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FINAL REPORT

IN VITRO GERMPLASM PRESERVATION OF *MUSA* SPP.

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1) SUMMARY

The study was performed in Israel with Cavendish bananas in four major lines: i) *in vitro* conservation at a temperature of 6°C; ii) effect of plant growth retardants on *in vitro* grown plantlets; iii) detection of somaclonal variants by different method and iv) selection of stable clones which do not produce undesired somaclonal variants.

In most experiments plantlets survived and resumed growth at a reasonable rate after an incubation period of not more than one to two weeks at a temperature of 6°C. Many experiments were done in order to elongate that period with a significant success. They included one or more of different supplements to the basic medium: activated charcoal, plant growth promoters and retardants, different mineral compositions of the media and different sequential combinations of temperatures before, during and after incubation at 6°C.

The potential use of plant growth retardants for conservation was studied with *in vitro* plantlets of cv. 'Williams'. The following retardants were tried: abscisic acid (ABA), p-chlorophenoxyisobutyric acid (PCIB), daminozide, chlormequat, and the antigibberellins uniconazole, paclobutrazol and ancymidol. The main differences found between regardants were the concentration needed to achieve retardance and the mode of retardance. Relatively high concentrations (>100 mg/l) were needed when PCIB, daminozide and chlormequat were used while low concentrations (1-5 mg/l) were found to be effective in reducing plantlets height when the antigibberellins were used. The other effect found by the antigibberellins included dramatic reduction in leaf size, plantlet compactness even in the dark and a high leaf production at the lower concentrations. At the lower concentration (1 mg/l) plantlets continued to develop and produce new leaves for a period of 4 months. These plants remained compact with small leaves and it was possible to dissect the upper part of the plants and transfer them to a new medium. It enabled to

get continuous growing shoots whereby the upper part is subcultured periodically. This method coupled with reduced temperature and incubation in the dark might expand the length of time between subcultures and might be effective in reducing somaclonal variation since no shoots of adventitious origin are used.

Different approaches were tried to develop monitoring methods for detecting somaclonal variants. One approach was to develop selective media. The two major somaclonal variants; dwarfs (DW) and mosaic like (MO) variants of cvs. 'Williams', 'Grand Nain'. and 'Nathan' as well as normal (NL) plants were established in culture and were used in different experiments. A certain difference in growth was found between DW when compared to NL or MO which was expressed in a higher compactness of the dwarf variants and a higher production of leaves. These differences were not big enough to be used as a detecting method even when the three kinds of plants were grown on media differing in their mineral composition plant growth retardants and promoters. One exception was the response to gibberellic acid where NL and MO plantlets responded with fast elongation and reduced leaf production as compared to DW plantlets. A better parameter to distinguish between DW and NL or MO plantlets was the ratio between height and number of leaves produced which was found to be in NL and MO about twice than that in DW plantlets.

By using the Random Amplified Polymorphic DNA (RAPD) technique it was possible to recognize between different cultivars and their dwarf *in vitro* somaclonal variants.

Stomata measurements of vitroplants at the *in vitro* stage showed that MO plantlets have significantly larger stomata than NL or DW plantlets. And since epidermal cells of MO plantlets are larger than those of NL or DW plantlelts recording of stomata on a per unit area basis was found to be most efficient method for distinguishing MO plantlets from NL or DW plantlets *in*

vitro. The values of NL or DW plantlets were found to be about twice than that of MO plantlets.

The hypothesis that the rate of occurrence of dwarf variants among 'Cavendish' vitroplants is governed by clonal-inherent genetic factors was studied. For this purpose, a selection in favour of highly stable clones(s) was performed. Six families that did not produce dwarf variants in a former study were further evaluated. At the first stage few hundred plants of each family were evaluated. Only one of the families did not produce any dwarfs. At the second stage this family was tested on a big scale. In that clone only five dwarf variants were recorded among more than 21,000 vitroplants evaluated in the field over a span of few years. During that period plants for evaluation were regenerated from newly initiated cultures with primary explants of the clone. The fact that plants of the clone remained stable in regard to restoring a negligible rate of dwarf variants with time, both *in situ* and *in vitro* is attributed to a genetic trait that was revealed by the selection procedure employed.

2) INTRODUCTION

Results obtained in this study are brought according to the main lines of research done in Israel. Part of the results were published already and others were submitted for publication. Results which are included in the last category are brought in details as appendixs in the form of the paper or manuscript, and only main results are referred in the report.

The main four lines of research were: i) *In vitro* conservation at a temperature of 6°C. ii) effect of plant growth retardants on *in vitro* grown plantlets. iii) detection of somaclonal variants by different methods and iv) selection of stable clones which do not produce undesired somaclonal variants.

3) *IN VITRO* CONSERVATION AT A TEMPERATURE OF 6°C

All plantlets used in this line of work were of clone 6/3 cv. 'Williams' which was found to be stable from the point of not producing *in vitro* dwarf somaclonal variants (Cahpter 6 below). A similar procedure was applied in all experiments done in this line. It included three stages of handling: i) multiplication of stock plants; ii) transfer of plantlets onto an elongation medium (EM) to get individual plants and iii) cutting back of roots and leaves of well developed plants that were transferred onto the final experimental medium (FM) and incubation at 6°C. In part of the experiments plantlets were transferred after the EM medium on a pre-medium (PM) and then on the FM medium. Except the main treatment (incubation at 6°C) there were in most experiments sub-treatments which included different media and temperature combinations at one or more stages of the experiments.

Multiplication medium (MM) of stock plants contained MS Salts (Murashige & Skoog, 1962, Physiol. Plant. 15: 473-497) supplemented with sucrose (3%) and (in mg/l) myoinositol (100), $NaH_2PO_4.2H_2O$ (380) thiamine HCl (0.5), adenine sulfate (160), l-tyrosine (200), IAA (2), BA (5) and agar (0.8% Difco Bacto agar or 0.65% Sigma agar). Liquid medium when used

contained the same composition without agar and supplemented with ancymidol (1mg/l). The pH of the multiplication media was adjusted before autoclaving to 5.8 of semisolid medium (MM-1) and to 5.0 of liquid medium (MM-2). Elongation was done on a medium (EM-1) which was similar to MM-1 except that tyrosine was omitted and kinetin (5) was used instead of BA. Multiplication in liquid was done in order to obtain small bulbils that were transferred onto different EM media before the FM media. Both multiplication and elongation was done at 28°C and a cycle of light:dark=16:8h. Incubation at 6°C was done in the dark.

After incubation at 6°C cultures were tramsferred back to 28°C where they were kept for few weeks in order to get a real information about their recovery potential after the cold treatment.

Generally for each subtreatment there were 10-20 test tubes (25 x 150 mm) containing 15 ml of medium with one plantlet per tube. Many of the experiments were repeated few times and typical reproducable results are brought below.

In order to be sure about the resumption of growth it was necessary to keep the plantlets for several weeks at 28°C after the incubation at low temperatures (Tables 1 to 3). In most experiments explants or plantlets survived and resumed growth at a reasonable rate after an incubation period of not more than one or two weeks (Tables 1 to 3). Many experiments were done therefore in order to elongate that limit of survival of only one to two weeks at 6°C.

Promising results were obtained by supplementing the FM medium with activated charcoal (Table 1). In repeated experiments no significant improvement was achieved in improving the survival of plantlets over two weeks at 6°C when EM and FM media were supplemented with activated charcoal and also with or without growth retardants (Table 4).

Other treatments which were not found to be significantly efficient included supplements of plant growth promoters and retardants to the FM medium (Tables 2 and 3) or to the EM and FM media as well various mineral composition alone (different ratios of N^{+5}/N^{T}, N/K, K+Na/Ca+Mg) or with growth retardants in the EM and FM media (Table 4).

In another set of experiments small bulbils were produced by suing MM2 medium which was developed for this purpose in this study. After multiplication the bulbils were transferred to an elongation medium (EM) supplemented by plant growth retardants and promoters as well activated charcoal followed by different sequential combinations of temperatures before, during and after incubation at 6°C (Table 5). In these experiments no advantage was found to the small bulbils over small plantlets used in other experiments.

Table 1; Survival rate of plantlets incubated at 6°C for different periods on media with (+) and without (-) activated charcoal.*

	days at different temperatures (No).								survival (%)	
	7	7	7	7	7	7	7	105	-	+
temperature (C°)	6	28	28	28	28	28	28	28	100	100
	6	6	28	28	28	28	28	28	87	97
	6	6	6	28	28	28	28	28	18	87
	6	6	6	6	28	28	28	28	5	80
	6	6	6	6	6	28	28	28	0	47
	6	6	6	6	6	6	28	28	0	0
	6	6	6	6	6	6	6	28	0	0

*Sequence of media used: MM1→EM1→FM=EM1 without (-) and with (+) activated charcoal (0.25%).

Table 2: Survival rate of plantlets incubated at 6°C on media with plant growth promoters and with different sequence of temperature applied before and after the low temperature.*

	days at different temperatures (No.)							survival on different FM media (%)				
								0	Ad	Ad+K+ IAA	Ad+K+ IAA+Ty	Ave
	7	7	7	7	7	7	66					
temperature (C°)	6	6	28	28	28	28	28	50	35	35	50	43
	6	6	6	28	28	28	28	0	0	0	0	0
	6	6	6	6	28	28	28	0	0	0	0	0
	16	6	6	28	28	28	28	80	60	40	56	59
	16	16	6	6	28	28	28	70	40	60	30	50
	16	16	16	6	6	28	28	80	40	30	0	38
	16	16	16	16	6	6	28	60	100	100	75	84
	28	6	6	28	28	28	28	100	100	100	100	100
	28	28	6	6	28	28	28	0	0	0	0	0
	28	28	28	6	6	28	28	0	0	0	0	0
	28	28	28	28	6	6	28	0	0	0	0	0
	28	28	28	28	28	28	28	100	100	100	100	100

*Sequence of media ussed: MM1→EM1→FM=EM1 supplemented with (in mg/l), Ad; adenine sulfate (160), K; kinetin (5), IAA; indole acetic acid (2), Ty; tyrosine (200).

Table 3: Survival rate of plantlets incubated at 6°C on media supplemented with plant growth retardents and with different sequence of temperatures applied before and after the low temperature.*

	days at differnet temperatures (No.)				survival on different media (%)				
	10	7	7	56	0	Pa	An	Pc	Ave
temperature (C°)	28	6	28	28	100	100	100	100	100
	28	6	16	28	100	100	100	100	100
	16	6	28	28	100	50	60	50	65
	16	6	16	28	15	15	10	5	14
	28	28	28	28	100	100	100	100	100

*Sequence of media used: MM1→EM1→FM=EM1 supplemented with (in mg/l), Pa; paclobutrazol (5), An; ancymidol (5), Pc; PCIB (100).

Table 4. Number of combinations of different media components at preapplication (PM) and final application (FM) of different sequential combinations of temperatures (TS) before, after and during incubation of small plantlets at 6°C in different experiments*.

Combinations (No.)					
PM media			at 6°C		
MN	AN	CH	FM	TS	Total
4			4	9	36
4	4		16	3	48
1	4	3	12	4	48
1	2	1	2	3	36

* Sequence of media used: MM1→EM1→PM (EM1+supplements)→FM (=PM).

MN; Different mineral composition, AN; Ancymedol, CH; Activated charcoal

Table 5: Number of combinations of different media components at the elongation stage (EM) and final application (FM) of different sequential combinations of temperatures (TS) before, after and during incubation of small bulbils at 6°C in different experiments *.

Combinations (No.)					
EM media			at 6°C		
AN (1)	CH (2)	BA (3)	FM	TS	Total
4	3		12	3	36
4	3		12	3	36
1		2	2	3	6

*Sequence of media used: MM2→EM (=EM1+ supplements)→FM (=EM).
AN; Ancymedol, CH; Activated charcoal, BA ; Benzylaminopurine

4) EFFECT OF PLANT GROWTH RETARDANTS

The effect of plant growth retardants on clone 6/3 of cv. 'Williams' was studied. The main results obtained were published are are brought in appendix No. 1 The following retardants were tried: abscisic acid (ABA), p-chlorophenoxyisobutric acid (PCIB), daminozide, chlormequat and the antigibberellins: uniconazole, paclobutrazol and ancymidol. All chemicals were found to be effective in suppressing growth as expressed by reduction in new leaf production and plant height.

Two main differences in response were found between retardants: concentration needed to achieve retardance and the mode of retardance. Relatively high concentrations (>100 mg/l) were needed when PCIB, daminozide and chlormequat were used, while relatively low concentration (1-5 mg/l) were found to be effective in reducing plantlets height when the antigibbberellins were used. The other effects found by the antigibberellins

included dramatic reduction in leaf size, plantlets compactness and a high leaf production at lower concentrations.

At the lower concentration (1 mg/l) of all 3 antigibberellins, plants continued to develop and produce new leaves for a period of 4 months. These plants remained compact with small leaves and it is possible to dissect the upper part of the plants and transfer them to a new medium. Further studies are needed to find out the optimal antigibberellin concentration for culturing *Musa* plantlets in the dark as continuous growing shoots whereby the upper part is subcultured periodically. This method coupled with a reduced temperature (15-18°C) might expand the length of time between subcultures. If this procedure will be found to be effective for conservation, somaclonal variation might also be reduced since no shoots of adventitious origin are used.

5) DETECTION OF SOMACLONAL VARIANTS BY DIFFERENT METHODS

Dwarf variants are the most common of variants found in Cavendish subgroup. The second most common off-type among banana vitroplants is mosaic-like variants. Attention was given therefore to these two variants and mainly to the dwarf variants.

Dwarf variants

Different approaches were tried to develop monitoring methods for detecting somaclonal variants. One approach was to develop selective media. Another approach was the use of DNA markers. By using the Random Amplified Polymorphic DNA (RAPD) technique it was possibile to recognize between different cultivars of the Cavendish banana group and their dwarf *in vitro* somaclonal variants. The results of this part of the study were summarized and submitted for publication as brought in appendix No. 2. The main findings were that one out of 10 primers generated polymorphic RAPD marker for each of three cultivars compared to their dwarf variants. The calculated degree of polymorphism determined by comparing the normal

cultivars one to each other revealed lower values than those observed for each cultivar and its *in vitro* somaclonal dwarf variant. This method can be used therefore for detecting dwarf somaclonal variants.

The line of work involved in developing selective media included media with different ratios between the major elements and plant growth regulators. The procedure applied in all experiments done in this line were similar to those described in Chapter 3 and included three stages of handling: i) multiplication of stock plants on MM-1; ii) transfer of plantlets onto elongation medium EM-1 and iii) cutting back of roots and leaves of well developed plants that were transferred onto the final medium (FM) and incubation at 28°C.

Plantlets of three 'Cavendish' banana cultivars as well as their dwarf and mosaic variants were established in culture and were used in the different experiments described below.

A certain difference in vegetative growth was found between dwarf variants and normal plants expressed in more compactness of the dwarf variants as a result of production a higher number of leaves and a lower stature. This difference was not big enough to be used as a detecting method even when normal and dwarf plantlets were grown on media differing in their mineral composition (Table 6) and plant growth retardants (Tables 7 and 8).

It was reported before that it is possible to detect dwards of 'Williams' in the flask by adding gibberellic acid to the medium. Normal plants responded with fast elongation and reduced leaf production, while dwarf variants were much less responsive. Other plant growth promoters did not induce the same rate of difference in response as GA_3 did (Table 9). Repeated experiments with other plant growth regulators as well with mevalonic acid and other gibberellins (GA_1, GA_4, GA_7) did not result with a stronger response as that obtained with GA_3.

It was found that in order to get a clear difference between normal and dwarf variants when media is supplemented with GA, evaluation has to be done

after a period of 7 to 8 weeks of incubation. At this time the number of newly formed leaves can be used as a good indicator. That long period of incubation is needed for tall varieties like 'William' and 'Grand Nain', while with lower varieties like 'Nathan' the difference can be observed at an earlier stage (Table 10).

A better parameter is the ratio between height and number of leaves produced which was found to be in normal plantlets about twice of that found in dwarf plantlets (Tables 9 and 11). The difference in ratio was kept even when antigibberellin like ancymedol was added to the medium together with gibberellic acid (Table 11).

Table 6: Effect of different nitrogen sources (N^{+5}/N^{T}) nitrogen to potassium ratio (N/K) and monovalent to bivalent cations (Na+K/Ca+Mg) on normal (NL) and dwarf (DW) 'Williams' banana plantlets grown *in vitro* for nine weeks.

Medium description			Plantlet type					
			NL			DW		
$\frac{N^{+5}}{N^{T}}$	$\frac{N}{K}$	$\frac{Na+K}{Ca+Mg}$	Height (H)**	Leaves (L)**	H/L	Height (H)	Leaves (L)	H/L
1.0	0.4	5.1	7.3	4.9	1.5	6.0	5.5	1.1
1.0	1.7	0.8	7.0	4.8	1.5	5.8	5.6	1.0
1.0	2.6	0.3	6.1	5.1	1.2	4.7	5.3	0.9
* 0.7	1.1	5.1	6.4	5.2	1.2	5.0	5.6	0.9
0.7	2.6	0.8	5.6	5.0	1.1	4.4	6.5	0.7
0.1	2.6	5.1	6.0	4.5	1.3	4.8	5.9	0.8
Average			6.4	4.9	1.3	5.1	5.7	0.9

*MS- medium ** H= Height of plantlets in cm, L= No. of new leaves.

Table 7: Effect of growth retardants (GR) on Normal (NL) and Dwarf (DW) 'Williams' banana plantlets grown *in vitro*.

GR		Plantlet type					
		NL			DW		
Kind	mg/l	Height (H)*	Leaves (L)*	H/L	Height (H)*	Leaves (L)*	H/L
0	0	4.4	3.9	1.1	3.1	3.0	1.0
CCC	0.1	4.9	4.2	1.2	3.4	3.5	1.0
CCC	0.5	5.8	3.5	1.7	4.0	4.0	1.0
CCC	1.0	4.5	4.0	1.1	3.7	3.7	1.0
CCC	5.0	4.5	3.8	1.2	3.2	3.5	0.9
ALAR	0.1	5.0	4.3	1.2	4.2	3.8	1.1
ALAR	0.5	5.0	3.7	1.4	4.1	3.8	1.1
ALAR	1.0	4.6	3.9	1.2	3.9	3.2	1.2
ALAR	5.0	4.6	3.8	1.2	3.7	3.5	1.1
Average		4.8	3.5	1.3	3.7	3.6	1.0

*H= Height of Plantlets in cm. L= No. of new leaves.

Table 8: Effect of growth retardants (GR) on Normal (NL) and Dwarf (DW) 'Williams' banana plantlets grown *in vitro* for six weeks.

GR		Plantlet type					
		NL			DW		
Kind	mg/l	Height (H*)	Leaves (L)*	H/L	Height (H)	Leaves (L)	H/L
O	0	2.3	3.0	0.8	2.0	4.0	0.5
ABA	1	1.0	1.1	0.9	1.1	1.4	0.8
ABA	5	1.0	0.7	1.4	1.0	1.1	0.9
ABA	10	1.2	2.1	0.6	0.8	0.8	1.0
Uniconazole	0.1	1.5	5.1	0.3	1.3	5.7	0.2
Uniconazole	0.5	1.2	5.6	0.2	1.1	5.9	0.2
Unizonazole	1.0	0.9	2.9	0.3	1.0	4.2	0.2
Average		1.3	2.9	0.6	1.2	3.3	0.5

* H= Height of plantlets in cm, L= No. of new leaves.

Table 9: Effect of Gibberellic acid (GA) and Kinetin (KIN) on Normal (NL) and Dwarf (DW) 'Williams' banana plantlets grown *in vitro* for nine weeks.

Parameter	KIN (mg/l)	GA (mg/l)							
		0		1		5		10	
		Plantlet type							
		NL	DW	NL	DW	NL	DW	NL	DW
Height (H)*		4.1	3.2	3.4	1.5	3.6	2.1	3.0	2.0
Leaves (L)*	0	4.8	4.9	3.1	3.8	2.2	3.5	1.9	3.4
H/L		0.9	0.7	1.1	0.4	1.6	0.6	1.6	0.6
Height (H)		5.0	5.0	4.1	3.9	3.2	2.9	4.9	2.7
Leaves (L)	1	5.5	4.9	2.9	3.9	2.3	2.9	1.6	2.8
H/L		0.9	1.0	1.4	1.0	1.4	1.0	3.1	1.0
Height (H)		2.4	3.4	1.6	3.0	1.5	2.3	2.1	1.6
Leaves (L)	5	4.0	4.2	1.6	2.8	1.0	1.5	0.9	2.3
H/L		0.6	0.8	1.0	1.1	1.5	1.5	2.3	0.7
Height (H)		3.0	2.5	1.9	1.0	0.9	1.8	0.8	1.7
Leaves (L)	10	2.7	3.5	0.7	0.8	0.3	2.0	0.4	1.8
H/L		1.1	0.7	2.7	1.3	3.0	0.9	2.0	0.9

*H= Height of plantlets in cm, L= No. of new leaves.

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Table 10: New leaves produced by normal (NL) and dwarf (DW) plantlets in response to gibberellic acid (GA) of 'Grand Nain' (GRN) and 'Nathan' (NAT) bananas after different time of incubation.

GA	Incubation time (days)															
	23				46				65				76			
	Cultivar and type of plantlet															
	GRN		NAT		GRN		NAT		GRN		NAT		GRN		NAT	
(mg/l)	NL	DW	NL	DW	NL	DW	NL	DW	NL	DW	NL	DW	NL	DW	NL	DW
O	1.4	1.5	1.4	1.8	2.9	3.4	2.8	4.1	3.9	4.3	4.0	5.5	4.9	5.0	4.8	6.5
1	0.3	0.6	0.5	1.1	0.9	1.8	1.3	2.7	1.6	2.5	2.1	3.8	1.7	2.7	2.7	4.7
5	0.4	0.6	0.6	1.5	0.9	1.4	1.5	3.0	1.0	2.3	2.2	3.9	1.1	2.5	2.6	4.5
10	0.2	0.4	0.3	1.0	0.6	1.5	1.0	2.5	0.9	2.1	1.5	3.4	1.1	2.4	1.7	4.0

Table 11: Effect of ancymedol (AN) and gibberellic acid (GA) on normal (NL) and dwarf (DW) 'William' (WIL), 'Grand Nain' (GRN) and 'Nathan' (NAT) banana plantlets grown *in vitrio* for 75 days.

Cultivar and type of plantlet	Parameter	Ancymedol and gibberellic acid (mg/l)															
		AN	GA	AN	GA	AN	GA	AN	GA	AN	GA	AN	GA	AN	GA	AN	GA
		0	0	0	5.0	0.1	0	0.1	5.0	0.5	0	0.5	5.0	1.0	0	1.0	5.0
WIL/NL	Height (H)*	7.3 ±0.3		6.8 ± 0.5		3.9 ± 0.2		7.2 ± 0.6		3.4 ± 0.2		7.1 ± 0.6		3.2 ± 0.2		6.0 ± 0.5	
	Leaves (L)*	4.8 ± 0.2		2.7 ± 0.2		10.6 ± 0.6		2.7 ± 0.2		13.5 ± 0.8		2.9 ± 0.2		15.4 ± 0.8		2.6 ± 0.2	
	H/L	1.5 ± 0.1		2.6 ± 0.2		0.4 ± 0.1		2.9 ± 0.3		0.3 ± 0.1		2.7 ± 0.2		0.2 ± 0.1		2.5 ± 0.2	
WIL/DW	Height (H)	5.6 ± 0.2		4.7 ± 0.4		3.7 ± 0.2		3.3 ± 0.2		3.3 ± 0.2		4.8 ± 0.4		3.2 ± 0.2		4.4 ± 0.4	
	Leaves (L)	5.9 ± 0.2		3.6 ± 0.3		10.8 ± 0.6		3.5 ± 0.2		13.6 ± 0.7		3.8 ± 0.3		15.4 ± 0.8		4.2 ± 0.2	
	H/L	1.0 ± 0.3		1.3 ± 0.1		0.4 ± 0.1		1.0 ± 0.1		0.2 ± 0.1		1.2 ± 0.1		0.2 ± 01		1.1 ± 0.1	
GRN/NL	Height (H)	7.1 ± 0.2		6.7 ± 0.5		3.9 ± 0.2		6.7 ± 0.6		3.6 ± 0.2		5.9 ± 0.4		3.2 ± 0.2		5.6 ± 0.5	
	Leaves (L)	5.1 ± 0.1		2.8 ± 0.2		10.6 ± 0.6		3.0 ± 0.2		13.6 ± 0.7		2.7 ± 0.2		14.7 ± 0.8		2.5 ± 0.2	
	H/L	1.4 ± 0.1		2.6 ± 0.2		0.4 ± 0.1		2.3 ± 0.2		0.3 ± 0.1		2.3 ± 0.2		0.2 ± 0.1		2.2 ± 0.1	
GRN/DW	Height (H)	6.1 ± 0.2		5.5 ± 0.5		4.2 ± 0.2		5.5 ± 0.4		3.7 ± 0.2		4.1 ± 0.3		3.8 ± 0.2		4.7 ± 0.4	
	Leaves (L)	5.8 ± 0.1		3.8 ± 0.3		11.4 ± 0.6		3.8 ± 0.2		14.4 ± 0.7		3.6 ± 0.2		15.2 ± 0.8		4.1 ± 0.3	
	H/L	1.0 ± 0.1		1.5 ± 0.1		0.4 ± 0.1		1.5 ± 0.1		0.3 ± 0.1		1.2 ± 0.1		0.3 ± 0.1		1.2 ± 0.1	
NAT/NL	Height (H)	5.9 ± 0.3		6.4 ± 0.5		3.9 ± 0.2		5.0 ± 0.4		3.7 ± 0.2		5.1 ± 0.4		3.2 ± 0.2		4.9 ± 0.5	
	Leaves (L)	6.2 ± 0.2		4.3 ± 0.4		11.4 ± 0.6		4.6 ± 0.3		14.4 ± 0.9		4.3 ± 0.3		15.9 ± 0.8		4.9 ± 0.4	
	H/L	1.0 ± 0.1		1.7 ± 0.2		0.4 ± 0.1		1.3 ± 0.2		0.3 ± 0.1		1.3 ± 0.1		0.2 ± 0.1		1.1 ± 0.1	
NAT/DW	Height (H)	4.9 ± 0.2		3.8 ± 0.3		3.5 ± 0.2		4.0 ± 0.2		3.2 ± 0.2		3.5 ± 0.2		3.6 ± 0.2		3.3 ± 0.2	
	Leaves (L)	7.2 ± 0.2		5.5 ± 0.4		11.2 ± 0.6		5.8 ± 0.3		14.0 ± 1.0		5.5 ± 0.3		15.3 ± 0.8		6.0 ± 0.3	
	H/L	0.7 ± 0.1		0.7 ± 0.1		0.3 ± 0.1		0.6 ± 0.1		0.2 ± 0.1		0.7 ± 0.1		0.2 ± 0.1		0.6 ± 0.4	

* H = Height of plantlets in cm, L = No. of new leaves.

Mosaic varaiants

A major group of variants which accounts for about 25% of the off-types includes plants characterized by leaves that are narrow, drooping, thick and rubbery in varying degrees and have a yellow-green variegation. Most of the mosaic variants (MO) yield small fruits of no commercial value. In a former study it was found that MO 'Williams' vitroplants are aneuploids and preliminary results were presented on the occurrence and appearance of stomata on normal (NL) and MO vitroplants. It was found that less stomata per leaf area were found on MO as compared to NL vitroplants.

In this study comparison was done between MO and NL *in vitro* grown plantlets of cvs. 'Williams', 'Grand Nain' and 'Nathan'. Stocks of plantlets was established and multiplied on MM-1 medium then transferred on an elongation medium EM-1 (media composition as described in chapter 3). Except MO and NL plantlets, dwarf (DW) plantlets were included in the study too. Stomata were measured from imprints of the leaf surfaces. Leaf surfaces were cleaned before a thin layer of translucent nail polish was painted onto the area to be studied. After drying, the nail polish impring was removed by applying a piece of translucent adhesive tape to it. The tape and imprint were then transferred to a slide for microscopic observation.

Another assay was a comparison between MO and NL plantlets grown on media supplemented with GA_3 as described above for NL and DW plantlets. In that assay no significant differences in response were found between NL and MO plantlets of the three cultivars compared.

Stomatal measurements of vitroplants of the three cultivars showed that MO have a significantly larger stomata than NL plantlets (Table 12). Epidermal cells of MO are larger than NL epidermal cells, and recording of stomata on a per unit are basis was found to be most efficient for distinguishing MO variants from NL plantlets *in vitro* (Table 12).

Stomatal measurements of DW plantlets differed a little bit from normal plantlets but were not significant (Table 12). It can be concluded that stomata size and their density can be used most efficiently for detecting MO variants at the *in vitro* stage.

Table 12: Stomatal length and density in normal (NL) mosaic (MO) and dwarf (DW) *in vitro* plantlets of 'Williams' (WIL), 'Grant Nain' (GRN) and 'Nathan' (NAT) bananas.

Cultivar and kind of plantlet	Leaf surface			
	Adaxial		Abaxial	
	Length (μm)	Density (No./mm^2)	Length (μm)	Density (No./mm^2)
WIL/NL	50.2 ± 0.9	17.8 ± 1.8	52.3 ± 1.1	77.0 ± 2.1
WIL/DW	44.7 ± 1.1	22.3 ± 1.6	48.8 ± 0.8	87.3 ± 3.7
WIL/MO	65.3 ± 1.2	8.5 ± 1.3	71.2 ± 0.9	38.3 ± 2.1
GRN/NL	49.2 ± 1.7	19.3 ± 1.2	52.0 ± 1.3	80.5 ± 3.7
GRN/DW	46.8 ± 1.6	19.7 ± 1.6	53.1 ± 1.0	82.2 ± 4.5
GRN/MO	64.8 ± 1.0	5.2 ± 0.9	69.1 ± 0.7	36.7 ± 2.2
NAT/NL	47.7 ± 0.2	19.2 ± 1.4	50.5 ± 0.8	82.3 ± 2.3
NAT/DW	44.1 ± 0.9	23.7 ± 1.7	51.5 ± 0.6	85.2 ± 2.4
NAT/MO	61.3 ± 0.7	8.5 ± 1.3	65.4 ± 0.9	36.2 ± 1.9

6) SELECTION OF STABLE CLONES WHICH DO NOT PRODUCE UNDESIRED DWARF SOMACLONAL VARIANTS

It was proposed that the rate of occurrence of the most common dwarf somaclonal variants among vitroplants is governed by clonal-inherent genetic factors. In order to verify this hypothesis a selection in favour of highly stable clone(s) was performed. Based on results obtained in an earlier study six families of cv. 'Williams' were selected for further large-scale multiplication and evaluation. After evaluation of few hundreds of plants of each of the families only two were tested further and expecially the one which exhibited the greatest stability. In that clone only five dwarf variants were recorded among more than 21,000 vitroplants over a span of a few years. The fact that plants of the clone remained stable in regard to restoring a negligible rate of dwarf variants with time, both *in situ* and *in vitro*, is attributed to a genetic trait that was revealed by the selection procedure employed.

Full details about this part of the study was summarized and submitted for publication as brought in appendix No. 3. The main finding was that it is possible to select banana clones that are stable in non-producing dwarf somaclonal variants when multiplied *in vitro*.

Response of *In Vitro* Banana Plantlets to Plant Growth Retardants

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Abstract

The potential use of plant growth retardants was studied for Musa conservation in the dark. This report describes the response of in vitro banana plantlets cv Williams to the following retardants: abscisic acid (ABA), p-chlorophenoxyisobutyric acid (PCIB), daminozide, chlormequat and the antigibberellins; uniconazole, paclobutrazol and ancymidol. All chemicals were found to be effective in suppressing growth as expressed by reduction in new leaf production and plant height.

Two main differences in response were found between retardants: concentration needed to achieve retardance and the mode of retardance. Relatively high concentrations (> 100 mg/l) were needed when PCIB, daminozide and chlormequat were used, while relatively low concentrations (1-5 mg/l) were found to be effective in reducing plantlets height when the antigibberellins were used. The other effects found by the antigibberellins included dramatic reduction in leaf size, plantlets compactness and a high leaf production at the lower concentrations.

The use of antigibberellins for Musa conservation is discussed from the viewpoint of keeping a continuously growing shoot in culture whereby its upper part can be dissected and subcultured periodically.

Introduction

The use of *in vitro* techniques for germplasm conservation has considerable advantages in the case of bananas and plantains where the majority is conserved in clonal form. Two approaches are taken to the storage of cultures; slow growth and cryopreservation as reviewed by Vuylsteke (1989) and Withers (1990). While cryopreservation is linked to success in plant regeneration via embryogenesis, slow growth can be

applied to existing procedures of shoot cultures. Slow growth can be achieved by maintaining cultures at reduced temperatures or in presence of osmotic or hormonal inhibitors (Withers, 1990). Of the two options available; modification of the nutrient medium or the physical environment in which cultures are incubated, the latter seems to be preferred by different operators (Schoof, 1990; Smith and Hamill, 1991; Vuylsteke, 1989). Slow growth was achieved by maintaining cultures at reduced temperatures (14-18°C) and low light level. The use of osmotica added to the culture medium is less common and the effect of plant growth retardants was less studied. The aim of this study was to observe the response of banana plantlets to plant growth inhibitors applied to cultures incubated under light or dark conditions. Dark incubation was the preferred option as it enables savings in space and energy used for conservation.

Materials and Methods

Shoot tips taken for cv Williams were established in culture on the following multiplication medium: Murashige and Skoog (1962) salts supplimented with (mg/l) $NaH_2PO_4.2H_2O$ (380), inositol (100), adenine sulfate (160), thiamine HCl (0.5), benzylaminopurine (5.0), indoleacetic acid (IAA) (2.0), sucrose (3%), agar (0.65%).

Before applying differential treatments, plantlets were incubated for 6-8 weeks on an elongation medium which contained kinetin (5 mg/l) instead of benzylaminopurine. Both stages were done under light (1,000 lux) at a temperature of 28 ± 1°C. Before transferring the elongated plantlets to differential treatments, their leaves and roots were cut back. Test tubes (15.0 x 2.5 cm) were used in all experiments with 15 ml of medium adjusted to pH 5.8 before autoclaving for 15 min at 121°C and a pressure of 1.05 kg/cm². Growth responses were measured in 10 plants per treatment. The length of plantlets was measured from their base to the crossing point of the uppermost leaves. The number of newly formed leaves was also counted.

Results and Discussion

Typical growth responses obtained with the different plant growth retardants are included in Table 1. The 3 antigibberellins: ancymedol, paclobutrazol, uniconazole, as well as ABA were found to be most effective in reducing plantlets height at relatively low concentrations (1-5 mg/l). PCIB was found to be effective only when a high concentration (200 mg/l) was used. Daminozide and chlormequat at a concentration of 25 mg/l did not affect plantlets height and only concentrations above 100 mg/l were found to be effective (data not presented).

Table 1. Effect of plant growth retardants on Williams banana plantlets grown *in vitro* for 6 weeks in the dark.

Growth Retardant		Height (H) (cm)		Leaves (L) (No.)		H/L (Ratio)	
Kind	mg/l						
0	0	6.9	abc[1]	3.0	c	2.3	abcd
IAA+KIN	2+5	8.1	a	3.4	c	2.6	abc
Ancymidol	1	4.8	defgh	6.8	a	0.7	gh
Ancymidol	5	3.8	fgh	6.7	a	0.7	gh
Paclobutrazol	1	5.1	cdefg	5.1	b	1.1	fg
Paclobutrazol	5	4.8	defgh	6.5	a	0.8	fgh
Uniconazole	1	4.0	efgh	6.6	a	0.8	fgh
Uniconazole	5	1.1	i	2.0	cd	0.3	h
ABA	1	6.2	abcd	2.7	cd	2.3	abcd
ABA	5	3.1	h	1.4	d	1.9	cde
PCIB	50	5.8	bcde	2.7	cd	2.2	bcd
PCIB	100	5.2	cdefg	3.2	c	1.8	de
PCIB	200	3.6	ch	2.2	cd	1.4	ef
Daminozide	5	7.2	ab	2.5	cd	2.8	ab
Daminozide	25	8.1	a	2.7	cd	3.0	a
Chlormequat	5	5.7	bcdef	3.2	c	2.0	cde
Chlormequat	25	7.6	ab	2.7	cd	2.9	ab

[1] Means within columns followed by the same letter are not significant using Duncan's multiple range test, P = 0.05.

The number of new leaves produced was associated with reduction in plantlets height when the following chemicals were used: ABA, PCIB, (Table 1) daminozide and chlormequat (data not presented). A completely different response in leaf production was obtained with the antigibberellins which was expressed in a high leaf production, except in the higher concentration of uniconazole (Table 1). Another effect of the antigibberellins was expressed in leaf shape and size which was much smaller and compact than in the control plants, both in the dark and under light (Fig. 1). This effect on leaf shape and size was not obtained with the other plant growth retardants, particularly in the dark

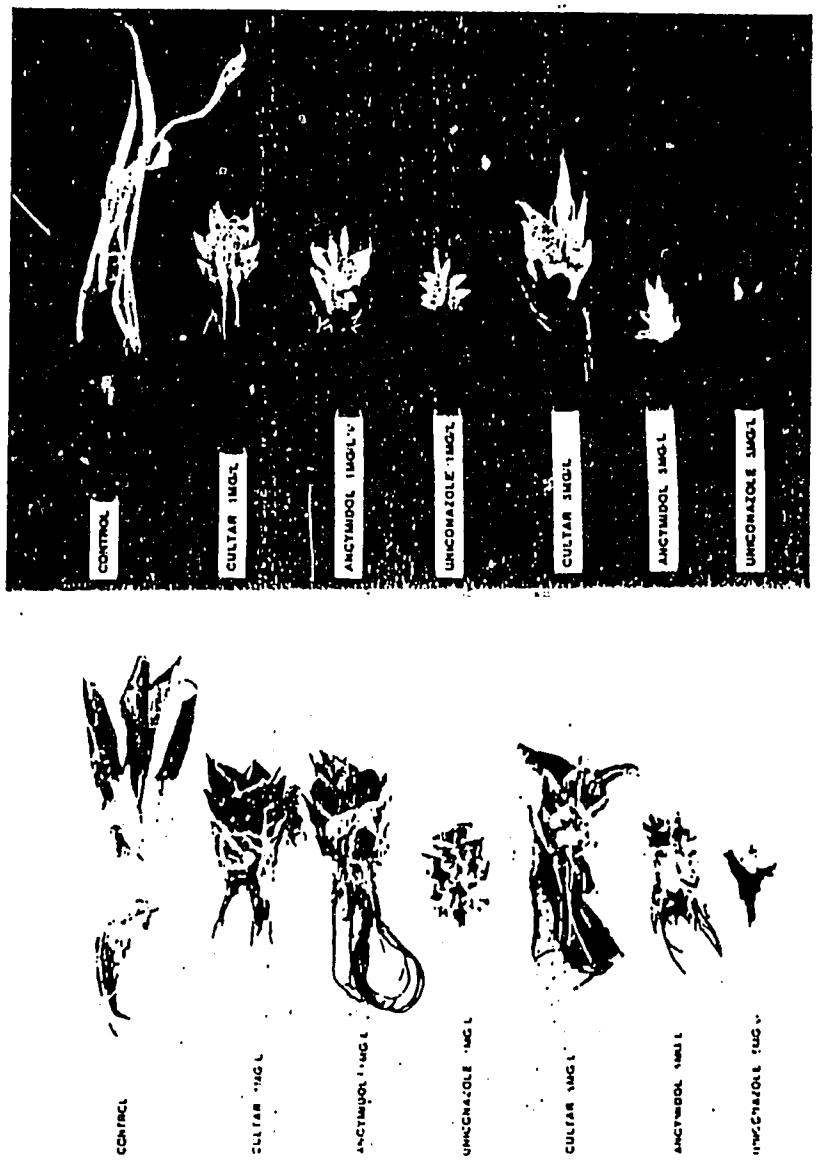

Fig. 1. Plantlets of banana cv Williams response to antigibberellins (1 and 5 mg/l) after 76 days in the dark (above) and under light (below).

where undesirable etiolated growth was obtained. This etiolated growth was similar to that found in the dark-grown control (Fig. 1).

In comparing the effect of the retardants on dwarfism as expressed in the ratio between plantlet height and number of leaves (H/L ratio in Table 1), it was found that all of them effected the rate of dwarfism obtained. This was particularly true with the antigibberellins as a result of higher leaf production.

It was found that there was a difference in activity of the different antigibberellins with uniconazole being the most active as shown by greater expression of effects at a lower concentration (Table 2). A high

Table 2. Effect of antigibberellins on Williams banana plantlets grown *in vitro* for different periods under light and the dark.

Antigibberellins		Days under light								
		65			98			128		
Kind	mg/l	H[1]	L	H/L	H	L	H/L	H	L	H/L
0	0	4.9	11.0	0.4	6.0	14.4	0.4	6.0	15.0	0.4
Uniconazole	1	3.2	16.0	0.2	3.8	22.2	0.2	3.7	23.0	0.1
Uniconazole	5	2.8	0.0	0.0	0.5	1.9	0.1	0.0	0.0	0.0
Uniconazole	10	2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Paclobutrazol	1	4.1	16.5	0.3	5.0	20.3	0.2	5.1	27.0	0.2
Paclobutrazol	5	4.7	15.0	0.3	5.5	18.0	0.3	3.1	15.1	0.1
Paclobutrazol	10	4.5	15.0	0.3	5.5	19.9	0.3	4.8	17.2	0.2
Ancymidol	1	4.0	16.5	0.2	6.2	19.0	0.3	3.2	17.7	0.1
Ancymidol	5	4.2	17.1	0.2	4.1	20.7	0.3	1.0	5.5	0.0
Ancymidol	10	3.4	18.1	0.2	3.6	20.1	0.2	1.5	6.3	0.1
		Days in the dark								
0	0	7.1	4.7	1.4	8.6	7.4	1.2	14.4	23.1	0.6
Uniconazole	1	4.0	10.2	0.4	4.0	15.5	0.2	4.7	23.1	0.2
Uniconazole	5	2.9	0.4	0.1	2.1	4.2	0.3	0.3	1.0	0.0
Uniconazole	10	2.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Paclobutrazol	1	3.9	9.9	0.4	5.1	15.5	0.3	5.6	18.8	0.3
Paclobutrazol	5	4.4	10.2	0.6	7.8	14.4	0.5	3.1	17.1	0.5
Paclobutrazol	10	3.5	8.2	0.3	4.9	12.7	0.3	5.5	14.4	0.3
Ancymidol	1	4.2	10.3	0.4	5.6	18.3	0.3	5.8	20.1	0.3
Ancymidol	5	3.7	10.6	0.2	4.4	16.4	0.2	5.5	22.1	0.2
Ancymidol	10	3.7	13.1	0.3	4.2	15.3	0.2	4.1	17.3	0.2

[1]H = Height in cm, L = No. of new living leaves.

concentration of antigibberellins brought about a reduction in living leaves with time, and in extreme cases led to the death of the plantlets, as in the case of uniconazole (Table 2). Over a period of time the reduction in growth and leaf survival was more pronounced under light than in the dark. At the lower concentration (1 mg/l) of all 3 antigibberellins, plants continued to develop and produce new leaves for a period of 4 months. These plants remained compact with small leaves and it was possible to dissect the upper part of the plants and transfer them to a new medium. Further studies are needed to find out the optimal antigibberellin concentration for culturing *Musa* plantlets in the dark as continuous growing shoots whereby the upper part is subcultured periodically. This method coupled with a reduced temperature (15-18°C might expand the length of time between subcultures. If this procedure will be found to be effective for conservation, somaclonal variation might also be reduced since no shoots of adventitious origin are used.

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APPENDIX 2

Detection of *in vitro* somaclonal dwarf variants of banana cultivars (*Musa*) by RAPD markers.

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Summary. One hundred different random 10 mer primers, were used to generate Random Amplified Polymorphic DNA (RAPD) markers in normal *'Williams', 'Grand Nein', 'Nathan'* and their *in vitro* somaclonal dwarf variants. One out of 10 primers generated polymorphic RAPD marker for each of the three cultivars compared to their dwarf variants. The calculated degree of polymorphism were 7.16%, 6.29% and 3.20% for *'Williams', 'Grand Nein'* and *'Nathan'* respectively, indicating an extensive genomic rearrangement that took place during the *in-vitro* propagation of the banana plants. The calculated degree of polymorphism determined by comparing the three normal cultivars one to each other, revealed lower values then those observed for each cultivar and its *in vitro* somaclonal dwarf variant.

Key words: Detection of somaclonal variants, *Musa,* banana, *in vitro* propagation, dwarf, *'Williams', 'Grand Nein'* and *'Nathan'*

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Introduction

In vitro shoot-tip culture is a basic technique used for *Musa* propagation, medium term conservation and germplasm exchange reviewed by Vuylsteke (1989). With the increased use of the technique, somaclonal variations were detected among field established banana plants (Reuveni and Israeli 1990). A description of banana somaclonal variants, their occurrence and the stage they can be detected was reported (Israeli et al. 1991). The detection and elimination of somaclonal variants represents an essential step in the application of the *in vitro* technique for propagation, germplasm exchange and conservation, where the maintenance of genetic stability is most important.

Dwarf offtypes is the most common variation in *Cavandish* bananas, where it accounts for about 80% of the total variants (Reuveni and Israeli 1990).The dwarf undesired variants are difficult to be detected at the *in vitro* and nursery stages. A reliable technique for their detection is therefore most important. Characterization of dwarf variants during the *in vitro* culture based on morphological identification is limited. It was possible to detect extra dwarf variants which were formed at high incidence when *'Nathan'* (a selection of dwarf *Cavandish*) was micropropagated (Israeli et al. 1991). Dwarf and normal plantlets of *'Williams'* bananas responded in a different way to gibberellic acid (GA_3). Normal plantlets reacted by internode elongation and suppression of leaf production, while dwarf plantlets were much less sensitive to GA_3 (Reuveni 1990). The earliest stage when dwarfs could be detected *in vitro* was at the end of their growth in the nursery. Differences in hight, distance between petioles, and leaf size could be distinguished (Israeli et al. 1991). The most promising selection criteria was petiole length, lamina length and petiole to lamina length ratio (Smith and Hamill 1993). Detection was effective when plants were growing vigorously and uniformally. The effect of GA_3 treatment was also tested on *'Grand Nain' in*

vitro plants during acclimatization (Cote et al. 1993). This treatment resulted in faster extension in normal compared to the dwarf plants. The disadvantage of these methods that are based on early morphological identification, is that they are prone to environmental effects. They are sufficient enough to detect undesired dwarfs in a commercial multiplication operation, but are not sufficient when *in vitro* germ plasm conservation is considered. Attempts were made therefor to use biochemical markers as reviewed by Novak (1992). Isosyme polymorphism were found to be reliable markers for discrimination among *Musa* clones of interspecific hybrids of *M. acuminata* and *M. balbisiana* but no difference was observed among different triploid clones of bananas and their *in vitro* somaclonal variants. It was reported recently (Kaemmer et al. 1992) that DNA oligonucleotide and amplification fingerprinting have been successfully used to detect genetic polymorphism in representative cultivars of the genus *Musa*. By using the Random Amplified Polymorphic DNA (RAPD) technique, it was possible to recognize between different cultivars of the *Cavandish* banana group and an induced mutant of the *'Grand Nain'* belonging to the same group. It was sought therefore studying the possibility of using the RAPD technique for detecting *in vitro* originated dwarf somaclonal variants in three cultivars of the *Cavendish* subgroup of bananas. Except for other characteristics, the cultivars differ in their stature: *'Williams'* being the tallest and *'Nathan'* (a selection of *'Dwarf Cavendish'*) the shortest.

Random Amplified Polymorphic DNA (RAPD) is a method based on the fact that when a relatively short primer is used to PCR (polymerase chain reaction) amplify genomic DNA, there is a high probability that DNA amplification products will be generated from several genomic regions that happens to be flanked by a pair of primers homologous to the priming sites (Williams et al. 1990). The amplification products are analyzed by electrophoresis. Genomic DNA from two different individuals, often produce different band patterns. A particular DNA fragment which is generated for one

individual, but not for another, represents a DNA polymorphism and can be used as a genetic marker.

Materials and Methods

Plant materials. Suckers of normal and dwarf somaclonal variants found in the field among *in vitro* propagated *'Williams' 'Grand Nain'* and *'Nathan'* bananas were transplanted to an experimental plot for observation for several additional cycles. It was done in order to verify that dwarfism was caused by genetic change transmitted to suckers for several ratoon crops (Reuveni and Israeli 1990). Suckers from such plants, that were found to be stable, were transplanted and grown in 50 l containers in a greenhouse at the Volcani Center for several further cycles. Samples were taken from different plants and on different dates from the middle part of fully expended leaves.

DNA Extraction and PCR amplification. Fresh leaves of banana plants were stored at -70 ^{0}C prior to DNA extraction. Total genomic DNA was extracted using the a modified CTAB DNA extraction procedure developed by Gawel and Jarret (1991). The DNA was quantified with fluorometer (Hoffer Scientific Inc.). Target DNA sequences were amplified by PCR in programmable thermocycler (M&J Inc.). PCR conditions were as described by Williams et al. (1990) except that 20 ng DNA was used per reaction. Taq polymerase was purchased from Promega Inc. Each reaction tube contained a single 10 mer random oligonucleotide purchased from Operon Technologies Inc. One hundred different primers (Kits A-E) were tested at least three times for all the primers that successfully generated bands in both normal and the dwarf mutants. PCR amplification products were analyzed by 2% Agarose/TBE gel electrophoresis (Sambrook et al. 1989), and stained with ethidium bromide. Degree of

polymorphism (DP) was defined as (1 - BS) X 100 = DP whereas BS is the "band sharing" calculated according to Vainstein et al. (1991).

Results and Discussion

One hundred different primers were tested. Fig 1. represents a typical DNA fingerprints generated by PCR of normal and *in vitro* somaclonal dwarf variants of *'Williams'* *'Grand Nain'* and *'Nathan'*. This picture do not reveal any significant polymorphism. Figure 2. represents typical RAPD markers obtained using five different primers and a comparison between cv. *'Williams'* and its *in vitro* somaclonal dwarf variant. Polymorphism is clearly observed between D1 and D2. Table 1 represents random amplified DNA polymorphism between the three banana cultivars and their dwarf vatiants. Between 56 and 73 primers (depending on the cultivar) successfully generated reproducible RAPD markers, at least three times using different DNA extraction batches. The other primers either did not produce reproducible RAPD markers in either the normal or the dwarf or in both. All the primers that established a polymorphic marker, generated at least one band in the normal and the dwarf mutant. The polymorphic band was either present or absent in the normal compare to the dwarf. Since the procedure depends upon a random primer, DNA amplification of polymorphic marker could be the result of either deletion or insertion of DNA (Baird et al. 1992). These types of mutational event have been proposed to take place in plant tissue cultures, and are responsible for somaclonal variation (Evans 1989). The calculated degree of polymorphism were 7.16%, 6.29% and 3.20% for *'Williams'*, *'Grand Nein'*, and *'Nathan'* respectively, indicating an extensive genomic rearrangements that took place during the *in vitro* propagation of the plants. Remarkably, the calculated degree of polymorphism determined by

comparing the three normal cultivars one to each other, revealed lower values then those observed for each cultivar and its *in vitro* somaclonal dwarf variant (Table 2.).

Recently Valles et al. (1993) have demonstrated the usefulness of RAPD markers in showing genetic stability of meadow fescue (*Festuca pratensis* Huds.) plants regenerated from suspension cultures and protoplasts. We have demonstrated that RAPD markers are useful also to detect genetic instability in *Musa* bananas propagated *in vitro.*

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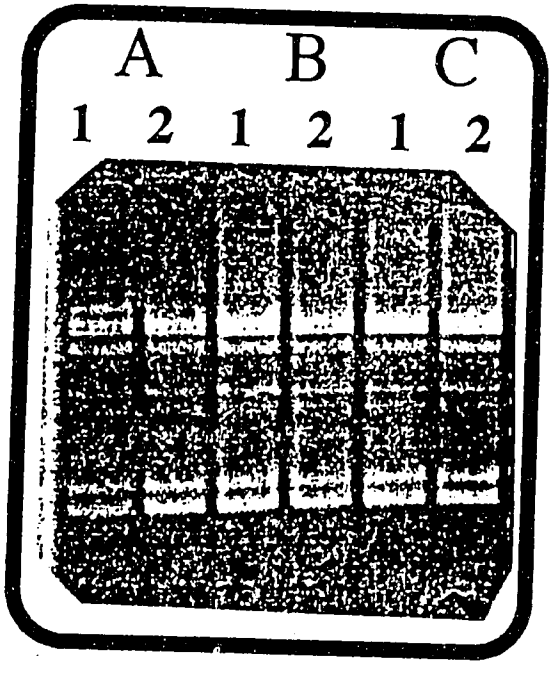

Fig. 1. RAPD analysis of normal (1) and *in vitro* somaclonal dwarf variants (2) of *'Williams'* (A), *'Grand Nain'* (B), and *'Nathan'* (C), using C18 primer.

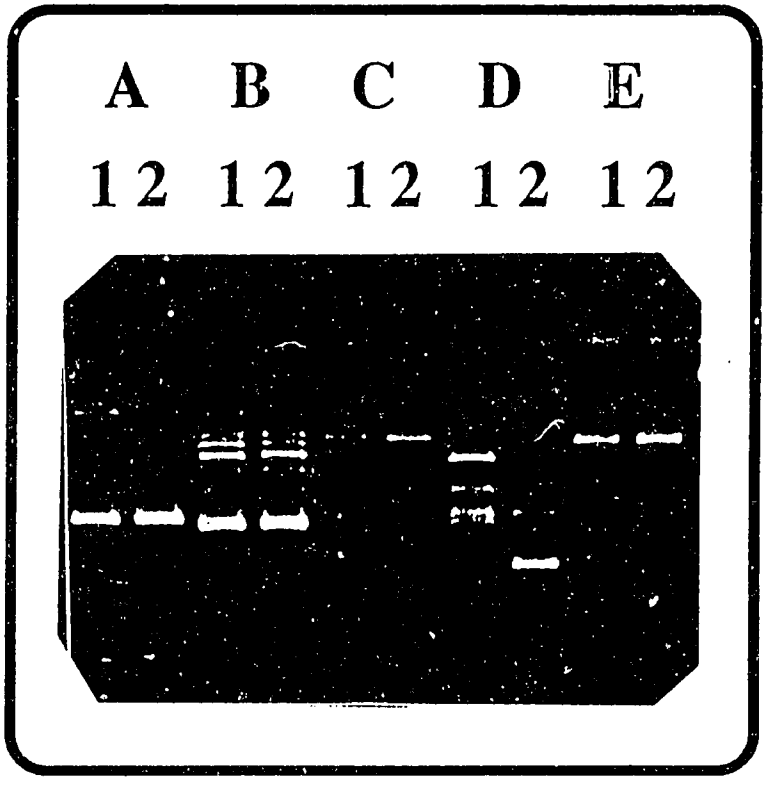

Fig. 2. RAPD analysis of normal (1) and somaclonal dwarf variant (2) banana cv. *'Williams'*. A, B, C, D, and E, represent primers C4, C5, C8, C11, and C14 respectively.

Table 1. Random amplified DNA polymorphism between three banana cultivars and their dwarf variants.

Parameter	*'Williams'*	*'Grand Nain'*	*'Nathan'*
Number of primers	73	56	61
Total number of bands	335	254	281
Polymorphism [%]	7.16	6.29	3.20

Table 2. Random amplified DNA polymorphism between three normal banana cultivars.

Cultivars	Polymorphism [%]	Number of primers	Number of bands
'Williams' X *'Grand Nain'*	0.82	58	266
'Williams' X *'Nathan'*	0.53	67	312
'Grand Nain' X *'Nathan'*	1.25	69	278

APPENDIX 3

Selection of stable banana clones which do not produce undesired dwarf somaclonal variants during *in vitro* culture

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Abstract: The use of shoot-tip culture for banana micropropagation, conservation and exchange of germplasm may be repressed by the occurrence of undesired somaclonal variants at high percentages. Dwarf variants which account for approximately 80% of the off-types among 'Cavendish' bananas are difficult to detect at the *in vitro* and nursery stages. A significant economic loss is caused when they are detected only at the production stage in the field.

In the present study the hypothesis that the rate of occurrence of dwarf variants among 'Cavendish' vitroplants is governed by clonal-inherent genetic factors was studied. For this purpose, a selection in favour of highly stable clone(s) was performed. Based on results obtained in an earlier study, 11 families of cv. 'Williams' were selected for further large-scale multiplication and evaluation. Six of the families that did not produce dwarf variants in the initial study are referred to as 'stable' families. Five of the families which did produce dwarf variants are referred to as 'non-stable' families.

At the first stage, a few hundred plants of each of the families were evaluated. All of the 'non-stable' families and four of the six 'stable' families produced dwarf variants. One of the 'stable' families produced a very low rate of dwarfs and one did not produce any dwarfs.

The two stable families were tested further, especially the one which exhibited the greatest stability. In that clone only five dwarf variants were recorded among more than 21,000 vitroplants over a span of 8 years. During that period plants for evaluation were regenerated from newly initiated cultures with primary explants of the clone. Some of the cultures were kept before evaluation for many cycles and for a longer period *in vitro* than the regular ones. The fact that plants of the clone remained stable in regard to restoring a negligible rate of dwarf variants with time, both *in situ* and *in vitro,* is attributed to a genetic trait that was revealed by the selection procedure employed.

The origin and causes of dwarf somaclonal variation in bananas is discussed. It is suggested that heterogeneity of the primary explants being chimiric, brings to the generation of dwarf somaclonal variants after dissociation of the chimeras.

Key words: Banana - Dwarf somaclonal variants - vitroplants - stable clones

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The use of shoot-tip culture for *Musa* micropropagation, conservation, and exchange of germplasm has been reviewed by Krikorian (1989); Vuylsteke (1989) and Novak (1992). With the increased use of shoot-tip culture for commercial micropropagation, undesirable somaclonal variants or "off-types" have been reported. The frequency of somaclonal variants in bananas has been found to vary over a very wide range from 0% to 90%, as reviewed by Cote *et al.* (1993); Israeli *et al.* (1995). A description of banana somaclonal variants, including the incidence, rate, and the stage they can be detected, was published

by Israeli *et al.* (1991). In the last study off-type plants were grown in the field for several cycles in order to check whether a variation found was transmissible by conventional vegetative propagation. Such an operation is necessary with vegetatively propagated sterile plants like bananas (Scowcroft 1984) in order to verify if a certain transmissible change was a result of a genetic change. It was found in that study and others (Cote *et al.* 1993) that most variations observed in banana vitroplants were transmissible and can be referred to therefore as somaclonal variants, which are known to occur in other plants as a result of a genetic change brought about by the *in vitro* culture (Larkin and Scowcroft 19811; Meins 1983; Swartz 1991). Such changes are undesirable when *in vitro* culture is used for multiplication and clonal fidelity is a prerequisite. In the case of *Musa,* the bulk of *in vitro* multiplication is done with dessert banana clones of the 'Cavendish' sub-group. In this group dwarfism is the most common undesired somaclonal variation, accounting for approximately 80% of the total variants (Israeli *et al.* 1991; Reuveni and Israeli 1990; Reuveni *et al.* 1993; Stover 1987). In certain cases a high rate of dwarfism was reported to prevent the commercial acceptance of banana vitroplants (Stover 1987; Smith and Drew 1990), while a low rate of variants did not repress the use of the technique on a big scale (Arias and Valverde 1987; Hwang *et al.* 1984; Israeli *et al.* 1991; Robinson *et al.* 1993).

Several features of the *in vitro* technique may raise the rate of somaclonal variation observed in plants (Scowcroft 1984). The composition of the culture medium and particularly its growth regulator content is often thought to be a factor that affects the rate of somaclonal variation (Smith 1988). Reuveni *et al.* (1993) did not find significant differences in somaclonal variation of cv. 'Williams' when cultured on 16 different media differing mainly in their rate of multiplication, kind and concentration of auxins and cytokinins. The overall rate of variation was still much higher than that obtained by conventional propagation. Factors which were found to affect the

rate of variation (Reuveni and Israeli 1990; Reuveni *et al.* 1993) were: (i) a high rate among plantlets suspected of originating, at least in part from adventitious meristems as opposed to those which developed from axillary buds; (ii) the rate of variants increased at each *in vitro* multiplication cycle; and (iii) large differences in variant rates in different families of cv. Williams suckers (family derived plants correspond to the entire *in vitro* progeny of one primary explant of a sucker).

The possibility of selecting 'stable' families which do not produce dwarf variants when multiplied *in vitro* was therefore sought. This paper presents the results obtained with field-grown banana vitroplants regenerated from families selected at an earlier stage as 'stable'; as well as 'non-stable' families which served as a control (Reuveni and Israeli 1990; Reuveni *et al.* 1993). The rate of somaclonal variation of the families and especially of those with a very low rate of dwarf variants was evaluated over several years on a large scale and through many multiplication cycles in order to confirm their stability.

Materials and methods

In a former study approximately 300 primary explants, each of them derived from a different sucker of cv. Williams, were randomly collected in the field and cultured on 16 different media (Reuveni *et al.*, 1993). During three to six multiplication cycles 30-100 plants of each family were planted for evaluation in the field. Independently of medium composition, 11 families were selected at the initial stage for further study. Six of the families which did not produce dwarf somaclonal variants were referred to as 'stable' families, and five of them which did produce dwarf variants were referred to as 'non-stable' families.

At the first stage a few hundred plants of each selected family were regenerated and planted in the field in different years for evaluation. The number of plants generated from each primary explant was low in order to

reduce the effect of duration in culture on somaclonal variation (Reuveni and Israeli 1990). At the second stage only families with a very low rate of dwarf variants were micropropagated and evaluated on a larger scale. This included repeated multiplication of many primary explants to a limited number of plants as well as some that were multiplied to large numbers.

Suckers 40-60 cm in height attached to fruiting mother plants were used as a source of shoot-tips, served as the primary explants. Mother plants were carefully inspected to make sure they were healthy and phenotypically true-to-type. After separating the suckers from the mother plant, their tops were cut back to approximately 30 cm, roots and dry leaf sheaths were removed, then washed with tap water and transferred to the laboratory. Outer leaf bases and corm tissue were trimmed to a size of approximately 25 x 50 mm cylinder with the shoot tip at the center, then surrface-sterilized by immersion in sodium hypochlorite (3%) for 20 min, rinsed three times with sterile water, after which shoot tips of approx. 5 x 5 x 5 mm, including a basal corm tissue, were excised aseptically. A second immersion in sodium hypochlorite (0.25%) for 10 min was followed by rinsing in sterile water (x3). Each tip was split longitudinally by two cuts forming four explants that were inoculated separately in test tubes (25 x 150 mm) containing agar medium (15 ml). The same medium was used for establishment and multiplication based on that recommended by Ma and Shi (1972). It included Smith and Murashige's (1970) mineral version supplemented with (in mg/l) myoinositol (100), L-tyrosine (100), thiamine HCl (0.5), adenine sulfate (100), benzylaminopurine (5), indole acetic acid (2); and (in gr/l) sucrose (30), and agar (7). Before acclimatization, explants were transferred to a similar medium supplemented with kinetin (5 mg/l) instead of benzylaminopurine. Before autoclaving for 20 min (103.4KPa at 121°C), pH was adjusted to 5.7. Cultures were maintained at 27°C under a 16:8 L:D cycle of 300 μmol $m^{-2}s^{-1}$ cool white fluorescent lamps, with 60-70% r.h. in the incubation room.

After 3-5 weeks of *ex vitro* acclimatization, plantlets were potted (1 lit) and grown in the nursery for another 6 weeks until their pseudostem reached a height of 20-30 cm. At this stage the first inspection for variants was conducted. A large part of the dwarf variants, most of the mosaic variants and other abnormal plants were detected (Israeli *et al.*, 1991) and eliminated from planting. The plants under observation were planted and treated in the field according to regular commercial practices. Three to four months after planting, the second inspection was conducted. At this stage most of the variants left could be detected. The third and last inspection was conducted after bunch emergence. Through all the *in vitro* and *ex vitro* stages, great care was taken to identify the individual plants by their selected family relation and their primary explant relation.

Results

At the first stage of selection, a few hundred plants were evaluated for each of 11 families. All the initially selected 'non-stable' families produced a considerable number of dwarf variants (the first five families in Table 1). Of the six families initially selected as 'stable', only two (13/7 and 6/3) produced a very low rate of dwarf variants (Table 1). Two other families in this group (3/28 and 8/19) produced a relatively very high rate of dwarf variants, and therefore it was decided at an early stage to stop their further evaluation (Table 1). The other two families (10/32 and 17/34) were evaluated for a longer period (Table 1), since the rate of dwarfs among vitroplants of these two families varied from year to year, and repeated tests were needed to draw the conclusion that they produce a high rate of variants.

Detailed results obtained in different years with a 'non-stable' (13/20) and a 'stable' (6/3) family are given in Table 2. Altogether 760 plants were evaluated of the 'non-stable' family. The plants were multiplied for a short time in culture and thus it was possible to detect dwarf variants in repeated batches

in different years. In the 'stable' family 1117 plants were evaluated by the same procedure, but no dwarf variants were found (Table 2).

In the second stage of selection the two initially selected 'stable' families, mainly one of them (6/3), were further tested on a larger scale. Only two dwarf variants were detected among some 12,485 plants regenerated from 125 primary explants that were cultured for a short period of time (Table 3). A negligible rate of dwarf variants was also found in batches kept in culture for a long period in different years before evaluation (Table 4). Altogether only five dwarf variants were found among 21,791 plants evaluated in the field of the selected family 6/3 (Tables 2, 3 and 4). It can be concluded that this clone is highly stable *in vitro* in producing negligible amounts of undesired dwarf somaclonal variants.

Further evaluation to a lesser extent of family 13/7 resulted in four dwarf plants out of 2548 evaluated in the years 1984-1992. A total of six mosaic variants was found in this family too. In this respect family 13/7 did not differ from family 6/3, and neither family differed from other families in the rate of mosaic vitroplants (Table 1).

Discussion

It has been reported in *Musa* that the extent of instability caused by *in vitro* culture can be related to cultivar (Daniells and Smith 1993; Johns 1994; Vuylsteke *et al.* 1991). Differences in somaclonal variation were also reported between clones of the same cultivars of Cavendish bananas (Johns 1994). In plantain, Krikorian *et al.* (1993) reported that the frequency of variants in a certain cultivar was confined to individual primary explants rather than clones. In previous publications (Reuveni and Israeli 1990; Reuveni *et al.* 1993) it was suggested that in 'Williams' bananas the primary explants heterogeneity could have been the cause of somaclonal percentage differences between families. Since some families were exceptional as regards the absence of dwarf variants at the initial stage, it was proposed that 'stable' families may occur, as a result

of a genetic trait. If so, this character has to be transmissible to the following cycles of vegetative propagation. Our results provide the evidence that primary explants heterogeneity does exist and it is possible to select banana clones that are stable in non-producing dwarf somaclonal variants when multipled *in vitro*. Our preliminary unpublished results with a family of another cultivar (5000 plants of 'Grand Nain') confirm this conclusion.

Another undesirable somaclonal variant is the mosaic type (Israeli *et al.* 1991). Heterogeneity in rates and incidence of that variant was reported to occur in different clones and batches of *in vitro* multiplied Cavendish cultivars (Johns 1994; Reuveni and Israeli 1990) as well as in this study. In the present study no effort was made to eliminate it. The possibility of selecting a stable clone which will not produce mosaic somaclonal variants is still open.

The origin and causes of variation in *Musa* were discussed previously on the basis of limited experimental data (Cote *et al.* 1993; Krikorian 1989; Novak 1992; Smith 1988; Vuylsteke *et al.* 1991). There are objective reasons for the limited data, mainly due to the difficulty of identifying dwarf variants at an early stage. As in other plants (Swartz 1991), variations in *Musa* shoot-tip cultures may be explained by three major mechanisms: (i) genetic changes that were already present in the tissue of the primary explants; (ii) genomic modifications in the vitroplants; and (iii) the *in vitro* culture environment which induces nontransmissible epigenetic modifications. Since the first two mechanisms are involved in forming heritable changes, they are relevant in the case of transmissible somaclonal variants.

Differences in rates of variants reported by different laboratories (Cote *et al.* 1993; Israeli *et al.* 1995), the results obtained by us in different years and with different primary explants in the same year (Reuveni and Israeli 1990), suggest that the high incidence and rate of dwarf somaclonal variants is caused by chimeric heterogeneity of the primary explants. Krikorian *et al.* (1993) referring to Maricongo plantain, claimed also that the heterogeneity of the

primary explants being chimeric was the cause of a high rate of differences in variation. Since chimerism is very common in plants propagated vegetatively for a long period (Swartz 1991; Tilney-Bassett 1986), its role in *Musa* somaclonal variation should be considered. Chimeric plants are easily dissociated *in vitro* and the regenerated plants could be somaclonal variants (Swartz 1991). In the case of cv. 'Red' banana which does not belong to the 'Cavendish' subgroup the situation is more clear. It is presumed that 'Red' is a periclinal chimera where a red outer layer covers a green core (Stover and Simmonds 1987). This chimera is dissociated *in vitro* and 'green red' plants are regenerated (Israeli *et al.*, 1991). This can be caused by the production of adventitious shoots in conjunction with axillary shoots (George and Sherrington 1984). Such a possibility was reported to occur in *Musa* (Banerjee *et al.* 1986; Novak *et al.* 1990), when plants were derived from axillary and adventive budding. This explanation is supported by the significantly higher rate of dwarf variants found among vitroplants regenerated from adventive budding (Reuveni *et al.* 1993).

Another causal mechanism involved in genetic modification expressed as somaclonal variation is associated with chromosomal variability of vitroplants (Lee and Phillips 1988). It has beem reported (Reuveni 1989) that the mosaic somaclonal variants possess aneuploid cells. Recent studies by Sandoval *et al.* (1993) showed that the mosaic somaclonal variants of cv. 'Grand Nain' bananas of the 'Cavendish' subgroup have 50% aneuploid cells, as compared with 14% in normal true-to-type and dwarf somaclonal vitroplants. Whether the high mixoploidy of the mosaic variants was induced by the *in vitro* environment, or pre-existed as cytochimeras in the primary explant, requires further study.

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Table 1. Total occurrence of somaclonal variants in 'Williams' vitroplants derived from 11 families at the first stage of selection. A relatively small number of plants was evaluated for each family.

Details of plants evaluated			Somaclonal variants					
			Dwarfs		Mosaics		Others	
Family	Years	Total (No.)	No.	(%)	(No.)	(%)	(No.)	(%)
6/32	1984-1989	523	34	6.50	2	0.38	19	3.63
13/20	1984-1990	760	15	1.97	4	0.53	0	0
17/27	1984-1990	887	12	1.35	6	0.68	0	0
10/17	1984-1990	731	11	1.50	4	0.55	0	0
10/10	1984-1990	472	14	2.97	5	1.06	0	0
3/28	1984-1987	148	6	4.05	1	0.68	2	1.35
8/19	1984-1987	167	12	7.19	1	0.60	1	0.60
10/32	1984-1990	1197	71	5.93	7	0.58	1	0.08
17/34	1984-1990	1101	37	3.36	5	0.45	0	0
13/7	1984-1989	914	2	0.22	1	0.11	0	0
6/3	1984-1989	1117	0	0	7	0.63	0	0

Table 2. Occurrence of somaclonal variants in 'Williams' vitroplants derived from initial 'non-stable' (13/20) and 'stable' (6/3) families in different years after a few multiplication (Multip) cycles in culture, at the first stage of selection.

Details of plants evaluated					Somaclonal variants					
					Dwarfs		Mosaics		Others	
Initiation (year)	Primary tips (No.)	Multip. cycles (No.)	Field planting (year)	Total plants (No.)	(No.)	(%)	(No.)	(%)	(No.)	(%)
13/20										
1984	1	3-4	1985	37	7	18.92	1	2.70	0	0
1986	1	6	1987	83	2	2.41	0	0	0	0
1987	4	5	1988	192	2	1.04	1	0.52	0	0
1988	4	4-5	1989	297	2	0.67	2	0.67	0	0
1988	4	6-7	1989	151	2	1.32	0	0	0	0
Total	14			760	15		4		0	
6/3										
1984	1	4	1985	40	0	0	0	0	0	0
1984	1	6	1986	47	0	0	0	0	0	0
1986	2	6	1987	225	0	0	2	0.89	0	0
1986	2	12	1988	140	0	0	1	0.71	0	0
1987	3	5	1988	211	0	0	3	1.42	0	0
1988	3	4-5	1989	275	0	0	1	0.36	0	0
1988	4	6-7	1989	179	0	0	0	0	0	0
Total	16			1117	0		6		0	

Table 3. Occurrence of somaclonal variants in 'Williams' vitroplants derived from a 'stable' (6/3) family in different years after a few multiplication (Multip.) cycles in culture, at the second stage of selection.

Details of plants evaluated					Somaclonal variants					
					Dwarfs		Mosaics		Others	
Initiation (year)	Primary tips (No.)	Multip. cycles (No.)	Field planting (year)	Total plants (No.)	(No.)	(%)	(No.)	(%)	(No.)	(%)
1989	38	4	1990	1061	0	0	6	0.56	1	0.09
1989	52	5-6	1990	4815	0	0	7	0.15	1	0.02
1990	19	5-6	1991	1827	2	0.11	8	0.44	4	0.22
1991	16	5	1992	4782	0	0	111	2.32	12	0.25
Total	125			12485	2		132		18	

Table 4. Occurrence of somaclonal variants in 'Williams' vitroplants derived from a 'stable' (6/3) family in different years after a large number of multiplication (Multip.)cycles in culture, at the second stage of selection.

Details of plants evaluated					Somaclonal variants					
					Dwarfs		Mosaics		Others	
Initiation (year)	Primary tips (No.)	Multip. cycles (No.)	Field planting (year)	Total plants (No.)	(No.)	(%)	(No.)	(%)	(No.)	(%)
1988	2	12	1990	1430	0	0	3	0.21	1	0.07
1988	1	18	1991	329	1	0.30	0	0	0	0
1989	10	10-12	1991	1240	0	0	13	1.05	0	0
1990	4	11	1992	4214	1	0.02	5	0.12	0	0.07
1989	1	13-16	1992	331	1	0.30	4	1.21	1	0.30
1989	1	18-20	1992	645	0	0	8	1.24	2	0.31
Total	19			8189	3		33		4	