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AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON D C 20523

DATE: 9/29/88

MEMORANDUM

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

FROM: AID/SCI, Victoria Ose

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

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 6.399
PR. II 9/29/88

Attachment

6.399

PROGRESS REPORT II

DEVELOPMENT OF METHODOLOGIES FOR COWPEA TISSUE CULTURE

GRANT NO. 936 5542 G-00-6044-00

Rec'd in SCI: JUL 23 1980

PROGRESS REPORT II (JUN-NOV. '87)
DEVELOPMENT OF METHODOLOGIES FOR COWPEA TISSUES CULTURES

GRANT NO. 936-5542-G-00-6044-00

PREPARED BY

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PRINCIPAL INVESTIGATOR

PREAMBLE TO THE PROGRESS REPORT II

This progress report summarizes the activities during the period of June through November 1987 of the existence of the project entitles "Development of methodolooies for Cowpea Tissue Culture" which later on will be referred to as the Cowpea Tissue Culture Project(CTP). The project is supported by the United States Agency for International Development under the Program on Science and Technology Cooperation*.

Although the CTP was granted since May 21 1986, its financial plan was not requested until December due to some administrative problems. Therefore, the CTP was actually carried out in full load in December 1986. As a consequence, the CTP first report appears in June 1987 which covers the activities during December 1986 through May 1987. And, this report which covers the activities during the period of June through November 1987 is considered to be the second one from the CTP.

This report described the research activities focussing on shoot bud formation from cultured of cowpea immature embryos via callus formation. The factors investigated include growth regulators, vitamins, minor elements, and amino acids.

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* Grant No. 936-5542-G-00-6044-00

SHOOT BUD FORMATION FROM CULTURED OF COWPEA IMMATURE EMBRYOS

INTRODUCTION

Cowpea(*Vigna unguiculata*), a grain legume species, is an extremely important source of plant protein for Africa. The plants can be grown under marginal moisture and poor soil conditions. It can be agronomically and nutritionally useful alternative for cassava. Thus, it is also a promising leguminous plant for the northeast region of Thailand Considerable efforts have long been put forward to agronomic studies of cowpea as well as to the use of cowpea as the food products for people in the northeast where protein malnutrition has become a big problem(Ngarmsak et al. 1982).

Although cowpea is becoming a promising leguminous plants for the northeast, the average yield of local varieties is low ranging from 200 to300 kg/ha due to their susceptibility to a number of diseases and insect pests(Fatokun and Singh, 1987) Therefore, various attempts have been made to improve cowpea varieties for their effective use. It is well recogized that developing technology related to tissue culture can be used to produce valueable agronomic traits. This has led to the consideration of the interjection of this technology into cowpea improvement programs.

For this approach to be useful in cowpea improvement program, it remains to be demonstrated that regeneration of cowpea plants from cells or tissues which have been in culture for several months is possible. Previous efforts of the in vitro culture of cowpea have been limited to embryo culture and anther culture(Ladeinde, 1982) which is sought as a solution to the hybridization problem and to shoot-tip culture (Kartha et al. 1981, Sebastian, 1983) which yielded whole plant, regeneration through callus or cell suspension cultures has never been reported. Therefore, the research on development of methodologies for cowpea tissue culture is undertaken in the hope that this study will definitely be useful to the cowpea breeding program.

The experiment reported herein is a part of the main project supporting by USAID . It presents evidence of adventitious bud formation on cotyledons as well as on embryonic shoot apex from cultured cowpea immature embryos.

SUMMARY

Attempts have been made to induce shoot bud formation from cultured of cowpea immature embryos. The immature embryos ranging in size 2.5-3.0 mm were cultured in modified Murashige and Skoog(MS) medium. Various categories of constituents were investigated including (a) growth regulators, (b) vitamins, (c) micronutrients, (d) FeEDTA, (e) inositol and (f) amino acid. The following results were accomplished.

1. Presence of cytokinin, benzyladenine (BA), in the medium was required for multiple shoot-bud formation. The optimum concentration appeared to be at 10 uM.

2. The budding percentage was increased by increasing concentration of thiamine HCl in the medium.

3. High level of micronutrients enhanced subsequent organogenesis.

4. High level of FeEDTA stimulated shoot growth.

5. Inositol did not have much effect on organogenesis.

6. Addition of amino acid, proline, caused a reduction of browning of callus tissues but did not have a pronounce effect on shoot bud formation.

7. Best root induction and root growth of cowpea shootlets obtained from in vitro culture were resulted from treatment contained 0.1 uM napthaleneacetic acid (NAA).

OBJECTIVES

The objective of this study was to develop an appropiate culture medium for cowpea plant regeneration via organogenesis from callus tissues of cultured immature embryos. The study was mainly involved with the effect of growth regulators, vitamins, inositol, micronutrients and amino acid on shoot bud formation.

MATERIALS AND METHODS

General Methodology

Immature embryos were selected as an explant source because of their juvenile nature and high regenerative potential. Cowpea plant (Vigna unguiculata cv. IT82E-889) were field grown at Khon-Kaen University. Pods were harvested and washed with soap. After a 20 sec soaking in 70% ethyl alcohol, the pods were dissected and the immature seeds removed and placed into sterile distilled water. These seeds were rinsed and transferred for further disinfection to a solution containing 5%(v/v) Clorox (commercial bleach possesing 5.25% sodium hypochlorite) plus a few drops of Tween 80 for 15 min. The seeds were subsequently rinsed three times in sterile distilled water for at least 5 min each time. The embryos ranging in size 2.5-3.0 mm were aseptically removed from the immature seeds under the stereomicroscope and transferred to 100 X 15 mm Petri dishes containing 25 ml of modified Murashige and Skoog medium (initiation medium, Table 2) (Murashige and Skoog, 1962 see Table 1). All media were adjusted to pH 5.8 and solidified with 0.7% Difco agar. Ten embryos were placed in a single dish which then was sealed with parafilm.

The cultures were incubated at 28°C in darkness to prevent the precocious germination effect of light, and were subcultured once at 4 weeks to the same initiation medium. Observations were taken at the end of 8 weeks by scoring on a regenerate/non-regenerate basis and whenever possible buds or shoots count were

taken. Thereafter, culrures containing smooth yellowish calluses and/or shoot buds were subcultured every 3-4 weeks to fresh MS medium containing 5 uM BA, 0.05 uM NAA(MS/5 BA) and kept in darkness for continued proliferation. Any soft watery callus that develop on the cultures during the first 8 weeks were discarded prior to the transfer of the tissue to the MS/5 BA medium. Cultures with well defined shoot bud structures were kept in continuous light for further growth and development.

Shoots with expanded leaves were transferred to 8 x 2 cm vials containing 5 ml of MS medium with various concentration of NAA for experiments on rooting. All experiments were repeated three times.

Experiment 1. Effect of growth regulators on shoot bud formation.

The immature embryos ranging in size 2-3 mm were culture in 100x15 Petri dishes containing the initiation medium in darkess. The combination of growth regulators, BA (5-30 uM)and NAA (0.05-0.5 uM), were employed in a factorial designed experiment. The cultures were incubated 8 weeks, then were scored for callus proliferation and regeneration.

Experiment 2. Effect of micronutrient levels in MS medium on shoot bud formation.

The immature embryos ranging in size 2-3 mm were cultured in 100x15 mm Petri dishes containing the initiation medium that has varied the relative concentration of minor elements(except FeEDTA). The effect of concentration of growth regulators was again tested to confirm the previous results.

Table 1: Murashige and Skoog's medium (MS)

Constituents	Amount (mg l^{-1})
Inorganic	
NH_4NO_3	1,650
KNO_3	1,900
$CaCl_2.2\ H_2O$	440
$MgSO_4.7\ H_2O$	370
KH_2PO_4	170
KI	0.83
H_3BO_3	6.20
$MnSO_4.4\ H_2O$	22.3
$ZnSO_4.7\ H_2O$	8.6
$Na_2MoO_4.2\ H_2O$	0.25
$CuSO_4.5\ H_2O$	0.025
$CoCl_2.6\ H_2O$	0.025
$FeSO_4.7\ H_2O$	27.80
$Na_2.EDTA.2\ H_2O$	37.30
Organic	
Inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2.0

Table 2: Initiation medium

Constituents	Amount (mg l^{-1})
Inorganic	
Major elements	
NH_4NO_3	1,650
KHO_3	1,900
$CaCl_2.2\ H_2O$	440
$MgSO_4.7\ H_2O$	370
KH_2PO_4	170
Minor elements	
KI	0.83
H_3BO_3	6.20
$MnSO_4.4\ H_2O$	22.3
$ZnSO_4.7\ H_2O$	8.6
$Na_2MoO_4.2\ H_2O$	0.25
$CuSO_4.5\ H_2O$	0.025
$CoCl_2.6\ H_2O$	0.025
$FeSO_4.7\ H_2O$	27.80
$Na_2.EDTA.\ 2\ H_2O$	37.30
Organic (B5 vitamins: Gamborg et al. 1968)	
Inositol	100
Nicotinic acid	10
Pyridoxine HCl	1
Thiamine HCl	10
Sucrose	30,000
Growth regulator	
Benzyladenine	10 uM
Napthaleneacetic acid	0.2 uM

Experiment 3. Effect of higher relative concentration of MS FeEDTA on shoot growth in callus cultures from immature cowpea embryos.

The immature embryos ranging in size 1-2 mm were cultured in 100 x 15 mm Petri dishes containing the initiation medium that had varied the relative concentration of FeEDTA. The cultures were incubated 8 weeks, then were scored for regeneration.

Experiment 4. Effect of proline on callus growth and shoot bud formation.

The immature embryos ranging in size 2-3 mm were cultured in 100 x 15 mm Petri dishes containing the initiation medium that had varied proline concentration (0, 10, 20 mM). The cultures were incubated 8 weeks, then were scored for regeneration.

Experiment 5. Effect of vitamins on callus growth and shoot bud formation

The immature embryos ranging in size 2-3 mm were cultured in 100 x 15 mm Petri dishes containing the initiation medium that had varied the composition of B5 vitamins as shown in Table 5. Each constituent was tested separately. The cultures were incubated 8 weeks, then were scored for regeneration.

Table 3. Variations of vitamin concentrations in the initiation medium

Nicotinic acid (mg/l)	Thiamine HCL (mg/l)	Pyridoxine HCl (mg/l)
0.5	6.0	0.25
1.0	8.0	0.50
2.0	10.0	1.00
4.0	12.0	1.50

Experiment 6. Effect of inositol on callus grwoth and shoot bud formation

The immature embryos ranging in size 2-3 mm were cultured in 100 x 15 mm Petri dishes containing the initiation medium that had varied inositol concentration(50,100,150,200 mg/l). The cultures were incubated 8 weeks, then were scored for regeneration.

Experiment 7. Effect of NAA on root formation and root growth

The effect of NAA on rooting ability of cowpea shootlets obtained from cultured immature embryos was investigated. Shootlets (15-20 mm in length) were cultured in 8 x 2 cm vials containing 5 ml of MS agar medium with various concentration of NAA(0.1, 0.3, 0.5 uM). The cultures were incubated 4 weeks, then were scored for root formation and root length.

RESULTS AND DISUSSION

Experiment 1. Effect of growth regulators on shoot bud formation

The earliest sign of growth from the embryos was noticeable within 2 weeks of culture. At all the hormonal concentrations tested, cowpea immature embryos differentiated giving rise into multiple buds accompanied by callus within 5-7 weeks. Multiple bud formation frequently occurred at embryonic shoot apex while bud formation from cotyledon region occurred at frequencies of 5-20%. The frequency of multiple bud formation was controlled by BA conc in the medium (Table 4). The average mean of percentage of embryo which formed buds from all NAA concentrations was quite the same (25%, 27%, 22%). However, the average mean of BA was increased from 15% at BA 3 uM to 30% and 29% at BA 10,30 uM respectively. Shoot buds occurred in cluster. Counting the quantity of shoot were employed under stereomicroscope. Nevertheless, obtaining precised number of shoot bud was painstaking job and it was also noticed that the quantity of shoot buds produced per embryo was highly variable. Thus,we will not use this parameter at this stage.

Higher level of NAA (0.50 uM) only increased callus proliferation.Increasing BA beyond 10 uM increased the cytokinin effect on shoot morphology, even though the number of shoots increased (data not shown), while lower levels of BA (3 uM) decreased the amount of regeneration.

Thus, the combination of 10 uM BA and 0.2 uM NAA was chosen as appropiate combination for all other experiments performed in order to induce multiple bud formation from cultured immature embryos.

Table 4. Effect of various concentration of BA and NAA on the percentage of embryos which formed buds from cultured immature cowpea embryos. Observations were taken eight weeks after the culture were initiated, using cv.IT82E-889 with 40 embryos per treatment on MS medium with B5 vitamins.

Hormone concentration, uM		Shoot-bud differentiation*
BA	NAA	(%)
3	0.05	14
10	0.05	30
30	0.05	32
3	0.20	18
10	0.20	34
30	0.20	30
3	0.50	14
10	0.50	27
30	0.50	25

Average mean of BA : at 3 uM = 15.6;10 uM = 30.3;30 uM = 29.0

Average mean of NAA : at 0.05 uM = 25.3;0.2 uM = 27.3;0.5 uM = 22.3

*Shoot buds occurred from embryonic apex

Experiment 2. Effect of micronutrient levels in MS medium on shoot bud formation.

The effect of increasing relative concentration of MS minor elements on regeneration percentage was shown in Table 5. The effect of BA was again confirmed. Higher relative concentration of minor elements enhanced the frequencies of multiple buds formation (x2, x4) while too high level (x6) slightly inhibited. Barwale et. al (1986) also reported the effect of increasing relative concentration of MS minor element on regeneration percentage in cultured soybean immature embryos. It was noticed that at x6 concentration the color of cultures turned brown very rapidly and callus formation was reduced. Shoot buds obtained from higher relative concentration of minor elements growed better than the control when subcultured them onto the regeneration medium(data not shown).

Experiment 3. Effect of higher relative concentration MS FeEDTA on shoot growth in callus from immature cowpea embryos.

The higher concentration of MS FeEDTA stimulated growth of embryonic shoot, however, too high concentration(x6) resulted in decreasing shoot growth (Table 6). It was also observed that at x6 FeEDTA callus formation was decreased as well as browning of callus was more pronounce. In radiata pine, the iron concentration in shoot elongation medium was doubled for good growth of micro-shoots(Horgan and Aitken, 1981). The effect of FeEDTA will be further studied especially on the growth of shootlets in regeneration medium.

Table 5. Effect of higher relative concentration of MS minor elements on the percentage of embryos which form buds in cultured immature cowpea embryos. Observations were taken eight weeks after the cultures were initiated, using cv. IT82E-889 with 4o embryos per treatment on MS medium with B5 vitamins supplemented with BA 10 uM NAA 0.2 uM. The minor elements are H_3BO_3, $MnSO_4$, $ZnSo_4$, KI, $Na_2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$ and $CoCl_2 \cdot 6H_2O$.

BA conc (uM)	Relative conc of micronutrients				
	x1	x2	x4	x6	$\bar{x}$
5	17	20	23	13	18
10	28	36	36	23	31
20	22	30	32	23	27
$\bar{x}$	22	29	30	20	

Table 6. Effect of higher concentrations of MS FeEDTA on shoot growth in callus cultures from immature cowpea embryos. Observations were taken eight weeks after the cultures were initiated, using CV. IT82E-889 with 40 embryos per treatment on MS medium with 13 uM BA, and 0.2 uM NAA.

Relative concentration of FeEDTA	Embryo size(mm)	
	1.0	2.0
1	1.97 ± 0.18[a]	2.10 ± 0.77
2	2.29 ± 0.41	2.41 ± 0.27
4	1.92 ± 0.73	2.44 ± 0.14
6	1.00 ± 0.00	1.25 ± 0.50

[a]Mean of shoot length±SD expressed as a relative unit : 1=1-5 mm, 2=6-10, 3=>10 mm.

Experiment 4. Effect of proline on callus growth and shoot bud formation.

The addition of proline to the initiation medium did not have much effect on the frequencies of shoot-bud formation (Table 6). It was observed that shoot buds occurred more frequently from the cotyledons of zygotic embryo (Fig. 4) than from the embryonic apex(Fig. 5). In addition, proline decreased degree of browning of callus tissues when used at 10 mM. Leaf like structures and embryoid like structures were found on the surface of cotyledons(Fig 6,7).

Table 7. Effect of proline on the percentage of embryos which formed buds from cultured immature cowpea embryos. Observations were taken eight weeks after the culture were initiated, using cv. IT82E-889 with 40 embryos per treatment on MS medium with B5 vitamins plus 10 uM BA and 0.2 uM NAA.

Proline conc (mM)	Shoot-bud formation (%)
0	54
10	60
20	52

Experiment 5. Effect of vitamins on callus growth and shoot bud formation

The stimulatory effect of vitamins on regeneration percentage was observed. Nicotinic acid at 0.5 mg/l was the best concentration for highest frequency of shoot bud formation and also for highest frequency of shoot bud formation occurred from cotyledons(Table 8, 9). At the end of 8 week culture, the callus had less degree of browning. Among the concentrations used, eventhough 0.5 mg/l was the best one, it should be pointed out that further study should be carried out with lower range of concentrations (ie. 0.1, 0.05, 0.001 mg/l) to verify this effect.

Thiamine was also found to be the imporcant facter for shoot bud formation from cultured immature embryos. It was found that regeneration was impossible when lowering thiamine concentration down to 8 and 6 mg/l (Table 10). Again, further study should be done on the effect of higher thiamine concentration (ie. 20, 25, 30 mg/l) on regeneration response.

The effect of pyridoxine on regeneration percentage was shown in Table 11. Among the various concentrations used, the amount of 1.50 mg/l gave lowest regeneration response.

Experiment 6. Effect of inositol on callus growth and shoot bud formation

Of all the concentration tested, the highest regeneration response from inositol treatment occurred at 200 mg/l (Table 12). However, the regeneration percentage at 150 mg/l appeared to be the lowest response. The fluctuation of regeneration percentage occurred needs further verification.

Table 8 Effect of various concentrations of nicotinic acid used in B5 vitamins on the percentage of embryo which form buds from cultured immature cowpea embryos. Observations were taken eight weeks after the cultures were initiated, using cv. IT82E-889 with 40 embryos per treatment on MS medium with B5 vitamins supplemented with BA 10 uM, NAA 0.2 uM.

Nicotinic acid (mg/l)	Shoot bud formation* (%)
0.5	52.5
1.0	31.7
2.0	8.0
4.0	0.0

*Shoot buds formation occurred from embryonic apex

Table 9 Effect of various concentrations of nicotinic acid used in B5 vitamins on the percentage of embryo which form buds from cultured immature cowpea embryos. Observations were taken eight weeks after the cultures were initiated, using cv. IT82E-889 with 40 embryos per treatment on MS medium with B5 vitamins supplemented with BA 10 uM, NAA 0.2 uM.

Nicotinic acid (mg/l)	Shoot bud formation* (%)
0.5	17.5
1.0	10.0
2.0	2.0
4.0	0.0

*Shoot buds formation occurred from cotyledons

Table 10 Effect of various concentrations of thiamine HCl used in B5 vitamins on the percentage of embryo which form buds from cultured immature cowpea embryos. Observations were taken eight weeks after the cultures were initiated, using cv. IT82E-889 with 40 embryos per treatment on MS medium with B5 vitamins supplemented with BA 10 uM, NAA 0.2 uM.

Thiamine HCl (mg/l)	Shoot bud formation* (%)
6	0
8	0
10	20
12	26

*Shoot buds formation occurred from embryonic apex

Table 11 Effect of various concentrations of pyridoxine HCl used in B5 vitamins on the percentage of embryo which form buds from cultured immature cowpea embryos. Observations were taken eight weeks after the cultures were initiated, using cv. IT82E-889 with 40 embryos per treatment on MS medium with B5 vitamins supplemented with BA 10 uM, NAA 0.2 uM.

Pyridoxine HCl (mg/l)	Shoot bud formation* (%)
0.25	32.5
0.50	22.0
1.00	36.6
1.50	14.0

*Shoot buds formation occurred from embryonic apex

Table 12 Effect of various concentrations of inositol used in B5 vitamins on the percentage of embryo which form buds from cultured immature cowpea embryos. Observations were taken eight weeks after the cultures were initiated, using cv. IT82E-889 with 40 embryos per treatment on MS medium with B5 vitamins supplemented with BA 10 uM, NAA 0.2 uM.

Inositol (mg/l)	Shoot bud formation* (%)
50	26.0
100	25.0
150	16.0
200	37.5

Shoot buds formation occurred from embryonic apex

Experiment 7. Effect of NAA on root formation and root growth

The effect of NAA on rooting ability of cowpea shootlets obtained from embryo axis was shown is Table 13.

Table 13. Effect of NAA concentrations on root formation and root growth in shootlets obtained from cultured immature cowpea embryos on MS FeEDTA medium. Observations were taken four weeks after the treatments, using cv. IT82E-889 with 20 shootlets per treatment.

NAA (uM)	Root number	Root length
0.1	2.15 ± 0.77[a]	2.00 ± 0.50[b]
0.3	1.72 ± 0.48	1.74 ± 0.45
0.5	1.40 ± 0.54	1.28 ± 0.38

[a] Mean of root number ± SD expressed as a relative unit : 1 = 1-3, 2 = 4-6, 3 = > 6.

[b] Mean of root length ± SD expressed as a relative unit : 1 = 1-10 mm, 2 = 10-20 mm, 3 = > 20 mm.

NAA concentration at 0.1 uM yielded the best root number and root growth. At 0.5 uM, root growth and root induction were markedly reduced and abnormal roots were observed.

From preliminary test, it was noticed that root formation and root growth occurred very easily without any auxin supplemnt. However, the addition of low level of auxin increased root growth and number of roots as well as growth of micro cuttings(data not shown). The cowpea plant obtained from in vitro was shown in Figure 8.

General discussion

Shoot bud formation from cotyledonary tissue can be induced from cultured immature embryos only, mature zygotic embryos were not able to do so (from our preliminary works). It was observed that shoot bud formation occurred more frequently from embryonic apex than from cotyledonary tissues. This suggests the possibility of correlative inhibition between embryonic apex and cotyledonary tissue. Within the cotyledon itself, shoot bud formation occurred only at the tip of the cotyledon. This leads us to think that we might use immature embryos that were too old. However, younger embryos did not work(data not shown). Therefore, we think that a gradient of regeneration potential may exist from the tip to the basal region of the cotyledon. And this need to be worked out.

Shoot buds originated both from callus and without callus formation, although this needs confirmation by histological studies. We observed under binocular stereomicroscope and found that in some cases direct shoot formation occurred at the tip of cotyledon after 2-3 weeks of incubation (Fig . 9).

Extensive variation in the regeneration response within treatment groups was observed in every experiments even though great care was excersised in producing uniformly explants (see Appendix). It is unclear why one embryo will regenerate while another in the same culture dish will produce only undifferentiated callus. In dealing with seed populations,genetics could be a factor, as well as the physiological condition of the cotyledons, or environmental factors during seed maturation.

Shoot bud formation from cultured cowpea immatured embryos reported by our group is the first study that be able to shown cowpea plant regeneration through callus formation. Attempts also have been made to induce shoot bud formation from mature zygotic embryos, however, there is no success yet. Cytological studies may need to be done in order to test for somaclonal variation of these cowpea plants. Since these plants were originated from cotyledonary tissues, we may expect them to have 3 n chromosomes. And we suspect that cowpea breeders may not want them. Therefore, we may need to apply our findings to another explant source such as young leaves or hypocotyl.

FUTURE PLAN OF EXPERIMENTS

1. Study on modification of the initiation medium based on initial findings.
2. Identify embryogenic and non-embryogenic callusses.
3. Exploration on the maintenance medium for high production of embryogenic callusses.
4. Develop the regeneration medium.
5. Study on correlation between passage number and regeneration potential.
6. Try other plant parts ie. young leaves, hypocotyl.

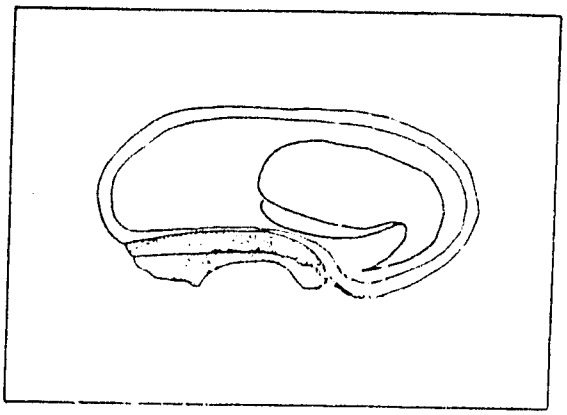

Figure 1. Diagramatic representation of embryo size and position in relation to rest of seed at time of excision

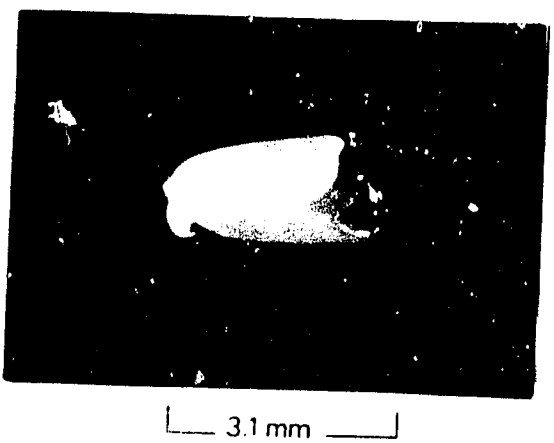

Figure 2. Cowpea immature embryo at the time of excision(7-day old)

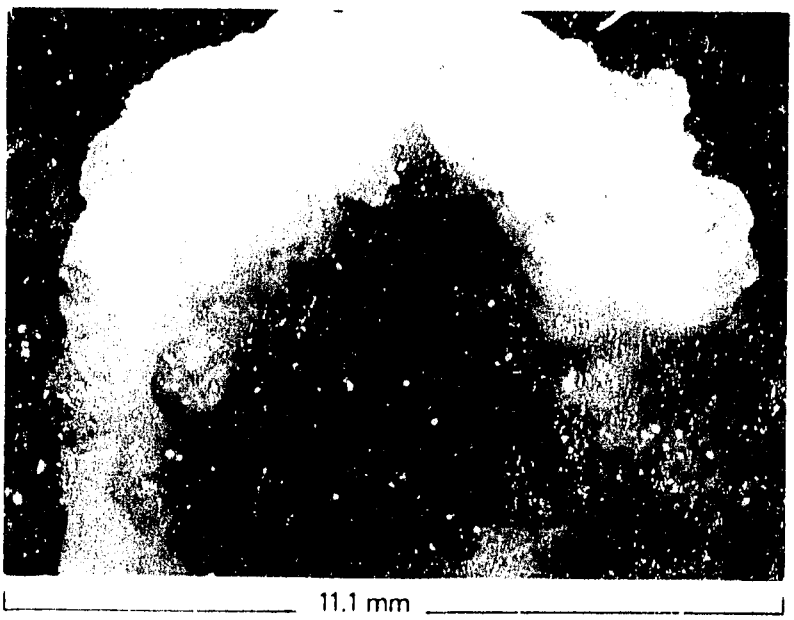

Figure 3. Callus formation in 3 week culture.

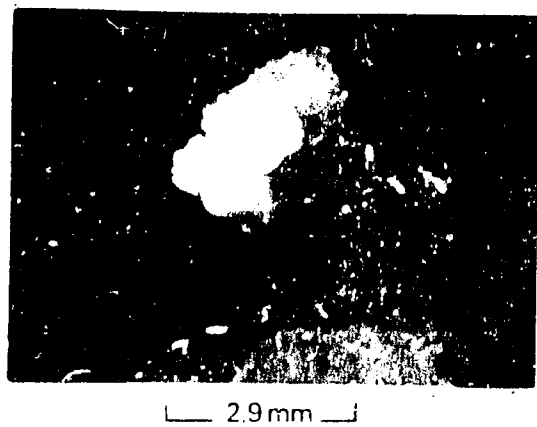

Figure 4. Multiple shoot bud formation from cotyledonary tissue.

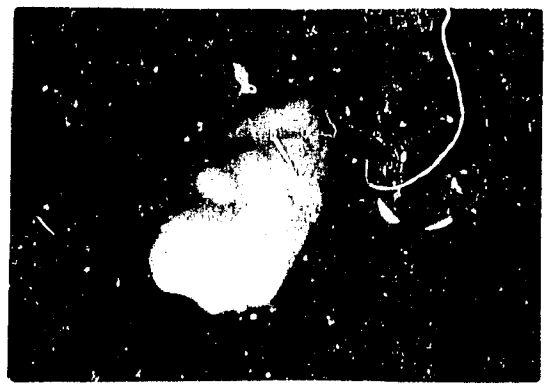

Figure 5. Multiple shoot bud formation from embryonic apex.

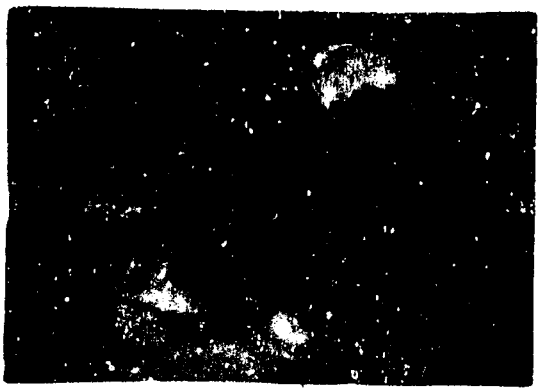

Figure 6. Leaf like structure occurred on cotyledonary tissue.

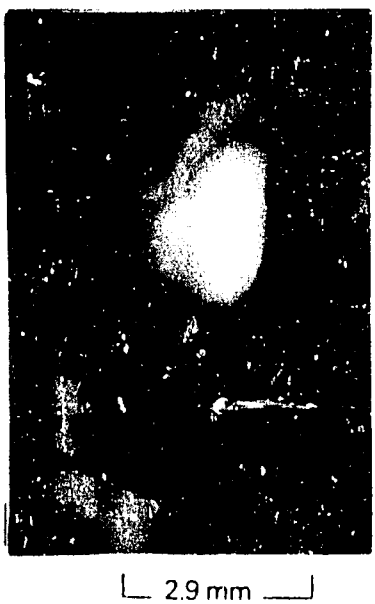

Figure 7. Embryoid like structure.

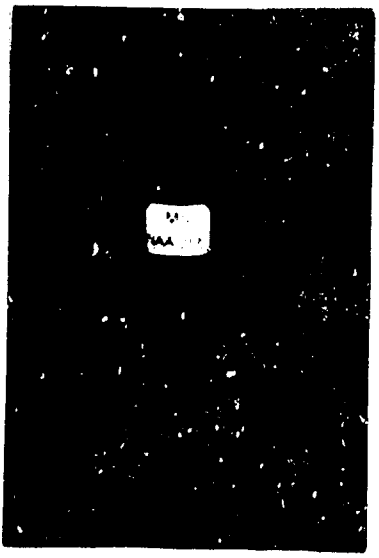

Figure 8. In vitro cowpea plant.

Figure 9. Shoot bud formation occurred directly on cotyledonary tissue.

REFERENCES

Barwale,U.B.,Kerns,H.R. and Widholm,J.M.(1986) Plant regeneration from callus cultures of several soybean genotypes via embryogenesis and organogenesis. Planta 167:473-481.

Durius, C.M. and Slaughter, J.C. (1980) The seed plants. In:Seeds and their uses. p 9 John Wiley & Sons.

Fatokun C.A. and Singh B.B. (1987) Interspecific hybridization through embryo rescue in cowpea. Proc. Second Ann. Conf. Int. Plant Biotech. Network:Biotechnology in the Development of Stress-Tolerant. Tropical Food Crops. No. 12

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

Hildebrand, D.F., Phillips, G.C. and Collins, G.B. (1986) Soybean [Glycine max (L.) Merr.]. In:Biotechnology in Agriculture and Forestry Vol. 2:Crops I. Bajaj, Y.P.S. ed. pp. 283-308. Springer-Verlag Berlin Heidelberg.

Hu,C. and Wang, P.(1986) Embryo Culture:Technique and Applications. In:Handbook of Plant Cell Culture. Vol. 4. Techniques and Applications (Evans, D.A., Sharp, W.R. and Ammirato, P.V. eds) pp. 43-96. Macmillan, New York.

Kartha, K.K., Pahl,F., Leung, L.L. and Mroginski, L.A.(1981) Plant regeneration from meristems of grain legumes:soybean, cowpea, peanut, chickpea, and bean. Can. J. Bot. 59:1671-1679.

Kerns, H.R., Barwale, U.B., Meyer Jr., M.M., and Widholm, J.M.(1986) Correlation of cotyledonary node shoot proliferation and somatic embryoid development in suspension cultures of soybean (Glycine max L. Merr.). Plant Cell Reports. 5:140-143.

Ladeinde, T.A.O. (1982) Anther and embryo culture in cowpea (Vigna unguiculata) breeding in Nigeria. In : Proc. of IAPTC 1982. Tokyo, Poster session.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth bioassays with tobacco tissue cultures. Physiol. Plant. 15 : 473-479.

Nagarajan, P., Mckenzie, J.S. and Walton, P.D. (1986) Embryogenesis and plant regeneration of Medicago spp. in tissue culture. Plant Cell Reports. 5 : 77-80.

Ngarmsak, T., Earle, M.D. and Anderson, A.M.(1982) Designing cowpea products for northeastern Thailand. IDRC Publication (Food drying : Proc. of a workshop. Edmonton, Alberta, 6-9 July 1981) no. 1[illegible], p. 36-39.

Power, C.J. (1987) Organogenesis from Helianthus annuus inbreds and hybrids from the cotyledons of zygotic embryos. Amer. J. Bot. 74 (4) : 497-503.

Sebastian, K.T.(1983) Shoot-tip culture and subsequent regeneration in cowpea. Sci. Hort. 20 : 313-317

APPENDIX

Study On Correlation Between Pod Size And Embryo Stage

Due to extensive variation within treatment on regeneration response of cultured cowpea immature embryos, the study on reproductive development and embryogeny in cowpea (*Vigna unguiculata* cv. IT82E-889) were accomplished. This finding helps in selection of pods that have the same developmental stage.

Seeds of *Vigna unguiculata* cv. IT82E-889 were planted in the field at the Agricultural Field Plot at Khon Kaen University. Cowpea flowers were tagged on the day of anthesis. After anthesis for 3 days, a representative group of pods, seeds, and embryos were selected every day until maturation for weight or length determinations.

The result shown in Table 1 illustrated the reproductive development in cowpea.

Table 1. POD WIDTH VS EMBRYO STAGE, SIZE AND AGE

POD WIDTH (MM)	EMBRYO STAGE	EMBRYO SIZE (MM)	EMBRYO AGE (DAYS)
2.00	PRE-HEART	0.14-0.20	3
2.40-3.20	HEART	0.30-0.55	4-5
3.75-9.00	COTYLEDONARY	1.00-11.00	6-17

The reproductive development of this variety required a total of 20-22 days after anthesis. From this study, embryo development was divided into 4 stages:pre-heart,heart, cotyledon, and mature. We were not able to detect the globular stage since excision of embryos younger than 3 day old age under binocular stereomicroscope was impossible. The three principal stages of embryo development of interest in this report,viz,pre-heart,heart, and cotyledonary were shown in Figure 1.

Fig 1. Illustration of various stages of embryo development

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Embryo development and pod size were correlated sufficiently to allow pod width to be used as a criterion for selecting pods that contain the stage of embryo development of interest(Fig. 2) In collecting the embryos used for culture, we will use pod with as an indicator. For example, pod width at 4-5 mm will give embryos of cotyledonary stage that are 1-1.8 mm in width and 2-3 mm in length and there will be some liquid endosperm in the milky stage. Thus, we use pod width as an indicator for selecting pod of desired stage of embryo delopment.

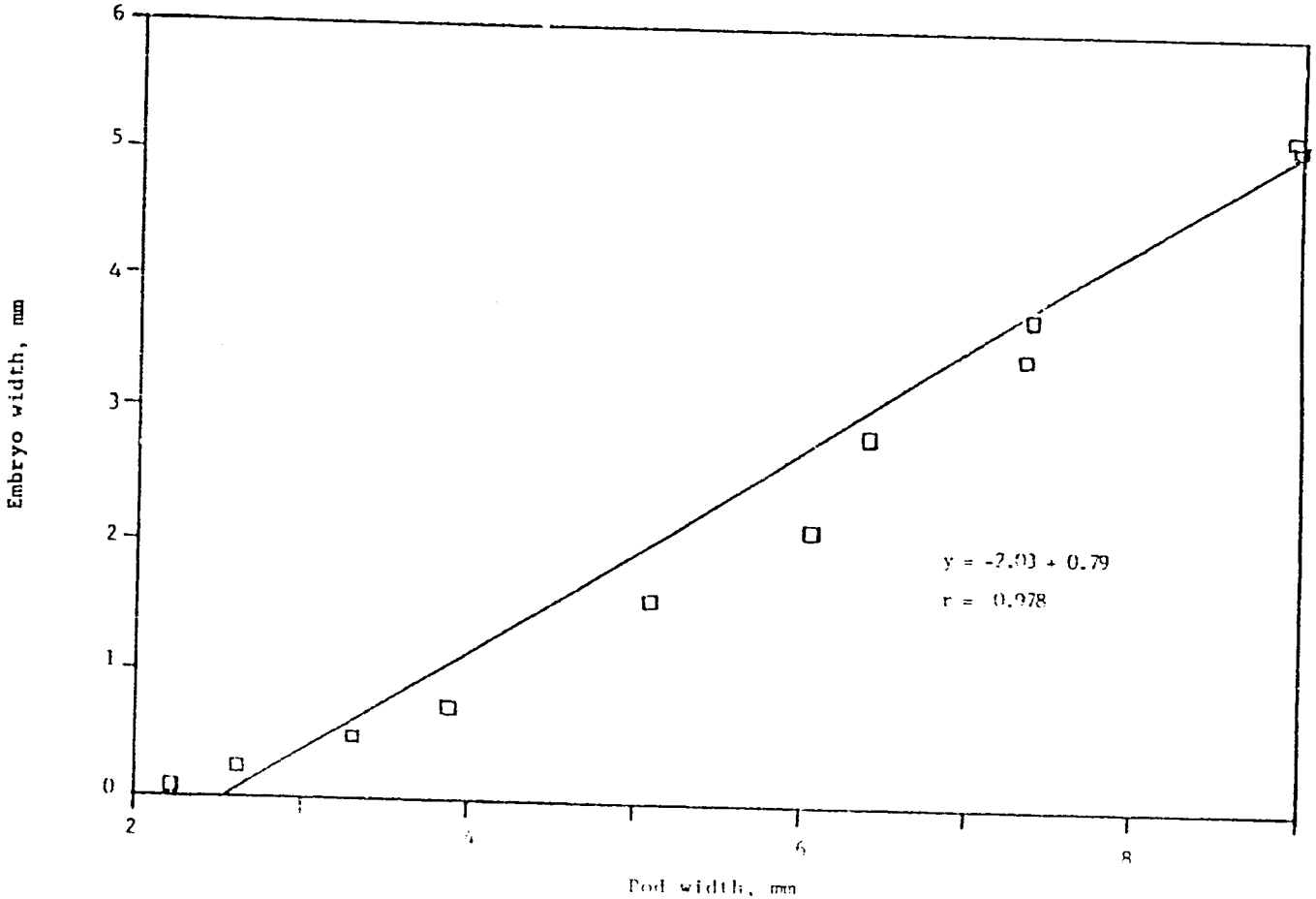

Figure 2. The relationship between pod width and embryo width for cowpea (*Vigna unguiculata*) cv. IT82E-[illegible].