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PROGRESS REPORT I

Callus Induction Techniques In Rice

Return to AID/SCI

From: Montakan Vajrabhaya
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B

PROGRESS REPORT

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

AID/SCI PROPOSAL 2A-15

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INTRODUCTION

Rice is the main agricultural product of Thailand. This is indicated by the fact that out of a total arable land area of 59 million acres, rice is grown in 24 million acres. As a result, Thailand produces over 17.8 million tons of rice annually, with 2.8 million tons left for export, which makes Thailand the second biggest rice exporter. This represents the largest income (one billion dollars) for any single agricultural product. (Anonym., 1979 a, Anonym., 1979 b, Anonym., 1980; Anonym., 1982)

However, Thailand is not without problems in rice production. A variety of problems results in low yield, i.e. the average yield in Thailand is 780 kilograms per acre, compared to the average yield of 1430 kilograms per acre in China, Indonesia and Malaysia whereas the United States, Japan and Korea produce 2296 kilograms per acre. (Anonym., 1979 b, Anonym., 1982.) Some of the problems that causes the low yields are:

1. IRRIGATION : Out of the total rice producing area of 24 million acres, only 6.4 million acres are in the irrigated zone; the rest depend on rainfall. (Boonsue, 1980; Anonym. 1982)

2. GROWER SEED PRODUCTION : Although there are a number of good rice varieties produced by the Department of Agriculture during the past twenty years, but the seed production of these varieties is inadequate, supplying less than 5% of the demand.(Boonsue, 1980; Senanarong, 1980.)

3. MANAGEMENT PRACTICES : A large proportion of the Thai farmers do not invest sufficiently in their farms to produce good crops. Even they learn how to manage their farms properly they still cannot practice effectively due to short of fund. Therefore they rely much of their fate in natural resources and phenomena rather than using newly technology.

4. SUITABLE AGRICULTURAL LAND : There is only a certain amount of land suitable for agriculture, the rest is not very suitable for most crops. It is estimated that there are one million acres of acid soil and 2.8 million acres of saline soil, representing 11% of the total rice producing land.

From the problem listed above it may become apparent that there are several causes several plausible solutions. In some of these cases, the solution must be applied concomitantly since the problems are inter-related. For

Figure 2 Selected varieties of rice are needed to increase yield in unfavorable agricultural land due to the excess salinity or acidity in soil . These are accounted for 11 percent of the arable land in Thailand. Most of the lands are not irrigated so the yield in such areas depends on the amount of rainfall each year. Without good irrigation system it is not possible to wash off salts or acids from the soil . Even a good breeding program have been established for almost thirty years, its produce about thirty varieties of rice with desirable characteristic suitable for certain area and methods of cultivation. More varieties are still needed to fit in certain areas such as the lands with excess salts or acids.

In the picture, the farmers are ploughing the field after a series of heavy rains and transplanting seedling to mewly prepared field.

example, the new high-performance varieties usually demand high fertilizer input and irrigation and are less tolerant to adverse conditions. This makes it impractical for farmers in poor soil area who depend solely on the monsoon rains and who may not have the financial resources to pay for fertilizers and pesticides.

The present project intends to deal only with the two world wide problems related to saline soil and acid soil. According to Carter (1975) the world arable land subject to salt and drought stress is around 25 percent. And another 25 percent of soil effected by high acidity which bring large amount of toxic metal into the soil solution. (Christiansen, 1979). In Thailand high salinity occurs where there are saltbeds underline rice growing land in the North East or where the sea water rises seasonally in the lowland near the gulf. Whereas excess soil acidity occurs natuarally in the tropic where the rainfall is high. Soil become acidic as a result of CO_2 dissolved in rain water which acelerates the replacement of cat-ion by H^+. This inturn brings usually insoluble ions into the solution which causes toxicity in plant tissues.

The current methods to correct these problems are to apply lime or marl in acid soils and intensive drainage system in saline soil . These methods require extremely high cost and are of temporary bases. Breeding for tolerant varieties have been done in several places producing some tolerant varieties with improved yields. This is the standard method for rice improvement for many decades. An alternative solution proposed in the present project is to find new rice varieties which are more tolerant to those conditions and produce acceptable yields. The new varieties may be used directly in the problem areas or may become breeding stocks for future varieties. This is a less expensive solution than correcting the soils. Furthermore, this remedial method is not a one shot affair but it will establish the base varieties for future development through either by tissue culture technique or by standard breeding methods.

The method employed in obtaining new varieties will be plant tissue culture selection which has already been successfully used to abtain salt tolerant cereal and other crop plants (Nabors, 1982). This technique bases on findings of several tissue culturists that the nuclei of cells often change during culturing which result in increased frequency of variability in whole plants (Murashige and Nakano, 1967; Vajrabhaya and Vajrabhaya, 1974; Vajrabhaya, 1975; Heinz et al., 1977). Even the chances of mutations and segregations are normally slim but the tissue culture method deals with multibillion actively

dividing cells in a limited space and time. It is believed that the resulting valuable variants come through screening will be many of these several will be really promising.

FACILITIES

The main research work of this project is in the Botany Building of Chulalongkorn University. The tissue culture section comprises of general preparation laboratory, a sterile transfer room with five transfer chambers which included two laminar flow transfer chambers and a temperature constant culture room. Each of these units equipped with standard equipment nescessary for plant tissue culture. These cover over one hundred square meters of floor area. Greenhouse space for rearing of young plants newly transfered from culture vessels. A separate insect screening lath house will be built in the future to accommodate facilities for saline and acid tolerance evaluation.

The Department of Agriculture, Ministry of Agriculture and Cooperatives offers to provide seeds of rice varieties for the experiments and will be responsible for field testing of selected mutants with noteworthy characteristics. Any variety proved to be salt or acid tolerance added to the typical characteristics of the specific variety will finally be released to famers.

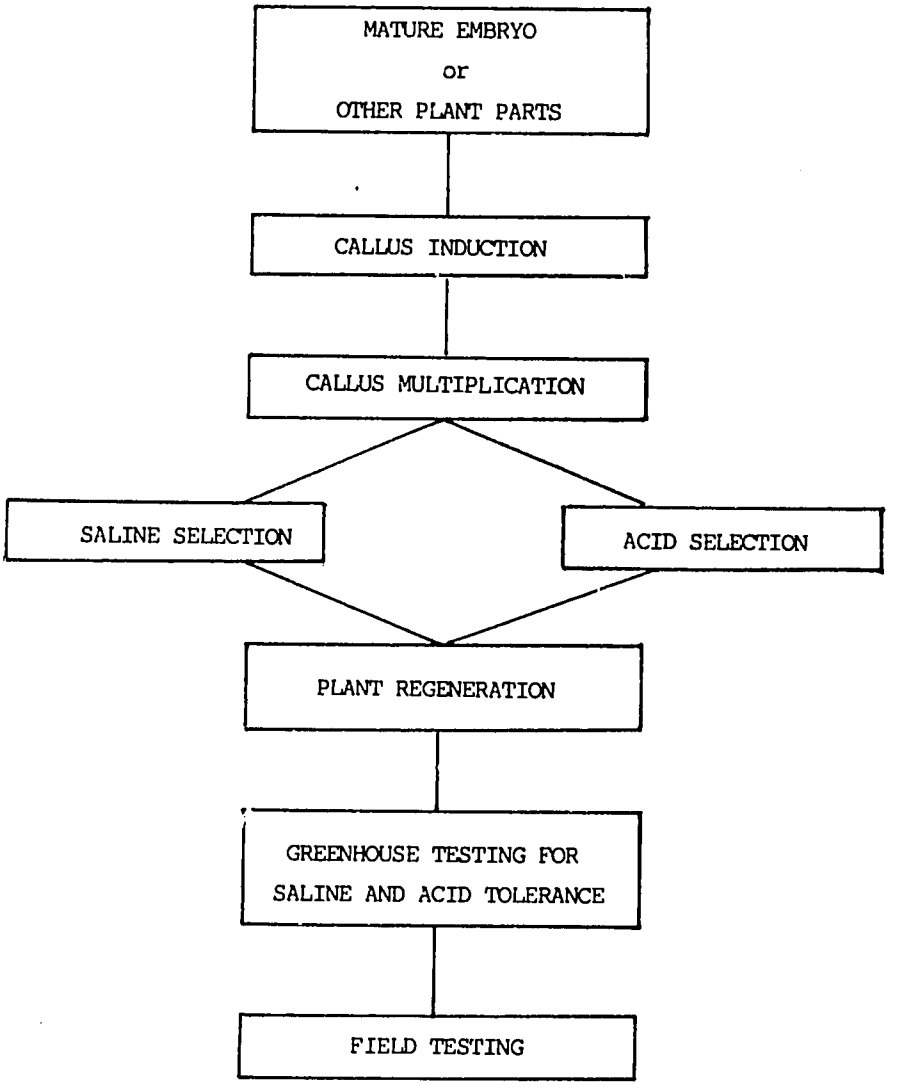
METHODOLOGY

A standard procedure for tissue culture is used in our laboratory. At present our work in rice is in the very early stages, only the induction of callus from mature embryo is carried out. The Linsmaier and Skoog's (1965) medium is selected as a general purpose medium at the moment. This medium is one of the high salt composition which generally gives good result on cereal Tissues whether they are in callus form or differentiated forms. The variation in organic additives and auxins have been tried to promote optimum growth for specific Tissues. The pH is adjusted to 5.5 prior autoclaving.

Sterilization is carried out by submerging shelled seeds in 95 % ethanol for one minute before transfering into 20 % chlorox solution for 20 minites. Then, concentration of chlorox is gradually reduced to 5 % in 25 minutes, and the seeds are rinsed thoroughly with sterilized distilled water. All the treated seeds are kept in the Petri dish until use. The seeds are then placed on a solid medium containing Linsmaier and Skcog's (1965) basic formula with

Figure 3 Rice tissue culture project facilities at Chulalongkorn University, Bangkok.

various concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4-D) ranging from 0.5 to 4 ppm. Kinetin concentration is kept at 0.3 ppm. for convenience. These culture vessels are kept in the dark at 24-26 °C during the first 4 weeks. The 100 ml. cylindical screw cap bottles are used throughout the callus induction experiments. The evaluation of callus induction is made by both weighting calli indirectly and by visual observation on morphogenesis, color and type of growth.



PROPOSED METHODS FOR PRODUCTION OF SALINE AND ACID TOLERANT RICE

IMPORTANCE OF TISSUE CULTURE IN PLANT IMPROVEMENT

Tissue culture of plant has been made possible for over 40 years but its applications to agriculture was first realized two decades ago. Morel (1960) could obtain virus-free Cymbidium orchids by regeneration of plants from stem-tip calli. He subsequently reported that unlimited proliferation also occurred during regeneration if tissues or plantlets are cut often enough (Morel, 1964). These two reports shed lights on the possibility of improving economic crops through tissue culture. The method mentioned above may be used either for eradication of diseases or mass sexual propagation of difficult varieties.

In 1963 an orchid firm in France began to offer orchid plantlets arising from tissue culture for sale. Since then asexual propagation of orchid relies largely on this method especially in cut flower industry. During the last two decades there have been so many reports on studies in tissue culture of many plants. The application of these findings in plant improvement is still in the early stages. Some have already been used with success but many are still in the experimental stages. The results reported upto the present indicate optimistic trend in using this technique for plant improvement but warning from some authors of this technique if not applied with care is worth noticing.

A.CLONING OF PROVEN VARIETY

Cloning is widely used in multiplying specific genotypes of many species or hybrids due to its simplicity. It is extremely useful for propagation of highly heterozygous individuals which lack natural propagation mechanisms like stolon, bulbil etc. or unable to respond well to artificial asexual propagation methods normally used today. These plants, for example, are orchids, palms, certain forest crops or newly introduced horticultural varieties. Cloning is particularly useful for monoecious species if only one sex or certain sex ration is needed. In the case of papaya only small number of male is needed as a source of pollination and only one sex is preferred by asparagus growers.

The success of cloning started when tissue culture technique was first introduced in Thailand by our laboratory in 1965. Since it was a boom to the

orchid cut flower industry, this method attracted the attention of many scientists as well as growers. During 1974 and 1977, six commercial laboratories established and began cloning service. At present, all laboratories propagating orchids by tissue culture produce five million plantlets per year. It is worth mentioning that some of these also produce ornamental plantlets upto 100,000 per year. The combination of cloning productive varieties of slow growing plants like orchids and breeding greatly facilitate the replacement of the new improved varieties in cultivation (Vajrabhaya, 1981).

Orchids have gained a superior status over the last 20 years, making Thailand the world's biggest exporter. The introduction of tissue culture technique not only to retain the firm leading role in orchid production and research for Thailand, but it is a stepping stone to the development of other plants of agricultural value. The use of tissue culture in cloning plants will not be completed without mentioning Malaysia, our neighbor, in cloning oil palm. Malaysia a leading country in palm oil production has been trying to established tissue culture technique for oil palm for a long time. Finally, their scientists overcame all difficulties built-in the palm family and obtained admirable result recently (Choo et al,1981). These are only the examples from South East Asia to demonstrate the effective use of tissue culture for cloning. There are many more in plant improvement through tissue culture in other parts of the world.

Cloning seems to be the most easily way to tackle problems in non reliable offsprings in sexual reproduction of many plants. At the same time we should not be over optimistic and overlook the danger that may arise in the practice. It should be pointed out that dealing with any group of organisms of similar biotypes is easy in many respects. But, if any trouble should arise these biotypes will respond the same way due to uniformity, it tends to cause damage more than the mixture of distantly related biotypes. The incidence of potato blight in 1846 and similar cases occurred intermittently due to uniformity of plants still registered in our memory. Therefore, we should make every effort to avoid recurrent of such problems.

Clonal variation occurs sporadically either under natural condition or in the test tube (Shamel and Pomeroy, 1936; Dermen, 1960; Murashige and Nakano, 1967; Shimada and Tabata, 1967). The chances are the variants may be either better or worse than the original clone. Naturally, the better ones are expected to be very few if started from already selected varieties. Even the

occurrence of the new characters are rare but it needs only once to ruin the majority, if it occurs in the early stages of propagation (Vajrabhaya, 1977). Care must be taken not to produce too many plants from any one stock or from any subsequent culture. This practice will limit the lost stemming from mutation arising during tissue culture. On the contrary, if this problem is taken into consideration seriously, we will find that the selection of mutation occurred in tissue culture is a new means of creating new varieties.

B. SEARCHING FOR NEW VARIETY

New varieties of plants can arise spontaneously or are deliberately induced in tissue culture (Murashige and Nakano, 1967; Shimada and Tabata, 1967; Vajrabhaya, 1977). In the past, most of the new cultivated varieties known were the result of laborious selection and breeding, only few were from spontaneous somatic mutations. After the world war II, ionizing radiations and some chemicals have been used to increase rate of mutation effectively (Sparrow and Konzak, 1958; Freese, 1971). These methods require ample space and high running cost to obtain only few improved varieties. Because only the changes in certain cells in meristem will have chance to express their new characters, while other affected cells elsewhere remain genetically inactive. In tissue culture, the number of actively dividing cells in a small test tube may exceed such comparable cells in a full grown tree. Each of these cells in a callus has a chance to be in a growing point or even become the whole plant later. If the single cell method is used,any mutation occurred in the one-celled stage will even express better. The treatment of ionizing radiations or chemical mutagens during tissue culture will certainly increase rate of mutation many times which facilitate further selection (Nickell, 1973)

Variation resulting from chromosomal mutation is very useful in plant improvement. The variant itself may become a new variety with desirable quality or if still lacks of certain characteristic it may become parent of next generation. Polyploids and aneuploids are common variants arising in tissue culture and are more numerous if treated with colchicine at certain stages (Torrey, 1967; Murashige and Nakano, 1967; Heinz, Mee, and Nickell, 1969; Vajrabhaya, 1977). In many cases, polyploid individuals have some specific phenotypes or improved fertility (when becoming alloploid) required by plant breeders. Whereas, occasional aneuploids arise in vitro proved to be useful plants provided that they are propagated asexually. Many polyploid and aneuploid orchids arising in tissue culture already win the places either in cut

flower industry or in the show. Not all of these variants mentioned earlier are better than the original ones, some may change the growth habit or flowering season. Genes are the ultimate determinants and an increase in number of poor gene due to chromosome duplication may even worsen the quality of already undesirable plants. Evaluation of the new variants arising this way should be practiced very carefully.

At present, we are able to create pure line through tissue culture techniques quite effectively instead of several generation of selfing. Microspore culture of many Angiosperm species give rise to monoploid sporophyte instead of usual three-celled gametophyte occurred in nature (Nitsch, 1970). This is an important advancement in botanical research, these plants not only useful in breeding but they can be used in the study of Genetics, Morphology and other aspects of biology. Once the monoploid plant is found, it can become a pure line easily if chromosome number is double by colchicine treatment. The plants arising this way are as good as or even better than purelines selected from laborious selfing of many generations.

At present, we are able to create pure line through tissue culture technique instead of several generation of selfing.

C. DISEASE ERADICATION

Viral disease or other diseases caused by virus-like agent are systemic, any division from diseased plants is certain to centain virus particles. No satisfactory method is yet known of eliminating a stable virus by inactivation with heat or chemicals in living plants without killing the host (Morel, 1960; Lawson and Hearon, 1973). However, virus-free plants can be obtained from infected ones, even those exhibiting severe symptoms (Morel, 1960). The method involves the excision of a very small tissue section (0.1 mm. thick) from the meristematic region of the shoot tip. This is then cultured on a nutrient medium. The combination of heat therapy and shoot-tip culture has been reported to be effective in elimination of various viruses in several plant species (Paludan, 1971). This should have some application in rescuing important clones which all members have been infected with virus.

D. PRESERVATION OF GERMPLASM

Among several methods of preserving germplasm, tissue culture is the

one that uses little resources and technology. This new technique of retarding the growth of whole plants, organs or tissue in a limited space is worth study further. Keeping tissue or plant regenerated from it in liquid nitrogen seems promising because it stops the growth and may stop any genetic aberration during the treatment. However, this technique is still to early to be evaluated.

E. GENETIC ENGINEERING

Transfering of genes or blocks of genes from one organism to another has been first observed by the latest Nobel laureate Babara McClintook for over thirty years ago. At present, many refined techniques for microorganisms have been established but only few have been successful in higher plants. However, fusion of protoplasts between related but sexually incompatible species has been done to create new hybrid. In this way a broad spectrum of resistance to disease or other desirable traits might be incorporated into commercial variety. Using recombinant DNA techniques to improve genomes of higher plants are progressing slowly. Cloned DNA is routinely transferred between microorgamisms, but lack of comparable vector systems has inhibited similar experiments in plants. However, progress is now being made in this area. These are some indications of success in creating useful symbiotic relationship between plants and microbes such as plant and microbial systems that provide their own nitrogen fertilizer (Abelson, 1983; Barton and Brill, 1983). The future of plant genetic engineering will be exciting. It is certain that many new varieties can be created by this method which is,otherwise, impossible by conventional means.

F. SUMMARY

The potential for improvement of crop plants through tissue culture seems vast. Although a few broad areas and limited examples have been cited in this article. Besides the obvious value of food production, advances in tissue culture will contribute to forestry, to fiber industry to ornamental plant, or to more efficient pharmaceutical production. Since the problem of food production is becoming more serious all over the world, crop improvement is urgently needed. Conventional methods of crop improvement still continue but plant tissue culture will help introduce more variability into the stream thus accelerating the process. In the future, it is hoped that workers in

plant tissue culture will begin to be more aware of the economy of the country and its problems in food production. It is hoped that further emphasis on crop improvement through tissue culture will follow.

CALLUS INDUCTION

The quantities of plant is a prime importance for selection of new rice varieties, therefore the methods of callus induction, callus multiplication and subsequent regeneration have to be studied in detail. Up to now, there is no such general procedure suitable for the methods stated above. The response of monocotyledonous tissues to the culture techniques varies appreciably from species to species and variation among varieties of rice have been found (Nabors, 1982). Therefore the first stage of the experiment is to find the efficient method of callus induction.

A. MATERIALS : Six varieties of rice were selected from the list of Rice Division, Department of Agriculture which recommend and distribute to the farmers. Among these four are ordinary rice widely grown in Thailand namely RD 23, RD 25, Kao-dok-mali 105 (KDML) and Kao-ta-haeng (KTH). Two varieties of glutinous rice (sticky rice), RD 8 and Neo-sun-pa-tong (NSPT), are also used in the experiments. Only embryos of mature seeds are used for callus induction experiments.

B. METHODS : The standard tissue culture techniques for callus induction are used as stated earlier in section Methodology. Two groups of chemical that play major role in callus formation are auxin and cytokinin. Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D), Naphthaleneacetic acid (NAA) and Indoleacetic acid (IAA) are used successfully for callus induction in rice (Nishi et al, 1973; Yan and Zhao, 1982). Only 2,4-D with varying concentrations (0.5, 1, 2, 4 ppm.) is selected for the experiments in callus induction because it seems to give consistent result with rice tissues (Yatazawa et al, 1967; Wu and Li, 1971; Davoyan and Smetanin, 1979).

There are several reports on using different chemicals including undefined organic additives which is essential for both monocot and dicot tissues in inducing cell division. Kinetin has been used as standard component of many culture media. Coconut water, the undefined organic additives used earlier, has advantage of having other organic supplements to the media and also

has synergistic effect on auxins. In addition, tryptophan is often used by some cereal tissue culturists (Steward, 1969; Vajrabhaya, 1978; Nabors, 1982; Siriwardana and Nabors, 1983). The three abovementioned materials were selected for the experiments, they are used either separately or in combination with others. Since the concentration of chemicals in this group is not as critical as of the auxin group. Only two concentration of tryptophan (50 and 100 ppm), 0.3 ppm of kinetin and 10 % V/V of coconut water are used throughout the experiments.

RESULTS AND DISCUSSION

The cultured embryos started swelling noticebly on the third day with shoots and roots elongation followed. The shoot and root were then removed on the seventh to tenth days, leaving middle parts on the media. Callus formation began few days after cutting, whereas the whole embryos rarely gave rise to calli. Fresh weight was determined thirty days after starting the cultures. Variations in size and shape of callus were clearly noticed at this stage.

Germination of embryos in all experiments ranged between 62.5 to 100 percent (Table 1). The difference in concentration and types of auxin and cytokinin did not have effect on the growth of embryos.

Callus formation of living organs was surprisingly high, it ranged from 51.43 to 100 percent with the average of 93.3 percent (Table 1). Concentation of 2,4-D have much effect on callus induction, number of calli obtained at the level of 2.0 to 4.0 ppm were obviously greater than at 0.5 or 1.0 ppm. This result is in agreement with Davoyan and Smetanin (1979).

Since the effect of 2,4-D on callus growth has been widely accepted in cereal tissue cultures, therefore 2,4-D was then selected as the auxin source in all experiments on callus growth. Four concentrations (0.5, 1.0, 2.0 and 4.0 ppm) of 2, 4-D were used in each experiment to facilitate analyses of the results. All cultures were kept in the dark at 23° to 26°C for four weeks prior to harvesting.

The effects of kinetin, tryptophan, and coconut water were studied (Table 2, 3, 4 and 5). Among these, the combination of kinetin and 2,4-D

Table 1 Embryo germination and callus formation from germinated embryos. (Each treatment average from 35-40 embryos.)

Medium	RD 23		RD 25		KDML		KTH		RD 8		NSPT	
	A	B	A	B	A	B	A	B	A	B	A	B
K/0.5	100.00	67.50	91.67	90.91	95.00	94.74	92.50	89.19	87.50	91.43	97.50	97.43
K/1.0	95.00	97.37	100.00	97.50	95.00	94.74	97.50	97.43	80.00	90.63	92.50	94.59
K/2.0	95.00	97.37	100.00	100.00	95.00	97.37	95.00	100.00	80.00	93.75	84.61	96.97
K/4.0	100.00	95.00	100.00	100.00	100.00	97.50	91.89	100.00	87.50	91.43	97.50	100.00
CW/0.5	95.00	0.00	100.00	0.00	100.00	92.10	92.10	94.29	71.87	95.65	95.00	0.00
CW/1.0	97.50	87.18	100.00	100.00	100.00	94.87	97.50	97.43	97.50	100.00	95.00	94.74
CW/2.0	95.00	94.74	100.00	95.00	100.00	100.00	100.00	100.00	87.18	100.00	97.50	97.43
CW/4.0	97.50	97.43	100.00	97.50	97.50	97.43	100.00	100.00	96.87	93.55	90.00	97.22
T_{50}/0.5	97.37	94.59	90.63	93.10	97.50	97.43	100.00	92.50	90.63	93.10	95.00	86.84
T_{50}/1.0	94.74	91.67	100.00	95.00	100.00	100.00	100.00	100.00	100.00	95.00	97.22	91.43
T_{50}/2.0	100.00	94.87	91.67	87.88	95.83	95.65	96.87	83.87	91.67	87.88	96.43	96.30
T_{50}/4.0	92.50	97.30	83.33	100.00	100.00	97.50	100.00	92.86	100.00	100.00	95.00	100.00

Table 1 Continue

Medium	RD 23		RD 25		KDML		KTH		RD 8		NSPT	
	A	B	A	B	A	B	A	B	A	B	A	B
$T_{100}/0.5$	97.50	87.18	94.44	97.06	100.00	100.00	97.50	94.87	84.62	78.79	90.00	97.22
$T_{100}/1.0$	100.00	97.22	100.00	100.00	90.00	100.00	100.00	87.18	82.05	93.75	100.00	94.59
$T_{100}/2.0$	97.50	100.00	89.74	97.14	100.00	97.37	100.00	100.00	92.50	94.59	100.00	97.30
$T_{100}/4.0$	100.00	94.87	89.74	94.29	100.00	94.44	95.00	92.11	85.00	88.23	100.00	94.59
CW+K/0.5	100.00	67.50	100.00	95.65	97.50	97.43	100.00	92.31	100.00	57.89	97.50	79.49
CW+K/1.0	100.00	82.50	100.00	94.29	97.37	97.30	92.50	97.30	100.00	95.00	95.00	92.11
CW+K/2.0	92.50	97.30	90.00	91.67	100.00	100.00	100.00	100.00	94.74	97.22	97.50	97.43
CW+K/4.0	97.50	97.43	95.00	80.00	97.50	100.00	95.00	94.74	96.87	100.00	100.00	97.50
$CW+T_{50}/0.5$	100.00	80.00	97.22	77.14	89.74	82.86	100.00	86.49	92.50	83.78	84.37	85.19
$CW+T_{50}/1.0$	92.50	86.49	92.50	100.00	97.50	94.87	94.12	100.00	100.00	84.61	100.00	93.94
$CW+T_{50}/2.0$	97.22	94.29	92.50	91.89	95.00	94.74	100.00	100.00	95.00	78.95	85.00	94.12
$CW+T_{50}/4.0$	94.87	94.59	90.00	91.67	97.43	81.58	96.30	100.00	97.50	92.31	100.00	100.00

Table 1 Continue

Medium	RD 23		RD 25		KDML		KTH		RD 8		NSPT	
	A	B	A	B	A	B	A	B	A	B	A	B
$CW+T_{100}/0.5$	87.50	51.43	88.89	81.25	97.50	87.18	67.44	79.31	65.00	0.00	85.00	76.47
$CW+T_{100}/1.0$	95.00	84.21	100.00	100.00	87.18	94.12	92.50	94.59	62.50	100.00	95.00	81.58
$CW+T_{100}/2.0$	95.00	97.37	73.68	85.71	87.88	82.76	94.87	78.38	100.00	100.00	95.00	100.00
$CW+T_{100}/4.0$	92.50	72.97	85.00	76.47	86.84	96.97	100.00	77.50	100.00	87.50	90.00	80.55

Notice : A = Percentage of embryo germination .

B = Percentage of callus formation from germinated embryos.

K = Kinetin 0.3 ppm

CW = Coconut water 10 % V/V

T_{50} = Tryptophan 50 ppm

T_{100} = Tryptophan 100 ppm

Each treatment varying concentrations of 2,4-D (0.5, 1.0,2.0 and 4.0 ppm)

Table 2 Effects of kinetin 0.3 ppm on callus growth in four concentrations of 2,4-D after 4 weeks in culture.

n = Number of replications (Each replication average from 4 embryos)

T = Total weight of callus $\bar{X}$ = Mean

Medium		Callus weight after 4 weeks (gm.)						Row total
		RD 23	RD 25	KDML	KTH	RD 8	NSPT	
K + 2,4-D 0.5 ppm	n	10	3	10	10	10	10	53
	T	0.3946	0.3042	0.8965	1.9350	0.3892	0.6774	4.5969
	$\bar{X}$	0.0394	0.1014	0.0897	0.1935	0.0389	0.0677	0.0884
K + 2,4-D 1.0 ppm	n	10	10	10	10	10	10	60
	T	0.4505	0.8814	0.8182	0.6781	0.9072	0.7156	4.4510
	$\bar{X}$	0.0451	0.0881	0.0818	0.0678	0.0907	0.0716	0.0742
K + 2,4-D 2.0 ppm	n	10	10	10	10	10	10	60
	T	0.6666	0.6968	0.5769	0.7218	0.4867	0.5063	3.6551
	$\bar{X}$	0.0667	0.0697	0.0577	0.0722	0.0487	0.0506	0.0609
K + 2,4-D 4.0 ppm	n	10	10	10	10	10	10	60
	T	0.4742	0.4877	0.5731	0.4121	0.5718	0.5117	3.0306
	$\bar{X}$	0.0474	0.0488	0.0573	0.0412	0.0572	0.0512	0.0505
Column total	n	40	33	40	40	40	40	233
	T	1.9859	2.3701	2.8647	3.7470	2.3549	2.4110	15.7336
	$\bar{X}$	0.0496	0.0718	0.0716	0.0937	0.0589	0.0603	0.0675

Table 3 Effects of tryptophan 50 ppm on callus growth in four concentrations of 2,4-D after 4 weeks in culture.

n = Number of replications (Each replication average from 4 embryos)

T = Total weight of callus $\bar{X}$ = Mean

Medium		Callus weight after 4 weeks (gm.)						Row total
		RD 23	RD 25	KDML	KTH	RD 8	NSPT	
T_{50} + 2,4-D 0.5 ppm	n	10	8	10	10	10	10	58
	T	0.2123	0.7130	0.5401	1.3504	0.3245	0.2756	3.4159
	$\bar{X}$	0.0212	0.0891	0.0540	0.1350	0.0325	0.0276	0.0589
T_{50} + 2,4-D 1.0 ppm	n	10	5	10	10	10	10	55
	T	0.3250	0.4793	1.2945	1.6615	0.2050	0.6992	4.6645
	$\bar{X}$	0.0325	0.0959	0.1295	0.1662	0.0205	0.0699	0.0848
T_{50} + 2,4-D 2.0 ppm	n	10	9	6	8	10	7	50
	T	0.1797	0.6994	0.2700	1.0404	0.2526	0.1371	2.5792
	$\bar{X}$	0.0180	0.0777	0.0450	0.1300	0.0253	0.0196	0.0516
T_{50} + 2,4-D 4.0 ppm	n	10	3	10	7	10	10	50
	T	0.2390	0.2452	0.3565	0.7577	0.1595	0.4070	2.1649
	$\bar{X}$	0.0239	0.0817	0.0357	0.1082	0.0160	0.0407	0.0433
Column total	n	40	25	36	35	40	37	213
	T	0.9560	2.1369	2.4611	4.8100	0.9416	1.5189	12.8245
	$\bar{X}$	0.0239	0.0855	0.0684	0.1374	0.0235	0.0411	0.0602

Table 4 Effects of tryptophan 100 ppm on callus growth in four concentrations of 2,4-D after 4 weeks in culture.

n = Number of replications (Each replication average from 4 embryos)
T = Total weight of callus $\bar{X}$ = Mean

Medium		Callus weight after 4 weeks (gm.)						Row total
		RD 23	RD 25	KDML	KTH	RD 8	NSPT	
T_{100} + 2,4-D 0.5 ppm	n	10	10	10	10	9	10	59
	T	0.1735	0.3709	0.5339	0.5706	0.2249	0.2610	2.1548
	$\bar{X}$	0.0174	0.0371	0.0534	0.0571	0.0250	0.0281	0.0365
T_{100} + 2,4-D 1.0 ppm	n	10	10	10	10	10	10	60
	T	0.1598	0.2946	0.3208	0.7071	0.1985	0.2830	1.9638
	$\bar{X}$	0.0160	0.0295	0.0321	0.0707	0.0199	0.0283	0.0327
T_{100} + 2,4-D 2.0 ppm	n	10	10	10	10	10	10	60
	T	0.2160	0.3441	0.4471	0.5316	0.3945	0.3658	2.2991
	$\bar{X}$	0.0216	0.0344	0.0447	0.0532	0.0395	0.0366	0.0383
T_{100} + 2,4-D 4.0 ppm	n	10	10	10	10	10	10	60
	T	0.2189	0.2963	0.4057	0.4145	0.4228	0.2589	2.0171
	$\bar{X}$	0.0219	0.0296	0.0406	0.0415	0.0423	0.0259	0.0336
Column total	n	40	40	40	40	39	40	239
	T	0.7682	1.3059	1.7075	2.2238	1.2407	1.1887	8.4348
	$\bar{X}$	0.0192	0.0326	0.0427	0.0556	0.0318	0.0297	0.0353

Table 5 Effects of coconut water 10 % V/V on callus growth in four concentrations of 2,4-D after 4 weeks in culture.

n = Number of replications (Each replication: average from 4 embryos)
T = Total weight of callus $\bar{X}$ = Mean

Medium		Callus weight after 4 weeks (gm.)						Row total
		RD 23	RD 25	KDML	KTH	RD 8	NSPT	
CW + 2,4-D 0.5 ppm	n	10	10	10	8	8	10	56
	T	0	0	0.3567	0.3235	0.1054	0	0.7856
	$\bar{X}$	0	0	0.0357	0.0404	0.0132	0	0.0140
CW + 2,4-D 1.0 ppm	n	10	10	10	10	10	10	60
	T	0.4339	1.5628	0.5432	1.4516	0.4679	0.5166	4.9760
	$\bar{X}$	0.0434	0.1563	0.0543	0.1452	0.0468	0.0517	0.0829
CW + 2,4-D 2.0 ppm	n	10	10	10	10	10	10	60
	T	0.2931	1.3813	0.5098	0.7292	0.1754	0.3700	3.4588
	$\bar{X}$	0.0293	0.1381	0.0510	0.0729	0.0175	0.0370	0.0576
CW + 2,4-D 4.0 ppm	n	10	10	10	10	10	10	60
	T	0.2308	1.2463	0.2968	0.7772	0.3259	0.3953	3.2723
	$\bar{X}$	0.0231	0.1246	0.0297	0.0777	0.0326	0.0395	0.0545
Column total	n	40	40	40	38	38	40	236
	T	0.9578	4.1904	1.7065	3.2815	1.0746	1.2819	12.4927
	$\bar{X}$	0.0239	0.1048	0.0427	0.0864	0.0283	0.0320	0.0529

Table 6 Effects of kinetin 0.3 ppm and coconut water 10 % V/V on callus growth in four concentrations of 2,4-D after 4 weeks in culture.

n = Number of replications (Each replication average from 4 embryos)

T = Total weight of callus $\bar{X}$ = Mean

Medium		Callus weight after 4 weeks (gm.)						Row total
		RD 23	RD 25	KDML	KTH	RD 8	NSPT	
K + CW + 2,4-D 0.5 ppm	n	10	6	10	10	8	10	54
	T	0.3533	0.1305	0.4050	0.6176	0.5907	0.2855	2.3826
	$\bar{X}$	0.0353	0.0218	0.0405	0.0618	0.0738	0.0286	0.0441
K + CW + 2,4-D 1.0 ppm	n	10	9	10	10	10	10	59
	T	0.2389	0.2372	0.3190	0.3771	0.3071	0.2652	1.7445
	$\bar{X}$	0.0239	0.0264	0.0319	0.0377	0.0307	0.0265	0.0296
K + CW + 2,4-D 2.0 ppm	n	10	10	10	10	10	10	60
	T	0.2230	0.3508	0.3120	0.6302	0.4245	0.3986	2.3391
	$\bar{X}$	0.0223	0.0351	0.0312	0.0630	0.0425	0.0399	0.0390
K + CW + 2,4-D 4.0 ppm	n	10	10	10	5	8	10	53
	T	0.2064	0.4000	0.2798	0.2067	0.3428	0.3999	1.8356
	$\bar{X}$	0.0206	0.0400	0.0280	0.0413	0.0429	0.0400	0.0346
Column total	n	40	35	40	35	36	40	226
	T	1.0216	1.1185	1.3158	1.8316	1.6651	1.3492	8.3018
	$\bar{X}$	0.0255	0.0320	0.0329	0.0523	0.0463	0.0337	0.0367

Table 7 Effects of tryptophan 50 ppm and coconut water 10 % V/V on callus growth in four concentrations of 2,4-D after 4 weeks in culture.

n = Number of replications (Each replication average from 4 embryos)

T = Total weight of callus $\bar{X}$ = Mean

Medium		Callus weight after 4 weeks (gm.)						Row total
		RD 23	RD 25	KDML	KTH	RD 8	NSPT	
T_{50} + CW + 2,4-D 0.5 ppm	n	9	9	10	10	10	8	56
	T	0.1876	0.5682	0.7373	0.2996	0.5054	0.1254	2.4235
	$\bar{X}$	0.0208	0.0631	0.0737	0.0300	0.0505	0.0125	0.0433
T_{50} + CW + 2,4-D 1.0 ppm	n	10	10	10	9	10	10	59
	T	0.1765	1.0028	0.4257	0.3116	0.2995	0.9080	3.1241
	$\bar{X}$	0.0177	0.1003	0.0426	0.0346	0.0300	0.0908	0.0530
T_{50} + CW + 2,4-D 2.0 ppm	n	10	10	10	10	10	10	60
	T	0.2326	1.4021	0.2564	0.4898	0.3801	0.4162	3.1772
	$\bar{X}$	0.0233	0.1402	0.0256	0.0490	0.0380	0.0416	0.0530
T_{50} + CW + 2,4-D 4.0 ppm	n	10	10	10	9	10	10	59
	T	0.1866	0.3844	0.3740	0.3317	0.2999	0.2437	1.8203
	$\bar{X}$	0.0187	0.0384	0.0374	0.0369	0.0300	0.0244	0.0309
Column total	n	39	39	40	38	40	38	234
	T	0.7833	3.3575	1.7934	1.4327	1.4849	1.6933	10.5451
	$\bar{X}$	0.0201	0.0861	0.0448	0.0377	0.0371	0.0446	0.0451

Table 8 Effects of tryptophan 100 ppm and coconut water 10 % V/V on callus growth in four concentrations of 2,4-D after 4 weeks in culture.

n = Number of replications (Each replication average from 4 embryos)

T = Total weight of callus $\bar{X}$ = Mean

Medium		Callus weight after 4 weeks (gm.)						Row total
		RD 23	RD 25	KDML	KTH	RD 8	NSPT	
T_{100} + CW + 2,4-D 0.5 ppm	n	10	5	10	10	10	10	55
	T	0.5194	0.1019	0.2396	0.1313	0	0.2166	1.2088
	$\bar{X}$	0.0519	0.0204	0.0204	0.0131	0	0.0217	0.0220
T_{100} + CW + 2,4-D 1.0 ppm	n	10	2	10	10	2	10	44
	T	0.1596	0.1265	0.4954	0.4440	0.0319	0.2200	1.4774
	$\bar{X}$	0.0160	0.0633	0.0495	0.0444	0.0160	0.0220	0.0336
T_{100} + CW + 2,4-D 2.0 ppm	n	10	5	10	10	3	10	48
	T	0.1634	0.2862	0.3964	0.4623	0.0616	0.2928	1.6627
	$\bar{X}$	0.0163	0.0572	0.0396	0.0462	0.0205	0.0293	0.0346
T_{100} + CW + 2,4-D 4.0 ppm	n	10	5	10	10	5	10	50
	T	0.3908	0.1405	0.2325	0.2960	0.0963	0.3147	1.4708
	$\bar{X}$	0.0391	0.0281	0.0233	0.0296	0.0193	0.0315	0.0294
Column total	n	40	17	40	40	20	40	197
	T	1.2332	0.6551	1.3639	1.3336	0.1819	1.0441	5.8118
	$\bar{X}$	0.0308	0.0385	0.0341	0.0333	0.0095	0.0261	0.0295

produced best overall result regardless of concentration of 2,4-D. However, calli grew best at lower concentration of 2,4-D (0.5 and 1.0 ppm) and the growth gradually declined in media containing higher concentration of 2,4-D (2.0 and 4.0 ppm). The 2,4-D seems to depress callus growth at higher concentrations (Davoyan and Smetanin, 1979) on the contrary it induces callusogenesis better at high concentrations (see Table 1). Therefore, the combinations of kinetin and 2,4-D at suitable concentration in the basic formular of Linsmaier and Skoog (1965) will serve as the best general culture media for both induction of callus and growth of callus.

The present of tryptophan in the media is interesting (Table 3). At 50 ppm, it gave the results almost as good as the ones in Table 2 but at 100 ppm (Table 4) the weight of callus is only half of those grew in 50 ppm. The cause of poor growth is not clearly understood. It is worth noting that callus formation is possible without exogenous cytokinin in this experiment. The present of tryptophan in the media may be useful in regeneration experiments later because it tends produce more regenerative callus (Nabors, 1982; Siriwardana and Nabors, 1983).

Coconut water, an undefined organic additive, which has been used in the early days of tissue culture gave fairly good result in both callus induction and growth (Steward, 1969; Vajrabhaya, 1978). The best growth was obtained the medium containing 1.0 ppm 2,4-D (Table 5). Besides having factors for promoting cell division, coconut water also contains some forms of sugar, amino acid and other chemicals useful for callus growth. Organic additives may have advantage over the defined media in supplying nutrients and necessary growth regulators for tissues. Furthermore, the present of coconut water in the medium seems to stabilize pH at an acceptable level for long time. This also reduces the work in reflasking and preparing of media. If ones just want to obtain calluses for other experiments, coconut water is recommended as additive due to its simplicity mentioned above.

The experiments shown in Table 6 to 8 were the attempt to use the combination of coconut water and kinetin or tryptophan. The results obtained from these experiments were clearly demonstrated that all the combination of coconut water and kinetin, coconut water and 50 ppm tryptophan, coconut water and 100 ppm tryptophan produced poor results. Adding coconut water to media containing kinetin does not promote growth any further but on the contrary high level of cytokinin may retard the growth of calli. Either coconut or kine-

tin at the given levels may already give sufficient growth factors to support growth of calli. Supplementing coconut water to media containing tryptophan also produced poor result (Table 7 and 8). At 100 ppm tryptophan alone (Table 4) caused callus to grow slowly, adding coconut water intensified the effect further. The present of coconut water and tryptophan at lower concentration (50 ppm) had similar effect as above but to lesser extent. Mixing of these growth factors mentioned above does not have synergistic effect yet they seem to either interfere with each other. Organic additives, in many cases, are useful but they may cause failure when combined with others in the media. Therefore, it is nescessary to investigate thoroughly before use.

SUMMARY

Callus induction of rice embryo was obtained at the average of 93.3 percent of living embryos cultured in the dark at 23 to 26 °C. The suitable medium for callus induction was the basic Linsmaier and Skoog (1965) supplemented with 2-4 ppm 2,4-D and 0.3 ppm kinetin. Maximum callus growth could be produced in similar nutrient media similar to the induction medium except the level of 2,4-D was kept at 0.5 or 1.0 ppm. Coconut water can be used in place of kinetin to obtain similar result in growing calli if simplicity is needed.

Responses of all rice varieties to all treatments was not significantly different.

Among the six varieties of rice, two of which are glutinous rice and four are ordinary ones. It is surprising that the response to various treatments above mentioned was not much significantly different. Their response to concentration of 2,4-D was consistent, lower concentrations (0.5 and 1.0 ppm) favor callus growth and higher concentrations (2.0 and 4.0 ppm) induce more callus. High concentration of tryptophan (100 ppm) tended to retard callus growth of all varieties. All varieties responded to kinetin and coconut water,when treated separately,well in all treatment and grew poorly in the mixture of these.

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