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IMPROVEMENT OF PLANT REGENERATION FREQUENCY IN VITRO IN INDICA RICE

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In order to improve the frequency of plant regeneration from callus in indica rice, the influences of different factors on plant regeneration were investigated. Supplement with cytokinins (KT, BAP, Zeatin or 2ip, 1mg / L) and NAA(1 mg / L), or supplement with thidiazuron (0.5 mg / L) in the induction medium or subculture medium; and partial desiccation of callus before transfer to regeneration medium have been found significantly increase the frequency of plant regeneration in indica rice. 5 – 14 folds more plants were obtained than untreated control by the combination of these treatments with indica varieties TN1, IR72 and IR64.

KeY WORDS: Indica rice, Tissue culture, Regeneration plant

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INTRODUCTION

Rice (*Oryza sativa* L) is one of the most important cereal crops. About 40% the world's population, especially the people in many developing countries, are living on this important crop. Therefore, the improvement of rice varieties is a pressing issue at the moment.

It is hoped that this task will be completed by directed genetic manipulation at the single cell, and tissue, or organ level, thereby shortening the time required for breeding plants with disease and insect resistance, improving nutritional quality and stress tolerance. It is therefore essential to develop efficient methods for the transformation of beneficial genes in rice plants and regeneration of rice plants.

Japonica and Indica rice are two major subspecies growing in different regions of the world. Successful plant regeneration of Japonica rice has already been reported using tissue culture procedures for transformation [14]. But the use of the same protocol with Indica rice varieties, such as IR72, IR64, and TN1, results in a very low regeneration ability. However indica rice varieties are of greater importance and irreplaceable, because they provide the principle food source in most of the world's tropical regions. Therefore we have to investigate the influence of different factors on plant regeneration in indica rice to increase the frequency of plant regeneration from rice calli. These fac-

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tors are the following: new hormone combination in induction medium or subculture medium or addition of TDZ; and partial desiccation treatment of calli and so on.

MATERIALS AND METHODS

1.1 Plant material:

Seeds of the indica rice varieties TN1, IR72 and IR64 wrer obtained from the International Rice Research Institute (Los Banos, Philippines). Mature and immature seeds were used as explants to induce callus.

Mature or immature dehuched seeds were surface sterilized with 70% ethanol for 1 minute, and 25% bleach of commercial for 45 minutes, rinsed 4–5 times with sterilized water, then placed on various induction media according to the experiment.

1.2 Medium

1.2.1 Induction Medium and Subculture medium The basic medium is NB medium (as described previously, i.e. N6 medium [5] macroelements, B5 medium [10] microelements and B5 medium vitamins), caseine enzymatic hydrolysate 300 mg / L, proline 500 mg / L, plus 2, 4–D 2mg / L, pH 5.8, agarose (Sigma) 4 g / L.

We have compared different hormone combination in the induction medium and subculture medium, such as 2, 4–D 1 or 2 mg / L with NAA 1 to 3 mg / L, and kinetin, BAP, zeatin, 2ip or thidiazuron (TDZ) 0.5 to 1 mg / L.

1.2.2 Regeneration Medium The basic regeneration medium is NB medium or MS medium [19] plus NAA 0.5–0.05mg / L BAP 3 mg / L, pH 5.8–6.0, and phytagel (Sigma) 2.5 g / L.

We have tried using kinetin, zeatin, 2ip and TDZ instead of BAP in the regeneration medium.

1.2.3 Medium for Growth of Plantlets

1 / 2 MS medium [19] salts, B5 medium [10] vitamins, NAA 0.05 mg / L, pH 5.8, phytagel (Sigma) 2.5 g / L.

1.3 Culture condition and treatment

1.3.1 After seeds were inoculated on induction medium in dark, at 26℃ for 2–3 weeks, the calli were transferred onto regeneration medium or propagated in the same condition for subculture.

The calli onto the regeneration medium under light were induced the differentiation of green spots and small shoots. Then the green calli were transferred on hormone–free medium or only plus 0.05 mg / L NAA medium in order to allow rooting and shoot growth in about three additional weeks.

1.3.2 Desiccation Before the calli were transferred onto regeneration medium, the calli were partially desiccated. To do so, calli were placed on one or two layers of filter paper in a Petri dish. The Petri dishes were kept at 26℃ in dark for 2 to 6 days (depending on the size of the callus), so that they lost about 40 to 65% of their water content (difference between the weight before and after the treatment). Then, the calli were transferred to regeneration medium.

Sometimes desiccation were also applied to the calli which did not regenerate, or the small shoots did not germinate on regeneration medium for long term.

1.3.3 Light microscopy observation The callis on different induction medium were fixed in HistoChoice Tissue Fixative (Amresco), then samples were dehydrated, embedded, cut sections and

stained with Periodic acid–Schiff (PAS) [8] . Then sections of callus were observed under microscope.

2. Results and Discussion

2.1 Effect of different hormone combinations in induction and subculture media

The calli induced on NB medium supplemented with 2, 4–D alone were transferred to regencration medium, only obtained a very poor regeneration frequency in indica rice (about 0–10%).

We found that supplement with NAA, Kinetin and 2,4–D in induction or subculture medium can improve later regeneration frequency.

Table 1

Effect of Supplement of KT and NAA in Induction Medium on Regeneration Frequency in TN1

Experiment	medium	Resource of Callus	No. of callus tested	No.of regenerated plant	Regenerated plant %
I	NB medium	Immature seed	11	0	0.0
	NBK medium		10	1	10.0
II	NB medium	Mature seed	30	0	0.0
	NBK medium		21	8	38.1
III	NB medium	Mature seed	54	0	0.0
	NBK medium		52	7	13.46
IV	NB medium	Subculture	89	0	0.0
	NBK medium		91	11	12.09
V	NB medium	Subculture	59	0	0.0
	NBK medium		65	6	9.23
VI	NB medium	Subculture	66	1	1.05
	NBK medium		69	18	26.09

Note: NB medium: +2,4–D 2mg / L; NBK: medium: +2,4–D 2mg / L + KT 1mg / L + NAA 1mg / L

The calli, which were induced and grown on NB medium containing 2, 4–D 2 mg / L, Kinetin 1 mg / L and NAA 1 mg / L, provided better regeneration frequencies than 2, 4–D 2 mg / L alone. This experiment has been repeated more than 6 times in TN1, one time was immature seeds as explants, two times were mature seeds as explants, tree times were subcultures, and the results of every time were the same (Table 1).

We have obtained similar results in IR72, IR64 and other varieties.

We found that supplied NAA and cytokinin in induction medium or subculture medium can improve the quality of the callus. The morphology of callus grown on the that on NB medium supplement with 2, 4–D, NAA and kinetin were more embryogenic, granular, and compact than NB

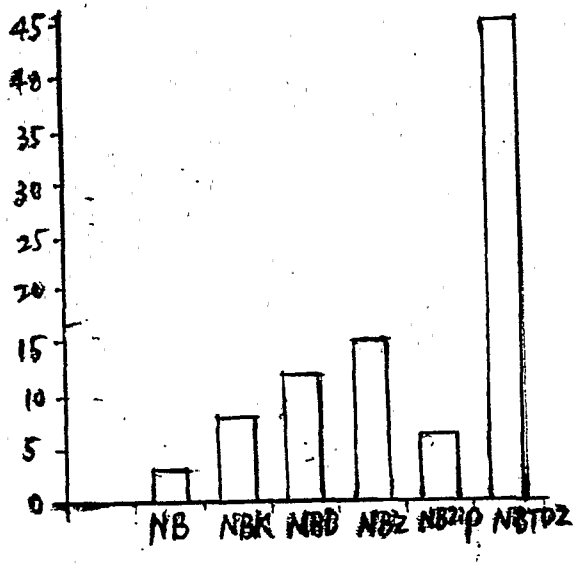
medium with 2, 4–D alone. (Fig. A and B)

From the histological section we can see that there were many old, empty cells or the cells with thinner cytoplasms, bigger vacuoles, and smaller nuclears in the calli induced on 2, 4–D alone medium. However the cells with thicker cytoplasms, bigger nucleus and some of them being in a vigorous mitosis in the calli induced on medium cantaining 2, 4–D, NAA and kinetin. (Fig. C and D)

The quality of callus is very importent for the regeneration, and improvement of the quality of callus in the induction and subculture medium is also very importent for increasing the regeneration frequency.

We have tried using zeatin, 2ip and thidiazuron (TDZ) as supplement in induction medium, or subculture medium most of them resulted in higher frequencis of plan regeneration from TN1, IR72 and IR64. For example, Fig 1. shows the effect of different hormone combination in induction medium on IR64 plant regeneration. The highest frequency (44.64%) of plant regeneration have been obtained from the calli induced on NB medium containing 2, 4–D 2mg / L, NAA1 mg / L and TDZ 1 mg / L.

Fig 1 Effect of Different Hormme Combination in Induction medium on Regeneration Frequency in IR64



Note: NB: NB medium + 2,4–D 2mg / L
NBK: NB medium + 2,4–D 2mg / L + KT 1mg / L + NAA 1mg / L
NBB: NB medium + 2,4–D 2mg / L + BAP 1mg / L + NAA 1mg / L
NBZ: NB medium + 2,4–D 2mg / L + Zeatin 1mg / L + NAA 1mg / L
NB2iP: NB medium + 2,4–D 2mg / L + 2:p 1mg / L + NAA 1mg / L
NBTDZ: NB medium + 2,4–D 2mg / L + TDZ 1mg / L + NAA 1mg / L

The thidiazuron is a substituted phenylurea, it exhibits strong cytokinin –like activity. TDZ

can induce cytokinin autonomy in callus cultures, and stimulate ethylene production. It has been demonstrated that TDZ can induce shoot differentiation, embryogenesis and organogenesis in tissue culture in bean [3,15], peanut [20], rubus [8], geranium. [11]; and white ash [1]; and cassava [17].

In our experament the calli in Indica rice grown on TDZ medium calli differentiated small shoots and roots after one week (Fig E), then were transferred to cytokinin–free medium, and plantlets were

grown up soon. (Fig F).

Further experaments of TDZ demonstrated that using TDZ alone in subculture medium was better for plant regeneration in Indica rice. Than TDZ combined with 2,4–D, or TDZ combined with 2,4–D, NAA, and 2,4–D alone (control) (Table 2).

David S. Koetje et al. (1989) reported that plant regeneration on N6 medium exhibited a dramatic 2,4–D dose response with increasing concentrations of 2,4–D above 0.5mg / L being increasingly inhibitory to plant development. Differential response to medium may also have resulted in an auxin: cytokinin ratio which inhibits embryo germination in some genotypes. So we suggesed that it is beneficial to keep certain auxin: cytokinin ratio in induction or subcultuce medium in indica rice. Sometimes, we need a higher concentration of cytokinin. For example we have obtained 93.7% plant regeneration frequency from the calli grown on the medium containing 2,4–D 2mg / L + KT1 mg / L + BAP 1mg / L + NAA 1mg / L.

However the cytokinin can promote chloroplast development and chlorophyll synthesis [9]. Sometimes we can get 100% green callus, but only a few plants can be obtained. As soon as green calli with unequal are formed differentiation, they should be transferred onto 1 / 2 MS hormone free medium or desiccated,so that they can regenerate or germinate.

Table 2
Function of TDZ in Induction Medium

Variety	Medium	No.of callus tested	No.of regene–rated plant	Regeneration %
TN1	1	41	2	5.13
	2	92	22	25.05
	3	75	30	39.61
	4	55	25	45.37
1R64	1	60	2	3.33
	2	64	10	15.63
	3	50	7	14.00
	4	56	12	21.43

Note: medium1: NB+2,4–D 2 mg / L
medium 2: NB+2,4–D 2mg / L+TDZO.5mg / L
medium 3: NB+2,4–D 2mg / L+ TDZ 0.5mg / L+NAA1mg / L
medium 4: NB+TDZ 0.5mg / L

2.2 The Effect of Partial Desiccation on Regeneration Ability: Tf callis undergo the partial

desiccation befer transfered to regeneration medium, plant regeneration frequency. could be obviously enhanced.

We have repeated 8 times with TN1, 4 times with IR72, and 2 times with IR64. Table 3 shows the efficient effect of partial desiccation. The plant regeneration frequency of TN1 with desiccation increased more than one fold, IR72 increased 3 folds, and IR64 increased neerly 3 folds.

Some of the green calli or small shoots on the regeneration medium or 1 / 2 MS medium can not differentiate to plantlets, or small shoots cannot germinate, We have to desiccate. About 70% regenerated plants have been obtained from these green calli or small shoots wich had beenkept in previous medium about two months.

Table 3
Effect of Partial Desiccation on Regeneration

	TN1		1R72		1R64	
	No Desic.	Partial desic.	No desic.	Partial Desic.	No Desic.	Partial Desic.
Seeds inoculated	520	903	279	316	235	213
Plants regenerate	165	609	43	193	44	157
% of regeneration	31.7	67.4	15.5	61.1	18.7 ± 2.9	73.7 ± 6.7
mean (standard devtation)	(+—15.1	(+—11.23)	(+—17.9	(+—17.2)		

Notc: Induction medium were NB, NBK and NBB.

Regeneration medium: NB+BAP 3mg / L+NAA 0.5 mg / L.

Somatic embryo desiccation has been reported to enhance development in grape [12], soybean [13], wheat [4], spruce [21] and cassava. Recently, Masayoshi Tsukahara and Takayasu Hirosawa (1992) reported that simple dehydration treatment promotes plant regeneration of rice callus in Japonica rice [16].

It has been reported that water stressed plants showed higher abscisic acid (ABA) biosynthetic activity [18,23,24], and, consequently a higher level of ABA content in water stressed plants, Brown et al. (1989) reported that ABA and mannitol promoted somatic embryogenesis in wheat [2]. However Massayoshi Tsukahara and Takayasu Hirosawa reported that the effects of ABA and mannitol in the regeneration medium on the regeneration frequency didn't have significant effects within the concentration range tested. We suppose that not only the reduction of the water content of callus in a certain duration, but also starvation of callus in a certain duration, by desiccation provoked the cell physiological and biochemical change, which is necessary for efficient regeneration.

2.4 The Effect of different cytokinin on differentiation

We have tried using different cytokinin (KT.BAP, zeatin, 2ip) and TDZ in the regeneration medium in TN1, IR64 and IR72.

We have repeated 4 times. The results of each time were varied. The responses of different varieties to different cytokinin were different. We think, due to cytokinin supplemented in induction medium or subculture medium, the regeneration function of medium was not very obvious, because some of calli have been differentiated in induction medium and subculture medium. Sometimes the calli were only transferred to hormone–free medium, they can regenerate and germinate. If the concentration of cytokinin was too high in regeneration medium, the calli maybe turned to brown and died.

For example, 80% regeneration frequency obtained from the calli of TN1 grown on NB me-

dium containing TDZ 0.5mg / L when were transferred to 1 / 2 MS medium but if transferred to RN regeneration medium (containing BAP 3mg / L) only 17.86% regeneration frequency has been obtained.

But when the concentration of cytokinin in cells is not enough, it needs supplying some cytokinin.

Conclusion

Combination of some of these treatments (to supply some cytokinin and NAA in induction medium or subculture medinm, or only using TDZ for improving the quenlity of callus, and before the callis were treansferred to regeneration medium, they were partially desiccated) lead to even higher regeneration efficiencies, and we have obtained 5–14 folds more plants than control. So far the best frequencies we have obtained are 93.7%, 74.4 and 79.1% respectively from TN1, IR72 and IR64.

We have to notice that the best medium and combinations of treatment differ for different cultivar. Therefore, there is not a highly efficient standard protocol for all indica varieties.

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Figures :

A. The callus grown on NB medium (+2, 4–D 2mg / L), showing more soft callus.

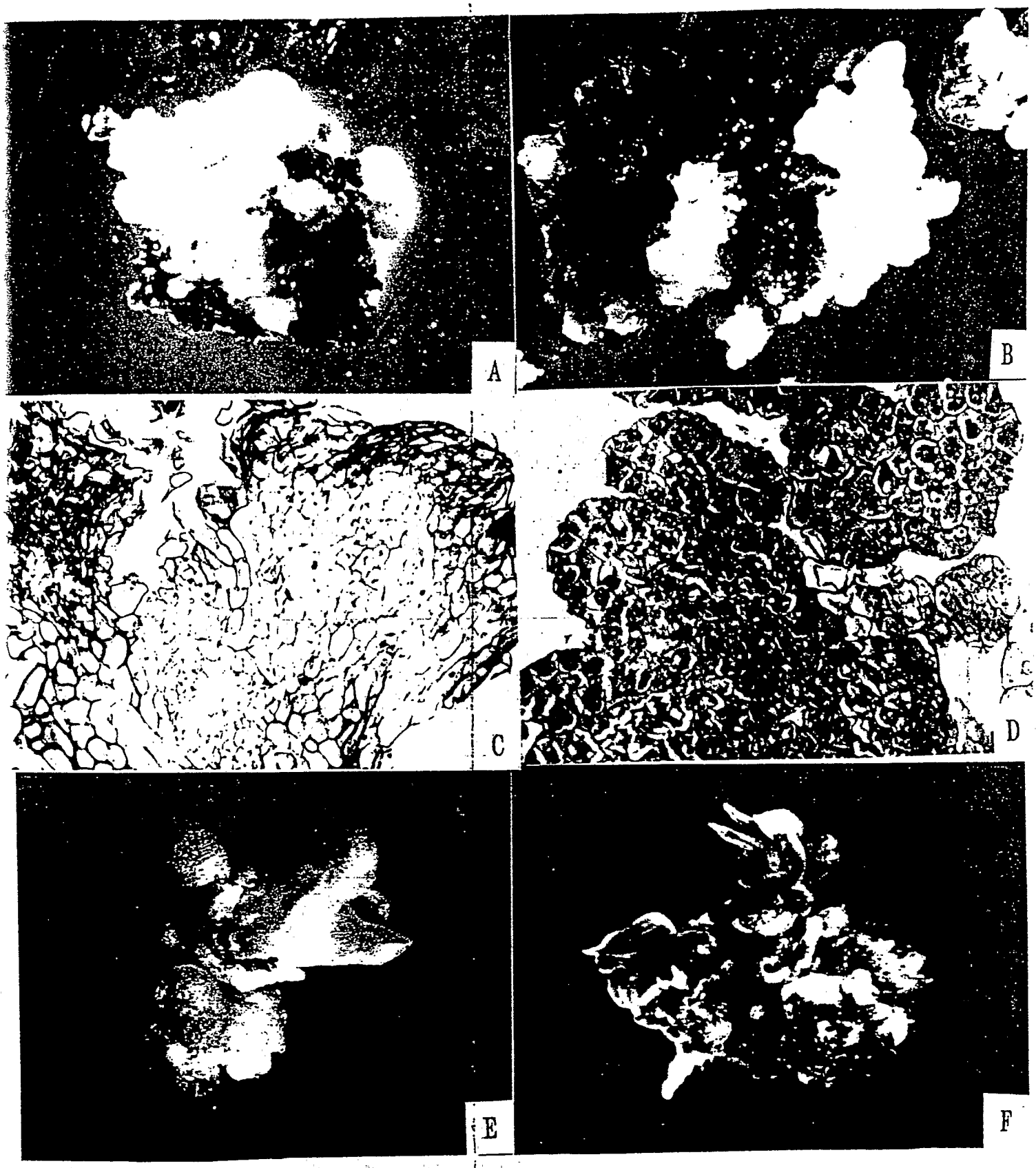
B. The callus grown on NBK medium (+2, 4–D 2mg / L + KT 1mg / L + NAA 1mg / L), showing more embryogenic, granular, and compact structure.

C. Light micrograph of the callus on NB medium, showing many old empty cells (10 × 40).

D. Light micrograph of the callus on NBK medium, showing many vgorous cells (10 × 40).

E. The callus grown on NB medium containing TDZ 0.5mg / L after one week differentiated small shoot.

F. The callus growm on NB medium containing TDZ 0.5mg / L after one week transferred to 1 / 2 MS cytokinin–free medium was grown up plantlet.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