EFFECTS OF COPPER SULPHATE AND COBALT CHLORIDE ON *IN VITRO* PERFORMANCES OF TRADITIONAL INDICA RICE (*Oryza sativa* L.) VARIETIES IN SRI LANKA

A.A.Y. Amarasinghe[1]

ABSTRACT

This study was carried out to investigate the effects of copper sulphate and cobalt chloride on the rate of callus proliferation and the in vitro regeneration in nine indica rice varieties and a Japonica variety. The disinfected seeds were inoculated in callus initiation medium composed of N6 basal elements, 1000 mg/l proline, 2 mg/l 2,4-D and 3% (w/v) sucrose to screen high responsive varieties. Calli of screened varieties were used to determine the effects of additives on in vitro responses in proliferation and regeneration media. Taipei 309, Pachcha Perumal, Heenati Vee and Suvandel were screened as high responsive varieties at $P \leq 0.05$. The rate of callus proliferation in the screened varieties was significantly higher in the medium supplemented with 5 mg/l copper sulphate and 5-10 mg/l cobalt chloride together ($P<0.05$). In regeneration, the highest number of normal plants with the least number of albino plants could be obtained in the media containing 5 mg/l copper sulphate in combination with 5 mg/l cobalt chloride. The results in this study indicate that the in vitro performance in Sri Lankan traditional indica rice varieties can be improved by using the media containing both copper sulphate and cobalt chloride.

Key words: *Rice, copper sulphate, cobalt chloride, Indica, Japonica*

INTRODUCTION

Rice is a monocot and one of the most important cereals in the world. More than half of the world's population consumes rice. Because of Asia's favorable hot and humid climate, about 90% of the world's rice is grown and consumed in Asia, where it contributes about 50 to 80% of dietary energy (Juliano, 1985). Rice belongs to the genus *Oryza*, which consists of 20 wild species and 2 cultigens. Although, uncountable numbers of subspecies are available, mainly 3 subspecies of *Oryza sativa* are more important and more common. Of which, subspecies Indica is tropical and produces longer grain and sticky rice compared to Japonica, which is short grain and in various degree of stickiness and grows in temperate climate. However, nearly all South East Asian rice is medium to long grain, but they are not sticky. They are known as Javanica, the third subspecies, which apparently originated in Java.

Regeneration from callus was achieved many years ago in Japonica varieties (Nishi *et al*., 1973). The potential for callus formation and regeneration has been reported to be a varietal characteristic. An efficient callus induction and proliferation followed by regeneration in Indica rice is still poses a major problem for genetic manipulation through innovative approaches (Toki, 1997). While it has been possible to obtain high callus induction, proliferation and plant regeneration frequencies in Japonica rice varieties, the success for reproducible fertile plant regeneration has been limited in Indica rice varieties so far (Kyozuka *et*

[1]*Senior Lecturer ,Department of Export Agriculture, Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka ,Belihuloya, Sri Lanka*

al., 1988; Raman *et al*., 1994). As a result, progress towards the transfer of useful genes into Indica rice has been slow. Many factors have been examined to improve the frequency of callus induction and plant regeneration in rice.

The quality of the calli of rice is one of the major factors to determine the rate of regeneration. It has been reported that one of the major factors to improve the quality of the calli is the composition of the *in vitro* media. Hence, this study was focused on the effect of additives, specially microelements, in the *in vitro* media used in rice.

MATERIAL AND METHODS

The variety and method for preparation of explants

Nine traditional indica rice varieties found in Sri Lanka and a japonica variety as a model rice variety, which had been used for many *in vitro* culture experiments (Xiong and Yang, 2004; Yang *et al*. 2000; Yang *et al*. 1999a; Yang *et al*. 1999b; Yang *et al*. 1999c; Jian and Jintaanankul, 1998; Yang and Jian, 1996) were initially used in this study. *In vitro* performances of traditional rice varieties were compared with those of the Japonica variety used. Mature dry seeds of the varieties were de-husked and then surface-sterilized with 70% (v/v) ethanol for 30 s, 0.15% (w/v) mercuric chloride for 10 minutes followed by three rinses with autoclaved distilled water.

Initiation and proliferation cultures of callus

The disinfected naked seeds were placed on the medium composed of N6 basal elements (Chu *et al*.1975), 1000 mg/l proline, 2 mg/l 2,4-D, 3% (w/v) sucrose and gelled with 0.75% (w/v) powdered agar at pH 5.8. After one month culture under darkness at 24-26^{0}C, compact and nodular calli produced in different quantities and qualities were collected and subcultured on fresh medium of the same composition for maintenance and proliferation of calli. Subculture of the calli was done for 3 times with three week interval.

Screening of high *in vitro* performing rice varieties

Proliferation rates were calculated for each variety in each subculture. The optimized subculture for callus proliferation out of three subcultures was identified. Comparing with the Japonica variety, three traditional rice varieties showing high *in vitro* performances at the optimized subculture were screened for further experiments. For comparative studies in the next set of experiments, the Japonica variety was also used with these three traditional rice varieties.

Use of copper sulphate and cobalt chloride in the medium

Calli of four screened varieties obtained from the optimized subcultures were cultured in the same basal medium used in the initiation and proliferation cultures of calli. However, the media were supplemented with different concentrations of copper sulphate or cobalt chloride or both. The control was the medium without additional microelements, copper sulphate and cobalt chloride. The cultures were placed in the culture room under darkness at 24-26^{0}C for 3 weeks before subculturing them.

Plant regeneration cultures

Calli of four screened varieties obtained from the established protocol where the basal medium containing 5 mg/l copper sulphate and 5 mg/l cobalt chloride were first dehydrated (Tsukahara and Hirosawa, 1992) to lose about 25% of the fresh

weight within 24h and incubated at this state for three days in the culture room for obtaining a better dehydration effect of enhancing plant regeneration. These dehydrated calli from each screened variety were then cultured on the media composed of N6 basal macro-elements, MS micro-elements, 3% sucrose, 500 mg/l casein hydralysate, 1 mg/l NAA and different combinations of copper sulphate and cobalt chloride for evaluation of their effects on plant regeneration. About 150 mg calli, dehydrated from 200 mg fresh ones, was cultured on 40-ml medium in one flask and five flasks were used per a treatment per variety. Then they were placed under 10/14 h light/dark photoperiod of about 20 μEM^{-2} s^{-1} provided by daylight fluorescent lamps at 24-26 ^{0}C. Results of the experiments were evaluated 30-days after inoculation of calli in the regeneration medium (Amarasinghe and Yang, 2005).

Experimental Design and Statistical Analysis

All the experiments were arranged in Complete Randomized Design with 5 replicates per variety in each treatment. Data were analyzed by Analysis of Variance (ANOVA) using the SAS system for Microsoft Windows, release 8.01 (SAS Institute, 1985). Significant differences among means were determined by Duncan's multiple range test at $P \leq 0.05$.

RESULTS AND DISCUSSION

Screening of high responsive varieties

Callus of the Japonica variety of Taipei 309 and the Indica variety of Heenati Vee could be observed two weeks after the initiation of the culture. Extreme variation existed among explants in the formation of callus, both quantitatively and qualitatively (Figure. 1). This extreme variation in callus formation seemed to be mainly due to the difference in the physiological state of the explants and some of the physical factors such as the position of the explants on the medium, since all the explants were of the same gene constitution.

Callus proliferation culture

Compact and nodular calli were selected and cultured to fresh medium for proliferation. Calli proliferated quite fast on the medium with a proliferation rate of 8-10 folds within a culture period of 21 days. In the proliferation culture, calli appeared with undesirable features, such as with hairy roots or brown colour (Figure 1), were removed.

However, the callus proliferation rates were calculated 3 weeks after inoculation of calli of ten rice varieties and the proliferation rates and the quality of calli of each variety were compared witheach other at each subculture. Calli of the Japonica variety, Taipei 309, and Indica varieties, Heenati Vee and Suvandel, were nodular and granular which can be directly used for the regeneration without going through several subcultures. Calli of other varieties used in this experiment were not compact and nodular at the first subculture where they had to be sub-cultured again to obtain high quality calli with high regenerative abilities.

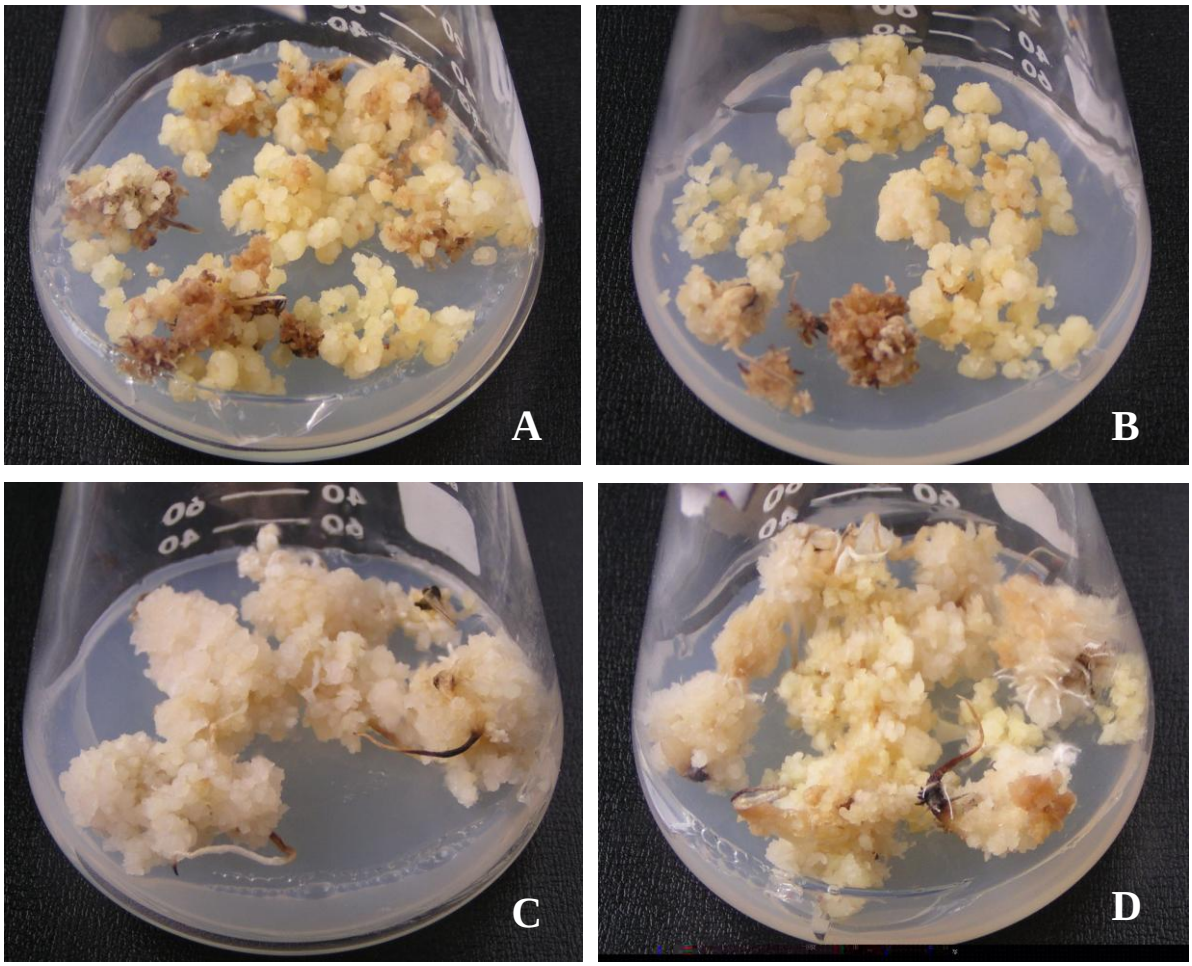

Figure 1: Variations in callus formation in rice, 3 weeks after inoculation of explants.

A – Taipei 309 (Japonica) B – Pachcha Perumal (Indica)
C – Murungakayan (Indica) D – Madathawalu (Indica)

Screening of varieties was done considering the rates of proliferation. The highest rates of proliferation could be observed through all subcultures in the Japonica variety, Taipei 309, even though the rates at different subcultures were not significantly different at $P \leq 0.05$. Comparing with the performances of the Taipei 309, the model variety, screening of the varieties was carried out for all other Indica rice varieties (Table 1). The compact and nodular calli were obtained in almost all the varieties at the second subculture.

The rate of proliferation of calli of Taipei 309 at the second subculture was significantly different ($P \leq 0.05$) from that of the other varieties at the same subculture. Hence, the second subculture is considered as the best to evaluate *in vitro* performances. Proliferation increases over the first subcultures and then it declines normally (Norton and Norton, 1989). Hamad and Taha in 2008 have reported the same for the shoot proliferation in pineapple. This specially depends on the changes in genetic materials, hormones in the tissues and nutritional status, and the physiological status in the explants. This study showed

that the second subculture was the best subculture for the highest *in vitro* performances in rice even though the first three subcultures did not show significant differences from the rate of callus proliferation in all the varieties. However, after the second subculture, it was obvious that the rate of proliferation declined in all rice varieties used in this experiment.

Table 1: Rate of callus proliferation of ten rice varieties at the end of the subcultures

Variety	Rate of Callus Proliferation (%)[1]		
	Subculture 1	Subculture 2	Subculture 3
Taipei 309	560.84 a A[2]	618.76 a A	595.22 a A
Rathran Podi Vee	372.47 bc A	456.76 b A	398.79 cd A
Suvandel	483.48 a A	484.24 b A	465.00 bc A
Rathdel	249.88 d A	326.86 c A	296.16 e A
Beheth Heenati	217.01 d B	324.26 c A	291.66 e AB
Kalu Heenati	297.93 cd A	325.16 c A	371.86 de A
Pachcha Perumal	485.64 a A	499.37 b A	494.61 b A
Murungakayan	16.24 e A	23.51 d A	13.82 f A
Madathawalu	35.92 e A	68.33 d A	65.70 f A
Heenati Vee	457.89 ab A	498.85 b A	474.53 cb A

1. Rate of proliferation was calculated at the end of each subculture by,

$$= \frac{\text{(Final weight of calli – Initial weight of calli)}}{\text{Initial weight of calli}} \text{ X } 100$$

2. Means followed by the same lower case letter in the same columns and by the same upper case letter in the same rows are not significantly different at 5% level in Duncan's multiple range test (n = 5).

As per the results obtained, the descending order of the rate of proliferation of varieties at the second subculture is Taipei 309, Pachcha Perumal, Heenati Vee, Suvandel, Rathran Podi Vee, Rathdel, Kalu Heenati, Beheth Heenati, Madathawalu and Murungakayan (Table 1). Hence, the top four varieties including the model variety showing high *in vitro* performance, Taipei 309, Pachcha Perumal, Heenati Vee, and Suvandel were selected for further studies.

The four high responsive varieties screened in this study were used to determine the effects of additives, copper sulphate and cobalt chloride, used in the proliferation media in different concentrations on the rate of callus proliferation. According to the results obtained, the rate of callus proliferation can be increased in the media containing lower concentrations of copper sulphate. However, the rate of callus proliferation was decreased when the concentration of copper sulphate in the media was

increased. High quality granular calli were produced in the media containing 5 mg/l copper sulphate. Similarly, Yang *et. al*., 1999 have reported that the copper increases the *in vitro* performances in Japonica rice varieties. Cobalt chloride alone as an additive used in the medium decreases the rate of callus proliferation. As the high performances of callus proliferation and the high quality calli production were obtained in the media containing the lower concentration of copper sulphate, 5 mg/l $CuSO_4$ was used with the different concentrations of cobalt chloride in the media as shown in the table 2.

Table 2: Rate of callus proliferation in the media containing different combinations of copper sulphate and cobalt chloride

Treatments	Rate of Callus Proliferation (%)[1]			
	Taipei 309	**Suvandel**	**Pachcha Perumal**	**Heenati Vee**
Control (no additives)	618.76 bc [2]	484.24 bc	499.37 b	498.85 cd
5 mg/l $CuSO_4$	687.54 b	558.21 b	575.53 b	584.54 bc
10 mg/l $CuSO_4$	550.84 cd	382.84 de	509.08 b	511.30 cbd
15 mg/l $CuSO_4$	568.23 c	383.40 de	492.36 bc	455.29 de
5 mg/l $CoCl_2$	531.42 cd	460.24 cd	507.38 b	523.48 bcd
10 mg/l $CoCl_2$	478.22 de	368.22 e	416.22 cd	489.28 d
15 mg/l $CoCl_2$	429.20 e	312.42 e	341.93 d	383.71 e
5 mg/l $CuSO_4$ + 5 mg/l $CoCl_2$	909.70 a	565.12 b	552.44 b	772.35 a
5 mg/l $CuSO_4$ + 10 mg/l $CoCl_2$	691.39 b	735.87 a	656.47 a	597.53 b
5 mg/l $CuSO_4$ + 15 mg/l $CoCl_2$	547.75 cd	305.34 e	489.89 bc	480.58 d

1. Rate of proliferation was calculated at the end of each subculture by,

$$= \frac{\text{(Final weight of calli – Initial weight of calli)}}{\text{Initial weight of calli}} \text{ X } 100$$

2. Means followed by the same lower case letter in the same columns are not significantly different at 5% level in Duncan's multiple range test (n = 5).

The results clearly showed that the rate of callus proliferation in all the varieties used in this experiment was significantly higher in the medium containing both additives at the concentrations of 5 mg/l $CuSO_4$ and 5-10 mg/l $CoCl_2$ ($P<0.05$). This value was higher than that in the media supplemented with no additives or with one additive alone. This indicates that the callus proliferation media containing both copper sulphate and cobalt chloride create a synergistic effect to increase the rate of *in vitro* callus proliferation in rice. Copper and cobalt are microelements essential to the physiological activities in cells. Callus proliferation is an energy consuming activity. Therefore, the rate of respiration in cells is normally higher during callus

proliferation and cell division to produce required energy. Micronutrients in plant cells, especially copper, iron, manganese and zinc, are important in the process of respiration where iron and copper are the functional parts of some oxidative enzymes contained in plant tissues (Sumner and Somers, 1953). Furthermore, manganese and zinc are essential cofactors in the process of glycolysis and Krebs' cycle (MacElroy and Nason, 1954). Therefore, these elements trigger the rate of respiration. Microelements also affect the endogenous oxygen uptake and the rate of photosynthesis in plant tissues (Fujiwara and Tsutsumi, 1962). On the other hand, cobalt is a transition element which is an essential factor in many enzymes and co-enzymes. It affects the growth and metabolism of plants in various degrees depending on the concentration of cobalt in the surrounding medium (Palit *et al*., 1994). Therefore, copper and cobalt together show the additive effect to trigger the rate of respiration in cultured cells. Through which the rate of callus proliferation may have been improved with synergistic effects when they are together in the medium. Hence, this protocol can be used to improve the *in vitro* responses even in traditional Sri Lankan Indica rice varieties which are considered as recalcitrant in *in vitro* performances.

The calli obtained from the media containing both additives, 5 mg/l copper sulphate and 5 mg/l cobalt chloride, were inoculated in the regeneration media containing different combinations of additives. The results of this experiment indicates that the maximum regeneration in all the varieties with the production of the least number of albino plants can be achieved in the media containing both additives at the rates of 5 mg/l (Figure 2). Comparatively, though the regeneration medium containing 5 mg/l copper sulphate shows the better results in regeneration, all other media containing one additive alone gives the poor results (Table 3).

Furthermore, the poor regeneration abilities were obvious even in the medium supplemented with higher concentrations of both additives together. The poor responding media has produced more albino plants too (Table 3). Strong hormones at high concentrations in the media may produce abnormal plants. Hence, the increased number of normal plants produced in the media containing both copper sulphate and cobalt chloride may be mainly due to two reasons.

The first reason may be the antagonistic effects of these microelements on the hormones, which may boost the albino plant production, added in these regeneration media. The second reason may be the synergistic effects of these microelements on improving the metabolic activities in shoot initiation and development. There are evidences which clearly shows that cobalt interact with other microelements to form complexes (Palit *et al*., 1994). The competitive absorption and mutual activation of the elements like copper in these complexes influence and activate the action of cobalt on various phytochemical reactions showing the synergistic effects.

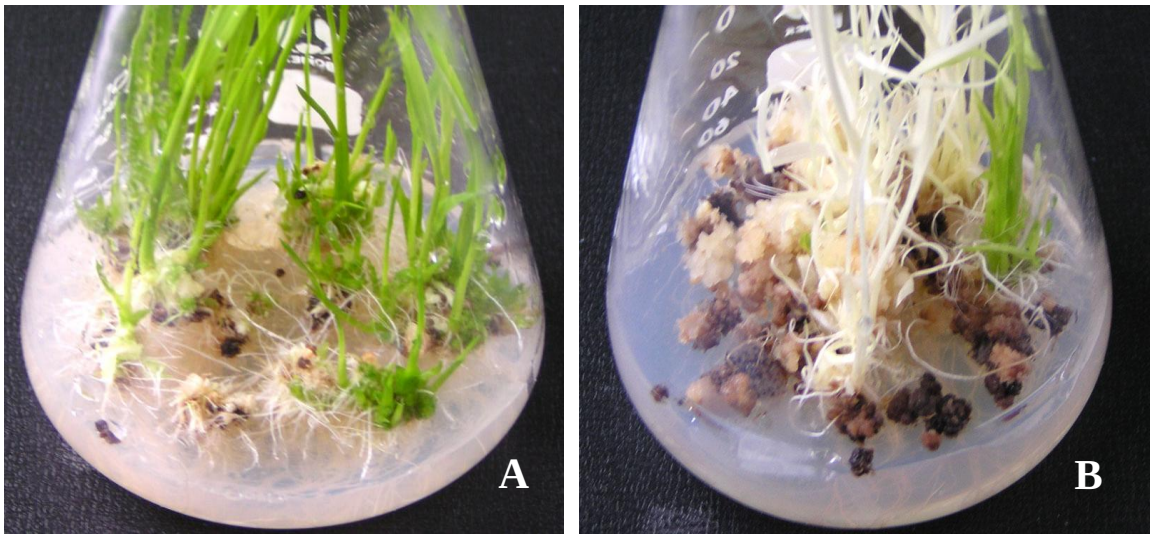

Figure 2: Regeneration of normal and albino plants

A – Albino plants produced in the control (without additives)
B – Normal plants produced in the medium containing 5 mg/l copper sulphate and 5 mg/l cobalt chloride

Table 3: Regeneration of plants from desiccated calli cultured on the media containing different combinations of additives

Treatments[1]	Mean number of regenerated plants[2]							
	Taipei 309		Suvandel		Pachcha Perumal		Heenati Vee	
	Normal	Albino	Normal	Albino	Normal	Albino	Normal	Albino
Control (without additives)	63.6	5.2	62.2	3.8	52.2	4.2	70.0	3.0
5 mg/l $CuSO_4$	101.8	1.4	110.8	0.8	92.4	0.0	72.6	0.8
10 mg/l $CuSO_4$	96.6	3.6	78.6	1.6	18.0	0.0	54.2	2.4
15 mg/l $CuSO_4$	8.4	4.8	10.2	3.0	2.2	3.2	15.2	2.4
5 mg/l $CoCl_2$	5.4	0.0	2.4	1.0	3.6	1.2	6.0	1.0
10 mg/l $CoCl_2$	1.8	2.0	0.8	2.4	2.0	2.2	2.2	1.0
15 mg/l $CoCl_2$	0.0	2.2	1.0	2.4	0.0	0.0	2.0	2.6
5 mg/l $CuSO_4$ + 5 mg/l $CoCl_2$	145.4	0.6	122.0	0.0	102.6	0.0	132.0	0.4
5 mg/l $CuSO_4$ + 10 mg/l $CoCl_2$	110.8	0.6	93.6	0.0	72.0	1.0	86.2	2.0
5 mg/l $CuSO_4$ + 15 mg/l $CoCl_2$	12.0	1.6	9.2	2.6	10.2	1.0	5.6	1.8

n =

1. Regeneration media were supplemented with different combinations of copper sulphate and cobalt chloride
2. Mean number of regenerated normal and albino plants were calculated, 30 days after inoculation of calli in the media (Five replicates per a treatment were used in each variety)

CONCLUSIONS

Even though it was reported that the Japonica rice varieties have showed higher *in vitro* performances comparing with Indica rice varieties, the *in vitro* performances could be improved in both Japonica and Sri Lankan traditional Indica rice varieties in the proliferation and regeneration media supplemented with both copper sulphate and cobalt chloride. Synergistic effects of 5 mg/l copper sulphate and 5-10 mg/l cobalt chloride together increase the rates of callus proliferation. The production of normal plants is also increased when copper sulphate and cobalt chloride are together in the media. Furthermore, the antagonistic effects of these microelements on the hormones added may have reduced the production of abnormal plants (Albino) when these microelements are together in the regeneration medium. These results can be used to establish a protocol for high efficient *in vitro* regeneration both in Japonica and Indica rice.

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