-1-

PD-AAX-191
54375

3.A-03

FIRST

PROGRESS REPORT

ON

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

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

for period ending January 31, 1984

Submitted to:

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

Dr. S. Venketeswaran
Principal Investigator
Department of Biology
University of Houston
Houston, Texas 77004

Dated February 23, 1984

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

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

The following is the first Progress report of the technical performance submitted to the Project Officer, Agency for International Development, Washington, D.C. 20523 on the above grant describing the accomplishment of work carried out from August 5, 1983 to January 31, 1984. The overall objectives and goals of this contract are towards isolation of strains, clones and regeneration of plants from single cells of the winged bean, *Psophocarpus tetragonolobus* (L) DC using a variety of procedures of tissue culture technology.

This report will describe experiments which have been carried out and which are in progress on six different varieties of the Winged Bean. The seed material for these investigations was acquired mainly from Sri Lanka during the P.I.'s visit to attend the International Seminar on Winged Bean at Sri Lanka and from domestic available sources. The work is presented mainly along the lines of: (i) establishment of callus and suspension cultures; (ii) induction of organogenesis in tissue and cell cultures; (iii) initial analyses of the effect of a gradient of NaCl concentrations incorporated into the culture medium on growth of callus cultures and (iv) anther culture to establish haploid tissues. Some of these observations are of a preliminary nature and detailed results from continuing experiments which are in progress are anticipated. Several of the experiments are further carried out to obtain quantitative data on the growth of

the tissues of the different varieties in various media composition containing different growth hormone combinations.

(i) Establishment of callus and suspension culture: Seeds of six different varieties of winged bean, Psophocarpus tetragonlobus (L.) DC were selected for the initial establishment of callus cultures. These are representatives of different geographical locations particularly from Thailand, Indonesia, Sri Lanka and Papua New Guinea (SL 50 var., LBNG 3 var., LBNC 1 Var., PNG ups 102, SLS 23 var., and Thailand var.). Seeds were prepared for germination by pretreatment with dilute sulphuric acid (1 volume of concentrated H_2SO_4: 8 volumes of water) for 25 minutes. The seeds were then surface-sterilized for 10 minutes in 12% commercial clorox and rinsed several times with sterile distilled water. They were placed for germination on a salt-sucrose culture medium indicated as B5 (Gamborg et al., 1968) or MS (Murashige and Skoog, 1962). Both these media are referred to in this report as Basal Medium (BM) with no growth regulators. Epicotyl segments (6-8 mm. long) from 15-25 day old in-vitro grown seedlings served as sources of explants. In addition, embryo segments (from seeds sown on MS + 2,4-dichlorophenoxyacetic acid - 2,4-D at 0.5 mg/l for 4-6 days) and stem segments (6-10 mm. long) from mature plants grown in the field (8 weeks) also served as explants (Table 2). These explants were placed on agar media for callus induction. The composition of the culture media is indicated on Table 1. The pH of the media was adjusted to 5.8 before autoclaving and dispensed to 100 x 15 mm. diameter plastic dispo-petri dishes. The cultures were incubated at 27 ± 1°C in the incubator illuminated with fluorescent lights (ca. 7,000 lux) to give a 12 hr. day.

Suspension cultures were initiated by placing 75-100 mg. of callus tissue into 50 ml. of MS or B5 liquid medium supplemented with 2,4-D (1.0 mg/l) in

Erlenmeyer flasks. The cultures were incubated on an orbital platform shaker maintained at 60 rpm.

Table 1. Compositions of plant culture media

	MS (mg/l)	B5 (mg/l)
NH_4NO_3	1650	
KNO_3	1900	2500
$CaCl_2$-$2H_2O$	440	150
$MgSO_4$-$7H_2O$	370	250
KH_2PO_4	170	
$(NH_4)_2SO_4$		134
NaH_2PO_4-H_2O		150
KI	0.83	0.75
H_3BO_3	6.2	3.0
$MnSO_4$-$4H_2O$	22.3	
$MnSO_4$-H_2O		10
$ZnSO_4$-$7H_2O$	8.6	2.0
Na_2MoO_4-2 H_2O	0.25	0.25
$CuSO_4$-$5H_2O$	0.025	0.025
$CoCL_2$-$6H_2O$	0.025	0.025
Na_2EDTA	37.3	37.3
$FeSO_4$-$7H_2O$	27.8	27.8

Supplements:		
Inositol	100	100
Nicotinic acid	0.5	1.0
Pyridoxine HCl	0.5	1.0
Thiamine HCl	0.1	10.0
Glycine	2.0	
Auxin[a]	1-5 ppm	1-5 ppm
Sucrose (gram)	30	20
Agar (gram)	8	8

[a]Auxins = 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthalene acetic acid (NAA).

The source of explants selected for experiments on 6 different varieties of Winged Bean represented 3 different stages of plant development. Segments of embryo represented initial stage where cell division activity is at its maximum, and epicotyl portion of seedling represented an intermediate stage where cells were still dividing and elongating, and finally the stem segments from mature plants (Fig. 1). The initial response of all the explants to culture media were uniform. The explants did not evoke any response in the BM alone, whereas, the growth regulators (2,4-D, α-NAA, 6-BAP*) when used alone or in combination resulted in callus formation (Table 1). The callus formed on 2,4-D (0.5 mg/l) medium was friable and white, whereas, α-NAA (0.5 mg/l) and 6-BAP (2.0 mg/l)

6-BAP = 6-Benzylaminopurine

combination supported proliferation of fast growing, compact green callus, in all the 6 varieties (Table 2).

The callus obtained from stem and epicotyl segments of seedlings (Thailand variety and PNG) on 2,4-D (1.0 mg/l) dissociated to single cells in suspension culture at the end of 2 week culture period. These single cells when plated on to agar MS medium alone, formed callus masses which finally differentiated to roots and shoot buds at the end of 4 week culture period (Fig 2). The callus cultures formed on MS + αNAA (0.5 mg/l) + 6 BAP (2.0 mg/l) medium were transferred to MS medium alone and BAP supplemented medium (at all concentrations for plantlet regeneration.

(ii) 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).

Previous observations on the tissue culture of winged bean was reported in an abstract (Venketeswaran and Huhtinen, 1978). The results reported here, although some of a preliminary nature, demonstrate that induction of organs and regeneration of plantlets can be obtained on the winged bean using explants as indicated in the text and with different media composition. Roots initiated from callus derived from epicotyl segments of var. PNG ups 102 cultured on BM + 2,4-D at 0.5 mg/l (Fig. 2). Embryo segments from seeds of var. LBNC 1 and var. LBNG 3 after being grown on MS + 2,4-D (0.5 mg/l) for 5 days and then transferred to MS alone, showed differentiation of roots, shoot-buds from callus

masses within 4 weeks (Figs. 3 and 4). In var. LBNC 1, regeneration of fully-differentiated plantlets were obtained (Fig. 5). These plantlets had well-developed root system and green leafy shoot system. Further experiments are in progress to determine whether these plantlets had differentiated directly from the callus or through the formation of somatic embryoids. These responses are indicated on Table 3.

(iii) NaCl tolerance:

To study the effect of a gradient of NaCl concentrations on callus cultures, 4-8 masses of callus (75-100 mg of each) originating from stem segments and epicotyl segments from seedlings (Thailand var and LBNG3 var), were placed on MS + α-Naphthalene acetic acid (NAA, 0.5 mg/l) + 6-Benzyl-aminopurine (6-BAP), 2.0 mg/l containing a gradient of salt concentrations (NaCl at 0%, 0.01%, 0.05%, 0.1%, 0.25%, 0.50%, 0.75%, and 1.0% w/v).

Figures 6 and 7 show the effect of a gradient of NaCl concentrations on callus cultures of 2 varieties of winged bean. These are preliminary experiments designed to know whether callus tissue which is fast growing on MS + NAA (0.5 mg/l) + 6-BAP (2.0 mg/l) medium, can withstand NaCl at concentrations ranging from 0% - 1.0% when incorporated into the medium. At the end of 6 week of culture period, the growth of the callus masses at various concentrations of NaCl compared with control was uniform and uninhibited even at 1.0% w/v (Figs. 6 and 7). Experiments are in progress to determine the salt tolerant level of callus in suspension cultures and also to select the salt tolerant lines and to obtain plantlet regeneration from these lines.

Table 2: RESPONSES IN CULTURE OF THE EXPLANTS OF 6 VARIETIES OF WINGED BEAN

Variety	Explant	Media composition	Response
1. Thailand var	stem segments from mature plant (old)	MS or B5 (BM)	Enlargement of segments
		BM + 2,4-D (0.5 mg/l)	Formation of friable, white callus
		BM + 2,4-D (0.5 mg/l) + Kinetin (2.0 mg/l)	Formation of fast growing, green callus at the end of 4 weeks.
		MS + NAA (0.5 mg/l) + 6-BAP (2.0 mg/l)	
2. LBNC 1 3. LBNG 3	Embryo segments from seeds sown on MS + 2,4-D (0.5 mg/l) for 5 days	BM	Formation of callus and plantlet differentiation at the end of 4 weeks.
		BM + 2,4-D (0.5 mg/l) + Kinetin (2.0 mg/l)	Formation of white callus.
4. SL 50	Epicotyl segments and sections of cotyledon from 15-25 day old seedling	BM	Slight proliferation from the cut ends
		MS + NAA (0.5 mg/l) + 6-BAP (2.0 mg/l)	Formation of fast growing, compact green callus at the end of 4 weeks.
LBNG 3	" "	" " "	" " "
5. SLS 23	" "	MS + NAA (0.5 mg/l) + 6-BAP (2.0 mg/l)	" " "
6. PNG ups 102	" "	" " "	" " "
Thailand var	Anther Segments	" " "	Formation of friable callus at the end of 4 weeks.

Table 3. ORGANOGENESIS AND REGENERATION IN CULTURE FROM EXPLANTS OF WINGED BEAN

Variety	Explant	Media composition	Response
LBNC 1	Embryo segments from seeds sown on MS + 2,4-D (0.5 mg/l) for 5 days	BM	Leafy shoots and roots in 3 weeks
LBNG 3	" "	BM	Roots and shoot buds in 3 weeks
LBNC 1	" "	BM	Fully differentiated plantlets in 5 weeks
PNG ups 102	Epicotyl segments	BM + 2,4-D (0.5 mg/l)	Root initiation and shoot buds in 4 weeks

No responses on SLS 23, SL 50 and Thailand var.

(iv) Anther Culture: To raise haploid cultures, anthers were excised from 6mm - 10 mm long flower buds, under aseptic conditions and placed on nutrient media (Table 1). Segments of anther (Thailand var.) responded to MS+NAA (0.5 mg/l) + 6-BAP (2.0 mg/l) medium, at the end of 20 day culture period by forming friable callus. The callus has been subcultured to regeneration medium to induce plantlet formation. The cytological analysis of these callus cultures is also in progress to determine the ploidy of callus cells.

REFERENCES

Gamborg, O. L., R. A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.

Gresshoff, P. M. and S. S. Mohapatra. 1981. Legume Cell and Tissue Culture. In: COSTED Symp. on Tissue Culture of economically important plants. Ed. A.N. Rao, pp. 11-24.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Phillips, G. C. and G. G. Collins. 1981. Induction and development of somatic embryos from cell suspension cultures of soybean. Plant Cell Tissue Organ culture 1: 123-129.

Venketeswaran, S. and O. Huhtinen. 1978. *In vitro* root and shoot differentiation from callus cultures of a legume, the Winged Bean, *Psophocarpus tetragonolobus* (L) DC. *In vitro* 14: 355 (Abstr.).

Collaborative efforts: Correspondence is in progress with Prof. Dr. Walter Herath, Deputy Director General of the Internaticnal Dambala (Winged Bean) Institute Sri Lanka, and Profs. H.P.M. Gunasena and Y.D.A. Senanayake of University of Peradeniya, Peradeniya, Sri Lanka to recruit two students from Sri Lanka so that they can be sent to my laboratory for tissue culture training.

These students trainees will become potential employees of the Institute after they finish training in U.S.A. The scientific collaborators are also in the process of sending specific seed varieties which will be of particular benefit for them in Sri Lanka for tissue culture experimentation, regeneration, etc.

Prof. Senanayake, who was visiting Texas A and M College Station on a sabbatical in September 1983 visited my laboratory in Houston and the scientific cooperation was initiated in person. A future visit by Prof. Walter Herath is contemplated.

Copies of relevant correspondence is included in Appendix.

PLATE I: Establishment of callus cultures, organogenesis and plantlet regeneration in winged bean.

Fig. 1: Embryo and 15-day old seedling (var. SL 50) as source of explant.

Fig. 2: Root initiation from callus cultures derived from epicotyl segments (var. PNG ups 102) cultured on BM + 2,4-D (0.5 mg/l).

Fig. 3: Differentiation of roots and shoot buds from callus cultures of embryo explants (var. LBNC 1) cultured on BM alone (seeds were sown on BM + 2,4-D (0.5 mg/l) for 5 days).

Fig. 4: Origin of roots and shoot buds from callus masses obtained from embryo segments (var. LBNG 3) on BM alone (seeds were sown on BM + 2,4-D (0.5 mg/l) for 5 days).

Fig. 5: Regeneration of fully differentiated plantlets (var. LBNC 1) from callus cultures of embryo segments on BM alone (seeds were sown on BM + 2,4-D (0.5 mg/l) for 5 days).

C = Callus; Co = cotyledon; e = embryo; ep = epicotyl r = root and Sh = Shoot

13a

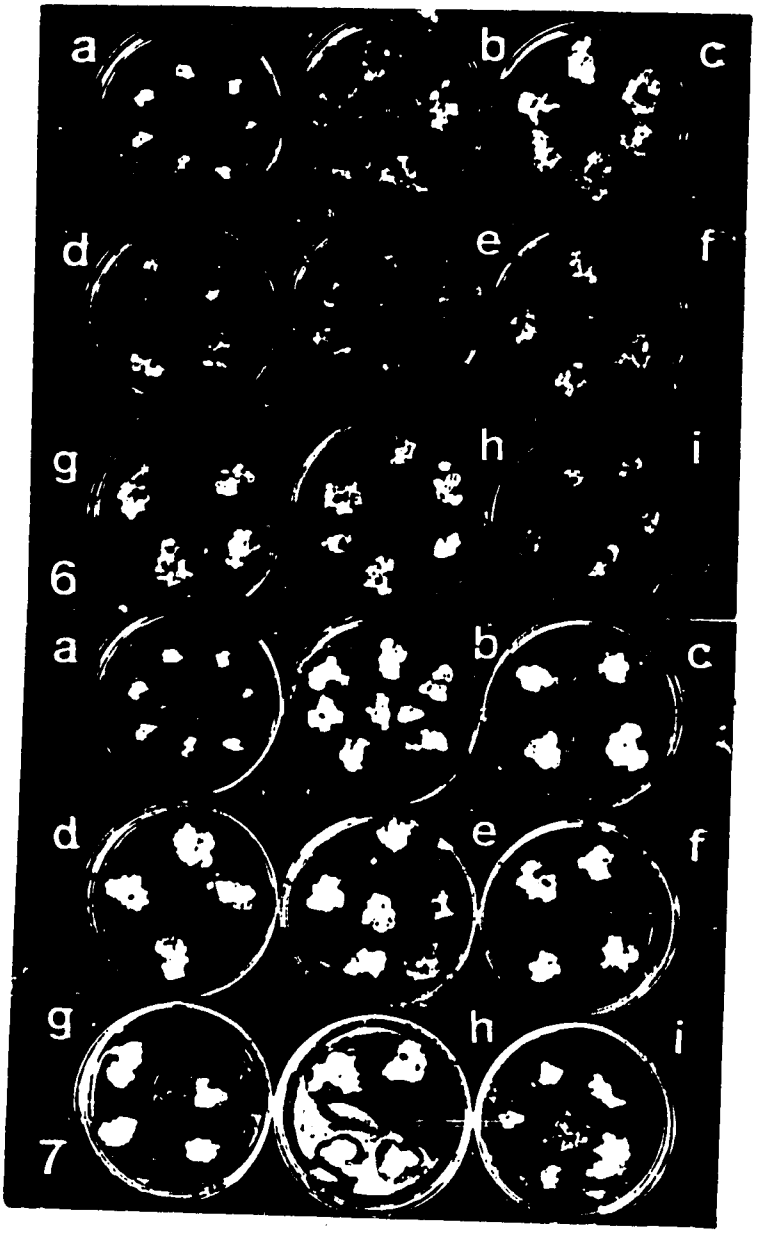
PLATE II: Effect of NaCl concentrations on callus cultures of winged bean.

Fig. 6: var. LBNG 3 and Figure 7: var. Thailand.

a. : Callus masses at the time of inoculation.

b. : Callus cultures at the end of 7 weeks of growth on MS + α -NAA (0.5 mg/l) and 6-BAP (2.0 mg/l) with no NaCl --- CONTROL

c.-i.: Callus cultures at the end of 7 weeks of growth on the above media + NaCl concentrations of 0.01; 0.05; 0.10; 0.25; 0.50; 0.75 and 1.0 o/o (w/v).


a
b
c
d
e
f
g
h
i
6
a
b
c
d
e
f
g
h
i
7

DEPARTMENT OF CROP SCIENCE
FACULTY OF AGRICULTURE, UNIVERSITY OF PERADENIYA
PERADENIYA, SRI LANKA.

Tele. : 08 — 8301 Ext. 320
Direct line :08 -8837

8th December, 1983.

Dr. S. Venketeswaran,
Associate Professor,
Department of Biology,
University of Houston (Central Campus),
Houston, Texas 77004,
USA.

Dear Professor Venketeswaran,

I received the copy of your letter dated October 11, 1983. I have spoken to Professor Walter Herath after he returned from his recent visit to USA. We should be able to identify two graduate students from here. Most probably one would be from the Staff of the Winged Bean Institute and one from our department.

Due to the unfortunate events that took place in Sri Lanka in July when I was visiting the different universities, the recruitment procedures at our end had got delayed. Therefore we may take a couple of months more to identify two good students. As soon as we have identified them we will keep you informed.

I'am sorry for not visiting you before we left Texas. Our University messed up our return air tickets and I was busy at College Station doing the last minute packing which did not give me time to come to Houston to see you again. At the end my wife and I had to fly into Houston on Saturday October 1st and pick our tickets at the airport for the connecting flight to Colombo within the hour.

I'am still trying to get settled down after returning. Although I brought all the material on the small research project that I completed in College Station, I have not had the time to sit down and write a paper on it.

Let me take this opportunity to wish you and your family the seasons greetings and good wishes for 1984.

Sincerely,
Don
Y. D. A. Senanayake,
Prof. of Crop Science.

cc. Dr. H. M. W. Herath

16

INTERNATIONAL DAMBALA (Winged Bean) INSTITUTE

Phone: 08-23568

PALLEKELE,
KUNDASALE,
SRI LANKA.

3rd January, 1984.

Dr.S.Venketeswaran,
Associate Professor,
Department of Biology,
University of Houston,
TEXAS 77004.

Dear Dr.Venketeswaran,

Greetings from Sri Lanka! Let me wish you and your family the the very best in 1984.

Thank you very much for your letter dated October 11, 1983. I am sorry for the delay in sending this reply.

Congratulations! I am so pleased to hear that you have received the grant for the Winged Bean Proposal submitted to USAID.

I am making arrangements to select two graduate students for for nomination to the project. I hope to do this in consultation with Dr.Senanayake and Dr.Gunasena. I would like these graduate trainees to be potential employees of the Insitute, since we are in the process of building up our staff. Does the salary of $650 per month indicated by you cover the 12 months period for each student?

I have not received the application forms and information regarding admission requirements for graduate students. Please send me two sets of these forms as soon as possible.

Contd /2 .....

17

Thank you for inviting me to visit you.
I shall certainly try to do so on my next trip to the states.
Would you be in a position to visit us soon? Please be assured that you are welcome at any time and that we will be pleased to have you ~~and~~ your wife and daughter as our guests.

Hoping to hear from you soon.

With best wishes and kind regards.

Yours sincerely,

Walter

Dr.Walter Herath.
DEPUTY DIRECTOR GENERAL.

WH/smf.