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AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 9/2/87

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 3.F.29

Final rec'd 9/2/87

Attachment

3 cy

PROGRESS REPORT

3. F-29

(Final)

Grant No. DFE 5542-G-55-4030-00

UTILIZATION OF PLANT PROTOPLAST BIOTECHNOLOGIES FOR THE TRANSFER OF ORGANELLES

by

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The original goal of this research project was to develop novel protoplast manipulation biotechnologies, which could be applied to crop improvement for developing areas. After discussions with experts of the International Potato Center (CIP) in Lima, Peru, we focussed on the transfer of cytoplasmic-male-sterility (CMS) to prospective Seed-Parents of F_1 hybrid true-potato-seeds (TPS). CMS Seed-Parents should vastly decrease the production costs of F_1 hybrid TPS, thus, rendering such seeds readily available to farmers in developing countries.

More fundamental investigations on protoplast-fusion mediated organelle-transfer in *Nicotiana*, performed in the frame of this project, were instrumental for the successful application of this biotechnology to potato crop improvement. We detailed the results of these efforts in our previous Progress Reports, in reviewed journal publications and in oral presentations (see list at the end of this Report).

As shall be described in this final Report we succeeded in transferring CMS to prospective Seed-Parents of TPS F_1 hybrids. We are therefore very pleased that the main goal of this Project was actually achieved.

The execution of the experimental work was mostly performed by Avihai Perl, a Ph.D. student in our laboratory.

TRANSFER OF CMS TO POTATO CULTIVARS

In coordination with CIP scientists, the potato cultivars into which cytoplasmic male sterility (CMS) was to be introduced were "Atzimba" and "Atlantic". The source of CMS was line Y245.7, a male sterile line obtained from CIP.

Briefly gamma-irradiated protoplasts, derived from aseptically grown Y247.7 shoots (the donor) were fused with either "Atzimba" or "Atlantic" protoplasts (the recipients). Prior to fusion, the recipient protoplasts were treated with iodoacetic acid (IA) or rhodamine 6-G (R6G) - a treatment which enhances the selection against recipient's organelles and thus increasing the chance of donor organelles to be prevalent in the established cybrids. This 'donor-recipient' methodology was developed by us during and before this research project and proved to be very successful in inducing CMS in *Nicotiana*.

Our own results with *Nicotiana* as well as other published information strongly suggested that mitochondrial/nuclear interactions are controlling the manifestation of CMS. We therefore concentrated on the mitochondrial analysis of cybrid potato plants.

Twenty one calli were recovered from two independent experiments in which "Atzimba" protoplasts were used as recipient and two calli were recovered in an experiment in which "Atlantic" protoplasts were used as recipient. At least 5 plants were regenerated from each callus. The shoots of these plants were further cultured and then each shoot was divided in half and the halves were propagated *in vitro*. One set of plants was then transferred to the greenhouse for further analysis. The other set of plants was sent, as shoot cultures, to CIP for flowering analysis.

The characters analysed in greenhouse plants included flower morphology, pollen fertility, chromosome number and mitochondrial composition as shall be detailed below.

POLLEN FERTILITY.

Pollen fertility was determined by a germination test. Pollen was collected from flowering cybrids at the beginning of anthesis and immediately tested for germination in liquid drops of a medium composed of 6% sucrose, macroelements and boric acid. Each plant was sampled several times. Plants in which no pollen germination was detected in any of the samples were considered sterile (Tables 1 and 2 and Figures 1-5).

The flower pigmentation of the CMS donor plants, Y245.7, is violet-white. Pollen maturation in these plants is arrested at the tetrad stage and therefore no pollen germination is evident (Table 1 Figure 1). The flower pigmentation of "Atzimba" (recipient) is white. Pollen maturation in "Atzimba" is normal and pollen germination is evident in every test (Figure 2). Out of 21 calli 19 produced cybrid plants with white flowers which were identical to those of the recipient "Atzimba" (Table 1). Twelve of these calli produced plants with non-germinating pollen. Among these 12 calli which produced apparently sterile plants, four calli, namely I-B, I-P, II-C and II-F produced only such sterile plants while eight others produced fertile and sterile plants. Only one of the sterile plants exhibit also anther malformation (cybrid II-F4, Figures 4 and 5, Table 1) reminiscent of CMS in *Nicotiana*.

In the experiment in which protoplasts of "Atlantic" were used as the recipients, one of the resulting calli regenerated only sterile plants (Table 2). The development of the other callus was slow; therefore the regenerated plants were only recently transferred to the greenhouse and did not flower yet.

In our previous fusion experiments with *Nicotiana* it was common to obtain different types of plants from the same callus. Similarly, the *Solanum* fusion experiments yielded calli each of which produced both sterile and fertile plants.

The provisional evaluation of sterility/fertility was based on pollen germination. Because pollen germination may be affected also by environmental factors (e.g. high temperature) it was important to add other tests. Below we describe two additional analyses namely, chromosome counts and mitochondrial DNA restriction profiles. We have also sent a set of shoots from the same cybrid plants to CIP, Peru, where they should be grown under optimal conditions for flowering. One of us (D.A.) will visit CIP in August 1987, to obtain detailed results obtained over there and to evaluate in collaboration with potato experts, the cybrids derived from our fusion experiments.

CHROMOSOME ANALYSIS

Somaclonal variations, including chromosomal abnormalities, are common phenomena in tissue-culture derived plants. Both male and female fertility may be affected by chromosomal changes. We therefore checked the chromosome numbers of several cybrid plants. Chromosomes were counted either at meiosis (n) or at mitosis (2n). In the former case, anthers were removed from very young flower buds and squashed directly in acetocarmine. In the later case, cut shoots were transferred into rooting medium; after 4-5 days, when the roots were about 1 cm long they were incubated in a bromonaphthol solution for 3 hours. The roots were then transferred into acetocarmine and squashed for chromosome analysis.

The results are summarized in Table 3. Twenty out of the 21 plants contained 2n=48 or n=24 chromosomes which is the normal tetraploid chromosome number of *S. tuberosum*. Only one plant had a higher chromosome number. Among the plants listed in Table 3 some had fertile pollen while others had sterile pollen. We therefore may conclude that at least in some cases sterility was not caused by a change in chromosome number.

MITOCHONDRIAL DNA ANALYSIS - CORRELATION WITH CMS

Mitochondrial (mt) DNA was isolated from each of the fusion partners namely cv. "Atlantic" cv. "Atzimba" and line Y245.7. The mtDNA was digested with one of several restriction enzymes, run on a gel and stained. *Sal*I was one of the restriction enzymes which gave a unique mtDNA pattern of the fusion-donor Y245.7. This pattern was distinctly different from either "Atzimba" or "Atlantic" (Figure 6). *Sal*I was therefore used to digest the mtDNAs of the three presumptive male sterile cybrids: II-C2 and II-M3, derived from the fusions where "Atzimba" was the recipient and I-A4, derived from the fusion where "Atlantic" was the recipient. The restriction pattern obtained for each of the three cybrids was different from either of the fusion-partners patterns (Figure 6). We therefore conclude that indeed all these three plants were cybrids, derived from fusion events. We also assume that donor and recipient mtDNAs interacted and provided novel restriction-patterns. Novel mtDNA restriction profiles in cybrid plants were previously described by us and by others for several plant genera (e.g. *Nicotiana, Petunia, Brassica, Citrus)* and attributed to mtDNA recombination.

Detailed analysis of cybrid *Nicotiana* plants indicated that novel mtDNA restriction patterns may be observed in either fertile or sterile cybrids. Only in rare cases was the exact pattern of donor mtDNA found in cybrids. In the latter cases the cybrids were male sterile. Most cybrids exhibited various rates of apparent mtDNA recombination and among the latter cybrids only some were male sterile. We assume that CMS occurs only when the interaction of the product of a given mitochondrial gene(s) with the product of a certain nuclear gene is faulty. Therefore unless this given mitochondrial gene(s) is transferred from the donor to the recipient, CMS will not be expressed. As for the present results with the *Solanum*, we have good indications that at least some of the fusion derived plants are indeed cybrids containing part of the donor's mtDNA. We have yet to receive the results from field studies in Peru to firmly establish that the transfer of part of the donor mtDNA resulted in functional male sterility under optimal conditions for flowering and to ascertain that in such cybrid plants the female ferility was not impaired. We hope to obtain the respective information in the near future.

To complete this study mtDNA analysis should be performed on other presumptive male sterile cybrids. To facilitate regeneration in fusion-derived calli we developed an O-coumarin treatment (Table 4). We also studied the ctDNA and mtDNA restriction profiles of prospective fusion partners (Tables 5 and 6).

Recently we applied the "donor-recipient" methodology to cv. "Desiree" as recipient and several wild *Solanum* species as donors (Table 7). The number of calli obtained from each fusion experiment varied between 4 and

58. In some experiments we have already mature plants while in others the calli just started to regenerate. Analysis of these experiments may shed more light on the mechanism of CMS. We also may discover a better source for CMS among the wild *Solanum* species. Two AID research grants, C7-189 (to D.A.) and C7-191 (to E.G.) to be implemented in the near future will enable us to bring the present project to a reasonable conclusion.

PUBLICATIONS AND ORAL PRESENTATIONS OF THE RESULTS OBTAINED IN THIS PROJECT

1. Accepted Journal Publications.

Aviv D, Chen R, Galun E (1985) Does pretreatment by rhodamine 6-G affect the mitochondrial composition of fusion-derived *Nicotiana* cybrids? Plant Cell Reports 5: 227-230.

Aviv D, Galun E (1986) Restoration of male fertile *Nicotiana* by fusion of protoplasts derived from two different cytoplasmic male-sterile plants. Plant Mol. Biol. 7: 411-417.

2. Oral presentations at International Scientific meetings.

Aviv D and Galun, E (1985) Restoration of male fertile *Nicotiana* by somatic fusion of two different cytoplasmic male sterile cybrids. 1st Intern. Congress of Plant Mol. Biology, Savannah, Georgia (November, 1985).

Galun E, Aviv D (1986). Objectives, strategies and experimental results in unidirectional plasmone transfer by donor-recipient protopalst fusion. The Netherland-Israel Binational Symposium on Non Conventional Approaches to Genetic Manipulation in Plnats (February 1986).

Galun E, Aviv D, Breiman A, Fromm H, Perl A, Vardi A (1987) Cybrids in *Nicotiana, Solanum* and *Citrus:* isolation and characterization of plastome mutants, prefusion treatments, selection and analysis of cybrids. NATO Adv. Study Inst. Copenhagen, June 1987.

Galun E, Aviv D (1987) Fundamental and applies aspects of plant-organelle transfer by protoplast manipulaiton. Intern. Congress of Plant Tissue Culture - Tropical Species. Bogota (Colombia) September 1987.

Perl A, Aviv D, Galun E (1987) Organelle transfer by protoplast fusion: application for potato improvement. Intern. Congress of Plant Tissue Culture - Tropical Species. Bogota (Colombia) September 1987.

Galun E, Perl A, Aviv D (1987) Protoplast-fusion mediated transfer of male-sterlity and other plasmone-controlled traits. Ciba Foundation Symposium No. 137, Kyoto (Japan), October 1987.

TABLE 1. ANALYSIS OF POTATO PLANTS DERIVED FROM THE FUSION: γ-IRRADIATED (10 Kr) Y245.7 PROTOPLASTS (CMS, DONOR) WITH PRE-FUSION TREATED (IA OR R6G) PROTOPLASTS OF THE CULTIVAR ATZIMBA (FERTILE, RECIPIENT)

CALLUS SOURCE OR DESIGNATION	FLOWER PIGMENTATION	NUMBER OF ANALYSED PLANTS		MALFORMATION IN POLLEN OR ANTHERS
		WITH STERILE POLLEN	WITH FERTILE POLLEN	
Y245.7 (Donor)	VIOLET/WHITE	(ALL PLANTS)	NONE	[3]TETRADES
"ATZIMBA" (Recipient)	WHITE	NONE	(ALL PLANTS)	
[1]I-B	WHITE	3	0	
I-C	WHITE	0	4	
I-E	WHITE	1	2	
I-F	WHITE	2	3	
I-G	VIOLET/WHITE	1	1	TETRADES
I-H	VIOLET/WHITE	5	0	TETRADES
I-P	WHITE	3	0	
I-S	WHITE	0	3	
I-X	WHITE	2	2	
I-8	WHITE	0	4	
[2]II-A	WHITE	1	3	
II-B	WHITE	1	1	
II-C	WHITE	2	0	SHRUNKEN POLLEN
II-D	WHITE	2	3	
II-E	WHITE	0	1	
II-F	WHITE	3	0	PETIOLATED ANTHERS
II-G	WHITE	1	4	
II-I	WHITE	0	2	
II-L	WHITE	0	2	
II-M	WHITE	1	3	SHRUNKEN POLLEN
II-N	WHITE	0	2	

[1]I-Prefusion treatment with R6G; [2]II-Prefusion treatment with IA. [3]-Pollen development arrested.

TABLE 2. ANALYSIS OF POTATO PLANTS DERIVED FROM THE FUSION OF Y245.7 (CMS) PROTOPLASTS WITH *S. TUBEROSUM* CV. ATLANTIC PROTOPLASTS

PLANT DESIGNATION	FLOWER PIGMENTATION	NUMBER OF ANALYSED PLANTS	
		WITH STERILE POLLEN	WITH FERTILE POLLEN
Y245.7 (DONOR)	VIOLET/WHITE	ALL PLANTS	—
"ATLANTIC" (RECIPIENT)	VIOLET	—	ALL PLANTS
I-A*	VIOLET	5	0
I-B		NOT TESTED	NOT TESTED

* Plants exhibiting similarity to Atlantic in respect to leaf and tuber morphology, in addition to similarity in flower pigmentation.

TABLE 3. CHROMOSOME ANALYSIS AND FERTILITY/STERILITY OF FUSION DERIVED PLANTS

PLANT DESIGNATION	CHROMOSOME NUMBER		MALE - FERTILITY/ STERILITY
	AT MIOSIS (n)	AT MITOSIS (2n)	
"ATZIMBA"	24	48	FERTILE
I-8-9	24		FERTILE
I-8-13	24		FERTILE
II-A-6	24		FERTILE
II-B-4	24		FERTILE
II-B-6	24		-
II-C-1		48	STERILE
II-C-2		48	STERILE
II-F-1		48	STERILE
II-F-3		48	STERILE
II-F-4	24		FERTILE
II-F-6		48	-
II-F-11		48	-
II-G-10	24		FERTILE
II-G-11	24		FERTILE
II-L-3	24		FERTILE
II-M-1	36		FERTILE
II-M-2	24		FERTILE
II-M-3		48	STERILE
II-M-4	24		FERTILE
II-N-3	24		FERTILE
II-N-4	24		FERTILE

TABLE 4. PROMOTION OF SHOOT REGENERATION FROM PROTOPLAST FUSION DERIVED CALLI BY O-COUMARIC ACID.
CALLI DERIVED FROM THE FUSION OF *SOLANUM ETUBEROSUM* OR *S. BERTHAULTII* (DONORS) PROTOPLASTS WITH *S. TUBEROSUM* CV. DESIREE (RECIPIENT) PROTOPLASTS. SHOOT REGENERATION WAS SCORED 21 DAYS AFTER TRANSFER TO REGENERATION MEDIUM.

O-COUMARIC ACID(μg/ml)	NUMBER OF CALLI		MEAN NUMBER OF SHOOTS PER CALUS
	WITH SHOOTS	WITHOUT SHOOTS	
0	0	26	0
1.5	0	22	0
2	12	14	5
3	8	19	3
4	20	9	10
5	4	27	2
20	2	32	1
25	0	34	0

TABLE 5. CT DNA AND MT DNA RESTRICTION PROFILES, FROM POTATO CULTIVARS AND WILD *SALANUM* SPECIES OBTAINED AFTER DIGESTION WITH VARIOUS RESTRICTION ENDONUCLEASES.

CULTIVAR OR SPECIES	RESTRICTION ENDONUCLEASES USED	
	FOR CT DNA	FOR MT DNA
S. tuberosum cv.Mirka	BI	Sa,BII,PI,PII
S. tuberosum cv.Atzimba	BI,II,Ba	Sa,BII,PI,PII,C, EV,SI,SII
S. tuberosum cv.Desiree	BI,Ba,EI,Xh	SI,Ba,EV,BII PI,PII,C,EV
S. tuberosum cv.Atlantic	Ba,PI,EI,II	BII,PI,PII,C,EV,SI, SII
S. tuberosum cv.Kufri-jyoti	Ba	Sa,Ba,BII,PII C,EV,SII.
S. tuberosum cv. Serrana	Ba,PI,Ps,II EI,xb	Sa,Ba,BII,PI,PII EV,SI,SII
S. tuberosum cv. 245.7	EI,Ba,II,Xh	Sa
S. tuberosum cv.LT-1		Sa
S. tuberosum cv.DTO-28		Sa
S. tuberosum cv.Ticausi		BI,PI,PII,C,EV
S. brevidens		PI,PII,C,EV,SI,SII
S. chacoense		PII,C,EV,SI,SII
S. berthaultii		C,EV,SI,SII
S. commersonii		EV
S. venturii		
S. nignum	*BI*	

BamHI - Ba; BglI - BI; BglII - BI; ClaI - C; EcoRI - EI; EcoRV - EV; HindIII - II; Pst - Ps; PvuI - PI; PvuII - PII; SacI - SI; SacII - SII; SalI - Sa; XbaI - Xb; XhoI - Xh.

TABLE 6. RESTRICTION ENDONUCLEASES WHICH PROVIDED RESTRICTION PROFILE DIFERENCES BETWEEN POTENTIAL DONOR AND RECIPIENT, USED IN FUSION EXPERIMEMNTS

POTENIAL RECIPIENTS IN FUSION EXPERIMENTS	POTENTIAL DONORS IN FUSION EXPERIMENTS				
	S.tuberosum Y245.7	S.tuberosum cv Serrana	Solanum brevidens	Solanum chacoense	Solanum commersonii
S. tuberosum cv Desiree	EI^{ct},Ba^{ct}, BII^{mt},PI^{mt}, C^{mt},SI^{mt}, SII^{mt}	BII^{mt},PII^{mt}, C^{mt},	BII^{mt},PII^{mt},	PII^{mt},PI^{mt}, C^{mt},SI^{mt}, SII^{mt}	C^{mt},SI^{mt}
S. tuberosum cv Atlantic	EI^{ct},$Ba^{ct,mt}$, Sa^{mt},BII^{mt}, PI^{mt},C^{mt} EV^{mt}	EI^{ct},Xh^{ct}, $Ba^{ct,mt}$ BII^{mt},C^{mt}	BII^{mt},PII^{mt}	PII^{mt},C^{mt}, SII^{mt}	C^{mt},SII^{mt}
S. tuberosum cv. Atzimba	Sa^{mt},BII^{mt} PII^{mt}		BII^{mt},PII^{mt}		

ct -differences in restriction profiles of ct DNA.
mt - differences in restriction profiles of mt DNA.
BamIII - Ba; BglI - BI; BglII - BII; ClaI - C; EcoRI - EI; EcoRV - EV; HindIII - H; Pst - Ps; PvuI - PI; PvuII - PII; SacI - SI; SacII - SII; SalI - Sa; XbaI - Xb; XhoI - Xh.

TABLE 7. DONOR-RECIPIENT FUSION EXPERIMENTS
(UPDATE-JULY 1987)

FUSION PARTNERS		AIM OF FUSION	NUMBER OF CALLI OBTAINED	STAGE OF EXPERIMENT
DONOR	RECIPIENT			
Y245.7 (CMS	*S.tuberosum* cv.Atzimba	Transfer of CMS	21	Flowering cybrids
Y245.7 (CMS)	*S.tuberosum* cv.Atlantic	Transfer of CMS	2	Flowering cybrids
Y245.7 (CMS)	*S.tuberosum* cv.Desiree	Nucl/cyto.compatibility	(many)	Calli in liquid medium
S.demissum	*S.tuberosum* cv.Desiree	Nucl/cyto compatibility	55	Mature cybrids plants
S. brevidens	*S.tuberosum* cv.Desiree	Nucl/cyto compatibility	6	Regenerated cybrids.
S.chacoense	*S.tuberosum* cv.Desiree	Nucl/cyto compatibility	10	Regenerated cybrids
S.commersonii	*S.tuberosum* cv.Desiree	Nucl/cyto compatibility	7	Regenerating cybrids
S.etuberosum	*S.tuberosum* cv.Desiree	Nucl/cyto compatibility	58	Regenerated cybrids
S.berthaultii	*S.tuberosum* cv.Desiree	Nucl/cyto compatibility	24	Regenerated cybrids
S. nigrum	*S.tuberosum* cv.Desiree	Nucl/cyto compatibility	(many)	Calli in liquid medium.

Figure 1. Pollen arrested at the tetrade stage, typical for the pollen of the CMS Y245.7 .

Figure 2. Normal (fertile) germinating pollen of "Atzimba".

Figure 3. Normal floral members of "Atzimba", see anthers with open pollen sacs.

Figure 4. Petiolated anther of the cybrid II-F-4 (see Tables 1 & 3).

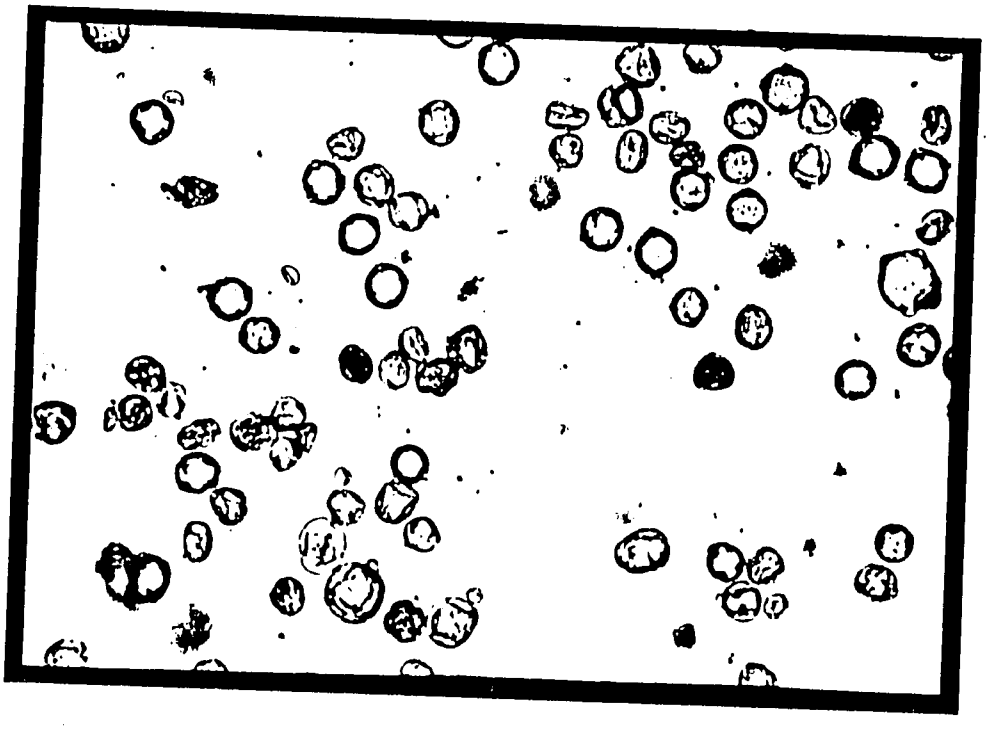

Figure 5. Shrunken pollen, which does not germinate, of the cybrid II-F-4 (see Table 1 & 3).

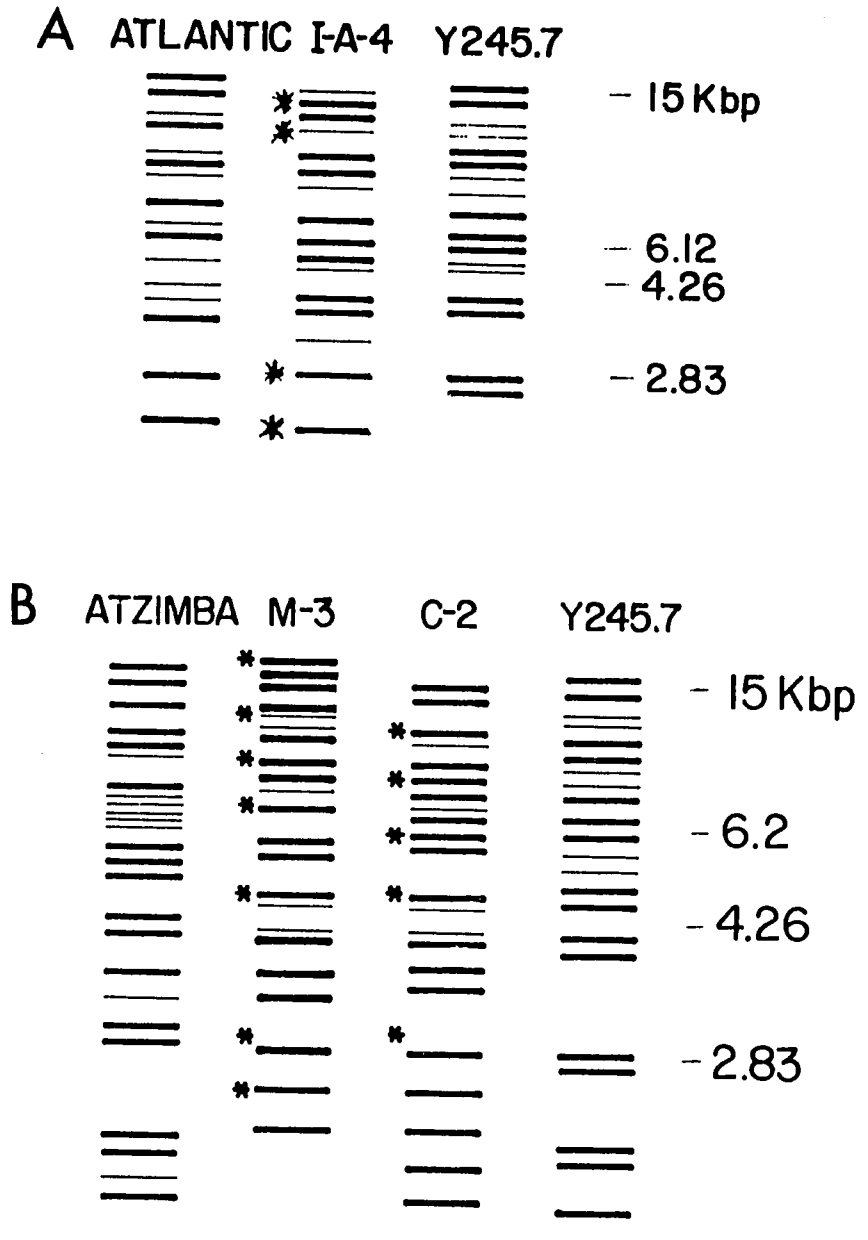

Figure 6. Schemes of mtDNA restriction profiles of fusion partners and their respective cybrids; Sal I digests. Asterisks denote differences between cybrids and their respective recipient parents.
A. Fusion partners: Y245.7 (CMS, donor) with "Atlantic" (recipient);
B. Fusion partners: Y245.7 (CMS, donor) with "Azimba" (recipient).