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PROGRESS REPORT V

Salt and Acid Selection

2A-15

PDAAL-180

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

Department of Botany
Chulalongkorn University
Bangkok

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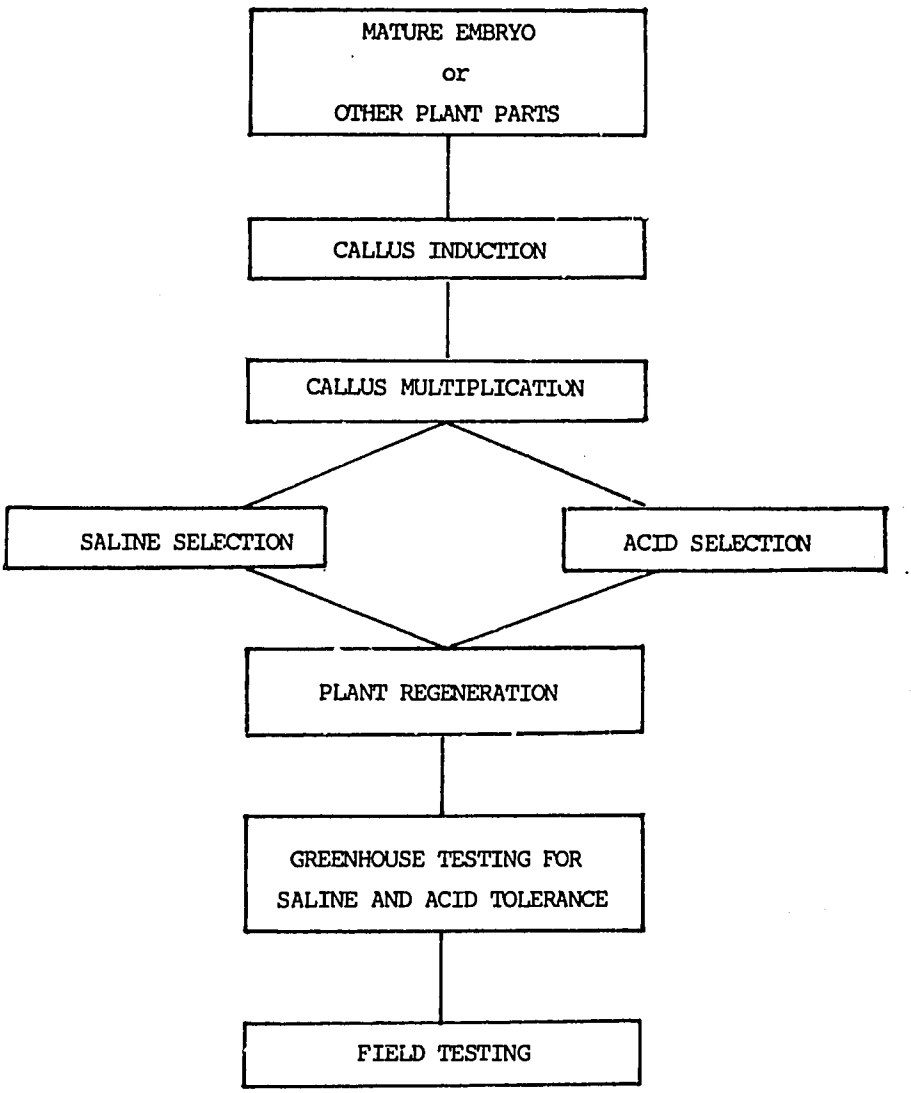
PROPOSED METHODS

FOR

PRODUCTION OF SALINE AND ACID TOLERANT RICE



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

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 $= \frac{\text{No of calli that produce plants}}{\text{No of total calli}} \times 100$

C — Visual rating : 1 = very poor; 2=poor; 3 = good; 4 = excellent

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Figure 1 Our new transfer room can house six plexiglass transfer hoods for a routine work and two laminar flow cabinets (not shown) for critical works in protoplast culture and fusion. Seven out of eight hoods can be used simultaneously. The addition of 50 m^2 to our laboratory space increases the total working area to 150 m^2 which will both increase the amount of work and improve performance of the laboratory.

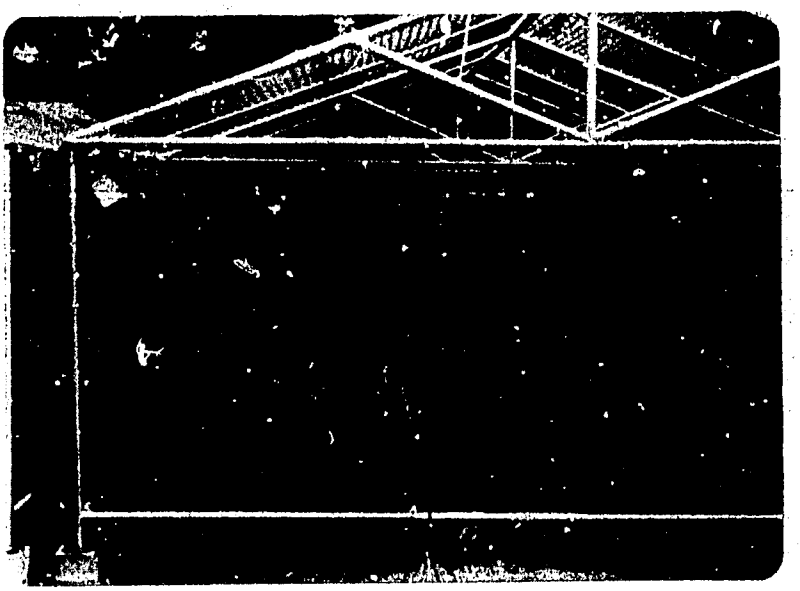

Figure 2 Regenerated plants of RD23 are in sand culture under plastic covered roofs. Some of these regenerated plants are from NaCl or acid treated calli and some are second generation resulting from screening for salt tolerant lines. Variations in morphology are being observed among the regenerated plants but the salt or acid tolerant characters will be screened in the next generation.

RD23 is one of the day neutral varieties which can be planted all year round, the harvest time is about 130 days after sowing. Theses characteristics greatly facilitate the handling of regenerated plants which they can be planted and harvested any time of the year. And now the techniques of plant regeneration has been refined to cope with the difficult variety such as the RD23.

The selection of material and technique is very important for this project. (This photograph was taken in July when the night lenght was still fairly long).

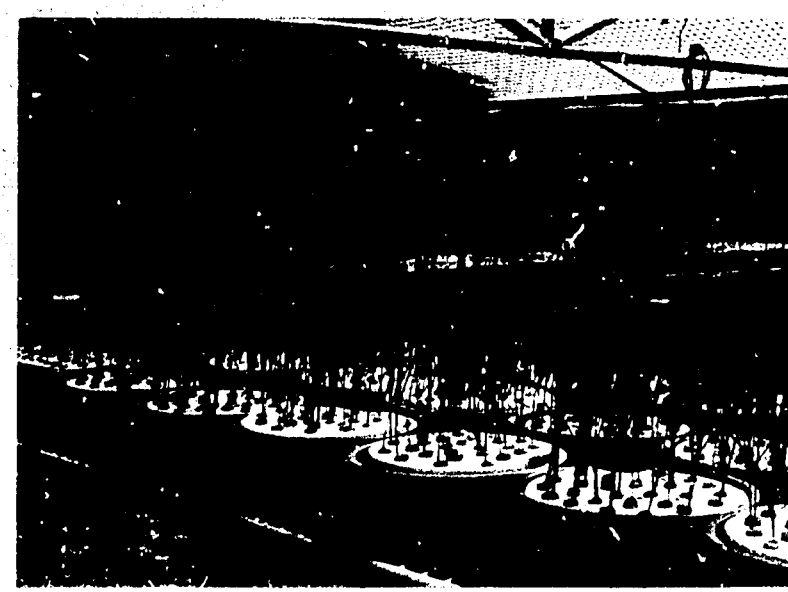

Figure 3 Seedlings from regenerated plants are being planted in styrofoam floating on nutrient solution. Most of these will be treated with 0.5% NaCl when reaching 4 to 5 leaf-stages, about 5% of the treated seedlings survive after one month of treatment. Only the best 2% are selected for further investigation.

The treatments are made under the plastic covered roof to prevent rain and to reduce excessive light.

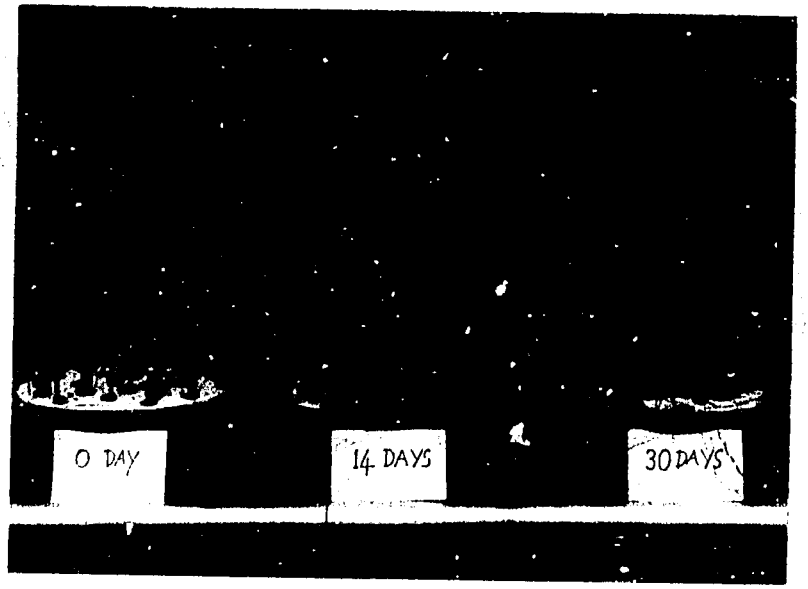

Figure 4 Responses of the KDML seedlings of a regenerated plant after 14 days and 30 days of 0.5% NaCl treatment. Normally, no symptom is observed during the first week, but is easily seen in the second week (middle). Old leaves of the sensitive ones turn light green and having brown tips indicating considerable number of death cells in such area. The brown area moves toward the base and finally whole leaf becomes brown, some of the plants die during this period.

The effect of NaCl becomes severe in the third and fourth weeks (right), most of the plants (up to 95%) die before reaching this stage. Two percent of the treated seedlings are selected for planting for further screening.

Figure 5 Investigators and technical staff of the project "New Varieties of Rice for Saline and Acid Soil Through Tissue Culture".

Front row from left to right : Rungrudee Nilvichien, Kritsana Jiamsamai, Montakan Vajrabhaya (principal investigator), Ourasa Tunvachkul (lab. manager), Kerri Wright (from TCCP),

Back row from left to right : Suriya Singnil, Vasana Sanit, Duangjai Boontiam, Surapong Vatekul, Piangjai Pongpaew, Chanida Khongchuensin Thavorn Vajrabhaya (investigator).

Figure 6 The preparation room is equipped with standard equipment and supplies necessary for tissue culture. Approximately 2000 culture bottles are prepared each week.

Miss Kerri Wright (far left) of the TCCP is now working in our laboratory to get acquainted with working condition and handling of materials in the tropic which are very different from what she has done before. She has been here for three months working mostly on callus induction and regeneration of Thai rices, she will continue the work for another two months to complete the experiments.

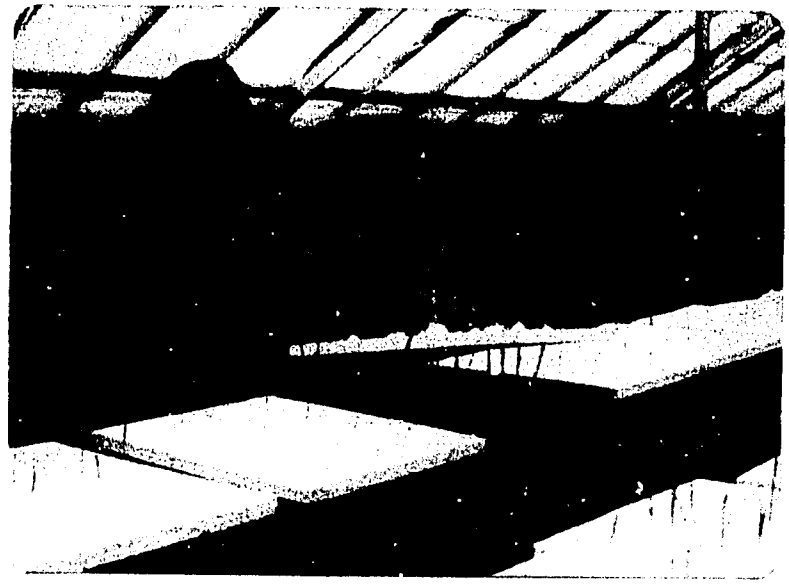

Figure 7 Miss Supaporn Tang, our first graduate student in the exchange program between Colorado State University and Chulalongkorn University, is discussing with her advisor on salt tolerant rice in the CSU greenhouse. Miss Tang is now working on the problem of regeneration of rice from callus, she will spend six months working under the supervision of Professor M.W. Nabors.

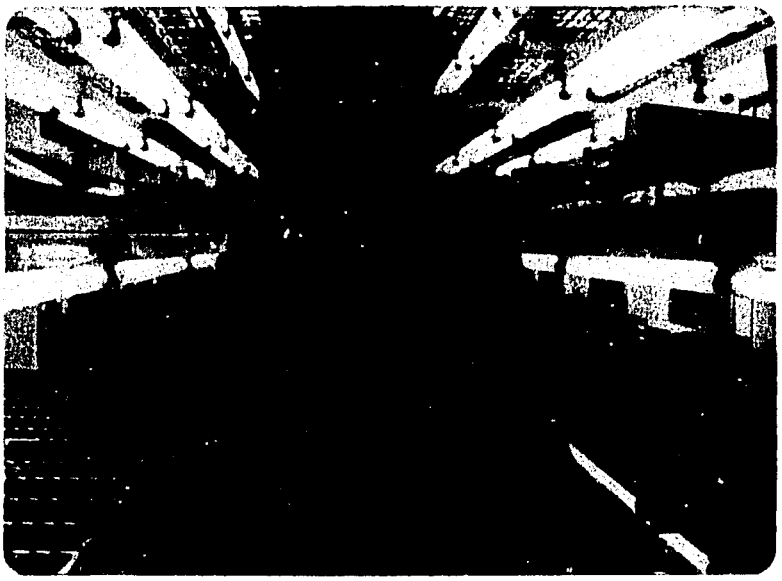

Figure 8 Miss Poungpet Poonsapaya, our former laboratory and Research Operation Manager, is showing cultures to the principal investigator of the "New Varieties of Rice for Saline and Acid Soil Through Tissue Culture" at the TCCP culture room. Miss Poonsapaya is now working for her Ph. D. in tissue culture at CSU.

PREAMBLE TO THE PROGRESS REPORT V

This report, in general, is similar to the Progress Report IV in presenting information on work done in selection of plant in stress conditions, some experiments on the improvement of regeneration, news and problems arising during the last six months.

Selection for salt tolerant plants has been done both in vitro and in natural conditions. Difficulties arose when acid was added to the medium which made it too soft to support callus. This problem has been overcome by autoclaving some ingredients separatey. This is a result from a series of experiments done previously. But the problem of acid soil is a complex one, toxicity may come from high concentration of some ions in the presence of acid rather than the effect of acid alone. At this stage, the regeneration trial is done in media with pH 4.0 with standard tissue culture ingredients. For salt selection out door, the treatments have been carried out in trays under plastic covered roof to protect the rain. Selection starts when seedlings reach five leaf stage and the treatments last for about one month. Only 2 to 5% of the most healthiest seedlings are transplanted to sand culture for further study.

Even work load of the staff has been geared toward routine works of salt and acid selection, some experiments on regeneration have been continued to search for better methods for regeneration. During this period altering macroelements and sugars are found to effect regeneration to certain extent.

Format of the report has been changed appreciably since the Progress Report IV. After we began to realize that there were two distinct groups of person who read our report, one group is the administrators who want to know the objectives of the works and results, another group is the academics who want to know the work in technical point of view and certainly the progress in this particular field for evaluation of the result.

To facilitate reading, first half of each experiment report are "Objectives" and "Conclusion" with some necessary technical data and the second half is "Results and discussion" for those who want to know more about the work. Some detail data are left out intentionally if they can be covered easily by discussion or when they are too lengthy comparing to its progress. Some negative results with merit are reported here too to complete the picture of the whole work.

Furthermore, each report has tables of references which readers can always refer to when needed. These are the lists of abbreviation of varietal names, chemicals, and terminologies; reference medium; standardized media; basic media; nutrient solutions etc. Several methods are still referred to the previous reports.

If any doubt on any part of the report should arise we are willing to answer the questions or elaborate on any specific points.

ACTIVITIES

During the past year, many activities besides routine works have been going on both in our laboratory and outside. Some activities are summarized as follow.

CONFERENCE, WORKSHOP ETC :

1. November 19 - 22, 1984. Kyoto Symposia on Bioscience : Prospect in plant cell science and technology. Associate Professor M. Vajrabhaya was invited to attend the symposia and chaired one of the session.

2. March 18 - 20, 1985. Workshop on Tissue Culture Technology, Bangkok. Sponsored by Asian Network for Biological Sciences. Professor T. Vajrabhaya and Associate Professor M. Vajrabhaya were the local organizers and they also presented the status of plant tissue culture in Thailand and the prospects of tissue culture in Asia.

3. September 1 - 9, 1985. Associate Professor M. Vajrabhaya paid a visit to Dr. K. Oono's laboratory at National Institute of Agrobiological Resource, Tsukuba. The visit was a fruitful one, for she could study the work and consulted with Dr. Oono and colleage on various problems in tissue culture.

4. September 10 - 13, 1985. Third International Cell Culture Congress, Sendai. After visiting Dr. Oono; she attended the conference as a representive of the Ministry of Science, Technology and Energy.

5. October 21 - 25, 1985. First Annual International Plant Biotechnology Network Conference, Fort Collins, Colorado.

We are one of the three members of an informal tri-lateral network which has been first established among the TCCP, IRRI in the Philippines and our unit cell. Associate Professor M. Vajrabhaya, the principal investigator was invited to attend the conference and named a chairlady for a group discussion. She also presented a paper on the Selection of Salt and Acid-Tolerant Rice, and another on the Cooperation between Chulalongkorn University and Colorado State University. Professor T. Vajrabhaya a member of Management Review Group of the IPBNET, was invited to attend an administrative meeting. He also presented a technical paper on the Effects of Coconut Water and Sucrose on Regeneration of Rice Callus.

At Fort Collins, a visit to Tissue Culture for Crop Project laboratory and the exchange of ideas in reseach and future cooperation were made with their principal investigator and staff.

6. November 25 - 29, 1985. Fifth Society for the Advancement of Breeding Researches in Asia and Oceania (SABRAO) Congress, Bangkok. Associate Professor M. Vajrabhaya attended the conference.

VISITOR : There have been several important visitors from oversea and many from other institutions in Thailand. Frequent seminars or lectures have been organized for the visitors who has time to discuss the problems with our staff and interested persons. Our regular visitors are Dr. E.J. Briskey, Director of the Office of Science and Technology, USAID, Thailand and Dr. M.W. Nabors, Principal Investigator of Tissue Culture for Crop Project and also our collaborating investigator. Dr. Nabors has his plan to visit

our laboratory approximately six days each year to disscuss on the technical problems in tissue culture, exchange of materials and personels and also travelling to other research institutes. Dr. M.S. Swaminathan, Director General, IRRI had just visited us in November and Dr. F.J. Zapata, a tissue culturist at IRRI also visited us earlier in September. Mr. Bob Barnes, a new officer of the USAID Thailand accompanied Dr. Briskey on the visit in October, to get acquainted with various projects supported by USAID.

Since our laboratory has been recognized as a "Unit Cell" by our university in 1983, there have been other scientists from different parts of the world visiting the loboratory.

AUSTRALIA	:	Dr. Jen McComb, Murdoch Univ.
		Dr. Elizabeth S. Williams, Univ. of Melbourne
CANADA	:	Dr. J.P. Kutney, Univ. of British Columbia.
INDIA	:	Prof. H.Y. Mohan Ram, Univ. of Delhi.
	.	Prof. G.M. Reddy, Osmania Univ.
INDONESIA	:	Dr. E. Noerhordi, Inst. Tech. Bundung.
JAPAN	:	Dr. T.Hara; Sugar Inst.
		Dr. H. Harada, Univ. of Isukuba.
		Dr. T. Irabu, Okinawa Agric. Exp. Satation.
		Prof. H. Ishikawa, Chief Advisor of R.F.D.
		Prof. T. Nakajima, Tamagawa Univ.
		Dr. Y. Yamada, Kyoto Univ.
MALAYSIA	:	Dr. S. Kadzimin, Univ. Agr.

NETHERLAND	:	Prof. H.J.J. Nijkamp, Free Univ.
PHILIPPINES	:	Dr. Aurora G.del Rosario, U.P. Los Banos
		the late Dr. S. Yoshida, IRRI.
SINGAPORE	:	Prof. A.N. Rao, Nat'l Univ. of Singapore.
U.K.	:	Dr. T. Flowers, Univ. of Sussex.
		Dr. M. Tribe, Univ. of Sussex
		Dr. J.M. Wilson, Univ. of North Wales.
U.S.A.	:	Mr. T.A. Dyke, Colorado State Univ.
		Dr. D.A. Evans, DNA Plant Technology Corp.
		Prof. B. Greenebaum, Acting Vice Chancellor
		Univ. of Wisconsin, Parkside.
		Prof. W.L. Kopp, Pennsylvania State Univ.
		Dr. J.S. Walker, USAID, Washington D.C.

EXCHANGE OF PERSONEL : Collaboration between the Tissue Culture for Crop Project at Colorado State University and the Plant Tissue Culture Unit Cell at Chulalongkorn University has been established since 1983. And this was followed by the agreement of graduate student exchange between the two graduate schools. Beginning last year, when the IPBNET was first formed, we were invited to join the network as one of the three core institutes. More exchange in personel or materials among the IPBNET members are expected in the future.

At present Miss Supaporn Tang, one of our graduate student, is now attached to the TCCP for her research project to gain more experience in cereal tissue culture. At the same time the TCCP sent Miss Kerri Wright, one of their technicians, to work in our

laboratory to get acquainted with the technical problems, both in materials and environments, that frequently occurred in the tropic. It is hoped that the exchange program will be more extensive among the IPBNET members in the near future.

Our former laboratory manager, Miss Poungpet Poonsapaya, got accepted in the Ph.D. program at the CSU and is now also working as a research assistant in the TCCP. The present manager, Miss Ourasa Tunvachkul will visit the IRRI for a short period in January, during this time she will be trained in working on embryogenesis of rice under Dr. Zapata.

LABORATORY : Fifty square meters of working space have been provided by the Department of Botany, this area will be used mainly as a transfer room and and a culture room. The new arrangement will also leave enough space in the preparation room which we have not had enough room for installing more equipment. At this writting, the installation of furnitures and electricity have been completed and the new space is expected to be fully utilized very soon. Besides the generosity of the staff in the Department of Botany who sacrificed their space for the project, the university also provided a grant amount of U.S. $10,315 for the expansion of working space to accommodate the ever increasing works in the present and future.

COOPERATION : The cooperation between our unit cell and other unit at other institutions for exchange of material, personel and technical know how or technical assistance is very

important in working with rice tissue culture. In the rapid progress in the improvement of plant through tissue culture, one cannot rely solely on gathering information through published journals or short communication, a direct contact must be made to prevent repetition of the work and help each other through transferring of technology in the changing world.

The cooperation between TCCP at Colorado State University has been established since 1983 when this project was first started. Since then the principal investigators of both project have been visiting each other laboratories regularly approximately once a year for discussion on the problems and plan for the future. Besides this, the exchange of personels, materials and numerous correspondence have been made.

Other cooperation is being done with the IRRI in the Philippines, several visits of the personel from both institution have been made. IRRI also agrees to train some of our staff for various aspects of rice technologies.

Cooperation between government bodies specializing in different aspects of rice reseach has also been done. Our materials used in reseach are obtained from Rice Division, Department of Agriculture. An informal agreement was made with Dr. Somsri Arunin the leader of the Soil Salinity Research Section at Land Development Department to field test our modified plants in different localities.

It is hoped that all activities that we have been doing would greatly help us to work efficiently in this project and the

final outcomes, new varieties of rice, would be our contribution to the people who need them.

WORK DONE : JULY – DECEMBER 1985

OBJECTIVES

To find an appropriate method for selection of acid tolerant plants in vitro; to select acid tolerant lines in vitro by lower the pH of Culture media; to continue selection of salt tolerant line by either incorporation of salt directly into culture media or adding salt into nutrient solution for the plant grown in sand culture; to further improve the method for plant regeneration by modification of culture media.

BACKGROUND

Up to now a number of plants regenerated under salt or acid stress in vitro have been obtained and grown to maturity. Some of which are from non-stressed proceedures which their seedlings are subjected to stress in the following generations. The question is, which method would have better chance of obtaining the wanted variants. At a first glance, plants regenerated under unfavorable condition seem to be better because the sensitive ones would be killed outright at the beginning leaving the ones that can tolerate such condition for further selections. It seems to have a major change in genetic make up of the survivors, if it is true we will be very lucky, but if it is just a kind of physiological adaptation or epigenetic change, the plants will revert back to normal in the next generation. Another look into this problem is to assume that genetic of stress tolerant is rather complex and the chances are very romote to have just a simple gene responsible for a given characteristic. If this is the case, many useful variants occurring during callus growth would be lost though stressing when the surrounding cells are killed. Furthermore, if a gene in question is a recessine one it would not have chance to express itself, or if an array of genes responsible for a specific character are of multiple type, this would further reduces chance of occurring down close to zero. By allowing regeneration to take its normal course and let the plants grow to maturity, this would facilitate gene recombination through sexual reproduction. Selection of seedlings from regenerated plants would be the combined results of gene mutation

during tissue culture and gene recombination which finally express in the organism level, this seems logical and has a direct application. However, subsequent selections are still needed to fix gene combination in case of multiple gene and to select out the undesirable characters arising in the process.

Since both methods have their own merits and drawbacks, we are working on both.

Acid selection is not as simple as one think, acid soils contain several toxic materials such as aluminum and iron compounds and at the same time they contain low level of phosphorus and calcium which both are essential for plant growth. Adding acid to culture medium or nutrient solution does not simulate the actual conditions in nature; putting soil extract in culture media increases the unknown up to a point that we cannot control the experiment. Only stabilizing the pH of culture media without changing the gelling property of agar took us a lot of time and materials for a series of experiments. However, at this moment we are able to cope with the stabilizing pH in the media and regenerating some plants from the treated calli.

Regeneration is still a problem that we have to put much of our time and efforts to obtain plants from only five to thirty percent of calli. The rest of the calli either turn brown and die after two weeks in regeneration media while some turn green or even produce shoots but eventally die. This means that, we still work five times more than we should to get the same number of regenerated plants. Other laboratories that we have visited

do not seem to do any better with the indica rices either. We wish to spend more time on fundamental works on regeneration of plant from somatic cell callus to establish the method for better regeneration not only for our own use but for any one who wish to utilize the results for their works in the future.

The statements above are the critical problems which we are facing during the past period but these are only a fraction of the total works. All accomplishments including a series of preliminary tests cannot be all presented in the written form only positive results or negative results with special value are summarized in these reports.

MATERIALS

Seven rice varieties namely, RD23, RD25, KDML, KTH, LPT, NMS and RD8 (the last one being glutinous rice) 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 in only 100 to 130 days after sowings (annonymous, 1983). Both of which can be grown upto three crops a year. This definitely accelerates 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 handle <u>in vitro</u>. On the contrary, the other five varieties, which are the improved strains of old established varieties, respond better in the <u>in vitro</u> method,

but their harvest times are only in November and December when the night lengths are sufficiently long. So far, we have not yet found the ideal experiment materials.

METHODS

1. SURFACE STERILIZATION : The method for surface sterilization used in these experiments were described in the Progres Report II, p. 10 - 11. Bacterial and fungal contaminations were found among 8 to 10% of seeds which were slightly higher in rainy season when the humidity was high.

2. STANDARDIZED CALLUS INDUCTION MEDIA : Due to varietal differences, two media were used for callus induction from embryos 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 I.

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 modifications e.g. the deletion of sodium sulfate or addition of ammonium sulfate. In addition, micro-elements (including iron) are used according to Murashige and Skoog (1962). Coconut water is the only undefined organic additive that gives consistent result for regeneration in many experiments done previously. (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. And the physical conditions are also listed in the Table 3.

5. MODIFIED WP NUTRIENT SOLUTION : All plants that grow in natural condition are planted either in water culture or sand culture. The nutrient solution used in all cultures is that of a modification of Wagner and Poesch of Ohio Agricultural Experimental Station since 1945 (Bentley, 1959), which all components can be mixed dry as the mixture will not deteriorate and can be stored and used as needed. The micro-elements are added according to Bentley's formula (1959) to insure the availability of micro-elements. Supply of iron is made by adding sodium ferric diethylenetriamine pentaacetate giving 5 ppm of available iron in working solution.

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

Table 4 Modified WP Formula

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	"
Zinc sulfate	0.0005	"
Copper sulfate	0.0005	"
Water	1 liter	

ON THE SELECTION OF STRESS TOLERANT PLANT

A series of experiments have been done for salt and acid selections in vitro since June 1984, some modifications have been made to suit our work. Difficulties arose when trying on low pH experiments and now some of these problems have been overcome. However, plant regeneration from callus has been a major problem in developing salt or acid tolerant lines through tissue culture. The rate of regeneration of indica rices in general is lower than the japonica types and the modern hybrids which have the IR blood in the back ground are found to be among the most difficult ones. This factor alone increases the work for almost ten times to obtain the same number of plants if it were the amiable ones. To illustrate this point, we have found that only 8 to 10% calli of RD23 regenerate into plant without stress and only 5 - 8% is obtain in KDML; extreme case is found in the RD25 which give us erratic results varying from 0 to 5%. At this time, the LPT is the most reliable one which gives 15 to 30% regeneration but, however, this happens to be a short day plant which will slowdown the work in subsequence selections in the field.

Selection in natural condition has been done by using seeds from regenerated plant and seeds from second generation of the stessed plants. The plants have been grown in plastic trays under plastic covered roof to prevent rain. Two to five percent of the survived plants are selected at the end of one month of treatment for further selection.

SALT SELECTION IN VITRO

OBJECTIVE : To select plant that can regenerate from callus under salt stress.

BASIC CULTURE MEDIUM : Modified Murashige and Skoog's medium (Table 1) using Macro and Micro-elements and add 0.4 mg/l thiamine. HCl.

RICE VARIETIES : KDML, KTH, LPT, NMS, RD8, RD23, RD25

LIGHT REGIME : 16/8 hrs. light/dark

NUMBER OF CALLI TREATED : 14133

NUMBER OF SHOOT OBTAINED : 7

CONCLUSION : Seven calli produced shoots from a total of 14133 calli treated, which is 0.17% regeneration. Number of total shoots were 24. KDML KTH and NMS did not produce any shoot.

DISCUSSION : Re :- Table 5. After establishing method in vitro selection, mass selection started in the August 1984 and some results were presented in the Progress Report IV, June 30, 1985. This report covers the work from April to October, 1985.

The methods used during the past period are the same as the one described in the Report IV. Calli used in the treatment were selected from only one third of the embryos cultured, some embryos were inviable some were contaminated with microorganisms and some were too small or having undesirable characters. Two-

Table 5 Shoot formation of callus of seven rice varieties after treated with 1.5% NaCl for four weeks

VARIETY	No of callus treated	Callus survival after 4 wks		Callus with shoot		Shoot	
		No	%	No	%	No	%
KDML	2639	1597	60.52	0	0	0	0
KTH	690	396	57.39	0	0	0	0
LPT	3352	1633	48.72	4	0.12	17	0.51
NMS-4	1140	540	47.37	0	0	0	0
RD 8	2050	1022	49.58	1	0.05	2	0.10
RD 23	2138	951	44.48	1	0.05	4	0.19
RD 25	2124	994	46.80	1	0.05	1	0.05
ALL VARIETY	14133	7133	50.47	7	0.05	24	0.17

week old calli in this period were treated with 1.5% NaCl for four weeks.

Seven varieties, namely, KDML, KTH, LPT, NMS, Rd8, Rd23 and RD25 were used in the treatment. A total of 14,133 calli were selected for the treatment, fifty percent of these survived at the end of four week of 1.5% NaCl treatment. Then the living calli were transferred to a regeneration medium (see Table 2) many of them stopped growing and died as a resalt of treatment. Some continued to grow and form greenspots but very few produced shoots. The LPT is still the best for regeneration in media with or without NaCl; four shoots were produced while others produced only one or none. The total number of calli of all varieties produced shoots were 7 from the total of 14,133 calli which was only 0.05% regeneration. Normally the LPT has rate of regeneration from 15 to 30%, after treated with salt only 4 out of 3352 calli had shoots which is 150 to 300 times differences. However a total of 17 shoots were obtained from these four calli. Some varieties, RD8, RD23 and RD25, produced one shoot per 2,000 calli while KDML, KTH and NMS did not produce any shoot during this period.

The results of selection shows variability in calli in salt tolerance in the cell level, which only tolerant cells were able to survive and grow and later differentiated into shoots at later stages. It is interesting to note that the whole process of regeneration occurred after treatment, which presents us with a problem whether the whole plant obtained by this method would still be tolerant or not. This can be tested in the following generations.

SALT SELECTION IN NATURAL CONDITION

OBJECTIVE : To select the best two percent seedlings from plant regenerated from callus.

NUTRIENT SOLUTION	:	Modified WP formula
RICE VARIETIES	:	KDML, RD23
NUMBER OF TREATMENTS	:	1; 2 varieties
NUMBER OF SEEDLINGS	:	KDML-2891; RD23 - 2500
LENGTH OF TREATMENT	:	4 wks. (approx.)

CONCLUSION : Seedlings began to show symptom of NaCl toxicity after one week of treatment, three to five percent died and many old leaves began to dry from the tip. Severe symptom was observed during second and third weeks with 25 to 45% of seedlings died in each tray, while relatively low percentage of plants died during the third and fourth weeks (2 to 12%). The best two percent of seedlings of the plants treated were selected for planting.

METHODS : Preliminary works on salt selection were done during January and June 1985 and some results were already presented in the Progress Report IV. A change in the method of treatment was made after doing some experiments; instead of using a sand basket submerging in a tray, water culture in a tray was used in later works. Water culture is found to be more convenience to handle and easy to control salinity than sand culture. Removal, of the dead plants can be done without disturbing others.

The selection of the whole plant in natural condition takes more time and space than the selection in vitro but the selective force is acted on the whole organism like plant growing in nature. Sometimes only a change in root function can limit transportation of salt into inner tissues which make the plant survive in high saline condition without having a change in cell or organs in other parts. Furthermore, any gene mutation or change in the function of some latent genes that arise during tissue culture will have a chance to express after gene recombination in the sexual phase.

Seeds collected from plant regenerated from callus without treating with NaCl or seeds from salt selected plant were used in the treatment. Germination was done by soaking seeds in tap water until they began to germinate which took about three days and then sowed the seeds on a large tray of sand. When the seedlings reached 2-leaf stage they were transplanted to water culture trays with a modified WP nutrient formula (Bentley, 1959). When most of the seedlings attained 5-leaf stage in appoximately 7 to 10 days, nutrient solution was drained off and a new solution with 0.5% NaCl was replaced. Electrical conductivity of the solution at the beginning was around 9 to 10 mMho/cm at 25 ^{o}C and tended to rise when day temperature was high due to evaporation. Whenever the conductivity was 10 mMho/cm or higher the level of nutrient was adjusted by adding tap water and if the conductivity was still lower than 10 mMho/cm, a nutrient solution with 0.5% NaCl was

then added. Every week the dead plants were removed and the solution was changed.

Termination of treatments was determined by the number of plants killed. When 90% or more plants treated died which happenned mostly during the third and the fourth week of treatment, all plants were removed from the trays and only the healthiest ones were selected for planting. In practice, 2% of the plants treated were saved and planted in sand culture for seed production.

Salt selection for this period started on the fourth of October when the heavy rain period passed. The treatment was done under the plastic covered roof to prevent rain water getting in nutrient solution. Two types of plastic tray were used,one type was the rectangular with 26 x 41 cm and another type was a round tray with 27 cm diameter. A cover lid was made of styrofoam with 52 holes for the retangular tray and 25 holes for a round one. The plants were held in place by a piece of synthetic sponge wraphed around the stem.

The treatment of NaCl was made with 2891 seedlings of KDML variety since October, 22 out of 1144 seedlings from the first lot were already selected and planted in pots, the rest would follow soon. The selection of RD23 was started in December which 1386 seedling were subjected to NaCl treatment and about the same number would be ready for another batch of treatment soon.

DISCUSSION : It is a little too early for the discussion of salt selection at present. However approximately 2% of the healthiest seedlings of the treated plants were selected for planting, up to now 40 seedling are being grown for further investigation. These seedling were among the best survivors that had green leaves and long roots after one month in nutrient solution with 0.5% NaCl added. The plants recovered quickly in few days when tranplanted into sand culture with nutrient solution, and now they are reaching 50 cm mark.

ACID SELECTION IN VITRO

OBJECTIVE : To select plant that can regenerate from callus under acid stress.

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

RICE VARIETIES : KDML, KTH, LPT, NMS, RD8, RD23, RD25.

CALLUS AGE : 2 wks.

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

NUMBER OF CALLI TREATED : 7115

NUMBER OF SHOOTS OBTAINED : 20

CONCLUSION : Twenty calli produced shoots from a total of 7115 calli treated, which is 0.28% regeneration. Number of total shoots were also 20 because no more than one shoot per callus was obtained, KDML, KTH and RD8 did not produce any shoot.

DISCUSSION : Re :- Table 6 and Exp. 1. Mass selection was based on the result obtained from Experement 1. This report covers the work from April to October 1985, the same period as salt selection. Methods used for callus induction and callus selection were the same as salt selection. Culture medium was prepared according to the experiment 1, which stabilized pH at 4.0 $\pm$ 0.1.

Same seven varieties of rice were used as in the salt selection. A total of 7115 calli were selected for the treatment, only ten percent of calli survived at the end of four week treatment which is five times lower than the salt tretment. During

Table 6 Shoot formation of callus of seven rice varieties after treated with pH 4.0 buffer acetate for four weeks

VARIETY	No of Callus treated	Callus survival after 4 wks		Callus with shoot		Shoot	
		No	%	No	%	No	%
KDML	1076	73	6.78	0	0	0	0
KTH	284	117	41.20	0	0	0	0
LPT	1840	205	11.14	14	0.76	14	0.76
NMS-4	948	49	5.17	2	0.21	2	0.21
RD 8	1151	152	13.21	0	0	0	0
RD 23	584	26	4.45	4	0.69	4	0.69
RD 25	1232	117	9.50	0	0	0	0
ALL VARIETY	7115	739	10.39	20	0.28	20	0.28

treatment most calli turned white or very pale yellow instead of light yellow in the normal ones in the first two weeks. After transferring calli onto regeneration medium considerable number of calli recovered and formed greenspots and later produced shoots. In spite of high mortality during the treatment more shoots were formed than calli in the salt treatment. A total of 0.28% of the treated calli of all varieties initiated shoots. This figure is 5.6 times higher than in the salt treatment.

The LPT, again, is the variety that gives best regeneration whether they are in normal condition or in salt or acid stress conditions. In this case, 14 out of 1840 calli produced shoots, which is 0.76% or 20 to 40 times less than in reference medium. The KDML and KTH calli still gave no regeneration in both salt and acid treatment during this period, these proved to be difficult varieties. The RD8 and RD25 gave no regeneration while they barely came through salt treatment, on the contrary, the NMS surprisingly produced two shoots in this acid treatment but never in salt treatment. The report will not be completed without mentioning about the steady RD23, the regeneration rate is around 8 - 10% with the method available and it tolerate not only salt and acid in these experiments, it also keep on regenerating in many experiments done previously.

The acid selection reported here is a priliminary work following the established method for stabilizing the pH. One should be aware that acid soils in nature are of several types,

which acid is the only common factor among them. Other components of the soil vary to a great extent according to soil formation and subsequent changes either by nature or by man. Deficiency of phosphorus and other nutrients are more prevalent which can possibly be corrected by adding phosphate fertilizer, but toxicity due to aluminum, iron or other ions at low pH are probably the most difficult to deal with. However, acid selection method should be the first step to establish before tackle on the more complex treatments.

EXPERIMENT 1

OBJECTIVE : To find a method of stabilizing pH during growth of callus at 4.0 or lower without changing gelling property of agar.

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

CALLUS AGE : 2 wks.

NUMBER OF TREATMENTS : 3 concentrations of acetate buffer, 3 varieties (KDML, LPT, RD8)

NUMBER OF REPLICATIONS : 24 calli

LENGTH OF TREATMENT : 4 wks.

CONCLUSION : Initial pH was 3.9 in all treatments after four weeks pH rose to 4.6 in 20 mM acetate buffer but in 30 and 40 mM they were between 3.9 to 4.1. Calli became pale yellow or white with greenspot forming, two plants were obtained from the experiment.

MATERIALS AND METHOD : Buffer stock of acetic acid and sodium acetate which would give 20, 30 and 40 mM in final solution were made and autoclaved. These were mixed with sterilized standard culture medium before pouring into sterils culture bottles. Some culture bottles were chosen to measure pH before inoculation, two weeks and four weeks after inoculation. Visual observations were also made during such period.

RESULTS AND DISCUSSION : Initial pH of all media was about 3.9 before inoculation. After two weeks only 50% of calli of LPT in 20 mM buffer formed greenspots, whill calli of other two varieties became pale yellow. In 30 and 40 mM buffer all calli became white or near white within two weeks and continued to increase size slowly toward the end of the fourth week. Only two plants were obtained from the LPT grown in 20 mM buffer. pH of the media with 30 and 40 mM remained almost constant (3.9 - 4.1) throughout the experiment, but the pH in 20 mM ranged from 4.0 to 4.6 due to more activity of calli. This results indicate that 20 mM buffer is sufficient to keep pH of the media down close to 4.0 without much effect on growth and differentiation.

EXPERIMENT 2

OBJECTIVE : To study the effect of iron on regeneration.

BASIC CULTURE MEDIUM : Modified White's medium (Table 2) using Macro-elements No. 2 and Micro-elements No. 1 (except iron compound), with 3 mg/l kinetin, 1 mg/l IAA.

RICE VARIETIES : KHT, LPT

CALLUS AGE : 2 wks.

LIGHT REGIME : 16/8 hrs. light/dark

NUMBER OF TREATMENTS : 4 different media; 2 varieties

NUMBER OF REPLICATIONS : 40 calli

LENGTH OF TREATMENT : 4 wks.

CONCLUSION : Concentration of iron according to Murashige and Skoog's (1962) is still the best for regeneration of rice.

RESULTS AND DISCUSSION : Re :- Table 7. Reduction of iron concentration still gave high percentage of greenspots but no further development was observed, higher concentrations (X2 and X4) progressively reduced numbers of calli with greenspots and shoots. Low level of iron may have effect on further development of shoot after initiation in form greenspot. Nitsch (1972) found that the development of tobacco embryoids from microspore stopped around the globular stage of embryogenesis. Higher concentration may have toxic effect on the normal function of the cells. Also Nitsch (1972) found embryogenesis of tobacco dropped sharply as the concentration of iron went over 10^{-4}M. This experiment shows that iron at the right concentration is also crucial for shoot development of rice from callus.

Table 7 Effects of iron concentrations on greenspot formation and shoot development from callus.

*Iron Conc.	Callus Age (Wk)	LPT					KTH				
		N	GS (%)	R (%)	SH (%)	C	N	GS (%)	R (%)	SH (%)	C
$X\frac{1}{2}$ Fe	4	40	20.0	0	0	3.8	36	38.9	0	0	2.9
	6	40	92.5	5.0	0	2.9	36	80.6	0	0	2.7
X1 Fe	4	40	40.0	2.5	0	3.3	32	46.9	0	0	3.2
	6	40	97.5	5.0	10.0	2.8	32	71.9	3.1	3.1	2.7
X2 Fe	4	40	22.5	0	0	3.4	28	39.3	0	0	3.0
	6	40	90.0	0	2.5	2.7	28	60.7	0	0	2.5
X4 Fe	4	40	0	0	0	3.0	24	16.7	0	0	3.1
	6	40	70.0	0	0	2.2	24	50.0	0	0	2.8

* Iron compound is made according to Murashige and Skoog's

EXPERIMENT 3

OBJECTIVE : To study the effect of phosphorus on regeneration.

BASIC CULTURE MEDIUM : Modified White's medium (Table 2, using Macro-elements No. 2 (except the amount of NaH_2PO_4), and Micro-elements, No. 2, with 3 mg/l kinetin, 1 mg/l IAA.

RICE VARIETY : KDML

CALLUS AGE : 2 wks.

LIGHT REGIME : 16/8 hrs. light/dark

NUMBER OF TREATMENTS : 5 different media; 1 variety

NUMBER OF REPLICATIONS : 40 calli

LENGTH OF TREATMENT : 4 wks.

CONCLUSION : Re :- Table 8. Increased concentration of NaH_2PO_4 ten times from the original White's formula increased regeneration from 15% in reference medium (Table 3) to 25% but in the presence of 1% sucrose the increase of NaH_2PO_4 by 20 to 30 times became effective but regeneration is inferior to the ones cultured in the reference medium.

RESULTS AND DISCUSSION : Re :- Table 8. White's macro-elements formular contains very low phosphorus if compare to most other formulae So far, a low salt formula, supplemented with coconut water in the absence of sucrose give better regeneration than others. Increasing NaH_2PO_4 provides tissues with more sodium and phosphate ions at the same time, therefore the increase in regeneration may be due to either sodium or phosphate ions or

posibly both. However, calli will normally die after two weeks if 1 to 6% of sucrore is added to the medium but in this experiment the media with high phosphate survived through this period and produced shoots. It is already difficult to explain the early degeneration of callus in the presence of sucrose in the first place and the problem is even more complicated that high phosphate in the medium reduced the effect of sucrose. However, for practical purposes increase phosphate ten times from the original formula is beneficial for plant regeneration.

Table 8 Effects of phosphorus concentrations on greenspot formation and shoot development from callus

Phosphorus conc.	Callus Age (Wk)	N	GS (%)	R (%)	SH (%)	C
OSu	4	40	100	12.5	25.0	3.5
X10P	6	40	100	10.0	25.0	3.0
1%Su	4	20	45.0	15.0	0	3.1
X1P	6	20	50.0	15.0	0	2.8
1%Su	4	20	50.0	20.0	0	3.2
X10P	6	20	60.0	25.0	0	2.8
1%Su	4	20	55.0	30.0	10.0	3.6
X20P	6	20	60.0	20.0	10.0	3.0
1%Su	4	20	35.0	30.0	5.0	2.8
X30P	6	20	50.0	30.0	5.0	2.8

* Su = sucrose; X1P = NaH_2PO_4 16.5 mg/l

EXPERIMENT 4

OBJECTIVE : To study the effect of potassium, nitrogen and coconut water on regeneration.

BASIC CULTURE MEDIUM : Modified White's medium (Table 2) using Macro-elements No. 2 (except the amount of potassium nitrate), and Micro-elements No. 2, with 3 mg/l kinetin, 1 mg/l IAA.

RICE VARIETIES	: KDML, RD25
CALLUS AGE	: 2 wks.
LIGHT REGIME	: 16/8 hrs. light/dark
NUMBER OF TREATMENTS	: 9 different media; 2 varieties
NUMBER OF REPLICATIONS	: 40 calli
LENGTH OF TREATMENT	: 4 wks.

CONCLUSION : Concentration of coconut water at 10% level was best for regeneration, at higher level (20 and 40%) calli turned brown and die earlier than the ones in 10% CW. Increased amount of potassium nitrate did not change the rate of regeneration appreciably.

RESULTS AND DISCUSSION : Re :- Table 9. Some literatures stress on the important of potassium and nitrogen in regeneration and embryogenesis (Kato and Takeuchi, 1966; Behrend and Mateles, 1975; Brown et al, 1976; Wetherell and Dougall, 1976; Nitsch, 1981). The results from this experiment did not show any specific requirement of these two elements in regeneration. One of the reasons is that all nine media are supplemented with coconut water which has fair amount of potassium and nitrogen in different forms and other

essential substances for growth and differentiation of callus (Steward et al, 1969). If coconut water supplies enough necessary ingredient for regeneration, adding more of these will not make any different but they may even be harmful to the tissue at high level. Unfortunately, such experiment cannot be carried out in the absence of coconut water because the regeneration would be extremely low without it. Toxicity of coconut water was noticed when 20 and 40% of coconut water were used. In this connection, the rich liquid endosperm which certainly contains some growth regulators can become inhibitory or upset the physiological balance of the tissues when the amount is excessive. In practice, the amount of potassium and nitrogen in the White's formula are certainly sufficient when combined with 10% coconut water. This means that our modified White's formula is on the right tract except the amount of phosphate which needs to be studied further (see experiment 3).

Table 9 Nine combinations of potassium nitrate and coconut water; forty calli of each of the KDML and RD25 after two weeks in callus induction medium were used for each treatment.

KNO_3 (K)	Coconut water		
	100 mg/l (X1 CW)	200 mg/l (X2CW)	400 mg/l (X4CW)
80 mg/l (X1K)	X1K / X1CW	X1K / X2CW	X1K / X4CW
800 mg/l (X10K)	X10K / X1CW	X10K / X2CW	X10K / X4CW
1600 mg/l (X20K)	X20K / X1CW	X20K / X2CW	X20K / X4CW

X1K = 80 mg KNO_3/l as used in White's formula

X1CW = 100 ml coconut water/l as used by F.C. Steward

EXPERIMENT 5

OBJECTIVES : To study the effect of high salt formula on regeneration; to find out whether some components of the formula have deleterious effects on callus or not; to find the different effect between high sucrose and low sucrose in the media.

BASIC CULTURE MEDIUM : Modified Murashige and Skoog's medium (Table 1) using Macro and micro-elements and add 100 mg/l myo-inositol, 0.4 mg/l thiamine. HCl, 3 mg/l kinetin, 0.1 mg/l IAA.

RICE VARIETY	:	KDML
CALLUS AGE	:	2 wks.
LIGHT REGIME	:	16/8 hrs. light/dark
NUMBER OF TREATMENTS	:	8 different media; 1 variety
NUMBER OF REPLICATIONS	:	40 calli
LENGTH OF TREATMENT	:	4 wks.

CONCLUSION : Reduction of the amount of nitrogen both in ammonium and nitrate forms did not improve regeneration; and the reduction of all chemicals in half did not make any significant difference.

RESULTS AND DISCUSSION : Re :- Table 9. Since Murashige and Skoog's (1962) mineral nutrient has been widely used in plant tissue culture throughout the world, it is the first high salt formula originated from a series of experiment on tobacco callus which later found to be very useful for many species. Rice callus, with no exception, can initiate from embryo, leaf and stem

and grows profusely for a period of one month without any difficulty. After this stage, with some exceptions,calli begins to turn brown and die whether they are left in the original vessel or are transferred to a new medium with or without dividing. Our results in previous experiments showed that calli grown in a low salt formulae formed more greenspots which later became shoots better than the ones cultured in the high salt medium. The differences in the low and high salt media seem to be mainly on the nitrogen and potassium salts.

Therefore, this experiment is aiming at the result of reduced amount of nitrogen in both forms together. The experiment 4 showed that the amount of potassium and nitrate nitrogen in the presence of coconut water did not have distinct effect on regeneration.

In this experiment, the reduction of each chemical used in the M.S. formula into half and the reduction of one component, NH_4NO_3 to $\frac{1}{5}$ and $\frac{1}{20}$, did not improve regeneration whether the media were supplemented with 3 or 6% sucrose. Calli in all treatment became brown during the first week which means that the effect of browning is not due to excess amount of nitrogen in the form of NH_4NO_3. This means that the presence of the nitrogen sources in either nitrate or ammonium forms is not the determining factor for low regeneration. And the reduction of all component does not help either.

Table 10 Six combinations of ammonium nitrate and sucrose ; 40 two-week old calli of KDML were used in each treatment.

NH_4NO_3 (N)	Sucrose 30 gm/l (X1SU)	Sucrose 60 gm/l (X2SU)
1650 mg/l (X1N)	X1N / X1SU	X1N / X2SU
330 mg/l ($X\frac{1}{5}$ N)	$X\frac{1}{5}$ N / X1SU	$X\frac{1}{5}$ N / X2SU
82.5 mg/l ($X\frac{1}{20}$)	$X\frac{1}{20}$N / X1SU	$X\frac{1}{20}$N / X2SU

X1N = 1650 mg NH_4SO_4/l as used in Murashige and Skoog's formula

X1SU = 30 gm sucrose/l as used in Murashige and Skoog's formula

EXPERIMENT 6

OBJECTIVE : To study the effect of organic additives on regeneration.

BASIC CULTURE MEDIUM : Modified white's medium (Table 2) using Macro-elements No. 3 and Micro-elements No. 1, with 3 mg/l kinetin, 1 mg/l IAA.

RICE VARIETIES	:	KDML, KTH, RD23
CALLUS AGE	:	4 wks.
LIGHT REGIME	:	16/8 hrs. light/dark
NUMBER OF TREATMENTS	:	3 different media, 3 varieties
NUMBER OF REPLICATIONS	:	40
LENGTH OF TREATMENT	:	4 wks.

CONCLUSION : Increase in regeneration was observed when thiamine and inositol were added, other additives were not effective.

RESULTS AND DISCUSSION : Re:-Table 11 and 12 coconut water provides both inorganic and organic additives to the tissue to certain extent (Steward et al, 1969) but it may not be sufficient for regeneration. If the tissue receives enough of these, they would grow equally well, when judging from greenspot formation, green area coverage and finally number of shoot, the media supplemented with thiamine and inositol grew and regenerated better. Callus induction from embryo in the medium supplemented with coconut water does not seem to require additional of thiamine and inositol probably the callus can absorb such substances from the existing

embryo. Or it is possible that initiation and growth of shoot apex require, high thiamine and inositol because tissues in 0.4 mg/l thiamine medium produced relatively larger area of greenspot and more shoots than those counterparts in 0.1 mg/l thiamine media.. Glycine, nicotinic acid and pyridoxine gave slightly better result in the KTH and RD23, but they gave negative effect on the KDML. These additives are not required by tobacco callus tissue for maintence of callus form (Linsmaier and Skoog, 1965), but one of these or all may be beneficial for regeneration of rice.

In Table 12 abc as discussed previously it showed slight improvement in regeneration when glycine, nicotinic acid and pyridoxine were added. Another test was made with other two varieties, LPT and RD25, which the RD25 being our most difficult one. For shoot development the results were the same, for the LPT 15% of calli produced shoots but no shoot as large as 1 cm was form in the RD25. It is worth noting that, when these additives were addled, green areas greenspots, and roots increase considerably, which means that the added substances improve growth and development of callus in general but unfortunately not specifically on shoot development. So far we have not really found any substance which effectively acts on shoot development in later stages.

Table 11 Combinations of organic additives used in the Experiment 6 (for results see Table 12)

Organic additives	Combination A (mg/l)	B (mg/l)	C (mg/l)
myo-inositol	-	100	100
glycine	-	2.0	-
nicotinic acid	-	0.5	-
pyridoxine.HCl	-	0.5	-
thiamine.HCl	-	0.1	-

Table 12 Effects of organic additives on regeneration of three rice varieties; 40 two-week old calli were used in each treatment.

Treatment	Callus age	KDML					KTH					RD23				
		A	GS	R	Sh	C	A	GS	R	Sh	C	A	GS	R	Sh	C
A	4 wks	15.9	67.5	0	2.5	3.9	22.4	62.5	7.5	0	3.3	21.1	22.5	2.5	0	3.1
	6 wks	32.4	95.0	2.5	5.0	3.5	30.4	62.5	7.3	0	2.7	38.2	27.5	2.5	0	2.5
	8 wks	38.3	90.0	7.5	7.5	3.4	32.3	65.0	7.5	0	2.5	51.7	30.0	2.5	0	2.0
B	4 wks	17.9	35.0	0	0	3.9	24.7	75.0	12.5	0	3.4	35.2	62.5	5.0	5.0	3.4
	6 wks	22.6	77.5	0	0	3.6	34.7	75.0	20.0	2.5	2.7	41.1	87.5	5.0	5.0	3.3
	8 wks	26.4	82.5	0	0	3.8	30.0	70.0	20.0	2.5	2.4	47.3	92.5	7.5	10.0	3.2
C	4 wks	15.0	80.0	0	2.5	3.9	25.8	65.0	2.5	0	3.5	26.3	87.5	2.5	7.5	3.4
	6 wks	30.6	85.0	7.5	2.5	3.4	41.7	72.5	5.0	0	3.0	52.6	97.5	5.0	25.0	3.3
	8 wks	33.5	77.5	10.0	7.5	3.1	42.1	72.5	5.0	5.0	2.8	66.0	95.0	10.0	27.5	3.2

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