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PROGRESS REPORT VI

Rec'd in SC SEP 29 1986

Salt and Acid Selection

CONTINUE 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

Department of Botany
Chulalongkorn University
Bangkok

INVESTIGATORS AND CONSULTANTS

PRINCIPAL INVESTIGATOR

Montakan Vajrabhaya, M. Sc.
Associate Professor
Department of Botany
Faculty of Science
Chulalongkorn University
Bangkok 10500, Thailand

INVESTIGATOR

Dr. Thavorn Vajrabhaya
Professor
Department of Botany
Chulalongkorn University

COOPERATING INVESTIGATORS

Dr. Murray W. Nabors
Professor
Department of Botany and Plant Pathology
Colorado State University, U.S.A.

Dr. Shouichi Yoshida (Deceased)
Plant Physiologist
International Rice Research Institute
Philippines

PROJECT CONSULTANTS

Dr. Preeda Boon-Long
Department of Botany,
Chulalongkorn University

Dr. Aruni Chantarasnit
Department of Botany
Chulalongkorn University

Chulaluk Komoltri, M. Sc.
Statistician
Clinical Epidemiology Unit
Mahidol University

PROJECT STAFF

TECHNICAL STAFF

Ourasa Tunvachkul B.Sc.

Laboratory and Research

Operation Manager

Rungrudee Nilvichian M.Sc.

GRADUATE STUDENT

Arunya Tantipunjaporn B.Sc.

Chaiwat Narchom B.Sc.

Kitti Photipatama B.Sc.

Porntip Lohvanichai B.Sc.

Siriluck Iamtham B.Sc.

Siriporn Jatapadma B.Sc.

Supaporn Tang B.Sc.

TECHNICAL SUPPORT STAFF

Bang-urn Suksomya

Duangjai Boontiam

Feungfa Sawangsilpa

La-ong Tamiyavanish

Lumduan Do-on

Piangjai Pongpaew

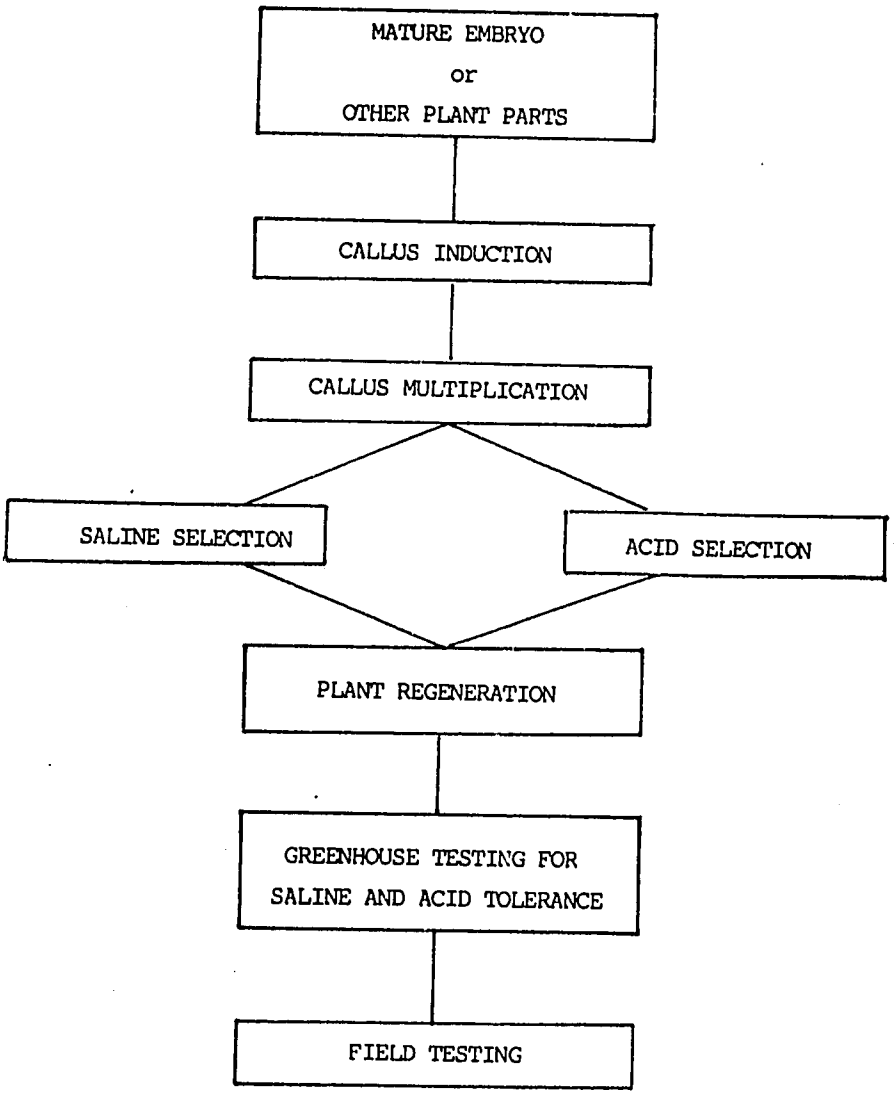
Suriya Singnil

Vasana Sanit

PROPOSED METHODS

FOR

PRODUCTION OF SALINE AND ACID TOLERANT RICE



PREAMBLE TO THE PROGRESS REPORT VI

This report comprises information on work done in selection of plant in stress conditions, some experiments on the improvement of regeneration, activities and news during the last six months.

From January and June are dry months in Bangkok which makes selection in the plastic covered roof convenience. Not only light rain which can be prevented by plastic sheets but also high light intensity throughout the period provides fairly uniform condition in which several experiments could be done at different times. However, during this period, we observed more birds visiting our experiments and did some damage to young seeds. Even the net were used to cover the side of the house but there was one or two birds were occasionally found. Insects could be controlled by spraying with malathion but fungal infeetion was retatively high during this period probably because of the frequent rains in April and May this year.

Changing method of culture from sand to water culture (as stated in the Report V) generally gives better result in seedlings or plantlets regenerated from tissue culture. This method is conuenience for salt treatment because whole nutrient solution with salt can be discarded completely and refilled with freshly prepared solution every week. However, this method has a drawback for growing plants to maturity because the plastic foam often topples when the plant reaches certain height. Various methods of preventing this are being tried at the moment. Furthermore, the plants in

water culture were often found to produce more empty seeds than those grown in the sand. The reasons of being this is not clearly known but it could be due to high mass of the plant to volume of nutrient solution which quickly upset the balance of the nutrient even it is added two or three times a week. Using larger containers or fewer plants may give better results.

This report is supposed to be the last one for this particular grant but the results up to now are still not conclusive due to most of the salt tolerant plants obtained are still in young stages and some are in the second generation. We would like to confirm our results in the third and fourth generation before handing them over to the Department of Agriculture and the Department of Land Development for field testing. We will be glad to write another report of the test in later generation and summary of the lines obtained with some specific characters.

ACTIVITIES

During January and June 1986, many activities have been going on both within the laboratory and outside. Some activities are listed as follow.

CONFERENCE, WORKSHOP .....ETC :

1. March 22 - 26, 1986. Professor Thavorn Vajrabhaya was invited to attend a closed Japan - Singapore Seminar on "Clonal Multiplication and Transformation of Useful Plants through Cell and Tissue Culture". at the University of Tsukuba, Japan. He was a convener on the first session and presented a paper "On the Selection of Salt Tolerant Rice from Tissue Culture". in the second session.

In Tsukuba and Vicinities, he also visited several research institutes dealing with plant tissue culture, owned by both government and private enterprises.

VISITOR : Since this grant has been awarded in June 1983, several important guests from govermental organizations and oversea institutions visited our laboratory (see Report V). These include Deputy Prime Minister, Our regular visitors are Dr. E.J. Briskey, former Director of the office of Science and Technology, USAID, Thailand, and Professor M.W. Nabors, Principal Investigator of Tissue Culture for Crop Project and our collaborating investigator. Visitors are often invited to give a lecture or presence in our small group discussion. Listed below are only the visitors to our laboratory during January and June 1986.

AUSTRALIA : Dr. Peter Dart
Research School of Biological Sciences
The Australian National University

GUATEMALA : Dr. Carlos Rolz
Central American Research Institute

INDONESIA : Professor Eddy Noerhadi
Bandung Institute of Technology

JAPAN : Associate Professor Michihiro Hara
Iwate University

: Associate Professor Toyoki Kozai
Chiba University

: Associate Professor Nobumasa Nito
Saga University

: Professor Gotaro Tomonaga
Tokai University

UNITED STATES : Dr. Oluf L. Gamborg
Colorado State University

: Dr. Kerri-Ann Jones
AID Washington D.C.

: Mr. Raymond E.B. Ketchum
Colorado State University

: Dr. Christy MacKinnon
Colorado State University

: Professor M.W. Nabors
Colorado State University

: Dr. J.M. Widholm
University of Illinois at Urbana-Champaign

LABORATORY : New sections of the laboratory which comprises two transfer rooms and a culture room have been in full operation since February 1986. At present, upto nine persons can work on transferring simultanaeoasly which is quite a relieve especially for graduate students who only have certain free period for laboratory work. The extra space also provides more space for culture room both for routine works on callus culture and plant regeneration and for special problems dealing with nutrition, various experiments in altering external environments and experiments on manipulation of tissues. Most of the latter parts are being worked out by graduate students either for their theses or special problems. All of the works in these areas are very valuable for the improvement of efficiency of regeneration.

COOPERATION : A complete picture of the cooperation between our unit and other units at other institution was already presented in the Report V. All of these are still going on actively except the connection with the IRRI. Due to internal difficulties in the Philippines, the plan to send our laboratory manager for training in embryogenesis under Dr. Zapata has to be delayed to the end of the year or even called off if the situation over there is not better.

WORK DONE ✣ JANUARY–JUNE 1986

OBJECTIVES

To continue selection of salt tolerant lines by incorporation of sodium chloride into the culture media during callus growth and development and by adding sodium chloride in the nutrient solution used in water culture; to continue selection of acid tolerant plants in vitro and to find an appropriate method for acid selection in natural condition; to further improve the method for plant regeneration by modifying the culture media.

BACKGROUND

The problems of plant regeneration from callus and the selection of stress tolerance have been discussed througroughly in the previous reports. Among these acid selection is the most difficult problem both in vitro and in natural conditions. Very low pH (4.0 or lower) will directly cause damage to root cells and other cells near vascular beendles which results in slowing down water uptake. At pH below normal ranges soils often release excess soluble toxic materials such as aluminum, iron, and copper compounds and at the same time these soils often contain very low level of phosphorus and calcium both of which are essential for normal growth. Adding acid to the culture medium or nutrient solution may not be sufficient to upset physiological processes stated above because availability of those elements mentioned above is limited which may never reach critical level. On the contrary, using extract of acid soil for testing may produce similar effect to acid soil in nature but the factors affecting the growth of the plant will not be discovered immediately unless soil analyses are readily available. In the experiments here the treatments of acid conditions were limited to lowering the pH in the defined nutrient solutions and saved the survivors at the end of experiments for further evaluation

MATERIALS

Seedlings of first(R1) and second(R2) generations of plant regenerated from callus were used in the selection program. Some seedlings were from NaCl-treated calli and several from untreated ones; some of the second generations were from NaCl-treated seedlings

and some from mother plants grown in sand or water culture with standard nutrient solution.

For callus culture, mature seeds were obtained from the Rice Division Department of Agriculture. Five varieties, RD23, RD25, KDML, LPT and NMS, were used regularly for callus culture either for experiments on plant regeneration or the profuction of plantlets, while RD23 and KDML were used in Nacl selection program (More information on each variety are in the Report IV and V).

METHODS

1. CALLUS INDUCTION : The methods for surface sterilization and callus induction were described in the Report II, p. 10 to 12. The standardized callus induction media are listed in Table 1.

2. REGENERATION : Three media for regeneration were used in the Reports IV and V, those were the results obtained from a series of experiments. In this report, only one standardized medium, has been used since November 1985, is listed in Table 2.

The new standardized medium, believed to be a better one, is being tested at this writing.

3. GROWING PLANT IN NATURAL CONDITION : 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 modified Wagner and Poesch's formula released 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 to insure

the availability of micro-elements. Supply of iron is made by adding sodium ferric diethylenetriaminepentaacetate giving 5 ppm of avail able iron in working solution (see Table 3).

Two-leaf-stage Seedlings were planted on 25 holed round styrofoam raft or in diameters floating in plastic trays containing three liter of nutrient solution. Larger rafts were also used in some experiments. During the growth period, alternate addition of fresh nutrient and water was regularly done to keep the water level high. Periodic measurement of conductivity was done and the nutrient solution was adjusted to 2.0 to 2.2 mMho/cm at 25 ^{o}C by adding the nutrient or water accordingly.

Some seedlings were planted in sand in clay jars filled with washed river sand. Nutrient solution was filled up between two to five centimeters above sand, and conductivity was adjusted as in water culture. This method is good for tall growing varieties which the plants will not topple easily. All experiments were done in the plastic covered roof houses to prevent occasional rain in dry season. Light intensity was reduced to appoximately half by were mesh amot plastic.

4. SALT SELECTION IN NATURAL COND ITION : Seedling were planted as in no. 3 when they reached for to five leaf stages, nutrient solution was drained off and a new solution with 0.5% NaCl added was replaced. This gave electrical conductivity around 9 to 10 mMho/cm at 25 ^{o}C. Whenever the conductivity was higher than 10 mMho/cm at 25 ^{o}C the level of nutrient solution was adjusted by adding tap water and if the conductivity was lower than 10 mMho/cm

at 25 ^{o}C, a nutrient solution with 0.5% NaCl was then added. Every week the dead plants were removed and the solution was changed.

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

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	

SALT SELECTION IN NATURAL CONDITION

OBJECTIVES : To select the most tolerant seedlings from plants regenerated from callus.

NUTRIENT SOLUTION : A modified WP formula with 0.5% NaCl added; Electrical conductivity, 9 - 10 mMhos/cm at 25 °C

RICE VARIETIES : A. KDML, 2839 seedlings of the first generation of regenerated plants (R1), Table 5, 6.

: B. RD23 4758 seedlings of the first generation of regenerated plants (R1), Table 7.

: C. RD23 647 seedlings of the first generation of regenerated under salt stress in vitro (R1/Na) Table 8.

: D. RD23 3761 seedlings of the second generation of regenerated plants (R2), Table 9.

: E. R25 176 seedlings of the second generation of regenerated plants (R2), Table 10.

LENGTH OF TREATMENT : 4 wks.

TIME OF YEAR : October 1985 to May 1986

CONCLUSION : During the first week of treatment most of the seedlings survived and showed little symptoms. After the second week many seedlings showed severe symptoms and die. At the end of experiments less than 15% of seedlings survive except the seedlings of the NaCl - treated mother plants. The survival of seedlings are listed below.

Survivors	:	KDML (R1)	14.1%
	:	RD23 (R1)	[illegible]
	:	RD23 (R2)	12.3%
	:	RD23 (R2)	4.5%
	:	RD23 (R1)	from NaCl - treated tissues *in vitro* 25.0%

The most tolerant plants with the least symptoms were selected for planting (usually 2 to 3% of the treated plants). The second and third generations are in the process of selection now.

The experiments even in the early stages show promising result that the R2 generation of RD23 is more tolerant than R1. Special attention should be drawn to the most tolerance of the seedlings from NaCl - treated tissue *in vitro*.

RESULTS AND DISCUSSION :

A.) KDML is a selected variety for salt tolerance which is recommended by the Department of Agriculture to be grown in several locations in the country for 27 years. Seedlings from the regenerated plants showed the same trend as the other seedlings from other plants. It is hoped that if favorable somatic mutations occur during callus culture and regeneration processes they can be screened by salt selection.

Among the 2839 healthy seedlings from seven different regenerates were treated, 400 plants survived through the end of fourth week, giving 14.1 percent survival. Table 5 showed variation in the responses among the seedlings from different mother plants, the percentage plant survival ranges from 3.2 to 31.9 percent. Such variations depended partly on the environments where the plant grew and internal factors of the plants themselves. If there is no new genes occurring, only gene recombinations may give favorable combinations for salt tolerance. But, if there is any gene for salt tolerance should arise de novo, it will persist and await further recombination. In this case the advantage of tissue culture will play major role in plant improvement.

Table 6 showed the survival of the plants each week (Note:- the treatment No. 1 and 2 are not included in the table). In the first week, most of the plants did not show any symptom of salt stress, but some had dried leaf tips. Only the weakest plants died during the first week. At the end of the second week, many plants

Table 5 Survival of five - leaf stage seedlings (generation R1) of a regenerated plant (RO - TC 1) when treated with 0.5 % NaCl in nutrient solution for four weeks.

Cultivar :- KDML, Electrical conductivity :- 9 - 10 m Mhos/cm.

Treatment No.	Number of plants treated	Plant mortality		Plants surviving	
		No.	%	No.	%
1	676	631	93.3	45	6.7
2	468	449	95.9	19	4.1
3	156	151	96.8	5	3.2
4	375	313	83.5	62	16.5
5	364	249	68.4	116	31.9
6	400	317	79.2	83	20.7
7	400	330	82.5	70	17.5
Total	2,839	2,439	85.91	400	14.1

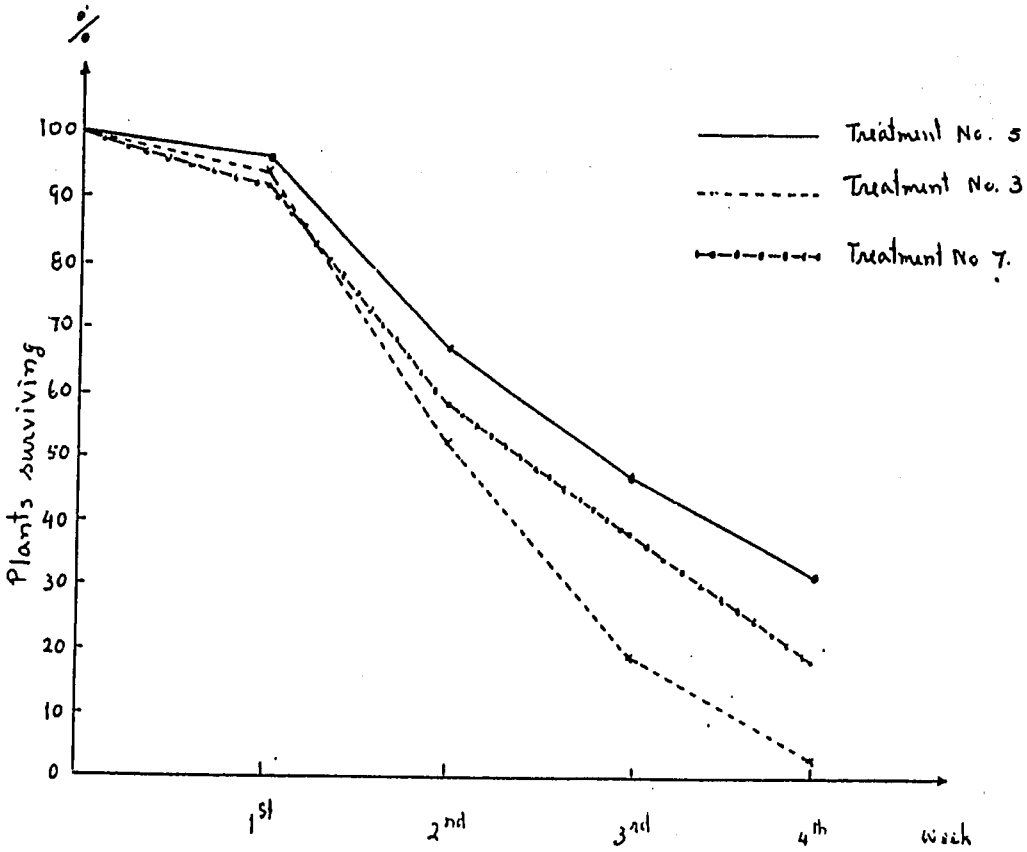
Table 6 Responses of seedlings (generation R1) of a regenerated plant (RO - TC 1) to the treatment of 0.5 % NaCl in nutrient solution.

Cultivar :- KDML ; Electrical conductivity :- 9 - 10 m Mhos/cm.

Stage of seedling development at the beginning of treatment :- five - leaf stage.

Treatment No.	Number of plants	Plants surviving (%)			
		1st wk	2nd wk	3rd wk	4th wk
3	156	93.6	51.9	19.2	3.2
4	375	89.6	57.1	31.1	16.5
5	364	96.4	67.8	47.6	31.9
6	400	92.7	58.7	38.0	20.7
7	400	95.0	74.7	51.7	17.5
Total	1,695	92.9	63.5	38.8	19.8

* No. of treatment corresponds to table 5



Figune 1: Responses of seedlings (generation R 1) of a selected regenerated plants (RO - TC 1), representing sensitive, intermediata and tolerant groups.

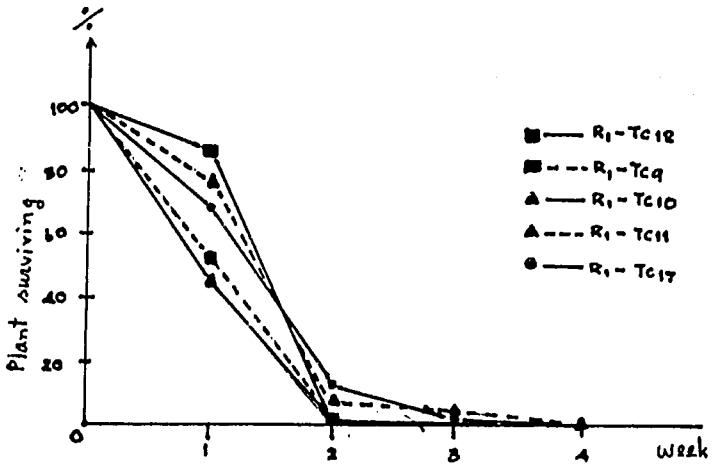
Re : Table 6, Cultivar :- KDML

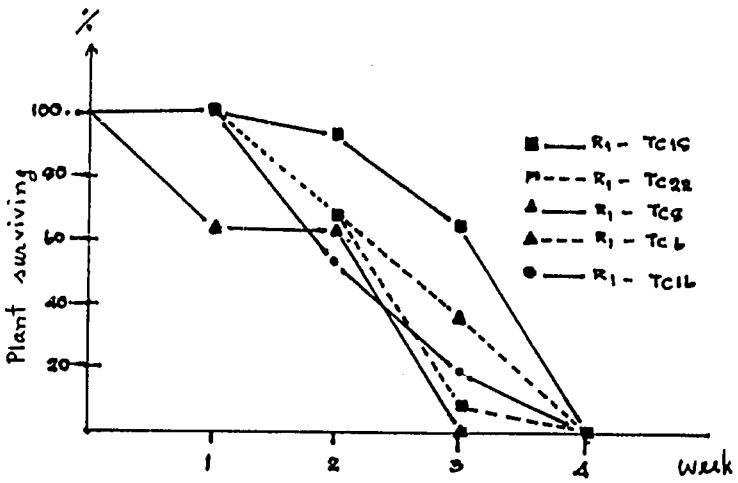
Electrical conductivity : 9 - 10 mMhos/cm.

Stage of seedlings development at beginning of treatment :- five - leaf stage.

Figure 2 Responses of seedlings (generation R1) of five selected regenerated plants (Tcs) representing sensitive, intermediate and tolerant groups.

Re:- Table 7 Cultivar:- RD23; Electrical conductivity :- 9 - 10 mMhos/cm; Stage of seedlings development at the beginning of treatment :- four - leaf-stage; R1 - TCX = Seedlings (generation R1) of regenerated plant no. x.


%
Plant surviving
100
80
60
40
20
0
1
2
3
4
week
R1 - Tc12
R1 - Tc9
R1 - Tc10
R1 - Tc11
R1 - Tc17


%
Plant surviving
100
80
60
40
20
1
2
3
4
week
R1 - Tc16
R1 - Tc22
R1 - Tc8
R1 - Tc6
R1 - Tc16

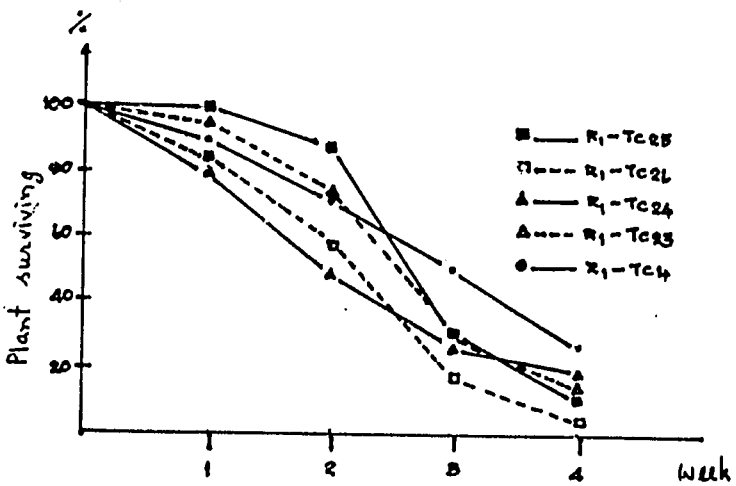

%
Plant surviving
100
80
60
40
20
1
2
3
4
week
R1 - Tc25
R1 - Tc21
R1 - Tc24
R1 - Tc23
R1 - Tc4

Table 7 Responses of seedlings (generation R1) of 21 regenerated plants (Tcx) to the treatment of 0.5 % NaCl in nutrient solution.

Cultivar :- RD 23 ; Electrical conductivity :- 9 - 10 mMhos/cm ; Stage of seedling development at the beginning of treatment :- four-leaf stage ; R1 - Tcx = Seedlings (generation R1) of regenerated plant no. x.

Treatment		Number of plant treated	Plant Surviving							
			1st wk		2nd wk		3rd wk		4th wk	
No.	Stock No.		No.	%	No.	%	No.	%	No.	%
1	R_1-Tc4	46	41	89.1	33	71.7	23	50.0	13	28.3
2	R_1-Tc6	150	150	100.0	99	66.0	56	37.3	4	2.7
3	R_1-Tc8	45	29	64.4	29	64.4	0	0.0	0	0.0
4	R_1-Tc9	200	107	53.5	7	3.5	4	2.0	0	0.0
5	R_1-Tc10	100	40	40.0	3	3.0	1	1.0	0	0.0
6	R_1-Tc11	225	176	78.2	20	8.9	9	4.0	0	0.0
7	R_1-Tc12	100	86	86.0	4	4.0	0	0.0	0	0.0
8	R_1-Tc16	379	379	100.0	208	54.9	71	18.7	0	0.0
9	R_1-Tc17	375	256	68.3	45	12.0	8	2.1	0	0.0
10	R_1-Tc18	398	398	100.0	369	92.7	264	66.3	5	1.3
11	R_1-Tc22	416	416	100.0	283	68.0	37	8.9	2	0.5
12	R_1-Tc23	600	569	94.8	437	72.8	175	29.9	98	16.3
13	R_1-Tc24	250	220	80.0	145	58.0	49	19.6	16	6.4
14	R_1-Tc25	225	222	98.7	188	83.6	69	30.7	32	14.2
15	R_1-Tc26	150	126	84.0	74	49.3	41	27.3	29	19.3
16	R_1-Tc27	75	75	100.0	4	5.3	00	0.0	0	0.0
17	R_1-Tc29	300	282	94.0	125	41.7	2	0.7	2	0.7
18	R_1-Tc30	40	39	97.5	26	65.0	0	0.0	0	0.0
19	R_1-Tc31	120	119	99.2	24	20.0	0	0.0	0	0.0
20	R_1-Tc33	309	285	92.2	37	19.9	0	0.0	1	0.3
21	R_1-Tc34	255	238	93.3	60	23.5	0	0.0	0	0.0
Total		4,758	4,261	89.6	2,220	46.7	809	17.0	202	4.2

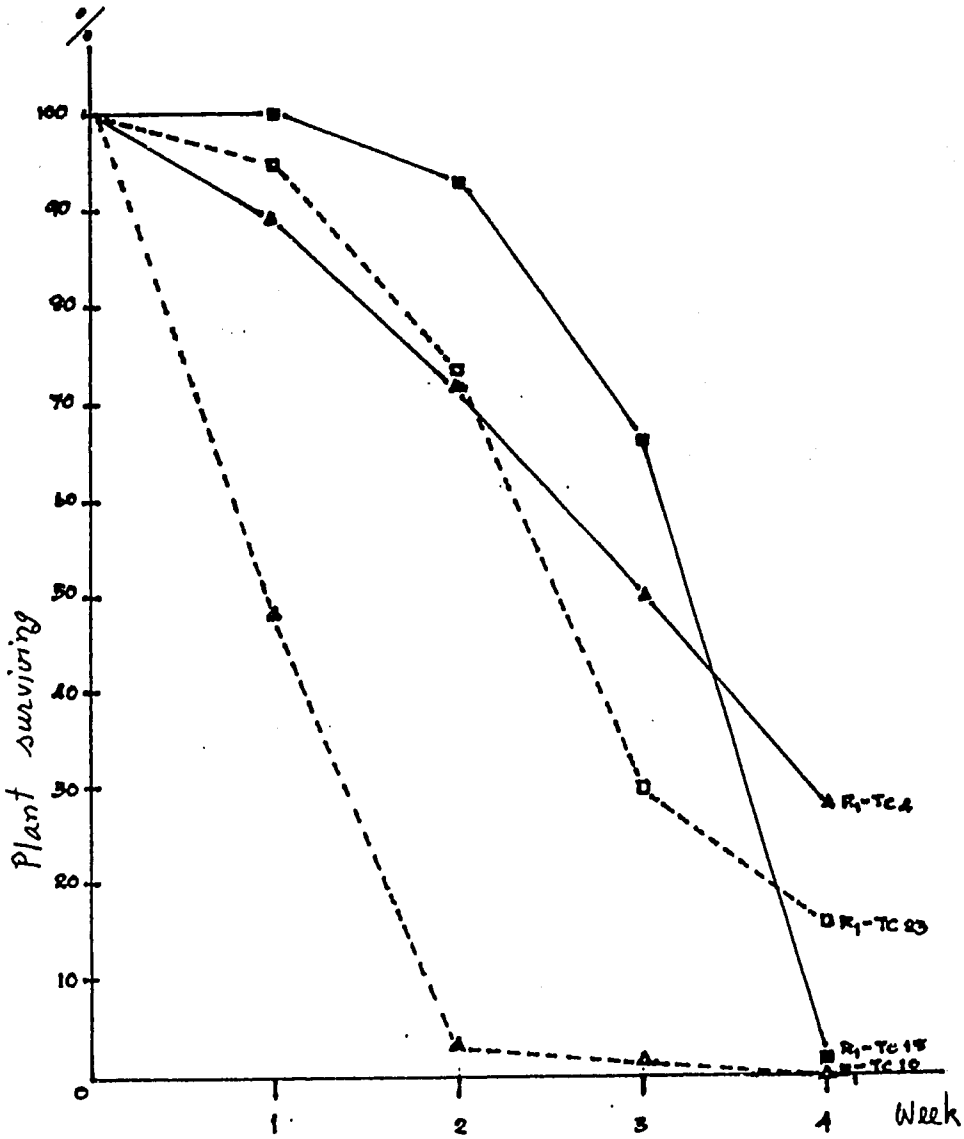


Figure 3 Responses of seedlings (generation R1) of four selecled regenerated plant (RO-TC4, RO-TC10, RO-TC18 and RO-TC23), representing sentitive, intermediate and tolerant groups

Re : Table 7, Cultivar : RD23 Electrical conductivity :- 9-10 mMhos/cm. Stage of seedlings development at beginning of treatment :- four-leaf stage.

began die and some showed severe symptoms of salt stress. The tips of the old leaves were effected first, the drying of the leaves moved toward the bases. Then the younger leaves began to show the same symptom and finally the whole plant died. A dead plant is defined as the one that both the leafblades and leaf sheaths have lost green color altogether. At the end of the second week upto 63.5% of the plants still survived and all of the plants in the central grew profusely. It is worth noting that some of the plants appeared to be normal like the ones in control.

During the third and fourth weeks the weak plants with severe symptom died leaving the stronger ones with little symptom growing. At the end of the fourth week 400 plants were still living,these included the plants with severe and mild symptom. Only the healthiest plants were saved for planting, these were about two percent of the plants treated.

b) RD23 is a new variety which has just been released by the Department of Agriculture in 1981. This is a very interesting variety for this experiment because it is a day neatral and short life cycle variety which can be grown up to three times a year. It is a short variety which can be grown all year round and it also has virus resistant character. If the salt tolerant character can be added to this variety it will be a very useful short-live variety for the rain fed localities.

A total of 4758 seedlings of 21 regenerates plants under <u>in vitro</u> condition were screened (Table 7, Fig. 3). The results

were interesting because the variation was very high, most of the treated seedling died at the end of the experiment. Seedlings from four mother plants had high survival rates over 14 percent, the highest being 28.3 percent. These are the plants that will be grown for second and third screenings. It is interesting to note that the treatment No. 1, which has the highest survival rate of 28.3%, demonstrates its ability to survive under salt stress in the second and third weeks as well. Others seem to follow suit (Treatments 12, 14 and 15) except treatment No. 2 and 10 which the plant surviving rates drop sharply during the fourth week giving only 2.7 and 1.3% respectively.

C.) Seedlings of the plants regenerated under salt stress in vitro are of interest. Generally, RD23 callus gives regeneration rates ranging from 8 to 10% but under 1% NaCl stress the regeneration rate is reduced to 0.14%. Furthermore, most of the plants regenerated under such method produce no viable seeds. Only three mother plants produced viable seeds which could be germinated and used for screening.

The result of the screening in quite satisfactory (Table 8), the plant surviving rates are 14.4, 29.3 and 33.2 respectively. The average surviving rate of the seedlings from unstressed mother plants is only 4.2% which is only $\frac{1}{6}$ of the figure obtained from the above population.

It is interesting to note that survival after the third week was at least 40% which is a very high value for the RD23. This

Table 8 Responses of seedlings (generation R1) of three plants (R0) regenerated under salt stress *in vitro* to the treatment of 0.5 % NaCl in nutrient solution

Cultivar :- RD 23; Electrical conductivity :- 9 - 10 mMhos/cm., Stage of seedling development at the beginning of treatment :- five-leaf stage;

Na1 = 1 % of NaCl in culture medium

Na2 = 2 % of NaCl in culture medium

Treatment No.	Stock No.	Number of plants	Plants surviving							
			1st wk		2nd wk		3rd wk		4th wk	
			No.	%	No.	%	No.	%	No.	%
1	R1 - TC7Na1	247	242	98.0	204	82.6	129	52.2	82	33.2
2	R1 - TC22Na2	150	144	96.0	122	81.3	69	46.0	44	29.3
3	R1 - TC142/2Na2	250	242	96.8	176	70.4	100	40.0	36	14.4
Total		647	628	97.1	502	77.6	298	46.0	162	25.0

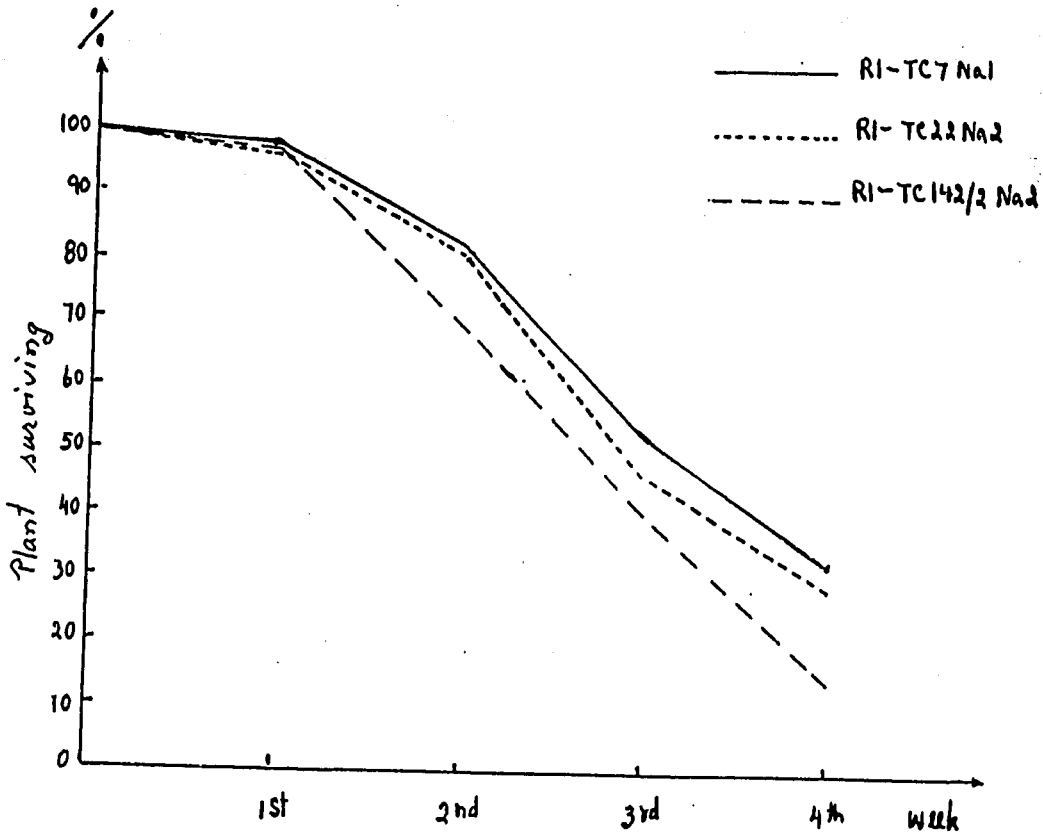


Figure 4. Responses of seedling (generation R 1) of plants (R 0) regenerated under salt stress *in vitro* to the treatment of 0.5% NaCl in nutrient solution.

Re:-Table 8, Cultivar:- RD 23

Electrical conductivity:- 9-10 mMhos/cm.

Stage of seedling development at the beginning of treatment:- five-leaf stage.

reflects the growth and development of calli during the salt stress. Under salt stress condition the cells which are sensitive will be come malfunction and die which affect the tolerant cells. If the population of tolerant cell cover large area they are likely to form shoot apex and finally become shoot. Therefore any shoot arising from such callus under stress should be tolerant.

The method of salt stress in vitro seems to be promising in selection of salt tolerant plants directly, this gives positive result in the first generation.

D.) Second generation (R2) of seedlings collected from four mother plants which had been grown in 0.5% NaCl for four weeks during four leaf stage in natural condition. The pattern of responses was not different from the R1 during the first two weeks. In the third week the number of survivors were rather high and none of the groups died completely. At the end of the fourth week the TC1a and TC1b showed tolerant trait clearly while the other two populations demonstrated sensitivity like the ones in the R1. However the percentage of the survivors were not better than the best of the R1 population (Table 9).

It is still too early to compare the two method of salt stress selection, one in vitro and the other in natural condition. The mother plants regenerated in vitro under salt stress seemed to give more seedlings with high salt tolerance than the unselected ones but only few fertile plants were obtained. On the contrary, more mother plants were obtained after selection under natural

Table 9 Responses of seedlings of the second generation (R2) of regenerated plants to the treatment of 0.5 % NaCl in nutrient solution.

Cultivar :- RD 23, Generation :- R2; Electrical conductivity :- 9 - 10 mMhos/cm; Stages of seedling development at the beginning of the treatment :- four-leaf stage.

Note : Survival of seedtings in the 3rd and 4th weeks indicates variation among populations

Treatment No.	Stock No.	Number of plants	Plants surviving							
			1st wk		2nd wk		3rd wk		4th wk	
			No.	%	No.	%	No.	%	No.	%
1	R2 - TC1a	1,386	1,386	100.0	934	67.4	564	41.1	340	24.5
2	R2 - TC1b	1,100	877	79.7	532	48.4	249	22.6	116	10.5
3	R2 - TC2	775	662	85.4	178	23.0	19	2.4	2	0.2
4	R2 - TC3	500	420	84.0	119	23.8	36	7.2	5	1.0
Total		3,761	3,345	88.9	1,763	46.9	873	23.2	463	12.3

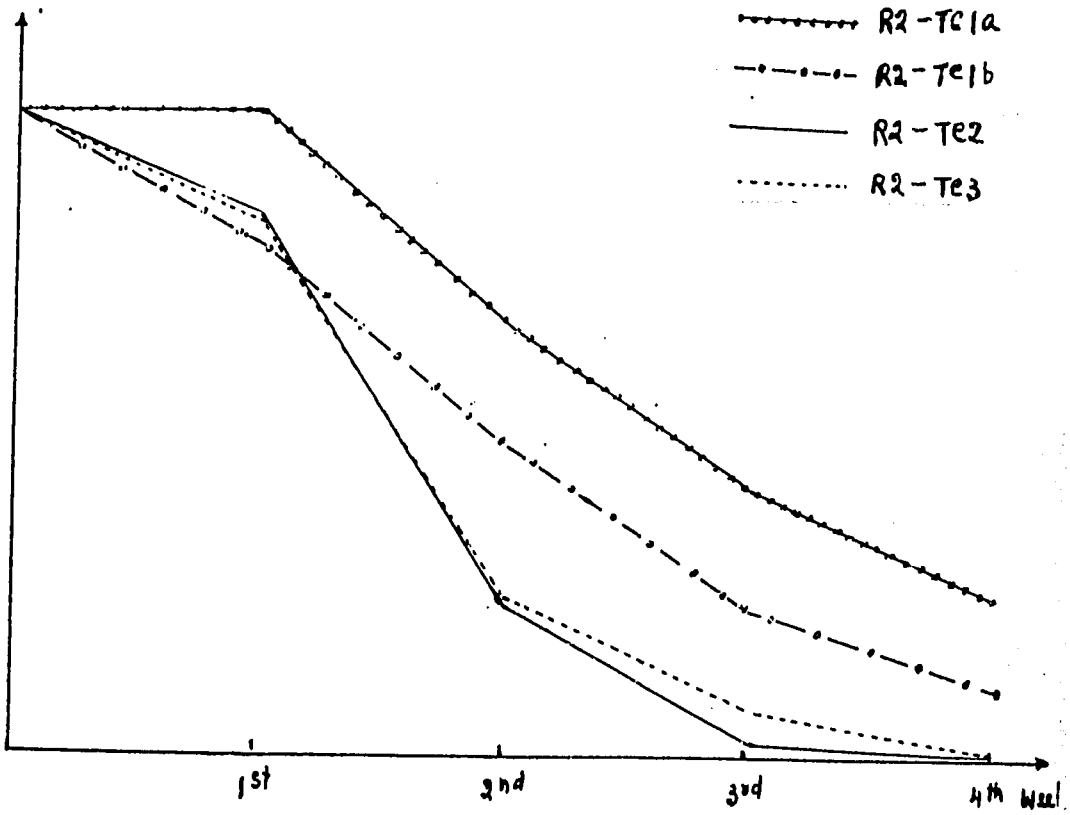


Figure 5 Responses of seedlings of the second generation (R2) of regenerated plants to the treatment of 0.5% NaCl in mutrient solution.

Re:- table 9, Cultivar:- RD 23

Electrical conductivity:- 9-10 mMhos/cm.

Stages of seedling sevelopment at the beginning of the treatment:- four-leave stage.

condition which gave a wide variation. However, the genetic of stress tolerance is rather complex and the chances are remote to have just a simple gene responsible for a given characteristics. If this is the case, many useful variants with favorable recessive genes occurring during callus growth would be lost through stressing when the surrounding cells are killed. Furthermore, if a gene in question is a recessive one it would not have chance to express it self, or if an array of genes responsible for a specific character are of multiple type, this would further reduces chance of occurring down to zero by selection in the callus stage. By allowing regeneration to take its normal course (without stressing) 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 result 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, subse quent selections are still needed to fix gene combination in case of multiple gene.

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

E.) RD25 is another new hybrid released by the Department of Agriculture five years ago. It is a day neutral, short growing and short live variety. This variety resists several pathogens and insects and can be grown three crops a year.

Unfortunately, this variety is a very difficult to handle both in the laboratory and in the field.

Table 10 Responses of seedlings of the second generation (R2) in regenerated plants to treatment of 0.5 % NaCl in nutrient solution.

Cultivar :- RD 25; Generation :- R2; Electrical conductivity :- 9 - 10 mMhos/cm; Stage of seedling development at the beginning of the treatment :- five-leaf Stage.

Note : All of the plants that survived the treatment died within on week after termination of the treatment

Treatment No.	Stock No.	Number of plants	Plants mortality		Plants surviving	
			No	%	NO	%
1	R2 - TC1	60	60	100.0	0	0.00
2	R2 - TC2	16	13	81.2	3	18.80
3	R2 - TC3	75	75	100.0	0	0.00
4	R2 - TC4	25	20	80.0	5	20.00
Total		176	168	95.45	8	4.55

Callus initiation seems to be normal but regeneration is rare. A callus with greenspot normally stops growing and die. Alteration of culture media have been tried but the improvement was not very much. However, some regenerates were obtained and their seedlings were treated with 0.5% NaCl during the R1 and R2 (Table 10).

The results of the treatment of the R2 were quite satisfactory upto the end of the fourth week. Two populations gave survivors upto 18.8 and 20.0 at the end of the experiment, but none of them survived when the plants were transferred to the standard nutrient solution. This variety has to be investigated in its physiology further in order to cope with the regeneration and methods of treatment.

EFFFCTS OF MACRO-ELEMENTS ON PLANTS REGENERATION

BACKGROUND : Plant regeneration from callus has been a major problem in tissue culture of Indica type rices. As reported earlier in the progress reports 3, 4 and 5 that the successful regeneration depended not only the techniques alone but on the interaction between the materials and techniques. The response of Japonicer type to the techniques used by many workers in the United States and Japan have not been successfully done in the Indica type. Due to scarcity of the literatures on regeneration from Indica callus, much of the time has been spent during the past two years to find basic causes of failure. The results obtained have been encouraging but we feel that there is much room for improvement.

There are many different media used by tissue culturists around the world at the present time. Most of this recommendations are from the workers that have worked out the method for specific groups of plants and can be applied to other plants for different purposes (White, 1954; Murashige and Skoog, 1962). Some derived a formula from a series of tests in both monocotyledonous and dicotyledonous tissues (Schenk and Hildebrandt, 1972). In addition to these, the recommended organic compounds and the combination of them are numerous. That renders a systematic testing of the effects of these compounds extremely diffecult. Therefore, we have selected some combinations for testing of plant regeneration from callus of Indica rices. (see Progress report 2, 3, 4 and 5).

This particular report is taken from a series of experiments on macro-elements. Previous experiments gave us a clue that

concentration of some macro-elements might have beneficial effects on plant regeneration in our regeneration medium based on White's supplemented with 10% coconut water.

OBJECTIVE : To find suitable concentrations of macro-element that give better regeneration from callus.

BASIC CULTURE MEDIUM : Re :- Table 2, using macro-elements No.1 and micro-elements No.1 with 1 mg/l. IAA, 3 mg/l. Kinetin, and 100 mg/l. coconut water.

RICE VARIETY : RD23

CALLUS AGE : 17 days in standardized callus induction medium No.1 (Table 1).

LIGHT REGIME : 16/8 hrs. light/dark

NUMBER OF TREATMENTS : 135

NUMBER OF REPLICATIONS : 30 - 60 calli

LENGHT OF TREATMENT : 6 wks.

CONCLUSION : 1. Best greenspot formation were obtained from media with NH_4-N at 3.2 mM.

2. Potassium at level of 6.64 mM. gave consistently good result.

3. NO_3-N at 34.23 mM gave best result in shoot formation but not in greenspot formation.

4. Other macro-elements P, Ca and Mg at concentrations tested (higher or lower than the original formula, failed to show significant difference in both greenspot formation and plant regeneration.

MATERIALS AND METHODS : Re :- Progress reports 3, 4 and 5; Table 2 of this report; and Basic culture medium shown above.

Modification of media were made by

a. adding $(NH_4)_2SO_4$ to make the concentrations of NH_4^-N to 0, 0.80, 3.20, 12.80 and 51.20 mM.

b. adding $NaNO_3$ to increase the concentrations of NO_3^-N to 3.33, 9.51 and 34.23 mM.

c. increasing $NaH_2PO_4.H_2O$ to increase the concentration of P to 0.12, 0.48, 1.92 mM.

d. increasing KCl to increase the concentrations of K to 1.66, 6.64, 26.56 mM.

e. adding $CaCl_2.2H_2O$ to increase the concentrations of Ca to 0.32, 1.27 and 5.08 mM.

f. increasing $MgSO_4.7H_2O$ to increase the concentrations of Mg to 0.73 and 2.92.

A total of 4230 calli were used in this study. The experiment is divided into 135 treatments each of which comprises 30 to 60 calli.

RESULTS AND DISCUSSION :

a. Greenspot formation : Regeneration is fairly difficult in rice, most of the calli grew to certain stages and turned brown and eventually died. Most of the calli reached greenspot formation stage which indicate early differentiation of shoot apex. Coverage of greenspots on the surface of callus normally shows the ability of shoot formation in later stages, the more greenspot forming the higher number of shoot usually resulted (see Tables 11 and 12). Table 13 shows greenspot formation in varying concentrations of NO_3 - N, NH_4 - N, P and K. It was quite clear that NH_4 - N at concentration of 0.8 and 3.2 mM gave best result in greenspot formation giving 67.49 and 62.96% greenspots respectively. Other components, NO_3 - N, P and K, did not show much difference in greenspot formation in different concentrations of those ions mentioned above.

The result of NH_4 - N in limited range of concentrations on greenspot formation and regeneration is in accordance with the results of Ohira et al (1973), Sargen and King (1974), Wetherell and Dougall (1976) in other plant tissues. The lack of NH_4^+ ions reduced the number of greenspots due to lack of organic nitrogenous compound rather than nitrogen because the NO_3 - N did not show much effect when varying concentrations of this were used. On the contrary, higher concentrations of NH_4 - N gave poor result which may be the result of accumulation of NH_4^+ ions in tissues giving inhibitory effect on ATP formation (Salisbury and Ross, 1977). Gamborg et al (1968) stated that the retarded growth of pea root callus was due to the interference of NH_4^+ to the Kreb's cycle.

Table 11. Effects of NO_3-N, NH_4-N, P and K on the formation of greenspot

Conc. of NO_3-N (mM)	No. of callus treated	Callus with greenspot						
		2nd wk		4th wk		6th wk		
		No.	%	No.	%	No.	%	
3.33	810	607	74.94	545	67.28	407	50.25	
9.51	810	588	72.59	524	64.69	424	52.35	(a)
34.23	810	570	70.37	539	66.54	431	53.21	
Total	2430	1,765	72.63	1,608	66.17	1,262	51.93	

Conc. of NH_4-N (mM)	No. of callus treated	Callus with greenspot						
		2nd wk		4th wk		6th wk		
		No.	%	No.	%	No.	%	
0	486	291	59.88	283	58.23	221	45.47	
0.8	486	376	77.37	375	77.16	328	67.49	
3.2	486	398	81.89	373	76.75	306	62.96	(b)
12.8	486	402	82.72	349	71.81	280	57.61	
51.2	486	298	61.32	228	49.91	127	26.13	
Total	2430	1,765	72.63	1,608	66.17	1,262	51.93	

Conc. of P (mM)	No. of Callus treated	Callus with greenspot						
		2nd wk		4th wk		6th wk		
		No.	%	No.	%	No.	%	
0.12	810	587	72.47	542	66.91	425	52.47	
0.48	810	587	72.47	545	67.28	433	53.46	(c)
1.92	810	591	72.96	521	64.32	404	49.88	
Total	2430	1,765	72.63	1,608	66.17	1,262	51.93	

Conc. of K (mM)	No. of Callus treated	Callus with greenspot						
		2nd wk		4th wk		6th wk		
		No.	%	No.	%	No.	%	
1.66	810	624	77.04	556	68.64	415	51.24	
6.64	810	598	73.83	536	66.17	433	53.46	(d)
26.56	810	543	67.04	516	63.70	414	51.11	
Total	2430	1,765	72.63	1,608	66.17	1,262	51.93	

Table 12 Relationship of NO_3-N, NH_4-N, P and K on greenspot formation (data were collected on the cna of second week)

(a)

Cone. of P (mM)	No. of callus with greenspot at different levels of NH_4-N (mM)					
	0	0.8	3.2	12.8	51.2	Total
0.12	96	123	136	140	92	587
0.48	99	130	124	127	107	587
1.92	96	123	138	135	99	501
Total	291	376	398	402	298	1765

(b)

Cone. of NO_3-N (mM)	No. of callus with greenspot at different levels of NH_4-N (mM)					
	0.	0.8	3.2	12.8	51.2	Total
3.33	100	126	135	135	111	607
9.51	95	123	138	133	99	588
34.23	96	127	125	134	88	570
Total	291	376	398	402	298	1765

(c)

Cone. of K (mM)	No. of callus with greenspot at different levels of NH_4-N (mM)					
	0	0.8	3.2	112.8	51.2	Total
1.66	109	133	137	137	108	624
6.64	105	120	135	143	95	598
26.56	77	123	126	122	95	543
Total	291	376	398	402	298	1765

(d)

Cone. of P (mM)	No. of callus with greenspot at different levels of K (mM)			
	1.66	6.64	25.56	Total
0.12	210	196	181	587
0.48	205	212	170	587
1.92	209	190	192	501
Total	624	598	543	1765

(e)

Cone. of NO_3-N (mM)	No. of callus with greenspot at different levels of K (mM)			
	1.66	6.64	26.56	Total
3.33	210	214	183	607
9.51	211	199	178	588
34.23	203	185	182	570
Total	624	598	543	1765

(f)

Cone. of NO_3-N (mM)	No. of callus with greenspot at different levels of P (mM)			
	0.12	0.48	1.92	Total
3.33	205	190	121	607
9.51	202	196	190	588
34.23	180	201	189	570
Total	587	587	501	1765

Note : Number of calli treated in each experiment were 270, except on the study of NH_4-N with other element were 162.

Total calli treated in this study were 2,530.

Little responses to NO_3 - N, P and K in varying concentrations may be due to sufficient supply of these ions partly from the White's formula and partly from coconut water. Over supply of these ions, not like NH_4^+, does not cause inhibitory effect on metabolism or toxicity in the tissue. Similar results were obtained in the experiments on Calcium and Magnesium.

b. Shoot formation : This is the most difficult stage of the work. Normally greenspots are formed with rudimentary shoot apices after callus attains certain size in media containing low auxin. If calli are left in the jar for a period of one month, some shoots appear and continue to grow and finally produce roots forming a complete plants. Unfortunately, the frequency of shoot formation is extremely low comparing to the number of greenspot on each callus. Changing composition of the media or physical factor seems to improve shoot formation.

Table 13 shows effect of NO_3 - N, NH_4 - N, P and K at various concentrations on shoot formation. Shoot formation often occurs at a low frequency for the RD23 variety, it usually ranges from 5 to 15 percent in our standard medium. In this experiment the best ten out of 135 media tested gave shoot formation ranging from 12.50 to 20.80 which shows significant improvement over the control (standard medium) which gave 5.56% in this experiment (Table 14).

The result of shoot formation is similar to greenspot formation in respect of NH_4 - N content. Concentration of NH_4 - N at 3.2 mM gave best overall result in both shoot formation and greenspot formation (Table 13). Even the bestten media shown in

Table 13 Relationship of NO_3-N, NH_4-N, P, and K on shost formation

(a)

Cone. of P (mM)	No. of shoot producing callus at different levels of NH_4-N (mM)					
	0	0.8	3.2	12.8	51.2	Total
0.12	4	8	23	20	3	58
0.48	5	13	38	11	1	68
1.92	6	34	18	7	0	65
Total	15	55	79	38	4	191

(b)

Cone. of NO_3-N (mM)	No. of shoot-producing callus at different levels of NH_4-N (mM)					
	0	0.8	3.2	12.8	51.2	Total
3.33	4	23	26	11	4	68
9.51	7	8	35	9	0	59
34.23	4	24	18	18	0	64
Total	15	55	79	38	4	191

(c)

Cone. of K (mM)	No. of shoot producing callus at different levels of MH_4-N (mM)					
	0	0.8	3.2	12.8	51.2	Total
1.66	10	14	26	12	1	63
6.64	3	28	37	15	3	86
26.56	2	13	16	11	0	42
Total	15	55	79	38	4	191

(d)

Cone. of P (mM)	No. of shoot producint callus at different levels of K (mM)			
	1.66	6.64	26.56	Total
0.12	22	28	8	58
0.48	19	26	23	68
1.92	22	32	11	65
Total	63	86	42	191

(e)

Cone. of NO_3-N (mM)	No. of shoot producing callus at different levels of K (mM)			
	1.66	6.64	26.56	Total
3.33	25	32	11	68
9.51	17	27	15	59
34.23	21	27	16	64
Total	63	86	42	191

(f)

Cone. of NO_3-N (mM)	No. of shoot producing callus at different levels of P (mM)			
	0.12	0.48	1.92	Total
3.33	29	20	19	68
9.51	16	26	17	59
34.23	13	22	29	64
Total	58	68	65	191

Note : Number of calli treated in each experiment were 270, except on the study of NH_4-N with other element were 162. Total calli treated were 2,530.

Table 14, they contain varying amount of NH_4 - N but other concentration do not give consistent results.

Potassium concentration at 6.64 mM again gave consistent results in shoot formation like in greenspot formation in the whole experiment with 135 different media. But among the best ten media the concentrations of K do not seem to have effect on shoot formation. This conflicting result may be due to the sufficient amount of K in the media to support normal growth of callus, and the role of K in tissue is not specific as in the whole plant. However Salisbury and Ross (1977); and Brown et al. (1976) stated that potassium played a major role in growth and development of the tissues but Ohira et al. (1973) found that potassium in wide range of concentration was beneficial for rice tissue but not to a great extent.

Nitrate nitrogen seems to have effect on shoot formation than greenspot formation. In the best 10 (Table 14), six out of ten media contain high nitrogen which this result is in agreement with the report from other tissue culturists working with coconut, wild carrat and rice (Eeuwens, 1976; Ohira et al., 1973; Yamada, 1982). Wetherell and Dougall (1976) found that embryogenesis of wild carrot were very poor when NO_3 - N was lower than 20 mM and the maximum yield was obtaned when NO_3 - N concentrations ranged from 20 to 40 mM. Our experiment showed 34.23 mM to be the best concentration for shoot formation of rice, but other concentrations were generally acceptable for shoot formation. However, it is interesting to note that there is relationship between the concentrations of NO_3 - N and P (see Table 13, lower right). This showed that increasing concentration of P gave better result when NO_3 - N concentrations increased progressively.

Table 14 Ten best formulae for shoot formation selected from 135 formulae tested; White's formulae was used as control.

Grade	Formulae No.	Conc. (mM)				n	Shoot producing callus (%)	Shoot (%)	Callus with greenspot at 2nd wk. (%)	Callus with root at 6th wk.
		NO_3^-	NH_4^+	$H_2PO_4^-$	K^+					
1	93	34.23	12.8	0.12	6.64	24	20.80	29.17	94.44	50.00
2	43	3.33	0.8	1.92	6.64	24	16.67	50.0	94.44	61.11
3	55	3.33	3.2	0.12	1.66	30	16.67	30.0	94.44	38.89
4	36	34.23	0.8	1.92	1.66	24	16.67	20.80	72.22	55.56
5	51	34.23	0.8	0.48	26.56	24	12.50	25.0	88.89	50.00
6	87	34.23	12.8	0.48	1.66	24	12.50	25.0	100.00	50.00
7	35	9.51	0.8	1.92	1.66	24	12.50	20.8	77.78	22.22
8	45	34.23	0.8	1.92	6.64	24	12.50	20.8	66.67	33.33
9	23	9.51	0.0	0.48	26.56	24	12.50	16.67	50.00	44.44
10	54	34.23	0.8	1.92	26.56	18	12.50	16.67	72.22	38.89
Control	1	3.33	0.0	0.12	1.66	54	5.56	9.26	83.33	33.33

n - number of calli in each experiment

Table 15 The effects of NO_3-N and NH_4-N on pH of the media (data were collected at the end of 6th wk)

Cone. of NO_3^- (mM)	Average pH at various conc. of NH_4^+					Total average
	0	0.8	3.2	12.8	51.2	
3.33	6.97	6.97	6.61	5.01	4.81	6.07
9.51	6.88	6.99	6.59	5.33	5.38	6.23
34.23	6.98	6.87	6.62	5.89	5.88	6.45
Total	6.94	6.94	6.61	5.41	5.36	6.25

Note :- Initial pH was 5.6

Table 16 Effects of Ca and Mg on formation of greenspot (data were collected at the end of 4th wk)

Conc. of Ca (mM)	Callus with greenspot at different levels of Mg (mM)		Total
	0.73	2.92	
0.32	116	110	226
1.27	112	112	224
5.08	106	116	222
Total	334	338	672

Note :- Each experiment : 150 calli.

Total : 900 calli.

Other elements, P, Ca and Mg, did not give consistent results in this experiment probably there were sufficient supply of these elements even at minimum concentration and these elements did not reach toxicity level at highest concentrations tested. Therefore the wide ranges of these elements contained in White's formula in combination of coconut water are sufficient for growth and development of callus, greenspot formation and shoot formation (see Table 13, 16, 17 and Figs.6,7)

When changing the composition of media not only the balance of the elements that will change but pH will be affected by concentration of salts. Most of the salts used in the experiment are more or less neutral in nature and they would not be affected by other added ions but the ammonium ions will affect the pH of the solution and will change in time. The measurements were made from the media that had different concentrations of ammonium salt and nitrate salts (Table 15). The results showed that without ammonium salt or at 0.8 mM of NH_4 - N the pH rose to around 6.8 which is unusually high for culture media. But when the concentrations of ammonium salt increased progressively the pH went down to 6.62 - 4.81 which the range is suitable for most tissue. Therefore the role of NH_4^+ will not limit to supplying NH_4^+ to tissue but it also has effects on acidity in the medium which in turn effect the absorbtion of other elements into tissues. And these will affect physiological activities which induce shoot apex initiation, growth, and development.

This experiment is only one facet of studies on regeneration which only the alteration of macroelements were done, other factors like relationship between auxin and cytokinen, physical enviorments

Table 17 Relationship of Ca^{++} and Mg^{++} on shoot formation (Data were collect at the end of 6th wk)

Cone. of Ca^{++} (mM)	No. of shoot producing callus cone. of Mg^{++} (mM)		
	0.73	2.92	
0.32	1	4	5
1.27	0	5	5
5.08	5	4	9
Total	6	13	19

Note :- Each experiment : 150 calli

Total : 900 calli

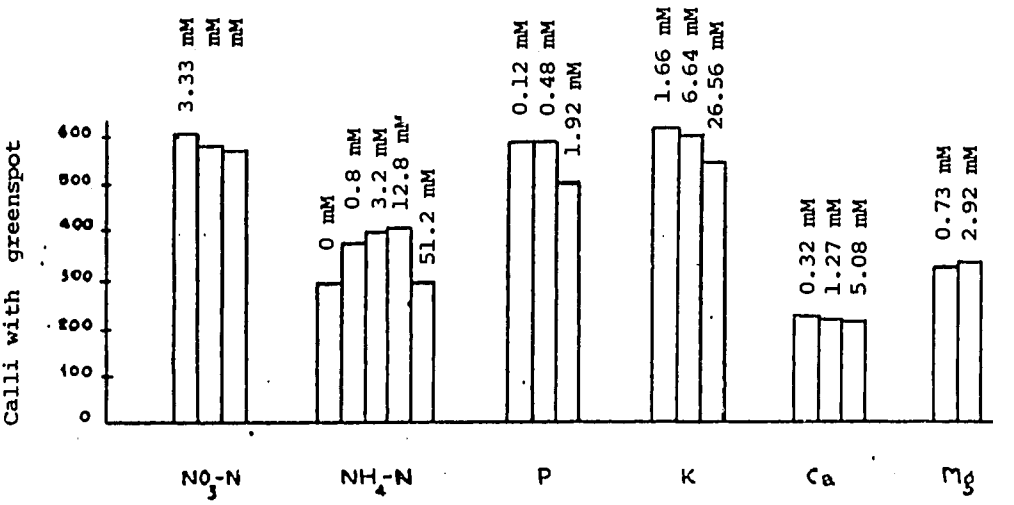


Figure 6 Effects of concentrations of ions on greenspot formation

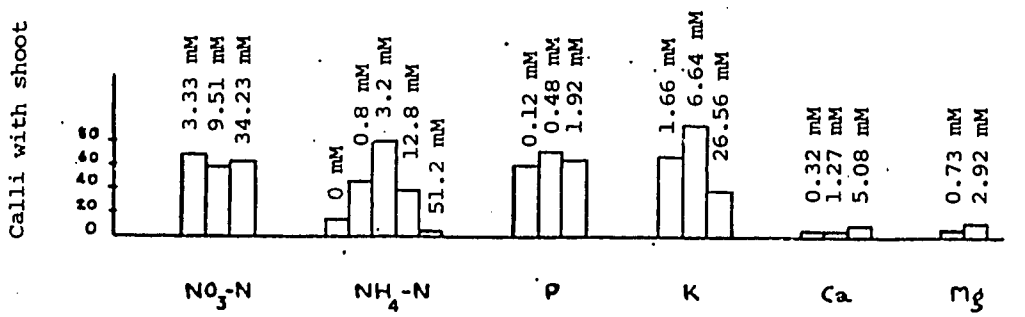


Figure 7 Effects of concentrations of ions on shoot formation

and others still need systematic study for better regeneration of Indica rice type from callus. The applied aspects of tissue culture would not be successful without fundamental search for appropriate methods for controlling and handling the specific materials.

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