'Keitt' and 'Hindi' mango, respectively. A preliminary study indicated that $12.5 \mu\text{g ml}^{-1}$ kanamycin sulfate was toxic to proembryo mass grown in suspension culture, while $200 \mu\text{g ml}^{-1}$ kanamycin sulfate was toxic in semi-solid medium (Mathews and Litz, 1990). Putative transformants could be confirmed based on histo-chemical staining with X-GLUX and southern hybridization. Complete transgenic plantlet could not be obtained due to hyperhydricity of embryos.

Cruz-Herandez et al. (1997) successfully transformed 'Hindi' mango cultures with an engineered disarmed strain of *A. tumefaciens* (LBA 4404) containing ACC oxidase, ACC synthase and alternate oxidase genes in antisense configuration. In addition, they could also raise some embryos to maturation, which were expected to express anti-ripening behaviour in fruits.

Gutierrez et al. (2001) isolated a mango cDNA homologue of ethene receptor gene ETR-1, which expressed transiently during fruit ripening as well as wounding of tissue. Cruz-Hernandez et al. (2000) optimized particle bombardment parameters and reported successful transient expression for beta-glucuronidase gene in polyembryonic mases. Stable trans gene expression was achieved using green fluorescent protein as reporter gene.

8. In vitro germplasm conservation

Mango seeds are highly recalcitrant and cannot be stored. Hence, tissue culture methods can be an ideal approach, (Engelmanns, 1991). Pliego-Alfaro et al. (1996a,b) cultured somatic embryos on mannitol and ABA supplemented medium, which suppressed the growth and the cultures could be extended up to three months. Further, they suggested that medium term germplasm storage can be made in mango through in vitro means. Wu et al. (2003) compared three techniques for cryopreserving the embryonic mango cultures. The best result was obtained with vitrification, while the encapsulation/dehydration yielded no recovery.

9. Conclusion and future thrusts

Biotechnology holds several promises in mango improvement. Tissue culture techniques like anther and

ovary culture can be exploited for raising homozygous lines. Likewise, genetic transformation to raise stable transformants for different characters is gradually been explored. Genetic markers are of special significance as it can aid in conventional breeding approaches in *Mangifera* spp.

Considerable success has been achieved in the development of regeneration protocols in several mango cultivars. Transformation of mango through repetitive somatic embryogenesis has also successfully been accomplished. Despite the successful regeneration of different genotypes, the conversion rate of somatic embryos into normal plantlets remains low. Future research must be focused on enhancing conversion frequency of somatic embryos into normal plantlets and regeneration of plantlets from shoot/nodal segments.

Most of the important mango varieties which dominate the world mango trade like Haden, Kent, Sensation, Alphonso, etc. have large canopies that disqualify them from being included in the concept of high density planting. Introduction of dwarfing gene(s) from Indian cultivars such as Amrapali, Kerela Dwarf, Manjeera could be a solution to induce dwarfing in an otherwise vigorous cultivars.

Another major problem confounding cultivation of many mango varieties is the occurrence of alternate bearing. This problem could be alleviated by introduction of flower-meristem-activity AGAMOUS-LIKE 20 (AGL20), APETALA1 (AP1) and LEAFY (Blazquez and Weigel, 2000) genes from *Arabidopsis*. AGL20 plays a pivotal role in floral evocation by integrating signals from several different pathways involving both environmental and internal cues (Borner et al., 2000). Once activated, AGL20 triggers the expression of LFY and LFY turns on the expression of AP1 (Simon et al., 1996). Likewise, the advances made in the field of biotechnology can also meet the challenges of abiotic and biotic stresses. Biotechnology is an important and invaluable asset to the breeders, which hold the greater promise to revolutionize the mango industry by development of altered variety(s) intended to serve the specific purpose through precise genetic manipulation, which was hitherto unachievable through conventional breeding.

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