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Molecular and Genetic Characterization of Somaclonal Variation in Micropropagated Bananas (*Musa* spp.)

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ABSTRACT

Banana is an economically important fruit as well as food security crop. Though it originated in the Southeast Asian region, it is widely grown around the world. Conventionally, bananas are vegetatively propagated by suckers or corms, while the seeds are employed for propagation only in breeding programs. Vegetative propagation is the major cause of introduction and spread of diseases to non-infected areas. To overcome this problem, tissue-cultured banana plants are being used as planting material. Somaclonal variation (SV) appears to be widespread among banana plants regenerated by tissue culture. A number of factors are known to induce SV which can be problematic during banana micropropagation, although, it may be utilized as an efficient tool in plant breeding. Somaclones have been identified based on their morphological or agronomic characteristics. Early detection of SV is, therefore, very useful. A morphological, biochemical, cytogenetic and/or molecular biology based approach of analysis would help throw light on the causes and detection of variants. Factors inducing SV, their detection and analysis have been discussed in this review.

Keywords: molecular markers, somaclonal variants, tissue culture, vegetative propagation
Abbreviations: AFLP, amplified fragment length polymorphism; **BA**, 6-benzyladenine; **GA_3**, gibberellic acid; **IRAP**, inter retrotransposon amplified polymorphism; **ISSR**, inter simple sequence repeat; **LTR**, long terminal repeat; **MSAP**, methylation-sensitive amplification polymorphism; **PCR**, polymerase chain reaction; **PGR**, plant growth regulator; **RAPD**, randomly amplified polymorphic DNA; **RDA**, representational difference analysis; **SAMPL**, selective amplification of microsatellite polymorphic loci; **SV**, somaclonal variation; **TC**, tissue culture

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INTRODUCTION

Banana, one of the most economically important fruit crops, grows well in tropical and subtropical regions of the world. It belongs to the genus *Musa*, which comprises of all (over 50 species) the edible bananas and plantains, in the family Musaceae. Banana ranks fourth in the world in terms of gross value of production exceeded only by rice, wheat and maize (Sahijram 2001). In fact, it is no longer considered just a fruit crop but is now categorized as a food crop providing nourishment to millions of people around the globe (Singh 2002). It is grown in more than 120 countries throughout the world and has an annual production of around 812 million tons (FAO 2007). India is the largest producer with a total annual production of 21.76 million tonnes followed by China, Philippines, Brazil and Ecuador (**Fig. 1**).

The earliest reference to banana dates back to about 500 BC (Pillay and Tripathi 2007). Edible bananas seem to have originated in the Indo-Malaysian region and its origin has been traced to Southeast Asia in the jungles of Malaysia, Indonesia or Philippines (Simmonds 1966; 1987; Morton 1987). Banana originated from two wild diploid ($2n = 22$) species, *viz*., *M. acuminata* Colla which has a genomic composition of AA and *M. balbisiana* Colla, with a genomic composition of BB (Cheesman 1948). *Musa acuminata* is a native of the Malay Peninsula and adjacent regions while *M. balbisiana* is found in India eastwards to the tropical Pacific (Simmonds 1966). Many wild varieties still exist in the natural vegetation of Southeast Asia, the center of origin (IPGRI 2000). From Asia, bananas and plantains are believed to have spread to Africa and throughout the tropics solely by humans through suckers (Simmonds 1962). Western and Central Africa harbors the world's greatest diversity of plantains and highland bananas and are considered to be the secondary centers of diversification of plantains and bananas (Swennen 1990) and have enriched the diversity of *Musa* with about 100 clones each

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Invited Review

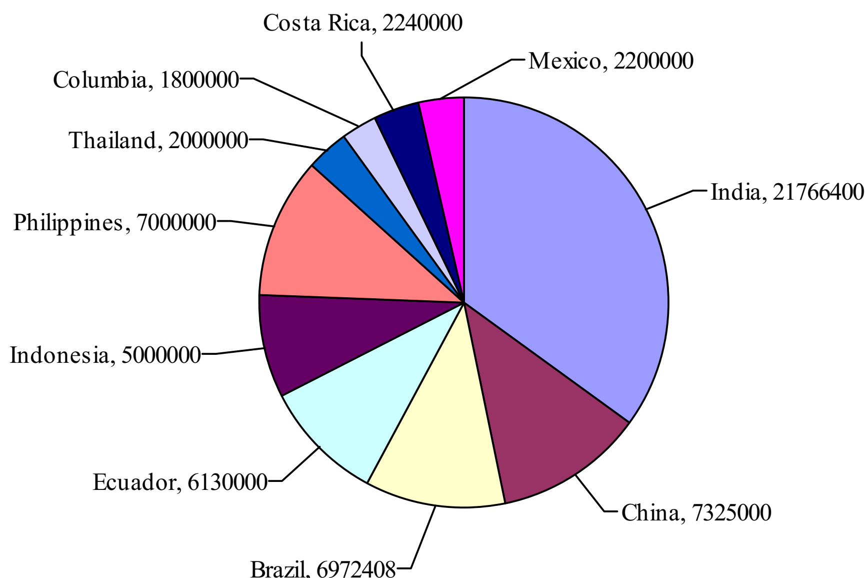

Fig. 1 Top ten banana producing countries of the world (the value is the production in tonnes, FAO 2007).

(Lescot 2000; Pillay and Tripathi 2007).

Banana is a perennial succulent having a juicy stem (pseudostem) which is a cylinder of leaf-petiole sheaths, reaching a height of 20-25 ft and arising from a fleshy rhizome or corm (Morton 1987). The leaves are either entirely green, green with maroon splotches, or green on the upper side and red purple beneath. They are tender, smooth, oblong or elliptic, fleshy-stalked, and 4-15 are arranged spirally. They unfurl as the plant grows and extend upwards and outwards becoming around 9 ft long and 2 ft wide. The growing point gets transformed into the inflorescence, which is a terminal spike, large, long-oval, tapering, purple at early stages. The flowers are white, slim, nectar-rich, tubular, toothed and clustered in whorled double rows along the floral stalk with each cluster covered by thick, waxy, hood-like bract which is purple outside and deep-red within (Morton 1987). The female flowers appear first and have large ovaries that develop into fruits. Female flowers occupy the lower 5 to 15 rows followed by some rows of hermaphrodite flowers while the male flowers are borne in the upper rows. Generally, shortly after opening, the inflorescence begins to bend downward. In most cultivated bananas, the fruits develop by parthenocarpy (Simmonds 1953). As the young fruits develop from the female flowers, they look like slender green fingers which turns from deep-green to yellow or red and ranges from 2-12 inch in length and ¾-2 inch in width, oblong, cylindrical and blunt to 3-angled, somewhat curved and hornlike. The bracts are shed and the fully grown fruits in each cluster become a "hand" of bananas with the number of "hands" varying with the species and variety. The stalk droops with the weight until the bunch is upside down (Morton 1987). The common cultivated types of bananas are generally seedless while the wild types may be filled with black, hard, rounded or angled seeds. In all bananas, the growing shoot dies after fruiting once and its life is perpetuated by means of suckers, which develop from adventitious buds produced on the corm (Simmonds 1962).

ECONOMIC IMPORTANCE OF BANANAS

Banana is utilized in a multitude of ways in the human diet. Its year round availability has made it an important food security crop as well as cash crop in the tropics (Jones 2000). Each country producing bananas has its own way of preparing and consuming it by way of its own traditional dishes and methods of processing (Frison and Sharrock 1998). It is consumed raw as a ripe fruit which is simply peeled and eaten out-of-hand to being sliced and served in fruit cups and salads, custards and gelatins or the ripe or raw fruit is cooked before consumption. Ripe bananas are also used in the manufacture of beer and wine (Sharrock 1997). Dried green plantains, ground fine and roasted, have been used as a substitute for coffee (Morton 1987). Non-fruit parts of the banana plant, including the corm, shoots, pseudostem and male buds are eaten as vegetables in Africa and parts of Asia (Simmonds 1962).

Banana rejects are fermented for the production of vinegar or supplemented with protein, vitamins and minerals and used as animal feed (Morton 1987). Leaves, pseudostems, fruit stalks and peels, after chopping, fermentation, and drying, yield a meal somewhat more nutritious than alfalfa press cake and has been considered for use as organic fertilizer (Morton 1987). Nutritionally, fresh bananas contain carbohydrates, fiber, protein and fat, major elements

Table 1 Food value per 100 g of edible portion of banana fruit.

Nutritional value	**Per 100 g of edible portion**
Energy	89 Kcal
Carbohydrate (g)	22.84
Fat (g)	0.33
Fibre (g)	2.6
Protein (g)	1.09
Water (g)	74.91
Vitamins (mg)	
Vitamin A	0.109
Vitamin B_6	0.367
Niacin	0.665
Riboflavin	0.073
Thiamin	0.031
Vitamin C	8.7
Vitamin E	0.1
Vitamin K	0.5
Minerals (mg)	
Calcium	5
Copper	0.078
Iron	0.26
Manganese	0.27
Magnesium	27
Phosphorus	22
Potassium	358
Selenium	0.001
Sodium	1
Zinc	0.15

Source: http://www.happyjuicer.com/Nutrition/bananas.aspx

such as potassium, magnesium, phosphorus, calcium, iron, and vitamins A, B_6 and C (**Table 1**, Robinson 1996). All parts of banana have medicinal applications. The fruits are beneficial in the prevention of intestinal disorders, constipation, arthritis, gout, anemia, allergies, kidney stones, tuberculosis and urinary disorders (Pederson 2009; http://www.banana.com/medicinal.html). The leaves are placed as poultices on burns and other skin infections. The flowers are used in bronchitis, dysentery, ulcers, diabetes, etc while astringent sap for hysteria, epilepsy, leprosy, fevers, hemorrhages, insect stings and bites, etc. The roots are administered for digestive disorders. Peel and pulp of ripe bananas also have antifungal and antibiotic properties. In Malaya, pigs fed with the pseudostems are less prone to liver and kidney parasites than those on other diets (Morton 1987).

Dried banana peel, which has 30-40% tannin content, is used to blacken leather while its ash, which is rich in potash, is used for making soap. Banana leaves can be used as thatching materials for houses, as plates, tablecloths, umbrellas, sleeping mats, animal feed and in food preparation. They serve as padding on the banana inspection turntables when they are split lengthwise. During transport, they are used to cushion the bunches. Banana leaves and pseudostems contain high quality fiber which is used for making ropes, string, thread, handicraft, baskets, table mats, wall hangings, lamp shades, carpets and manufacturing of banana paper (Sharrock 1997). It is also highly valued for fishing lines and is woven into a thin, transparent fabric. Banana plants also provide shade for crops that grow better in shade conditions such as cocoa, black pepper, coffee and vanilla. They maintain the soil structure and cover throughout the year, protecting it from wind and rain erosion.

PROPAGATION OF BANANAS

Banana seeds are employed for propagation only in breeding programs. Conventionally, bananas are propagated vegetatively by suckers or corms (reviewed by Baiyeri and Aba 2007). The sucker first emerges as a conical shoot which opens and releases leaves that are mostly midribs with only vestiges of blade and just before these suckers produce wide leaves resembling those of the mature plant but smaller, it has sufficient corm development to be transplanted (Morton 1987). In the field, multiplication has been artificially stimulated by removing the soil and outer leaf sheaths covering the upper buds of the corm, packing soil around them and harvesting them when they have reached the right sucker stage. In the greenhouse multiplication is artificially stimulated by cleaning and injuring the corm to induce callus formation from which new plants develop. However, field and greenhouse multiplied suckers are many a times infested with various pests and pathogens which affect the growth and in turn the quality and yield of the bananas.

Diseases are often spread by vegetative propagation of bananas, and this has led to creation of disease-free planting material on a large scale by tissue culture (TC). To avoid the introduction of pests in an area where the bananas are grown for the first time, TC derived plants can be used. Micropropagation has been achieved by shoot-tip and apical meristem culture (**Fig. 2**, Cronauer and Krikorian 1984; Banerjee and De Langhe 1985; Tripathi *et al.* 2003). Plant regeneration through somatic embryogenesis has been achieved by establishing embryogenic cell suspension cultures using explants such as leaf sheaths, corm sections, sections from highly proliferating shoot tip cultures, immature zygotic embryos and male flowers (Escalant and Teisson 1989; Novak *et al.* 1989; Dhed'a *et al.* 1991; Marroquin *et al.* 1993; Escalant *et al.* 1994; Cote *et al.* 1996; Navarro *et al.* 1997; Sági *et al.* 1998a; Becker *et al.* 2000; Ganapathi *et al.* 2001; reviewed by Xu *et al*. 2008).

Establishment of micropropagation and somatic embryogenesis have revolutionized banana cultivation and has replaced the use of conventional vegetative suckers in many of the intensive banana-growing regions. The technique

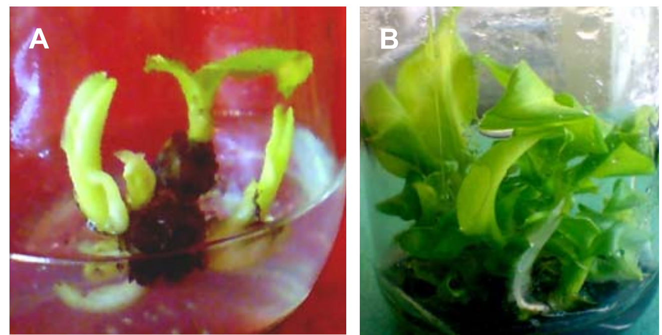

Fig. 2 Formation of multiple shoots from shoot apex explants of banana cv. 'Grand Naine'. (**A**) Early stage. (**B**) Advanced stage.

enables the mass production of clones at a far greater rate than by the collection of suckers from growing plants. TC regenerated plantlets are tender and require good management in the weeks following removal from the growing medium. However, once planted in the field and established, their growth and ultimate yield are superior, with dense and compact fruit bunches having well shaped fingers for better marketability (Vuylsteke and Ortiz 1996). This may be due to the juvenile nature of the material and the increased photosynthetic efficiency, increased functional leaf area, increased root vigor and total dry mass accumulation in TC derived plants (Robinson 2000). TC derived plants also enable flexibility in accordance with the planting season and marketing demand.

To improve the germination efficiency of seeds for breeding, hybrid embryo rescue techniques by excising the hybrid zygotic embryo and growing it *in vitro* on defined media has been developed (Bakry 2008). Banana breeders also use shoot-tip culture for multiplication of newly bred genotypes (Vuylsteke *et al*. 1998).

SOMACLONAL VARIATION IN BANANAS

Though TC has been widely exploited, plants clonally propagated *in vitro* are known to exhibit some off-types, frequencies varying from 1 to 74%. This variation among plants regenerated from TC is termed somaclonal variation (SV) and the off-types as somaclones (Larkin and Scowcroft 1981). Shoot-tip cultures are known to preserve genetic stability with far greater fidelity than callus or cell suspension cultures, yet variation appears to be widespread among plants regenerated from banana shoot-tip cultures. SV results from both pre-existing genetic variation within the explant and variation induced during the TC phase (Evans *et al.* 1984). Variations for karyotype, isoenzyme, precocity in bearing, ploidy level, growth, yield, quality, pigment, disease incidence and abiotic resistance in somaclones have been reported in different plant species (Patil and Navale 2000). Banana is known to produce somaclonal variants (Krikorian *et al.* 1993), but only those plants that show the same type of variation in side-shoots are considered as 'variants' (Rodrigues *et al.* 1998). SV can be problematic during micropropagation, *in vitro* conservation and in genetic transformation of crop plants. For those primarily looking for clonal fidelity, this can be a serious problem and strategies have been developed to reduce such variations to a manageable level. Though SV is a hindrance to clonal uniformity, particularly for populations used to establish commercial banana plantations, this phenomenon may also provide a useful source of genetic variation to the plant breeder (Nwauzoma *et al*. 2002) and has been used as an effective tool in the genetic improvement of horticultural traits in banana (Tang 2005). However, the major drawback of SV is the fact that it is a random process and hence it is non-directional. The kind of variation and the frequency of occurrence are unpredictable and not controllable. Also the occurrence of a useful variant is very low (Tang 2005).

Genetic transformation has also been used to introduce

foreign genes of horticultural traits in bananas (Chakrabarti *et al*. 2003; Atkinson *et al*. 2004; Sunilkumar *et al*. 2005). SV is an important scientific concern that relates to the TC component of genetic transformation of banana (Sági *et al*. 1998b). Regeneration systems requiring a minimum time in culture, but still compatible with transformation, are needed. It should be noted that SV is inherent to *in vitro* culture and is not related to the transformation techniques (Sági *et al*. 1998b).

Factors responsible for SV in bananas

To date, SV affecting *in vitro* propagated banana is not well understood, suggesting a complex underlying genetic basis. A number of factors are considered to be responsible for inducing SV in bananas. Choice of explants may influence the induction of SV. Highly differentiated tissues such as roots, leaves and stems, when used as explants or explants-source, produce more variation than axillary buds and shoot tips which have pre-existing meristems (Duncan 1997). Shoot tip culture of banana gave rise to higher rate of somaclonal variation compared to cell suspension cultures initiated from immature male flowers (Shchukin *et al.* 1997, 1998; Cote *et al*. 2000). This may be due to the presence of pre-existing variation such as endopolyploidization, somatic mutations, etc. in the explants used to initiate tissue culture (Veilleux and Johnson 1998). There is no evidence that plant growth regulators (PGRs) routinely used in TC *directly* affect the rate of variation; however, there have been reports where the use of high concentration of cytokinins might have induced SV in banana (Shepherd *et al.* 1996; Trujillo and García 1996; Wang and Liu 1997). These exogenously applied PGRs possibly trigger cell-cycle disturbance leading to the induction of SV (Peschke and Phillips 1992). Age of the culture enhances variability among regenerated plants and has been found to be positively related to the generation number (i.e. the number of subculture cycles). Though no somaclonal variants were observed in shoot-tip cultures of 'Nainco' at the end of three subcultures, they appeared after the fifth subculture and their frequency increased after five, seven, nine and eleven subcultures (Rodrigues *et al.* 1998). *In vitro* culture conditions and rapid multiplication may affect genetic stability of the explants by increasing mutation rate per cell-generation and accumulating it over a period of time (Duncan 1997; Martínez *et al*. 1998). Type of TC-induced response also plays an important role in the induction of SV. Callus cultures characterized by extensive dedifferentiation induced by high concentration of PGRs, in contrast to regeneration of shoots from pre-existing meristems, may influence the induction of SV. However, in banana, it has been observed that shoot-tip derived cultures exhibited a higher rate of SV as compared to plants regenerated by somatic embryogenesis (Shchukin *et al.* 1997, 1998). In bananas, SV may also be genotype dependent (Ventura *et al.* 1988; Pancholi *et al.* 1996; Martínez *et al.* 1998). Labile portions are known to exist in the genome rendering it susceptible to variation. These portions get modulated when cells undergo the 'stress' of TC, resulting in higher rearrangement and mutation rates than other portions of the genome (Thomas *et al.* 2002). Occurrence of hot-spots of mutation and recurring-menus of alternative alleles is consistent with this response being limited to a sub-fraction of the genome. Other factors such as ploidy level, transposable elements, genomic status of donor plant and DNA methylation status may be responsible for the induction of SV (Sahijram *et al*. 2003). Recent literature suggests that hypo- or hyper-methylation of DNA triggering genome-wide changes may be the underlying cause (James *et al.* 2007).

Types of somaclones observed in bananas

i) Morphological somaclones

Somaclones with different types of plant morphology have been reported in banana (**Fig. 3**, Israeli *et al.* 1991; Vidhya and Ashalatha 2002; Uma *et al.* 2002; Martin *et al.* 2006). In 'Grand Naine', various somaclones such as dwarf, giant and massada (mosaic variegation on the leaves) were reported (Walther *et al.* 1997). Variants for plant stature, abnormal leaves, pseudostem pigmentation, persistence of flowers and split fingers were observed in seven *in vitro* propagated cultivars of 'Cavendish' bananas (Israeli *et al.* 1991). Israeli *et al.* (1991) reported the rate of dwarf mutations *in vitro* was, by far, the most common somaclone followed by mosaic while giant and extra dwarf occurred in a much lower frequency. On the other hand, Arias and Valverde (1987) and Ventura *et al.* (1988) reported the appearance of the 'giant' somaclones in higher frequencies than dwarf

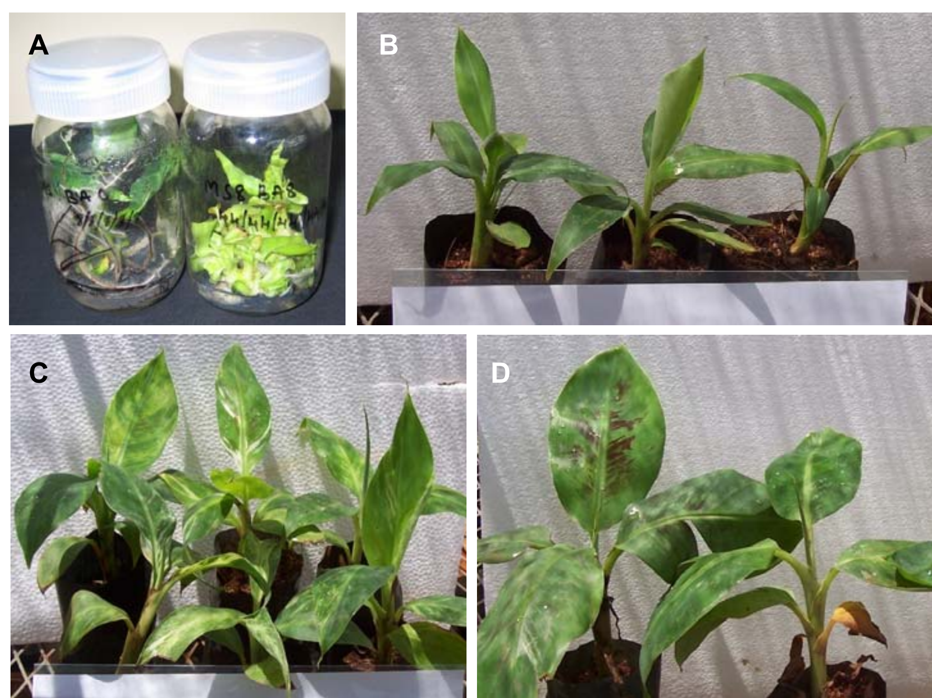

Fig. 3 Somaclones observed in banana cv. 'Grand Naine'. (**A**) *In vitro* culture without the addition of BA showing normal growth with roots (left) and with the addition of BA 8 mgL^{-1} showing stunted multiple shoots. (**B**) Acclimatization of stunted off-types in the greenhouse. (**C**) Acclimatization of streaked leaf off-types in the greenhouse. (**D**) Acclimatization of oval leaf off-types in the greenhouse.

indicating that the type and incidence of somaclonal variants are influenced by either the environmental conditions, the specific clone or both (Walther *et al.* 1997). Differences in rates of variants reported by different laboratories with different primary explants also suggests that the high incidence and rate of dwarf somaclonal variants is caused by chimeric heterogeneity of the primary explants (Reuveni and Israeli 1990; Cote *et al.* 1993; Krikorian *et al.* 1993; Israeli *et al.* 1995). In 'Red' variety, green variants lacking anthocyanin pigmentation in leaf sheath, petiole, midrib and fruit rind were observed (Vidhya and Ashalatha 2002).

In plantains, French and Monganga inflorescences, distorted lamina, drooping leaves and leaf variegation were observed (Vuylsteke *et al.* 1988). In 'Grande Naine', Martin *et al.* (2006) reported somaclones with variegated leaf, pseudostem, bracts, ovary of the male flower and fruits, reduced height, decreased lamina length and breadth, and early flowering. The effect of PGRs in inducing SV has been debated for a long time. In banana, PGRs have shown no effect (Reuveni *et al.* 1993) as well as direct (Stover 1987) and indirect (Karp 1994, 1995; Damasco *et al.* 1998a) effect on the rate of SV induction. PGRs may affect SV indirectly by increasing multiplication rate (Bairu *et al.* 2006). It was also found that adventitious origin of the meristem, contrary to axillary origin, high number of multiplication cycles and clonal origin were the major factors affecting the rate of SV (Reuveni and Israeli 1990; Reuveni *et al.* 1993; Israeli *et al.* 1996). It was also demonstrated that the frequency of variation was genotype dependent with some groups of plants giving rise to no variants independent of the medium, rate of multiplication and type of plantlet produced in culture (Reuveni and Israeli 1990; Damasco *et al.* 1998a). Other somaclones such as thin and sickly looking tall plants, abnormal pseudostem pigmentation with or without blotching, twisted and crinkly leaves, narrow or drooping leaves, leaves with unusually wavy margins, abnormal bunch orientation, small, narrow elongated or bloated male bud, absence of male buds or its reversion, small bunch with short/twisted fingers, variation in hand and finger orientation on the bunch, persistent or deciduous floral bracts, hairiness of bunch peduncle and fruit, warty fruits with ugly eruptions have also been observed in banana (Uma *et al.* 2002).

ii) Useful somaclones

In banana, appearance of somaclones during *in vitro* multiplication process is a serious impediment for mass propagation. However, banana being a vegetatively propagated crop that is recalcitrant to conventional breeding, this variation can be useful in selecting for clones with improved agronomic characteristics. A somaclonal variant 'CIEN-BTA-03' displayed resistance to yellow sigatoka, caused by a pathogenic fungi (Trujillo and García 1996). Two somaclones, viz., 'Tai-Chiao No. 1' and 'Formosana', were obtained from the wilt-susceptible 'Giant Cavendish' variety. Formosana had a higher level of resistance to *Fusarium oxysporum* f.sp. *cubense* (*Foc*) race 4 than 'Tai-Chiao No. 1' and also produced a bunch about 40% heavier than that of its progenitor 'Pei Chiao' (Hwang and Tang 1996; Hwang 2002). However, the growing cycle of 'Formosana' is about a month longer than 'Pei Chiao' (Tang 2005). From 'Tai-Chiao No. 1', a somaclone TC1-229 was obtained which was semi-dwarf, had the advantages of ease in field management, wind tolerance and retained the resistance to *Fusarium* wilt and was registered as 'Tai Chiao No. 3' (Tang *et al.* 2000; Tang 2005). 'Tai Chiao No. 3' also gave rise to two somaclones; 'TC3-600' with higher yield and 'TC3-1035' which had a shorter growing cycle (Tang 2005). Lopez (1995) obtained two somaclones of *Musa* spp. with tolerance to black Sigatoka which showed very high yield. Stover (1987) reported a few 'Dwarf Cavendish' types with superior bunch quality.

Tang (2002) also used SV for target breeding in banana, for resistance to *Fusarium* wilt, shorter plant stature, early flowering and large bunch size. Forty six putative resistant/tolerant clones were selected and evaluated. Of them, 'GC-1089' was identified as a new resistant/tolerant clone which showed low levels of susceptibility to disease (6-15%) in the infected orchards for a period of 3 consecutive cycles. 'GC-138' is a somaclone with 1-1.5 month shorter growing cycle than that of its parental cultivar 'Pei-Chiao'. 'TC2-425' was another somaclone selected for larger bunch size from a semi-dwarf cultivar 'Tai-Chiao No. 2', the average bunch weight/plant of 'TC2-425' being 19.4% higher than that of its parental cultivar. 'AO 2B2-2', a somaclone of 'Agbagba' expressed lower susceptibility to the black Sigatoka disease and compared to its progenitor had a higher bunch weight, more fruits per bunch with higher average weight, greater average length and greater average girth (Nwauzoma *et al.* 2002). Damasco *et al.* (1997) observed that micropropagated 'Cavendish' banana (*Musa* spp., AAA) off-type plants showed better tolerance to low temperature and low light with improved growth. Thus, the breeding strategy based on SV is a useful source of variation for the selection of both disease resistance and improved horticultural traits including earliness, dwarfism, and yield-potential in banana (Hwang 2002; Tang 2005).

Detection of somaclonal variation

i) Morphological, biochemical and/or cytological screening

Morphologically, the dwarf and giant off-types of banana plantlets have been optimally detected by using the leaf lamina length: width ratio and the pseudostem height: perimeter ratio (Siverio-Grillo *et al.* 1998). The dwarf off-types of 'Williams' have a leaf index of less than 2 while normal plants had a leaf index of 2.2 or higher (Damasco *et al.* 1998b). Another study took the petiole length also into consideration for the detection of somaclones (Cote *et al.* 1994). However, visual detection of off-types in micropropagated bananas is time consuming, laborious and expensive since it is done 3–4 months after field establishment (Israeli *et al.* 1991). Earlier detection of off-types in the nursery by inspecting individual plants is possible but also laborious and needs optimal and uniform growth conditions for all plants (Smith and Hamill 1993). Dwarf off-types can be detected at an earlier stage in the nursery by carefully measuring differences in plant height, petiole length and leaf morphology. Mosaic and leaf variants are comparatively easier to detect. Also, visual screening is neither completely efficient nor error-free. In addition, the appearance of the plants can be heavily influenced by the environmental conditions during hardening (Siverio-Grillo *et al.* 1998). Highly distinct variants such as extra dwarf, mosaic and variegated plants can sometimes be identified even during micropropagation (Israeli *et al.* 1991). The extra dwarf off-types could be identified by their compact appearance and the formation of rosette-like leaves and can be screened out easily during the hardening and nursery stages. Giants can be detected at the nursery stage as they are 30% taller than normal plants. The first distinguishable symptom of mosaic variants is mosaic spots on the lamina which can be detected at the *in vitro*, hardening as well as nursery stage. Extreme mosaic can be detected at the hardening stage or immediately after potting. The first detection of variegated leaves depends on the intensity of symptoms. Extremely variegated leaves could be detected in the test tube or at the very beginning of the hardening stage. Less intense symptoms could be detected at the nursery stage before transplanting to the field (Israeli *et al.* 1991).

Dwarfs of 'Cavendish' banana, induced by micropropagation, are often gibberellic acid ($GA_{3)}$ non-responsive (Reuveni 1990; Cote *et al.* 1993). Application of GA_3 to detect dwarf off-types was attempted *in vitro*, at deflasking or following establishment in soil. Elongation in normal plants was found to be 2-fold greater than in dwarf plants. When the screen was undertaken on the first leaf produced

after GA_3 application taken from plants at deflasking, separation of normal and dwarf plants was complete. However, there was at least 10% overlap with dwarf plants, indicating that 10% of normal plants would have to be discarded before it was clear that only normal plants remained (Damasco *et al.* 1996a, 1996b).

Cytogenetic analysis and relative nucleic acid content estimation by flow cytometry could be utilized to detect mutations of the 'CIEN BTA-03' somaclonal variant and showed that it is a tetraploid clone (Giménez *et al.* 2001). Plantlets of banana cv. 'Williams' micropropagated from shoot tips indicated that the first ratoon of some variants had a higher level of soluble proteins than their donor plants, while, the reverse was true for the total amino acid content (El-Tarras *et al.* 1995). El-Dougdoug *et al.* (2007) used isozymes to analyze SV in TC-derived banana plants tested for banana bunchy top virus and cucumber mosaic virus. Cytological studies of root tips at metaphase revealed that chromosome number differed among somaclonal variants (El-Tarras *et al.* 1995). However, none of the above methods of screening were completely reliable making the development and use of efficient and reliable methods for detecting off-types of prime importance to the banana industry.

ii) Molecular screening

A number of different molecular techniques are currently available to detect sequence variation between closely related genomes such as those between source plants and somaclones (Oh *et al.* 2007). These include randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), sequence characterized amplified region (SCAR, **Fig. 4**), inter retrotransposon amplified polymorphism (IRAP), methylation sensitive amplification polymorphism (MSAP), representational difference analysis (RDA), selective amplification of microsatellite polymorphic loci (SAMPL), etc.

RAPDs have been extensively used to detect SV in bananas. Pancholi *et al.* (1996) showed by RAPD analysis that SV was relatively high in banana TCs. Damasco (1997) identified RAPD Primer OPJ-04 (5′-CCGAACACGG-3′) to be specific to dwarf off-type. Primer OPJ-04 consistently amplified a fragment of approximately 1,500 bp which was present in normal (true-to-type) but absent in the dwarf off-type plants of two Cavendish cultivars including 'New Guinea Cavendish' and 'Williams'. It has been used to identify height-variants (extra-dwarf, dwarf and giant) originating from *in vitro* cultures of 'Grand Naine' and 'Petite Naine' (Martín *et al.* 1998). The RAPD marker was converted into a SCAR marker to develop a PCR-based detection system for the dwarfism genotype (Damasco *et al.* 1998a). However, use of a SCAR marker on its own has a major limitation as the absence of a PCR product from dwarf DNA cannot be distinguished between the off-type condition or as a result of a problem with PCR amplification. Hence, a positive internal PCR control to the SCAR marker was added in a multiplex PCR and also the technique was adapted for use with small amounts of fresh *in vitro* leaf materials as PCR template (Ramage *et al.* 2004).

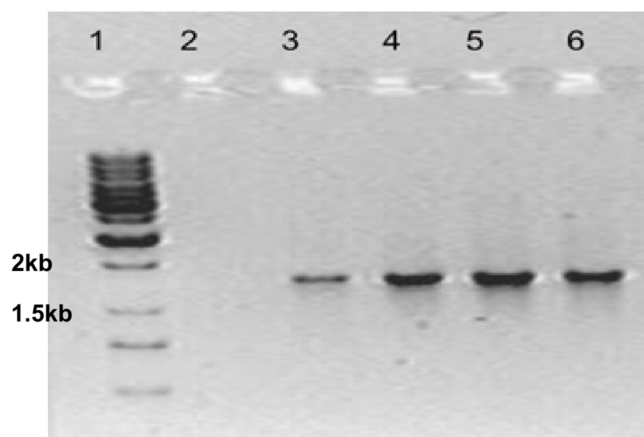

Fig. 4 SCAR analysis of somaclones of banana cv. 'Grand Naine' obtained after subculture cycle 7 (Sahijram *et al.*, unpublished data). Lane 1: 1 kb DNA ladder; Lane 2: BA 2 mg/l; Lane 3: BA 4 mg/l; Lane 4: BA 6 mg/l; Lane 5: BA 8 mg/l; Lane 6: BA 10 mg/l.

Bairu *et al.* (2006) also identified a RAPD Primer OPC-15 (5′-GACGGATCAG-3′) which could also consistently amplify a fragment of approximately 1,500 bp which was present in normal (true-to-type) but absent in the dwarf off-type plants. Although both fragments are similar in size, it is necessary to sequence both fragments to determine their base composition and identify similarities and differences. The sensitivity of the RAPD technique for detecting polymorphism among the somaclonal variant 'CIEN BTA-03' (resistant to Yellow Sigatoka), the parental plant (Williams), two susceptible somaclones obtained by clonal propagation of 'Williams', and two naturally resistant clones to the disease has also been tested (Vidal and García 2000). El-Dougdoug *et al.* (2007) used RAPD markers and found that the frequency of SV was dependent on the number of subculture cycles and were able to detect SVs only in 7-month old cultures. They also observed that morphologically abnormal shoots and visual changes in chlorophyll content showed genetic variations at the molecular level too.

RAPD analysis has been used to differentiate between a gamma irradiation-induced mutant of 'Grand Naine' and its original parental line (Kaemmer *et al.* 1992). RAPD primers were also able to detect SV among the parental plants as well as regenerated plants of the first, third, fifth, seventh and ninth sub-cultures. The presence of specific band/loci in the parental plants and loss of it in the regenerated plants of different subcultures indicates the loss of certain loci during TC due to SV (Sheidai *et al.* 2008). 'CUDBT-B1', a morphological variant showing variegated leaf, pseudostem, bracts, reduced height, decreased lamina length and breadth and early flowering, were assayed using RAPD which showed a marker DNA band of 1650 bp and differential band intensity between 'CUDBT-B1' and the normal clone (Martin *et al.* 2006). The mutations of the somaclonal variant 'CIEN BTA-03' which is resistant to Yellow Sigatoka could be detected by RAPD analysis (Gimenez *et al.* 2001).

RAPD and IRAP markers were used to investigate genetic variation in *Fusarium* wilt resistant and susceptible somaclones of 'Rastali'. It was observed that RAPD markers could successfully detect genetic variation within and between individuals of the clones while IRAP markers, either be a single primer or a combination of primers, based on long terminal repeat (LTR) orientation amplified different retrotransposons dispersed in the *Musa* genome detecting new events of insertions (Muhammad and Othman 2005). Kaemmer *et al.* (1992) used RAPD and microsatellite fingerprinting to construct phylogenetic dendograms of *Musa* spp., and characterize a somaclonal variant of 'Grand Naine' named Novaria. Radiation induced and TC derived dwarf off-types could be differentiated by a SCAR marker (Suprasanna *et al.* 2008). Venkatachalam *et al.* (2007a) developed a micropropagation protocol for *Musa acuminata* var. 'Nanjanagudu Rasabale' using a high concentration of cytokinins. They evaluated the genetic stability of these plantlets using RAPD and ISSR and showed the absence of genetic instability in these plantlets. They (Venkatachalam *et al.* 2007b) also screened a large number of micropropagated plantlets of 'Nanjanagudu Rasabale', developed from 10 year–old axillary shoot bud explants, with RAPD and ISSR markers and were not able to detect any genetic variation. To assess the genetic variability in micropropagated plants of 'Robusta', 'Giant Governor' and 'Martaman' obtained from shoot-tip cultures, RAPD and ISSR were utilized and were successful in identifying genetic variability in 'Robusta' and 'Giant Governor' (Ray *et al.* 2006). Amplified fragment length polymorphism (AFLP) fingerprints could be generated showing polymorphisms between true-to-type (dwarf) and off-type

(medium) 'Curare enano' plants obtained after *in vitro* culture suggesting that this technique could find use in early detection of SVs *in vitro* (Engelborghs *et al.* 1998).

To detect dwarf off-types, two different and complementary approaches are also being developed. The first one involves a "shot gun" method using the AFLP technique while the second involves a more physiological approach involving the expression of genes revealed by differential display. The second approach is complemented with the study of the gibberellin biosynthesis pathway genes as gibberellins are known to be involved in processes causing dwarfism in plants (Engelborghs *et al*. 2000).

The hypothesis that there are identifiable and predictable DNA markers for the early diagnosis of SV has been tested using RDA to isolate unique fragments of DNA (difference products) between visible culture-induced off-types and normal TC banana plants (Oh *et al.* 2007). The sequences and primers developed by RDA have the potential to be developed into a robust diagnostic DNA marker for SV. SAMPL primers were tested for their applicability to fingerprinting of DNA of banana cultivars and somaclonal variants and seemed to be promising for the distinction and characterization of commercially important cultivars and promising somaclonal variants (Gimenez *et al.* 2005). DNA methylation is a heritable epigenetic feature that is associated with transcriptional silencing, X-chromosome inactivation, genetic imprinting, and genomic stability. In order to initiate epigenetic studies of the level of DNA methylation in 'Grand Naine' micropropagated using sucker and male inflorescence explants, MSAP was used, which is a relatively new modification of the AFLP. In plants micropropagated from the male inflorescence explants, 14 (3%) DNA methylation events were polymorphic while plants micropropagated from the sucker explant produced 8 (1.7%) polymorphisms. No DNA methylation polymorphisms were detected in conventionally propagated banana plants. These results demonstrated the usefulness of MSAP to detect DNA methylation events in micropropagated banana plants and indicate that DNA methylation polymorphisms are associated with micropropagation (Peraza-Echeverria *et al.* 2001).

CONCLUDING REMARKS

In a micropropagation program, SV is not desirable as it defeats the purpose of clonal reproduction. It is a potential hindrance for *in vitro* propagation since SV may result from genetic changes due to mutation, epigenetic changes or a combination of both. Commercial micropropagation industry needs an in-depth analysis to minimize or eliminate somaclonal variants, to render TC propagation an attractive and safe alternative to conventional propagation technologies. To effectively use SV as a selection tool in conventional breeding programs, it is important to know where and how variability is elicited during the *in vitro* process as well as how cell competence for totipotency, somatic embryogenesis and regeneration capability are genetically controlled and inherited (Duncan 1997). Overdose of cytokinins, culture frequency/number (or both) and length of culture passage have been shown to cause SV. However, genome is the predominant predisposing factor for occurrence of somaclonal variants may be due to the presence of pre-existing variation or labile portions that render it susceptible to variation.

Banana TCs are proving to be rich and novel sources of variability with a great potential in crop improvement without resorting to mutations or hybridizations. In banana, SV has been exploited for generating disease-resistant clones, dwarfism, better yield, etc. SVs have been successfully detected using various morphological and cytological methods. Visual screening at *in vitro* culture stages as well as at prehardening and hardening phases in the greenhouse also help in detecting putative off-types. However, these methods are labor intensive, expensive, tedious and time consuming and are not always reliable. To overcome these disadvantages, recently, various molecular markers such as RAPDs, ISSRs, etc. have been successfully used to detect SV at an early stage. The molecular markers found useful in SV detection have also been sequenced to study the nucleotide variations. SV being random and non-directional, is unpredictable, not controllable and poses the disadvantage of giving rise to very low number of useful variants (Tang 2005). Determination of expected mutation rates for desirable *in vitro* traits would help in determining the sizes of plant populations necessary for selecting improved variants in the field.

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