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Mango Research and Biotechnology

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ABSTRACT

Revolutionary modern biotechnology can complement conventional breeding and expedite mango improvement programmes through studies involving *in vitro* culture and selection, micropropagation, embryo rescue, genetic transformation, marker-assisted characterization, and DNA fingerprinting, etc. *In vitro* culture and somatic embryogenesis of several different genotypes have already been achieved. The nucellus excised from immature fruitlets is the appropriate explant for the induction of embryogenic cultures. A specific Random Amplified Polymorphic DNA (RAPD) marker is used for segregating polyembryonic and monoembryonic mango cultivars. Other techniques, such as amplified fragment-length polymorphism (AFLP), macrosatellites, and microsatellites, have also been used to identify mango cultivars and rootstocks and to assess their genetic relationships. Genetic transformation using *Agrobacterium tumefaciens* has been reported. Genes that are involved in fruit ripening have been cloned and there have been attempts to incorporate these genes into plants. The present review highlights the information on biotechnological studies conducted in mango, presenting some potential biotechnological solutions to concrete problems for the improvement of mango production.

Keywords: biotechnology, genetic transformation, *in vitro* propagation, *Mangifera indica* L., markers, molecular biology, somatic embryogenesis

CONTENTS

INTRODUCTION

The mango (*Mangifera indica* L.) is native to southern Asia, where it has been cultivated since ancient times. Movement of mango germplasm has been a consequence of its cultivation throughout tropical and subtropical regions of the world. In the last 15 years, there has been a tremendous increase in areas dedicated to mango production throughout the world, especially in the United Sates of America and in Europe. In particular, the largest-volume European importer of fresh mangos is the Netherlands (63,000 t), followed by France (30,000 t), Germany (24,000 t) and the United Kingdom (23,000 t) (FAOSTAT 2005; UCI 2006). Florida mango cultivars have come to dominate the mango-export industry of the western hemisphere due to their versatility, beauty, relatively strong disease resistance, and good handling characteristics. Today, the search continues for additional Florida cultivars offering greater manageability, a smaller tree size, plus superior quality and horticultural traits (Campbell 2004). Spain is the main European producer of subtropical fruits, with approximately 1,400 ha dedicated to mango (Durán *et al.* 2003, 2006). In particular, the coast of Granada and Malaga (Andalusia) has a large potential area for the cultivation of tropical and subtropical trees, with a favourable year-round climate and infrequent frosts. Meanwhile, India continues to produce large volumes of numerous cultivars, Southeast Asia remains strong with yellow-skinned cultivars, and China continues to increase the production volume of its many cultivars.

Despite its importance and worldwide production and distribution, mango suffers from a long juvenile period, erratic flowering, and alternate bearing habits. In fruit crops, control of flowering is a critical aspect in the production system, since it determines the seasonality of fruit supply to the market. One of the great advantages of the tropics is the possibility of producing over the entire year, but fruit pro-

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ducers and the market nevertheless face major challenges to supply high-quality fruits throughout the year. Biotechnology can potentially be used to manipulate existing cultivars by targeting specific genetic traits, such as flowering behaviour, since it is crucial to improve the biotechnology of somatic embryogenesis in terms of yield and quality of somatic embryos. Improving mango by means of biotechnology is predicated upon the ability to regenerate elite selections of mango from cell and tissue cultures. In this context, somatic embryogenesis has been reported in some mango cultivars, most of which are polyembryonic (Litz and Gray 1995; Litz and Lavi 1997; Litz 2004). Moreover, mango production is affected by high cross-pollination and, owing to the fact that most marketable mango clones are monoembryonic, sexual propagation does not ensure true-to-type plant reproduction. Historically, Indian types (monoembryonic) and Southeast Asian types (predominantly polyembryonic) have been credited with having given rise to Florida hybrids. Early molecular data including isozyme and Randomly Amplified Polymorphic DNA analysis supported the hybrid origin (Olano *et al.* 2005). Florida varieties, although adapted to Florida conditions, perform well across many different environments, and several, including 'Tommy Atkins', 'Keitt', 'Osteen', 'Haden', 'Irwin', 'Sensation', 'Parvin', are produced commercially in tropical and subtropical countries (Durán *et al*. 2004; Galán *et al.* 2004; Durán and Franco 2006; Durán *et al*. 2006). Furthermore, production problems are associated with both the scion and the rootstock. Scion cultivar problems include biennial bearing habit, large tree size, susceptibility to major pests and diseases, short post-harvest life and many physiological disorders (anthracnose, malformation, spongy tissue, etc.) (Prakash 2004). For maximum return on rootstock breeding, priority should include tolerance of various soil-related stresses, induction of dwarfing and high degree of polyembryony to ease rapid multiplication (clonal plants).

Most marketable mango varieties are not amenable to hi-tech cultivation practices and do not meet the requirements of modern horticulture, such as early bearing, dwarfing, regularity in bearing high fruit yield, resistance to pests and diseases or physiological disorders, and good keeping quality. The need for such an ideal phenotype cannot be met by conventional breeding because of many problems (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). Moreover, given the meagre information on inheritance of important quantitative traits, biotechnology has a great potential to correct the genetic flaws of existing varieties.

The recent development of molecular biology and genetic markers for mango and their application to classical breeding offer tremendous potential for mango breeding. The introduction of specific genes for disease resistance from cultivated varieties and wild species into popular cultivars should soon be a reality. Although genetic transformation of mango with selectable and scorable marker genes is not a limiting factor (Mathews and Litz 1992), very few genes have actually been isolated from mango. Genes coding for such horticultural important traits as tree size, yield and fruit quality are not yet available.

Mango micropropagation has not met with the commercial success as obtained in other fruit crops (banana, pineapple, strawberry, etc). This is due to many impediments associated with it, such as latent microbial infection, excessive polyphenol exudation, early explant necrosis, etc.

MANGO MICROPROPAGATION

Micropropagation is the science of plant multiplication *in vitro.* The process includes many steps – stock-plant care, explant selection and sterilization, manipulation of the medium for proliferation, rooting, acclimation, and growing into plantlets. Particularly with mango, many studies have indicated various inherent problems associated with *in vitro* culture (phenol exudation, medium discolouration, explant browning, deep-seated systemic contamination, and *in vitro* recalcitrance of tissues).

Several attempts have been made to regenerate mango using leaf (Raghuvanshi and Srivastava 1995) and shoot explants (Thomas and Ravindra 1997; Rocha *et al.* 2002; Sharma and Singh 2002). Though these methods were found inefficient, they ensure the availability of explants year-round unlike the explants provided by fruitlets during only certain parts of the year. For the reduction of phenolic exudation (activation of oxidative enzyme system during the excision of mango plants) during *in vitro* culture, Raghuvanshi and Srivastava (1995) suggested pre-treatment of leaf explants using liquid shaker culture. Sharma and Singh (2002) reported that etiolated mango shoots when cultured *in vitro* registered a marked decline in polyphenol oxidase (PPO) activity and phenol content at the pre-culture stage, and phenolic exudation at the post-culture stage, which in turn increased the explant survival in Murashige and Skoog's (MS) culture medium.

Another important limiting factor for culture initiation using vegetative plant parts of mango is the presence of deep-seated contamination, which frequently leads to failure of cultures. Latent fungal infections reported in mango include *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 healthy cultures. These latent contaminants may be distributed in the intra- and inter-cellular parts of plant tissue such as cortex, phloem, xylem, and parenchyma cells. The isolation and identification of endophytic organisms would enable the use of specific antibiotics against contaminants for maintaining aseptic cultures of mango (Reuveni and Golubowicz 1997).

Another problem is recalcitrance, the inability of plant cells, tissues and organs to respond to tissue culture (Benson 2000). This is greatly influenced by factors such as the whole-plant physiology of the donor, *in vitro* manipulation, and *in vitro* stress physiology.

It is extremely important 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 has been observed only in immature zygotic nucellar embryos (juvenile tissue), while other explants, such as shoot tips, nodal segments, leaf (mature tissues), etc., showed limited success. Also, the outcome is greatly influenced by genotype, the growth status of the donor plants, and the developmental stage of the embryo in isolation (Thomas 1999; Laxmi *et al.* 1999).

In vitro manipulation of different components, such as inorganic or organic compounds, amino acids, enzymes, phytohormones, carbon source, gelling agents, and other additives, can help alleviate recalcitrance. Gaspar *et al.* (1996) stated that one of the most important approaches for overcoming this problem is to optimise the plant growth-regulator regime. Different combinations of auxins and cytokinins [e.g. naphthaleneacetic acid (NAA), indoleacetic acid (IAA), benzylaminopurine (BA), and kinetin] in MS medium supplemented with 1% polyvinylpyrolidone in 250 ml conical flasks on an automated shaker at 75 rpm to obtain multiple mango shoots from the callus derived from mature leaf explants were used by Raghuvanshi and Srivastava (1995).

Another important factor affecting the recalcitrance of mango tissues include light regime and *in vitro* plant-stress physiology. However, the most important *in vitro* stress factor is the oxidation of explants in the culture, owing to the high phenol content (Sharma and Singh 2002; Murti and Upreti 2003; Chandra *et al.* 2004; Singh *et al.* 2004). In comparison to the situation in many other crops, oxidation is more prevalent in mango, which has high levels of phenols associated with secondary thickening and lignification.

IN VITRO PROPAGATION

The main attribute of successful *in vitro* rearing systems is that they will permit the production of species not currently available by *in vivo* methods. Also, *in vitro* systems offer the potential for reducing costs with respects to *in vivo* systems, and provide better product standardization and formulation. Therefore, *in vitro* propagation facilitates rapid multiplication of superior clones within a short span. *In-vitro* selection has the potential to select mango types that have positive mutation/variation through somaclonal variation. Various regeneration protocols have been developed, including callus induction, somatic embryogenesis and organogenesis using different explants (cotyledons, nucellus, leaf disks and shoot tips of mango).

Particularly, polyembryonic mango rootstocks are propagated exclusively from seed that give rise to a limited number of clonal seedlings. In this context, different explants of mango cultivars were explored for *in vitro* plantlet regeneration on MS and Gamborg's (B5) medium. Only zygotic embryos of cv. 'Chaunsa' initiated callus formation and successful plant regeneration with MS medium supplemented with 1 mg L^{-1} of 2,4-Dichlorophenoxyacetic acid (2,4-D) (Usman *et al.* 2005). Calluses were also observed from zygotic embryos of mango but could not regenerate into plantlets. An open challenge for researchers is to identify the potentialities of micropropagation in order to resolve the problem of clonal mango rootstocks.

Somatic embryogenesis

The mango plant presents problems for tissue culture. Jana *et al.* (1994) performed a method for rapid production of somatic embryos having normal developmental morphology from the nucellar tissue of three monoembryonic Indian mango varieties ('Alphonso', 'Mundan' and 'Baneshan'), development being complete in 75 to 80 days. In this sense, the nucellus was used as the primary explants, which are generally free from viruses and other endophytic disease-causing organisms, due to the absence of a vascular connection between the surrounding maternal tissue and the nucellus. That is efficient recovery of somatic embryos in monoembryonic mango cultivars would eliminate pathogens and avoid loss frequently occurring in clonally propagated genotypes owing to diseases and environmental stress. This scenario has been studied by many authors (Litz *et al.* 1982; Dewald *et al.* 1989a; Litz *et al.* 1998; Pateña *et al.* 2002) in polyembryonic and monoembryonic genotypes (Litz 1984; Jana *et al.* 1994; Pliego *et al.* 1996a; Thomas 1999; Ara *et al.* 2000a; Chaturvedi *et al.* 2004a, 2004b; Rivera *et al.* 2004).

In polyembryonic genotypes, adventitious embryos differentiate from competent cells, which are present within the nucellus (Litz 2003). Litz *et al.* (1984) pointed out the suitable conditions for the induction of embryogenic cultures from nucellus of monoembryonic mango cultivars. Induction of embryogenic cultures from the excised nucellus of polyembryonic mangoes was described on a medium supplemented with 2 mg L^{-1} 2.4-D, and large numbers of somatic embryos could be produced in liquid medium (Litz *et al.* 1984). Xiao *et al.* (2004) regenerated plantlets from immature zygotic embryos of mango through direct somatic embryogenesis after 4 weeks of culture on the conversion medium containing 5 mg L^{-1} (23 μM) kinetin with minor problems of browning of cultures.

On the other hand, the problems of early germination and progressive necrosis have also prevented the efficient recovery of mango plantlets. Many authors (Dewald *et al.* 1989a; Ara *et al.* 2004; Chaturverdi *et al.* 2004a) investigated the suitable conditions for optimising the induction and maintenance of embryogenic cultures and for maturation of somatic embryos. In broad terms, the protocols employed have four stages: (1) induction of embryogenic culture from nucellus, (2) maintenance of embryogenic culture, (3) maturation and the development of morphologically normal embryos, and (4) germination of somatic embryos into well-developed plantlets. Early germination of somatic embryos could be minimized by the addition of 3.0 μM ABA and 6.0% (w/v) sucrose in the medium (Dewald *et al.* 1989b). In this context, according to Dewald *et al.* (1989a) the growth of embryogenic cultures in suspension was more efficient than on solid medium; however, subculture on solid medium was essential for high-frequency production of morphologically normal somatic embryos, being Gelrite being more effective than Difco Bacto-agar. In addition, modified B-5 basal medium was better for culture maintenance and for production of morphologically normal somatic embryos than either MS or Woody Plant Medium (WPM). Also, coconut water (20%, v/v) enhanced somatic embryo production by 18%, and other complex organic addenda alone or in combination with it were either ineffective (i.e. casein hydrolysate) or highly inhibitory (yeast extract) in comparison with basal medium alone. Despite the higher rate of embryogenesis from nucellar tissues, it has had considerable success with cv. 'Chaunsa' but not with cv. 'Anwar Rataul', which was attributable to excessive phenolic exudation from nucellus explant in the medium (Usman *et al.* 2005).

Wu *et al.* (2007) described the direct somatic embryogenesis both from immature cotyledon cuts and from nucelli with mango cv. 'Zihua'; these researchers studied the effect of cryopreservation growth conditions of embryogenic cultures (EMs) and compared the cryopreservation response of EMs induced from these two different explants. Their results showed that EMs from either nucelli or cotyledon cuts could be maintained in liquid medium or on solid medium and cryopreserved using a vitrification procedure. According to these authors success of cryopreservation of EMs depended on the dehydration treatment and the defined growth conditions, and when EMs were sampled during their exponential growth phase in liquid medium and dehydrated with PVS_3 solution (5 min), survival of the EMs induced from cotyledon cuts and nucelli reached 77.7 and 80%, respectively, after cryopreservation in liquid nitrogen (24 h). Thus, cryopreservation did not affect the plant-regeneration potential of EMs through somatic embryogenesis.

Embryogenic cultures

According to many authors (Litz *et al.* 1982; Dewald *et al.* 1989a; Ara *et al.* 1999; Sulekha and Rajmohan 2004) the 30- to 60-day-old fruits harvested after pollination are suitable for the induction of somatic embryogenic culture from the nucellus. Florez-Ramos *et al.* (2007) used nucellar tissues from immature fruits as the starting explant, the best results corresponding to cv. 'Keitt' with 48% of embryogenic culture induction. On the other hand, embryogenic nucellar cultures of two polyembryonic mango cultivars, 'Hindi' and 'Carabao' were selected for resistance to the culture filtrate and phytotoxin of a virulent strain of *Colletotrichum gloeosporioides* Penz (Jayasankar and Litz 1998). Mycelium growth was inhibited when the pathogen was co-cultured with the selected resistant embryogenic cultures, confirming that extracellular antifungal compounds were involved in the defence response.

Ara *et al.* (2004) transferred aseptically dissected monoembryonic ovules to an MS medium consisting of half-strength major salts and chelated iron plus full-strength microsalts and vitamins, 6% sucrose, 400 mg L^{-1} L-glutamine, 100 mg L^{-1} ascorbic acid, 0.8% agar, and 1 mg L^{-1} 2,4-D. For further optimisation of 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, 1994).

Chaturvedi *et al.* (2004a) observed that nucellar tissue at different developmental stages responded differently to the same gelled nutrient medium used for induction of em-

bryogenesis. 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} 2-isopentyl adenine (2-iP) was sufficient for embryogenesis. Litz *et al.* (1894) removed nucellus and globular adventitious proembryos from 2-month-old fruits of mango cv. 'Ono' and 'Chino', and cultured with solid MS medium modified as follows: half-strength major salts and chelated iron; 20% (v/v) coconut water; 6% sucrose; 100 mg L^{-1} ascorbic acid and 400 mg L^{-1} glutamine (Litz *et al.* 1984). Then the embryogenic explants were sub-cultured after 4-6 weeks in liquid modified MS medium containing 2 mg L^{-1} of 2,4-D instead of coconut water. Somatic embryogenesis was induced following sub-culture from MS medium using 2,4-D to MS without growth regulators and with or without activated charcoal (0.5%). Thus, according to Litz *et al.* (1894) germination of somatic embryos appeared to be enhanced by 1 mg L^{-1} benzyladenine (BA); however, most of the germinating embryos became embryogenic.

The embryogenic response is highly cultivar dependent. In this context, 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'). These researchers also demonstrated that nurse culture derived from cv. 'Parris' can improve the embryogenic response of non-embryogenic cultures. Likewise, Manzanilla *et al.* (2000) compared the induction responses of three cultivars and observed that cv. 'Ataulfo' (polyembryonic) was more embryogenic than either cv. 'Tommy Atkins' and cv. 'Haden' (monoembryonic). In this context, 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*.

The embryogenic mango culture consists entirely of proembryogenic masses (PEMs). According to Litz (2003), sustained proliferation in embryogenic cultures in most mango cultivars is possible on induction medium; however, regular subculture at 3- to 4-week intervals is essential to prevent darkening of tissues. The synchronization of embryogenic cultures is difficult to obtain on semi-solid medium due to the polarity within the culture relative to the accessibility of 2,4-D. Embryogenic cultures of many cultivars exhibit significantly higher proliferation in suspension. In addition, Litz and Gómez (2005) reported that the embryogenic suspension cultures can be synchronized according to size of PEMs by passage through different grades of filtration fabrics.

Litz (2003) considered both cytokinin and auxin to be important for proliferation and maintenance of mango globular embryogenic masses. Though cytokinin is not essential for induction, it is important for stimulating organization of the apical meristem during maturation. Deore *et al.* (2000) reported callus induction by supplementing medium with 0.1 mg L^{-1} kinetin plus 2.0 mg L^{-1} 2,4-D. Ara *et al.* (2004) pointed out the possibility of rapid multiplication of cv. 'Ampari' suggesting the use of 1.0 mg L^{-1} 2,4-D (2,4-dichlorophenoxy acetic acid) and 1.0 mg L^{-1} NAA (α-naphthalene acetic acid), either alone or with 1.0 mg L^{-1} of kinetin for enhancement of proliferation of the PECs (proembryogenic calluses) in liquid as well as solid media; however, proliferation was around 5-fold more profuse in liquid medium. Nevertheless, the differentiation followed by callusing of globular somatic embryos was observed in liquid medium, while on semi-solid medium, the globular somatic embryos successfully developed into the cotyledon stage. Moreover, according to Dewald *et al.* (1989b) developmental abnormalities (polycotyledony, the absence of polarity, hyperhydricity, etc.) are encountered in somatic embryos that developed in liquid medium in comparison with solid maintenance medium.

Serious obstacles to normal development of somatic embryos include early germination, secondary embryogenesis, etc. from hypocotyl of germinating embryos and the absence of bipolarity (Dewald *et al.* 1989b; Litz *et al.* 1994). 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 the establishment of an embryogenic culture in monoembryonic mango genotypes.

The initiation of maturation including the development of bipolarity in globular embryos followed by the differentiation of cotyledons can be stimulated by the transfer of embryogenic cultures from maintenance to maturation medium. In this context, Thomas *et al.* (1999) reported that the high frequency somatic embryogenesis from nucellar explants of mango cv. 'Arka Anmol' by culturing half ovules with intact nucellus gave a higher percentage of callusing and more embryonic cultures than using excised nucellus. Among the basal media, the Rugini Olive (RO) medium followed by B5 was superior to the MS medium for culture establishment and embryo induction, however, B5 medium was better than the others for embryo conversion and further development. Thomas *et al.* (1999) proposed the embryonic calli formed on establishment medium [RO, 6% sucrose, 2 g L^{-1} activated charcoal (AC), 2 g L^{-1} phytagel, 5 mg L^{-1} each of 2,4-D and GA_3] on transfer at 2-3 weeks to expression medium [RO with 6% sucrose, 2 g L^{-1} AC, 2 g L^{-1} phytagel, 400 mg^{-1} glutamine (Gl), 1-3 mg L^{-1} 2,4-D and 10 mg L^{-1} GA_3] showed pro- and globular embryos in 2-3 weeks. Further embryo development through heart and early cotyledonary stages occurred in a conversion medium (half strength B5 salts, full RO organics, 4% sucrose, 400 mg L^{-1} Gl, 2 g L^{-1} phytagel, 2 g L^{-1} AC, 100 mg L^{-1} casein hydrolysate, 20% coconut water, 0.5 mg L^{-1} 2,4-D and 10 mg L^{-1} GA_3). Proliferating embryonic cultures could be maintained in this conversion medium giving a continuous supply of embryos, and embryo maturation was attained with 1 mg L^{-1} abscisic acid (ABA). In this study the germination with shoot and root development was achieved with 0.01 to 0.1 mg L^{-1} thidiazuron (TDZ). Litz and Gómez (2005) suggested the use of filtration fabric with 1000 μm opening size to decant the suspension culture for the separation of smaller fractions, which are to be transferred either into liquid or onto semi-solid media without 2,4-D. Also, Laxmi *et al.* (1999) observed maximum embryo production on half-strength MS medium supplemented with 20.0 μM BAP devoid of 2,4-D.

Maturation of somatic embryos has often been accompanied by gradual necrosis of the cotyledon and hypocotyl. According to Litz (1984) the addition of malt extract, casein hydrolysate, and reducing agent is not effective, while yeast extract was inhibitory. Medium supplemented with coconut water (20% v/v) delayed necrosis and enhanced the production of somatic embryos. Maturation of mango somatic embryos was also achieved in liquid basal medium, and germination occurred following the transfer of apparently mature somatic embryos onto semi-solid medium supplemented with 4.4 μM BA. Plantlets could not be recovered, and secondary somatic embryogenesis was reported to occur from the transitional zone between the hypocotyls and radicle in the germination of somatic embryos (Litz *et al.* 1984). Dewald *et al.* (1989a) stated that among the different concentrations of sucrose tested, 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 than in medium supplemented with 3.0% sucrose.

Maturation of somatic embryos is usually controlled either by embryogenic cultures on semi-solid or liquid medium. The application of growth regulators, particularly cytokinin, offers further aid in this process. Lad *et al.* (1997) found a no clear relationship between developmental age of the nucellar explants and induction of embryogenic cultures, the culture initiation requiring a minimum pulse of 7-14 d with 2,4-D, a maximum being reached after a 56-d pulse; however, embryogenic competence was optimum after a minimum of 28 d exposure to 2,4-D. Somatic embryogenesis occurred directly from the nucellar explants at

low frequencies. And somatic embryo maturation occurred only after the planting of suspensions onto semisolid medium, and was stimulated by 2.4-4.8 μM kinetin and 4.4 μM (BA). On the other hand, Litz and Gómez (2005) pointed out that the addition of kinetin at 4.65 μM or BA at 4.44 μM to the maturation medium stimulated the development of cotyledons and hastened the maturation period.

The maturation of nucellar embryos excised from polyembryonic ovules and raised *in vitro* was reported by Pliego *et al.* (1996a). When *in vitro* systems for highly embryogenic cultivars are optimised as suspension cultures, the early cotyledonary somatic embryos, which develop in suspension, are hyperhydric. In this sense, somatic embryos that demonstrate this physiological disorder are unable to reach maturity and ultimately become necrotic (Mathews *et al.* 1992; Monsalud *et al.* 1995).

According to Pliego *et al.* (1996b) early germination in mango somatic embryogenesis can be inhibited by the addition of 100 μM ABA. Monsalud *et al.* (1995) described the control of early germination by the use of ABA (500 μM), and permitted somatic embryo maturation. The transfer of somatic embryos from medium containing ABA onto medium devoid of this acid stimulates highly synchronized germination, irrespective of the embryo-developmental stages.

Thomas (1999) suggested the reduction of sugar with 1.0 mg L^{-1} ABA for the maturation of early or late heart stage and early cotyledonary embryos. In this sense, Litz and Gómez (2005) also advocated reducing the sucrose in maturation medium from 6 to 4%. Furthermore, cessation of mango somatic embryo elongation at maturity has also been detected in the presence of a high sugar concentration.

Plantlet development

The transfer of somatic embryos to liquid medium [i.e. half-strength B5 macrosalts and 1.0 mg L^{-1} gibberellic acid (GA_3) (Ara *et al.* 2000a)] is essential for converting them into plantlets. Although Ammirato (1993) stated that the physical state of the medium has little effect on embryogenesis, there are instances in which maturation and germination of SEs are affected. The germination medium contains no filter-sterilized coconut water and has a reduced sugar concentration (Thomas 1999; Litz 2003). The germination of somatic embryos into plantlets on B5 medium was improved by lowering of sucrose concentration and by adding both GA_3 (1.45 mM) and N^6-benzylaminopurine (BAP; 2.2 mM) (Laxmi *et al.* 1999). The beneficial effects of BAP on somatic embryo germination have also been reported in different mango cultivars, as in Litz *et al.* (1984) with cv. 'Ono' and 'Chino' and Jana *et al.* (1994) with cv. 'Alphonso', 'Mundan' and 'Baneshan'.

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

As part of a recalcitrant crop, mango somatic embryos cannot be desiccated because partially dehydrated immature somatic embryos (about 4-7 mm long) remained viable for only 32 days in the absence of maturation medium under high RH (Monsalud *et al.* 1995). However, by optimum manipulation of different factors such as somatic embryo-induction medium, dehydration, ABA concentration, gelling agents, encapsulation medium, addition of fungicides-preservatives, etc., might be possible to keep partially dehydrated immature, recalcitrant somatic embryos in medium-to long-term storage (Gill *et al.* 1994; Monsalud *et al.* 1995; Datta *et al.* 1999).

In this context, Ara *et al.* (1999) attempted encapsulation of cotyledon stage somatic embryos regenerated from nucellar tissue individually in 2.0% alginate gel. Successful plant regeneration took place when encapsulated somatic embryos were germinated on agar-gelled medium containing B5 macro-salts (half strength), MS micro-salts (full strength), 3% sucrose, and 2.9 μM GA_3.

Chaturvedi *et al.* (2004b) stressed the need to use the liquid state of nutrient medium for synchronized development, maturation, germination (visible plumule with developed root) and convertibility (plantlet formation) of cotyledonary embryos. According to Chaturvedi *et al.* (2004b) the presence of 0.1 mg L^{-1} ABA together with 100 mg L^{-1} polyethylene glycol and 0.1 mg IAA L^{-1} in BM2 basal medium promoted development and maturation of embryos followed by their germination. Pateña *et al.* (2002) developed a protocol for somatic embryogenesis and plantlet regeneration in mango: eight strains of cv. 'Carabao' and two unidentified varieties (PHL 12384 and PHL 12378).

Xiao *et al.* (2004) claimed 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. In addition, secondary somatic embryogenesis could also be achieved on hypocotyls of mature primary somatic embryos cultured on the conversion medium. In this context, Ara *et al.* (2000a) described a protocol for regenerating plantlets isolated form PEMs in a suspension culture derived from mango nucellar callus. The dividing protoplasts were transferred to a medium with growth regulators for microcalli development that later produced somatic embryos. Finally the mature somatic embryos were germinated into well-developed seedlings and subsequently transferred to soil.

Organogenesis

Rao *et al.* (1981) pointed out the first study on organogenesis by inducing roots from callus that was initiated from mango cotyledons on MS medium supplemented with kinetin (11.6-23.2 μM) and NAA (26.9 μM); however, shoot development was not observed. Later the induction of callus on different explants (epicotyl segment, leaf petiole and shoot tip excised) from aseptically germinated embryos was reported by Singh *et al.* (1991), concluding that the maximum callus induction was on epicotyl segments, while direct root organogenesis was noted in epicotyl and shoot tip culture with low levels of 2,4-D.

Thomas and Ravindra (1997) attempted to establish shoot-tip culture in some mango genotypes (cvs. 'Alphonso', 'Totapuri', 'Banganapalli' and 'Arka Anmol'), showing that several problems such as phenolic exudation, medium discolouration and explant browning are interrelated and are influenced by different factors such as medium, genotype, explant, season and decontamination treatment. Less phenolic exudation and better explant survival were observed in 1/2MS medium than full MS and in semi-solid than in liquid medium. Also, among explant factors, age and shading of sheets, length, thickness, and scaling injuries on explant and lower cut petioles coming in contact with medium were important, but not the age of the tree or the number of leaves on the shoot.

The problems associated with field-grown shoots can be obviated by using shoots grown *in vitro*, which are more responsive to culture conditions and 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 [indole-3-butyric acid (IBA), inodolacetic acid and α-naphthaleneacetic acid], IBA was found most effective with respect to rooting and root growth. Maximum rooting (89.7%) was found on auxin-free agar-gelled rooting medium after 24 h pulse treatment with 5.0 mg L^{-1} IBA in liquid root induction medium in dark. The dark condition favoured root induction and root growth, whereas light incubation (16 h photoperiod with 60 μE m^{-2} s^{-1} light intensity) was inhibitory.

The somatic embryogenesis preceding rooting of microshoots obtained from somatic embryos compromises the chance of this approach to be used for commercial interests.

Table 1 Achievements on somatic embryogenesis and organogenesis for some mango cultivars.

Author(s)	Mango cultivar	Explant used	Media (PGRs)	Response
Ara *et al.* 1999	'Amrapali'	Nucellus	Modified MS (4.5 μM 2,4-D)	Somatic embryogenesis and plantlet regeneration from encapsulated SE
Ara *et al.* 2000a, 2000b	'Amrapali' and 'Chausa'	Nucellus	Modified MS (1.0 mg L^{-1})	Somatic embryogenesis and plantlet regeneration
Laxmi *et al.* 1999	'Amrapali'	Nucellus	MS and B_5 media (5-20 μM BAP)	Somatic embryogenesis and germination
Raghuvansi and Srivastava 1995	'Amrapali'	Leaves	MS medium (1.1 μM IAA + 13.0 μM Kinetin + 1.0% PVP)	Indirect organogenesis and plantlet regeneration
Ara *et al.* 1998	'Amrapali'	Microshoots from nucellar somatic embryos	Root initiation medium (5.0 mg L^{-1} ABA)	Rooting of microshoots
Chaturvedi *et al.* 2004a	'Ambalavi'	Nucellus	Modified medium (0.25 mg L^{-1} BAP+1.0 mg L^{-1} NAA; 0.5 mg L^{-1} 2ip)	Somatic embryogenesis and plantlet regeneration
Litz 1984	Florida cultivars	Nucellus	Modified MS (1.0 mg L^{-1} 2,4-D)	Somatic embryogenesis and plantlet regeneration
Xiao *et al.* 2004	'Carabao' and 'Manila Super'	Immature Zygotic embryos	Modified MS medium (5.0 mg L^{-1} IBA; 5.0 mg L^{-1} kinetin)	Direct somatic embryogenesis
Litz *et al.* 1982	'Carabao', 'Ono', 'Chino', 'Cambodiana', 'Sabre' and Others	Ovules	Modified MS + 100 mg L^{-1} ascorbic acid + 400 mg L^{-1} glutamine (1-2 mg L^{-1}; 20% coconut water)	Somatic embryogenesis and early germination
Litz *et al.* 1998	'Hindi', 'Nam Doc Mai', 'Lippens', and 'Tommy Atkins'	Nucellus	Modified B_5 (4.52 μM 2,4-D)	Induction of somatic embryogenic competence
Monsalud *et al.* 1995	'Hindi'	Ovular halves	Modified medium (500 μM ABA)	Inhibition of early germination and normal somatic embryo maturation
Dewald *et al.* 1989a	'Parris' and 'Saigon'	Ovular halves	Modified B_5 (4.5 μM 2,4-D)	Somatic embryogenesis

On the other hand, the balance between the use of explants derived from field-grown trees and *in vitro*-grown shoots can be struck by employing greenhouse grown shoots. Yang and Ludders (1993) employed the shoot tips of greenhouse rootstocks for *in vitro* culture, being the shoot proliferation higher in G medium containing a combination of BA (1.0 mg L^{-1}), zeatin (1.0 mg L^{-1}), 2iP (2.0 mg L^{-1}), IAA (1.0 mg L^{-1}) and IBA (0.5 mg L^{-1}) than on B5 basal medium or Woody Plant Medium (WPM). On the contrary, Shahin *et al.* (2003) reported that stem node explants were better than shoot tips with respect to shoot proliferation. The highest proliferation was noted on modified woody plant medium 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.

Table 1 shows a summary of studies for some mango cultivars in relation to somatic embryogenesis and organogenesis.

MOLECULAR BIOLOGY

Molecular biology concerns itself chiefly with understanding the interactions between the various systems of a cell, including the interrelationship of DNA, RNA, and protein biosynthesis as well as learning how these interactions are regulated. Recently, reliable genetic markers have been developed and introduced for mango-cultivar identification. These include isozymes, random amplified polymorphic DNAs (RAPDs) and variable-number tandem repeats (VNTRs). Genetic markers are not only to be useful for cultivar identification but also for the purpose of mango breeding, being a useful tool for characterizing the genetic diversity/relatedness among different cultivars or species of mango, for identifying genes of commercial interest, improvement through gene-transfer technology, creation of variations in existing cultivars *in vitro*, overcoming reproductive isolation barrier via protoplast fusion, etc. These new molecular techniques coupled with increased computational abilities are greatly improving management efficiency by providing better scientific evaluation, preservation, and utilization of genetic resources.

Authentication of cultivars and DNA markers

The wide range of tropical and subtropical conditions prevailing in different mango-growing areas, widespread hybridization, and recombination of characters have contributed to the existing diversity. For efficient and effective utilization of plant genetic resources, the characterization of germplasm is indispensable. 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 patterns of AFLP markers in the F1 progenies derived from crossing mango cultivars (e.g. 'Keitt' and 'Tommy Atkins'). These researchers observed high polymorphism in F1 progenies, while the average frequency of segregation was 37.16%. Isozymes have been widely used to differentiate among fruit cultivars, to determine their parentage, to characterize seedling populations following controlled pollinations, and to construct genetic linkage maps. In this sense, Gan *et al.* (1981) reported that genetic variation can occur within putative mango cultivars in South-east Asia. Later, Degani *et al.* (1990), working on different enzymes, such as GPI (EC 5.3.1.9), TPI (triosephophate 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.

Jintanawongse and Changtragoon (2000) used several enzyme systems to identify mango hybrids and true hybrids resulting from hybridization using 11 isozyme systems. In addition, for identification and characterization of diversity in mango, modern biotechnological tools can be utilized for determining the aetiology of dreaded diseases, which may affect mango cultivation. A disease called mango malformation appears both at the vegetative and flowering stages. In this sense, Freeman *et al.* (1999) provided unequivocal evidence that *Fusarium subglutinans* indeed provoked mango malformation by transforming wild isolates of pathogen with a GUS (β-glucuronidase) reporter and hygromycin-

resistant genes followed by their inoculation into healthy vegetative and reproductive mango buds. Some 6-8 weeks after inoculation, typical symptoms of malformation were noted. These symptoms were further corroborated by the presence of GUS-stained mycelia of the pathogen within plant organs (floral and vegetative buds). Later, genetic diversity among 74 *F. subglutinans*-like isolates from malformed mango in different producer countries throughout the world was reported by Zheng and Ploetz (2002). Aiming to characterize *Gibberella 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) examined 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. Besides the assessment of genetic diversity in *F. subglutinans*, other plant pathogens (e.g. *Colletotrichum gloeosporioides*), which cause mango anthracnose have also been examined for genetic diversity, employing RAPD analysis (Davis 1999).

Work has also been performed on the characterization of toxins produced by pathogenic organisms such as *Pseudomonas* (Arrebola *et al.* 2003). In this sense, Cazorla *et al.* (2003) reported the production of an antimetabolite toxin (named mangotoxin), primarily by strains of *Pseudomonas syringae* pv. *syringae* pathogenic to mango.

The fact of low polymorphism levels prompted researchers to rely more on DNA markers. Different DNA markers have been employed in mango in order to study monogenic and polygenic traits (Lavi *et al.* 1998; González *et al.* 2002). According to Ramirez *et al.* (2004), 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. These authors used different protocols for DNA extraction and purification from leaves of guava (*Psidium guajava* L.), avocado (*Persea Americana* Mill.), mango (*Mangifera indica* L.) and coconut (*Cocos nucifera* L.). The well-established CTAB protocol of Doyle and Doyle yielded excellent DNA templates for PCR amplification with mango and coconut, but not so with guava and avocado. In addition, a simple and efficient method for isolating DNA from of healthy and malformed floral tissues of *M. indica* was devised by Gomathi *et al.* (2005). van Wyk *et al.* (2005) confirmed the identity of *Ceratocystis fimbriata* in Oman based on DNA sequences, providing the evidence that this mango pathogen might have originated in Brazil.

Variable-number tandem-repeat sequence (VNTRS) and single-sequence repeats (SSRs)

Variable-number tandem repeats (VNTRs) are short nucleotide sequences ranging from 14 to 100 nucleotides long, organized into clusters of tandem repeats, usually repeated 4 to 40 times per occurrence. Clusters of such repeats are scattered on many chromosomes. Specifically for mango, Adato *et al.* (1995) analysed DNA fingerprint patterns of some genotypes, using minisatellite multilocus probes. Also, genetic analyses have been made of the progeny of the cross 'Tommy Atkins' × 'Keitt', for which the six bands of the parents showed polymorphism and the average transmission frequency was 65 and 81% for maternal and paternal specific bands, respectively.

Simple-sequence repeats or SSRs, also known as microsatellites of DNA markers are advantageous over many other markers as they are highly polymorphic, highly abundant, co-dominant in inheritance, analytically simple, and readily transferable. Microsatellite markers to study genetic diversity in *M. indica* were developed using a genomic library enriched for (GA)*n* and (GT)*n* dinucleotide repeats (Lavi *et al.* 1998; Barbieri *et al.* 2005). Nineteen SSR loci with clear scorable patterns were chosen to assess diversity in the mango germplasm bank of cv. 'Guadalupe' (FWI). The number of alleles ranged from three to 13 with observed heterozygosity levels ranging from 0.059 to 0.857 (Duval *et al.* 2005). Eiadthong *et al.* (1999a) examined 22 mango cultivars for 40 SSR-anchored primers of 15 to 18 oligonucleotides. Seven primers produced reproducible polymorphic DNA patterns. Also, Viruel *et al.* (2005) pointed out the usefulness of microsatellite markers for mango studies on identification, variability, germplasm conservation, domestication, and movement of germplasm.

Barbieri *et al.* (2005) reported the sequence and variability parameters of 16 microsatellite primer pairs obtained from two mango genomic libraries after digestion of DNA from cv. 'Tommy Atkins' with *Hae*III and *Rsa*I and enrichment in CT repeats. Although no significant differences were recorded between the two libraries in the informativeness of the markers obtained, the *Rsa*I library was shown to be more useful than the *Hae*III one, taking into account the efficiency of the library and the feasibility of clone sequencing.

Amplified fragment-length polymorphism (AFLP)

Amplified Fragment-Length Polymorphism (AFLP) information is used to identify mango cultivars, for studying the genetic relationship among cultivars and rootstocks and for the construction of a genetic-linkage maps. According to Kashkush *et al.* (2001), the AFLP markers are quite suitable for cultivar identification, estimation of genetic relationships, and mapping of QTLs in mango. Yamanaka *et al.* (2006) pointed out that the information generated by AFLP analysis regarding genetic relatedness and diversity existing in the mango genepool are useful for breeding of improved mango varieties. On the other hand, Ravishankar *et al.* (2000) investigated the genetic relatedness of 18 mango cultivars grown in different Indian growing areas through RAPD markers, concluding that most of cultivars were evolved from a local mango gene pool and were later domesticated.

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. In addition, Fang *et al.* (1999) constructed a fingerprinting of two mango cultivars viz., 'Keitt' and 'Tommy Atkins' employing AFLP.

Molecular markers Random Amplified Polymorphic DNA (RAPD) Restriction Fragment Length Polymorphism (RFLP), and Inter-Simple-Sequence Repeats (ISSR)

Mango has been the subject of many analyses using different molecular-marker types as isoenzymes (Degani *et al.* 1993; Aron *et al.* 1997), RAPD (Schnell and Knight 1993; Schnell *et al.* 1995a; López *et al.* 1997; Ravishankar *et al.* 2000), minisatellite and microsatellites (Zietkiewicz *et al.* 1994; Sharon *et al.* 1995; Eiadthong *et al*. 1999a, 1999b), and AFLP (Eiadthong *et al.* 2000). But, the identification or differentiation of zygotic plantlets among nucellar ones in polyembryonic seeds was tested only with isoenzymes (Degani *et al.* 1993) or selecting a fragment related to polyembryony (López *et al.* 1997) using RAPD.

A study using RAPD markers, supported the 'Haden' parentage of 'Eldon', 'Lippens', 'Tommy Atkins', and 'Zill'; however, the parentage of 'Glenn' and 'Osteen' was questioned (Schnell and Knight 1993; Schnell *et al.* 1995a). Using DNA fingerprint DFP markers Adato *et al.* (1995) confirmed the pedigree of many of the 'Haden' seedlings. Most recently, Viruel *et al.* (2005) used microsatellite markers to confirm pedigree information of the 'Haden' family, these agreeing with previous analyses (Adato *et al.* 1995; Schnell *et al.* 1995a) except for one case: the clone of 'Zill' was not resolved as a seedling of 'Haden'. On the other

hand, Jayasankar *et al.* (1998) used RAPD to characterize fungal-toxin-tolerant mutations (resistant to *Colletotrichum gloeosporoides*) from embyrogenic cultures of 'Carabao' and 'Hindi' mango. Souza and Lima (2004) examined 40 mango genotypes of the Embrapa Meio-Norte mango collection for RAPD markers with 32 random primers. Karihaloo *et al.* (2003) used RAPD analysis for 29 Indian mango cultivars comprising some popular and advanced cultivars. Rocha *et al.* (2006) in Brazil identified the zygotic among nucellar plantlets from polyembryonic seeds of 'Rosinha' variety by using RAPD markers, concluding that the elevated taxa of vigorous zygotic plantlets could be explained the great variability in tree height in mango orchards.

Kumar *et al.* (2001) estimated genetic diversity in 50 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 different trends. Earlier, Chunwongse *et al.* (2000), by employing RFLP and AFLP markers, constructed a molecular map of mango cvs. Alphonso and Palmer. Eiadthong *et al.* (1999b) confirmed the phylogenetic relationship in *Mangifera* by RFLP and amplification of cpDNA, denoting the geographic distribution of several *Mangifera* species.

Ravishankar *et al.* (2004), by genomic DNA and chloroplast DNA RFLP analyses, investigated genetic relatedness among 10 polyembryonic and monoembryonic cultivars traditionally grown along the west coast of southern India. According to their results 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.

The internal transcribed spacer (ITS) is a sequence of RNA in a primary transcript that lies between precursor ribosomal subunits and is removed by splicing when the structural RNA precursor molecule is processed into a ribosome. In this sense, the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) can be used to analyse phylogenetic relationships among different species (Harrington *et al.* 2005; Prasongsuk *et al.* 2005; Slippers *et al.* 2005; Limkaisang *et al.* 2006). Yonemori *et al.* (2002) compared sequences of ITS region of nrDNA to reveal phylogenetic relationships of *Mangifera* species.

Microsatellites or SSRs are characterized as short sequences 1 to 5 bp long. These monomer units display tandem organization representing hypervariable loci where the variations arise due to the differences in the number of repeating units forming the repeat array. ISSR technique allows the detection of SSR-derived polymorphisms directly from the genomes without the need to isolate and characterize these sequences.

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. In this context, González *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 exis-

Table 2 Molecular studies in mango breeding.

Author(s)	Biotechnological resource	Study
Fang *et al.* 1999	AFLP	Construction of fingerprints of mango
Yamanaka *et al.* 2006		Estimation of genetic relationship and diversity of mango species
Adato *et al.* 1995	DNA marker RAPD	Identification and analysis of mango genotypes
Jayasankar *et al.* 1998		Characterization of fungal toxin tolerant mutants
Bally *et al.* 1996		Examination of genetic diversity in mango cv. 'Kensington'
Lopéz *et al.* 1997		Geographic differentiation and embryo type identification
Deng *et al.* 1999		RAPD analysis of mango
Karihaloo *et al.* 2003		Assessment of genetic diversity in land races and advanced cultivars
Souza and Lima 2004		Grouping of varieties based on their similarities and divergence
Gomathi *et al.* 2005		Procedure for extracting genomic DNA from mango
Mathews *et al.* 1992, 1993; Samanta *et al.* 2007	Genetic transformation	*Agrobacterium* mediated genetic transformation of mango embryogenic cultures
Cruz *et al.* 1997		Transformation of cv. 'Hindi' with *Agrobacterium tumefaciens*
Cruz *et al.* 2000		Optimisation of a system for transient and stable transformation studies
Bojorquez and Gómez 1995	Cloning of genes	Isolation of peroxisomal thiolase cDNA
Cruz and Gómez 1996b		Isolation of cDNA, which codes for alternate oxidase
Lycett *et al.* 1997		Isolation of novel ripening specific cDNA clone from fruits
Chaimanee *et al.* 1999		Gene expression during fruit ripening
Suntornwat *et al.* 2000		Construction of cDNA using purified m-RNA of endo-polygalacturonase from fruits
González *et al.* 2002	ISSR	Identification of primer sequences showing clear polymorphism between cultivars
Eiadthong *et al.* 1999a	SSR	Identification and genetic variation in mango
Olano *et al.* 2005		Microsatellite markers, Pedigree analysis of Florida mango cultivars
Barbieri *et al.* 2005		Geographic differentiation in mango with microsatellites
Viruel *et al* 2005		Embryo type and geographic differentiation with microsatellites
Duval *et al.* 2005		Assessment of diversity in mango germplasm
Gan *et al.* 1981	Molecular and isozyme markers	Assessment of genetic variation in mango using isozyme markers
Degani *et al.* 1990		Identification of different loci with 17 allelomorphs in 41 cultivars
Schnell *et al.* 1995b		Isolation and characterization of 15 microsatellites loci from mango
Aron *et al.* 1997		Segregation distortion and linkage of mango isoenzyme loci
Jintanawongse and Changtragoon 2000		Identification of mango hybrids resulting from hybridization
Eiadthong *et al.* 1999b	RFLP	Confirmation of phylogenetic relationship and geographic distribution of *Mangifera sp.*
Kumar *et al.* 2001		Estimation of genetic relatedness among some poly-and mono-embryonic cultivars

AFLP, Amplified Fragment-Length Polymorphism; RAPD, Random Amplified Polymorphic DNA; ISSR, Inter-Simple-Sequence Repeats; SSR, Single-Sequence Repeat, RFLP, Restriction Fragment-Length Polymorphism

ting populations, and characterization of rootstocks.

Chavarri *et al.* (2004) studied the biobalistic genetic transformation of mango somatic embryos of cv. 'Haden', 'Madame Francis', and 'Kent', showing that the selected clones of cv. 'Kent' survived all phosphinothricin (PPT) concentrations (0, 0.5, 1.0 and 2.0 mg L^{-1} PPT) for three weeks, while non-transformed embryos survived only at 0.5 mg L^{-1}. The cv. 'Haden' and 'Madame Francis' were sensitive to the bombardment and selection conditions, and did not survive the; process therefore, enzymatic activity of transient expression of the GUS gene was observed and the incorporation of the GUS and BAR genes was demonstrated by means of PCR.

Table 2 shows some of the achievements made in mango breeding through molecular biology.

GENETIC TRANSFORMATION

Genetic transformation is the process by which the genetic material carried by an individual cell is altered by the incorporation of foreign (exogenous) DNA into its genome. In concrete, for plants this provides the means of modifying single horticultural traits in perennial plant cultivars without altering their phenotype. Many authors have pointed out the great importance of genetic transformation of mango (Mathews *et al*. 1992; Cruz *et al*. 1996a; Gómez and Litz 2004). In this context, genetic transformation of mango has been based upon embryogenic cultures derived from the nucellus of young fruit (Litz *et al.* 1982; Litz 1984). This capacity 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 (Gómez and Litz 2004).

Agrobacterium tumefaciens-mediated mango transformation

Genetic transformation, developed for many species, is usually based on the introduction of certain foreign genes into plant cells, integrating the genes into plant genomes, and observing the expression of the genes in the regenerated plants. Numerous DNA-delivery systems have been reportted. The preferred method of gene for dicots is *Agrobacterium tumefaciens*-mediated transfer. Mango tissue appears to be relatively amenable to *in vitro* generation, as pointed out by many authors with reference to the considerable success with somatic embryogenesis for mango cultures. In this sense, *Agrobacterium* transformation is successful after co-cultivation of an embryogenic mango culture. Although it had taken a very long generation time, the prospects for gene transfer in mango seem quite good. Success has been achieved in genetically transforming mango embryogenic cultures using *A. tumefaciens* (Mathews *et al.* 1992, 1993). Engineered *Agrobacterium* strains, (1) C 58CI with plasmid pG3850::1103 with selectable marker NPT II, and (2) A208 containing pTiT37-SE:: pMON9749 (a co-integrate vector with genes encoding for NPT II and GUS) were used for transformation of cvs. 'Keitt' and 'Hindi', respectively. According to Mathews and Litz (1990), 12.5 µgm L^{-1} kanamycin sulphate was toxic to proembryo masses grown in suspension culture, while 200 µgm L^{-1} kanamycin sulphate was toxic in semi-solid medium (Mathews and Litz 1990). Putative transformants could be confirmed based on histochemical staining with X-GLUX and Southern hybridization. Samanta *et al.* (2007) reported a protocol to regenerate shoots through somatic embryogenesis in the polyembryonic mango cv. 'Vellaikolumban', discussing the feasibility of gene transfer using the β-glucuronidase (GUS) reporter gene. Among the various stages of nucellar culture tested, embryogenic callus was found to be amenable to *Agrobacterium*-mediated gene transfer. And the maximum transformation was achieved using 150 µl bacterial cultures with 3 d of co-cultivation. Thus, the expression of the reporter transgene was confirmed by GUS assay (Samanta *et al.* 2007).

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

Gutiérrez *et al.* (2001) isolated a mango cDNA homologue of the ethelene-receptor gene *ETR-1*, which was expressed transiently during fruit ripening as well as wounding of tissue. On the other hand, Cruz *et al.* (2000) optimised particle-bombardment parameters and reported successful transient expression for the *GUS* gene in polyembryonic masses.

The flower-meristem-activity *LEAFY* gene from *Arabidopsis thaliana* (Weigel and Nilsson 1995) was found to stimulate flower initiation in transgenic poplar (*Populus* sp.). Under the control of an appropriate developmental promoter, it could be possible to overcome the problem of alternate bearing in mango by genetic engineering. According to Blazquez and Weigel (2000) alternate bearing could be partially alleviated by the introduction of flower-meristem-activity *AGAMOUS-LIKE 20* (*AGL20*), *APETALA1* (*AP1*), and *LEAFY* genes from *Arabidopsis*.

The control of the tree canopy could be possible by introduction of the gene *rol*C in mango transgenic plants, thereby producing dwarf plants. The gene *rol*C of *Agrobacterium rhizogenes* has been reported as a potential tool for controlling tree size (Oono *et al.* 1987; Koshita *et al.* 2002; Boase *et al.* 2004).

Ripening in most fruits involves cell-wall degradation and is characterized by progressive depolymerisation of pectic and hemicellulosic polysaccharides of the cell wall (Koslanund *et al.* 2005; Chourasia *et al.* 2006). A large number of genes involved in membrane trafficking have been identified and characterized in animal and yeast systems, which appear to be involved in controlling membrane fusion. A full-length c-DNA clone from mango fruit has homology to the rab1/YPT 3 class of small GTPases. The corresponding rnRNA is expressed in the fruit, only during ripening.

In postharvest physiology, one of the main concerns for prolonging the shelf life of fresh fruits is to stop ethylene action and this is usually achieved by the use of controlled atmospheres or such ethylene absorbents as potassium permanganate or activated charcoal/vanadium oxide (Maekawa 1990).

Mango is a climacteric fruit and highly perishable due to over ripening and increased susceptibility of mature fruit to development of anthracnose. Genetic transformation of mango has the potential for prolonging fruit shelf life (Michalczuk 2004). Recent achievements in the transformation techniques will permit testing the function of specific hydrolytic enzymes for extending the shelf live of mango fruit. This development is particularly relevant because it will probably be the first tropical fruit for which the ripening pattern may be genetically manipulated (Hemanth *et al.* 2007). The mango ACC synthase and ACC oxidase genes are currently being used in mango-transformation experiments. According to bin Zainal *et al.* (1996) a *rab11*-like gene is regulated in ripening mango fruit, the corresponding mRNA is expressed in fruit, only during ripening.

These are the first mango genes that have been cloned in an antisense strategy to stop ethylene production and extend the fruit shelf life. With regard to insect pests, considerable variation is also known to occur among mango cultivars with respect to their susceptibility to attack and injury. Transgenic plants provide better insect control as well as resistance from a range of fungal and bacterial infections, resulting in an overall lower loss of fruit yield (Carozzi and Kosiel 2007).

Preservation of *Mangifera* spp. germplasm for long-

term conservation should also be feasible by means of cryopreservation of embryogenic cultures (Engelmann 1991; Wu *et al.* 2003). Of the hundreds of mango cultivars, only some 25 to 40 are of commercial importance. There is considerable confusion regarding cultivar nomenclature, since similar cultivars grown in different areas are known by different names. For example 'Alphonso', probably the best mango cultivar grown in India, has been referred to as 'Badami', 'Gundu', 'Patnam', 'Jathi', 'Khader', 'Aphus', and 'Hapus' (Lakshminarayana 1990). Furthermore, different cultivars sometimes appear under the same name. Mango cultivars are currently identified on the basis of morphological traits such as leaf and fruit characteristics. The first step to preservation is accurate identification of the cultivars, for which molecular methods may be the key.

CONCLUSION AND FUTURE PERSPECTIVES

Mango is a fruit tree with a great economic importance, especially in tropical zones. However, its fruits are susceptible to many pathogens that threaten yield in both traditional and new growing areas (subtropics). Many difficulties arise in establishing aseptic mango cultures from mature explants, associated with phenolic browning, greatly hindering the micropropagation of mango. Biotechnology holds the promise to revolutionize the mango industry by development of altered varieties fore specific purposes through precise genetic manipulation, such aspirations being hitherto unachievable through conventional breeding. Breeding objectives in which biotechnology could play a part include disease and pest resistant, post-harvest problems, cold tolerance, canopy shape and size, and, above all, excellent fruit colour, shape, and taste.

Tissue-culture techniques such as anther and ovary culture can be exploited for raising homozygous lines. Also, genetic transformation to raise stable transformants for different characters is gradually being explored. Genetic markers are of special significance as they can aid in conventional breeding approaches.

Transformation of mango through repetitive somatic embryogenesis has also been accomplished. However, the successful regeneration of genotypes, i.e. the conversion rate of somatic embryos into normal plantlets, remains low. The future research needs to be addressed on promoting conversion frequency of somatic embryos into plantlets and the regeneration of them from shoot/nodal segments.

Introduction of dwarfing gene(s) could solve problems related to most worldwide marketable cultivars (e.g. 'Haden', 'Tommy Atkins', 'Osteen', 'Kent', 'Sensation', 'Alphonso'), as these have large canopies that hamper high-density planting.

Mangifera spp. originated in South-east Asia. Vast areas have been completely or partially deforested for expanding agriculture. This has caused great genetic erosion within many species and genera, including mango. Because of the loss of natural habitat, the establishment of *ex situ* and *in situ* germplasm collections of *Mangifera* species is considered to be imperative. Sources of resistance to mango malformation, anthracnose, powdery mildew, etc. are urgently needed and can be exploited from other species of *Mangifera*. Moreover, the biotechnology advances can also meet the challenges of abiotic and biotic stresses. Thus, through biotechnology, mango should continue to expand as a major world crop.

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