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PROGRESS REPORT IV

Rec'd in SCI: JUL 17 1985

Improvement of Plant Regeneration

Return to A 2. A-15

B

PROGRESS REPORT

NEW VARIETIES OF RICE FOR SALINE AND ACID SOIL THROUGH TISSUE CULTURE

AID/SCI PROPOSAL 2A-15

GRANT NO. 936-5542-G-SS-3037-00

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U.S. International Development
Cooperation Agency
Bangkok

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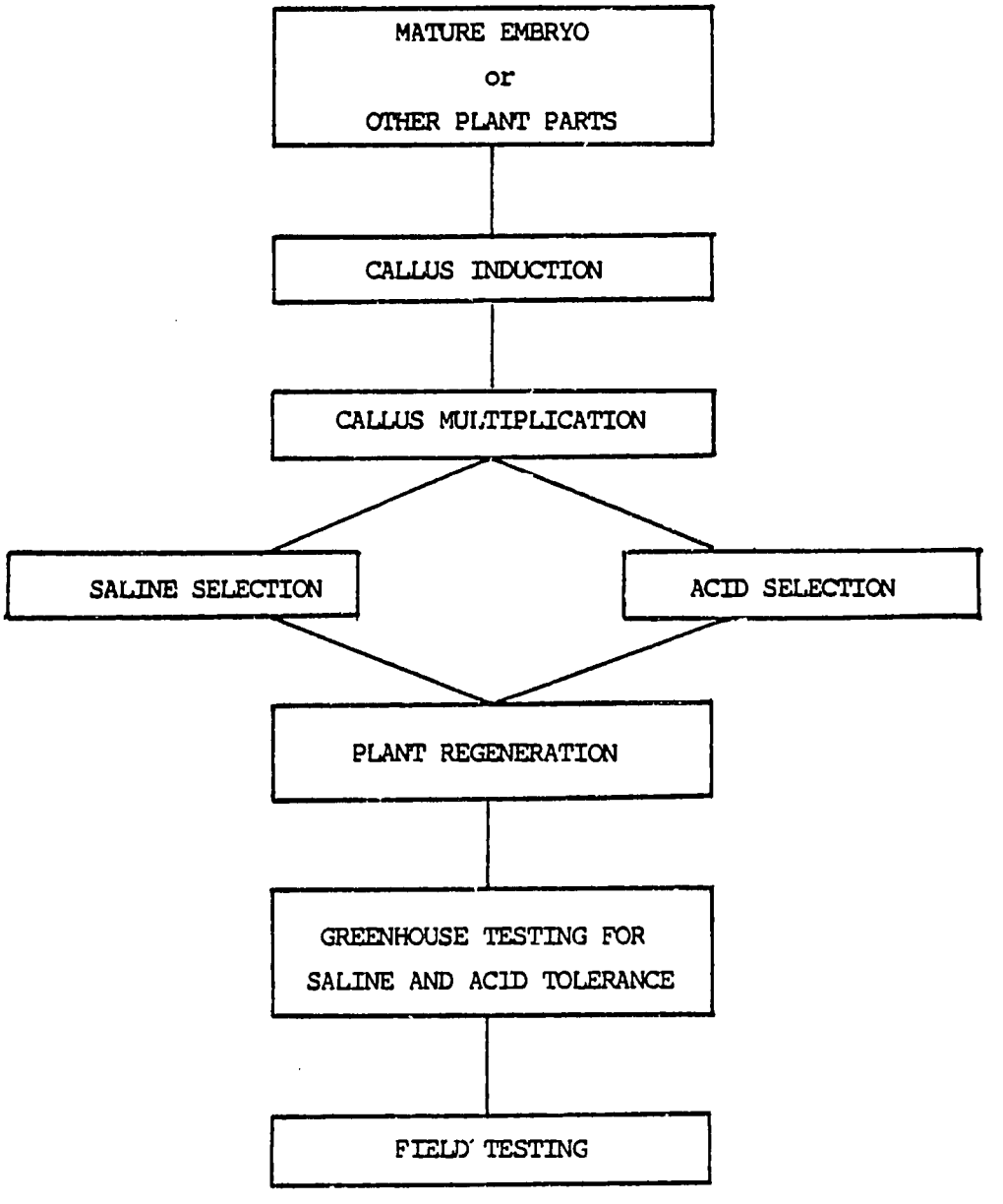
PROPOSED METHODS

FOR

PRODUCTION OF SALINE AND ACID TOLERANT RICE



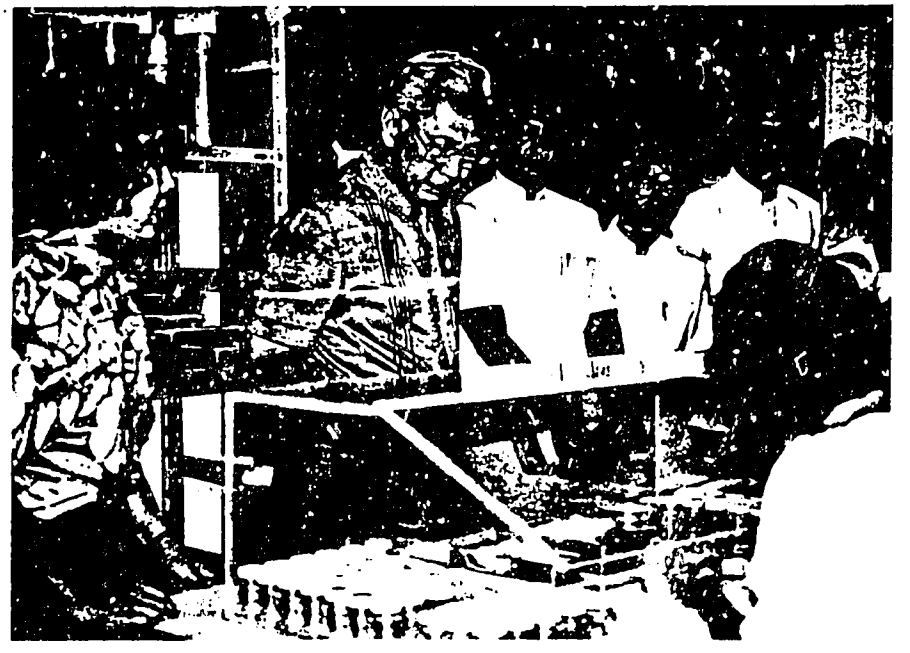


Figure A. Deputy Prime Minister, Mr Bhichai Rattakul who is also responsible for science and technology section, paid a visit to our laboratory in April, 1985.

Biotechnology is favorably placed among the top priorities in the program for development of science and technology in Thailand. Furthermore, types of biotechnology involving in food production will definitely have their place in the future where the world food shortage is becoming more serious.

Figure B. Professor Murray W. Nabors, Project Director of Tissue Culture for Crops Project at Fort Collins, Colorado and Mr. Kirby J. De Mott, Manager of Rice Program paid us a visit in February, 1985. Prof. Nabors is also our Cooperating Investigator.

Front row from left to right:

: Professor Thavorn Vajrabhaya, Investigator.

: Miss Nipa Laksuntarakorn, Project Staft.

: Miss Poungpet Poonsapaya, Laboratory and Research Operation Manager.

: Associate Professor Montakan Vajrabhaya, Principal Investigator.

Back row from left to right:

: Mr. Kirby J. De Mott.

: Professor Murray W. Nabors.

: Mr. Chana Opaskornkul, Project Staff.

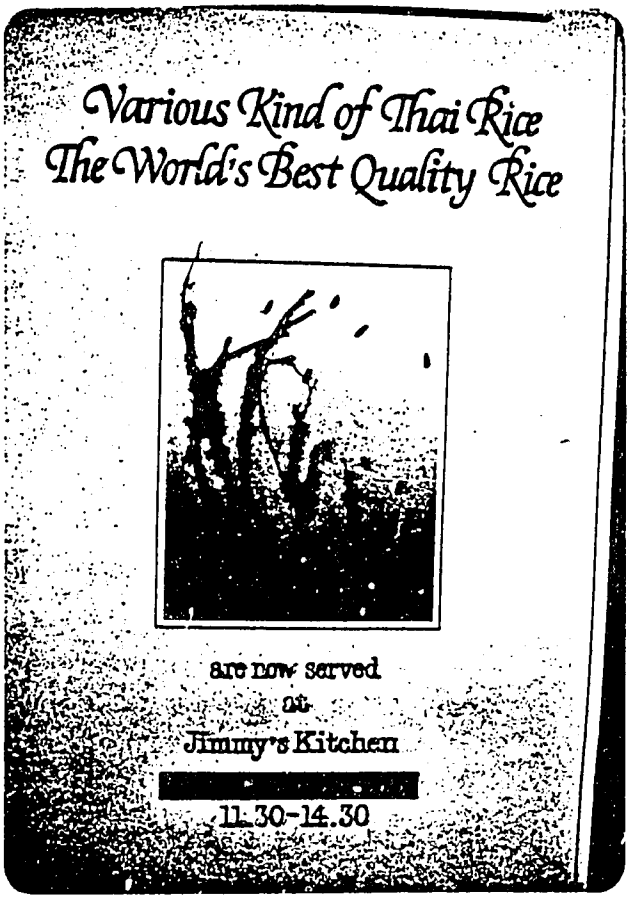


Figure C. An exhibition of Thai rice was held in March 1985 in the Ambassador Hotel, Bangkok, to acquaint consumers with the quality rices.

Figure D. The bags shown in this photograph are Kao-dok-mali (KDML), perhaps the best quality, collected from different provinces of Thailand. This particular variety not only has characteristic fragrant when cooked, it is also one of the fairly tolerant varieties to saline condition. Yet, the more adaptive variants of this variety to such condition is being searched for by means of tissue culture.

Figure E. Plant regeneration from LPT calli are ready to be grown in normal condition.

F. Plantlets regenerated from NaCl treated calli. Calli were cultured in the medium containing NaCl one month before transferring into regeneration medium. Such treatment reduces rate of regeneration drastically. Left, NSPT, 0.8% NaCl; middle, LPT, 1.0 % NaCl; right, LPT, 2.0 %.

K

Figure G. KDML plants from ordinary seeds in sand culture. This photograph was taken in December 1984 when night lengths had been long enought for flower bud initiation and fruit setting.

H. KDML plants from seeds collected from a plant regenerated from callus. Seedlings of the next generation will be subjected to selection under salt stress condition. Photograph was taken in March 1985 at the age of 82 days.

L

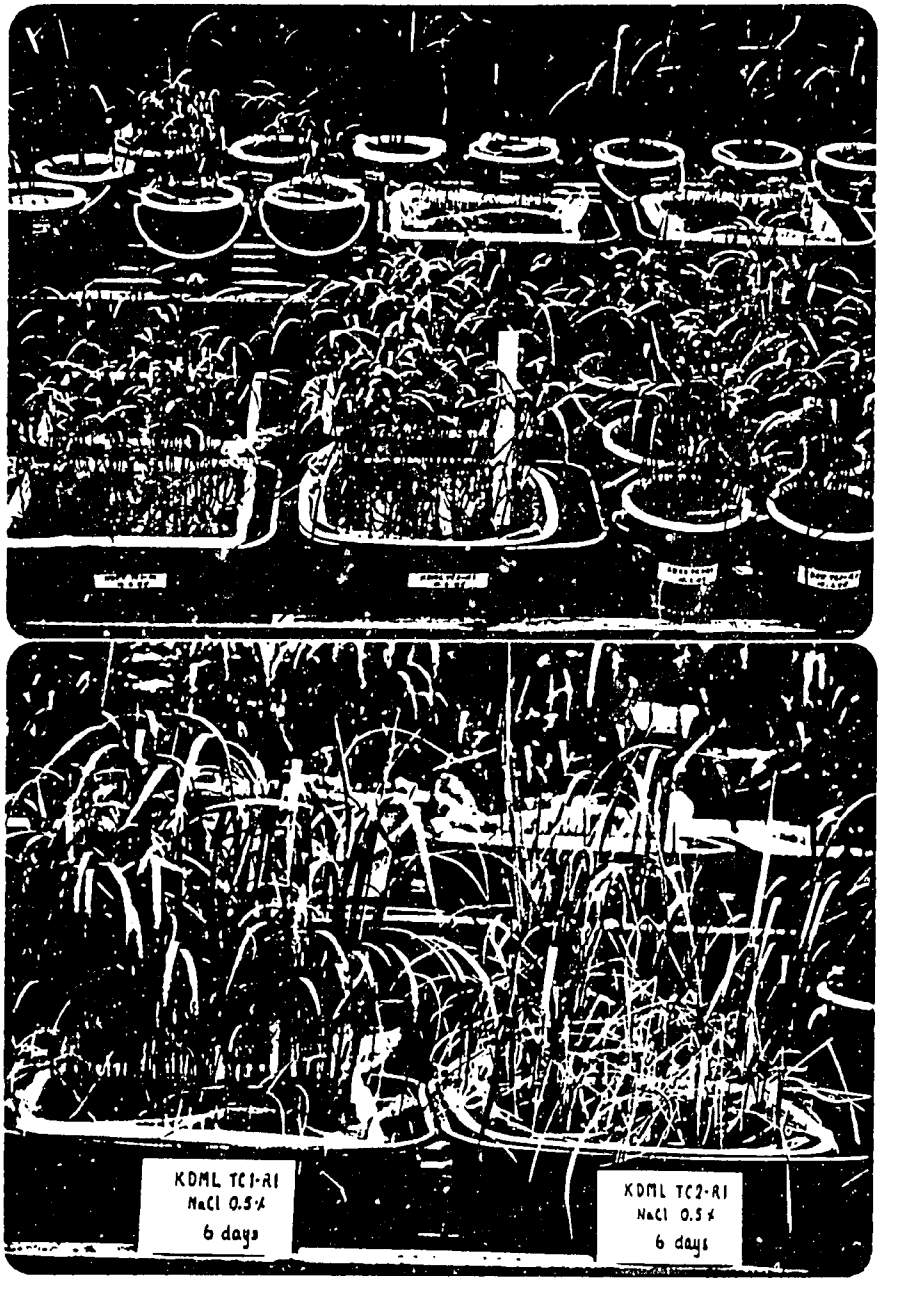


Figure I. Seedlings obtained from seeds of plants regenerated from callus. These five-leaf stage seedlings are ready for NaCl treatment.

On lower bench : left trays, NSPT; right trays, KDML.

: left buckets, RD 23; right buckets, RD 25.

J. Seedlings of KDML in sand culture with 0.5 % NaCl. After six days, the differences in sensitivity to salt among seedlings obtained from the mother plants regenerated from different calli were apparent.

Figure K. Responses of the RD 23 seedlings of a regenerated plant after six days of 0.5 % NaCl treatment. Note the differences in sensitivity to salt among seedlings from the same mother plant, the severe ones in the right bucket produce narrow and light green leaves before turning brown.

L. The differences among RD 25 seedlings are not so great when given the same treatment as the RD 23.

PREAMBLE TO THE PROGRESS REPORT IV

First part of this report contains a summary of the first generation of salt tolerant plants after a long strugling for plant regeneration. Hopefully, some of these will be the forerunners of salt tolerant lines in the future. The methods used for plant regeneration at present are the result of continuous searching for the suitable media and other environments for differentiation and growth of shoot apices during the culture (see Report II, III, and this Report). Now, most calli (50-80%) of the varieties cultured produce greenspots (early stages of shoot apex formation) in our improved methods which is a great advancement since the methods used in the Report II. However, relatively few of the shoot apices develop further and become shoots, this gives us a hint that the requirements for initiation and further development of shoot apex must be widely different. Further investigation on the development of shoot apex is indeed needed. During this period, five to thirty percent of calli form shoots, subsequently produce adventitious roots and finally become complete plants which can be grown in natural condition. When treated with salt <u>in vitro</u> the number of plants regenerated is reduced to 14 plants per 10,000 calli due to the damage of the shoot apices of the sensitive ones during initiation and development.

In growing rice outdoor, inorder to avoid variation in soil composition which may affect the results, when treated with salt or acid, sand culture method has been established with satisfactory result. Plastic protection can be installed on the roof only to prevent rain but it cannot prevent insects or birds. The climate in Bangkok is very warm and humid which bars the use of plastic on the side walls. Covering plastic on the side of the house will generate

too much heat and humidity, which causes damage to the plant directly and indirectly due to excessive temperature and help fungal diseases to spread rapidly, without protection rice plants attract some insects and birds. Insecticides and fungicides are being used only when necessary partial covering of the side wall with fish nets is then used to keep the birds out.

Activities in the laboratory still go on actively to produce more plants regenerated in salt stress and acid stress conditions. During the last eight months (Aug. 84 to March 85) over 27,000 calli were subjected to salt treatment which ranged from 0.8% to 4.0% sodium chloride incorporated in the media. Preparation of media for acid stress experiments are rather complex because of interaction between acid and other components in the medium and differential absorbtion of ions by calli. However, some successful results have been obtained after a series of experiment atempting to stabilize the pH during culture. At the same time we still continue working on the improvement in shoot development following shoot apex initiation in order to obtain more plants for rigorous treatment.

Recently, the university has recognized our laboratory as a unit cell for research and human resources development. To this end, the university just granted us US $ 10,315 for the development of our laboratory by expansion of working space which adding 50 m^2 of working space. This will enable us to improve our working condition, which, in turn, will incresae efficiency of our laboratory in the future.

ABBREVIATIONS USED IN THIS REPORT

RICE VARIETIES

RD 8	Rice Department No.8
RD 23	Rice Department No.23
RD 25	Rice Department No.25
KDML	Kao-dok-mali-105
KTH	Kao-ta-haeng
LPT	Lueng-pa-tiew-123
NMS	Nang-mol-S-4
NSPT	Neo-sun-patong

MACRO AND MICRO-ELEMENTS

Na_2EDTA	Disodium ethylene diaminetetraacetate
NaFeEDTA	Ferric monosodium ethylenediaminetetraacetate

GROWTH REGULATORS

IAA	Indole-3-acetic acid
2,4-D	2,4-Dichlorophenoxy acetic acid
BAP	6-Benzyl amino purine
K	Kinetin

TERMINOLOGIES

A — Green area coverage $= \frac{\text{Area covered with greenspots}}{\text{Total callus surface area}} \times 100$

GS — Greenspot $= \frac{\text{No of calli with greenspots}}{\text{No of total calli}} \times 100$

SH — Shoot $= \frac{\text{No of calli with shoot}}{\text{No of total calli}} \times 100$

R — Root $= \frac{\text{No of calli with roots}}{\text{No of total calli}} \times 100$

Regeneration $= \frac{\text{No of calli that produce plants}}{\text{No of total calli}}$

WORK DONE DURING JANUARY - JUNE 1985

OBTECTIVES

To select salt tolerant lines by using two different methods, one is to incorporate salt directly into the regeneration medium in *in vitro* and the other is to add salt into nutrient solution for the regenerated plants grown in sand culture; to further improve the methods for callus induction, callus culture and plant regeneratior by modification of culture media, altering environments or using various organs as sources for callus culture; to find an appropriate method for selection of acid tolerant line.

BACKGROUND

Regeneration has been a major problem in developing salt tolerant lines in rice through tissue culture. Previous reports (Progress Report II and III) have been dealing with composition of culture media and some physical environments for better regeneration. Upto now, even the results are not upto our expectation but our improved methods are quite sufficient for a routine work. A number of plants regenerated under salt stress *in vitro* have been obtained and are now being grown for further studies.

Salt treatments of the plants regenerated from callus under normal laboratory condition are being practiced to weed out the sensitive ones and to retain the ones that survive. One of these might be a chance mutant that ever appears in the cell lines during culture, if this is the case it will stand subsequent tests.

Improvement of regeneration is still a focal point of this project, since the number of plants regenerated is likely to determine the success in obtaining wanted mutants. Therefore, works on composition of media and improving environments still go on.

MATERIALS

Eight rice varieties, RD23, RD25, KDML, KTH, LPT, NMS, RD8 and NSPT (the last two being glutinous rices) were used in the experiments; these are among the recommended varieties of the Rice Division, Department of Agriculture. RD23 and RD25, the recently introduced varieties, are day neutral plants which their seeds can be harvested only in 100 to 130 days after sowing (annonymous, 1983). Both of which can be grown three crops a year. This definitely accelerate the work in the laboratory by having both continuous supply of materials and reduced generation time. But unfortunately, these are among the most difficult varieties to regenerate *in vitro*. On the contrary, the other six varieties in which five are improved old established variaties and one of hybrid origin respond much better in the *in vitro* method, but their harvest times are only in November and December when the nights are sufficiently long. So far we have not found the ideal experimental materials yet. While the immature seeds and young organs were obtained from our own pot grown plants.

METHODS

1. SURFACE STERILIZATION : The method of surface sterilization described in the Progress Report II, p.10-11. were used for mature seeds, slight modifications were made to suit immature seeds or young organs. Bacterial and fungal contaminations were found among 8 to 10% of seeds which is considered satisfactory.

2. STANDARDIZED CALLUS INDUCTION MEDIA : Due to varietal differences, two media were then used for callus induction from embryo of different varieties. A preliminary report is in the Progress report II, p.12. For

convenience the components of the media are listed in Table 1

3. BASIC COMPONENTS OF CULTURE MEDIA FOR REGENERATION. :

All media for regeneration used in this report are based on White's macro-elements with some modification eg. the deletion of sodium sulfate or addition of ammonium sulfate. But micro-elements (including iron) are used according to Murashige and Skoog (1962) in full or in half strength. Coconut water is the only undefined organic additive that gives consistent results for regeneration in many experiments done previously, therefore it is used at the rate of 100 ml/l. (see Table 2)

4. REFERENCE MEDIUM AND PHYSICAL ENVIRONMENT FOR REGENERATION : For convenience in discussion, the medium H_2 in the experiment 4 of the Progress Report III, p. 44-47 is selected as a reference medium. Since it gave good overall results in three varieties in the experiment, and subsequent tests indicated that this medium was still a useful one. The physical conditions are also listed in the Table 3 along with the reference medium.

5. MODIFIED WP NUTRIENT SOLUTION (Bentley, 1959) :

Potassium nitrate	0.58	gram
Ammonium sulfate	0.10	"
Magnesium sulfate	0.45	"
Triple superphosphate	0.25	"
Calcium sulfate	0.50	"
Sodium ferric diethylenetriamine pentaacetate	0.05	"
Manganese sulfate	0.005	"

Boric acid	0.005 gram
Zinc sulfate	0.0005 "
Copper sulfate	0.0005 "
Water	1 liter

A. ON THE SELECTION OF SALT TOLERANT PLANT

Preliminary studies were divided into two parts, one was made *in vitro* during callus growth and the other was made in natural condition when the plants produced four to five leaves. Treatment during growth would damage the sensitive cells, only the tolerant ones would continue to grow and differentiate into shoot and finally the whole plants. This method selects cell lines that tolerate high concentration of NaCl but it gives very low regeneration. Although salt tolerant cell lines have been selected *in vitro*, but it is no garantee that tolerance selected in culture will be expressed at the whole plant level. The plants obtained by this method must be evaluated further for salt tolerance and genetic stability.

On the other hand, the treatment of plants at four to five-leaf stages was also done in natural condition. This method has an advantage that the selection is done directly at the whole plant level which in some cases only the change in root functions can limit transportation of salt into inner tissues. In such treatment, if any favorable mutation should occur either in the cell lines or in root function the plant will tolerate high salinity. Such treatment was used in treating seedlings derived from plant regenerated *in vitro*.

1. SELECTION *IN VITRO* : During August 1984 to March 1985 (8 months) 81,756 embryos were surface sterilized and cultured in the Standardized Callus Induction Media for two weeks in the dark (see mothods p.10). At this stage, only healthy calli were cut off

from other tissues which they were originated from and transferred into media similar to the previous one except the presence of 1.5% or 2.0% NaCl in them. The number of calli in this treatment was reduced to 27,688 due to selection of only suitable calli before transferring to the new media. These culture vessels were now kept in the light for four weeks, at the end of this period only 10,682 calli survived.

These treated calli were then transferred to a modified White's medium, using macro-elements no 1 and micro-elements no 1, for regerneration. The culture vessels at this stage were moved to the shelves which the physical conditions were described in the Table 3. Only 0.09% of the treated calli were able to regenerate in the form of plantlets (with root, stem and leaf), while the untreated calli were able to give 8.3% regeneration in KDML, 10% in RD23, and 30% in LPT when grown in the reference medium. It is quite obvious that NaCl at the stated concentrations has deleterious effect on callus both in survival of the cells or differentiation. The regenerated plants from such treatment are most likely to come from the tolerant cell lines resulting from somatic mutation occurring during callus initiation and growth rather than later developmental stages.

2. SELECTION IN NATURAL CONDITION : Seeds collected from plants regenerated from callus, without treating with NaCl *in vitro* were soaked in tap water for 3 to 5 days until they germinated. Approximately one hundred seeds were planted in the 25 x 31 cm baskets containing coarse sand. The baskets were submerged inside the plastitrays of 26 x 41 cm where a modified WP nutrient formular (Bentley, 1959;) and methods in this report was used. The solution was kept within 1 cm below or above the sand level, addition of nutrient was done when necessary. When the seedlings has four to five leaves,

usually in two weeks, the nutrient was drained off and replaced with a fresh nutrient solution with 0.5% NaCl added. The solution was changed once in the next twenty four hours, the level of nutrient was kept at 0.5 to 1.0 cm above sand surface. A conductivity meter was used at three to four days interval to measure electrical conductivity of the solution. At the beginning, the electrical conductivity of fresh solution read between 7 to 8 mMho/cm at 25°c the reading increased gradually to 10 mMho/cm in 5 to 10 days. Whenever the conductivity went up above 10 mMho/cm, the solution was replaced by a fresh solution.

The treatments were terminated when 90% or more of the plants treated died which began around the third weeks. The living plants were removed from the baskets and planted in sand in either plastic trays or clay jars using the same nutrient solution described earlier. Some of the plants never recovered from the shock and died during the first week but the rests seemed to grow normally and began to produce spikes at this writing.

A total of 93 plants in different stages of development have been obtained upto now. Hopefully, some of the tolerant plants are the result of real somatic mutation, which would expressed themselves better in the next and subsequent generations.

Table 1. Standardized Callus Induction Media

Concentration in mg/l			
NO 1.		No 2.	
Mineral components of Murashige and Skoog's (1962)			
Sucrose	30,000		
Agar	8,000		
Myo-inositol	100		
Thiamine. HCl	0.4		
2,4-D	1	2,4-D	2
Kinetin	0.3	Coconut water	100 (ml/l)

Table 2. Basic components of culture media for regeneration

Macro-elements	Concentration (mg/l)		
Component	NO 1	NO 2	NO 3
$MgSO_4.7H_2O$	———	720	———
$Ca(NO_3)_2.4H_2O$	———	300	———
KNO_3	———	80	———
KCl	———	65	———
$NaH_2PO_4.H_2O$	———	16.5	———
Na_2SO_4	200	0	0
$(NH_4)_2SO_4$	0	0	200

Micro-elements	Concentration (mg/l)		
Component	NO 1	NO 2	NO 3
Na_2EDTA	——— 37.3 ———		18.65
$FeSO_4.7H_2O$	——— 27.8 ———		13.9
$MnSO_4.H_2O$	16.9	8.45	
$ZnSO_4.7H_2O$	8.6	4.3	
H_3BO_3	6.2	3.1	
KI	0.83	0.415	
$Na_2MoO_4.2H_2O$	0.25	0.125	
$CuSO_4.5H_2O$	0.025	0.0125	
$CoCl_2.6H_2O$	0.025	0.0125	

Others			
	Agar	8000	mg/l
	Coconut water	100	(ml/l)

Table 3. Reference medium and physical conditions for plant regeneration

Reference medium : (see Table 2)

Macro-elements	No 1	
Micro-elements	No 3	
Agar	8,000	mg/l
IAA	1	mg/l
Kinetin	3	mg/l
Coconut water	100	ml/l

Physical conditions :

Culture vessels : screw-cap bottle of 7 cm high and 4.2 cm wide containing 12-15 ml of medium

Light sources : Dark for callus induction

: Fluorescent lamps; one or two Philips Tl 40 W/33, 23-30 cm above callus

Light regime : 16/8 hrs.light/dark, except otherwise stated in some experiments

Temperature : 21-27°C

EXPERIMENT 1

OBJECTIVES : To find out whether the immature embryo has a any advantage over the mature one in the production of callus or not; to determine varietal differences in response to the treatment.

BASIC CULTURE MEDIUM : Standardized callus induction medium no 1 (Table 1)

RICE VARIETIES : LPT , NMS

CALLUS AGE : 0

LIGHT REGIME : 16/8 hrs. light/dark

NUMBER OF TREATMENTS : 2 (immature and mature embryos), 2 varieties

NUMBER OF REPLICATIONS : 28-40 embryos

LENGTH OF TREATMENT : 6 wks

CONCLUSION : Calli induced from immature embryos of NMS grew better than the ones from mature embryos. It is interesting that calli of LPT behaved in the same pattern as the variety mentioned above during the first four weeks but the growth of calli from immature embryos was very much retarded during the fourth and sixth weeks while the ones from mature embryos grew normally throughout the treated period. This may be caused by varietal differences or stages of development of embryos.

RESULTS AND DISCUSSION : Re : p. 13-21, Progress Report II; Table 4; Graph 1.

In this experiment, the results from RD 25, KDML and NSPT in the Progress Report II are referred to as reference culture. The newly introduced varieties, LPT and NMS, were used in the experiment. Calli

from immature embryos of NMS grew better than the ones from mature embryos throughout the period of experiment like the KDML and NSPT. Calli of the LPT from both immature and mature embryos grew at similar rates upto four weeks, then suddenly some calli of immature embryos grew at much slower rates and some seemed to stop growing entirely, while the calli of the mature ones continued to grow at the same rate until sixth weeks. Similar growth pattern was also observed in the culture of RD 25 callus.

Dormancy of the whole embryo after harvest has been reported (Rice Institute, 1983), which indicated the accumulation of some inhibitory substances during the process of embryogenesis. This may affect the growth of callus if an embryo in taken out at a particular stage of development. The differences of growth patterns of calli may simply due to the stage of development of embryo during the initial stage or it may be a built-in characteristic of certain varieties.

Even the immature embryo of some varieties produces better callus but the practical use of immature embryo for large-scale callus production is questionable for three reasons. First, inconsistency of callus growth has not been studied in detail as discussed above, second, it involves growing own plants especially the photosensitive ones that need photoperiodic treatments, and the last is regeneration obtained from calli from immature embryo is not better than the ones from mature embryo in spite of larger and healthier calli. Mature embryos can easily be obtained from stored seeds at 5°C in a refrigerator. This saves time and efforts in acquiring materials prior to beginning of the culture.

Table 4. Responses of immature and mature embryos to callus induction treatment.

Variety	Mean callus weight per embryo after two four and six weeks in culture (gram)							
	Immature embryo				Mature embryo			
	n	2Wks	4Wks	6Wks	n	2Wks	4Wks	6Wks
RD 25	40	0.0927	0.2566	0.2360	40	0.0796	0.1563	0.2578
NSPT	40	0.0703	0.2101	0.3242	40	0.0404	0.0716	0.1477
LPT	28	0.0383	0.1142	0.1196	40	0.0399	0.1024	0.1804
NMS	36	0.0598	0.1317	0.2084	40	0.0400	0.1089	0.1677

n = number of callus

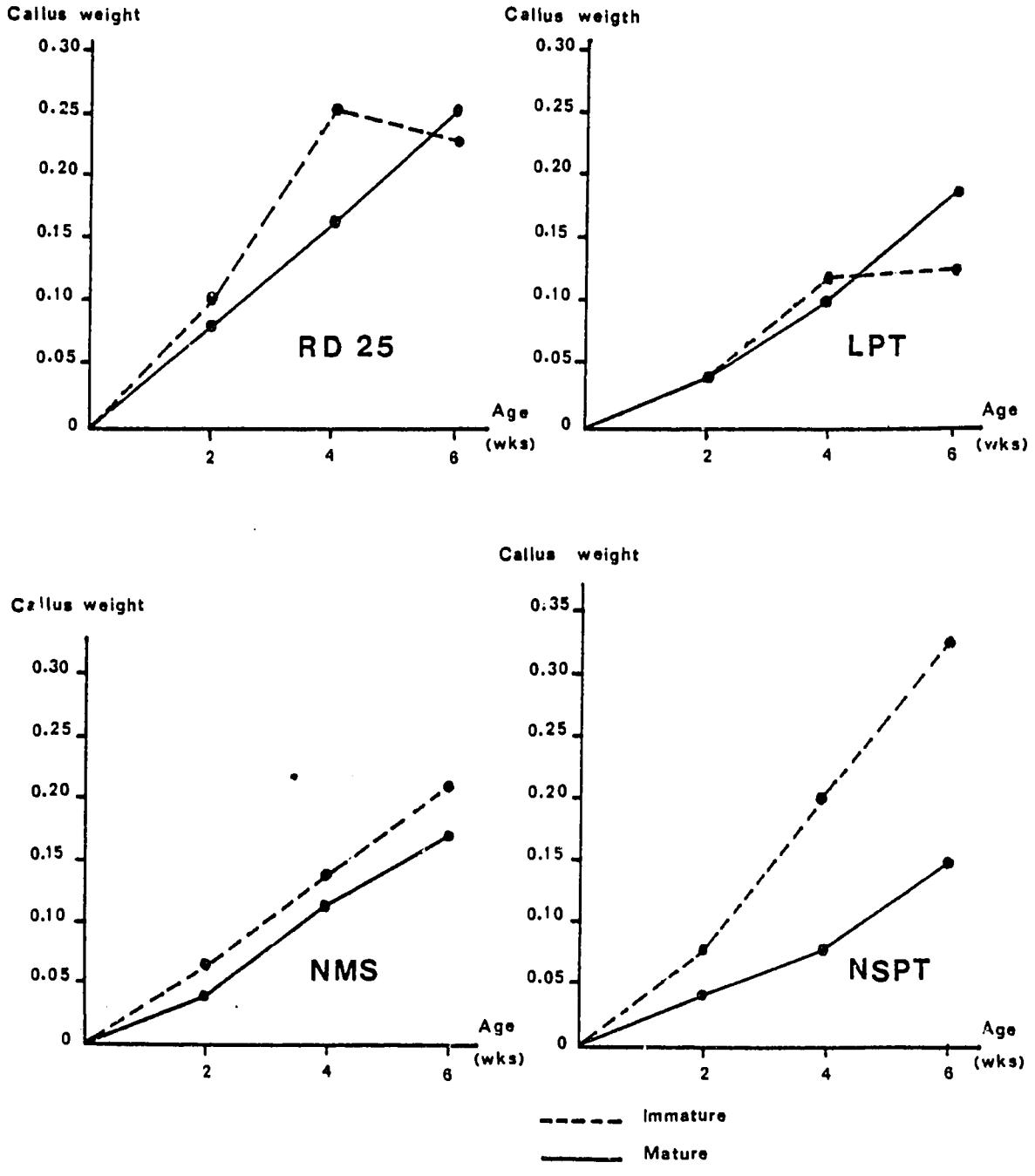


Figure M. Callus growth from immature and mature embryos of RD 25, LPT, NMS and NSPT.

EXPERIMENT 2

OBJECTIVES : To induce callus from young spikelet, node and young leaf; to find if callus initiated from such organs has any advantage over callus from embryos.

BASIC CULTURE MEDIUM : Standardized callus induction medium no 2 (Table 2).

RICE VARIETY : RD25

CALLUS AGE : 0

LIGHT REGIME : 16/8 hrs. light/dark

NUMBER OF TREATMENTS : 3

NUMBER OF REPLICATIONS : spikelets 13; nodes 6; young leaves 5.

LENGTH OF TREATMENT : 44 days

CONCLUSION : Calli were obtained from spikelets, nodes and young leaves when cultured in a standardized callus induction medium. Most of the calli arisen from all organs treated grew slower than those from the mature embryos.

RESULTS AND DISCUSSION : Other organs of rice, spikelet, node, and leaf, can be used as sources for callus production. Calli began to appear from the culture of spikelets and nodes kept in the dark for 15 days. Two weeks later in the light 84% of these inocculae formed calli which some parts of these transformed into greenspots or roots. Calli from young leaves were observed after they were taken out into light after 15 days and only 40% of the inocculae had calli. Some of these leaf isolates might include shoot apices because they produced new leaves directly and some calli might have originated from young stem below rather than from leaves. Therefore the actual rate of callus formation of young leaf must be slightly lower than 40% as stated above.

Subsequent growth of calli from the three organs mentioned above was not encouraging. These calli enlarged and produced some greenspots, roots and only one plantlet during the end of the first month but the growth rate declined afterward and began to die during the fifth and sixth weeks. All calli obtained from this experiment grew slower than calli originated from mature embryos and regeneration was much poorer. Therefore these organs are not suitable for callus production with the existing method.

EXPERIMENT 3

OBJECTIVES : To find suitable concentrations and ratios of auxin to cytokinin for regeneration; to find sources of cytokinin for better regeneration; to improve regeneration since it was reported in the experiment 4 in the Progress Report III.

BASIC CULTURE MEDIUM : Modified White's medium (Table 2) using Macro-elements no 2 and Micro-elements no 3.

RICE VARIETIES : RD23, KDML

CALLUS AGE : 2 wks.

LIGHT REGIME : 16/8 hrs. light/dark

NUMBER OF TREATMENTS : 6 different media; 2 varieties.

NUMBER OF REPLICATIONS : 40 calli

LENGTH OF TREATMENT : 4 wks.

CONCLUSION : Marked varietal difference was observed, KDML responded best when used 0.5 ppm IAA and 0.4 ppm kinetin but the RD23, the difficult variety, required 0.5 ppm IAA and 0.1 ppm BAP for good regeneration. However, more consistent results were obtained from different varieties when 1 ppm IAA and 3 ppm kinetin were used (see Report III and Reference medium in this report) and 4 ppm IAA and 3 ppm kinetin in this report gave a fair result.

RESULTS AND DISSION : Re : experiment 4, p.44-47, Progress Report III; Table 5, 6 in this report

A series of tests on using different concentrations of hormones in the modified White's media was made earlier and found that 1 ppm IAA and 3 ppm kinetin gave fairly uniform results in regeneration of both difficult and easy varieties of rice (see Progress Report III).

Therefore, the H_2 medium in the Progress Report III will be refered to as a reference medium from now on.

In the treatments A, B and C concentration of kinetin was fixed at 3 ppm and of IAA varied at 2,4 and 8 ppm. Shoot formation of KDML in the treatments A, B and C of this experiment was 5%, 7.5% and 5% respectively; and of RD23, the difficult variety, were 0, 5% and 0. Whereas, the reference medium gave 10% and 8.3% regeneration in RD23 and KDML respectively, younger calli of KDML were able to produce upto 15% regeneration

Table 5. Concentrations of auxin and cytokinins used in the experiment; our reference medium contains IAA 1 ppm, kinetin 3 ppm.

Growth regulator / Treatment	Kinetin (ppm)	BAP (ppm)	IAA (ppm)
A	3.0	0	2.0
B	3.0	0	4.0
C	3.0	0	8.0
D	0	0.1	0.5
E	0	0.4	0.5
F	0.4	0	0.5

Table 6. Responses of calli to different concentrations and sources of auxin and cytokinins

Medium	Age of callus	XDML					RD23				
		N	% GS	%A	%R	%SH	N	%GS	%A	%R	%SH
A	6	40	30.0	12.5	22.5	0	12	0	0	0	0
	8	40	65.0	17.7	32.5	2.5	12	83.3	32.0	100.0	0
	10	40	72.5	21.4	42.5	5.0	12	83.3	39.0	100.0	0
B	6	40	52.5	13.8	27.5	0	20	0	0	0	0
	8	40	70.0	16.1	37.5	5.0	20	70.0	48.0	100.0	5.0
	10	40	70.0	22.5	40.0	7.5	20	70.0	46.0	100.0	5.0
C	6	40	30.0	11.7	22.5	0	20	0	0	0	0
	8	40	90.0	14.4	22.5	0	20	80.0	37.0	100.0	0
	10	40	75.0	18.3	35.0	5.0	20	80.0	34.0	100.0	0
D	6	40	30.0	12.5	25.0	0	16	0	0	0	0
	8	40	85.0	15.0	30.0	0	16	93.8	27.0	100.0	0
	10	40	77.5	20.3	45.0	2.5	16	93.8	35.0	100.0	12.5
E	6	40	60.0	1.0	25.0	0	20	0	0	0	0
	8	40	87.5	12.6	30.0	0	20	95.0	41.0	100.0	0
	10	40	92.5	25.9	40.0	7.5	20	95.0	42.0	100.0	0
F	6	40	67.5	13.7	12.5	0	20	0	0	0	0
	8	40	87.5	16.0	35.0	10.0	20	70.0	29.0	100.0	0
	10	40	92.5	20.5	37.5	17.5	20	50.0	38.0	100.0	0

The requirements of cytokinins and auxins for regeneration are complex which they depend largely on the varieties of rice in questions. So, the result obtained by different workers varied according to varieties used and sources of auxin and cytokinins (Davoyan and Smetanin, 1979; Nabors, 1982; Oono, 1981; Vajrabhaya et al, 1984a; 1984b). Nabors (1982) divided rice into two groups according to the requirements of auxin levels for formation of embryogenic callus while low auxin favors non-embryogenic callus and the other group in just vice versa. Moreover, it was found that one variety produced endogenous auxin sufficient for growth of non-embryogenic callus, adding a little auxin promoted embryogenic callus. In some experiments there were some indications that BAP gave better result than kinetin. However, not only the concentrations of either auxins or cytokinins affect regeneration directly, but the relationship of the two seems to be very important in regeneration which makes the problem more complicated.

In the treatments D, E and F the concentration of IAA was fixed at 0.5 ppm, while BAP was used as a source of cytokinin at 0.1 and 0.4 ppm. In addition to this, 0.4 ppm kinetin was also used for RD23 a low concentration of BAP (0.1 ppm) induced 12.5% regeneration which seemed to be a marked improvement for difficult variety. However, KDML responded to 0.4 ppm BAP better than 0.1 ppm, but better regeneration (17.5%) was obtained when 0.4 ppm kinetin was used in place of BAP.

From our previous experiments, it was found that cytokinin in coconut water was not sufficient to support good regeneration, then adding kinetin gave better results by prolonging browning of callus and induced more greenspot and shoot to form. Low concentration

of BAP (0.1 ppm) seems to be beneficial for regeneration of the difficult varieties such as RD23 at it is surprising that KDML responds so well in both types of cytokinins at widely different concentrations. However, the effects of kinetin and BAP on regeneration depend on other factors like auxins, macro-elements and physical environments, such effects need to be re-examined before establishing standardized regeneration medium.

EXPERIMENT 4

OBJECTIVE : To test concentrations and ratios of IAA to kinetin and IAA to BAP recommended by others against our reference medium (1 ppm IAA to 3 ppm kinetin).

BASIC CULTURE MEDIUM : Modified White's medium (Table 2) using Macro-elements no 1 and Micro-elements No 1.

RICE VARIETIES : RD23, KDML, NMS

CALLUS AGE : 2 wks.

LIGHT REGIME : 16/8 hrs. light/dark

NUMBER OF TREATMENTS : 3 different media; 3 varieties

NUMBER OF REPLICATIONS : 20, 40 calli

LENGTH OF TREATMENT : 4 wks.

CONCLUSION :

The combination of IAA and BAP or kinetin at low concentrations did not give better regeneration than our reference medium.

RESULTS AND DISCUSSION : Re : experiment 4, p 44-47, Progress Report III. Table 5, 6 in this report.

Low concentrations of IAA and BAP have been reported by Nabors (1982) to be effective in inducing regeneration of some varieties. This experiment was carried out according to their recommendation and the substitution of BAP with kinetin at the same concentration was also tried. For control, calli of these three varieties were cultured in the reference medium along with the other treatments.

In the experiment 3, when 0.5 ppm IAA and 0.4 ppm kinetin were used in the regeneration medium, 17.5% of KDML calli produced shoots

which considered to be high percentage of its variety. But in this experiment, only 5% of calli produced shoot in such medium while 17.5% of calli in the reference medium formed shoots. This result in just a revense of what obtained in the experiment 3, however the low concentration of IAA in conbination with low kinetin are useful for KDML. For RD23, only calli cultured in reference medium produced shoot. On the contrary, five percent of NMS calli were able to produce shoots in all three treatments.

Even the response of different varieties to concentrations of auxin and cytokinins varies widely, the concentration of IAA and kinetin found in the H_2 medium in the Progress Report III still gives consistent result in all varieties tested. This proves that our reference medium (H_2) still stands the tests upto now.

Table 7. Concentrations of auxin and cytokinins used in the experiment 4; our reference medium contains IAA 1ppm and kinetin 3 ppm as in the treatment A.

Growth regulator / Treatment	Kinetin (ppm)	BAP (ppm)	IAA (ppm)
A	3.0	0	1.0
B	0.4	0	0.5
C	0	0.4	0.5

Table 8. Responses of calli to different concentrations and sources of auxin and cytokinins

Medium	Age of callus	RD23					KDML					NMS				
		N	GS (%)	R (%)	SH (%)	C	N	GS (%)	R (%)	SH (%)	C	N	GS (%)	R (%)	SH (%)	C
A	2	40	0	0	0	4.00	40	0	0	0	4.00	20	0	0	0	4.00
	4	40	25.0	12.5	5.0	3.95	40	60.0	10.0	12.5	3.80	20	90.0	0	0	2.95
	6	40	85.0	22.5	7.5	2.95	40	97.5	20.0	17.5	3.33	20	85.0	0	5.0	2.85
B	2	32	0	0	0	4.00	40	0	0	0	4.00	20	0	0	0	4.00
	4	32	59.4	21.9	0	3.19	40	17.5	2.5	2.5	3.15	20	55.0	5.0	0	2.85
	6	32	68.8	18.8	0	2.84	40	27.5	2.5	5.0	3.03	20	55.0	5.0	0	2.75
C	2	32	0	0	0	4.00	40	0	0	0	4.00	20	0	0	0	4.00
	4	32	62.5	6.3	0	3.03	40	42.5	0	2.5	3.13	20	70.0	0	5.0	3.15
	6	32	50.0	15.6	0	2.97	40	45.0	2.5	5.0	3.00	20	65.0	5.0	5.0	2.80

EXPERIMENT 5

OBJECTIVE : To determine the effect of ground rice used as an organic additive; to study the effect of ground rice with and without embryo.

BASIC CULTURE MEDIUM : Modified White's medium (Table 2) using Macro-elements no 2 and Micro-elements no 3.

RICE VARIETIES : KDML, LPT

CALLUS AGE : 2 wks.

LIGHT REGIME : 16/8 hrs. light/dark

NUMBER OF TREATMENTS : 2 different media; 2 varieties

NUMBER OF REPLICATIONS : 36, 40 calli

LENGTH OF REPLICATION : 4 wks.

CONCLUSION : Ground rice with embryos inhibited greenspot formation of both varieties but the effect was not as clear in regeneration. However, the regeneration in the media supplemented with both kinds of ground rice was definitely poorer than in the reference medium.

RESULTS AND DISCUSSION : Re : Table 9.

In an attempt to add organic compounds to the culture medium for promoting regeneration, only coconut water is found to be definitely effective, others tend to cause adverse effects on regeneration (Progress Report III, 1984). Another attempt was made again in this experiment to add ground rice seed, a more natural form of additive, to the medium. This resulted in the delay of greenspot formation during the first four weeks in the medium supplemented with ground rice with embryos. During the fourth and sixth weeks greenspots of

the LPT increase rapidly in the medium with embryos but regeneration was only 5.6% comparing to 20% in the medium without embryos. It is interesting to note that, greenspots of KDML in the medium with embryos were only half of the calli grown in the other medium but shoot formation was twice as high. This means that the inhibitory effect resulting from embryos is confined to greenspot formation more than shoot formation.

However, shoot formation is still affected by ground rice either with or without embryos because RD23 and LPT gave 5.0% and 20% regeneration respectively while our reference medium gave 10% and 30%. Therefore ground, rice is not only an ineffective additive it also has an adverse effect on greenspot formation and regeneration.

Table 9. Responses of calli to ground rice with and without embryos as organic addition our reference medium gave 8.3% regeneration for KDML and 30% for LPT.

Treatment	Age of callus	KDML					LPT				
		N	GS (%)	A (%)	R (%)	SH (%)	N	GS (%)	A (%)	R (%)	SH (%)
With embryo	2	40	0	0	0	0	36	0	0	0	0
	4	40	25.0	10.0	15.0	2.5	36	38.9	10.0	2.8	5.6
	6	40	25.0	14.0	12.5	5.0	36	91.6	19.7	13.9	5.6
Without embryo	2	40	0	0	0	0	40	0	0	0	0
	4	40	52.5	12.9	12.5	2.5	40	80.0	10.9	17.5	7.5
	6	40	42.5	15.9	10.0	2.5	40	95.0	27.4	22.5	20.0

EXPERIMENT 6

OBJECTIVES : To re-examine the effect of organic additives, yeast extract and casein hydrolysate, in the modified White's medium (similar study was reported in the Progress Report III using Murashige and Skoog medium as a basic culture medium).

BASIC CULTURE MODIUM : Modified White's medium (Table 2) using Macro-elements no 2 and Micro-elements no 3.

RICE VARIETIES : RD23, KDML

CALLUS AGE : 2 wks.

LIGHT REGIME : 16/8 hrs. light/dark.

NUMBER OF TREATMENTS : 3 different media; 2 varieties

NUMBER OF REPLICATIONS : 20, 40 calli

LENGTH OF TREATMENT : 4 wks.

CONCLUSION : Supplementing media with yeast extract, casein hydrolysate or both consistently gave low regeneration than in the reference medium (Table 3).

RESULTS AND DISCUSSION : Re : experiment 1, p.3-22, Progress Report III; Table 10, 11 in this report.

Similar experiment has been reported in the Progress Report III, when using macro-element component of Murashige and Skoog's (1962) as the main inorganic sources. During that time it was also found that a low salt formula like White's (1954) worked better in the induction of regeneration. Therefore a modified White's medium was used in this experiment.

The result of this experiment was not encouraging, only the medium supplemented with casein hydrolysate supported regeneration of only 2.5% of KDML calli, other media with casein hydrolysate, yeast extract or a combination of the two did not induce regeneration in either RD23 or KDML. While our reference medium gave 10% regeneration in RD23 and 8.3 in KDML.

This experiment, as the previous one, again is not in agreement with those of Davoyan and Smetanin (1979); Oono (1981); Siriwardana and Nabors (1983); and Yatazawa et al (1967) that they obtained good results in regeneration by using casein hydrolysate, yeast extract or a combination of the above. This experiment confirms the experiment 1 in the Progress Report III that casein hydrolysate and yeast extract are not the organic additives suitable for regeneration of the rice calli tested.

Table 10. Concentrations of casein and yeast extract used in different media; our reference medium contains neither casein hydrolysate nor yeast extract.

Treatment	Casein hydrolysate (ppm)	Yeast extract (ppm)
A	0	3000
B	2000	0
C	2000	3000

Table 11. Responses of calli to casein hydrolysate and yeast extract used as organic additives; our reference medium gave 10% regeneration for RD23 and 8.3% for KDML.

Treatment	Age of callus	RD23				KDML			
		N	GS (%)	R (%)	SH (%)	N	GS (%)	R (%)	SH (%)
A	6	20	0	0	0	40	17.5	30.0	0
	8	20	0	95.0	0	40	0	45.0	0
	10	20	0	95.0	0	40	0	47.5	0
B	6	20	0	0	0	40	32.5	20.0	0
	8	20	65.0	100.0	0	40	42.5	22.5	0
	10	20	50.0	100.0	0	40	22.5	3.0	2.5
C	6	20	0	0	0	40	17.5	7.5	0
	8	20	5.0	95.0	0	40	0	17.5	0
	10	20	10.0	100	0	40	0	22.5	0

EXPERIMENT 7

OBJECTIVE : To study the effects of light quality and duration on regeneration in varying concentration of iron, IAA and kinetin.

BASIC CULTURE MEDIUM : Modified White's medium (Table 2) Macro-elements no 2 and Micro-elements no 2 or no 3.

RICE VARIETIES : RD23, RD25, KDML

CALLUS AGE : 2 wks.

LIGHT REGIME : 16/8, 24/0 light/dark

LIGHT QUALITIES : Fluorescence lamps Philip Tl 40/33, fluorescence lamps Philip Tl 40/33 + 40W incandescent bulbs operating at 110V. (normal voltage is 220V.)

NUMBER OF TREATMENTS : 2 light qualities, 2 light duration, 3 different media, 3 rice varieties.

NUMBER OF REPLICATIONS : 20, 40 calli

LENGTH OF TREATMENT : 4 wks.

CONCLUSION : Light regime of 16/8 light/dark favored regeneration than continuous light or continuous light supplemented with red light from incandescent bulbs regardless of concentration of iron, IAA or kinetin. Lower concentration of iron seemed to favor regeneration in this experiment but other experiments gave inconsistent results. Supplementing the media with 1 ppm IAA and 3 ppm kinetin induced fair regeneration in calli of the RD23 and KDML, the moderately difficult varieties. On the contrary, calli of RD25, the most difficult variety, regenerated better in a low concentration of IAA (0.1 ppm) and a low concentration of cytokinin (from coconut water only).

RESULTS AND DISCUSSION : Re : Table 12, 13

Physical environments have influence on growth and development of plants, and their effects are even more pronounced in tissue culture. The problems of regeneration is rather complex, it involves interaction between cells and environments. In this experiment, light qualities and duration are selected as physical environments for testing. However, the roles of hormones and some elements presence in the immediate environs must not be neglected, so a limited variation in the media was also incorporated in this experiment.

It was found that, light regime of 16/8 light/dark favored regeneration than either continuous light or continuous light supplemented with red light from incandescent bulbs regardless of concentration of iron, IAA or kinetin. Regeneration may depend on photoperiod, longer light period may inhibit shoot development, or it is possible that light itself may give off excessive heat which directly affect regeneration rather than the light duration. It is very easy for heat to built up in the closed bottle. If this is the case low room temperature may cancel the effect.

Concentration of irons seems to affect regeneration of haploid plant from microspore (Nitsch, 1981). In this experiment 2.9 ppm of iron seemed to favor regeneration but other experiments indicated that regeneration was also good at 5.8 ppm of iron. It is safe to say that the amount of iron presence in the media was sufficient for normal growth and development.

Concentration of 1 ppm IAA and 3 ppm kinetin still favored regeneration of RD23 and KDML calli, which are moderately difficult varieties. On the contrary, calli of RD25, the most difficult variety,

required only 0.1 ppm IAA and only cytokinin from coconut water for regeneration in all three light conditions, while higher concentrations of both IAA and kinetin, regeneration was limited to the ones grown under 16/8 light/dark condition.

Table 12. Concentrations of indole acetic acid, kinetin and FeEDTA in the media tested; our reference medium contains 1 ppm IAA, 3 ppm kinetin , 18.65 g/l, Na_2EDTA, 13.90 g/l $FeSO_4.7H_2O$ which is similar to the medium C.

Treatment	IAA (ppm)	Kinetin (ppm)	FeEDTA	
			Na_2EDTA (g/l)	$FeSO_4.7H_2O$ (g/l)
A*	0.1	0	18.65	13.90
B	0.1	0	37.30	21.80
C	1.0	3.0	18.65	13.90
D	1.0	3.0	37.30	27.80

*All calli were culture in the medium A for two weeks for inducing greenspot before transferring into other media for regeneration.

Table 13. Shoot formation in the media B, C and D under three light conditions; our standard light duration is 16/8 light/dark, and the reference medium is close to C.

Light condition (light/dark-hrs)	RD23			RD25			KDML		
	B	C	D	B	C	D	B	C	D
16/8 fluorescent bulbs	0	0	5.0	6.2	7.5	4.6	0	40.6	5.0
24/0 fluorescent bulbs	0	4.1	0	4.1	0	0	10.0	19.4	12.5
24/0 fluorescent bulbs + incandescent bulbs	0	0	0	2.3	0	0	5.0	3.1	0

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