Mitotic instability in banana varieties. I – Plants from callus and shoot tip cultures

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ABSTRACT
More than 1 600 chromosome counts were made from root tip cells of banana plants of diverse varieties and origins. Even in conventional triploid plants there were some cells with numerical errors or with evident chromosome breakage, defects which were very much more common in plants from meristem cultures employing 5 mg/l of benzylaminopurine (BAP). Plants derived from callus cultures showed extreme chromosome loss.

Instabilité mitotique des variétés de bananier. Plantes issues de cultures de cals et de méristèmes.

RÉSUMÉ
Plus de 1 600 comptages de chromosomes ont été effectués sur des cellules de pointes de racines de bananiers appartenant à diverses variétés et origines. Même dans les plantes conventionnelles triploïdes, il y a eu des cellules présentant des nombres de chromosomes aberrants ou révélant la cassure évidente d'un chromosome ; ces défauts ont été bien plus courants dans les plantes obtenues à partir de la culture de méristèmes sur milieu contenant 5 mg/l de BAP. Des plantes obtenues à partir de la culture de cals ont montré une grande diminution du nombre de chromosomes.

Instabilidad mitótica de las variedades del banano. Plantas procedentes de cultivos de callos y de meristemas.

RESUMEN
Fueron efectuadas más de 1 600 cuentas de cromosomas en células de ápices de raízes, de bananos de origines diversos. Hasta en las plantas convencionales triploides hubo algunas células con errores numéricos o con rompimiento evidente de un cromosoma, pero estes defectos fueron mucho más comunes en las plantas que provenieron del cultivo de meristemas, con el empleo de 5mg/l de BAP. Otras plantas, obtenidas a partir del cultivo de callos, mostraron una pérdida extrema de cromosomas.

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KEYWORDS
Musa, in vitro culture, chromosomes, karyotypes, chromosome number.

MOTS CLÉS
Musa, culture in vitro, chromosome, caryotype, nombre chromosomique.

PALABRAS CLAVES
Musa, cultivo in vitro, cromosomas, cariotipos, número de cromosomas.

introduction

Somaclonal variation in bananas is not as well documented as it might be, but it is said to have reached sometimes an unacceptable incidence in the largescale multiplication of banana clones (STOVER, 1987; KRIKORIAN, 1988, 1989). In those plantain clones where variations frequently arise in conventional propagation, the incidence is further increased by shoot tip culture in vitro (VUYLSTEKE and SWENNEN, 1990).

KRIKORIAN (1989) has argued strongly that much more information is needed on the fidelity of in vitro banana plants and on how this relates to the methodology of culture. He has also listed a number of ways in which chromosomal changes might have a place among the causes of offtypes, including polyploidy, aneuploidy and translocation, as well as other disturbances not normally visible in chromosome studies of *Musa*. LEE and PHILLIPS (1988), in their review of chromosome modifications in tissue and cell culture, do refer to the occurrence of polyploidy and aneuploidy in cultures of cereal plants and of breakage-induced deficiencies, perhaps associated with delays in the replication of heterochromatin.

Numerical instability has in fact been observed over the years in many new hybrid banana genotypes produced in the breeding programme at the National Centre for Research in Cassava and Tropical Fruits (CNPMF, Brazil). Some of these arose from zygotes that should have been exact triploids or tetraploids, but morphological abnormalities gave a first indication of mitotic irregularities. Occasionally, such an abnormal plant could revert to a normal aspect.

Cytological investigation of clonal material at the CNPMF also began from curiosity about obviously abnormal plants. These were derived from callus cultures received from EMBRAPA's National Genetic Resources Centre (CENARGEN, Brazil). Research was subsequently amplified to a more general study of mitotic behaviour in banana clones, but now in plants not previously identified as abnormal.

The objective of this paper is to report data on chromosomal aberrations in the callus-derived plants and in others from shoot tip cultures, alongside some preliminary results from conventional plants of the same cultivars.

material and methods

The callus cultures from CENARGEN that initiated this work were derived from already differentiated inner leaf sheath tissues of the AAA group, Cavendish subgroup cv Nanicão by the joint use of picloram and the cytocinin 2iP in high dosages (MATSUMOTO, personal communication). On arrival at the CNPMF, the few petri plates already showed an abundant proliferation of shoots. After much difficulty in inducing rooting, a large number was established in a greenhouse and transferred either to plastic potting bags of about 1 l capacity or, for the more vigorous ones, to rigid plastic pots of about 2 l.

The triploid plants studied, either as vitroplants or as conventional ones, were all initially supposed to have 33 chromosomes and came from the CNPMF germplasm bank. Those not from Brazilian sources had been received at the CNPMF in the form of shoot tip cultures, but had since spent some years in the field. The genotypes were as listed in table I.

For shoot tip cultures, explants of terminal buds of offshoots (small sword suckers) were of about 1.0 to 1.5 cm in each dimension. They were set initially in darkness in a basal medium, that in use at the CNPMF having a Murashige and Skoog base supplemented with the following organic components per litre: sucrose 30 g, agar 8 g, inositol 100 mg, nicotinic acid 0.5 mg, piridoxine 0.5 mg, thiamine 0.1 mg and glycine 2.0 mg. From the first subculturing the medium was changed to one with the addition of 5 mg/l of benzylaminopurine (BAP), the only growth regulator used in the production of the vitroplants here analysed. Earlier explants were maintained whole but later ones were divided longitudinally. Further subculturing was at intervals of 30 to 45 days. Excised shoots were placed again in basal medium for rooting.

The number of vitroplants studied varied between accessions and some individual ones are identified in later sections, and in tables III and IV, by two letters representing the parent triploid clone and a two digit number for certain individual plants. These were all transplanted and maintained in a greenhouse at the CNPMF, in rigid plastic pots of 2 to 3 l capacity, for the provision of material for cytology.

Chromosome counts have been made in root tip squashes, prepared by a simplified orcein schedule developed in Jamaica in the 1960s. Among c-mitotic agents tested, 8-hydroxyquinoline has been found to be the least inconsistent in its effect on mitosis in bananas, used mostly at 0.03% (0.002 M). Root tips are collected early in the day and kept in this solution for a period varying between 4 and 9 h, at a temperature between 20 and 25 °C. They are then simultaneously fixed and macerated, usually overnight, in a mixture of four parts glacial acetic acid, five parts water and one part ethyl alcohol (95%). Finally, the apical portion of about 1.5 mm is squashed and flattened between slide and cover glass in a drop of 2% orcein in lactophenol (equal parts of lactic acid, phenol, glycerol and water). The stain needs about 2 days to intensify and provide good contrast, presumably because of its high solubility in this solvent.

All counts were made using a Zeiss Docuval microscope, with its advantage of a continuously variable magnification control. Cells included in the record fell into three classes, which were: class 1: all chromosomes separate and with little dispersion between levels of focus; class 2: some superimposition of pairs of chromosomes, again with little vertical dispersion, provided that all units were sharply and unequivocally defined; class 3: with minor defects of definition or of vertical displacement, such that the chance of error should still be minimal.

As an added precaution, almost all counts were agreed by two and, at times, three independent observers, aided by a rough drawing. Disagreement resulted in the removal of the cell from the record. It is hoped that these details may be of use to other laboratories contemplating parallel research.

Table I
Banana genotypes used in the mitotic instability study.

Cultivar	*Group*	*Subgroup*	*Source*
Highgate	AAA	Gros Michel	Honduras
Nanicão	AAA	Cavendish	Brazil
Maçã	AAB		Brazil
Malbhorg	AAB		Martinique
Mysore	AAB		Brazil
Pacovan	AAB	Pome	Brazil
Ice Cream	ABB		Hawaii
Gia Hui	ABB	Awak	Martinique
Muisa Tia	ABB	Awak	Martinique
Namwa Daeng	ABB	Awak	Thailand
Namwa Khom	ABB	Awak	Thailand
Tai	ABB	Awak	Thailand

results

A common difficulty encountered was in the failure of the chromosomes to scatter after paralysis of the mitotic metaphase; some materials were particularly obstinate. When extreme strictness was also imposed on the quality of cells selected, the productivity achieved in terms of clear counts was low indeed, little more than one per root tip excised. Of all counts reported, the frequencies noted under classes 1, 2 and 3 were, respectively, 11.4, 48.4 and 40.2%.

sizes and forms of chromosomes

The technique employed resulted in chromosomes much contracted and usually intensely stained, not permitting the observation of centromeres except in rare instances. In addition, the fact of working at the same time with diverse genotypes was not conducive to the establishment of standard clonal karyotypes. In general, chromosome lengths appeared to vary from about 0.7 to 1.5 times the median size, where the rather longer form was not recognized more than once per cell.

Varying frequencies were observed of chromosomes of much reduced size ('minichromosomes'), which could only be interpreted as products of breakage. The 'mini' was typically rectangular in outline and sometimes relatively weakly stained (fig 1). Clearly, the minis identified were only the more deficient chromosomes

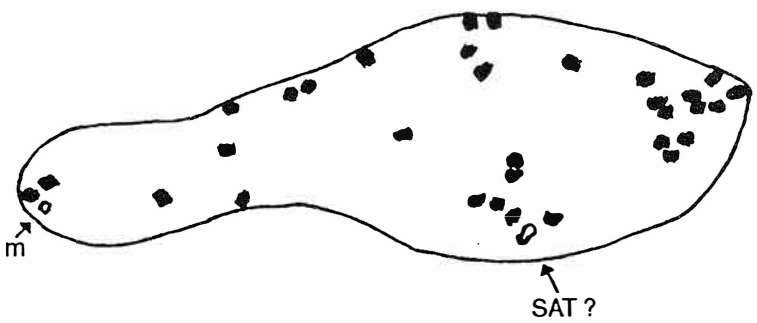

Figure 1
Root tip cell of plant TI01 (Tai, ABB Awak type) with 34 chromosomes including one with a possible satellite (SAT?) and an angular, weakly stained "mini" (m).

and their frequencies may have been underestimated.

A satellite was often visible, depending on its degree of isolation from the chromosome bearing it. In the better cells, its identity could not cause confusion, even when it was already divided, because of its quite small size and rounded outline. Occasionally, doubt arose as to whether a body was a satellite or a mini and such cells were rejected.

plants from callus cultures

When shoots were transferred from callus culture to basal medium, rooting was weak and slow. Plants also failed in the greenhouse from the same cause. Many were established, however, supposedly of Nanicão, and these were of two general types:

– type 1 ('Stunted'): the great majority were of this type, weak and having much reduced internodes, short broad leaves with:

. inrolled margins and petioles either very short or absent,

. leaf mortality kept pace with leaf production;

– type 2 ('diploid'): a small number of plants, less than 10%, showed better and taller growth with normal petioles and rather erect, narrow leaves, again few in number at any one time.

Chromosome counts made are shown in table II, where it is evident that the diploid plants fell into two subcategories. In most plants with useful cells, chromosome numbers found ranged from 26 to 30, in which respect this 'diploid A' subtype agreed with the few counts achieved from 'stunted' plants; cells with 28 and 29 chromosomes were commonest in both cases. In the 'diploid B' subtype, 22 was the commonest count, although perhaps of no euploid significance, and no cell had more than 25 chromosomes. These findings reflected a massive level of chromosome loss, relative to the original number of the cultivar that gave rise to the callus.

vitroplants of triploid clones produced at the CNPMF

The only plants available of the AAA group, arising from cultures with 5 mg/l of BAP, were of Highgate, an exceptionally difficult clone for cytological analysis. The strangest result from this material was for plant HG05 (table III), where all ten cells from two roots contained a mini and six had 34 chromosomes instead of 33. Summing all plants, more than half the cells had an identified mini, although an exceptional plant had only one among eight cells from three different roots.

In the AAB group, three plants of Maçã have been excluded from table III because all the 11 cells counted had 23 chromosomes. They were moderately vigorous in pots and so closely similar in appearance that they must be considered as the identical products of a single major accident. Otherwise, the few culture plants of Maçã and its synonym Malbhog were relatively stable except for plant MB06, where 34 chromosome cells predominated among the small number counted from two roots. Minis were quite infrequent.

Mysore, of the same group, was relatively unstable as meristem culture material, in contrast to its stability in conventional suckers. Of the 122 cells counted from roots of 16 plants, only 62% contained 33 chromosomes. The most remarkable result was from plant MY15 but, exceptionally, all 19 counts came from the same root. The general count of 32 and the high frequency of minis were not necessarily typical of the plant as a whole. None of the other plants of Mysore manifested frequent minis but there was a marked tendency towards chromosome gain (table III).

Table II
Chromosome counts from callus-derived plants of Nanicão.

Plant type	*Plant number*	*Useful roots*	*Cells counted*	*Cells with 18-21*	*22-25*	*26-30*
Stunted	4	4	7			7
Diploid A(1)	7	7	15			15
Diploid B(1)	2	4	17	5	12	

(1) Subclasses distinguished by the ranges of chromosome numbers found.

Still in AAB, Pacovan has stood out for its capacity to gain a chromosome or two in the conditions of culture practised. From a clone whose normal cells evidently have 33 chromosomes, every one of 12 in vitro plants now has 34 as the standard number (table III).

The ABB group cultivar Ice Cream, has performed unexpectedly in that its in vitro plants were scarcely less stable in total than their conventional counterparts (table IV). However, the number of cells counted of the latter were rather few and further work could reveal a divergence in behaviour.

The Awak type of this same group has been studied very intensively because of the availability of many plants when the work started. In this case even conventional plants showed a standard chromosome count of 34 instead of the expected 33. The higher number was found in 85% of the cells from seven conventional plants of five accessions, also in 77% of the cells from in vitro plants. Yet table IV shows large variations between individual vitroplants, some showing a majority of cells with 35 chromosomes (GH23, NK17 and TI21) while others were without numerical anomalies (GH18 and 21, NKll and 12, TI19). Also to be noted are the many cells with at least one mini, 23.6% in all, and the irregularity of their presence between plants, varying from a high proportion of cells (GH18 and 23, NK04, 08, 11 and 12) to none at all or hardly any (GH02, 03 and 17, MT02, NK07 and 17, TIO1 and 19). The plant TI19, on a joint evaluation of both aspects, of instability, seems to be even more normal than conventional material of the Awak type.

discussion and conclusion

Whatever may be the questions still to be answered, it now seems probable that a triploid banana cannot ever be totally stable in its somatic karyotype. In root tip meristems, inasmuch as these are representative of the whole plant, cells can be found with numerical errors and chromosome breakage can be detected.

The relative frequencies of these two types of aberration have been found to vary between triploid clones in the form of conventional offshoots. It is also evident that the in vitro culture of vegetative shoot tip meristems, for the purposes of multiplication, can seriously aggravate the tendency to either kind of aberration, so that in the most extreme case studied, that of Pacovan, the standard chromosome number has been effectively changed.

The type of culture system has been shown to be important. While only one example has been

Table III
Chromosome counts from plants of AAA and AAB group cultivars derived from meristem cultures with 5 mg/l of benzylaminopurine (BAP).

Cultivar	*Group*	*Total plants*	*Plant number*	*Cells counted*	*Cells with 32*	*33*	*34*	*35*	*Cells with minichromosomes*
Highgate	AAA	17	—	100	4	78	17	1	50
			HG05	10		4	6		10
Maçã[1]	AAB	7[2]	—	66	3	55	8		4
			MB06	6		2	4		1
Mysore	AAB	16	—	122	22	64	31	5	28
			MY02+MY04	13		3	10		4
			MY15	19	17	2			12
		+ 3[3]	—	15		14	1		2
Pacovan	AAB	12	—	233		31	182	20	58
		+ 2[3]	—	26		19	5	2	2

(1) Excludes three possibly identical plants with 23 chromosomes.
(2) Includes plants of the synonym Malbhog.
(3) Conventional plants for comparison.

examined of plants derived from a callus culture, it must now be suspected that any such cultures, as well of those of cells or protoplasts, may be subject to major dislocations of the normal mitotic process.

Returning to the case of plants derived from proliferating whole or split meristems, there must be stressed the typical chimerical nature of chromosomal aberrations, even within roots.

No knowledge exists about the longterm result of competition between normal and aberrant cell lines in the same tissue or in adjacent ones. Do new errors arise continuously to compensate the possibly reduced viability of modified cells or does the plant tend to 'purify' itself as in the observed reversions to normal aspect in hybrids? This is a difficult question to answer in the greenhouse because vigorous root systems are not readily maintained in a container of conveniently small size. Field assessment is needed and especially for the less stable plants; side shoots can later be collected and returned to the greenhouse for a second analysis.

The field stage acquires additional importance in that no attempt has been made, in the studies reported, to link cytological with morphological anomalies. STOVER (1987) has stated that the latter cannot be identified confidently in small containers, although exceptions might be made. Indeed, plants that have spent an excessive time with restricted root space almost always look abnormal.

An alternative approach to establishing a link would obviously be to first identify offtypes in medium to largescale field plantings, with subsequent cytological analysis of side shoots. Here it is important that other centres who already have off-types identified and preserved should seek cytological data from them.

Whether or not there is found to be a high degree of correlation between off-types and chromosome accidents after in vitro culture, it can at

Table IV
Chromosome counts from plants of ABB group cultivars derived from meristem cultures with 5 mg/l of benzylaminopurine (BAP).

Cultivar	*Total plants*	*Plants number*	*Cells counted*	*Cells with 32*	*33*	*34*	*35*	*36*	*37*	*Cells with minichromosomes*
Ice Cream	5	—	73	2	47	23	1			21
	+ 2(1)	—	39		26	11		1	1	10
Awak type:	79	—	848	1	34	642	163	8		212
Gia Hui		GH02	14			13	1			0
		GH03	12		4	7	1			0
		GH17	10		1	5	4			0
		GH18	10			10				8
		GH21	12			12				3
		GH23	38			12	26			28
Muisa Tia		MT02	18			15	3			0
Namwa Khom		NK04	15		1	14				12
		NK07	16			14	2			0
		NK08	13		2	9	2			10
		NK11	13			13				10
		NK12	9			9				7
		NK17	11			5	6			0
Tai		TI01	11			6	5			1
		TI19	23			23				2
		TI21	14		1	4	9			5
	+ 7(1)	—	123		3	105	14	1		8

(1) Conventional plants for comparison.

worst be hoped that they have common causes. In such a case, cytological analysis of products could serve as a valuable tool or indicator in the assessment of genetic fidelity, in relation to explant culture methodology. Although admitted to be a laborious task, chromosome counting does have the merit of giving results before the stage for field planting. Such an activity would use from preference triploids relatively responsive to 8-hydroxyquinoline and varying in their known relative stability in meristem culture.

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