Int.J.Curr.Microbiol.App.Sci (2019) *8*(3): 82-82

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 03 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.803.012

Regeneration of *Indica* Rice (*Oryza sativa* L.) from Calli Derived through Immature Embryos

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Keywords

Oryza sativa, Regeneration frequency, BAP, Kinetin, NAA, Proline

Article Info

Accepted:
04 February 2019
Available Online:
10 March 2019

A B S T R A C T

A new and rapid method for optimum callus production was established in 6 rice (*Oryza sativa*) varieties. Immature seeds were used as a starting material for callus induction using different concentrations of BAP, NAA, and Kinetin. Higher percentage of callus induction was obtained in MS + 0.5mg/l BAP + 1.0mg/l NAA + 3% sucrose. Healthy calli emerged which were compact in texture and globular, light yellowish in colour and also it was observed that the calli of the variety, INH1001 shows early and rapid growth in callus size within 14 days of incubation as compared to other varieties used in this study. For calli regeneration, induced calli of higher efficiency INH1001, along with other five varieties were treated with MS media supplemented with various concentrations of BAP (0.5-2.0mg/l), NAA (0.1-1.0 mg/l) and 5mg/l proline. The shoot regeneration was found maximum in MS media supplemented with 2.0 mg/l BAP+ 0.1 mg/l NAA + 5mg/l proline within 1 week of culture; it was observed that the calli of INH1001 variety shows early regeneration within 7 days of incubation as compared to other varieties. However, other calli responded to late shoot regeneration.

Introduction

Rice is the major food crop in the world from the initiation of the human civilization. It is a cereal crop of amazing economic importance. Rice is a monocot plant belongs to the genus *Oryza* under the family *Gramineae*. It is the major food crop in the Asian country and the rest of the world, and thus, varieties with improved characteristics are desired using the rice verities takes as a model, monocot system is critical for food safety, hunger eradication and poverty alleviation (Coffman *et al.*, 2004). The last 30 years shows the production of the rice has increased and output comes in doubling of the yields.

The Asian countries are demanding for rice for which increase of rice is predicted to be 781 million tons in 2050, which is related with the world population of 9.3billion (Nguyen, 2009). Rice has been considered as

the third economical crops in the world (Abdullah *et al.,* 2016). It is the most important source of employment and income for rural people (Hossain and Pingali, 1998). The Rice production has increased with the increasing rate of the population growth. New studies suggest that an additional increase of 50-70% of the current supply is needed to meet the demand until 2025 (Pingali *et al.,* 1997). In India, it occupies an area of about 44.00 million per hectare respectively (Annual Project Director's Report, IIRR, 2011). As the land resources are shrinking, the present trends suggest that tomorrow's rice land will be under even greater pressure (Greenland, 1997).

Rice is considered as the best model plant for molecular studies from the cereals category (Mukhopadhyay and Tyagi *et al.,* 2004). Throughout the cereals, rice and maize are largely response to tissue culture and have the efficient of regeneration under *in vitro* conditions (Ganeshan *et al.,* 2006). Basically, *in vitro* regeneration occurs in two ways that includes organogenesis and somatic embryogenesis.

Somatic embryogenesis has developed the bipolar structures that derived from haploid or diploid somatic cells and formed through an embryological stage without fusion of gametes that are not connected to the primary vascular tissue of the mother calli. Somatic organogenesis is a special technique in plants and has considerable significance for biotechnological application such as clonal propagation, production of synthetic seeds and genetic transformation (Quiroz- Figueroa, 2006). The method of the somatic organogenesis is integrated with the classical breeding programs and molecular biology techniques provides a valuable tool to enhance the genetically improvement of crop species (Quiroz- Figueroa, 2006). In rice, somatic organogenesis is the best and common regeneration method and has been obtained from roots, leaf bases of young seedlings, mature embryos, immature embryos, caryopses, coleoptile, cell suspension protoplast and young inflorescence (Hoque and Mansfield, 2004; Ramesh *et al.,* 2009; Naqvi *et al.,* 2006; Noouri-Delawar and Arzani, 2001. Gairi and Rashid, 2004; Valedez *et al.,* 1996; Akter and AL-Forkan, 2010; Jelodar *et al.,* 2002; Chen *et al.,* 1985; Meneses *et al.,* 2005; Ge *et al.,* 2006).

Several factors are responsible for the growth of the plant *in vitro* including regulators, explant, culture conditions, plant genotypes, light, charcoal and other biochemical factors such as amino acids of the rice plant regeneration from calli (Deo *et al.,* 2010). The production of rice is severely affected by several biotic and abiotic stresses. Conventional plant breeding methods (Bögre, Stefanov *et al.,* 1990) like selection, hybridization, polyploidy and mutation breeding failed to address the problem as donors for resistance against these stresses are not available in rice gene pool (Bonman *et al.,* 1992). To circumvent the problem, transgenic approach offers the choice of an unlimited gene pool for transfer and expression of desirable genes between any two species irrespective of their evolutionary and taxonomic status.

The transgenic technology provides the researchers a unique opportunity for incorporation of agronomically useful genes into commercially popular cultivars having wider adaptability. The genetic enhancement of host plant resistance through genetic engineering approach is a practical, viable and eco-friendly approach as application of chemicals can be minimized. The nutritional properties of the grain can also be enhanced through incorporation of novel traits that can effectively address the malnutrition issues (Ye

et al., 2000). The totipotent character of plant cells allows that any differentiated cells that retains its nucleus has the ability to regenerate an entire new plant by organogenesis (Reynolds, 1997).Keeping the above facts in view, the present investigation focuses on organogenesis of *indica* rice varieties with the following studied was screening of rice genotype for organogenesis and to access the effect of different hormone concentrations on callusing and shoot regeneration of rice.

Materials and Methods

The present investigation was carried out at National Rice Research Institute (NRRI), Cuttack, Odisha, India.

Plant Materials

Six high yielding rice varieties were used for the present study.

Explant source

Immature grains of *Oryza sativa* were collected from the plant grown at the field of NRRI, Cuttack, Odisha.

Preparation of culture media

The MS medium described by Murashige and Skoog's (1962) was used as basal medium throughout this investigation as it is the most widely used medium for tissue culture. For convenience and in order to avoid the time-consuming process of weighing individual ingredients each time, concentrated stock solution of mixtures of selected components of the medium were prepared and stored in a freezer. At the time of medium preparation, they were brought to room temperature and mixed in the recommended proportion to get the desired media. Sucrose and agar-agar used were at 3% (w/v) and 0.6% (w/v), respectively. 100 mg of inositol was added per litre of medium. The pH of the media adjusted 5.7 to 5.8 using 0.1N HCl or 0.1N NaOH.

Hormone Preparation

NAA (α-naphthalene acetic acid)

Firstly, weighed 20mg, NAA and dissolved in absolute alcohol and then added double distilled water and made up volume up to 80ml.

2, 4-D (2, 4-dichlorophenoxy acetic acid)

Firstly, weighed 20mg, 2, 4-D and dissolved in absolute alcohol and then added double distilled water and made up volume up to 80 ml.

BAP (6-benzyl adenine or 6-benzyl aminopurine)

Firstly, weighed 20mg BAP and dissolved in 0.1N HCl first and then add distilled water and made up volume up to 80 ml.

Kinetin

Firstly, weighed 20mg kinetin and dissolved in 1N NaOH first and then added double distilled water and made up volume up to 80 ml.

Sterilization

Surface sterilization of explants/seeds

Dehusked seeds were surface sterilized with 70% ethanol for 30 seconds and rinsed with sterile distilled water followed by treatment with 4% sodium hypochloride and two drops of Tween 20 for 10 mins. Thereafter, the explants were washed with 0.1% mercuric chloride ($HgCl_2$) for 5mins followed by washing with sterile distilled water for 3-4 times prior to inoculation.

Sterilization of equipment and glassware

The transfer cabinet was first wiped clean with 70% ethanol and sterilized by germicidal ultraviolet light (40 W, UV TL, Philips) for 20 min. All metal instruments, glass wares and other accessories were steam sterilized in an autoclave. The standard sterilization cycle was for 20min at 121^{o}C with 15 lb/in^{2}.normal steam pressure.

Sterilization of culture media

Before sterilization, the gelled media (25ml/tube) were poured into culture tubes and closed properly with cotton plugs with capping of paper. Usually, the sterilization was carried out in an autoclave for 20minutes at 121^{o}C with 15lb/in^{2}.

Inoculation of seed

The most important step in tissue culture technique is the inoculation of explants. This operation was performed in the laminar flow cabinet at the culture room of NRRI. Before the operation surface sterilization of the laminar flow unit was carried by UV-light for ten minutes. After that hands were disinfected with 70% ethanol to prevent chance of contamination. After proper sterilization, the dehusked immature seeds were then inoculated into test tubes under aseptic condition in laminar flow unit into the tubes containing semisolid media. Each tube was inoculated with 4 seeds and 4 replicates of each treatment. Injured seed were rejected. To minimize chance of infection the instruments were dipped in disinfectant after every operation.

Culture initiation

Callus culture medium

Four different callus induction media were tried with different combinations and concentrations of kinetin, BAP, and NAA.

Callus culture

The dehusked sterilized immature seeds were inoculated into the MS medium with different concentration of hormone as given above and kept at about 25^{o}C in complete darkness for a period of 21 days. Callus induced from the embryo of the swollen seed in these medium within 15days of incubation.

Organogenesis medium

Three different shoot regeneration medium were tried. Different compositions of media supplemented with MS medium are depicted in (Table 2).

Root and shoot regeneration

Friable yellowish brown calli were cultured in the media and then were transferred onto fresh MS media with particular composition and concentration for shoot regeneration (which shows early shoot bud initiation and high efficiency of shoot regeneration).

Incubation and maintenance of cultures

All the cultures were incubated in the racks inside a culture room in dark for first 48 hrs and then under controlled condition of light and temperature. The cultures were exposed to continuous illuminations (3000 lux), cool white fluorescent lights (Philips, India) in a photoperiodic cycle of 16 hr light and 8 hr dark at 25±2^{o}C for the period of 4 weeks.

Results and Discussion

A total of 6 varieties were taken in the present study for assessment of callusing and regeneration potential.

Optimization of media for callus induction

The MS (Murashige and Skoog, 1962) medium, widely used basal medium, was used

for callus induction in rice. Four treatments (Table 1) were formulated and tested for callus induction. Different combinations and concentrations of plant growth regulators such as BAP, Kinetin, and NAA were supplemented to the MS basal medium for better growth performances.

Effect of plant growth regulators on callus induction

The present study showed that callus produced from the scutelum region and were clearly visible after seven days of culture. Different types of textures, size and colours of calli were found with respect to the media. Callus induced from the embryo of the swollen immature seeds in these medium within 12 days of culture. Compact, light yellowish colour calli were obtained after 15 days of culture.

Differential response for callusing was observed in all the four treatments used for calli response study. The result showed that MS media supplemented with 0.5mg/l of BAP and 1.0mg/l of NAA gives 100% response in INH 1001 followed by87.5% in G409. Moreover, increase of NAA from 1.0 to 1.5 mg/l and decrease of BAP from 0.5 mg/l to 0.1mg/l showed 80% callusing in INH1001 and 62.5% inG409. However, kinetin in combination with BAP and NAA showed 68.75% and 43.75% callus induction in G406 and G1931 respectively. A variety, INH 1001 showed high callus induction in MS media supplemented with 0.1mg/l BAP and 1.5mg/l NAA while in MS media in combination of 0.1mg/l Kinetin and 2.0mg/l NAA showed the list percentage efficiency in callus induction. Among the 4 treatments used, it was observed that combination of high concentration of BAP and NAA or lower concentration of kinetin showed the maximum calli induction. Hence, it was inferred that MS media in combination of BAP and NAA were best studied media for callus induction in a number of varieties used for study.

Shoot regeneration

The calli derived from immature seeds were transferred to the shoot regeneration media for which the basal media was MS. The Calli turned into yellowish or light brown colour after 12 days of culture; three different types of shoot regeneration media namely RegA, Reg B, Reg C were used for the study mostly in media with the concentration of hormones BAP 1.5mg/l, NAA 0.1mg/l (Reg B) and BAP 1.5mg/l, Kn 1.0mg/l, NAA 0.1mg/l, Glutamine 5mg/l (Reg C). However, the frequency of shoot regeneration was found maximum in media with the concentration of hormones: BAP 2.0 mg/l, NAA 0.1mg/l, Proline 5mg/l (Reg A) suggesting being the best suited media combination for shoot regeneration. It was observed that percentage efficiency of shoot regeneration was found maximum in the varieties like INH 1001, G406, G409, G1931 and B-16. From the data it was evident that the lower concentration of NAA (0.1mg/l) along with a higher level of cytokine in BAP (2.0mg/l) and proline (5.0mg/l) was found to be suitable for early shoot initiation and rapid elongation.

Morphological signs in calli for shoot initiation

Considering the above-mentioned characters, it was concluded that in the media Reg A with the concentration with BAP 2.0mg/l, NAA 0.1mg/l and Proline 5.0mg/l (Reg A) showed early greenish shoots spots initiation and high percentage efficiency of regeneration within 6 days. In the media, Reg B with concentration of BAP 1.5mg/l, NAA 0.1mg/l showed 60% of regeneration efficiency and greenish shoots spots showed within 9 days. However, in the media Reg C with the concentration of BAP 1.5mg/l, Kn 1.0mg/l, NAA 0.1mg/l,

glutamine 5mg/l showed no greenish shoots or spots owing to its very low percentage efficiency of regeneration (Table 6). Likewise, media concentration with BAP 1.5/2.0mg/l, NAA 0.1mg/l and proline 5.0mg/l were found to be effective for shoot initiation and proliferation for varieties like INH 1001,G406,G409,G1931, B -16. However, varieties like B-16, NHN132 in RegC with hormonal concentration of BAP 1.5mg/l, Kn 0.1mg/l, NAA 0.1mg/l, glutamine 5mg/l showed minimal percentage efficiency of regeneration. Therefore, it can be inferred media with BAP 2.0mg/l, NAA 0.1mg/l, proline 5mg/l was found to be the best for shoot initiation.

Root formation

It was observed that callus showing root initiation after 10 days of callus culture into the shoot regeneration mostly in all the varieties used for the study except the cultivar NHN132.

Efficiency of root formation of calli in percentage (%) in different types of media combination data recorded, as par data highest percentage callus showing root initiation are in these varieties like INH1001, G1931, G406, 40%, 38.70%, 35.71% in media Reg A and Reg B. whereas cultivar NHN 132 did not show any sign of root initiation 0%.

Necrosis of calli

The 3 week old calli of various varieties were transferred from the callus induction media onto the shoot regeneration media turned to brown and necrotic which is ultimately died after 15 days of inoculation mostly in media with the concentrations of hormones BAP 1.5mg/l NAA 0.1mg/l, Kinetin 1.0mg/l, glutamine 5.0mg/l (Reg C) (Table 7). However, the frequency of shoot regeneration were found maximum in media with the concentration of hormone BAP 2.0mg/l, NAA 0.1mg/l, along with proline 5.0mg/l (Reg A) suggesting to the best suited media combination for shoot regeneration.

Variety used for study along with ecotype and source

SL NO.	FIELD NAME	PLANT VARIETY	ECOTYPE
1	MP110	INH 1001	Irrigated
2	MP701	G406	Irrigated
3	MP702	G409	Irrigated
4	MP721	G1931	Irrigated
5	MP97	B-16	Irrigated
6	MP116	NHN132	Irrigated

Table.1 Details of Media Concentration of Growth Regulators Supplemented with MS Medium for Callus Induction of *Indica* Rice varieties

TREATMENT	BAP(mg/l)	NAA(mg/l)	Kn (mg/l)
MS 1	**0.1**	**1.5**	**_**
MS 2	**_**	**2**	**0.1**
MS 3	**0.05**	**2**	**0.05**
MS 4	**0.5**	**1**	**_**

Table.2 Details of Media Concentration of Growth Regulators Supplemented with MS Medium for Shoot Regeneration of *Indica* Rice Varieties

Treatment	BAP (mg/l)	NAA (mg/l)	Proline(mg/l)	Glutamine	Kinetin(mg/l)
Reg A	2	0.1	5	_	_
Reg B	1.5	0.1	_	_	_
Reg C	1.5	0.1	_	5	1

Table.3 Effect of Growth regulators on callus development from seeds of various rice varieties

Sl.no.	varieties	After 5days	After 12 days	After 21 days	Callus colours
1	INH1001	2mm	5mm	8mm	whitish yellow
2	G406	2mm	4mm	6mm	yellowish
3	G409	1.5mm	3mm	5mm	whitish yellow
4	G1931	1mm	4mm	5.5mm	whitish yellow
5	G-16	1.5mm	2.5mm	4.5mm	yellowish
6	NHN132	1mm	1.5mm	3.5mm	yellowish

Table.4 Frequency of calli Induction in MS media containing different concentration and combination of growth regulators from seeds of rice varieties

Sl.No.	Genotype/variety	Callus induction (%) after 21 days of culture			
		MS1	MS2	MS3	MS4
1	INH1001	100	50	62.5	100
2	G406	87.5	31.25	68.75	75
3	G409	62.5	18.75	81.25	87.5
4	G1931	50	25	43.75	75
5	G-16	50	25	25	62.5
6	NHN132	37.5	0	50	50

Table.5 Effect of growth regulators on shoot regeneration from calli derived from rice varieties after 21 days

Sl.no.	Varities	Morphological sign up shoot regeneration in different types of media			Efficiency shoot regeneration
		Reg a	Reg b	Reg c	
1	INH1001	greenish shoot and spot	greenish shoot and spot	no green spot	85%
2	G406	greenish shoot and spot	greenish shoot and spot	no green spot	70%
3	G409	greenish shoot and spot	greenish shoot and spot	no green spot	75%
4	G1931	greenish shoot and spot	no green spot	no green spot	60%
5	G-16	no green spot	greenish shoot and spot	no green spot	20%
6	NHN132	no green spot	no green spot	no green spot	0%

Table.6 Root Formation of calli (%) in different types of media combination

SL NO	Varieties	TOTAL CALLUS	NO of callus showing root initiation	No of Root initiation	Media showing root initiation
1	INH 1001	50	20	40	Reg A, Reg B
2	G 406	42	15	35.71	Reg B
3	G409	40	12	30	Reg C
4	G 1931	31	12	38.7	Reg A
5	B16	13	4	30.76	Reg C
6	NHN132	11	0	0	_

In the present study, significant genotypic response to NAA was observed in callus induction. For primary callus induction from immature embryo, NAA was considered as critical at concentration between (1.0 – 2.0mg/l) in cereals (Jia *et al.,* 2008) Moreover, difference in NAA concentration required for callus proliferation may be due to the genetic variation in endogenous levels of phytohormones, which influence the callus proliferation (Deo *et al.,* 2009). However, at higher concentration of NAA, all the cultivars showed similar tendency in decrease of callus induction. In our study, all the cultivars showed good proliferation of calli in MS media supplemented with BAP 0.5mg/l+ NAA 1.0mg/l (MS4). Among all the varieties used, INH1001 showed highest proliferation in MS4 medium followed by G409, G406, and G1931. The present result was similar to the earlier reports (Kaur D *et al.,* 2009).

In the present study immature seed callusing was observed in INH1001, G406 in MS medium supplemented with NAA 1.5mg/l + BAP 0.1mg/l. The hormone NAA has been reported to promote somatic embryogenesis and plays important role in dedifferentiation and cell division in rice (Panjaitan *et al.,* 2009 and Meneses *et al.,* 2005). These genotypic variations are probably due to differential expression depending upon the spatial and temporal distribution, their physiological and developmental stages.

Immature seed callusing and organogenesis, both are triggered by auxins and cytokinins (Jia *et al.,* and Liu *et al.,* 2008; Chaudhury *et al.,* 2000). In the present investigation, proline was found to be effective for induction of immature seed callusing. Role of cytokinins (BAP) on immature seed calli has been explained by enhanced cell division of pre-embryogenically determined cells (Kintzios *et al.,* 2002). Besides, proline enhances callus proliferation and regeneration by influencing mitosis, cytokinins, total protein synthesis, lignin biosynthesis, vascular differentiation and differentiation of mature chloroplast from protoplastids (Wan *et al.,* 1988).

After establishing proper callusing conditions the calli were transferred to different media with various hormonal compositions. Usually high 2, 4- D along with BAP treatment is used to increase the callus induction frequency in anther based cultures (Rout *et al.,* 2016). Contradictory to this, in our study we observed a negative effect of high concentration of BAP on the immature seed based callus. The healthy callus of rice seed (var. INH 1001) were transferred to the regeneration medium, RegA for organogenesis. Subsequently, these calli showed organogenesis: INH1001> G409> G406> G1931> B16> NHN132 in the decreasing order of response for shoot regeneration. Similar to this RegA also showed high frequency of root induction in

the order of INH1001> G1931> G406> B16> G409> NHN132.

It is concluded that MS media (MS4) supplemented with BAP 0.5mg/l + NAA1.0mg/l was responsive for callus induction in six rice varieties. Besides, MS+ 2.0mg/l BAP+ 0.1mg/l NAA+5mg/l proline (Reg A) was found suitable for early shoot regeneration with more efficiency in INH1001 variety. Similar to the shoot regeneration response, the root regeneration was also enhanced in the RegA medium with INH1001 showed the highest root regeneration percentage.

Role of auxin and cytokinin in callusing and regeneration could be focused for different varieties of rice in the media which were used for study. Immature seed organogenesis is novel approach used in *indica* variety quite recently and should be analysed at molecular level in future.

Acknowledgement

The author is thanks to Barsita Swain, Sangeeta Kujur, Jagdish Jenafor their help in this work. We are grateful to the National Rice Research Institute, Cuttack, Odisha, India.

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How to cite this article:

Bhakti Prakash Mallik, Barsita swain, Sangeeta Kujur and Jagadish Jena. 2019. Regeneration of *Indica* Rice (*Oryza sativa* L.) from Calli Derived through Immature Embryos. *Int.J.Curr.Microbiol.App.Sci.* 8(03): 82-82. doi: https://doi.org/10.20546/ijcmas.2019.803.012