Mitotic instability in banana varieties. IV. BAP concentration and effects of number of subcultures

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ABSTRACT
Mitosis in root tip cells of vitroplants of Pacovan (AAB), derived from meristem cultures without BAP, was no less stable than in conventionally propagated plants of this variety. Yet, in Caipira (Yangambi Km 5, AAA) and Gia Hui (ABB), these behaved in the same manner as plants from cultures with from 2.5 to 10 mg/l of BAP. In none of four triploids studied was there evidence of an increase in instability with increasing dosage of this cytokinin, except that three hexaploids were found in the case of Mysore (AAB) from cultures with 7.5 mg/l of BAP. In Caipira and, even more, in Prata Ana (AAB), the prolongation of culture beyond five months (and five subculturing cycles) resulted in a considerable increase in the frequencies of numerical and structural errors.

KEYWORDS
Musa, triploidy, plant propagation, in vitro culture, culture media, chromosome aberrations.

Instabilité mitotique des variétés de bananier. IV. Influence de la concentration en BAP du milieu de culture et du nombre de repiquages des vitroplants

RÉSUMÉ
Les mitoses étudiées sur des cellules isolées dans les pointes de racines de bananiers du cultivar Pacovan (AAB), issus de la culture de méristèmes sur milieu sans BAP, se sont révélées aussi stables que celles provenant de plants de la même variété multipliée de façon conventionnelle. Cependant, dans le cas de Caipira (Yangambi Km 5, AAA) et de Gia Hui (ABB), ces plantes se sont comportées de la même façon que celles issues de vitroculture, avec des doses allant de 2,5 à 10 mg/l de BAP. Pour aucun des quatre diploïdes étudiés, l'instabilité mitotique ne s'est montrée corrélée avec l'augmentation du taux de BAP dans le milieu de culture ; exceptionnellement, pour Mysore (AAB), trois hexaploïdes ont été trouvés à partir d'un milieu contenant 7,5 mg/l de BAP. Pour Caipira, et plus encore pour Prata Ana (AAB), la poursuite de la vitroculture au-delà de 5 mois (ou de cinq cycles de repiquages) a augmenté considérablement la fréquence des erreurs numériques et structurelles.

MOTS CLÉS
Musa, triploïdie, multiplication des plantes, culture in vitro, milieu de culture, aberration chromosomique.

Instabilidad mitótica de las variedades de banano. IV. Influencia de la concentración en BAP del medio de cultivo y del número de transplantes de las vitroplantas.

RESUMEN
La mitose en células de ápices de raizes de vitroplantas de Pacovan (AAB), derivadas de culturas de meristemas sin BAP, no fué menos estable que en plantas convencionales de la misma variedad. Sin embargo, en Caipira (Yangambi Km 5, AAA) y en Gia Hui (ABB) estas plantas mostraron el mismo comportamiento de las plantas obtenidas a partir de culturas con 2,5-10 mg/l de BAP. Entre quatro triploides no fué observado ningún aumento claro y progresivo de la inestabilidad según la concentración del producto, con excepción de tres plantas hexaploides de Mysore (AAB), estas a partir de culturas con 7,5 mg/l. En Caipira y, más aún, en Prata Ana (AAB), la prolongación de la cultura por más de cinco meses determinó un aumento considerable de los errores numéricos y estructurales.

PALABRAS CLAVES
Musa, triploidia, propagación de plantas, cultivo in vitro, medio de cultivo, aberración cromosómica.

introduction

In the first paper of this series, it was shown that banana vitroplants of diverse triploid cultivars, produced in shoot-tip cultures with a standard dosage of 5 mg/l of the cytokinin benzylaminopurine (BAP), tended to be more mitotically unstable than plants of the same cultivar propagated by conventional techniques (SHEPHERD and SANTOS, 1996). These vitroplants were derived from relatively young cultures, that is, after a few monthly subculturings. The degree of instability varied between clones in both types of plant material; this variation between cultivars under conventional propagation was later emphasized in a subsequent contribution (SHEPHERD and SILVA, 1996).

The aim of this paper is to show the relative mitotic instability of vitroplants produced without growth regulator in the media or with four concentrations of BAP: 2.5, 5, 7.5 and 10 mg/l. Furthermore, mitotic instability was also evaluated for plants isolated after two to seven monthly subculturings.

materials and methods

As in earlier papers, these results were achieved in the laboratories and greenhouses of EMBRAPA's Centro Nacional de Pesquisa de Mandioca e Fruticultura Tropical (CNPMF, Brazil).

material

for testing various BAP concentrations

For the first aspect studied, concentration of BAP in the media, cultivars were selected on a broad genetic basis to embrace the three triploid genomic groups (AAA, AAB and ABB). Knowledge of the relatively good scattering of their chromosomes in the presence of 8-hydroxyquinoline was also taken into account for this choice.

The vitroplants of the first three cultivars listed, established from up to the third subculture, were produced by the second author with the protocol described elsewhere (SOUZA, 1994). Those of the latter were earlier products of the laboratory but derived by closely similar methods:

– Yangambi Km 5 (AAA group), now in process of liberation in Brazil as Caipira, was identified by this latter name ; it was used in a study of five different BAP concentrations, from 0 to 10 mg/l of BAP;

– plants of Pacovan (AAB group, a mutant of Prata) were available from up to three subcultures containing 0 to 7.5 mg/l of BAP ; no viable plants were recovered from the highest dosage of 10 mg/l;

– for Gia Hui (ABB group, Awak subgroup), the five BAP concentrations (0 to 10 mg/l) were represented in the chromosome counts, but the numbers of cells from two of them (5 and 7.5 mg/l) had to be combined because of the few roots that provided counts;

– plants of Mysore (AAB), including its Thai clone Thap Meio, were obtained from cultures containing only three BAP concentrations of 2.5, 5, 7.5 mg/l. The two names are treated as one entity (Mysore).

the effect of number of subcultures

Two clones were used for studying the effect of in vitro subcultures with 2.5 mg/l of BAP in the media on mitotic instability:

– plants of Caipira isolated from the second to the seventh monthly subcultures were evaluated (SOUZA, 1994),

– plants of Prata Ana (AAB) were donated by a colleague A DA S SOUZA and came from the third to the seventh cycle of vitroculture. Because of the small number of individuals available for the study, vitroplants issuing from media with very minor differences in BAP concentrations were grouped.

cytological methods

Cytological methods were the same as those previously employed, including the extreme care in admitting cells as valid data sources. The parameters employed have been principally the same, namely the frequencies of apparently normal cells, of supernumerary chromosomes and of deficient ones ('minis', fig 1), as well as the ranges of numbers found in different cells. In a few cases very low frequencies of numerical deficiencies were also recorded.

results

testing various BAP concentrations

plants from short-term cultures

Table I shows details of mitosis in vitroplants derived from young cultures and based on different BAP dosages. Treatments have sometimes been combined to improve the sample size. The frequencies of normal cells are more or less variable among the treatments. No genuine differences appear in mitotic instability of plants coming from media with various BAP concentrations within each variety. There is some suggestion of a decline in stability with increasing BAP concentrations in Caipira, but the results from plants not exposed to BAP (table II) throw doubt on this, as do the cells analyzed from old cultures and treated in a later section.

Data obtained from these vitroplants have then been grouped, whatever the BAP concentration (2.5, 5, 7.5 or 10 mg/l) of the culture media (BAP +), and are compared with plants derived from media without BAP (BAP –) or with conventionally propagated plants (CV) (SHEPHERD and SILVA, 1996) (table II). In this case, striking differences were observed between the four cultivars for all parameters studied.

cells with numerical or structural abnormalities

Cells with numerical deficiencies were absent in Pacovan and otherwise quite rare in the others

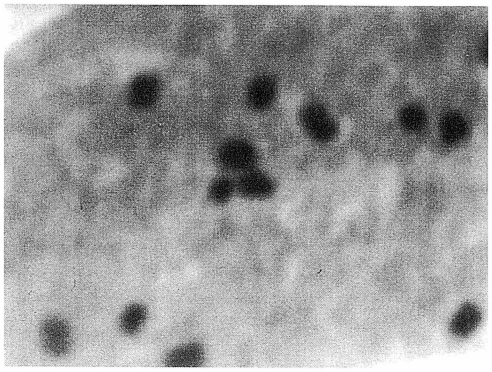

Figure 1
Enlarged part of 34-chromosome cell of SOUZA (1994); one of the 12 chromosomes included, in a group of three, is a very conspicuous 'mini'.

(F_D ranging from 0.00 to 0.003) except for vitroplants from Gia Hui produced with BAP (F_D = 0.11). They were always fewer than cells with extra chromosomes (F_S ranging from 0.03 for Mysore up to 1.12 for Pacovan), doubtless reflecting differential survival rates.

Plants from shoot-tip cultures of Mysore were generally more stable than in an earlier report, where values for both chromosome loss and gain were higher (SHEPHERD and SANTOS, 1996). However, three hexaploids were now identified among plants from cultures with the highest BAP concentration (7.5 mg/l). No other variety has doubled its chromosome number in meristem culture at the CNPMF, although rare hexaploid cells have been seen.

With Caipira, we noticed a high incidence of supernumerary chromosomes in treatment without BAP. This result would be scarcely credible as

Table I
Chromosome counts from vitroplants established from up to the third subculture, with a range of concentrations of BAP (CNPMF 1992-1994).

Cultivar	*mg/l*	*No of*	*Useful*	*Total*	*Chromosomes*			*'Minis'*	
(group)	*BAP*	*plants*	*roots*	*cells*	*Range*	F_N	F_S	*Most*	F_M
Caipira	2.5	16	24	50	32-34	0.40	0.24	2	0.40
(AAA)	5.0	23	31	50	32-35	0.30	0.38	2	0.62
	10.0	19	21	50	32-35	0.24	0.48	3	0.76
Pacovan	2.5	3	5	34	33-35	0.12	1.03	3	0.58
(AAB)	5.0	4	6	27	33-35	0.07	1.30	2	0.56
	7.5	5	11	36	33-37	0.11	1.08	2	0.47
Gia Hui	2.5	7	20	116	33-36	0.69	0.11	2	0.21
(ABB)	5.0 & 7.5	9	22	68	33-35	0.46	0.31	2	0.40
	10.0	5	15	113	33-36	0.57	0.28	2	0.23
Mysore	2.5	6	22	79	32-34	0.75	0.13	1	0.14
(AAB)	5.0 & 7.5	16	19	65	32-34	0.83	0.08	2	0.14

F_N: cells with the standard chromosome number (34 in the case of Gia Hui) and without 'minis'; F_S: supernumerary chromosomes; F_M: 'minis'resulting from breakage deficiencies.

Table II
Chromosome counts from conventional plants of some triploids (CV)[1] and of vitroplants from up to the third subculture, with BAP absent (BAP –) or present (BAP +) (CNPMF 1992-1994).

Cultivar	*Plant*	*No of*	*Useful*	*Total*		*Chromosomes*			+
(group)	*type*	*plants*	*roots*	*cells*	*Range*	F_N	F_D	F_S	F_M
Caipira	CV	3	17	100	32-35	0.45	0.03	0.25	0.47
(AAA)	BAP –	11	23	50	32-36	0.28	0.02	0.68	0.54
	BAP +	58	76	150	32-35	0.31	0.03	0.37	0.59
Pacovan	CV	2	25	100	33-35	0.51	0.00	0.44	0.34
(AAB)	BAP–	3	4	27	33-34	0.48	0.00	0.52	0.26
	BAP +	12	22	97	33-37	0.10	0.00	1.12	0.56
Gia Hui	CV	2	5	34	34-35	0.91	0.00	0.06	0.03
(ABB)	BAP –	5	13	110	33-36	0.46	0.04	0.27	0.34
	BAP +	21	57	297	33-36	0.59	0.11	0.22	0.26
Mysore	CV	5	24	115	33-34	0.90	0.00	0.03	0.08
(AAB)	BAP –	0							
	BAP +	22	41	144	32-34	0.78	0.02	0.10	0.14

[1] Data adapted from SHEPHERD and SILVA (1996).
F_N: cells with the standard chromosome number (34 in the case of Gia Hui) and without 'minis'; F_D: numerical deficiencies (never more than one per cell); F_S: supernumerary chromosomes; F_M: 'minis' resulting from breakage deficiencies.

a genuine treatment difference. Indeed earlier findings pointed to the potential distortion of results caused by differences between plants of the same treatment, or even between different roots from the same plant (SHEPHERD and SANTOS, 1996). For this variety, the frequencies of chromosome breakages ('minis') were remarkably similar for all three types of plant (BAP –, BAP + and CV).

In Pacovan, the rather small sample of cells from the few vitroplants obtained without BAP surprisingly performed like the conventionally propagated ones. Plants from cultures including BAP had about twice as many extra chromosomes and 'minis' (F_S = 1.12 and F_M = 0.56); in fact they were a little more unstable than those previously reported for the cultivar (SHEPHERD and SANTOS, 1996).

Vitroplants of Gia Hui behaved similarly whether produced with or without BAP and were moderately unstable, in contrast with the highly stable conventional plants.

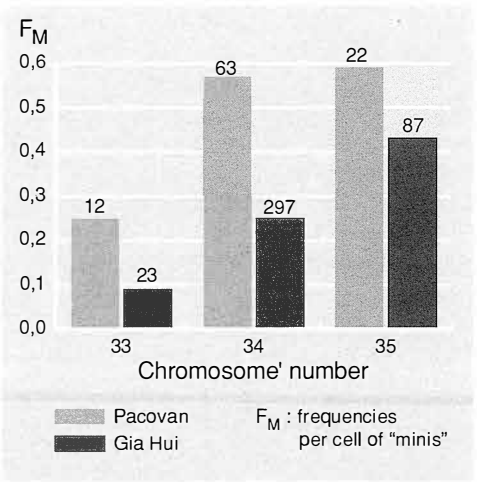

Figure 2
Interaction between frequencies of numerical changes and 'minis' in vitroplants from up to the third subculture of two triploids (CNPMF 1992-1994). Pacovan data exclude plants from cultures without BAP.

correlation between frequencies of supernumeraries and 'minis'

There has sometimes been a consciousness that the frequencies of extra chromosomes and of 'minis' might be correlated. This hypothesis has been tested with the cultivars Pacovan and Gia Hui (figure 2), where we observed an obvious difference for frequencies of 'minis' between cells with normal chromosome numbers and those with supernumerary chromosomes. The results show that frequencies are positively correlated with the presence of supernumerary chromosomes in the cells. Consequently, an additional chromosome is more likely to be a broken one; however, the interpretation of such findings still remains uncertain.

plants of Caipira from longer-term cultures

Chromosome studies of Caipira plants established from the seventh subculture are listed in table III. There is no increase of mitotic instability correlated to BAP concentrations in the media, in contrast with results from plants of young subcultures (table I). It should be emphasized here that there was no visual difference in the greenhouse between these plants and those from earlier subcultures.

the effect of number of subcultures

vitroplants of Caipira

Table IV compares the effects of short, intermediate and long periods of Caipira culture in medium containing 2.5 mg/l of BAP.

No difference in instability was observed with vitroplants emerging from the third to the fifth cycle of subculturing. At the seventh cycle, however, the frequencies of normal cells decreased (F_N = 0.14) while those of both supernumerary chromosomes and 'minis' increased (F_S = 0.54 and F_M = 0.74). From these results, it seems that the critical duration for this genotype is about 150 days, after which the frequencies of chromosome abnormalities increased appreciably.

vitroplants of Prata Ana

Counts reported in table V have been grouped with the previously reported comparative figures from conventional plants (SHEPHERD and SILVA, 1996). Among the 277 cells classified, only two had 32 chromosomes and these were of plants from the third and fourth subculturings.

Results from Prata Ana resemble those from Caipira in that mitotic instability increased rapidly beyond the fourth subculturing. There is a difference, however, in that the parent material has a relatively unstable mitosis even under conventional propagation, with up to four 'minis' in a single cell, as well as frequent extra chromosomes. Stability worsened in culture on both parameters, to the extent that the mean chromosome number now approximated to 34 instead of 33 in plants

Table III
Chromosome counts of cultivar Caipira (AAA group) in plants derived from the seventh monthly subculture, with varying dosages of BAP (as mg/l) (CNPMF 1992-1994).

Dosages	*No of*	*Useful*	*Total*	*Chromosomes*			*'Minis'*	
of BAP	*plants*	*roots*	*cells*	*Range*	F_N	F_S	*Most*	F_M
0.0, 2.5	27	47	50	32-36	0.12	0.62	2	0.96
5.0	23	31	50	33-35	0.16	0.52	2	0.92
7.5, 10.0	34	40	50	32-37	0.10	0.64	3	0.94

F_N: cells with the 33 chromosomes and without 'minis'; F_S: supernumerary chromosomes; F_M: 'minis'resulting from breakage deficiencies.

Table IV
Effects of three durations of culture, with 2.5 mg/l of BAP on chromosome counts of vitroplants of cultivar Caipira (AAA) (CNPMF 1992-1994).

Subcultures		*No of*	*Useful*	*Total*	*Chromosomes*			*'Minis'*	
Days	*Cycles*	*plants*	*roots*	*cells*	*Range*	F_N	F_S	*Most*	F_M
Up to 90	3	5	7	50	32-35	0.30	0.32	2	0.52
120-150	4-5	4	4	50	32-35	0.24	0.34	2	0.52
210	7	7	13	50	32-35	0.14	0.54	2	0.74

F_N: cells with 33 chromosomes and without 'minis'; F_S: supernumerary chromosomes; F_M: 'minis'resulting from breakage deficiencies.

Table V
Chromosome counts of cultivar Prata Ana in vitroplants derived from different monthly subcultures, compared with conventionally propagated plants (CV) (CNPMF 1992-1994).

Subcultures	*No of plants*	*Useful roots*	*Total cells*	*Chromosomes*			*'Minis'*	
				Range	F_N	F_S	*Most*	F_M
3rd	15	21	74	32-36	0.14	0.78	3	0.76
4th	6	7	68	32-37	0.25	0.74	4	0.85
3rd and 4th	21	28	142	32-37	0.19	0.76	4	0.80
6th	9	23	64	33-36	0.02	1.27	5	1.42
7th	11	16	71	33-35	0.06	0.93	5	1.49
6th and 7th	20	39	135	33-36	0.04	1.09	5	1.46
CV	3	10	96	32-35	0.32	0.50	4	0.56

F_N : cells with 33 chromosomes and without'minis'; F_S : supernumerary chromosomes ; F_M :'minis'resulting from breakage deficiencies.

from subcultures six and seven. One cell in each of these treatments had as many as five 'minis', with an average per cell much higher than that found in vitroplants of Caipira with the same duration in culture. In some cells two extremely small 'minis' appeared to be co-orientated, suggesting either a recent longitudinal division or a new breakage.

In the greenhouse, growth rates were slow for all Prata Ana vitroplants, but those established from the sixth and seventh subcultures were decidedly poorer than those from earlier ones. They were always stunted, except for a few which had longer internodes and narrower leaves than the norm. It was readily deduced that these had lost the dwarfing gene of the original stock, but it was not possible to make a separate mitotic analysis of them, because of the scarcity of vigorous roots.

discussion and conclusions

This presentation reveals principally that the number of subcultures of stem tip explants may be a much more important determinant of mitotic instability in triploid banana cultivars than the concentrations of BAP used to stimulate proliferation. For two cultivars, this instability can be most conspicuously detected after about 150 days of culture, corresponding to four to five successive cycles of reculturing.

It must be emphasized that the practice, as in earlier papers of this series, has been to retire already formed plantlets at each subculturing. It is not impossible that the renewed division and culture of these plantlets could aggravate the problems.

Once again, important differences have been found between cultivars in basic chromosomal stability and in how this affects the results of varied culture procedures. In this respect, the rare occurrence of hexaploids in Mysore is scarcely relevant, since these can be very easily recognized at an early stage. More critical are the shifts towards aneuploid numbers and the higher incidences of deficiencies, implying a gene loss which was well demonstrated in one instance.

Nevertheless, the CNPMF biotechnology laboratory team still has a lack of useful knowledge of the field performance of its products, although the effects of long culture duration in Prata Ana were clearly visible in the greenhouse. This correlation with field data is the final step needed to demonstrate that somaclonal variation can indeed have a chromosomal basis.

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