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INTRODUCTION

As stated in the document "CIAT in the 1980s Revisited", the Biotechnology Research Unit (BRU) will deal primarily with those new technologies that can significantly increase the efficiency of plant improvement methods or help resolve problems that escape solutions through traditional procedures. While emphasis is on applied research, the Unit also assumes responsibility for selected research, with high potential pay-off in terms of technology development. In the development of these activities, close interaction with CIAT commodity programs, especially with the breeders, will be established. On the other hand, the Unit will seek complementarity with advanced institutes and national program scientists through special research projects.

In 1985, the major activity in the Unit involved research in cell and tissue culture for clonal propagation and for the generation of useful variability. This activity included work carried out at CIAT and at collaborating institutions abroad. Work at CIAT continued to develop tissue culture applications with CIAT crops. Research was initiated abroad to tackle potentially useful areas that require higher specialization at this time.

Recovery of Healthy Clones

The Technique

The disease elimination Technique developed previously consists in culturing 0.2-0.3 mm (width) by 0.1-0.2 mm (height) meristem tips comprising the apical dome plus one or two of the youngest leaf primordia , which are excised from apical buds sprouted at 40°C day and 35°C night (thermotherapy) for 3-4 weeks. In cases where no stakes are available, e.g. germplasm introduced to CIAT *in vitro*, thermotherapy is applied *in vitro*. Rates of elimination of a given virus strain depends to a large extent on the size of the explant used and on whether thermotherapy has been applied or not (see Cassava Program Ann. Report, 1982). In general, the following relative order of difficulty has been observed: frog skin disease (less difficult), cassava common mosaic (most cases), cassava caribbean mosaic, latent virus and some strains of common mosaic (more difficult).

Cleaning by this technique needs to be validated through proper virological indexing. Virologists in the Cassava Program have been working in this area. Since 1983, the Program's Virology Section has checked up a number of clones introduced *in vitro* from various countries as well as clones from the field collection maintained at CIAT. Table 1 summarizes the results of such tests with 461 varieties; with the exception of materials from Colombia and the elite clones, all were transferred to CIAT *in vitro* from various countries. Because of lack of facilities at the National Programs, heat treatment was not applied prior to culture in all those cases; in addition, the explants obtained for *in vitro* transfer had to be large enough (0.5-0.8 mm) in order to secure survival of the small sample brought to CIAT (3-4 test tubes/ clone). In the case of Paraguay for example, 9 clones out of 50 tested had at least one plant reacting possitively for a CCMV strain; when these same clones were exposed to thermotherapy *in vitro*, and again tested using

Table 1. Results of virological testing cassava varieties after meristem cloning with and without thermotherapy.

Procedence of material	No. vars. tested	Heat treat.	No. vars. with possitive (+) * reaction				
			Nieves	BPL	WF	FSD	Graft
Brazil	78	no	1	0	-**	-	-
Argentina	16	no	0	-	-	-	-
Costa Rica	102	no	0	-	-	-	0
	13	no	0	-	-	-	-
Peru	64	no	0	-	-	-	-
Panama	18	no	0	-	0	-	-
Guatemala	86	no	0	-	1	-	-
Colombia	11	yes	0	0	0	-	-
Elite clones	23	yes	0	0	0	0	-
Paraguay	50	no	9***	-	-	-	3
	9***	yes	0	-	-	-	-
TOTAL	461		10	0	1	0	3

* (Virus testing carried out by the Cassava Program's Virologists) Nieves and BPL are isolates of CCMV; WF is a latent virus; FSD = frog skin disease; grafting for latent virus.

** Non-tested.

*** The same varieties with and without thermotherapy.

ELISA, the virus was eliminated from all 9 varieties at 90% rate. This procedure is now applied to all newly introduced clones prior to potting and field transplanting.

Routine cleaning

The cleaning technique continued to provide healthy material for: (a) distribution of elite clones to national programs; (b) recovery of pest/disease/damaged clones from the field collection; (c) upgrading important regional varieties for use in trials by the Cassava Program. Table 2 shows the number of clones cleaned from various diseases during 1985; including these clones, a total of 1300 cassava clones infected with viruses, bacteria and fungi and others apparently disease-free clones, have been processed by this technique in the last 5 years.

Table 2. Cassava clones cleaned by thermotherapy and meristem culture in 1985.

Cleaning for	No. Clones
Frog skin disease (CIAT field collection)	167
Super elongation disease (CIAT field collection)	4
Elite clones (preventive cleaning)	39
Regional varieties (preventive cleaning)	7
Total	217

Germplasm Exchange

Distribution from CIAT

In 1985, a total of 200 elite cassava clones were shipped *in vitro* to 10 countries (Table 3). In several instances, the same hybrid or variety was sent to more than one country. In every case, the material was accompanied by a Colombia Phytosanitary Certificate, a CIAT Phytosanitary Statement which describes the procedures, treatments and pathogen testing carried out on the material in preparation for shipment; a list of clones, and a recommended handling procedure.

For an appropriate handling of cultures upon arrival to destination, personnel with minimum training is required, especially for shipments lasting more than two weeks. Feed back information on handling has been received from various countries. Table 4 shows that after nearly 3-week trip to VISCA, Philippines, plants were recoveredfrom all but one of 9 clones in spite that several of them developed medium to severe chlorosis during the trip. Utilization of a micropropagation technique by node cuttings and appropriate potting can be pointed out as two main factors for

Table 3. Distribution of elite clones *in vitro* by country and institutions in 1985.

Country	Institution	No. clones
Argentina	Ministry of Agriculture, Resistencia Chaco	12
Barbados	CARDI, St. Michael	15
Bolivia	IBTA, Cochabamba	7
China	Ac. Sinica, Kwangchow	19
	SCATRC, Hainan Island	22
Ecuador	INIAP, Portoviejo	9
Honduras	Esc. Nac. Agric., Catacamas	4
Mexico	INIA, Zacatepec	19
	INIA, Huimanguillo	6
Panama	IDIAP, Rio Hato	51
Philippines	VISCA, Leyte	6
	UPLB, Los Baños	14
Trinidad	CARDI, St. Augustine	16
	Total	200

Table 4. Results of handling first *in vitro* shipment of cassava elite clones to VISTA, Leyte, Philippines*.

Clones	No. tubes per clone	General arrival condition	No. cultures dead at arrival	No. node cuttings for micropropag.	No. Potted plants
MBra 12	5	Severe chlorosis	1	4	0
CM 681-2	5	Slender, green	0	16	2
CM 982-20	5	Green	0	12	3
MCub 74	4	Med. chlorosis	1	6	5
CM 342-170	5	Green	2	8	5
MVen 156	5	Green	0	10	5
CM 981-8	5	Green	3	10	7
CM 523-7	5	Med. chlorosis	1	7	6
CM 507-37	5	Med. chlorosis	0	13	5

* Information kindly supplied by N. T. Thanh-Tuyen, VISCA.
Date of shipment: Feb. 10, 1984
Date of arrival at VISCA: Feb. 29, 1984

success. In another shipment to the SCATC, Hainan Island, China, out of 114 tubes shipped this year, only 6 arrived with deteriorated or dead cultures. An earlier shipment to South China Institute of Botany, at Guangzhon was effeciently micropropagated, pottedand two clones were pointed out as high yielding in preliminary trials (Fig. 1). Micropropagation by node cuttings was also used at CATIE, Costa Rica to yield 100 plants per each of the 24 clones shipped *in vitro* in 1980. Furthermore, most of the 42 clones shipped *in vitro* to Mexico in the last five years have been distributed (as stakes and/or potted plants) from the recipient INIA Center (CAEZACA, Zacatepec) to two INIA Stations located in Veracruz and Guerrero (Table 5); and in Panama, 18 clones introduced from CIAT via *in vitro* techniques have been multiplied at the IDIAP Station in Rio Hato for use in regional trials.

Introduction to CIAT

Following procedures established earlier (see Tissue Culture, Cassava Program, Ann. Report 1983), 148 cassava clones were introduced *in vitro* to CIAT in 1985 as part of a collaborative effort with the IBPGR. These materials comprised: 28 clones (collected from Junin, Pasco, San Martín, Huanuco and Ucayali) sent by G. Delgado, a former CIAT trainee from Univ. Pedro Ruiz Gallo, Lambayeque, Peru; and 120 clones from Paraguay (collected in 1983 and 1984), including 113 cassava varieties and 7 wild species.

As referred to above, after a short establishment phase, these

Figure 1. Micropropagation, potting and field trial at Guagdong, China of clone CM 321-188 shipped <u>in vitro</u> in 1983. Photos kindly supplied by K. Chun-Yen, South China Institute of Botany, Guangzhon, China.

Table 5. Distribution within Mexico of cassava elite clones shipped *in vitro* from CIAT to CAEZACA, Zacatepec, from 1981 to 1985*.

Clones	Dates of arrival in Mexico**	Recipient INIA Stat. ***	
		CAECOT	CAEIGUA
1. CM 321-188	1	X	X
2. CM 342-55	1	X	X
3. CM 430-37 (x)	1, 5, 6	X	
4. CM 489-1 (x)	1, 2, 5	X	X
5. CM 507-37 (x)	1, 2, 5, 6	X	X
6. CM 523-7 (x)	1, 2, 5, 6	X	X
7. MCol 22	1, 2	X	X
8. MCol 1468	1, 2	X	X
9. MCol 1684	1, 2	X	X
10. MCol 1940	2	X	X
11. MMex 17	1	X	X
12. MMex 59	1, 4	X	
13. MVen 23 (x)	1, 5, 6		
14. CM 91-3	2, 3	X	X
15. MCol 638	2	X	X
16. MCub 51	2	X	X
17. CM 517-1	2	X	X
18. CM 391-2	3	X	X
19. MCol 12	3	X	X
20. MCol 1964	3	X	X
21. MCol 1964	3	X	X
22. MCol 2058	3	X	X
23. MBra 5	3	X	X
24. MPan 97	3	X	
25. CM 981-8	4	X	
26. CM 1305-3	4	X	
27. MCol 72	4	X	
28. MCol 1522	4	X	
29. MCol 2061	4	X	-
30. MBra 12	4	X	-
31. MEcu 72 (x)	4, 5, 6	X	-
32. MPer 245	4	-	-
33. MVen 77	4	-	-
34. MVen 156	4	X	
35. CM 849-1	5	-	-
36. CM 1335-4 (x)	5, 6	X	
37. MCol 1917	5	-	-
38. MCub 74 (x)	5, 6		
39. MVen 25 (x)	5, 6	X	
40. HMC-1 (x)	5, 6	X	
41. MCol 1914 (x)	6		

* Data kindly supplied by Ing.R. Méndez, CAEZACA (INIA), Zacatepec, Morelos.

** Arrival dates of *in vitro* cultures: 1 = Oct.18, 1981; 2 = August 12, 1982; 3 = Dec.7, 1982; 4 = Dec.15, 1983; 5 = Dec.14, 1984; 6 =Jul.11, 1985.

*** INIA Stations receiving stakes and/or potted plants: CAECOT (Cotaxtla, Veracruz), CEIGUA (Iguala, Guerrero).

(X) Material remaining at CAEZACA, Zacatepec.

__ Means: clones lost.

cultures will be subjected to thermotherapy *in vitro*, subcultured twice, and moved to a glasshouse for virological testing.

Germplasm Conservation

Minimum growth storage

This year, the number of cassava clones placed into *in vitro* storage has reached 2,783 clones. As shown in Table 6, nearly 40% are Colombian varieties, 26% are Brazilian, followed by Paraguay, Peru, Costa Rica and Guatemala; the remaining, comprises materials from other 12 Latin American, Caribbean and Southeast Asian countries.

Under current storage conditions. (24-25C° day and 18-20C° night and 12 hr. of 1500 lux illumination), cultures are transferred to fresh medium every 18-24 months. Previous research on the effect of various treatments, e.g. lower temperature, mineral starvation and medium osmotic concentration, demonstrated the feasibility of further extending the culture transfer period. Addition of mannitol, as osmoticum, to the medium has been investigated more throughly. Earlier it was found that low mannitol concentration caused growth reduction and was not detrimental to culture viability as long as it was accompanied by sucrose (Tissue Culture, Cassava Program, Ann. Report, 1983). In order to elucidate the question of wheather the effect of mannitol is purely osmotic and independent from the effect of sucrose as a carbohydrate source, work was initiated this year using two varieties and a series of sucrose/mannitol combinations in order to obtain three total molarities, i.e. 0.1, 0.2 and 0.3 M. Table 7 shows preliminary results of this work with MCol 22. Culture growth increases with sucrose only, but viability tends to be higher at the lower sucrose concentrations (Treats. 1 and 2) than the control (No. 3) which has a total molarity of 0.1. If this molarity is maintained by adding mannitol to the medium a significant reduction in culture growth rate, and high viability in terms of both leaf retention and propagation potential (No. tips per culture), is achieved (Treat. No. 5). This treatment contains the same sucrose level as No. 2 plus mannitol. Increasing the total molarity of the medium with sucrose and/or mannitol resulted in much reduction in growth rate and very low viability.Thus, appropriate storage conditions are provided by reduced carbohydrate availability, about 1/3 of the standard medium concentration. When mannitol is added to this medium, the total molarity should not exceed 0.1 M in order to achieve similar

Table 6. No. of cassava clones by source country maintained in the *in vitro* gene bank as of December 1985.

Source country	CIAT code	No. clones
Argentina	M. Arg.	16
Bolivia	M. Bol	1
Brazil	M. Bra	730
Colombia	M. Col	1101
Costa Rica	M. Cr	135
Cuba	M. Cub	23
Dominican Republic	M. Dom	2
Ecuador	M. Ecu	18
Guatemala	M. Gua	92
Malaysia	M. Mal	3
Mexico	M. Mex	35
Panama	M. Pan	29
Paraguay	M. Par	215
Peru	M. Per	191
Philippines	M. Fil	2
Puerto Rico	M. Ptr	3
Thailand	M. Tai	1
Venezuela	M. Ven	50
Hybrids (CIAT)		136
	TOTAL	2,783

Table 7. Effect of sucrose and mannitol concentrations on growth and viability of stem-tip cultures maintained at 24-25°C day and 18-20°C night, 12 hours of 1500 lux illumination.

	Treatment			Growth*	Viability*	
No.	Sucrose (M)	Mannitol (M)	Total (M)	cm per month	% leaf retention	No. tips per culture
1	0.01	0	0.01	0.4	78	1.5
2	0.03	0	0.03	0.6	88	2.3
3**	0.10	0	0.10	1.3	58	2.0
4	0.01	0.09	0.10	0.6	42	1.0
5	0.03	0.07	0.10	0.5	59	2.5
6	0.20	0	0.20	0.4	26	1.3
7	0.01	0.19	0.20	0.2	41	1.8
8	0.03	0.17	0.20	0.2	0	1.2
9	0.30	0	0.30	0.3	10	1.3
10	0.01	0.29	0.30	0.1	0	0.8

* Average of 4 replications/treatment.

** Represents standard medium for cassava micropropagation (control).

results. The fact that this result can not be obtained solely by increasing sucrose concentration, suggests that in addition to low sucrose levels, a reduction in water and nutrient uptake by the cultures is necessary in order to maintain slow growth and fairly high viability. Mannitol seems to exert this effect when used at low concentrations.

Cryogenic storage

This research has been conducted during the last three years at the Biotechnology Institute, Saskatoon, Canada with the support of IBPGR and CIAT's collaboration.The project aimed at developing methods for preserving cassava shoot-tip cultures in liquid nitrogen (LN). This project finished this year; the following conclusions can be drawn: a) the main achievement was the recovery of cassava plants from shoot-tips stored in liquid nitrogen; b) the procedure which resulted in this achievement consisted in placing the tips in microdroplets (0.2 ul) of a freezing medium containing 1 M Sorbitol, 9% sucrose, and 5% dimethyl sulfoxide (DMSO) in the Murashige and Skoog liquid medium, without growth hormones. Prior to

cooling, the meristems were allowed to achieve equilibrium in: 9% sucrose for 18 hrs., 0.4M Sorbitol and 9% sucrose for 6 hrs. and 1 M Sorbitol and 9% sucrose for 18 hrs. using the same liquid medium. The best cooling rate was 0.5 C/min to - 30°C prior to immersion in liquid nitrogen. After 1-3 hrs in LN, rapid thawing and washing in the liquid medium was carried out at 37°C. Regrowth of meristems was obtained by transferring them to cassava plant regeneration medium; c) this procedure was carried out with 12 cassava genotypes, with variable success levels; although tissue survival following retrieval from LN was high (up to 60%), only 2 to 20% plant regeneration was achieved with these genotypes. A large part of the surviving tissues grew into callus without shoot formation; d) variability in response was attributed to the physiological condition of donnor plants and the range of cell types comprising each shoot-tip.

This work provides the basis for further research into cryopreservation of cassava. Among the factors which should be looked at, preconditioning of plants and tissues seems important. Since it isnow possible to regenerate cassava plants by somatic embryogenesis (see below, this Report), these embryos should provide an appropriate explant for freezing; in theory such embryos ought to be physiologically more adapted for standing cooling and freezing than shoot-tips.

Material of 4 varieties retrieved from LN storage have been multiplied at CIAT and planted in the field for evaluation of phenotype stability. Results of this trial will be available next year.

Evaluation of Clones Retrieved from Minimum Growth Storage

Evaluations of the first cycle plants (C_1 plants) showed no changes in morphology and biochemistry in 5 varieties which could be attributed to *in vitro* storage. Increase in yield and changes in morphology of two varieties was attributed to the elimination of frog skin disease by *in vitro* culture (see Tissue Culture, Cassava Program Ann. Report, 1984).

Stakes, obtained from plants of the C_2 generation, and from their non-stored meristem culture-propagated and traditionally-propagated counterparts, were planted in the field (C_2 plants) for an evaluation. Table 8 shows the results of this evaluation.

Except for total plant height, which does not significantly change with the propagation technique, in all other variables, meristem culture micropropagation technique,

Table 8. Evaluation of cassava plants grown from cultures stored *in vitro* in comparison to meristem-micropropagated and stake-propagated plants*.

Variables	Treatments	M.Mex 20	M.Col 650	M.Col 2197	C M 305-38	C M 323-375
Plant height (m)	Stakes	1.4±0.3	2.1±0.2	1.4±0.3	1.7±0.2	1.7±0.1
	Meristem	2.1±0.2	1.9±0.2	1.3±0.2	1.6±0.2	1.8±0.1
	Storage	2.1±0.4	2.0±0.2	1.3±0.2	1.7±0.2	1.8±0.1
Height, 1st.branch (m)	Stakes	0.7±0.4	1.4±0.5	0.5±0.1	0.6±0.5	0.3±0.2
	Meristem	1.3±0.5	1.5±0.2	0.5±0.1	1.2±0.4	0.7±0.6
	Storage	1.3±0.5	1.7±0.1	0.8±0.4	1.0±0.5	0.7±0.6
Root F.W./ plant (kg)	Stakes	0.3±0.4	8.0±1.4	3.1±1.0	15.0±4.9	12.5±2.1
	Meristem	15.0±3.0	8.6±2.3	3.2±1.5	12.5±4.5	14.7±2.3
	Storage	13.1±5.0	10.0±1.8	2.0±0.7	11.1±2.0	15.5±1.9
No.Stakes/ plant	Stakes	5.1±3.3	6.7±1.8	5.3±2.1	6.0±1.8	5.4±1.5
	Meristem	7.3±2.6	8.1±3.1	5.7±2.2	5.5±1.4	7.6±2.0
	Storage	8.6±4.2	9.0±2.4	5.1±2.1	5.8±1.4	7.7±2.3

* Second year propagation (C_2 plants).
Data represents average and standard deviation of 20 plants in 4 replications.

resulted in increments; plants derived from *in vitro* stored cultures tend to be equal or slightly higher than meristem propagation. This is especially stricking for the var. M. Mex 20, which showed symptoms of frog skin disease in the stake-propagated plants. Thus, the height of first branching, yield of fresh roots and commercial stakes increase as a consequence meristem micropropagation.

Thus, it can be concluded that the phenotypic changes observed in the plants derived from *in vitro* storage are due mainly to the elimination of diseases which occur in the process, and that *in vitro* storage *per se* does not change the phenotype of the five varieties studied.

Cassava Genotype Identification by Electrophoresis

As the world collection increases, it is becoming expensive and time-consuming to identify and catalogue varieties on the basis of classical vegetative criteria, since morphological characters usually vary with variable agroclimatic conditions. Assuming that each cultivar is genetically distinct, it should be possible to use electrophoretic patterns of cassava proteins/enzimes for identifying crop cultivars. On a short term basis, these methods could be applied to sort out duplicates in

the collection, cluster germplasm accessions and helping to identify particular genotypes from clonal mixtures.

With the support of IDRC, a research project was established between the University of Manitoba, Winnipeg, and CIAT. This project has been underway for one year and the progress achieved at the University of Manitoba now follows: Out of 16 enzymes extracted from each of 5 different tissues and runned under 5 different buffer systems, four enzymes: EST (α, β esterase), GOT (glutamate oxaloacetate transaminase), PGI (phosphoglucoisomerase) and ACP (acid phosphatase), were selected on the basis of band quality, relative mobility and number of bands. Out of these EST resulted the best isozymne system. Root tissue grown from stakes produced the most intense and clear band patterns both on starch and polyacrylamide gels, but 12% polyacrylamide gels gave better band resolution (Fig. 2). Basic extraction and trisborate electrode buffer were found the best. It was found that genotypes showing very similar root enzyme patterns could be discriminated or confirmed by extraction from a mixture of young shoot parts (apical bud, leaves with petioles, and stem), (Figs. 2B and C). This technique should be further tested with more cassava varieties. Computarization of the data, after conversion of the analog readings into a digital system, should speed up analysis and data storage.

Computarization of Cassava in vitro

As described before, the number of cassava clones being processed in vitro is steadily increasing. Over 1300 varieties have been cleaned up from various diseases; more than 2,700 clones are currently included in the in vitro gene bank and hundred of clones are exchanged every year with various institutions around the world. A systematic treatment of these operations should greatly speed up the entering and retrieving of data at any time, link and in vitro operation with another or with the main files of the Cassava Program, and would also reduce risks of human errors in any of the number of steps that comprise the in vitro operations.

With the collaboration of the Data Services Unit and the Cassava Program, the preparation of a data base package for cassava in vitro was initiated this year. This package can be linked to be cassava germplasm collection files, passport and evaluation packages (Fig. 3), and comprises the following individual items: (a) disease/pest cleaning up

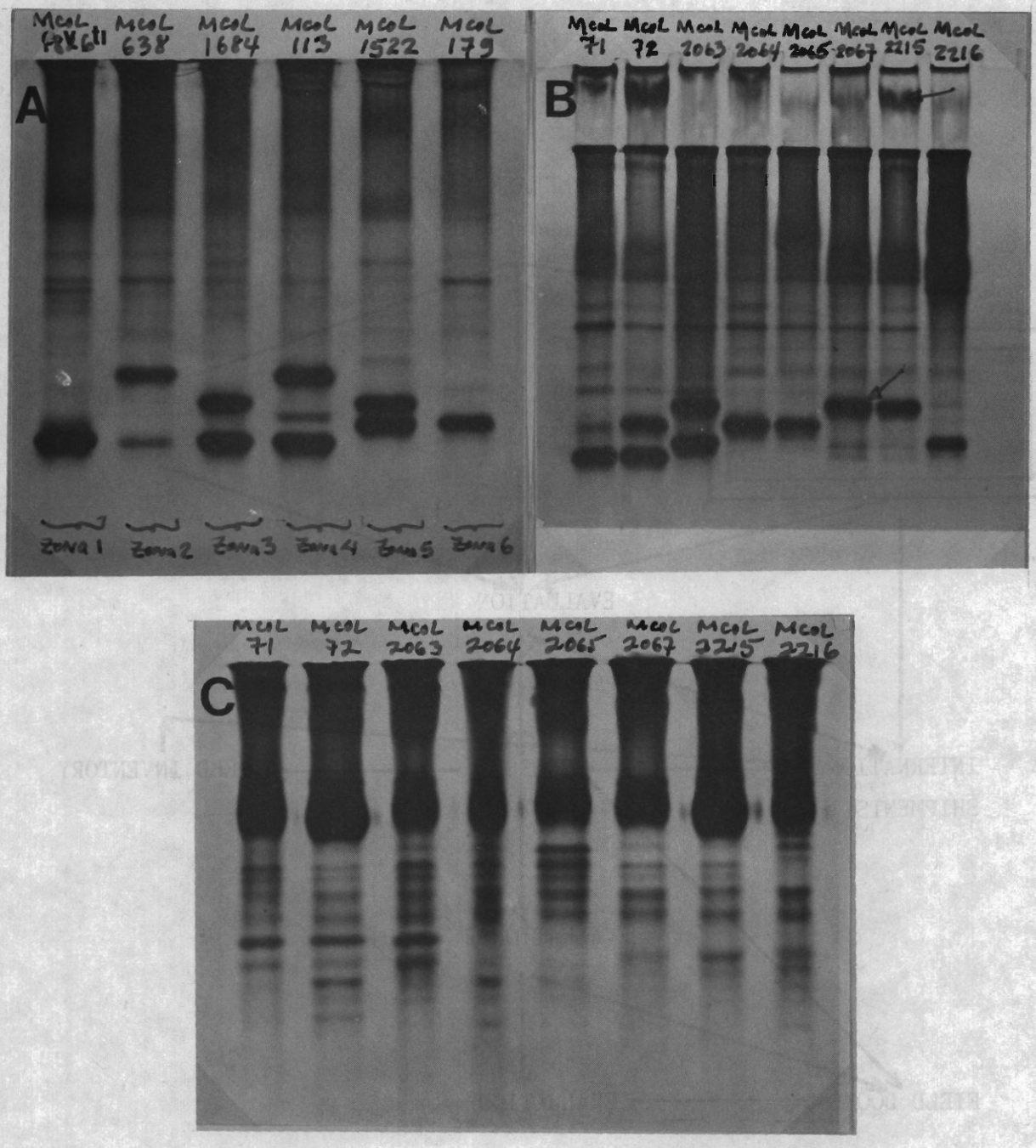

Figure 2. α, β - esterase (extracted from root tissues) electrophoretic patterns of six cassava cultivars in polyacrylamide gels.

A. From left to right, each cultivar represents the Agroclimatic zones 1 to 6, according to Cassava Program's classification. Note the very thick and thin bands characteristic for each genotype.

B. Root tissue banding patterns of 8 cassava cultivars that grew in agroclimatic zone No. 1. Note the stricking similarity of M Col 2067 and 2215.

C. Shoot tissues (apical bud, leaf with petiole and stem) banding patterns of the same 8 cassava cultivars from zone 1, as at B. Note similarities between M. Col 2067 and 2215 still exist but a very small difference in migration of the thickest band can be seen.

(Photos kindly provided by W. Bushuk, A. Hussain and H. Ramirez, Univ. of Manitoba, Winnipeg, Canada).

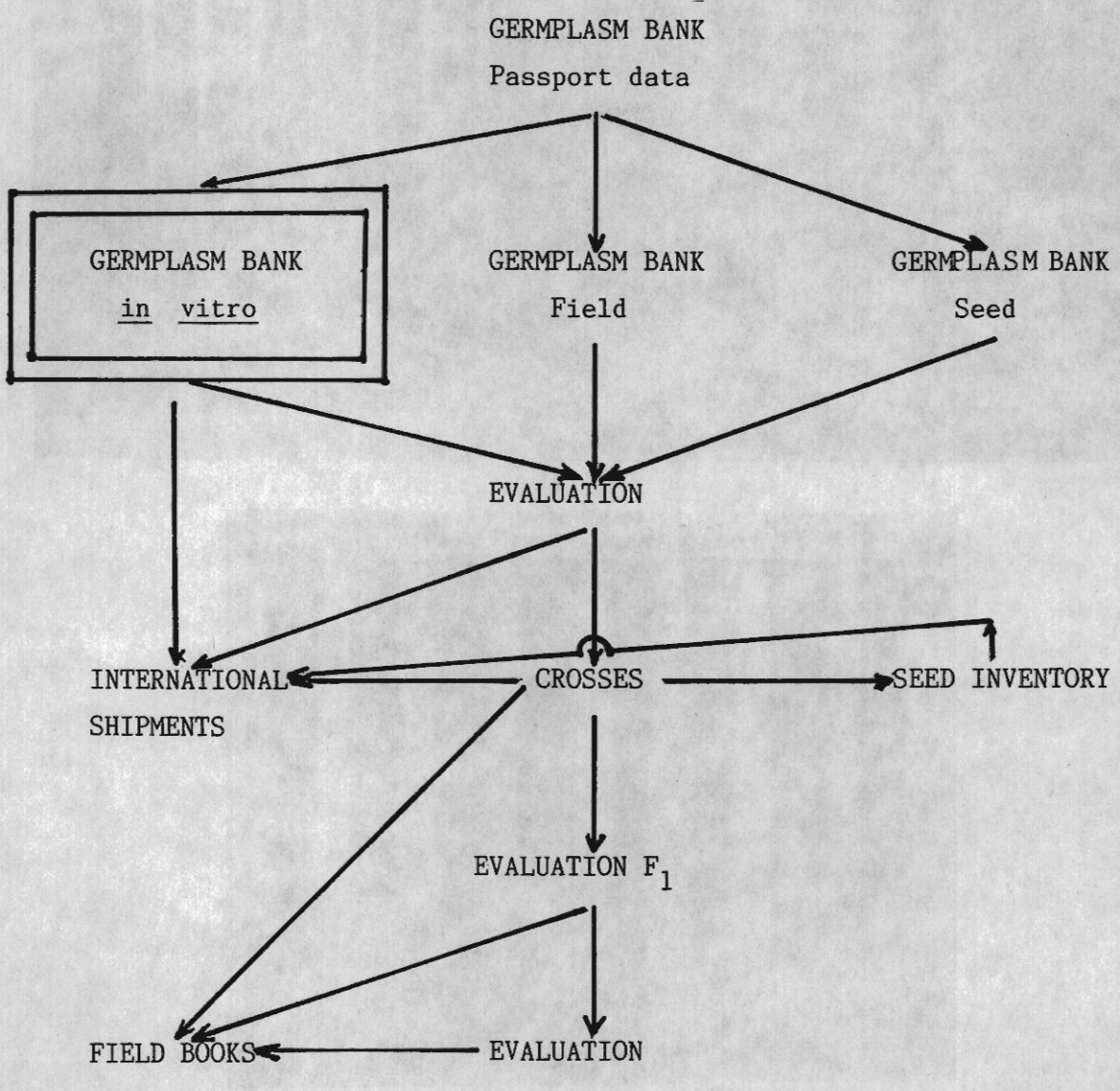

Figure 3. Inter-relations of Cassava in vitro data base with germplasm bank and breeding data of Cassava Program.

operation; (b) *in vitro* gene bank operations, and (c) evaluation of stored material. The germplasm *in vitro* exchange data is already included in the Cassava Program's data base along with germplasm exchange in the form of seed and stakes.

The *in vitro* gene bank package will include: entry number, CIAT code, species, origin and procedence of material, virus testing results, date of introduction to CIAT, culture medium, number of tubes in storage, number of tubes retrieved from storage, date of retrieval, reason for retrieval, number of tubes sub-cultured, observations. These data can provide various types of information for users, e.g. permanent current inventory, comparisons of storage time for varieties, mvoement of entries and retrievals, etc. In addition, the system should allow a constant checking for accuracy.

Tissue Culture Regeneration of Cassava

Tissue culture is the route through which the various forms of *in vitro* genetic manipulation should pass in transition from the laboratory to the field. Therefore, the ability to regenerate cassava plants from non-meristem tissue cultures (e.g. callus, cells and protoplasts) is a necessary first step for the utilization of these techniques.

Regeneration of cassava plants by means of two methodologies are being studied at CIAT: somatic cell embryogenesis and protoplast culture.

Somatic embryogenesis of cassava

As shown previously, somatic embryogenesis was induced when immature leaf segments were cultured on a medium containing high concentration of 2,4-D. The induced somatic embryos "germinated" into plantlets when transferred to a regeneration medium containing a cytokinin and very low levels of 2,4-D. The induction of secondary embryogenesis, when the somatic embryos were sub-cultured in the induction medium, was also reported (see Tissue Culture, Cassava Program, Ann. Report, 1984).

This year, research in this area aimed at: establishing conditions for sustained proliferation of somatic embryos, germination of embryos into plantlets, transfer of plantlets to pots, field evaluation of plants, and response of embryogenetic cultures to stress applied *in vitro*.

Following induction on solid medium, continuous proliferation of secondary embryos occurred when the embryogenic cultures were maintained in the induction medium in darkness (Fig. 4A and B). Upon transfer of these cultures to light, in the

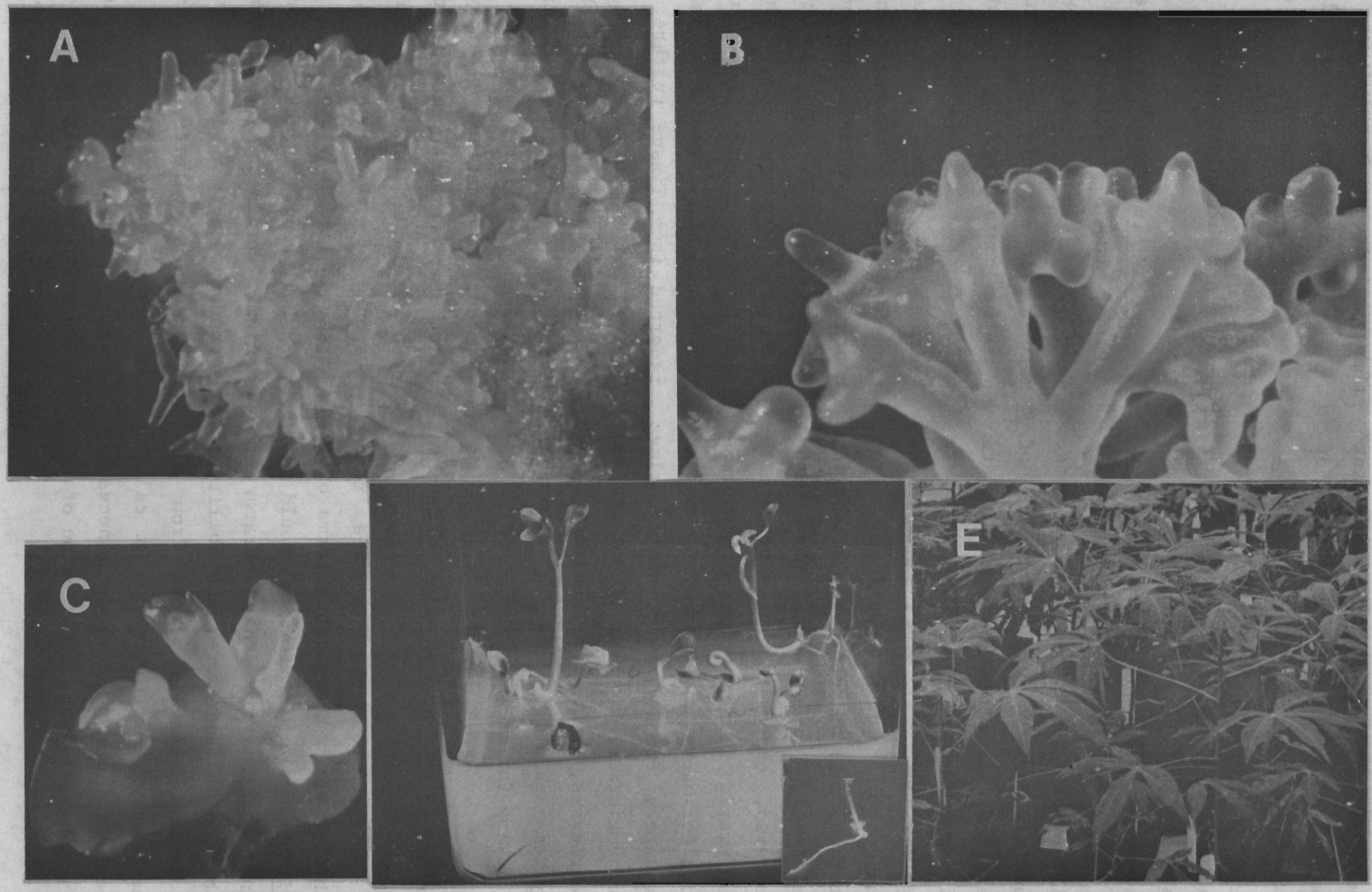

Figure 4

Figure 4. Regeneration of cassava plants by somatic cell embryogenesis.

A. Continuous proliferation of secondary embryos in induction medium in darkness

B. Finger-like secondary embryo initials at tip of primary embryos.

C. Development of cotyledonary embryos upon transfer of cultures, as at A, to light.

D. Germination of somatic embryos on solid, regeneration, medium. Inset: individual plantlets, each grown from a single somatic embryo.

E. Potted, somatic embryo-derived cassava plants ready for field transplanting.

regeneration medium, the embryos stop proliferation and developed structures (e.g. cotyledons) characteristic of incipient germination (Fig. 4C). With the addition of a cytokinin, complete individual plantlets formed (Fig. 4D). These plantlets were potted in the greenhouse which after 1-1.5 months (Fig. 4E), were transplanted to the field.

It was also found that the number of leaf explants forming embryogenic structures increases with the use of very high 2,4-D concentrations (Table 9); and agitation of liquid medium in a shaker helped proliferation by separating small embryogenic structures being formed on the initial explant.

Long-term embryogenic cultures have been established and maintained for over 12 months without loss of the plant regeneration potential. This is significant since genetic manipulations with somatic cells require protracted regenerative capacity during treatment application and selection.

Table 9. Effect of auxin concentration on the induction of somatic embryuogenesis in three cassava varieties (percent of explants forming embryogenic structures).

Cassava varieties	2,4-D concentrations mg/l				
	1	2	4	8	16
M. Col 1505	19	31	46	64	86
M. Ven 25	15	17	40	70	50
CMC-40	14	38	38	38	50

The question of whether induction of somatic embryogenesis, on the one hand and embryo proliferation on the other, can occur under specific selective conditions, e.g. high salt concentration, was also investigated. Although embryogenic callus could be formed on leaf explants of MCol 1505 cultured in the induction medium with up to 1.0% NaCl, embryos differentiated only up to 0.5% NaCl (Table 10). On the other hand, when embryogenic structures of MCol 22 and MCol 1505, induced on standard medium, were transferred to the same liquid medium but stressed for 2 months with NaCl, proliferation of embryos (as measured by fresh weight) occurred up to 1.0% NaCl in MCol 1505 but proliferation continued even at the highest, 2.0% NaCl in MCol 22.

Thus, MCol 22 cultures seem to tolerate higher salt concentration than MCol 1505. More work is needed to establish the threshold concentrations above which salt stress can induce only tolerant cells grow to form somatic embryos and plants.

At this stage it is important to know if regenerated cassava plants through somatic embryogenesis are true

Table 10. Induction of somatic embryogenic structures on leaf explants of M. Col 1505 maintained in NaCl - supplemented induction medium during one month.

Structures induced	Concentration of NaCl (%)						
	0.0	0.05	0.1	0.2	0.5	1.0	2.0
Amount embryogenic callus/explant	++++	++++	++++	++++	+++	++	-
% explants with embryos	30	20	50	15	20	0	0

++++ Callus formed on whole explant.

+++ Callus formed on 3/4 explant.

++ Callus formed on ½ explant.

— Without callus formation.

to type. If not, the extent to which useful variability (intra-clonal) can be recovered would be worth to investigate. Regenerated plants have been moved to the field after 1, 2, 3, 6 and 12 months subculture periods. Since these experiments have been initiated this year, only partial data is available from plants regenerated immediately, i.e. without sub-culturing. The performance of the somatic embryo plants is being compared with micropropagation (using meristem tips), stored cultures in vitro, rapid propagation (two node cuttings) and stakes. Table 11 shows preliminary results obtained after 6 months field evaluation of MCol 1505.

Out of 9 morphological descriptors studied, no differences were found in the color of petioles nor in the form of the central lobe. The largest differences in the remaining 7 descriptors were for the color and pubescence of unopened apical leaves, petiole length, and the length of the central lobe (Table 11). From Table 11 a hierarchy of variability (C.V.) among the various methods of plant reproduction can be established: somatic embryogenesis (highest variability among plants), in vitro conservation, rapid propagation, stake propagation and micro-propagation (lowest variability among plants). These results should be taken with caution at this stage until a final evaluation at harvest is carried out as well as in a second propagation cycle. Agronomic, e.g. harvest index, fresh and dry weights, and biochemical, e.g. isozyme electrophoretic patterns,

Table 11. Phenotypic stability of cassava plants regenerated by somatic embryogenesis, in vitro stored-, stake-, and rapidly propagated-plants (M. Col 1505)*.

Morphological descriptors**	Somatic embryog.		In vitro conservation		Micro-propagation		Rapid propagation		Stakes	
	X	CV%	X	CV%	X	CV%	X	CV%	X	CV%
A. Color stem	1.2	24	1.0	0	1.0	0	1.0	0	1.0	0
B. Color mature leaves	4.6	15	4.7	12	5.0	0	5.0	0	5.0	0
C. Color unopen apical leaves	4.8	42	3.6	31	3.0	0	3.0	0	3.3	17
D. Pubescence young leaves	3.3	29	5.0	5	5.0	0	4.9	5	5.0	0
E. Length petiole	4.7	22	5.5	12	6.9	7.0	6.7	10	7.0	5
F. No. lobes mature leaves	6.6	8	7.2	4	7.0	0	6.9	4	7.0	0
G. Length central lobe	13.9	7	16.1	8	16.7	8	15.6	12	17.9	7

* Average obtained of 27 plants in 3 replications.

** A: silver green, 2. light grey, 3. dark grey; B and C: 3. light green, 5. dark green, 7. purple green, 9. purple; D: 0. abscent, 3. little, 5. moderate, 7. hight; E: 3. short, 5. med., 7. long; F: number; G: length (cm).

analyses will complement these data. What occurs with protracted subculturing of embryogenic structures in terms of stability is still to be determined. A highly stable system would be useful as a mean for mass vegetative propagation; perhaps the possibility of embryo encapsulation procedures can be studied later.

Cassava protoplast culture

Protoplasts have been isolated from leaf mesophyll cells and shoot-tips of test tube plantlets, and from somatic embryos. Isolated protoplasts could resynthetize cell wall and divide with frequencies up to 35% (see Tissue Culture, Cassava Program, 1984). Rates of protoplast division frequency was variable for the various types of explants and genotypes used; these results and the formation of cell colonies which ensued following protoplast division are shown in Table 12. Highest divisions and callus/colony formation occurred with leaf explants followed by shoot-tips. Somatic embryos and induced leaf lobes yielded small, highly dense, protoplasts which divided with great difficulty.

A series of experiments were carried out attempting to induce morphogenesis in the protoplast-derived cell colonies of 12 cassava genotypes. Two media of different mineral salt composition (MS and B5) were supplemented with a range of combinations of the following additives: casein hidrylyzate (100 and 200 mg/l), glutamine (30 m M and 600 mg/l), proline (50 mM), coconut milk (4 and 5%), sucrose (0.1, 0.3, 1, 2, 3, and 5%), charcoal (0.5%), indol acetic acid (0.1 and 1 mg/l), α-naphthaleneacetic acid (0.02, 0.1, 0.3, 1 and 2 mg/l), 2,4-dichlorophenoxyacetic acid (0.01, 0.1, 1, 4, 8 and 12 mg/l), kinetin (0.5, 3 and 5 mg/l), zeatin (0.5, 1 and 2 mg/l), benzy- laminopurine (0.04, 0.1, 0.5, 1, 2, 5 and 20 mg/l), gibberellic acid (0.05, 0.1, 0.2 2.4 and 10 mg/l), and abscisic acid (0.1 and 0.3 mg/l). No morphogenesis has been observed with these treatments to this date.

Protoplasts have been isolated from shoot tips and immature leaf lobes. As shown above, tissues of these organs have the capacity to form somatic embryos. It is reasonably to expect the expression of the embryogenic capacity of protoplast-derived colonies originated from these tissues.

Isolated protoplasts from these organs were usually smaller and more compact when compared to leaf mesophyll protoplasts; sometimes they contained a few chloroplasts, more frequently they were colorless. When fractionated in Ficoll step gradients (0-20% Ficoll), 1000 rpm for 10 min.

Table 12. Protoplast division frequency and callus/colony formation from various explant types in ten cassava genotypes.

Genotype	Explant type	Division frequency	Callus/ colonies
MCol 22	- leaf	up to 38%	callus
	- shoot-tip	2-4%	callus
	- induced leaf lobes*	-	
MCol 1505	- leaf	3-6%	callus
	- shoot-tip, leaf lobes	1-3%	colonies
	- induced leaf lobes	few	few colonies
	- somatic embryos	-	
	- cotyledon	1-2%	few colonies
MCol 1940	- leaf		callus
	- leaf lobes	several	colonies
	- somatic embryos	-	
MMal 1	- leaf	3-5%	callus
	- induced leaf lobes	several	colonies
MCol 1438	- leaf	5-12%	callus
	- shoot-tips	1-2%	callus
MPer 302	- leaf	10-18%	callus
	- induced leaf lobes	5-7%	callus
MCol 2017	- leaf	up to 30%	callus
CMC 40	- leaf	3-8%	callus
	- leaf lobes	-	
	- induced leaf lobes	few	few colonies
MBra 739	- leaf	up to 30%	callus
M. cecropiaefolia	- leaf	3-5%	colonies

* Induction of embryogenic structures with 2,4-D.

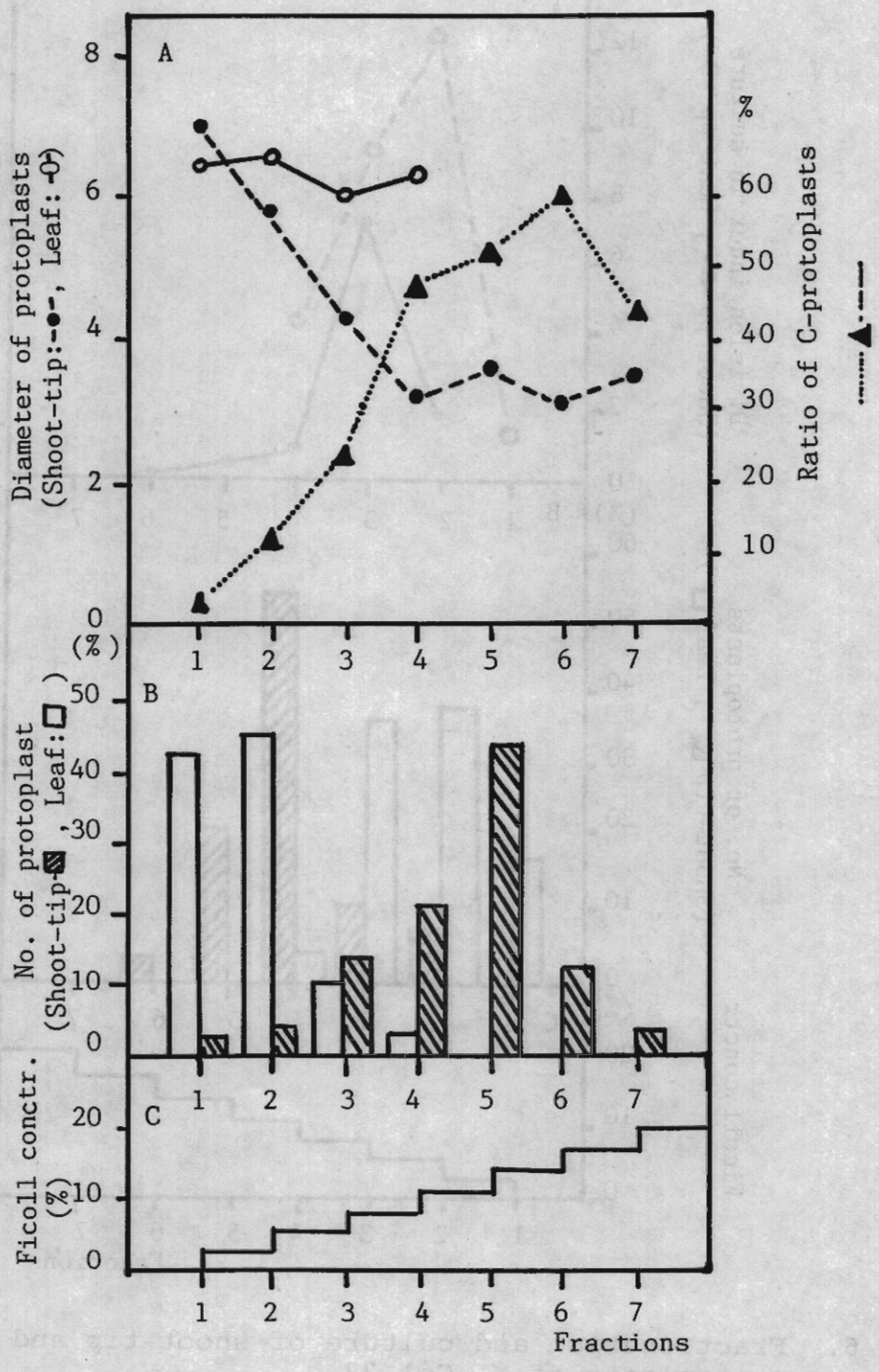

Figure 5. Fractionation of shoot-tip and leaf-derived protoplasts of M. Col 1940 in Ficoll gradients.

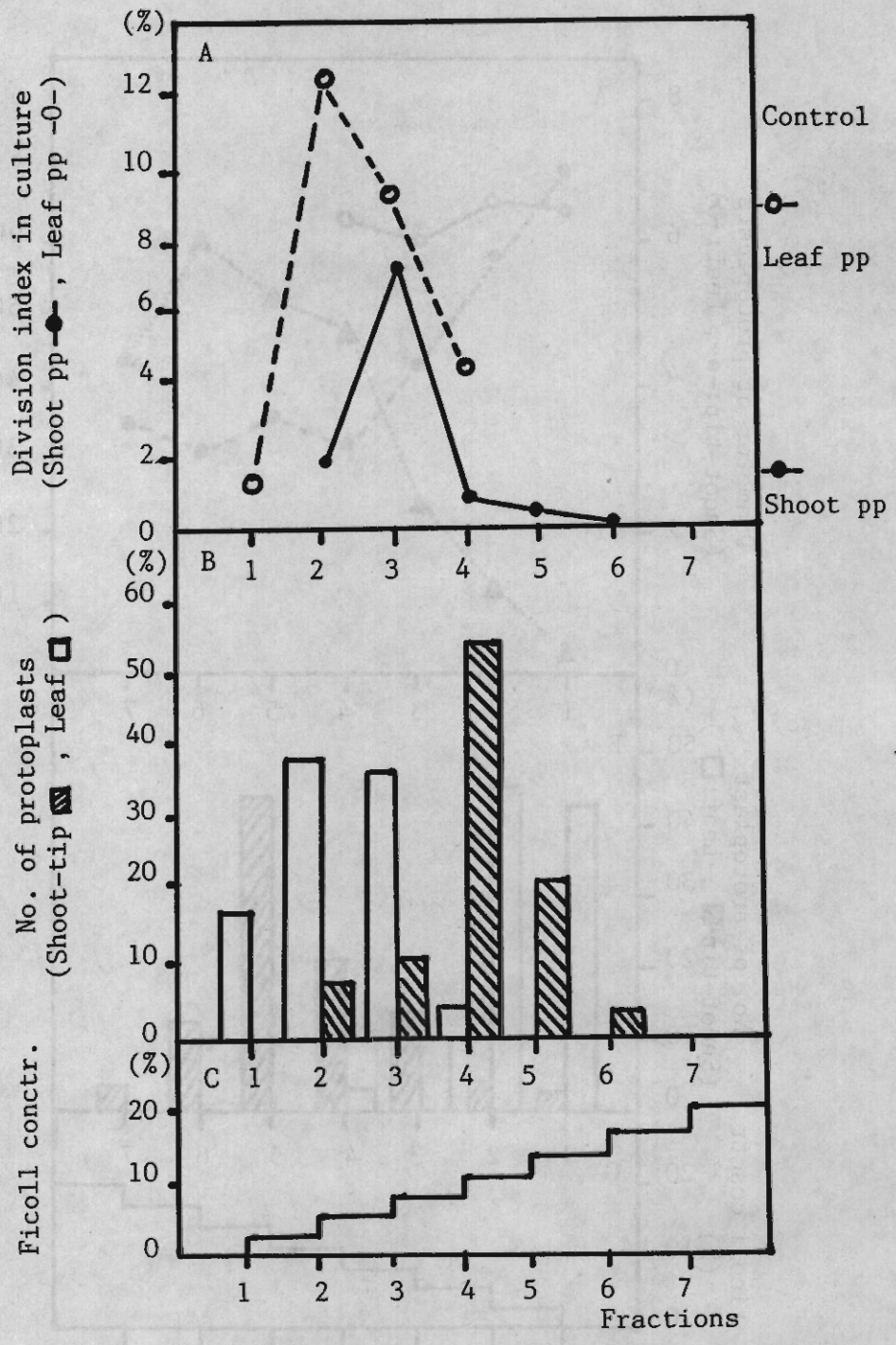

Figure 6. Fractionation and culture of shoot-tip and leaf-derived protoplasts of M. Col 22.

centrifugation, the majority of the shoot-tip protoplasts were recollected at 10-15% Ficoll concentrations (Figs. 5B, C and 6B, C). Leaf mesophyll protoplasts formed sharp bands at 2.5-7.5% Ficoll. This difference is due to the higher density and smaller diameter of the shoot-tip protoplasts (Fig. 5A). There was a compact, cytoplasm rich, type of protoplastd in the more dense fractions (C-protoplasts). The C-protoplasts had no chloroplasts most probably they were released from meristem cells and comprised 50-60% of the protoplast population (Fig. 5A) in the dense fractions. Isolated shoot-tip protoplasts resynthetized cell walls and divided when cultured. There were marked differences in the division capacity of each protoplast fractions; leaf protoplasts divided at higher frequencies than shoot-tip protoplasts (Fig. 6A). Dividing protoplasts formed colonies and callus.

Experiments are in progress to (a) attempt induction of morphogenesis in shoot-tip derived protoplastas, and (b) to screen a wide range of cassava genotypes for their for their morphogenetic capacities.

BEANS

Plant Regeneration in Tissue Culture

The development of a tissue culture cycle in Phaseolus vulgaris is the first essential step for applying most biotechnological tools in the species. A tissue culture cycle involves the establishment of a more or less dedifferentiated cell or tissue culture under defined culture conditions, proliferation for a number of cell generations and the subsequent regeneration of plants. In other words, a period of essentially dedifferentiation cell proliferation is impossed between an explant (initial plant part) and the next plant generation. While induction of dedifferentiation can be done readily, beans have resisted the regeneration phase. Since regeneration seems to depend primarily on the genotype, the kind of tissue used as explant, and the response being modulated by the culture environment, a systematic approach to this problem in beans, is appropriate.

Results of work initiated this year at CIAT are now reported. Using embryo axes from mature seeds of four genotypes, sterile plantlet cultures were established in a medium routinely used for cassava meristem culture. Shoot tips and nodes (0.5 cm in size) were excised from actively growing plantlets and used as explants in four culture media (Table 13), under low illumination (1000 lux) during 4 weeks. Both type of explants reacted in a similar fashion; a mass of nodular callus grew around and underneath the explant (Fig. 7Aa), leafy tissue developed at some points of the nodular mass where differentiation of buds occurred (Fig. 7Ab). The amount of budding in media 3 and 4 was twice as much as in 1 and 2, while the amounts of leafy tissue and nodular mass were about the same in all media, and all genotypes responded in a similar way, being G07060 somewhat better (Table 13). The nodules could be isolated and sub-cultured in the same media. In medium 1, and to some

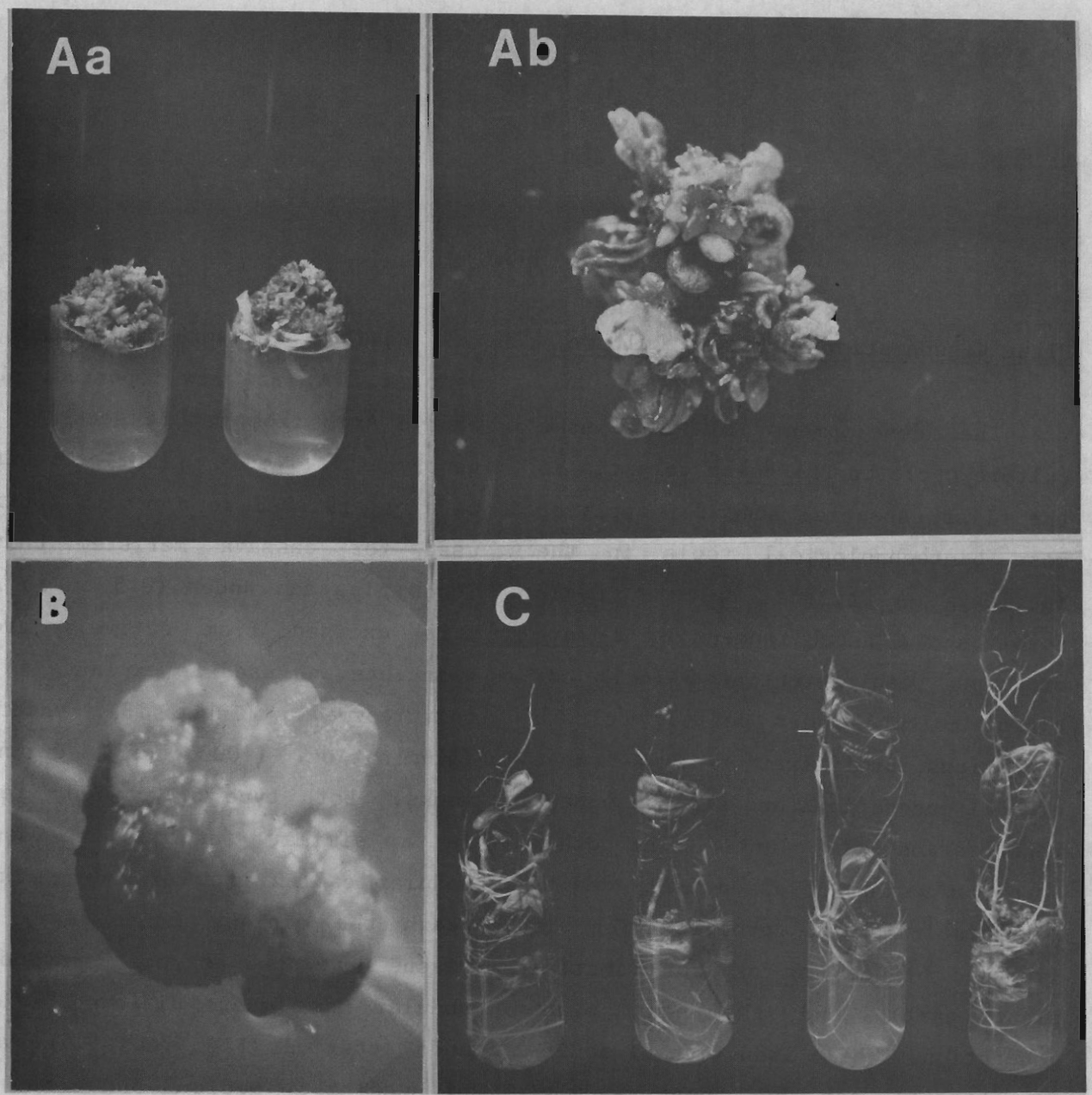

Figure 7. Regeneration of bean plants in tissue culture.

Aa. Nodular "callus" mass formed from shoot-tip and node explants.

Ab. Development of multiple budding from a nodular mass as at Aa.

B. Buds differentiating on an isolated nodular structure.

C. Plant regeneration from the buds as at B.

Table 13. Responses of shoot-tip and node explants from bean *in vitro* plantlets to four media of different composition.

	Additives to basal* medium (mg/l)				Cultures with responses (%)**		
	BAP	NAA	IAA	L-cysteine	Callus	Leafy tissue	Budding
1.	0.02	0.18	-	-	78	27	12
2.	0.02	0.18	-	100	80	27	10
3.	-	-	0.18	-	94	24	30
4.	-	-	0.18	100	82	31	38

* Basal medium for 1 and 2: MS + 3% sucrose; for 3 and 4: ½ MS + 3% sucrose.
** Average of 8-10 cultures per treatment per genotype (G 07060, G 00012, G 15637).

Table 14. Recovery of bean healthy plants by means of thermotherapy of cultured embryos.

	Thermoperiod*					ELISA test for BMMV**	
	I		II			Embryo + cotyledons	Embryo - cotyledons
1.	25°C	25°C	25°C	25°C			
					G 08060	+	+
					G 12491	+	+
					G 00012	+	+
2.	35°C	35°C	40°C	35°C			
					G 07060	+	-
					G 12491	-	-
					G 00012	+	-

* I. 25 days; II. 7 days; 12 hr. photoperiod.

** Treat. 1: 3 seeds per genotype.
Treat. 2: 3-6 seeds for embryos without cotyledons;
2 seeds for embryos with cotyledons.

extent in 2, these nodules would become phenolic and die; however in media 3 and 4, many buds would differentiate on each nodule (Fig. 7B). Upon transfer of these nodular cultures to a low salt medium, with higher cytokinin and low auxin, a single plantlet would grow from each culture (Fig. 7C) due to dominance of the oldest bud. Since it was possible to go from a somewhat undifferentiated structure (nodular callus mass) to plantlets via a "budding" process, these results should set the beginning of more work in bean tissue culture regeneration. The origin of buds, whether adventitious or axillary, and the use of liquid, shake, cultures for increasing the proliferation of such nodular structures should be investigated.

Tissue Culture for Recovering Germplasm Accessions

In the handling of bean germplasm, two frequent constraints to the flow of materials are (a) slow multiplication of materials procedent from the so called high risk countries, and (b) very few and/or deteriorated seeds available in many accessions.

In collaboration with the GRU, work was carried out to find out (a) if healthy plants can be recovered by embryo culture of virus infected seed, and (b) if viable plants can be recovered by embryo culture of rare and unique Phaseolous spp. which are represented in the GRU bank by 1-3 seeds only.

Seeds of three accessions with BMMV were used for embryo culture. Embryos accompanied by the cotyledons and embryonic axes only (without cotyledons) were explanted individually in test tubes containing the medium routinely used for cassava meristem culture; immediately, one set of tubes was maintained continuously at 25°C, with a photoperiod of 12 hrs and another set was exposed first to continuous (day/night, 12 hr each), 35°C for 25 days and then to alternating, 40°C day and 35°C night, temperatures for 7 days. At the end of these treatments, the embryo-derived shoots were sub-cultured in a single medium, at 25°C day/night, where rooting occurred in 1-2 weeks. The plantlets were potted and then used for serological (ELISA) evaluation. Table 14 shows clearly that healthy clones could be recovered only if embryonic axes were cultured at high temperature; neither thermotherapy or culture of embryos without cotyledons was sufficient to produce healthy plants. Seed was collected from these plants for virus retesting.

One seed from each of the following

seven accessions of rare Phaseolus spp. were received from the GRU: DGD 1510 P. neglectus, DGD 1516 P. scabrellus, DGD 1513 P. xanthotrichus, DGD 1523 P. pedicellatus, DGD 1522 P. neglectus, DGD 1520 P. neglectus, DGD 1509 P. glaucocarpus. Following surface sterilization with 70% ethyl alcohol for 0.5 min, and a rinse in sterilized distilled water, 10 min. immersion in 5% sodium hypochlorite, and throughly washing (4-5 times) with sterilized distilled water, the seeds were slightly scarified and explanted in the medium routinely used for cassava meristem culture. After about 3 weeks of culture under low light intensity, 25°C, 12 hr photoperiod, embryo-derived shoots were transferred to a simpler medium for rooting. These cultures will be ready for potting in 2-3 weeks.

These results demonstrate that valuable, rare, bean germplasm as well as completely infected materials can be saved, making them available for use.

Genotype Identification by Electrophoresis

The IDRC supported collaborative project referred to in the Cassava Section of this Report, also includes the development of genotype fingerprinting for Phaseolus bean germplasm.

Research with beans at the University of Manitoba began a few months ago using germplasm accessions provided by the GRU. Initial trials with seed isozymes did not show good discrimination between varieties. However, seed proteins separated in poly-acrylamide acid gels did show different patterns for different varieties with white, black and yellow color seeds (Fig. 8A), and even accessions with very similar patterns could be identified within white seeded accessions (Fig. 8B). The resolution of the banding pattern was further improved by a sequential extraction procedure of residual proteins (Fig. 8C).

These results, though promising, are still preliminary. Work now underway should provide a workable procedure, quick, and consistent for handling large accession numbers. The research team will move to CIAT early in 1986 to adapt the techniques to CIAT conditions.

Inoculation of Beans with Agrobacterium

As research in techniques for genetic engineering important crop plants progresses in advanced laboratories, the tissue culture component of these new approaches can be developed at CIAT. The large

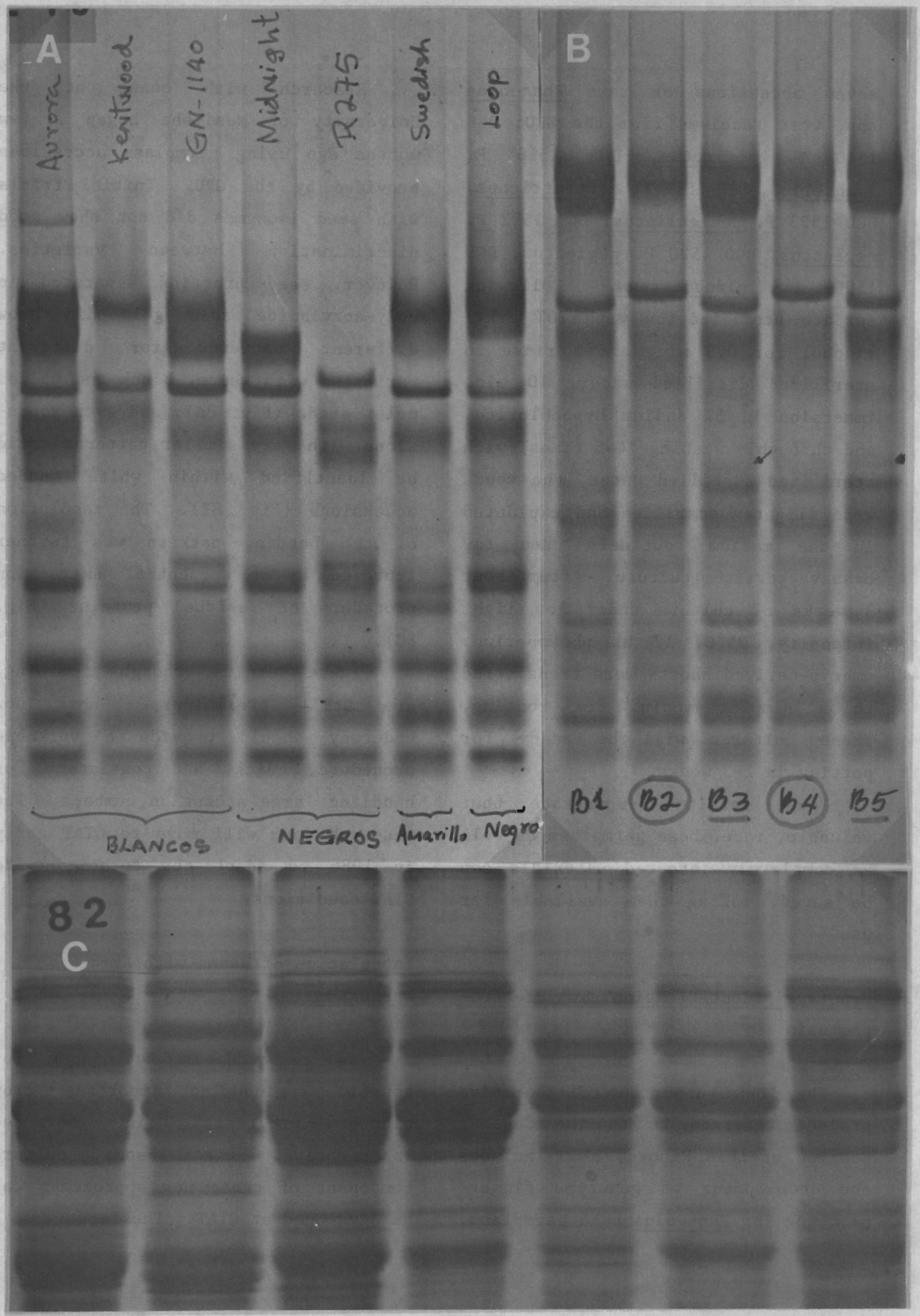
A
Aurora
Kentwood
GN-1140
Midnight
R275
Swedish
Loop
BLANCOS
NEGROS
Amarillo
Negro
B
B1
B2
B3
B4
B5
82
C

Figure 8

Figure 8. Electrophoretic characterization of bean germplasm.

A. Seed proteins of seven bean varieties separated in polyacrylamide acid gel. Note striking differences between lanes; each lane represent one variety.

B. Same as at A for five white seeded variedies. Note similarities between lanes B_2 and B_4 and between lanes B_3 and B_5. Each lane represents one variety.

C. The same seven varieties as at A, but using sequentially extracted residual proteins in polyacrylamide gel. Note the much higher band resolution obtained with this procedure.

(Photos kindly provided by W. Bushuk, A. Hussain and H. Ramirez, University of Manitoba, Minnepeg, Canada).

germplasm collection and tissue culture capabilities available at CIAT provide a comparative advantge in this area.

In order to develop the basic methodologies of gene transfer techniques with beans, a collaborative effort has been initiated with scientists at Louisiana State University. At LSU, a synthetic DNA sequence (sp DNA) has been constructed and put under the control of the nopaline synthase promoter, and introduced into Agrobacterium rhizogenes plasmid vectors. The sp DNA was shown to code for a polypeptide rich in essential amino acids, especially the sulfur-containing ones: up to 23% lysine, 12% tryptophan, 11% methionine.

Three A. rhizogenes strains, 10-3-2, 10-3-3 and 10-3-4, containing the plasmid with the sp DNA, plus a wild type R 1000, were obtained from LSU for use in the inoculation trials. Using the wild type, (R-1000), several bean varieties were inoculated, with the result that ICA Viboral (G 12722) and Calima gave the best response.

During the infection process, A. rhizogenes transfers to the host cell genome a portion of its plasmid DNA which elicits root growth at the point of inoculation. Thus, root formation is a signal for successful inoculation with A. rhizogenes. In this trial, control plants only developed a small callus, but no roots, at the point of inoculation. Next, various inoculation techniques were tried, being the immersion of excised embryos in bacterial suspension for 24 hrs, and the inoculation of in vitro plantlets by a cut on the stem, the ones that yielded highest root development (Fig. 9A). It was also found that the A. rhizogenes strain 10-3-3 induced root formation in up to 50% of the embryos and 70% of the in vitro plantlets in contrast to 10-3-2 and 10-3-4 which could only induce 20-30% rooting. In addition, embryos treated with the strain 10-3-3 gave rise to plantlets with twice as much roots than shoots, as compared to strain 10-3-2 and the wild type which yielded almost equal roots and shoot mass, and the control (non-treated) having about half root mass (Table 15). There is probably a high A. rhizogenes strain/bean genotype specificity for transformation.

After 15 days from inoculation, the roots were excised from the plant and cultured in a medium with 250 mg/l cefatoxine to eliminate the bacteria. Upon sub-culturing root tips, transformed roots branched and grew more than the control (Fig. 9B); close examination of laterals revealed the typical "hairy root" syndrome caused by A. rhizogenes (Fig. 9C). LSU

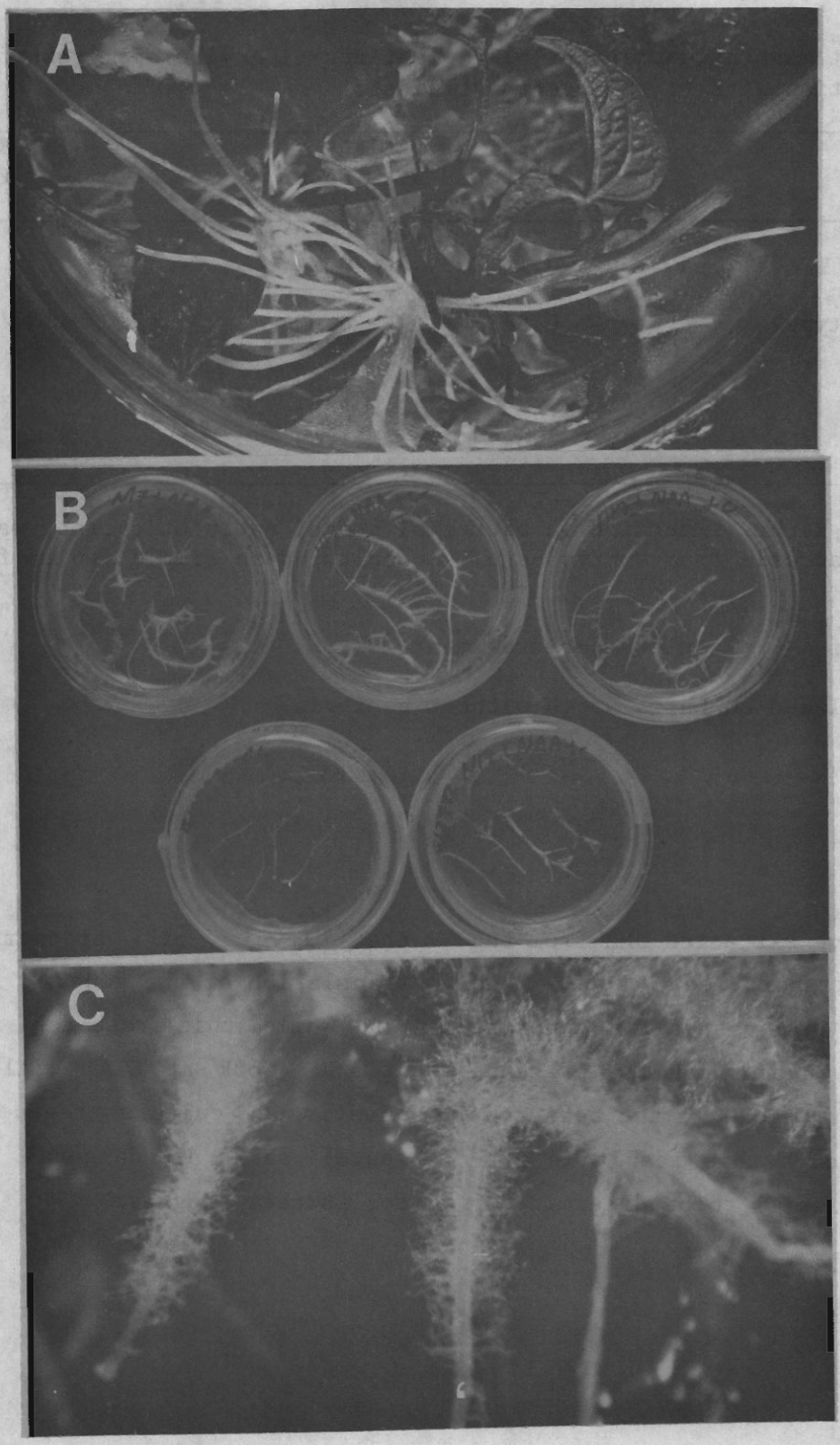

Figure 9. Response of beans inoculated with Agrobacterium rhizogenes.
A. Root formation at the point of inoculation on the stem.
B. Proliferation of induced roots (top row) as compared to control (non-treated) roots (lower row).
C. Close up of B to show typical "hairy root" syndrome caused by A. rhizogenes cell transformation.

Table 15. Root mass production by bean plantlets (ICA Viboral) grown from embryos after inoculation with A. rhizogenes strains*.

A. rhizogenes strains	X F. W. (gr)** Root	Shoot	Root/shoot F.W. ratio
Control***	0.15	0.24	0.64
R 1000 (wild type)	0.36	0.33	1.09
30-3-2	1.51	1.55	0.97
10-3-3	2.84	1.49	1.91

* Following 24 hr. immersion in bacterial broth, antibiotic treat was used to rid the bacteria from embryos and then cultured in simple medium for plant growth.

** Average of 4-6 plantlets per treatment.

*** Embryos placed on Luria Broth without bacteria.

scientists have extracted DNA from the putatively transformed roots, and using a DNA probe specific for the root genome, have shown the presence of the synthetic gene in the bean root genome. Although this result is promising, it is still to be determined the level of transcription and translation of the gene.

While this research is conducted at LSU, work at CIAT will concentrate on tissue culture regeneration (see above) as a fundamental prerequisite for further progress.

Tissue Culture Regeneration of Stylosanthes spp.

In the last few years, methodologies for plant regeneration of S. guianensis, S. capitata and S. macrocephala accessions from leaf and epicotyl-derived callus and cell suspensions have been developed at CIAT. This year, the techniques for consistent regeneration of S. guianensis var. pauciflora (CIAT 2243) and var. guianensis (CIAT 136) have been standardized. Thus, 4-8 mg/l BAP and 0.5 mg/l NAA were found optimal for callus induction from leaf segments, and 0.05 mg/l BAP, 0.01 mg/l NAA and 0.05 mg/l GA for organogenesis. Regenerated shoots could be easily rooted in a simpler salt-medium and transferred to pots and to the field readily.

Phenotypic Stability of Regenerated Plants

In collaboration with the Breeding Section of the TPP, an experiment was set up to determine the extent of variability in tissue culture regenerated (S. guianensis, CIAT 2243, Bandeirante) plants.

Two types of explants were obtained (a) leaf segments (1 x 1 cm) from plants (R_o plants) grown in the greenhouse; (b) portions of hypocotil (0.5 x 0.5 cm) from germinated seed (R_o plants), and cultured in a single callus induction medium. Sixty Petri plates (5 x 1 cm) containing 3 explants each, were used per explant type. One third of the calluses were transferred to a medium for regeneration (S_o) and the remaining was sub-cultured in callus medium. After one month, half of the calluses were removed for regeneration (S_1) holding the remaining in a fresh callus medium; on the second month, half of the calluses were transferred for regeneration (S_2). A total of 96 regenerated plants (R_1 plants) were potted and grown to maturity in the glasshouse.

At a later date another set of 44

R_1 plants were regenerated from sub-cultures S_3 and S_4 of CIAT 2243. Along with this, 31 plants regenerated from leaf-derived callus and 36 plants from gamma-irradiated suspension cultures of CIAT 136 were also potted for evaluation.

Preliminary evaluations of the 96 R_1, CIAT 2243, plants comprised root-tip chromosome counts, morphology, seed production and seed size; the reaction to inoculation with Colletotrichum gloeosporioides strains was also studied.

The first stricking finding was that 25% of the regenerated plants have doubled their chromosome complement from 20 (2X) to 40 (4X) chromosomes; there was no difference in the percentage of chromosome doubling between the two explant types, but there was a tendency for increasing doubling frequency with sub-culturing (Table 16). Some of the most obvious morphological changes observed were associated with the increase in ploidy, e.g. taller plants but with fewer stems, as well as wider flower buds and more pubescence of 4X than 2X somaclones and control plants (Table 17 and Figs. 10 and 11). Seed production is an important agronomic parameter in Stylosanthes. Most 2X and all 4X somaclones produced (on two month harvest basis) less than 400 seeds per plant; a few 2X somaclones yielded up to 800 seeds per plant as compared to an average of 300 seeds per control plant. The 4X clones had, however, larger seeds than the 2X clones and the control plants (Tables 18 and 19). Thus, 4X plants yielded lower number but larger seeds, while some diploid somaclones had higher seed production than the control plants. In addition, there were some 2X and 4X somaclones which did not set seed at all in spite of having flower buds; this may be a case of sterility.

Since anthracnose is a very important problem in Stylosanthes, a total of 95 2X and 4X somaclones and the six control plants were inoculated in the greenhouse with three strains of Colletotrichum gloeosporioides with the collaboration of the TPP's Pathology Section. Table 20 shows the evaluation of anthracnose symptomatology. Diploid somaclones and the control plants showed in general a similar reaction distribution to the less (A), medium (B) and Highest (C) pathogenic strains of the fungus. However, there was a clear tendency to a higher tolerance reaction of the tetraploid somaclones inoculated with the three fungal extracts than diploids and controls.

Since these are the first observations ever made on phenotypic stability of Stylosanthes regenerated plants, it was worth to make a

Table 16. Number of diploid and tetraploid S. guianensis, CIAT 2243, plants (R_1) regenerated from callus cultures, with three sub-cultures and two explant types.

Explant type	Sub-culture	No. plants			4X %
		Total	2X	4X	
Leaf	S_0	42	35	7	
	S_1	16	8	8	
	S_2	3	2	1	
Sub-total		61	45	16	26
Hypocotil	S_0	27	22	5	
	S_1	3	3	0	
	S_2	5	2	3	
Sub-total		35	27	8	23
TOTAL:		96	72	24	25

CONTROL: 6 plants = 20 chromosomes (2X).

Table 17. Morphology of diploid and tetraploid S. guianensis, CIAT 2243, plants (R_1) regenerated from callus cultures in relation to control plants.

No. plants		Plant height	Internode length	No. shoots per stand	Ratio width/length		
					Leaf	Flower	Pubescence*
Control	6	1.2m	3.6cm	23	0.20	0.50	2
2X	72	1.3	4.5	23	0.13	0.46	3
4X	24	1.5	5.5	17	0.21	0.60	4

Evaluation in 5 month old plants.

* 1 = abscent; 2 = low; 3 = moderate; 4 = high; 5 = very hign.

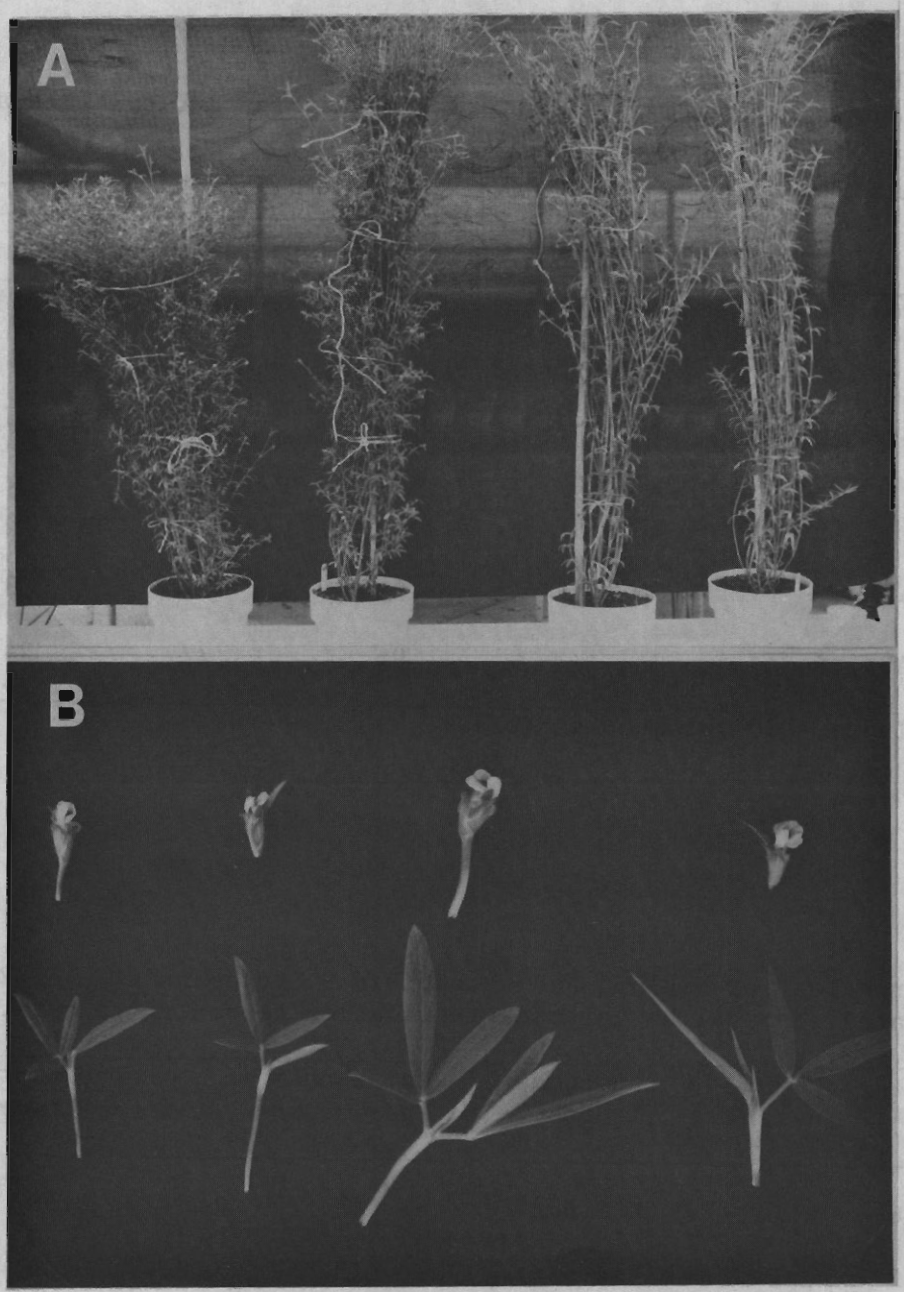

Figure 10. Variability in morphology and ploidy of S. guianensis, CIAT 2243, (R_1) plants regenerated from callus cultures.

A. Left to right: Control plant grown from seed. Three somaclones: diploid (2X = 20), tetraploid (4X = 40) and diploid, respectively.

B. Flower buds and apical leaves of plants as at A. From left to right: control; three somaclones: diploid, tetraploid and diploid, respectively.

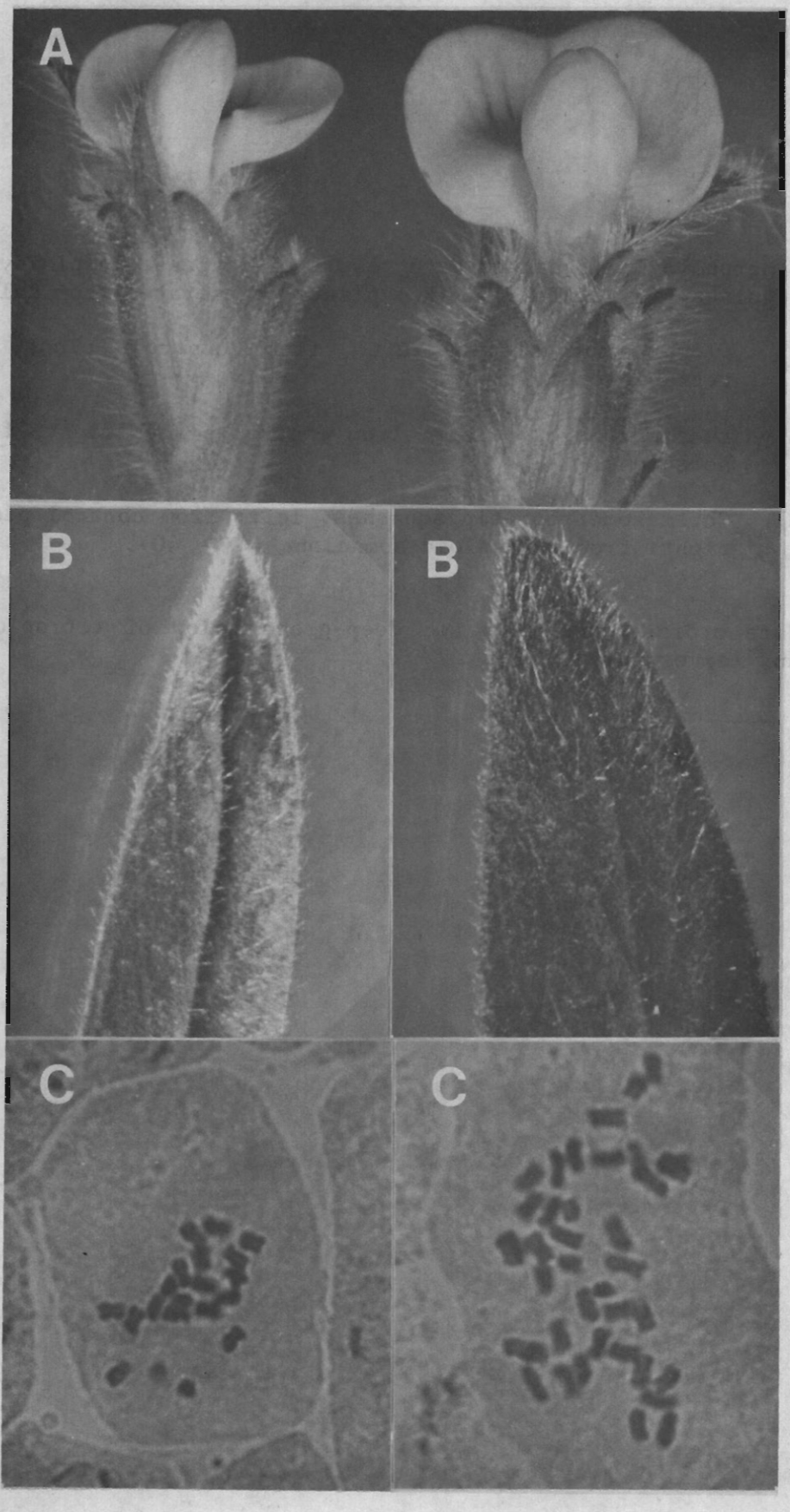

Figure 11

Figure 11. Morphological changes as a result of increase in ploidy of S. guianensis, CIAT 2243, (R_1) plants regenerated from callus cultures.

A. flower morphology, left: from control plant; right: from tetraploid somaclone.

B. Leaf morphology, left: from control plant; right: from tetraploid somaclone.

C. Chromosome root-tip squashes, left: from control plant (2X = 20); right: from tetraploid somaclone (4X = 40).

Note profuse pubescens and deep-green color of tetraploid sepals (A) and leaves (B).

Table 18. Seed production* of S. guianensis, CIAT 2243, plants (R_1) regenerated from callus cultures and distribution by ploidy.

Range seed No. per plant**	Total No. plants	No. Plants 2X	4X
1 - 100	23	16	7
101 - 200	16	14	2
201 - 300	13	10	3
301 - 400	6	6	0
401 - 500	-	-	-
501 - 600	1	1	0
601 - 700	2	2	0
701 - 800	2	2	0
Total	63	51	12

* Seed harvested for two months. CONTROL X = 300 seeds.

** To Dec. 1985, 9 diploid and 4 tetraploid somaclones did not set seeds in spite of having formed flower buds.

preliminary recording of them. The changes observed in the R_1 plants may or may not pass to the sexual offspring (R_2 plants); on the other hand, selfing of R_1 plants to produce the R_2 generation may untap changes not seen in the R_1 plants, especially if these are recessive. Collaborative work with the TPP's Breeding Section is underway to look at the sexual generation of the R_1 plants in the field.

Stylosanthes Protoplast and Cell Suspension Cultures

The successful agricultural application of various somatic cell genetic technologies depends, to a considerable extent, on having an efficient technique for plant regeneration from isolated protoplasts. Until now, reports on legume protoplast cultures have been scarce and no reported results on leaf mesophyll protoplast culture of Stylosanthes are available. Leaf mesophyll tissue can provide a genetically uniform protoplast source with good morphogenetic potential. Work in this area was carried out this year at CIAT.

Cell suspension cultures

Callus cultures were readily obtained from leaf explants. Pipetable suspension cultures could be produced from friable callus in one week (Fig. 12A). When planted on agar-solidified medium and cultured in light, colonies produced multiple green shoots and these gave rise to plants (Fig. 12B).

Protoplast isolation and culture

Protoplasts were isolated using pectolyase Y23, Onozuka R_{10} cellulase, and hemicellulase for digesting cell walls of mesophyll cells (Fig.

Table 19. Size of seeds* of S. guianensis, CIAT 2243, plants (R_1) regenerated from callus cultures and distribution by ploidy.

Weight of 100 seeds (gr)	Total No. plants	No. plants 2X	4X
0.24 - 0.26	46	46	0
0.29 - 0.32	6	0	0

* Seed harvested for two months.
CONTROL: 0.26 gr.

13A) and suspension cells (Fig. 13B), respectively. Protoplast yields were good when young, fully expanded, leaves of two month old seedlings were used and when the cell cultures used as protoplast source were sub-cultured twice weekly. After washing, protoplasts were cultured in a more complex medium in which 30-50% of them regenerated cell walls within 1-3 days. First cell division were observed after 2-3 days of culture (Fig. 13C). At 12 days after isolation, 4% of the cultured protoplasts divided; and serial addition of fresh medium was found necessary for subsequent divisions and rapid growth of protoplast-derived colonies (Fig. 13D). When sub-cultured on solid medium, the colonies produced yellow (CIAT 2243) or yellow and light green (CIAT 136) calluses. The efficiency of callus formation from protoplast-derived colonies was greatly influenced by the gelling agent used to solidify the medium. Agar (Difco bacto agar) was always inferior than agarose (Sigma, Type VII) or gelrite (Kelco). Gelrite gave similar or better results than agarose (Table 21).

Table 20. Reactions of S. guianensis, CIAT 2243, plants (R_1) generated from callus cultures, to inoculation with extracts of three strains of Colletotrichum gloeosporioides in the greenhouse, and distribution by ploidy.

Reaction scale*	(A) 46B - CAP			(B) 2315			(C) 1808 CPAC		
	Control	2X	4X	Control	2X	4X	Control	2X	4X
A	0	1	8	0	0	0	0	0	0
B	4	25	11	1	0	3	0	1	3
C	2	36	3	4	19	17	2	6	8
D	0	11	0	1	54	2	5	66	11
E	0	0	0	0	0	0	0	0	0

* A = No damage; B = small lesions; C = medium size lesion; D = large lesions and defoliation; E = tissue death.

** Total No. plants inoculated: Control = 6, 2X = 73; 4X = 22.

(Inoculation and evaluations carried out with the collaboration of the TPP's Plant Pathology Section).

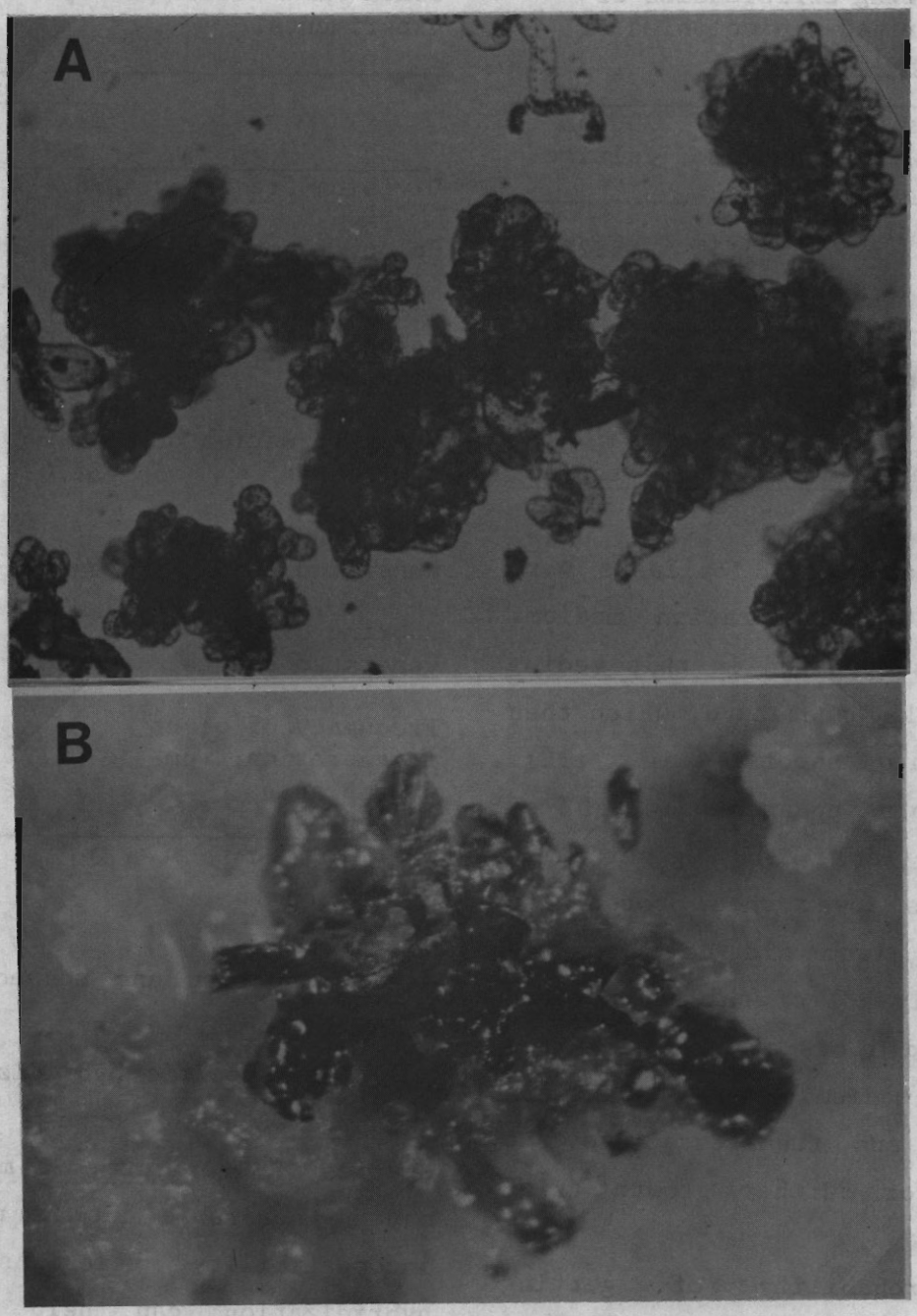

Figure 12. Regeneration of Stylosanthes guianensis, plants from cell suspensions.

A. Cell suspension culture showing pipetable cell clusters (CIAT 2243).

B. Shoot differentiation from cell suspension colonies (CIAT 136).

Table 21. Effect of three gelling agents on plating efficiencies of protoplast derived minicolonies of Stylosanthes guianensis*.

Gelling agents	0.8% agar	0.5% agarose	0.15% gelrite
Plating efficiency	2.5%	31.5%	55.2%

* CIAT 2243 and CIAT 136.

Table 22. Efficiencies in two representa tive leaf protoplast culture experiments.

	S. guianensis	
	CIAT 2243	CIAT 136
Division frequency	6.4 %	5.5 %
Colony formation	1.5 %	2.5 %
Growth of mini-colonies on solid medium	42.0 %	39.0 %
Shoot formation on p-calluses	32.0 %	46.0 %
No.of shoots on re-generated shoots	84.0 %	80.0 %
Rooting of re-generated shoots	84.0 %	80.0 %
Frequency of plant regeneration from protoplasts	0.17%	0.39%

Plant regeneration from protoplast cultures

Protoplast-derived calluses were transferred to regeneration medium. Green patches appeared in this medium after 2-4 weeks of culture, which then developed into shoots (Fig. 13E). Numerous shoots appeared on 40-50% of the calluses (Table 22). Regeneraation frequency was lower when protoplasts were derived from old cell suspensions. Nearly 90% of the regenerated shoots formed roots when transferred to a rooting medium. Rooted plantlets were potted and transferred to the glasshouse for further growth (Fig. 13F).

The probability of getting variants from protoplast derived callus cultures may be high. A total of 31 CIAT 2243 and 66 CIAT 136 plants regenerated from protoplasts have been transplanted to the glasshouse for seed production and subsequent evaluation in the field.

Interspecific hybridization of S. guianensis with other Stylosanthes species (S. capitata, S. macrocephala) may be desirable but is prevented by incompatibility reactions. Somatic hybridization can help bypass the incompatibility barriers. Recently, shoot initiation of protoplast-derived callus has been obtained with S. capitata at CIAT.

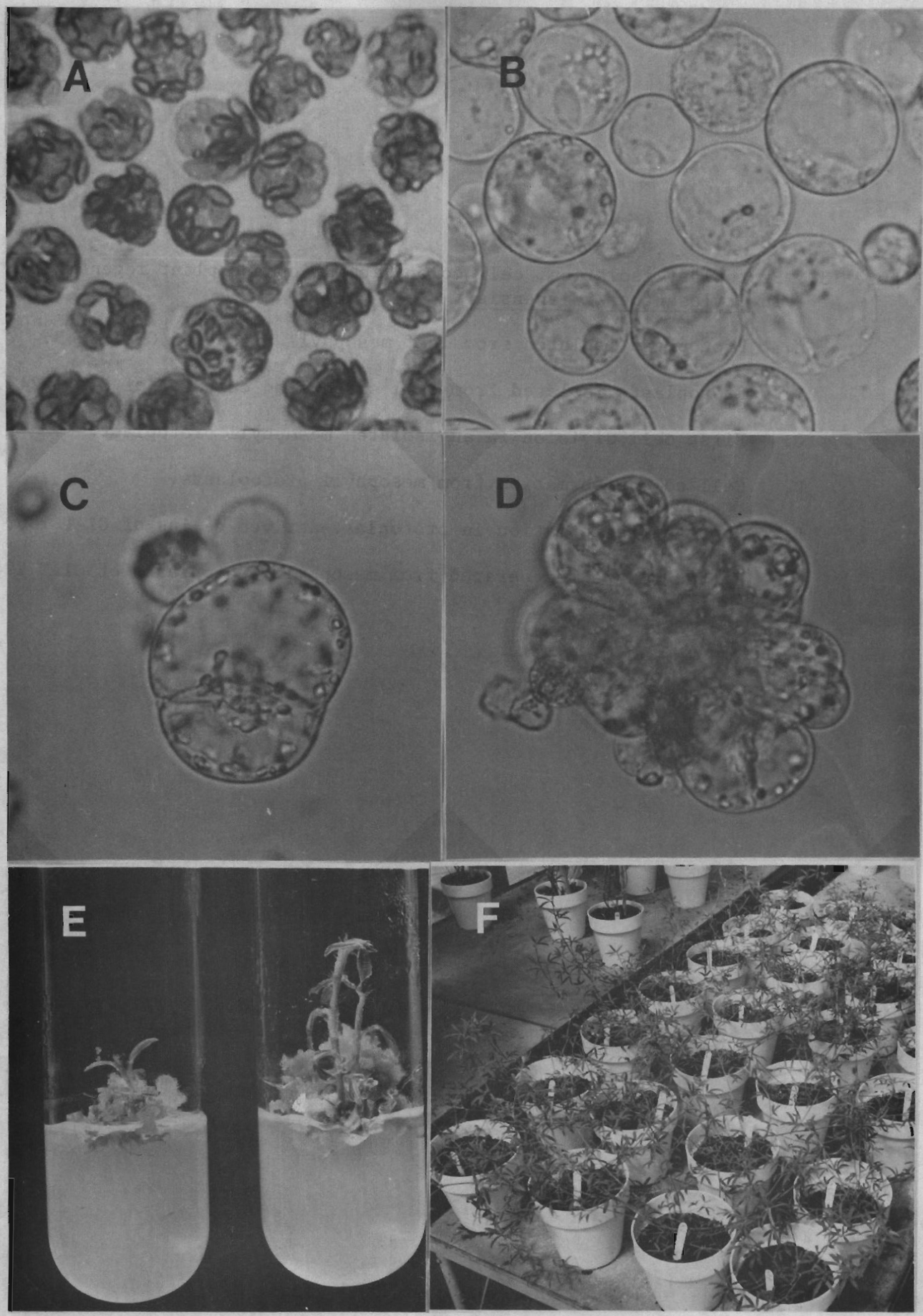

Figure 13

Figure 13. Protoplast isolation, cell colony formation and plant regeneration in Stylosanthes guianensis.

A. Protoplast isolated from leaf mesophyll tissue of CIAT 2243.

B. Protoplasts isolated from cell suspension cultures of CIAT 2243.

C. First cell division in an isolated mesophyll protoplast.

D. Cell colony formation from mesophyll protoplasts.

E. Shoot differentiation in protoplast-derived callus of CIAT 136.

F. Potted plants regenerated from mesophyll protoplasts of CIAT 2243.

Response of Stylosanthes Cell Suspensions to Pathogenic Stress

Agronomic characteristics can be affected by several different mechanisms, some acting at the cellular level, and others only at the whole plant level. If a trait is expressed by the whole plant as well as by cultured cells, it is possible to develop selection systems for alterations of these cellular functions. On the other hand, if a toxin produced by a microbial pathogen is the primary responsible for disease symptoms, the challenge of large cell populations with the pathotoxin, or with less purified culture extracts, can be used to select cells expressing tolerance to such substance.

In collaboration with the TPP's Pathology Section, culture filtrates of Colletotrichum gloeosporioides were mixed with cell culture medium in different concentrations, and cell suspensions of S. guianensis were plated on Petri dishes using these toxic media. Such filtrate of C. gloeosporioides has been used in the TPP for inoculation of seedlings and found to be toxic. Necrosis and death of the cultured cells indicated the toxicity of the fungal culture filtrate at higher concentrations (Fig. 14). Decrease of plating efficiencies of cell suspensions of four S. guianensis genotypes were compared (Fig. 15). The less sensitive was CIAT 10136, a genotype which is regarded as one of the most anthracnose resistant ones. Of the other, CIAT 2243 was the most sensitive to Q 136 toxin, and CIAT 2312 was very sensitive to LVE-Seca toxin.

These experiments show that anthracnose tolerance and sensitivity of the various S. guianensis genotypes is expressed to a certain extent at the cellular level. Work is necessary to further refine these correlations using fine cell suspension cultures with the view to use in vitro selection of anthracnose tolerant cells. This approach could be rewarding for selecting sexual recombinants from F_1 microspore cells.

Transfer of Tropical Grass Germplasm in vitro

In collaboration with the TPP, a collection of grass germplasm from Africa was transferred to CIAT in the form of shoot-tip cultures in test tubes. A total of 431 accessions comprising 35 grass species, from five African countries (Table 23) were put into culture for transfer. The in vitro work was carried out in ILCA for the material procedent from Rwanda, Burundi and Ethiopia; at the

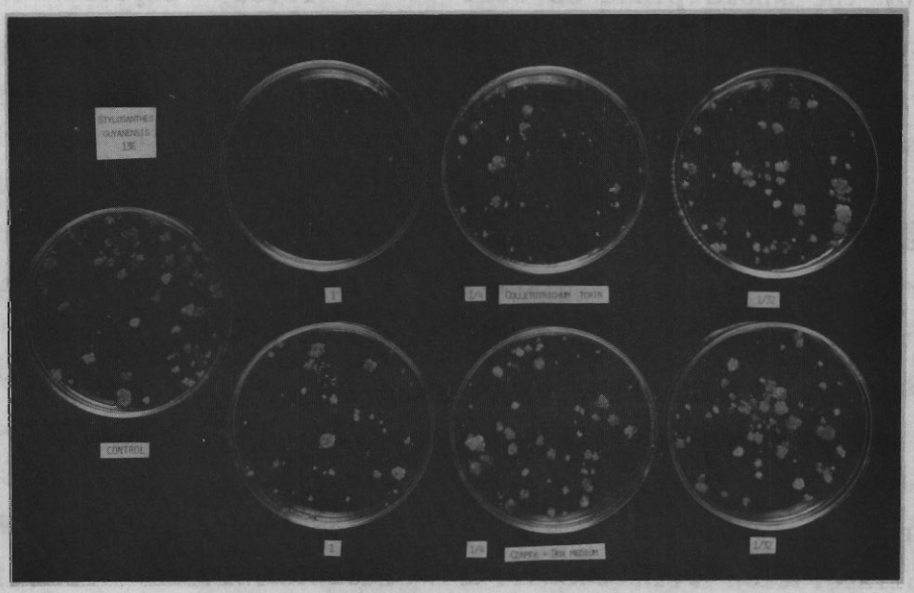

Figure 14. Effect of Colletotrichum gloeosporioides culture filtrate mixed in various concentrations with cell culture medium on the plating efficiency of S. guianensis cell suspensions.

Top: Gradual increase in plating efficiency with dilution of fungal filtrate (left to right).

Botton: Almost no effect of medium used for fungal culture without the toxin.

Control: Standard medium for cell colony formation.

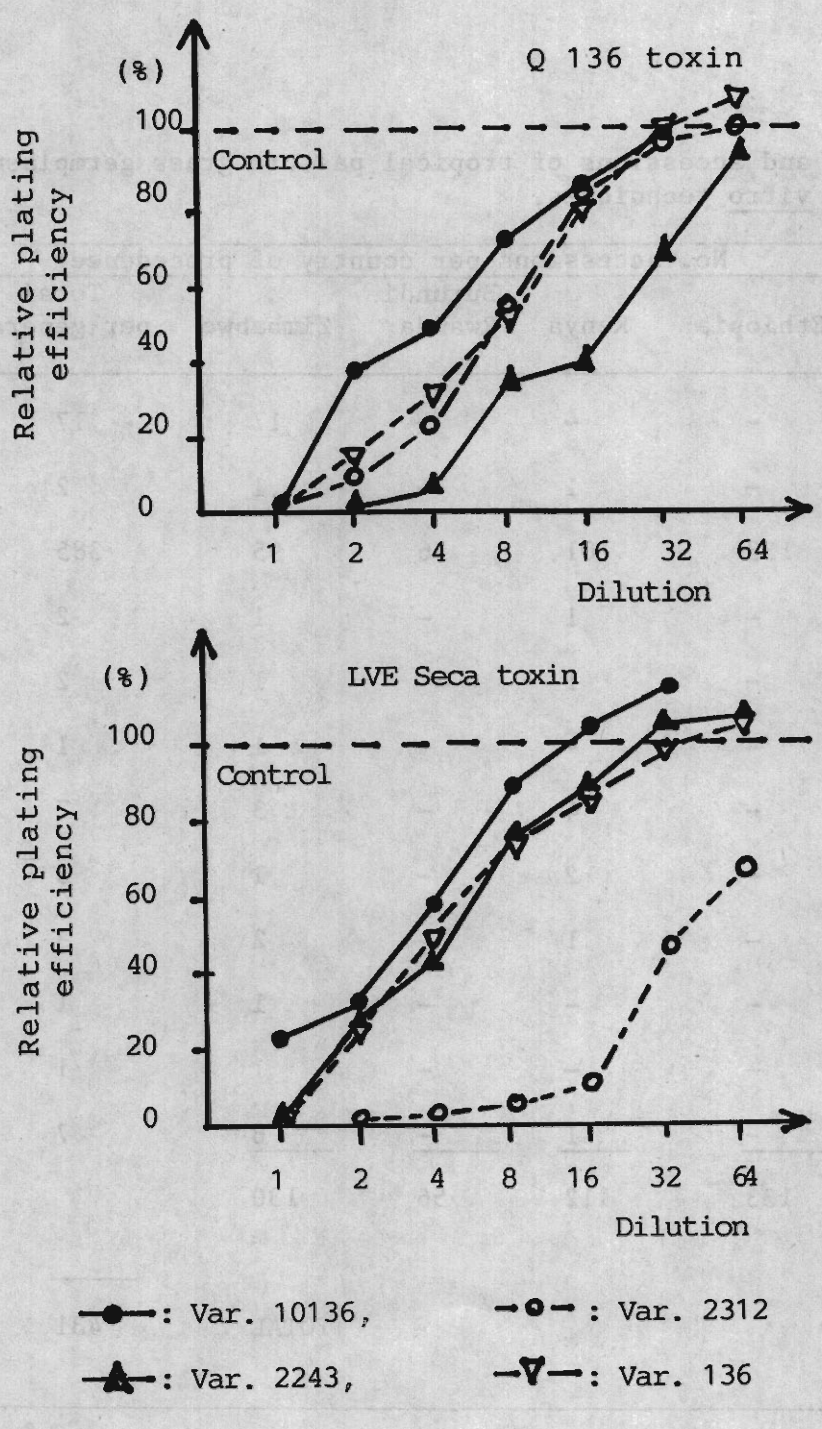

Figure 15 . Comparison of relative plating efficiencies of Stylosanthes guianensis cell suspensions in the presence of Colletrotrichum toxins.

Table 23. Number of species and accessions of tropical pasture grass germplasm introduced to CIAT using *in vitro* techniques.

Genera	No. of Species	No. accessions per country of procedence				Total per genera
		Ethiopia	Kenya	Burundi Rwanda	Zimbabwe	
Andropogon	2	-	-	-	17	17
Bothriochloa	2	-	1	-	1	2
Brachiaria	14	133	101	56	95	385
Eragrostis	2	-	1	-	1	2
Hyparrhenia	2	-	1	-	1	2
Ischaemum	1	-	-	-	1	1
Panicum	4	-	4	-	3	7
Paspalum	3	-	2	-	1	3
Setaria	3	-	1	-	2	3
Stereochlaena	1	-	-	-	1	1
Urochloa	1	-	-	-	1	1
Unknown grasses		-	1	-	6	7
Total/country	35	133	112	56	130	
					TOTAL	431

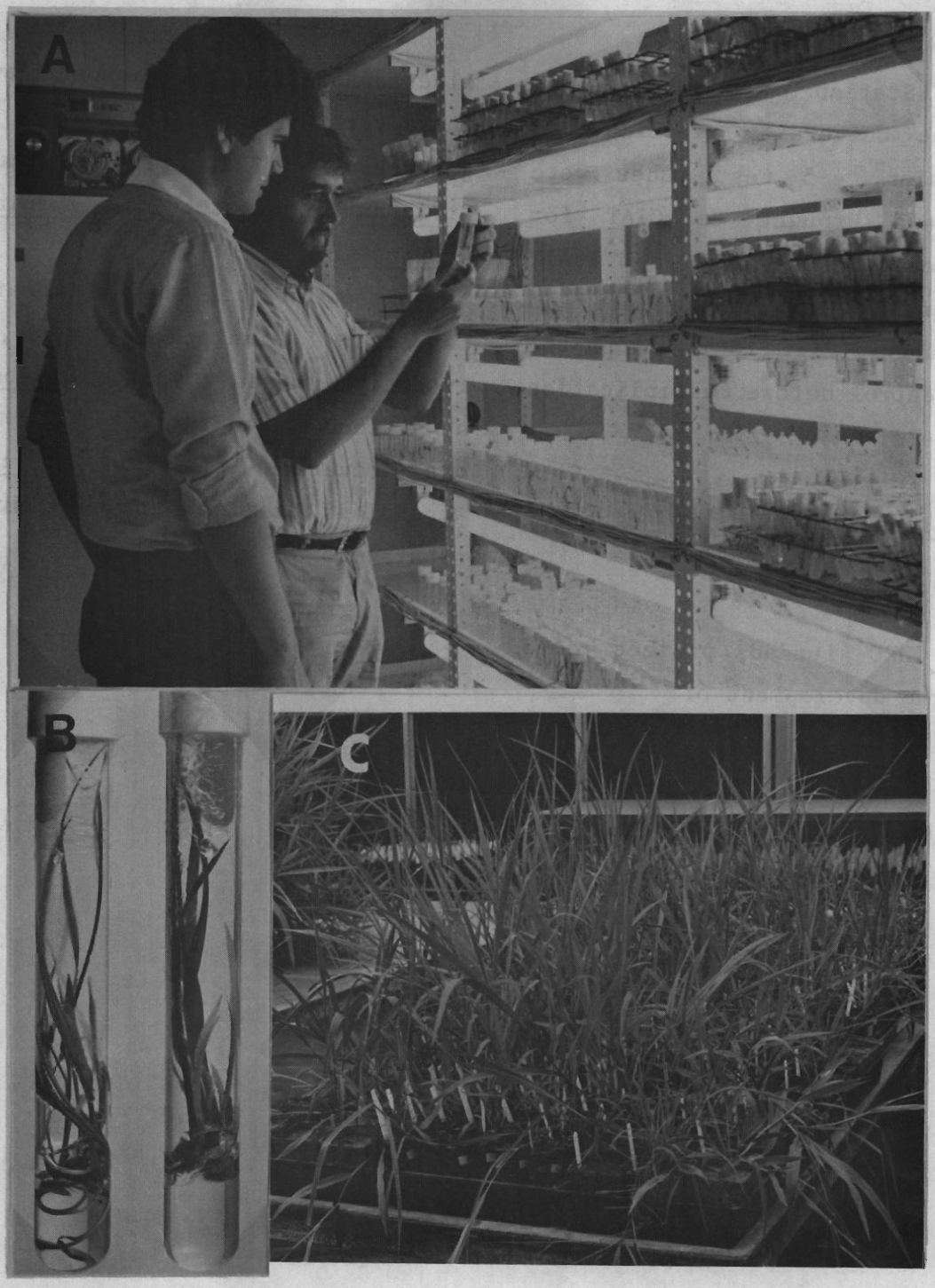

Figure 16. Transfer of tropical pasture grass species germplasm from Africa to CIAT using *in vitro* techniques.

A. Collection of 432 grass accessions in culture tubes under controlled growth at CIAT.

B. Three-week old culture ready for potting.

C. Potted plants of *Brachiaria* spp. growing under phytosanitary conditions in the glasshouse.

Quarantine Station of Maguga, Kenya and at the University of Zimbabwe. Per accession, 4-5 test tubes were prepared and brought to CIAT for further growth in the lab (Fig. 16A). Potted plantlets are being placed in the glasshouse (Fig. 16C) for phytosanitary control before multiplication for the field.

Genotype Identification by Electrophoresis

The IDRC funded project also includes the development of electrophoretic techniques for the characterization of legume forage germplasm. The project includes Stylosanthes, Desmodium, Zornia, and Centrosema.

Research at the University of Manitoba, Winnipeg, Canada, has recently began with Stylosanthes accessions. Figure 17 shows discrimination of six S. capitata accessions based on polyacrylamide gel separation of seed proteins.

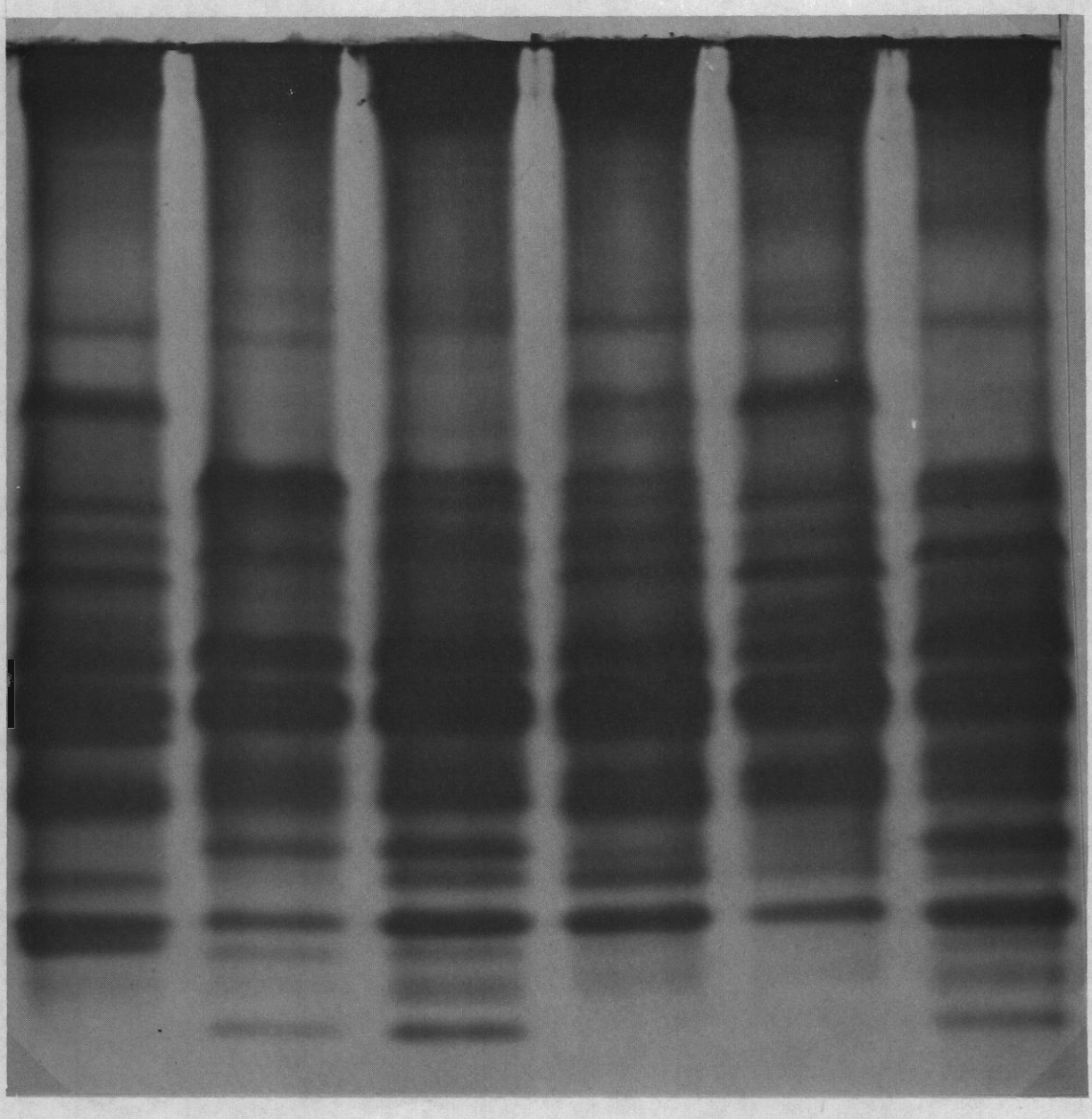

Figure 17. Electropherogram showing discrimination of S. capitata genotypes by electrophoretic separation of seed proteins in polyacrylamide gel. Note the differences in banding pattern between lanes. Each lane represents a S. capitata accession.

RICE

Improvement of Rice Anther Culture Methodology

The application of anther culture to rice breeding requires scaling up the production of diploid homozygous plants to obtain about 150 R_2 lines per cross, and the Rice Program's estimations are approximately 100 crosses per year needed for any given target ecology.

The collaborative activities of the BRU with the Rice Program in rice tissue culture in 1985, comprised two main areas: (a) improving laboratory facilities for scaling up the anther culture work and, (b) improvement of various aspects of the anther culture technique itself.

The non-illuminated shelf area now has a capacity to hold over 4,000 callus-induction, bottles; and the illuminated shelving can hold up to 1800 regeneration-flasks at anyone time. If 15 calluses can be cultured per flask, the potential capacity of the room (3 x 5 m) is 27,000 calluses. A transfer room to accommodate six laminar flow cabinets and sterilizing/ washing facilities are also being adapted.

Regarding the technique for anther culture, trials were carried out to evaluate factors, and inter-relations of these, which influence the balance between callus induction and plant regeneration e.g.: (1) residual effects of callus induction conditions (media composition, cold shock, etc.) on plant regeneration; (2) effect of number and size of callus produced from the anthers, as well as phenolic-type browning of callus, on plant regeneration; (3) effect of callus age and size on plant regeneration; (4) effect of gelling agents and other type of substrata, for solid media, on plant regeneration.

This methodology improvement work was conducted by the Rice Program personnel assigned to the project during periods of minimum routinary work.

Other constraints to the anther culture technique that will be tackled are: high frequency of albinism in some materials, and microbial contamination and physiological deterioration of panicles stored prior to culture.

To increase the number of doubled haploids, especially in genotypes with low regenerative capacity, work on chromosome doubling will be carried out. As the straight forward anther culture technique is used by the Rice Program routinely, other potentially useful tissue culture methods, e.g. somaclonal variation, should be explored for application to rice.

COLLABORATIVE PROJECTS
(1985-86)

1. Title: Development of *in vitro* culture methods for the propagation and conservation of wild *Manihot* species.
   Institution: IBPGR
   Scientist: W.M. Roca (CIAT)
   Period: 1983-85

2. Title: Cryopreservation of cassava meristems
   Institution: IBPGR/Plant Biotechnology Institute, Saskatoon, Canada
   Scientist: K.K. Kartha
   Period: 1983-85

3. Title: Electrophoretic characterization of genotypes in the world collections of *Phaseolus*, *Manihot*, *Stylosanthes*, *Centrosema*, *Desmodium* and *Zornia*
   Institution: IDRC/University of Manitoba, Winnipeg, Canada
   Scientists: W. Bushuk, A. Hussain (University of Manitoba); W.M. Roca, H. Ramirez (CIAT)
   Period: 1984-87

4. Title: Cassava tissue culture research
   Institution: University of Bath, U.K.
   Scientist: G.G. Henshaw
   Period: 1984-86

5. Title: Inter-specific hybridization in *Phaseolus*
   Institution: USAID
   Scientists: R. Hidalgo (GRU-CIAT); W.M. Roca (BRU-CIAT)
   Period: 1985-86

6. Title: Use of plant transformation techniques to increase the protein of cassava and modify the protein quality of common beans
   Institution: Louisiana State University, Baton Rouge, Louisiana, U.S.A.
   Scientist: J. M. Jaynes

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(as of December, 1985)

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