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Phenotypic characters of various off types identified in laboratory, primary and secondary hardening in tissue cultured banana var. *Grand naine*

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An integrated study was undertaken on micropropagation of Grand nain variety of banana (Musa paradisiaca) from elite plants to identify and segregate (i) 3 types of off plants at the end of growth room phase development, (ii) 5 types of off plants at the end of primary hardening phase and (iii) totally 9 types (which included common 5 types observed at the end of primary hardening) at the end of secondary hardening phase. The morphological profiles of normal and off type plants were studied as a function of the above mentioned growth phases. The 9 off types identified after secondary hardening were finally subjected to field trials along with normal plants to serve as control using an optimized protocol of fertilizer application and irrigation. Their outcome indicated that off types, regardless of the type, could not sustain either transplantation shock or survived for not more that 3-6 months, giving poor growth/ no fruiting/ poor yield in quality and quantity. The composite data indicated that these off types were somoclonal variants and were not the result of epigenetic factor(s), as ruled out from studies of factors likely to affect their growth. It is, therefore, prudent to segregate them rigorously at each developmental phase so that delivery of only healthy normal plants to farmers was assured, limiting to minimum, if any, inadvertent contamination of off type plants due to limitation in their identification.

Keywords: Banana; *Grand naine;* off types; somoclonal variation; morphological profiles; tissue culture

Introduction

Jain Irrigation Systems Ltd. (JISL) initiated tissue culture (TC) work on banana var. *Grand naine* since 1990. Upon optimization of its protocol, production of these plantlets started in 1994 with a modest supply of 45,000 plants. Simultaneously, a package of agronomic practices, especially (i) plant spacing on the field, (ii) quantum, quality and frequency of application of fertilizers and (iii) volume of water/its frequency for irrigation through drip irrigation, was standardized. The cumulative outcome of these efforts led to (i) fetching 3 crops in 30 months as against 2 crops by traditional planting method, (ii) 27 kg average yield per bunch *vis-a-vis* 15 kg by traditional planting method and (iii) shape and quality preferred by export market, not feasible by traditional planting material. As a result, production of tissue culture plants metamorphosed from 45,000 plants in 1994 to more than 12 million plants in 2007. During this period, anomalies, especially generation of off type plants, was noted and its implication on TC plantlet quality was examined. This theme constitutes the focus of the present article.

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Materials and Methods

Elite Planting Material

Sword suckers from the elite plant, cut from the pseudostem 15 cm above the base level, weighing 500-1500 g were used as starting material for micropropagation.

Virus Indexing

For this purpose, enzyme linked immuno-sorbent assay (ELISA) technique was used[1]. Plants free from virus infection as determined by ELISA, were subjected to explant disinfection.

Explant Disinfection

The indexed sword suckers were thoroughly washed with chlorinated tap water and rinsed in a 0.001 *M* Teepol solution. Residual Teepol was removed by repeated washings with de-mineralized (DM) water. Older leaves and extraneous sucker tissues were carefully chopped off with a stainless steel knife. From such trimmed suckers, soaked in 0.5% Bavistin

(fungicide) solution and 0.05% streptocycline (antibiotic) solution for 6-8 h, shoot tips, containing several sheathing leaf bases and enclosing the axillary buds with underlying sucker tissue, measuring 2.5-3.5 cm in length were isolated. These shoot tips were soaked in 0.05% cetrimide (bactericide) solution for 15-20 min, surface-sterilized with chlorine-saturated DM water in an aseptic flow chamber for further 15-20 min and finally traces of chlorine were removed by repeated washings with sterile distilled water.

Explant Preparation

From the sterilized shoot tips, explants were prepared using sterilized stainless steel scalpels. For this purpose, cut surface of the sucker tissue and leaf bases was further trimmed so that shoot tips finally contained at least 6-8 overlapping leaf bases, enclosing axillary buds. The explants measuring 0.5-1.0 cm (3-4 g) were aseptically inoculated into a sterile MS basal medium (BM) in the culture bottles and preserved in a clean growth room optimized for 25°C, 3000 lux light and 60% RH.

Induction of Growth

It was induced in 4 wks at 25±2°C, with 16 h photo-period and 40-60% RH in growth room, illuminated by cool, white fluorescent lamps (4', 40 Watts, emitting 30-50 μ $E.m^2sec^{-1}$ light intensity), when explants swelled up, turned green, showing intense morphogenetic activity for sub-culturing in the multiplication medium.

Multiple Shoot Induction and Rooting

The multiplication medium (*p*H 5.0-8.0) comprised of benzyladenine (2-5 mg/L), IAA (1-2 mg/L), sucrose (20-30 mg/L) and agar (0.7%). In 4-8 wks of subculturing, the explants evinced enhanced axillary branching, heralding the appearance of tiny, creamy, white protuberances, which were progenitors of multiple shoots. About 10-15 shoot buds were induced from a single explant and up to 15 cycles of "true to type" sub-culturing generated a sizable population of plantlets.

At the end of multiple shoot generation cycles, individual shootlets were carefully excised and transferred to semi-solid BM, containing indole butyric acid (IBA) (1 mg/L) for inducing rooting within a week. Thus, fully differentiated *in vitro* plantlets were ready for primary hardening.

Primary Hardening

The plantlets, placed in portrays containing peat mix as a growth matrix, were allowed to harden in green house for 45 d, optimized at 27°C, 70% RH and 15,000 lux[2].

Secondary Hardening

The plantlets from primary hardening, placed in 18 × 15 cm black polythene bags containing soil and organic manure as a growth matrix, were allowed to harden in shade house for 45 d at 50% light cut and atmospheric temperature and RH[3].

Identification and Classification of Off Type Plants

The plantlets which appeared varying from normal were segregated at the end of growth phase, primary hardening and secondary hardening.

Field Trials

The off type plantlets after secondary hardening, were subjected to field trials along with normal plants as control. They were given fertilizer dose and irrigation of similar amount and at the same frequency. Their survival and fruiting profile was studied.

Results and Discussion

Identification of an Elite Mother Plant

To qualify to be an elite mother plant, it exhibited attributes like (i) disease-free and vibrant plant, (ii) on an average 13-15 photo-synthetically active leaves at every growth stage, (iii) pseudostem sturdy enough to support weight of the plant and fruit peduncle, (iv) an early fruit bearer, reflecting a shorter cropping cycle, (v) higher fruit bunch mass, as compared to population mean, (vi) bunch orientation representative of the cultivar and (vii) well-spaced identical size of hands. Plants with the above attributes were subjected to virus-indexing.

Virus-indexed Plantlets

In India, four viruses that infect banana crop are: (i) banana bunchy top virus (BBTV), (ii) cucumber mosaic virus (CMV), (iii) banana bract mosaic virus (BBMV) and (iv) banana streak virus (BSV). For producing healthy plants, these viruses were eliminated before multiplication and validated through sero-diagnostic method[4].

To ensure (i) disease-free propagules, (ii) uniformity in growth and (iii) simultaneous harvesting for avoiding prolonged farm management, *in-vitro* clonal propagation with an emphasis on viral indexing was accorded. Besides these advantages, this approach offered (a) better field establishment, (b) uniformity in flowering, (c) shorter crop duration and (d) increased yield, cumulatively affording higher returns. Thus, the use of virus-indexed, vibrant planting material led to effective restoration of vitality to banana plantation and enhanced productivity.

Merits of Tissue Cultured Plantlets

For inherent merit of providing the same genotype and phenotype characteristics in growth and yield, micropropagation has become the most preferred alternative method of rapid multiplication of plants[5]. Our studies over 15 years have proved that tissue cultured plants of *Grand naine* variety exhibited (i) 97-98% survival on farms upon transplantation, (ii) subsequent vigorous growth and (iii) finally superior qualitative and quantitative yield as compared to the same variety raised by conventional method[6]. These observations by thousands of farmers operating under widely differing geo-climatic conditions are in concurrence with the superiority of tissue cultured plants over conventional planting material, even in other varieties of banana like *Nendran*[7], *Dwarf Cavendish*[8], *Robusta*[9], etc.

Identification of Variants

Identification of variants (off types) in a multiplying culture was indeed difficult due to lack of finite differentiating characters. Therefore, on the basis of morphological features, normal plantlets and variants were identified.

Plantlets in Growth Rooms

Normal Plantlets

These were green, erect, healthy, growing leaves having an alternate leaf arrangement and pointed leaf tips (Fig. 1a). These plantlets served as control for comparison.

Off Type Plantlets

These are those plants which differed temporarily or permanently from the elite mother plant. It was thought that their occurrence could be the result of either (i) physiological (epigenetic) effect, characterized by a non-heritable change and reversible or (ii) permanent types (somaclonal variants), which were an inheritable genetic expression of the pre-existing variation through undetermined factor(s) from the plant source. Their occurrence at every growth stage and every year, in spite of ever-improving and tighter quality specifications made us aware to examine this phenomenon, its possible causes and its implications on the quality of plantlets to be supplied to the farmers for large-scale commercial plantation. Three off type plantlets (A, B and C) were identified at the end of growth phase.

Off Type A

These were dark green plantlets, small sized, psuedostem thicker than normal, leaves emerging from bottom or top, long petiole and lamina thicker than normal, undulating or rounded (Fig. 1b).

Off Type B

These were dark green plantlets, leaves emerging from their basal region, long petiole, two leaves fused together and curved in the middle, rounded tip and leaf at times bigger than that of the normal plant (Fig. 1c).

Off Type C

These were occasionally a few plantlets, leaves bearing white or yellow patches, their morphology conformed to the normal ones and exhibited normal rooting and elongation (Fig. 1d).

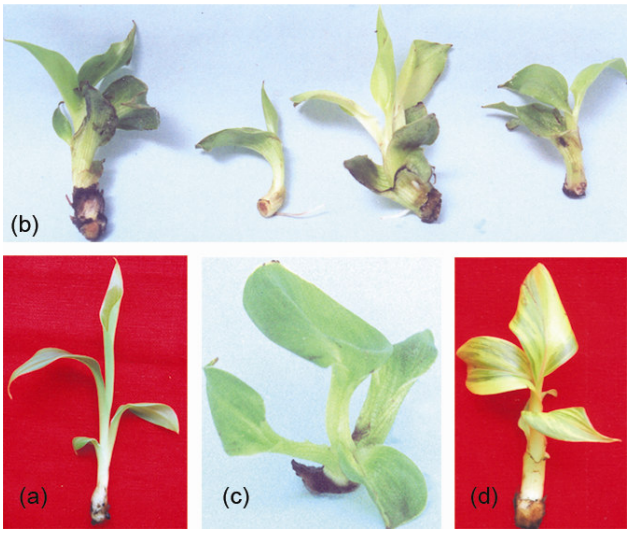

Fig. 1 — a-Normal plant; b-Type A; c-Type B; & d-Type C.

Morphological profiles of these normal as well as off type plantlets emerging upon 4-8 wks growth in growth rooms are summarized in Table 1. It is clear from the Table that off type A, B and C not only differed from the normal plantlets, but also differed amongst themselves in height, psuedostem girth, leaf length/width, arrangement and colour of leaves, besides distinguishing characteristics discussed above. While the occurrence of off types was not on a large-scale, regularity of occurrence from the batch to batch and year to year has puzzled. That their distinguishing morphological features are not a reflection of virus infection was not only ruled out by virus indexing[4], but subsequently validated also by random sampling. Apparently, it was neither the effect of an microbial infection (as they were grown under most hygienic conditions), nor an effect of nematode infection (being grown on synthetic sterilized growth matrix). Since normal and off types were grown under identical set of physico-chemical conditions, the variation could not be attributed to physiological factor(s), especially due to occurrence from year to year. Therefore, it appeared to be an expression of inherent somoclonal variation.

Plantlets after Primary Hardening

The plantlets rigorously inspected and made free from off type characteristics were subjected to primary hardening. Segregation of these plantlets into normal and off types was carried out. Their details are given below.

Normal Plants

These plants were sturdy, in good health, green, with alternate leaf arrangement, better leaf area, roots entangled in the growth medium and formed a root ball from young roots. As against these characteristics, five off type plants with the following characteristics were identified.

Off Type Plants

(i) Tall Plants

These were (a) approximately 2-3 times taller than the normal plants, (b) tapering and (c) with pointed leaves, internode distance more as compared to the normal ones (Fig. 2a).

(ii) Dwarf Plants

These were most common off types, (i) difficult to identify during primary hardening, (ii) identified mostly towards the end of secondary hardening and (iii) remained stunted even after providing either optimum growth conditions or a longer period or both than that provided to normal (Fig. 2b).

(iii) Plants with Variegated Leaves

These plants (a) apparently looked normal, if their age was not known, (b) in the later phase of their growth, remained stunted compared with normal ones, (c) possessed leaves of normal shape but smaller size compared with normal plants of the same age, (d) showed pale green or whitish yellow patches on their surface, (e) in some cases had leaf lamina either deformed or thicker and rubbery, (f) leaves as grew older, showed pale green patches, gradually turned black and the whole leaf dried and (g) internodal distance was less than normal (Fig. 2c).

(iv) Plants with Mosaic Leaves

This was a common variant with (a) one side of the midrib greenish yellow and deformed lamina, (b) other side green and normal, (c) deformed area of the leaves thinner than normal and (d) rubbery and loosely arranged leaves on the psuedostem (Fig. 2d).

(v) Plants with Extremely Mosaic Leaves

This type had (a) pronounced mosaic characteristics, felt by touching its surface, resembling with virus-infected plants, (b) drooping leaves, lamina extremely narrow and little interpetiolar distance gave them a bunchy appearance, (c) curled leaves, giving the plant a water-stressed look, (d) loose leaf sheath, (e) deformed and wavy lamina leaving the basal portion of the psuedostem, (f) petiole broader near the psuedostem and narrower towards the leaf, with effect such plants remained dwarf (Fig. 2e).

Table 1—Morphological profiles of normal and off type plantlets in growth rooms

Plantlets	Height (cm)	Psuedostem girth (cm)	Leaf length (cm)	Leaf width (cm)	No. of leaves	Leaf arrangement	Leaf colour	Occurrence %
Normal	9.0	0.5	5.5	2.8	6.0	Alternate	Green	98.8
Type A	6.0	0.4	4.0	2.1	5.0	Whirl at base	Dark green	0.8
Type B	7.0	0.6	7.0	3.2	6.0	Whirl at base & then alternate	Dark green	0.2
Type C	8.5	0.5	5.5	2.7	6.0	Alternate	Variegated	0.1

Growth room conditions: 30-50 μ $E.m^2sec^{-1}$ light intensity; 25 ± 2^{o} C; 40-60% RH, number of plants surveyed 2,00,000.

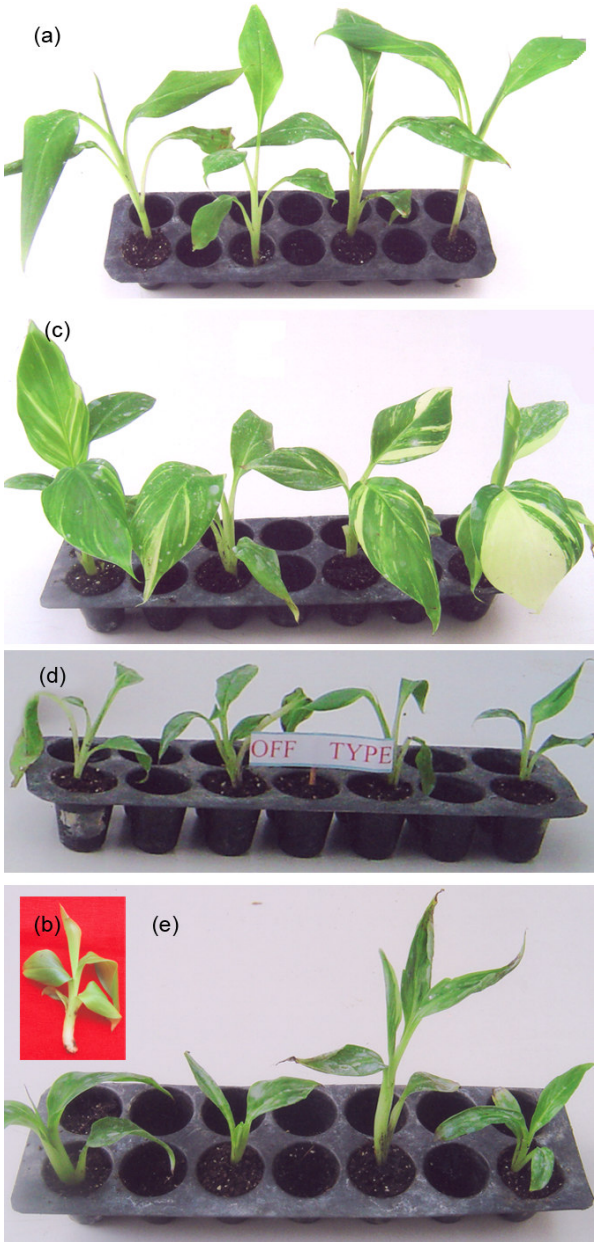

Fig. 2 — A-Tall plant; B-Dwarf plant; C-Variegated leaves; D-Mosaie leaves; & E-Extermely mosaic leaves.

Morphological profiles of these normal as well as off type plants at the end of primary hardening are summarized in Table 2. It is clear from the Table that plantlets appearing apparently normal at the end of the growth phase in laboratory (growth rooms), exhibited additional distinguishing features at the end of primary hardening. Not only they differed from normal plants, they also strikingly differed amongst themselves in several parameters (height, girth, leaf length/width, arrangement and colour), presumably reflecting late expression of the genes of inherent variation.

Plantlets after Secondary Hardening

The plantlets apparently free from off type characteristics selected at the end of primary hardening were subjected to secondary hardening. Segregation of these plantlets into normal and off types was carried out. Segregation of the plantlets at the end of secondary hardening indicated that besides off types identified at the end of primary hardening (Table 2), 4 more off types were expressed. Their characteristics were as below:

(i) Plants with Deformed Lamina

The leaf blade of these plants was deformed so much so that only half portion of the leaf was visible, as if another half portion was eaten up by caterpillars (Fig. 3a).

(ii) Plants with Extremely Deformed Lamina and Psuedostem

These plants exhibited (a) prominently deformed lamina, (b) an unusually long petiole, almost equaling to the leaf length, (c) abnormally thick and brittle midrib and petiole, (d) incomplete lamina with cuts, (e) separate petiole right from the base, rendering almost missing psuedostem and (f) a bushy appearance (Fig. 3b).

(iii) Plants with Highly Pigmented Leaves

This was a very rare variant, characterized by (a) appearance of dark red pigmentation on the entire leaf, especially at the nursery stage and (b) normal growth during secondary hardening (Fig. 3c).

Table 2—Morphological profiles of normal and off type plants upon primary hardening

Plant	Height (cm)	Psuedostem girth (cm)	Leaf length (cm)	Leaf width (cm)	No.of leaves	Leaf arrangement	Leaf colour	Occurrence %
Normal	10.0	1.8	8.8	4.0	5.0	Alternate	Green	99.48
Tall	15.9	1.6	12.0	2.5	4.5	Alternate	Green	0.07
Dwarf	3.7	1.2	3.3	1.7	3.0	Alternate	White patches	0.23
Variegated leaves	7.6	1.6	9.5	5.2	4.4	Alternate	White patches	0.13
Mosiac leaves	7.5	1.8	10.0	5.1	4.2	Opposite	White patches	0.08
Extremely mosaic leaves	7.0	2.1	10.5	3.6	4.3	Opposite	White patches	0.01

(iv) Plants with Dark Brown/Black Pseudostem

This rare off type was characterized by (a) dark coloured psuedostem, (b) difficulty in identifying during secondary hardening and (c) short survival in the field (Fig. 3d).

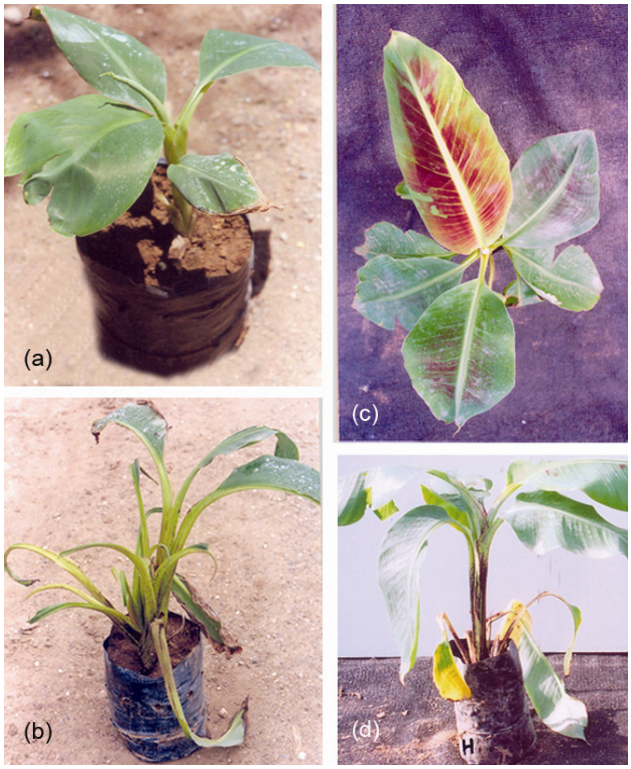

Fig. 3 — A-Deformed leaves; B-Extermely deformed lamina and pseudostem; C-Highly pigmented leaves; & D-Dark-brown/black pseudostem.

Morphological profiles of normal and off type plants emerging at the end of secondary hardening are summarized in Table 3 and the frequency of their occurrence is summarized in Table 4.

From Table 3, it is obvious that off types observed and segregated at the end of primary hardening showed up again comparable characteristics after secondary hardening also. This occurrence is not due to oversight in segregation at the primary hardening stage; it is due to genuine difficulty in identifying them. In all probability, their characteristics were still in dormant condition, not detectable *per se* and they have surfaced distinctly upon relatively harsh conditions (37°C, 30000 lux and 35% RH, 45 d) of secondary hardening. This observation confirms recurrence of somoclonal variation rather than epigenic effect.

From Table 4, it is clear that the frequency of occurrence of individual off types in total population is very little, maximally 0.47-0.50% (dwarf), variegated and mosaic each less than 0.1% and other 6 types cumulatively accounting for not more than 0.1%. While their occurrence is abysmally low, given the identical condition of their growth along with normal, the cause of their occurrence appears to be a somoclonal variation. Such phenomenon has been earlier noticed in cultivar Willaams (*Musa* sp. AAA, subgroup Cavendish)[10].

Field Profiles

After secondary hardening, the off types were transplanted on the farm at 180 cm × 150 cm spacing

Table 3—Morphological profiles of normal and off type plantlets upon secondary hardening

Plant	Height (cm)	Psuedostem girth (cm)	Leaf length (cm)	Leaf width (cm)	No. of leaves	Leaf arrangement	Leaf colour
Normal	22.9	16.3	22.6	11.2	8.4	Alternate	Green
Tall	37.3	16.0	35.9	12.9	6.8	Alternate	Green
Dwarf	5.2	6.7	7.6	3.8	4.0	Alternate	Light green
Variegated leaves	10.0	10.5	13.1	6.4	6.2	Alternate	White patches
Mosaic leaves	13.4	11.8	15.1	5.9	6.4	Opposite	White spots
Extremely mosaic leaf	9.8	17.1	15.4	4.6	7.4	Opposite	White spots
Deformed lamina	5.6	13.9	8.6	3.8	7.2	Irregular	Light green
Extremely deformed lamina	14.0	28.2	18.4	8.6	7.6	Irregular	Light green
Highly pigmented leaf	23.0	15.3	29.0	15.0	6.0	Alternate	High pigment
Dark brown/black psuedostem	17.0	16.0	23.0	13.0	8.0	Opposite	Pale green

along with the normal plants. They were given identical treatment of organic manure (13 kg/plant) and chemical fertilizers (N 210 g, P 80 g and K 300 g/plant through drip irrigation), irrigation at the same frequency, weeding identically and their performance was observed. The results are summarized in Table 5. It is clear from the Table that (i) normal plants showed 100% survival, normal growth and yield, (ii) highly pigmented leaf off type too showed 100% survival, without fruiting, (iii) dark brown/black psuedostem off type plants showed 100% survival only up to 6 months, (iv) mosaic and variegated off type plants showed 60±10% survival up to 3 months without economic yield and other off types gave unacceptable percentage of survival and were no good due to either early death or no fruiting. At best, they could be considered as ornamental plants. In conclusion, therefore, all the off types were inherently weak, possibly lacked certain vital enzymes required for normal morphology, physiology and vigor to absorb transplantation shock, muster 100% survival, register sustainable growth and give an impressive output.

Somaclonal Variation

Occurrence of somaclonal variations in banana is not a new observation. In fact, it has been reported earlier also[11,12]. Subsequently, basis of somaclonal variation was traced to chromosomes[13]. Eventually, measures were suggested to reduce somaclonal variation in *in vitro*-propagated banana[14]. These comprised of (a) carefully selecting true-to-type plant material for propagation, (b) minimizing the number of transfers in culture, (c) screening out unusual off types at nursery stage and (d) investigating the possibility of selecting stable families for *in vitro* propagation. In spite of putting all these factors in practice over a period of 10 years (1998-2008), we observed that the occurrence of off types was not eliminated. Although minimization of the number of transfers in culture appeared to be a possibility, in practice, it raised practical difficulties as to how much

Table 4—Frequency of occurrence of off type plants up secondary hardening

S.No.	Types/Year No. of plants surveyed	2005-06 503570			2006-07 301080		
		No. of off types identified	% in total	% contribution of total off types	No. of off types identified	% in total	% contribution of total off types
1	Dwarf	2356	0.47	64.99	1492	0.50	63.54
2	Variegated	422	0.08	11.64	243	0.08	10.35
3	Mosaic	356	0.07	9.82	220	0.07	9.37
4	Tall	169	0.03	4.66	119	0.04	5.07
5	Deformed lamina	108	0.02	2.98	123	0.04	5.24
6	Extremely mosaic	103	0.02	2.84	95	0.03	4.05
7	Extremely deformed lamina	98	0.02	2.70	56	0.02	2.39
8	Dark brown/black psuedostem	8	0.00	0.22	0	0.00	0.00
9	Highly pigmented leaves	5	0.00	0.14	0	0.00	0.00
	Total	3625	0.72		2348	0.78	

Table 5—Profiles of off types after transplantation in the field

Type	% survival after transplantation in field	Remarks
Normal	100	Normal fruiting (30± 5 kg/plant)
Highly pigmented leaf	100	No fruiting
Dark brown/black psuedostem	100	100% died after 6 months
Mosaic	98	70% died after 3 months, delayed flowering with small bunch size and mosaic fruits
Variegated	97	50% died after 3 months, delayed flowering, small bunch size
Tall	83	delayed flowering and very small bunch size
Deformed lamina	67	No fruiting
Extremely mosaic	52	No fruiting
Extremely deformed lamina	42	No fruiting
Dwarf	6	delayed flowering and very small bunch size

the frequency of minimization was necessary to reduce their occurrence to zero and after reducing the number of transfers to bare minimum, was it feasible to propagate millions of plants needed for commercial plantation? However, this also does not altogether eliminate off types. Thus, it appeared that the occurrence of somaclonal variants limits the expansion in the use of *in vitro*-propagated plants, which otherwise have outstanding advantages of synchronization in growth, flowering, fruiting, shorter duration of harvesting and higher yield.

Somaclonal Variation is Wide Spread

It seems that the occurrence of several kinds of off types of *in vitro*-propagated banana plants of several cultivars (*Red* AAA; *Williams* AAA; *Giant Cavendish* AAA; *Nathan* AAA; *Mons Mari* AAA; *Grand naine* AAA; *Figue Sucree* AA; *Maricongo* AAB; *Dwarf Horn* AAB; *Bobby Tannap* AAB; *Ntange-2* AAB; *Obino L'Ewai* AAB; *Agbagba-4* AAB; *Ubok Iba* AAB; *Big Ebagnga* AAB; *Bise Egome-2* AAB; *False Cuerno-5* AAB and *Saba* ABB) is a reality repeatedly observed by several researchers[15]. These off types being seen in different cultivars, under different geo-climatic conditions, showing odd characteristics (green-red, dwarf, giant, extra dwarf, etc.), tend to indicate that their occurrence is certainly not due to epigenetic factor. In fact, most of the researchers have arrived at the conclusion that they represent somaclonal variations. Naturally, *Grand naine* was no exception to occurrence of off types, presumably the somaclonal variants.

Frequency of Off Types

The frequency of off types reported earlier varied from 9-25%[12] to as low as 3%[11] or even 1%[16]. Even the low frequency (3-1%) is much higher than those found (2 mutants per million) in clonal propagation.

Efforts have also been made to determine factors, likely to influence the occurrence of these variations such as (i) composition of the medium of their growth, (ii) rate of multiplication, (iii) length of time in culture and (iv) bulbous or non-bulbous rhizome. However, none of these factors appeared to the cause of variation. Having excluded these factors, many researchers thought that it was the initial explant which determined the occurrence of somaclonal variants. We considered that perhaps number of sub-culturing might be responsible for off types' occurrence[17]. To our surprise, although the frequency of occurrence reduced reasonably upon reduction of sub-culturing from one elite plant by 50%, it did not altogether abolish the variance. At present, there is no way that these variants could be totally abolished. Until a cause and effect relationship of somaclonal variation is deciphered, from a practical point of view, it is desirable to identify, segregate and cull these off types after growth room, primary hardening and secondary hardening so that their frequency in the field is reduced to almost zero. To be doubly sure, the only practical way is to finally subject tissue cultured plants after secondary hardening to rigorous inspection and select only the healthy ones for giving to the farmers to weed out the possibility of inadvertently giving the plantlets which may turn out off types in field trials.

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