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FINAL REPORT

ON

ISOLATION OF STRAINS, CLONES AND REGENERATION OF PLANTS FROM SINGLE CELLS OF WINGED BEAN

Grant number: DPE-5542-G-SS-3056-00

August 1983 - January 31, 1986

Submitted to:

Project Officer Dr. L. Schulze
Agency for International Development
S and T/AG
SA - 18, 413
Washington, D. C. 20523

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ISOLATION OF STRAINS, CLONES AND REGENERATION OF PLANTS FROM SINGLE CELLS OF WINGED BEAN

AID GRANT #. DPE-5542-G-SS-3056-00

LAYMAN'S ABSTRACT:

Winged bean, is a very common vegetable in the tropical regions of Asia and Africa. It is rich in protein, oil, carbohydrates and minerals. The plant is grown from seeds in backyards or in fields. Using tissue culture technology, it is now possible to grow winged bean in the laboratory in artificial media as "test tube plants" from a single cell or a group of cells by adding the right combinations and concentrations of plant growth hormones. Therefore, it is now possible to obtain several thousands of plants in the laboratory from a few cells and transfer them to the fields all year round. This method requires very little space, time and effort and thereby greatly reduces costs. Seeds used for planting can thus be used for consumption, production of milk or other protein-rich foods. This technology also provides methods to produce new varieties of winged bean plants with various other desirable qualities like higher food-value or plants which can grow in less favourable soil or climatic conditions. The applications and uses of this research are very significant because these techniques can now be transferred world-wide, particularly to LDC nations where production of protein-rich food is of primary importance.

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ISOLATION OF STRAINS, CLONES AND REGENERATION OF PLANTS FROM SINGLE CELLS OF WINGED BEAN

AID GRANT #: DPE-5542-G-Ss-3056-00

TECHNICAL SUMMARY

The winged bean has a great potential as a protein-rich major food crop, and regeneration using tissue culture is of paramount significance. Successful plantlet regeneration from callus tissue of the winged bean has been achieved routinely on three different varieties. Callus tissue establishment was carried out either on a Murashige Skoog basal medium or B_5 salt-sucrose mixture supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), α-Naphthalene acetic acid (NAA), and kinetin, in combination of two both at 1 mg/l from segments of embryos, cotyledons, epicotyls of in-vitro grown seedlings or stems, roots and leaves of mature field-grown plants (6-8 mm.). Callus tissue transferred to a liquid medium grew as suspension cultures. Filtered suspensions of single cells and cell aggregates when plated on agar petri dishes formed round callus clumps. Calli from above sources developed leafy shoots on medium containing 1 mg/l of 6-benzylaminopurine (BAP) and/or kinetin at 0.1 or 1.0 mg/l. Subsequent transfer of the leafy shoots to a medium containing 1 mg/l indoleacetic acid (IAA) alone or with 1 mg/l indole-butyric acid (IBA) produced well-developed roots. Transfer of the fully-developed plantlets from asceptic cultures to the growth chamber and to greenhouse were very successful. Experiments were also carried out to induce somatic embryogenesis on liquid suspension cultures (with some preliminary success) and on those experiment on the analysis of salt tolerance and recovery. Analyses of the effect of a gradient of NaCl concentrations (1.0% - 10.0% - w/v) incorporated into the culture medium on growth of callus cultures showed that the winged bean callus tissue can tolerate a concentration of upto 2% (2 g [illegible]).

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A graduate student trainee from Sri Lanka arrived here on January 1985 and another in August 1985 and they brought several varieties of winged bean (international and ones which are useful to Sri Lanka) seeds. Because of quarantine limitations, only a few seeds (small number have been obtained). These seeds were grown in the greenhouse in order to have enough quantities of seeds for other physiological experiments.

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ISOLATION OF STRAINS, CLONES AND REGENERATION OF PLANTS FROM SINGLE CELLS OF WINGED BEAN

Grant #: DPE-5542-G-Ss-3056-00

1. INTRODUCTION AND OBJECTIVES OF THE GRANT:

This is the final report submitted to the Agency for International Development, Washington, D.C. 20523 for the grant period, August 1983 - January 1986 on the above grant. This report will describe the experiments that were carried out and described in the previous 3 reports (AID Reports, I II, III, 1984-1985) and of those experiments that have been carried out since then.

The advent of biotechnology and its applications to agricultural plants has opened up new avenues for crop improvement, particularly of cereals, seed and forage legumes, etc. When researches talk about "biotechnology" in plants, what they usually mean is tissue culture. Bollinger (1980) calls "tissue culture" as the "botanical equivalent of the laser" suggesting the vast area of potential applications of tissue culture to a variety of agricultural crops towards crop improvement. Therefore, the primary aims considered "a priori" for intensive research on crop plants such as the winged bean was to understand the mechanisms of genotypic differences in plant regeneration of tissue cultured cells and developing methods to overcome such differences by appropriate manipulations. The objectives of this grant were directed to: (i) establish tissue cultures from different varieties of Winged Bean and determine the response on different hormonal consideration and concentrations; (ii) isolate strains and clones from callus and suspension cultures; (iii) determine optimal conditions that will permit calli derived from various sources to

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undergo differentiation and regeneration of plants for subsequent plant propogation and (iv) establish methodology for the Selection of traits to tolerate stress conditions such as salt tolerance.

2. IMPLICATION AND RELEVANCE TO DEVELOPMENT:

This research on the protein-rich winged bean played a dominant role in the short and long range development and improvement in food production in LDC nations, particularly, Sri Lanka and other countries in Asia and Africa. In Sri Lanka, at present, the winged bean is grown in the backyard as a vegetable crop by individuals for their daily cooking. The establishment of the International Winged Bean Institute in Sri Lanka demonstrated that winged bean plays a vital role in expanding its resources for commercial production and for various industrial uses. In the Philippines, a national program on the winged bean has been targeted. The Govt. of Indonesia has taken winged bean as a priority research crop. In Ghana, the bean is adapted as a crop for its green pod and extensive research on agronomy, dietary use, etc. are being undertaken. In India, the green pod is grown for use as a vegetable in certain regions and states and some research is carried out to expand its germplasm resources. These are just a few of the tremendous impact and interest that the winged bean holds as a potential high-protein crop for the tropics, and for LDC nations.

This program provided valuable knowledge on the biotechnology and its applications on research on the winged bean to AID and helped in the development, application, and transferring technology of this knowledge to Sri Lanka and other LDC nations. The development of *in vitro* culture techniques on the winged bean offered an alternate method in comparison to the conventional plant breeding programs because with this method, it is possible to handle much larger populations of cells under

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standardized conditions. In effect, the tissue culture vessel containing tissues and cells (viz., test tubes or petri plates) is comparable to a plant breeder's field of heterozygous plants where conventional plant breeding programs require large hectares of land and longer periods of time in comparison to the tissue culture laboratory where selection can be made within limited space and short period of time. Selection of plant materials (cells and tissues) to stress tolerance and regeneration of plants from these which are resistant to NaCl will be advantageous to LDC nations.

The basic development goals of most LDC in the world include methods for increased food production and the development of high protein-rich food crops. The winged bean shows a promising potential towards this goal. To achieve this, the LDC need extremely large quantities of seeds (particularly for countries in Africa). Every effort which can lead to increased seed production or development of plants in large numbers for mass propagation using tissue culture, viz., organogensis or somatic embryogensis, will relieve the shortage of plant stock materials. Particularly, through somatic embryogenesis (some initial success was achieved in this laboratory and generally very difficult with legume crops), it is possible to produce thousands and thousands of potential plants which can be transferred to the greenhouse and eventually transported world-wide to different regions. The selection of stress-resistant plants will increase an added dimension to this program.

The application of this research on the winged bean and development of technologies to analyze variation and selection of stable variants will offer the farmers and research workers worldwide. This research which was carried out on the different varieties and cultivars from Sir Lanka and other representative regions of the world and the utilization of the results will be very significant worldwide.

The LDC nations need tremendous economic resources, trained manpower and assistance to convey the know-how and practical applications to the farmers. Most of

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several of the LDC lack this or have a tremendous shortage of such assistance. After the training which the graduate students and/or research personnel obtain here, they will return to their native land and enter the work force there, e.g. in Sri Lanka, at the International Winged Bean institute. Such training assistance (short term) or long-term research can be offered to participants from other LDC also, according to their needs.

Expansion of winged bean cultivation is of vital importance, in view of the immense potential the crop has in improving the standard of nutrition in LDCs like Sri Lanka. It is unique in its potential for village scale development in view of the numerous possible uses of the crop, for "food, feed, fodder and fertilizer".

This research on the winged bean and tuber cultivation will also lead to the creation of many village scale industries for the production of winged bean milk and milk-based products like yogurt, also winged bean coffee and other beverages. Further, large scale industrial projects for the manufacture of winged bean seed oil, leaf protein concentrates and pellets for animal feed are other possible benefits.

Winged bean also has potential in rehabilitation and development of denuded or degraded lands as it can be grown as a live mulch or cover crop either singly, or in combination with commercial perennial crops. The marginal tea lands in the mid-country of Sri Lanka can be rehabilitated by this means, provided adequate quantities of desirable planting material are available. The experiments on 'salt resistance' will help develop plants that could be grown on saline/alkaline soils. These soils are increased in extent in LDCs. Thus the availability of plants that could grow in such soils would reclaim areas with salinity problems and thereby increase land availability and productivity.

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3. INNOVATIVE ASPECTS AND SIGNIFICANCE:

This research was innovative because: (i) the winged bean, which is an important legume crop plant, specifically suitable for LDC nations as a high-protein source, was established in tissue culture, particularly as liquid suspension cultures and plating of cell types, both of which offered the additional advantage to expose the cells uniformly to mutagenesis, stress conditions, etc.; (ii) the recent initial observation of somatic embryogenesis in liquid suspension cultures and therefore raising the possibility of producing plants in very large numbers; and (iii) the development of a successful procedure to regenerate plants routinely. Therefore, stable variants (natural or induced) could be analyzed, selected and new plant types with desirable characteristics could be obtained. This was a significant contribution to the area of modern plant tissue culture biotechnology, since most legume plant systems generally do not respond or show regeneration capacity.

This research has lead to biotechnological development of new legume types, rapid multiplication techniques and selection for tropical stresses such as salt. Since incompatabilities have been identified in interspecific as well as intraspecific hybridizations, this research could lead to approaches of overcoming the barriers to crossing. Unlike other legume crops the tuber tissues of winged bean would also be available for microprogagation and genetic engineering.

This work involved a new approach to the breeding and development of winged bean. It involved a more intimate contact with individual cells rather than whole tissues (e.g. seeds or gametes).

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4. CURRENT LITERATURE REVIEW ON TISSUE CULTURE OF WINGED BEAN:

The earliest report of the tissue culture of the winged bean was recorded by Venketeswaran and Huhtinen (1978). Since then only a very few other articles have appeared (Bottino et al., 1979; Gregory et al., 1980; Brunel et al., 1980; Mehta and Mohan Ram, 1981; Trin et al., 1981; Venketeswaran, 1981). Other botanical and agronomical aspects of the winged bean has been well-documented (Report, 1984; The winged bean, 1977; The winged bean, 1978; The winged bean, 1981; The Winged Bean Flyer(s) 1977-1983).

5. TECHNICAL WORK PLAN:

(i) Materials: Seeds of several varieties of winged bean, *Psophocarpus tetragonolobus* (L.) DC were obtained from the seed collections of the International Winged Bean Institute and the University of Peradeniya, Sri Lanka. These seed varieties represented material from different geographical locations in the world, particularly from Papa New Guinea, Nigeria, Sir Lanka, Thailand and Indonesia. Our present collection included 11 from Sri Lanka, 4 from Papa New Guinea, 3 from Nigeria and 2 from Thailand (Appendix III).

Experiments were carried out on as many varieties as possible (depending on the particular nature of the experiment and the response of the variety). Special emphasis were placed on those varieties which are of significant importance to Sri Lanka, Papa New Guinea, and Nigeria and those 6 varieties in which certain experiments have been carried out already (AID Reports, I and II, 1984). Plants from all these varieties were also grown in the greenhouse to obtain research material (stems, leaves, flowers, etc.) and new seeds for various experimental procedures. The

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following are the varieties:

SLS 23	(Sri Lanka)	UPS 122 (Papua New Guinea)
SLS 1	(Sri Lanka)	TPT-1 (Nigeria)
SLS 44	(Sri Lanka)	LBNC 844 (Indonesia)
SLS 93	(Sri Lanka)	TpT-2 (Nigeria)
UPM 282		TPT-B
Other Sri Lanka vars.		Thailand var. (2)
Chimbu		

Although several of the experiments on various varieties were be initiated and continued here, efforts were also made to initiate collaborative research on these and others at the Winged Bean Institute and at University of Peradeniya, Sri Lanka where a tissue culture laboratory was being started.

(ii) Culture medium: The culture medium which was employed on the winged bean was the modified Murashige and Skoog (1962) medium, referred to as MS medium. In certain experiments, a modified mineral salt solution (Gamborg et al., 1968), referred to as B_5 medium was employed. These two media were routinely used in experiments with addition of the different growth hormones at appropriate concentration (mg/l) to obtain the necessary response (Appendix I). Certain modifications to this medium, like $\frac{1}{2}$ MS, or replacement of certain salts with others, omission of agar (for liquid suspension cultures), addition and/or deletion of certain chemicals according to a particular experiment were made.

(iii) Callus Establishment, Organogenesis Regeneration Experiments: As reported earlier (AID Reports I and II, 1984), standard aseptic tissue culture techniques (Venketeswaran, 1981) were employed and as shown in the protocol (Appendix II). The three explants used (a) hypocotyl axis from embryos of soaked seeds; (b) 1 cm. segments from elongated regions of germinated seedlings and (c) 1 cm. segments from stem of plants. In addition, other plant parts like, thin slices of cotyledons, punched out circular leaf segments, buds, etc. were used for comparison. After asep-

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tic procedure, the explants were induced to form callus masses in mainly three different media. Histological observations were made to observe the origin of the callus. After 6 weeks, the callus masses were transferred to shooting media (SM) with four different hormonal combinations and concentrations, to rooting media (RM), and the callus maintenance media (CMM)(See: Protocol for regeneration). Other manipulations for controlled initiation of organ primordia and eventually regeneration of plantlets were followed, since each tissue has its own requirement and the phenomenon is dependent on a number of factors like genotype, varietal differences, age, endogenous growth hormone levels + other physical factors. [Table 1 shows the responses of the different winged bean varieties which were selected for these experiments. As is evident, callus production was very pronounced in TPT-1, TPT-2, UPS-122 and SLS-1. Organogenesis, vz., root production was also observed in these varieties. Some of these responses are illustrated in plates 1,2 and 3].

(iv) Liquid suspension cultures, Isolation of strains Regeneration: Techniques were perfected to grow liquid cultures of winged bean routinely which form suspensions of cell aggregates, small clumps of cells and single cells, etc. and isolation of "clones" was possible in some varieties. These methodologies were extended to as many of the other seed varieties and cultivars which were available. Organogenesis and eventually regeneration of plantlets were analyzed from small clumps of cells and single cell clones, by variation in growth hormone (change of auxin: kinetin ratio). The regenerated plants were examined for different phenotypic expression and desirable characteristics. Particular attention was paid to the frequency of subcultures, viz., age of the culture, time after the subculture, the history of the suspension culture, etc.

Success with somatic embryogenesis on legume suspension cultures are limited (Phillips and Collins, 1980; Cheng et al., 1980; Kimball and Bingham, 1973).

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Christianson et al., (1983) refers to them as incomplete somatic embryogenesis. No convincing histological evidence has been presented.

Some embryogenesis at random, was observed in this laboratory in suspension cultures of winged bean.

A flow chart depicting the procedure to induce somatic embryogenesis is presented in the next page. Appropriate changes and/or modifications were made as and when necessary.

Table 2 shows the response of three varieties, viz. TPT-1, TPT-2 and UPS-122 to culture medium combination to form somatic embryoid or embryoid-like structures in liquid suspension cultures. These embryoid-like structures often isolated in the culture and when transferred to solid agar media grew normally. These results suggest that regeneration of plantlets through somatic embryogenesis is another possibility with winged bean cultures.

(v) Organogenesis and regeneration in tissue and cell cultures: Although the induction of organs and regeneration of plants have been attempted in several forage and seed legumes, there has been only limited success by various investigators (Gresshoff and Mohapatra, 1981). In soybean, which has been studied most extensively in tissue culture, there is yet no routine procedure reported or available to obtain regeneration of plants (Phillips and Collins, 1981).

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TABLE 1. Response of winged bean varieties to different combinations of hormones.

Media/Variety	TPT-1		TPT-2		SLS-1		SLS-44		25-01		UPM-282		UPS-122	
	C	R	C	R	C	R	C	R	C	R	C	R	C	R
MS	+		+						++	SHOOT			+	
MS + 1 D	++		+		++								+++	
MS + 2 D	+++				+++		+		+++				++++	
MS + 5 D	+++		+++		+				+				++++	
MS + 1 N	+++		+				+						+++	
MS + 2 N	+++		+++		+++		+						++	
MS + 5 N	+++		++		+++								++++	
MS + 1 I					+++				+				+	
MS + 2 I	++				+		+						++	
MS + 5 I	++				+++				++				++	
MS + 1 D + 1 N	++++		+		+				+++		+++		+	
MS + 1 D + 2 N	+++		+		+				++				+++	
MS + 1 D + 5 N	+++		++		+				+				+++	
MS + 2 D + 1 N	++++		+++		+				++				++	
MS + 5 D + 1 N	+++				++								++++	
MS + 1 D + 1 I	+		+		+				+++				+	
MS + 1 D + 2 I	++				+									
MS + 1 D + 5 I					+++		+						+++	
MS + 2 D + 1 I	+++		++		+++				++				+++	
MS + 5 D + 1 I	+++								+++				+++	
MS + 1 N + 1 I	+++		+++						++				+++	
MS + 1 N + 2 I	+++		+++		+				+++				+++	
MS + 1 N + 5 I	++		+++		+++								++	
MS + 2 N + 1 I							+++				+		++++	
MS + 5 N + 1 I	+++		++		+++								++	
MS + 1 K	+++		+++		+				++		++		+++	
MS + 2 K			+		+++		+		++		++		+++	
MS + 5 K	++-		++		+++		+		++++					
MS + 1 K + 1 I	+++		+						++					
MS + 1 K + 1 N	+++		+		+++				+++		+		++++	
MS + 1 K + 1 D	+++		+		+++				+++				++++	
MS + 1 I + 1 N + 1 D			+		+				+		+		+	

LEGEND FOR TABLES

MS - Murashige - Skoog Medium
D - 2, 4-Dichloro Phenoxy Acetic Acid (2,4-D)
N - Napthalene Acetic Acid (NAA)
I - Indol. Acetic Acid (IAA)
1, 2, 5 - (Prefixes) - Hormone conentrations in mg/l
C - Calli formation + = Little, ++ = Fair, +++ - Good, ++++ = Excellant
R - Root V = Roots formed

Varieties:

TPT-1, TPT-2 Nigerian varieties
SLS-1, SLS-44 - Sri Lankan varieties
UPS -122, Papua New Guinea varieties

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FLOW CHART

INDUCTION OF SOMATIC EMBRYOGENESIS IN WINGED BEAN

Seeds —4 wks→ Calli Initiation in MS + INAA + 1 2,4-D Agar Medium —4 wks→ MS + 1 2,4-D Agar Media —4 wks→ 1/2 MS and MS liquid Culture media —3-4 wks→ Embryoid

4 wks

Sub Culture

in MS + 1 NAA + 1 2,4-D

Agar Medium

(Calli maintenance media)

TABLE 2. Media which induced embryoid or embryoid structure formation.

Variety	Initial Medium Solid	First Subculture Medium Liquid
TPT - 1	MS + 5 NAA	MS + 1 2, 4-D
	MS + 2 4-D + 2 NAA	MS + 1 2, 4-D
UPS - 122	MS + 1 2, 4-D	1/2 MS
	MS + 5 2, 4-D	MS
	MS + 5 NAA	MS + 1 2, 4-D
TPT-2	MS + 1 IAA + 2 2, 4-D	MS

Previous observations on the tissue culture of winged bean was reported in an abstract (Venketeswaran and Huhtinen, 1978; AID Reports I, II III). The results reported here, demonstrate that induction of organs and regeneration of plantlets can be obtained on the winged bean using explants as indicated in Table 3 and with different media composition.

Roots and shoots developed and subsequently, plantlet formation was possible in several varieties. The results obtained on 3 varieties are shown in Table 3. It was also possible to obtain plantlets from callus through manipulation of growth hormones. A possible protocol for regeneration of plantlets from callus is suggested although different varieties and different sources of callus show different response with no definte predictability.

PROTOCOL FOR REGENERATION OF PLANTLETS

Step I:	Callus masses growing as stock cultures in culture medium (CMM) (1 mg/l each of 2,4-D and Kinetin) (1 mg/l eachof NAA and 2,4-D)	
Step II:	Transfer to shoot root-inducing medium (SM) (1 mg/l to 2 mg/l BAP)	shoots formed in two to three subcultures
Step III:	Transfer shoots to root-inducing medium (RM) (1 mg/l of IAA or IBA)	roots formed in 3-4 weeks

Step IV:	Transfer plantlets with shoots and roots from agar to liquid medium (cheese cloth base)	2-3 weeks
Step V:	Remove plantlets, wash in water several times non-asceptically -- let stand 2-3 hours. Transfer to vermiculite-perlite soil mixture (nonasceptic). To minimize bacterial and fungal growth and contamination, dip roots in $CuSo_4$ + antibiotic solution before planting. Maintain plants in incubator for production of new healthy green leaves	2-3 weeks
Step VI:	Transfer to growth chamber (4 weeks) to greenhouse.	

(vi) Selection of Stress tolerance, viz., Salt: A number of different procedures have been developed to demonstrate and use selection procedures for stress tolerance like salt, drought, metal ion like Aluminum, etc. (Dix and Street, 1976; Croughan et al., 1978, Meredith, 1978; Nabors, 1980; Tal, 1983; Bressan et al., 1981). In general, either callus masses or suspension cultures or plated cells on agar surface are used and they are exposed to different concentrations of NaCl. The use of suspension cultures and plating of cells have an obvious advantage since the cells are freely exposed to the selection treatment and such a procedure will be used in this study. The following procedures were adopted:

Table 3. Organogenesis response of different varieties and explants* of different origins to benzyl amino purine. (BAP)

Variety	Explant* Origin	Seed Incubation	Calli Initiation	1st SOB. CUI	Response
UPM-282	Shoot	MS + 1 BAP	1/2 MS + 1 BAP	1/2 MS	1 Shoot 5 Roots
	Root	DO	DO	MS	Plenty of Roots
	Shoot	DO	DO	MS + 1 BAP	11 Shoots
	Shoot/Root Collar	DO	DO	MS + 1 BAP	6 Shoots
	Shoot	MS + 0.5 BAP	DO	MS	8 Roots
	Shoot	DO	DO	MS + 1 BAP	CALLI.
	Shoot	DO	DO	MS + 5 BAP	CALLI.
	Root	DO	DO	MS + 1 BAP	CALLI.
	Shoot/Root Collar	DO	DO	1/2 MS	1 Plantlet 3 Shoots 11 Roots
	Shoot/Root Collar	1/2 MS + 1 BAP	DO	MS	3 Plantlets
	Shoot	DO	DO	MS + 5 BAP	CALLI.
SLS-1	Shoot	MS + 0.5 BAP	DO	MS + 1 BAP	CALLI.
	Root	DO	DO	MS + 1 BAP	CALLI.
	Shoot/Root Collar	DO	DO	1/2 MS	1 Shoot
TPT-8	Shoot	MS + 1 BAP	DO	MS	Roots
	Shoot/Root	DO	DO	1/2 MS	3 Shoots Plenty of Roots.
	Shoot	DO	DO	MS + 1 BAP	CALLI.
	Shoot	DO	DO	MS + 5 BAP	CALLI.

* Explant obtained from seeds which germinated in the seed incubating medium for 7 days.

Callus masses (200 - 500 mg.) each derived from established tissue cultures (derived from stem and epicotyle segments of seedlings var. LBNG 3) were transferred to MS medium + 0.5 mg/l NAA and 2.0 mg/l BAP containing NaCl concentrations of 0.0%, 1.0%, 2.0%, 5.0% and 10.0% - w/v). Appropriate quantities of NaCl were weighed and dissolved in the culture medium before autoclaving. The above medium was poured into petri plates and allowed to gel. The experimental set-up consisted of 4 petri plates with 4 individual callus pieces in each petri plates were sealed around with para-film. Cultures were allowed to grow in a growth chamber maintained at 24 $\pm$ 1°C with 12 hrs./day illumination. After 4 weeks, the weight of each of the individual callus mass were determined for each concentration. The results were tabulated and analysed statistically.

The histogram and Figures show the quantitative data and the growth of the callus pieces of winged bean in control medium and with different concentrations NaCl incorporated into the culture medium. The quantitative data was obtained by recording the initial and final fresh weight of each of the individual callus masses and determining the amount of growth in four weeks. The callus pieces appeared healthy and green and grew extremely well in the control medium after four weeks of growth. At a concentration of 1.0% NaCl, several of the cultures grew reasonably well and appeared comparatively healthy although the amount of growth was reduced. In comparison, at a concentration of 2.0% NaCl, the growth of the callus declined very considerably to the extent that there was very little growth. At 5.0% and 10.0% concentrations of NaCl, the callus masses turned brown and died off.

These experiments suggest that the callus cultures of winged bean can tolerate NaCl concentration levels up to 2.0% and it may be possible to obtain salt-tolerant callus tissue within the concentration level of 1.0% - 2.0%.

(vii). ELECTROPHORESIS OF WINGED BEAN CALLUS PROTEINS

Electrophoresis of winged bean callus protein extracts was carried out using the SDS/PAGE Bio Rad method. The results obtained were compared with electrophoresis patterns of tissues from which callus were obtained, namely, seeds, leaves, petioles and stems. Procedures adopted and preliminary results obtained are given below.

(a) Sample Preparation - Protein Extraction: Plant parts (2 - 3 gm dry wt.) were ground with glass beads using a chilled mortar and pestle placed in ice. A 0.1% SDS solution was found to be the most suitable extraction solvent. Water, 0.1 M KCl, 1 M NaCl and K Phosphate buffer were also tried. The extract was filtered through Whatman's #2 filter paper and the filtrate was centrifuges for 15 mts at 3000 rpm, to separate any debris. The supernatant was then treated with excess 20% TCA to precipitate proteins and then centrifuged at 3000 rpm for 30 mts. The supernatant was discarded and the protein pellet was redissolved in 0.1 M NaOH. The protein solution was mixed with sample reducing buffer at a ratio of 1:4. The reducing buffer had the composition recommended in the BIO RAD protocol.

(b) Electrophoresis: The recommended protocols for making gels (BIO RAD method) we adopted. Initially, 7.5% gel were used. However, it was observed that resolution was poor, with bands generally being faint or absent or concentrated as a single band at the bottom of the gel or dye front. Variation of running conditions (current, duration of run, buffer dilutions) did not have any beneficial effect. The presence of the single protein band at the dye front indicated that the sizes of the proteins were small and that they were being carried through, without being separated. This problem was overcome to a large extent by reducing pore size of the gel, which can achieved by increasing the amount of Acrylamide to 12%. A variety of running conditions were tried and the following was found to be the most satisfactory, with respect to resolution and running time; viz., 100 V - 1 hr; 200 V - 4 hr;

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300 V - $\frac{1}{2}$ hr. Higher voltages/currents (eg 500 V) gave good resolution of high molecular weight proteins, but low mol. wt. protein bands tended to be faint and distorted. Lower currents/voltages gave poor resolution of large proteins, which tended to give a dense band at the top of the gel and also took over 6 hours. A 4.0% gel was found to be suitable for the stacking gel.

(c) Staining Destaining: Overnight staining in 2% Coomassie Blue in 40%:10% methanol/acetic acid aqueous solution was necessary to give prominent bands. Stained gels were destained for 4 - 6 hours in 10%: 7.5% methanol/acetic acid aqueous solution until bands were clear. Complete removal of the stain from regions of the gel other than the protein bands required soaking in water for 2 days. However, such gels were fragile. A reduction in intensity of staining too was also evident. Silver staining using the Bio Rad kit was successful. This method is more sensitive than Coomassie Blue staining and indicated the presence of more protein bands. However, estimation of molecular weights was found to be difficult as the protein standards used gave more bands than they should have. Hence, more pure protein standards need to be used if silver staining is to be routinely adopted.

(d) RESULTS FROM CALLUS EXTRACTS: Comparison of bands from calluses of 3 varieties, viz. SLS 40 (Sri Lanka), UPS 122 (Papa New Guinean) and TPt 2 (Nigerian) showed distinct differences. UPS 122 had one unique protein (estimated mol. wt. 70,000), TPt 2 three (mol. wt = 400,000; 25,000 and a peptide of MW 1800) and SLS 40 two (mol. wt. = 340,000 and 27,600) Table 4. The origin (plant part) of calluses of TPt 2 and UPS 122 were seed cotyledon and that of SLS 40 was leaf petiole. This callus had both similar (one band) and dissimilar (one band) proteins when compared to leaf petiole extracts. The number of bands observed for leaf petiole extracts was more than in callus extracts, indicating that not all the proteins synthesized by leaves petioles were being synthesized by callus. This could be due to the absence

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of green pigmentation and photosynthesis in callus cells.

Table 4

Estimated molecular weights of distinct proteins observed in calli:

UPS 122	TPt 2	SLS 40
70,000	400,000	340,000
	25,000	27,600
	1,800	

These results indicate that isozyme studies will provide interesting results with regard to varietal origin and also genetic stability of calluses and callus derived plants.

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LEGEND FOR PLATES

Plate 1: Callus initiation from cotyledons of varieties TPT-1, UPS-122 and SLS-1 in hormones kinetin (K), α-Naphthalene acetic acid (NAA), Indol acetic acid (IAA) and 2,4-dichloro diphenoxy acetic acid (2,4-D)- (Refer Table I).

Plates in the photographs

Columns - left to right	1st - "K" treatment
	2nd - "NAA" treatment
	3rd - "IAA" treatment
	4th - "2,4-D" treatment
rows - top to bottom	1st - 1 mg/litre concentration of hormone
	2nd - 2 mg/litre " " "
	3rd - 5 mg/litre " " "

Plate 2: Response of the same varieties (as in plate 1) to combinations of hormones, in calli initiation.

Plate 3: (7) Roots formed in a clump grown in MS salt only liquid culture of which the calli was initiated with NAA (1 mg/litre)

(8) Root formation in primary culture of cotyledons.

(9) Embryoids isolated from liquid culture.

Plate 4: Root formation in liquid culture.

Plate 5: Regenerated plants transferred to soil, perlite 3:1 media and finally to greenhouse (18).

PLATE 1

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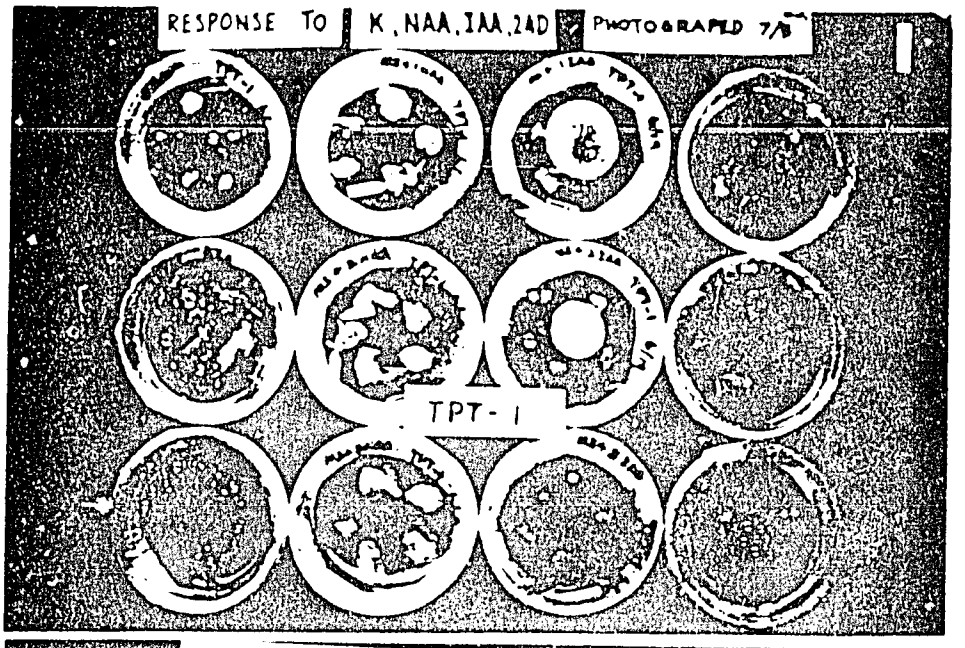

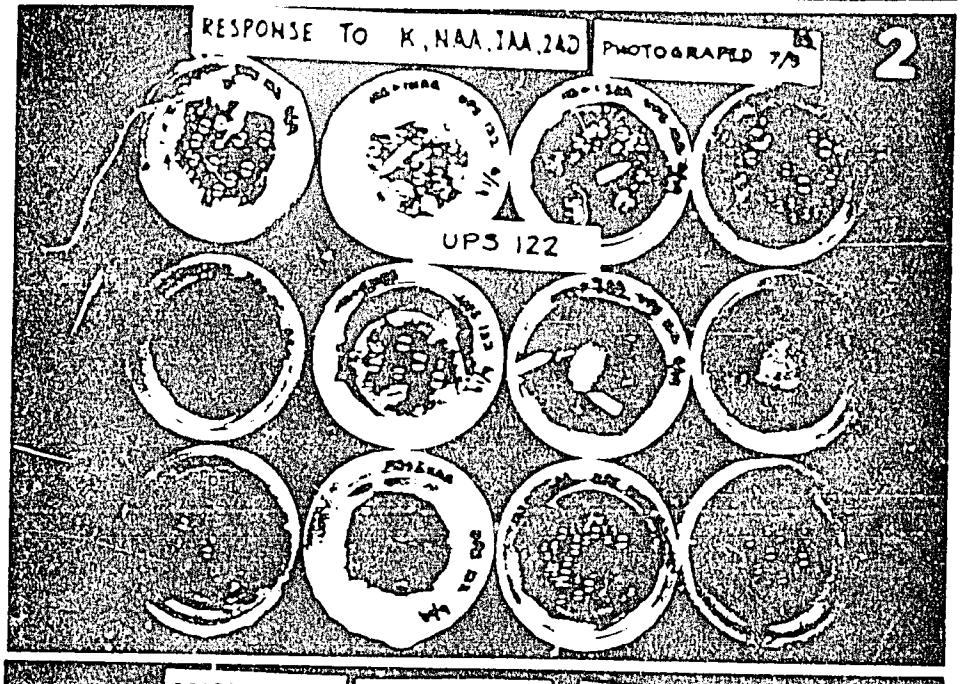

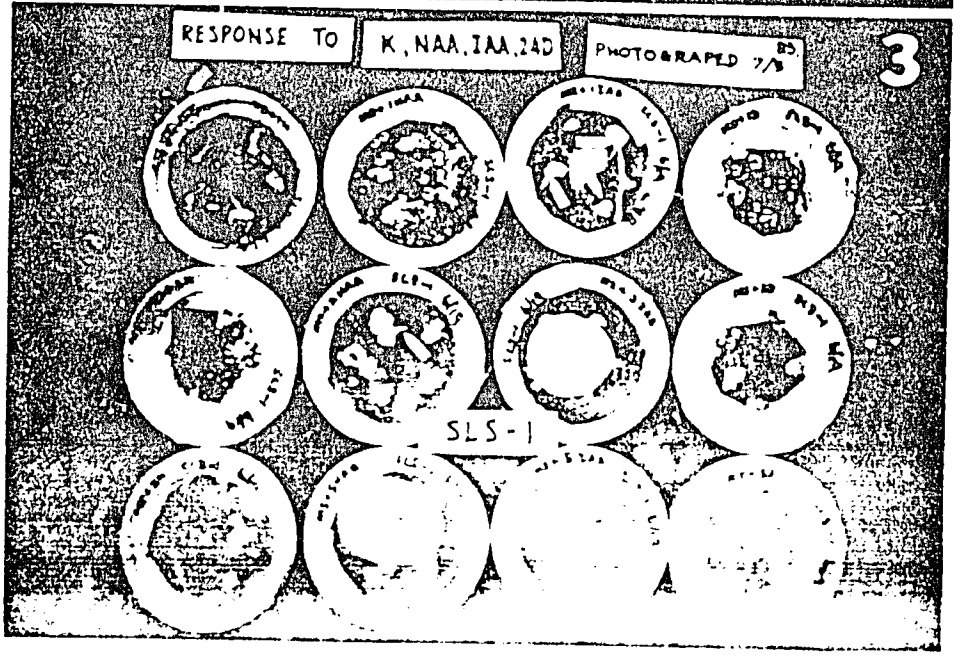

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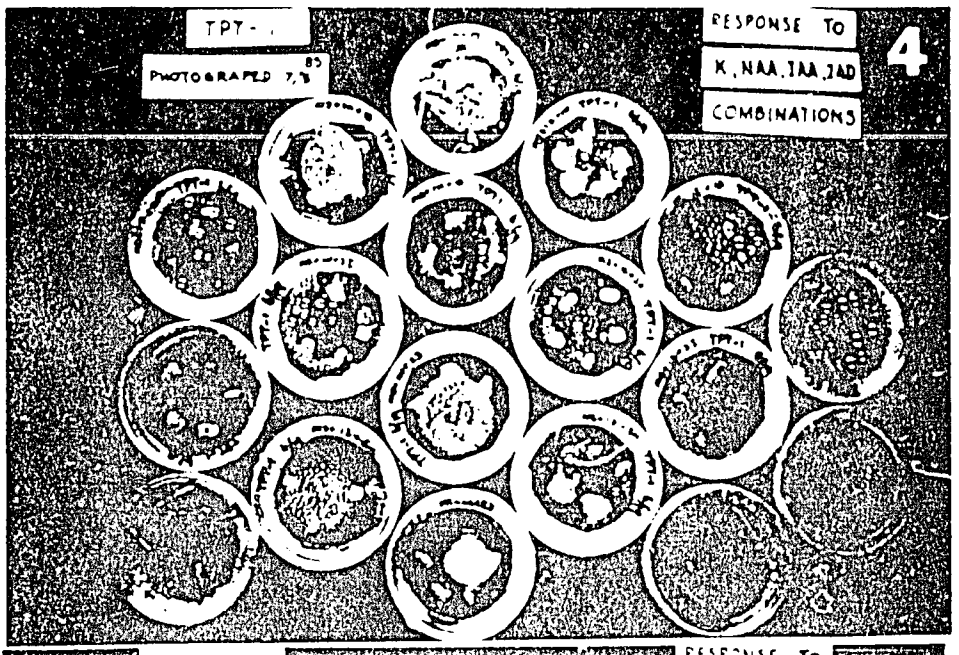

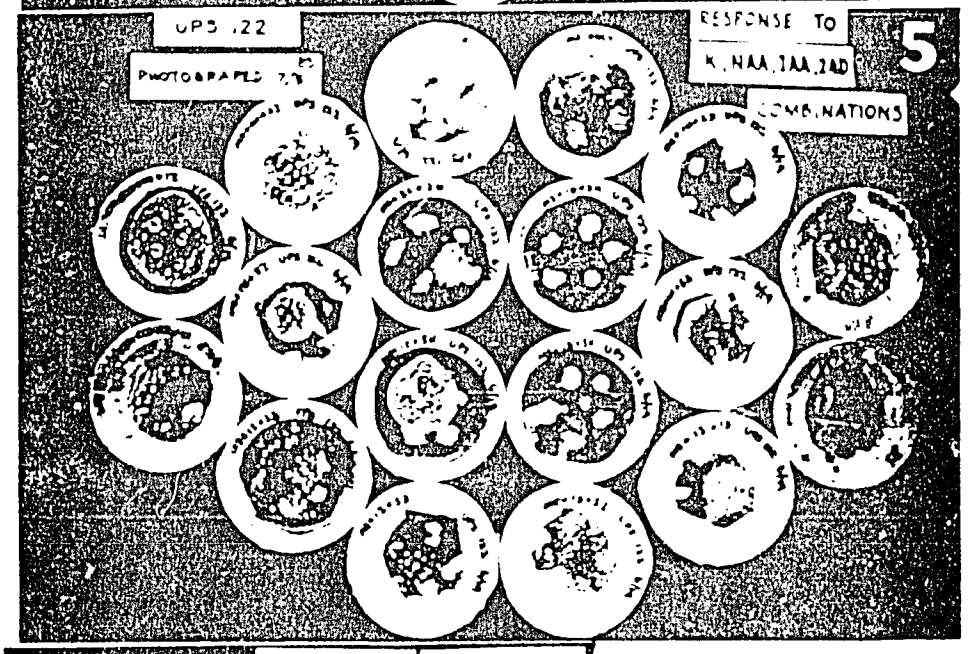

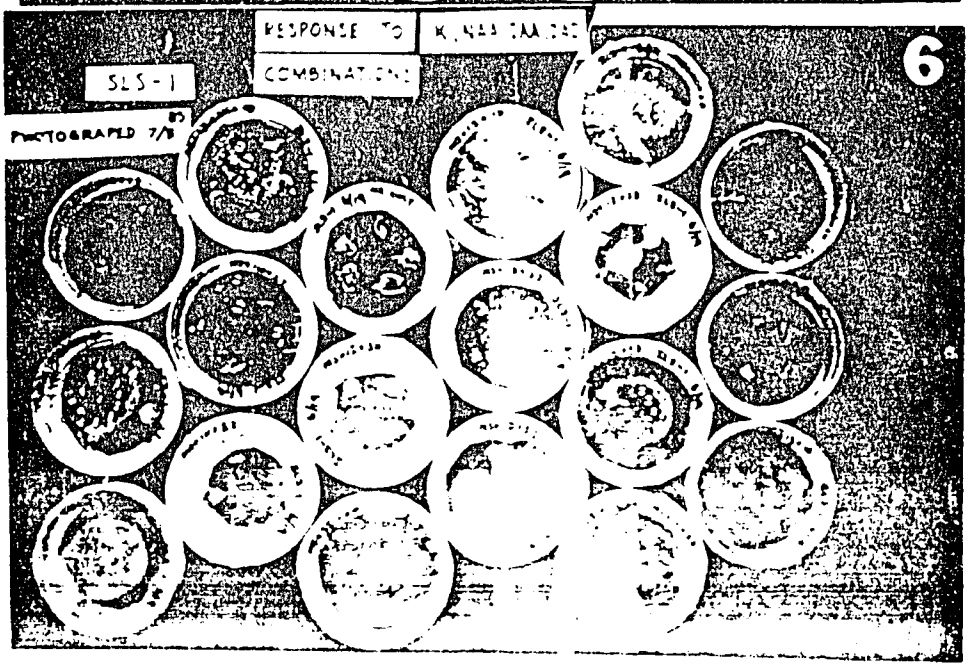

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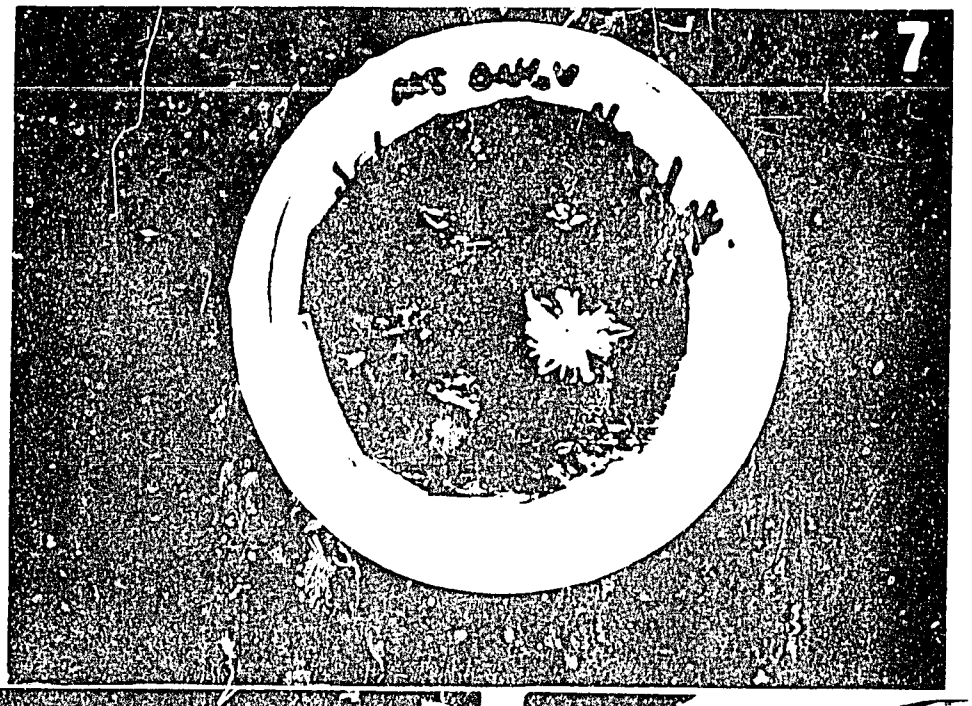
PLATE 3



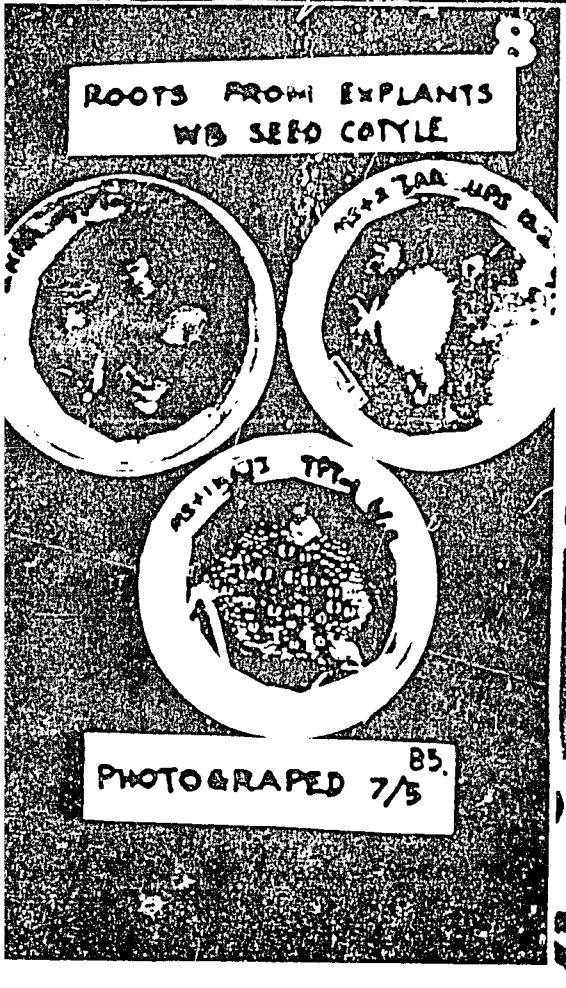


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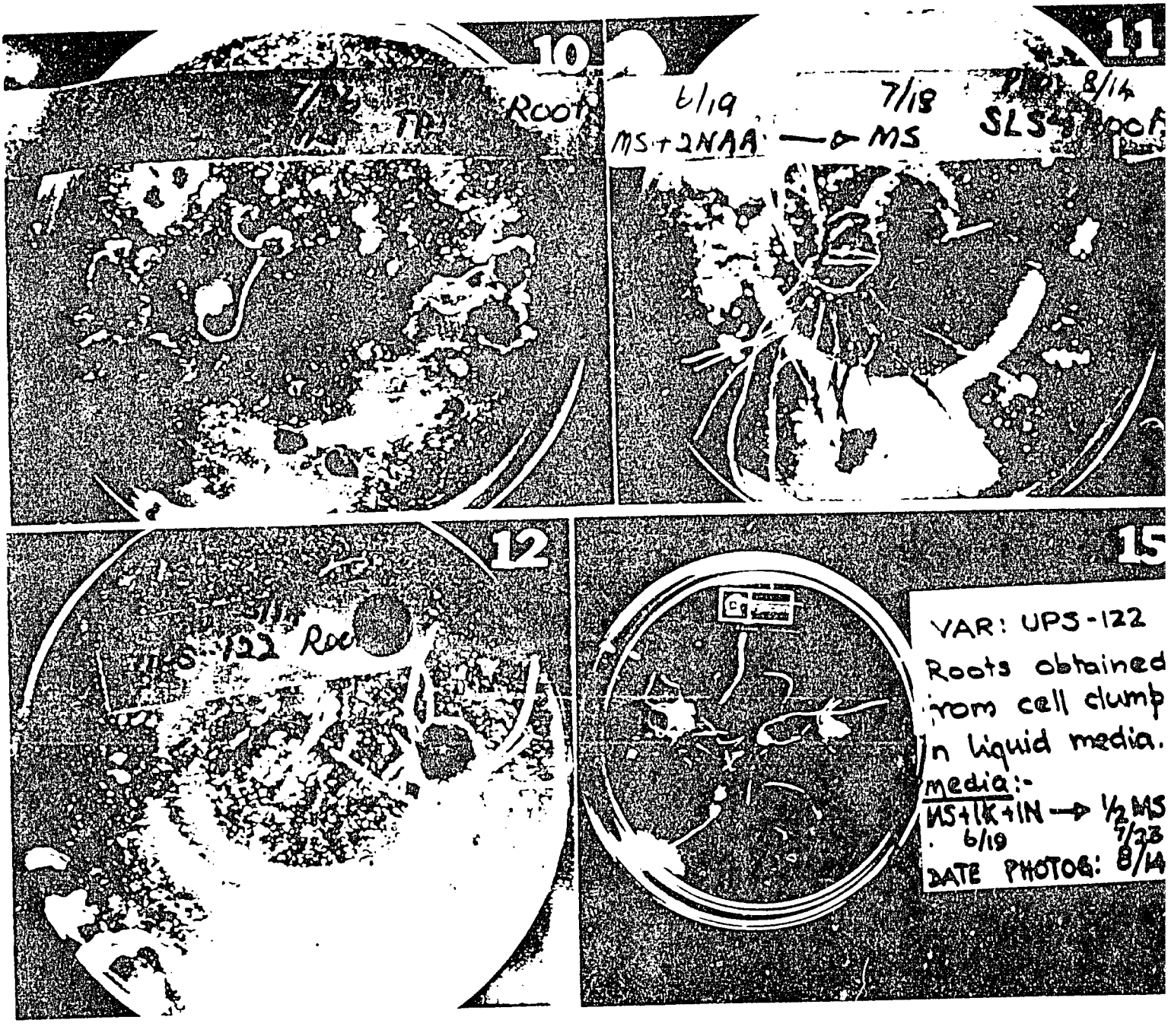
PLATE 4



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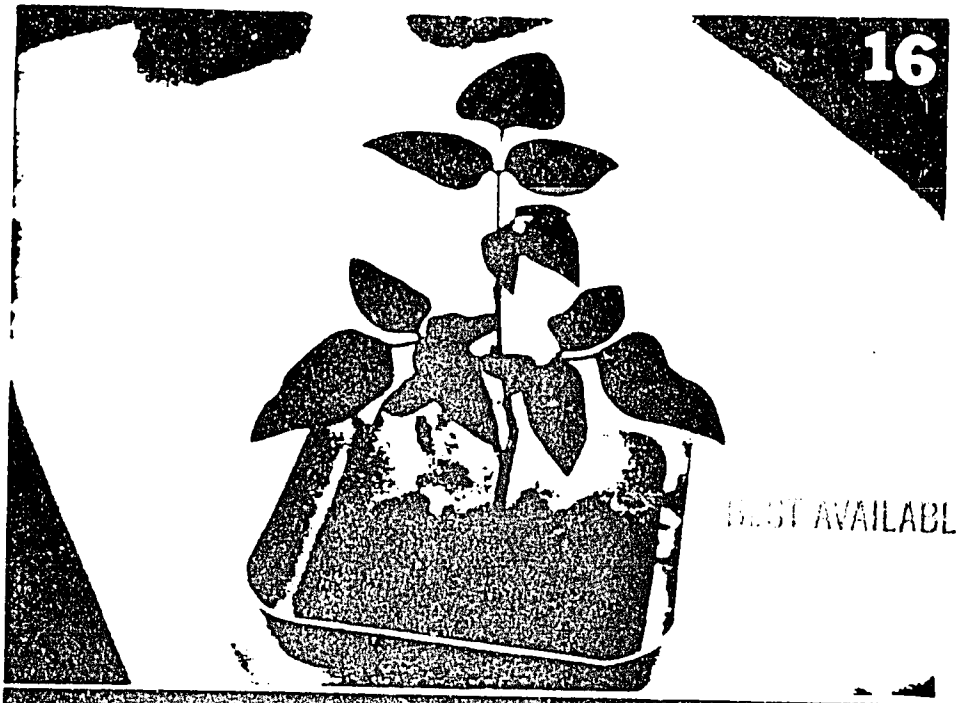

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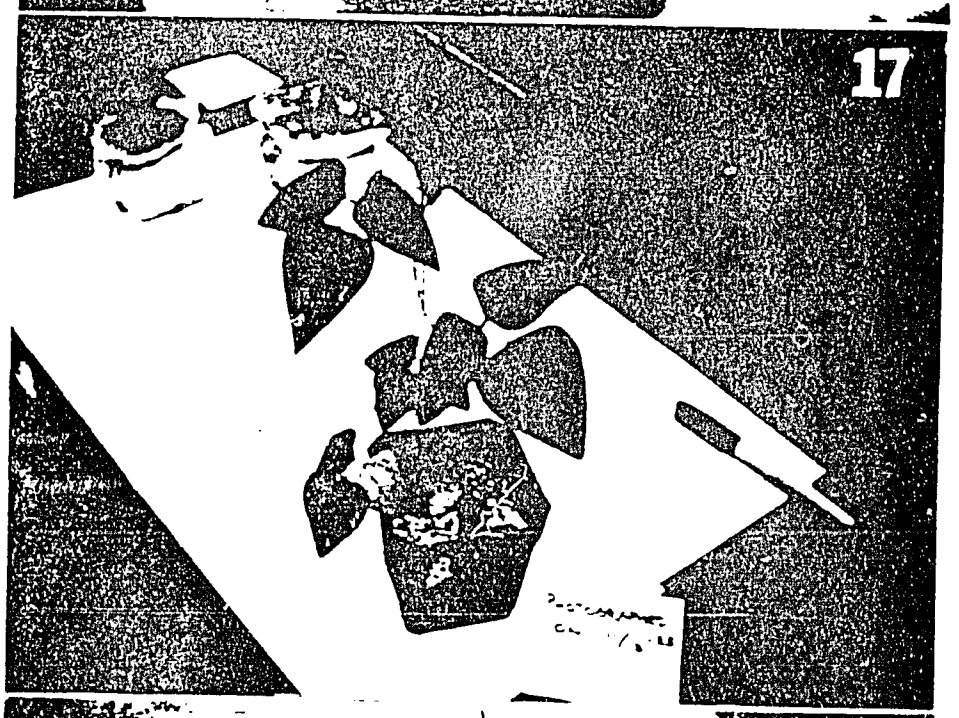

7. TIME SCHEDULE AND WORK PLAN CARRIED OUT DURING THE GRANT PERIOD

1st Year: Establishment and maintenance of callus in solid media and as suspension cultures of as many varieties as possible. Plating experiments, single cell isolation, cloning for isolation of strains, etc. and routine growth analyses, cytology, etc. Somatic embryogenesis and regeneration from somatic embryos.

2nd Year: Regenerated plants from all the above transferred and maintained in growth chamber, greenhouse, etc. Observational records and analysis of the variability.

Experiments on stress conditions, viz., salts. Electrophoresis of tissue cultures and plants.

8. SCIENTIFIC COLLABORATION

This was established with Prof. Dr. Walter Herath, Deputy General of the International Dambala (Winged Bean) Institute and Profs. H.P.M. Gunasena and Y.D.A. Senanayake of the University of Peradeniya, Sri Lanka, for the past two years. After advertisement and through selection procedures in Sri Lanka, two graduate students were recruited for training in tissue culture in this laboratory (University of Houston). Mr. Dilip Dias arrived here in January 1985 and is currently enrolled as a graduate student in the Department towards an M.S. Degree (2 years). The second student, Mr. Sultanbawa arrived in August and started in the Fall 1985 (September) towards his Ph.D. program while working in this laboratory. Both the students took courses (as advised by the P.I. and colleagues in the Department) which will enable them to be highly trained in Plant Physiology, Tissue Culture, Cell Biology, Biochemistry and other courses related to Biotechnology and the appropriate instrumentation. Mr. Dias worked on the tissue culture aspects of winged bean and both the students will be doing their dissertation topics for their respective degrees on specific aspects of tissue culture, embryogenesis, regeneration, variability and selection of variants, etc. on the winged bean. The education and training of these students and others enabled them to learn the most modern biotechnological aspects of cell culture and in turn very valuable for them as teachers and research workers when they return to Sri Lanka, particularly to the Winged Bean Institute, and/or University of Peradeniya, where they will be absorbed in the work force (as indicated in the letter). Mr. Dias was working at the University of Peradeniya prior to his coming to the U.S.A. and Mr. Sultanbawa was working at the Winged Bean Institute.

As indicated earlier, most of the proposed tissue culture work were carried out initially at the University of Houston. The P.I. (S.V.) was working 9 months of the

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year (15% time) at no salary and 3 months in summer (100% time) setting up experiments and administering the research, while at the same time supervising, advising and training the graduate students. The graduate students, the P.I. and technical help carried out the experiments in the laboratory and in the greenhouse raising plants from tissue culture and maintaining stock varieties of plants, etc. the collaboraters in Sri Lanka, viz., those at the Winged Bean Institute and the University of Peradeniya grew winged bean plants in the field and supplied seeds for experiments. Copies of all correspondence are included at the end of the report.