CANADIAN JOURNAL OF BIOLOGICAL AND MICROBIOLOGY RESEARCH

Journal homepage: www. http://mcmed.us/journal/cjmbr

STUDIES ON PRODUCTION OF *ORIZA SATIVA* VARIETIES APMS-6B AND BPT-5204 WITH CALLUS INDUCTION VIA MICROPROPAGATION

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Article Info

Received 25/10/2013
Revised 15/11/2013
Accepted 18/11/2013

Key words:
Regeneration,
Micropropagation,
Crop improvement.

ABSTRACT

Rice (*Oriza sativa*) is a monocotyledonous cereal crop plant produces an edible grain. Limitations in conventional breeding arise because of the lack of resistance genes in cultivated rice germplasm (*Oriza sativa)* and inadequate understanding of phenotypic variability. So efficient plant regeneration through in-vitro propagation is very essential for the successful utization of Biotechnology in rice crop improvement. Callus induction is the best way to create variations in crop plants through Micropropagation. The objective of this study was to develop a tissue culture system for micropropagation of rice plant to produce a healthy and completely free from any type of limitations for further use too to make rice plant which show resistance to all type of biotic and abiotic stresses.

INTRODUCTION

The population of rice eaters are increasing day by day and the number of rice consumers will probably two fold by the year of 2020 [1]. Rice is composed of essential food components, therefore more than two billion people in the globe depend on rice for more than half of the proteins and calories they consume [2]. Crop improvement through tissue culture techniques is easier and more often in use as compared to conventional plant breeding [3].The application of biotechnology in combination with conventional breeding methods may help to increase food production properly. One of the most exciting and important aspects of *in vitro* cell and tissue culture approach is the capability to regenerate and propogate plants from cultured cells and tissue. Micropropagation involves the production of plants from very small plant parts, tissue, or cells grown aseptically in test tubes or other containers where the environment and nutrition can be rigidly controlled [4, 5]. Efficient plant regeneration through in vitro micro propagation is very essential for the successful utilization of biotechnology in rice crop improvement [6]. The identification and screening of useful cultivars for embryogenic callus formation and subsequent plant regeneration through in vitro system is a key step in rice genetic improvement programme [7, 8]. It has been known that the potential for callus induction and regeneration in rice tissue culture depends on a number of factors, such as the genotype of the donor plant, the type and physiological status of the explant, the composition and concentration of the basal salt, and the organic components and plant growth regulators in the culture medium.

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Plant stem cells naturally present in the root and shoot apex of intact plants are considered 'pluripotent', since they are able to form cell and tissue types present in either root or shoot tissue. In contrast, the term 'totipotent' is applied to somatic cells cultured in vitro to produce embryogenic cells, which give rise to somatic embryos and regenerated whole plants [9]. Factors influencing in vitro adaptability and regeneration are varied, ranging from genotype, origin of explant, culture conditions, and hormonal effects. Plant tissue culture-mediated micropropagation is also referred to as 'clonal propagation' implying that all the progenies generated as a result of this asexual method in vitro are 'clones' or 'true to types'.
Micropropagation have evolved as in vitro experimental models for studying cell division, differentiation and morphogenesis, which are important in key developmental processes such as meristem formation and embryogenesis [10] and stress-related genome plasticity in plants Genetic changes frequently associated with in vitro regenerated plants lead to stable, lasting modifications to the genome that are inherited in subsequent generations [11].

MATERIALS AND METHOD

The research work for micropropagation in rice was counducted at the tissue culture laboratory of DRR, (ICAR) Hyderabad (A.P.). The procedure of this research work has been divided in the following categories.

Plant and Media Preparation

Seeds of APMS -6B and BPT -5204 varities of rice was taken as explants source for callus induction. Healthy and mature seeds selected by physical apperance and they were dehusked manually. For *in-vitro* production of rice Murashige and Skoog's medium was used with various concentrations of plant growth regulators [12].

Surface Sterilization of Seeds

Surface sterilization is necessary in order to disinfect the explant before it was placed over media. For this following steps are performed:

- The seeds were placed in different conical flask and covered with net + cotton and washed for 30 minutes under running tap water to remove all the dust particles and microbes from the surface.
- In the next step seeds were sterilized in 35% clorex supplemented with 2-3 drops of Tween – 20 for 30min. by gentle shaking.

Sterilization procedure under aseptic condition

After the surface sterilization seeds were taken inside the laminar flow hood. Here 2-3 washings were given with sterile double distilled water. Further seeds were surface disinfected with freshly prepared 0.1% (w/v) aqueous solution of mercuric chloride for 5 minutes. They were then thoroughly washed for 3-4 times with sterile double distilled water to remove any traces of mercuric chloride for 5 minutes (two cycles). The explants were immersed in 70% ethanol for 5 min. This was followed by at least three rinses in double distilled water. Rinsed explants were inoculated in prepared Murashige and Skoog's medium (MSM) [13].

Glasswares

The glassware used for culture work comprised of 6"Õ 1" borosil test tubes, 250ml, 500ml and 1000ml borosil flasks. In addition other glassware includes graduated measuring cylinder, Petri dishes,Conical flask, beakers, bottles and a range of pipettes. Before use, glasswares were thoroughly brushed with alkaline detergent teepol and then washed in running tap water. It was then treated with hot chromic acid (mixture of K2Cr2O7 + H2SO4 + H2O) followed by very thorough washing with tap water. All vessels were then inverted in a clean tray and left to dry. Copper distilled water (5-10ml) was then poured into every culture vessel which was tightly plugged. Plugs were made out of absorbent surgical cotton wrapped in muslin. Glassware was then steam sterilized in an autoclave at a pressure of 15 lb\in2 (1210C) for 15 to 20 minutes [14].

Culture conditions

The cultures were maintained in culture tubes and conical flasks and were kept in the culture room at a temperature of 25±2ºC, relative humidity (RH) of 60-70% and a light intensity of approx. 2500 lux provided by cool, white, fluorescent tubes under a photoperiod of 16/8 hr (light/dark).

Subcultures

Cultures were maintained through regular monthly subcultures. The cultured tissues were aseptically transferred on to fresh media without being subjected to chemical sterilization.The adhering agar and necrotic tissues were removed without damaging the shoot primordial/buds. These units were finally recultured on to fresh media having appropriate plant growth regulators (PGRs). Subcultures were also performed as and when necessary after evaluation of growth changes.

Inoculation of Sterilized Seeds

The most important step in tissue culture technique is the inoculation of seeds. This operation was performed in the laminar flow cabinet at the culture room of tissue culture. Before the operation surface sterilization of the laminar flow unit was carried by UV-light for two minutes. After that hands were disinfected with 75% ethanol to prevent chance of contamination. Dried seeds were then inoculated into test tubes under aseptic condition in laminar flow unit. To minimize chance of infection the instruments were dipped in disinfectant after every operation.

Callus Induction Medium

M.S and N6 basal media were used for callus initiation. These media were prepared with sufficient

amount of macronutrients, micronutrients, iron source and vitamin. The proposed medium was supplemented with (a) either concentration of growth hormones 2, 4-D (2.0, 2.5, 3.0 and 3.5 mg/L) for APMS-6B and BPT-5204 on MS medium.(b) or combined with 2, 4-D and cytokinin (0.35, 0.60 and 2.00 mg/L) on $N_6$for APMS-6B and BPT- 5204, having 4% sucrose and 0.9% agar and different concentrations of sucrose (3, 4,5 and 6%), carbon source (4% sucrose or maltose), casein hydrolysate (200, 300, 400,500 and 600 mg/L) different gels[0.9% agar, 0.4% agarose and 0.4% phytagel and 550 mg/l L-proline and 500 mg/l LGlutamine to study the relative importance of these factors affecting the callus production potential. The pH of the media was adjusted to 5.78 – 5.80 before autoclaving. Two independent experiments were performed for each of the factors studied. Each experiment was regarded as one replicate for further data analysis. The callus induction efficiency was computed on the basis of percentage from two experiments.

Shoot regeneration medium

MS medium was supplemented with different conc. of BAP (6- benzyladenopurine) and gibberellins i.e. 0.00, 1.0, 1.5,2.0,2.5,3.0 and 3.5 for APMS-6B and 0.00, 0.1,0.5,1.0, 1.5,2.0and 2.5 for BPT- 5204 with 4% sucrose, with 0.7or 0.8% phytagel respectively while maintaining 0.6 mg/L of NAA as constant. The pH of media was adjusted to 5.78 – 5.80 before autoclaving. Two independent experiments were performed for each of the factors studied. Each experiment was regarded as one replicate for the calculation of number of shoots per induced callus. The culture was performed at 25 ±2 ℃ under a cycle of 16 hours light/8 hours dark for a month, after which the frequencies of shoot regeneration were calculated, based on the appearance of shoots over 0.9 cm in height. The frequencies of emerging shoots were derived based on the percentage taken from the average of regenerated shoots from two experiments [15].

Root induction media

Shoots that developed in liquid multiplication medium were transferred individually into half strength MS agar (0.7%) medium supplemented with IAA (1.0 mg/l). The shoots were maintained for a month under the same culture condition as for development of roots. After this time, the percentage of rooted shoots was recorded. The plants with healthy roots, when grown to a leaf height of 15–20 cm, were transplanted to pots, filled with sterilized mixture of sand, vermicompost and soil (1:1:1) and grown for 5 weeks in greenhouse under a temperature of 27^{o}C conditions to determine the percentage of plants that survived and until the seed was harvested.

Transplantation

The rooted plantlets were first transferred to plastic pots containing garden soil, sand and peat moss (1:1:1), covered with transparent polythene bags and placed in acclimatization room at 28±2°C with 70-90% relative humidity, after five days temperature was increased from 28 to 32°C. After two weeks, transparent bags were removed from pots for proper hardening. After four weeks, the plants were then shifted in greenhouse and in field under low light intensity. The data for various growth attributes were recorded such as percent explants regeneration, multiple shoot formation, shoot length, percent plantlets rooted, number of roots per plantlet, and survival of plants during acclimatization and in the field was recorded.

RESULTS AND DISCUSSIONS

Callus induction frequency was observed for both the varieties APMS -6B and BPT – 5204 at different conc. Of 2,4-D on MS and Gibberellin and Cytokinin on N_6.The maximum callus formation was recorded at 3.0mgL^{-1} of 2,4-D at MS for variety APMS -6B and at 2.0mgL^{-1} of 2,4 –D at MS for BPT -5204. It was also noted during experiment that the callus frequency percent start decrease after increasing the conc. above 3mgL^{-1} 2, 4-D on MS for APMS -6B and the callus frequency percentage goes in decreasing order by adding more conc. Of 2,4-D on MS for BPT- 5204 variety of rice seed (Table -1). So we can show that the 2, 4 –D shows differential effect by changing the variety of seed of rice. In case of APMS – 6B more good result was observed than the BPT – 5204 variety of seed.

By using N_6 media supplemented with different conc. of Gibbrellin and Cytokinin for callus formation, we got results that range from 25 ± 0.32% to 41 ± 0.35% for APMS- 6B and from 60 ± 0.54% to 50 ± 0.33%.(Table -2). But inverse effect was observed for both the varieties. In case of APMS -6B the callus induction percentage increased by adding more conc. of Gibberellins and cytokine but the callus formation get start declined in case of BPT-5204 variety of rice seed .The highest results were observed at the conc. of Gibbrellin and Cytokinin 2.5 mgL^{-1} for APMS -6B and at 0.35 mgL^{-1} conc. for BPT -5204. Callus obtained from both the varieties were, fragile, granular and yellowish in color.

Table 1. Callus induction frequency % APMS -6B and BPT-5204 seeds of Rice Varieties

Different Conc. of 2,4-D in MS (mgL^{-1})	Callus Induction Frequency percent In	
	APMS – 6B	BPT – 5204
2.0	45 ± 0.22	65 ± 0.32
2.5	51 ± 0.25	62 ± 0.42
3.0	55 ± 0.65	60 ± 0.53
3.5	52 ± 0.55	55 ± 0.32

Table 2. Callus Induction frequency% for Rice varieties APMS -6B and BPT – 5204 on N_6 at different Conc. of 2,4-D and Cytokinin

Different Conc. of 2,4-D and Cytokinin on N6 (mgL^{-1})	Callus Induction frequency percent	
	APMS- 6B	BPT- 5204
0.35	25 ± 0.32	60 ± 0.54
0.60	28 ± 0.41	58 ± 0.32
2.0	35 ± 0.55	55 ± 0.42
2.5	41 ± 0.35	50 ± 0.33

Table 3. Effect of BAP and Gibberellins on regeneration of shoot of rice (APMS - 6B and BPT- 5204)

BAP and Gibberellins Conc. mgL^{-1}		Shoot induction percentage	
APMS -6B	BPT - 5204	APMS -6B	BPT – 5204
0.00	0.00	28.73	38.42
1.0	0.1	36.78	45.52
1.5	0.5	42.22	65.61
2.0	01.0	56.56	78.56
2.5	1.5	62.44	80.12
3.0	2.0	64.82	82.02
3.5	2.5	72.52	85.92

Table 4. Effect of different conc. of IAA, IBA and NAA in MS medium for root formation

Growth regulators(mg/l)	Root Formation (%)			Average length (cm)mean ±S.E		
IAA+IBA+NAA	IAA	IBA	NAA	IAA	IBA	NAA
0.5	32	25	30	2.5±0.5	2.2±0.6	2.5±0.5
1.0	45	46	45	3.2±0.8	3.4±0.5	3.5±0.3
1.5	56	53	41	7.5 ±0.5	3.6±0.7	4.5±0.2
2.0	45	28	26	4.3±0.5	5.0±0.4	4.8±0.3

Fig 1. Seed Inoculation

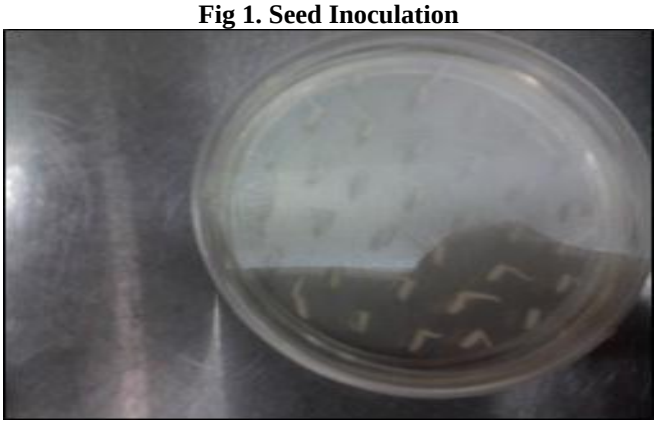


Fig 2. Callus Formation

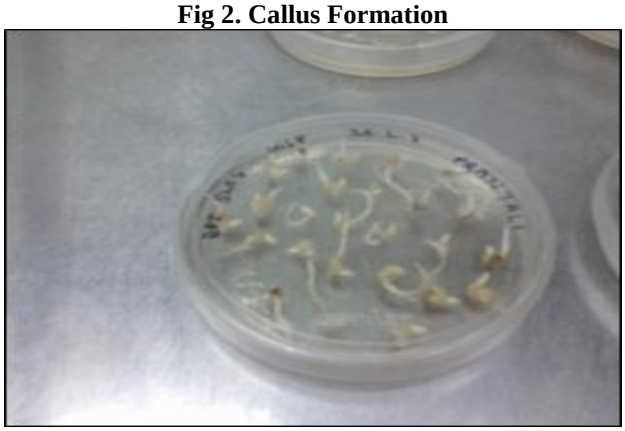


Fig 3. Shoot Induction

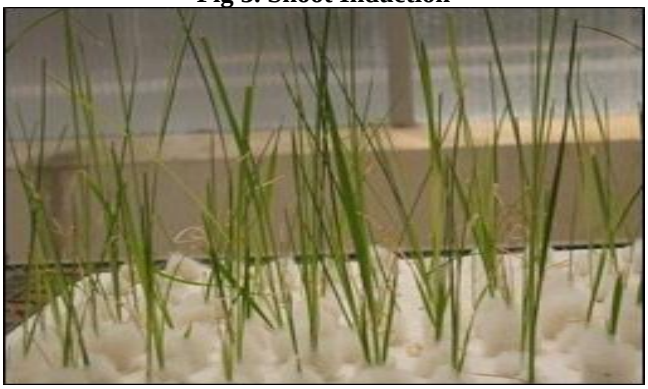


Fig 4. Root Regeneration

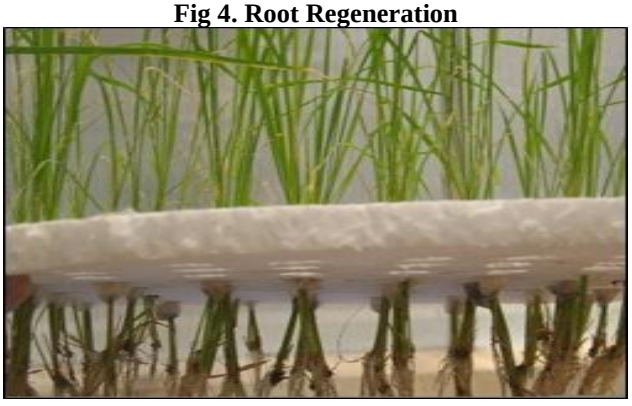
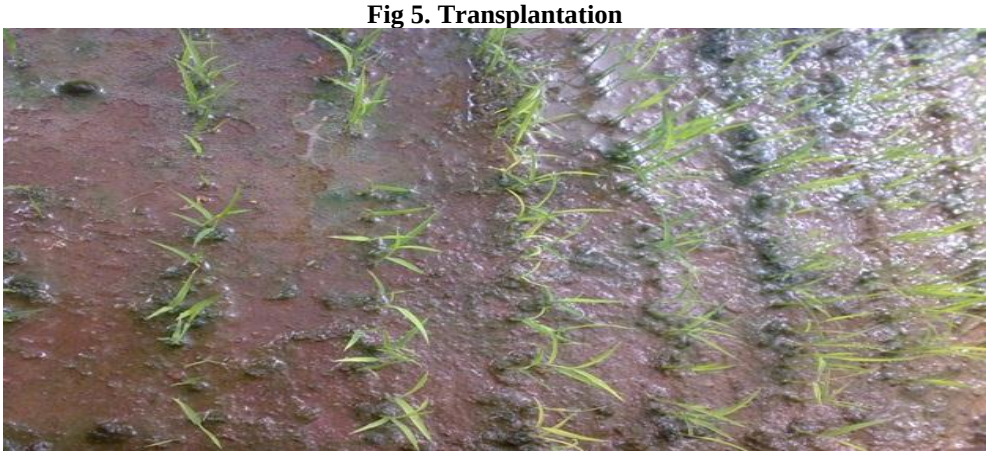


Fig 5. Transplantation



Gibberellinis a plant growth regulator that also play an important role in initiation and elongation of shoot from callus of rice by using at different conc. on MS (1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 percent) for APMS – 6B and at conc. of 0.1, 0.5, 1.0, 1.5 and 2.0 mgL^{-1} for BPT – 5204.

Table-3 shows the effect the different Gibberellins conc. for shoot induction. The shoots show bipolar structure by normal growth in rice seeds of both the variety. The highest results were observed at conc. of 3.5 mgL^{-1} of Gibberellins i.e, 72.52% APMS – 6B and for BPT- 5204 it was highest at the same conc. of Gibberellins, i.e 85.92%.

Table -4 shows that when different conc. of IBA, IAA and NAA is supplemented in MS medium then it proved to be best in rooting from regenerated shoots in all the cultivars which showed 100 percent shooting efficiency.

CONCLUSION

The present work investigated the role of MS media and N6 media supplemented with different conc. Of plant growth regulators for somatic embryogenesis in two different varieties of rice (APMS – 6B and BPT- 5204). Rice is a cereal crop of tremendous economic important. Firstly the callus formation was obtained from explants by using different conc. Of 2, 4-D and then shoot induction was observed by supplemented media i.e. Gibbrallin and Cytokinin. These shoots showed 100 percent root generation ability. The *in –vitro* regenerated plantlets after attaining a size of 2-3 inches were noted.

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