Journal of Applied Biology & Biotechnology Vol. 9(4), pp. 1-9, July, 2021
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2021.9401

Optimization of culture variables for efficient callus induction and rapid plant regeneration in zinc rich rice (*Oryza sativa* L.) cv. "Chittimuthyalu"

Swapan Kumar Tripathy*

Department of Agricultural Biotechnology, Odisha University of Agriculture & Technology, Bhubaneswar, Odisha, India.

ARTICLE INFO

Article history:
Received on: January 09, 2021
Accepted on: February 27, 2021
Available online: July 10, 2021

Key words:
Callus induction,
Plant regeneration,
In vitro zinc rich rice.

ABSTRACT

Sterilized kernels of a zinc-rich rice variety "Chittimuthyalu" were initially cultured in modified MS with 2.5 mg/l 2, 4-D + 0.5 mg/l Kn for callus induction. The callus induced was transferred to a modified MS medium supplemented with 0.5 mg/l NAA + 2.0 mg/l BAP for study of plant regeneration response. A series of culture variables at varying levels were tested as independent experiments to optimize callus induction and morphogenetic response of the variety. There was a continuous improvement in callusing and plant regeneration response in subsequent follow-up steps. Supplementation of 500 mg/l casein hydrolysate, 150 mg/l proline, 3% sucrose, 0.3% agar + 0.2% phytagel revealed rapid callus induction and highest callusing response under dark condition. Somatic embryogenic plant regeneration was improved at 2.5% sucrose under 12-h photoperiod using 4 week old partially desiccated calli. The optimized high throughput callus induction and regeneration system realized in this study can be suitably used for genetic transformation and *in vitro* mutagenesis in zinc-rich rice.

1. INTRODUCTION

Rice is the major staple food of more than half of the world population. Feeding hungry with nutritious rice seems to be a lasting solution to food and nutritional security. Rice is grown in more than 120 countries, with a total harvested area of approximately 167 million hectares with a production of 496.1 mill tons in 2019–20 (https://www.statista.com/statistics/271972/world-husked-rice-production-volume-since-2008/). China and India are the top leading countries which contribute 50% of global rice production. Asia alone meets 90% of global milled rice requirement. At least 60% increase in food production is needed in next 30 years to feed the world (https://www.eitfood.eu/blog/post/sustainably-feeding-the-world-in-2050-are-efficiency-and equity-the-answer) which is indeed a challenging task. Zn is a trace mineral and it serves as cofactor of more than 300 enzymes involved in cellular metabolism [1]. In animals, Zn deficiency leads to loss of immunity to diseases, stunted growth, impaired learning ability, wound healing, and reproduction; and increased risk of infection, DNA damage, and cancer [2]. Therefore, there is a need for Zn-biofortified rice in the food chain. In plants, Zn is needed for plant growth and resistance to biotic and abiotic stresses [1]. Grain Zn content is a complex polygenic trait with high G x E interaction [3]. Available Zn status in soil, influx to roots, presence of Zn-transporter genes, exudation of phytosiderophores, inherent physiological mechanism of Zn uptake, transport, and remobilization to sink (seed), and metal homeostasis determine the grain Zn content. Innovative breeding strategy coupled with biotechnological approaches can pave the way for development of high Zinc rice variety.

In cereals, various *in vitro* culture techniques are being applied for varietal development among which matured dehusked seed culture is often used for genetic transformation and creation of novel genetic variants. However, its application is limited by genotype, media supplements, and culture conditions [4] to sustain growth of calli, subsequent plant regeneration and survival as fertile plants. It is often difficult to induce embryogenic calli and to regenerate plants from the callus cultures specially those belonging to *Indica* subspecies [4]. The recalcitrant nature of this sub-species has, in fact, been a major limiting factor in transfer of valuable genes [5] and creation of somaclonal variation and mutagen induced genetic variation(*in vitro* mutagenesis). An efficient callus induction and reproducible rapid regeneration system can achieve the success. Therefore, the present experiment was undertaken to optimize media supplementation and culture conditions in a popular zinc rich rice variety "Chittimuthyalu."

Chittimuthyalu is a semi-dwarf, short bold grain land race of Andhra Pradesh (India) maturing within 135 days in wet season. "Chittimuthyalu" retains 23.45 ppm zinc, 4.05 ppm iron, and 9.31% protein in polished rice, and it is considered as quality check entry in biofortification trials of All India Co-ordinated Research Project on

*Corresponding Author:
Swapan Kumar Tripathy, Department of Agriculture Biotechnology, Odisha University of Agricultural and Technology, Bhubaneswar, Odisha, India.
E-mail: swapankumartripathy@gmail.com

Rice. In addition, it has high head rice recovery (66.1%), relatively low glycemic index (~50) and suitable status of amylose content (23.81%) that fetch consumer's preference [Supple. Table 1].

2. MATERIALS AND METHODS

2.1. Plant Material

Genetically, pure seeds of a zinc rich rice variety "Chittimuthyalu" were used for *in vitro* culture in this study.

2.2 *In vitro* Culture

Mature healthy dehulled kernels of cv. "Chittimuthyalu" was washed with 2% bavistin (w/v) for 30 min and surface sterilized with 70% ethanol for 2 min followed by washing (2×) with sterilized distilled water with a drop of Tween 20 with continuous shaking for 10 min. Further, the seeds were treated with 0.1% (w/v) $HgCl_2$ solution for 6 min followed by rinsing (5×) with sterile distilled water and blot dried on sterilized filter paper before inoculation on culture medium. Sterilized kernels were aseptically cultured in modified MS medium with 2.5 mg/l 2, 4-D + 0.5 mg/l Kn for callus induction. Calli induced were transferred to modified MS (R)medium supplemented with 0.5 mg/l NAA + 2.0 mg/l BAP to study plant regeneration response. The pH was adjusted to 5.7 with 0.1N NaOH or 0.1N HCl after addition of the plant growth hormones and autoclaved at 121°C for 15 min. All the cultures were maintained in a sterilized culture room and incubated at 25±1°C and relative humidity of 60±5% under specified photoperiod conditions for standardization.

Sub-culturing of calli into fresh MS media (MS with 2.5 mg/l 2, 4-D + 0.5 mg/l Kn for organogenic calli and MS with 2 mg/l 2, 4-D + 0.5 mg/l Kn for somatic embryogenic calli) was done at an interval of 4 weeks for maintenance of callus growth. For this, the calli were partially desiccated in two layers of whatman-1 filter paper on Petri dish sealed with parafilm and kept at 25±1°C in dark for 48 h to attain partial dehydration. Regenerated plantlets were transferred to half-strength basal R medium supplemented with 1 mg/l NAA and varying levels of BAP (0.1–0.5 mg/l) for rhizogenesis. Each step of *in vitro* culture was repeated, at least twice. Each experiment comprising individual culture variable at varying levels was laid out in completely randomized design with 24 replicates. Observations were recorded for callusing response, callus growth, morphogenetic potential, and plant establishment; and analyzed statistically as per Dafaallah [6].

2.3. Culture Variables

The culture variables included key media supplements, for example, casein hydrolysate (CH) (0–1000 mg/l), proline(25–500 mg/l), carbon sources (glucose, sucrose and maltose: 2–4% each), gelling agents (agar, gelrite, phytagel, and their combinations), and culture conditions, for exa,ple, photoperiods (light/dark : 0/24–24/0 with increment of 4 h exposure to light), desiccation (no and partial desiccation), and age of calli (after 1–4 passages of subculture) for optimization of *in vitro* culture of cv. "Chittimuthyalu."

3. RESULTS AND DISCUSSION

Zn content is a highly complex trait, and the variety "Chittimuthyalu" is known to have stable performance for grain Zn content over years; hence, the genotype seems to be a best candidate material to explore genetic variation at cellular level (somaclonal variation) or genetic manipulation using *in vitro* mutagenesis and genetic transformation for biotic and abiotic stresses and yield *per se*. For this, optimization of culture variables is a priori to develop a high throughput rapid plant regeneration system.

Callus induced from mature seed scutellum can follow either organogenic or somatic embryogenic plant regeneration on a suitable modified MS media. In this study, a large number of small embryoids were formed on each callus. Many of them did not develop and as such their germination was poor and irregular. Nevertheless, some callus gave rise to more than 10 plantlets. Hence, optimization of culture variables was needed to improve the number of viable plants from embryogenic calli. In fact, we tested a series of culture variables at varying levels as step by step independent experiments to optimize callus induction and morphogenetic potential of an upland rice variety "'Chittimuthyalu" in MS media with 1000 mg/l NH4NO3 + 2830 mg/l KNO3 as nitrogen source. There was a continuous improvement in callusing and plant regeneration response in subsequent follow-up steps. The optimized level of each culture variable is shown in Table 1. Figures 1 and 2 depict high throughput callusing response, growth of callus and follow-up green plant regeneration under optimized combination of media supplementation and culture conditions.

Table 1: Optimum MS media composition and culture conditions for embryo culture in rice cv. "Chittimutyalu".

Series of culture variables	Optimum condition	Callus induction[a]			Plant regeneration[b]	
		Days first callus observed	CIF (%)	Callus growth	Organogenic response (%)	Somatic embryogenic response (%)
Casein hydrolysate	500 mg/l	10	79.9±0.42[c]	+++++[d]	62.8±0.55*	74.4±0.35[c]
Proline	150 mg/l	10	82.5±0.15	++++	68.8±0.63	76.8±0.65
Sucrose	25000mg/l	12	75.2±1.05	++	70.2±1.00	78.2±1.02
	30,000 mg/l	10	83.6±0.35	++++	75.5±0.47	71.6±0.28
Agar+phytagel	3000+2000	10	85.2±0.51	+++++	78.2±0.45	82.0±1.02
Photoperiod (light/dark in h)	0/24	9	89.6±0.91	+++++	25.8±1.05	25.0±1.00
	12/12	12	72.4±0.90	+++	80.2±0.88	82.2±1.03
Dessication	No dessication	NA	NA	NA	80.5±1.02	82.3±0.87
	Partial dessication	NA	NA	NA	82.7±1.16	85.7±1.30
Age of calli	After 4 weeks	NA	NA	NA	85.2±1.01	88.2±0.18

[a]MS media with 2.5 mg/l 2,4-D+0.5mg/l Kn, [b]MS media with 2.0 mg/l BAP+0.5 mg/l NAA, [c]Values are mean+S.E. [d]+, ++, +++, ++++, +++++ indicate poor, feeble, average, good, and excellent callus growth, respectively, NA: Not applicable

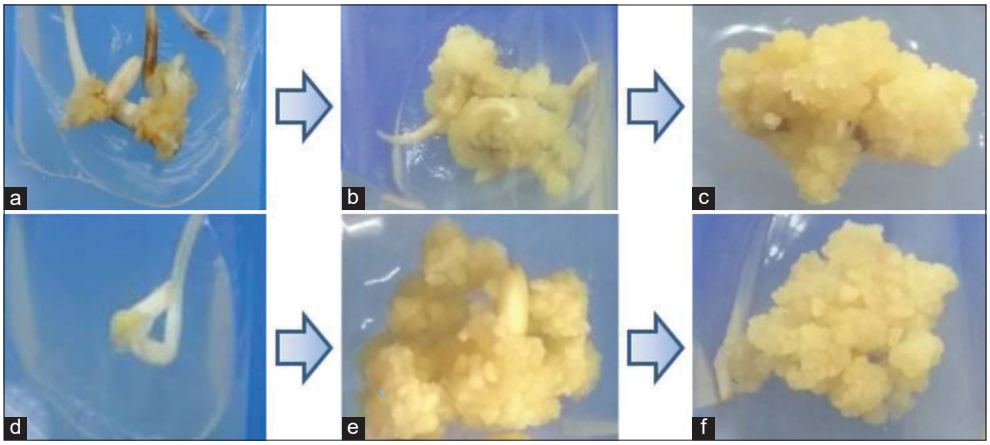

Figure 1: Organogenic callus induction (a and b) and callus proliferation upon subculture(c) in modified MS + 2.5 mg/l 2, 4-D + 0.5 mg/l Kn with addition of 500 mg/l CH, 150 m/l proline, 3% sucrose and 0.3% agar + 0.2% phytagel under complete dark condition in rice cv. "Chittimutyalu." Somatic embryogenic callus induction (d and e) in modified MS + 2.5 mg/l 2, 4-D + 0.5 mg/l Kn and callus proliferation upon sub-culture (f) in modified MS + 2 mg/l 2,4-D+ 0.5 mg/l Kn with addition of 500 mg/l CH, 150 m/l proline, 3% sucrose and 0.3% agar + 0.2% phytagel under complete dark condition in rice cv. "Chittimutyalu."

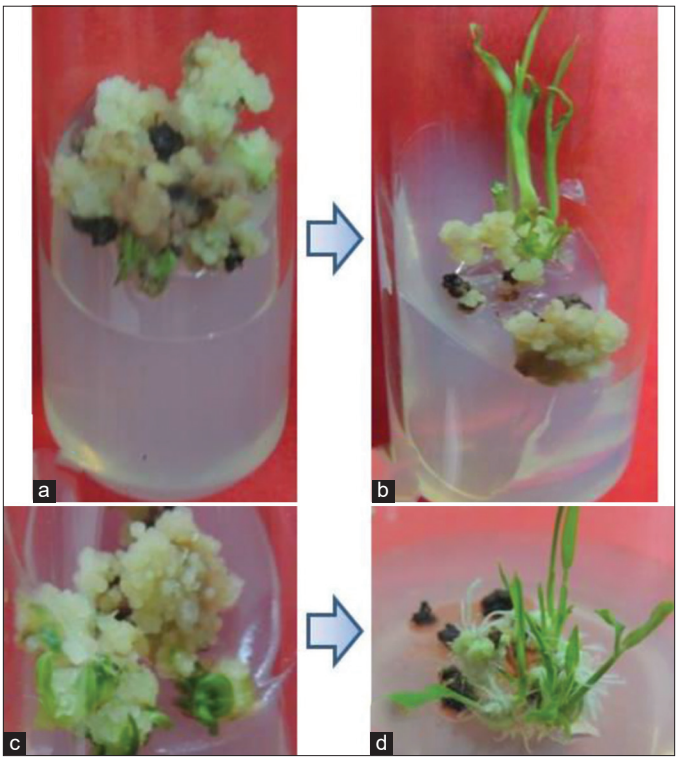

Figure 2: Organogenic (a and b) and somatic embryogenic (nodular and friable) (c and d) shoot bud differentiation and plantlet regeneration in modified MS + 0.5 mg/l NAA + 2 mg/l BAP with addition of 500 mg/l CH, 150 m/l proline, 2.5% sucrose and 0.3% agar + 0.2% phytagel under 12 h photoperiod using 4 week old partially desiccated calli in rice cv. "Chittimutyalu."

3.1. Optimization of Media Supplementation

3.1.1. CH

CH determines the quality and quantity of callus proliferation [7]. CH induces somatic embryos formation in callus culture of indica rice [8]. In the present investigation, CH concentration till 200 mg/l did not result any difference than media with no CH. However, CH at 500 mg/l elicited satisfactory callus induction (79.9±0.42%) and plant regeneration through organogenesis (62.8±0.55%) and somatic embryogenesis (74.4±0.35%) [Supple. Table 2]. At such concentration, calli were induced as early as 10th day of primary culture [Table 1] and were proliferated with rapid growth. However, Abiri *et al.* [9] reported that a much lower concentration of CH (100 mg/l) stimulated somatic embryogenesis and plant regeneration in Malaysian rice cv. MR 219.

3.1.2. Proline

Auxin-induced somatic embryogenesis in the presence of proline is well documented [8]. Proline is reported to have role in the initiation and maintenance of embryogenic calli [10]. Free proline acts as an osmoticum, a nitrogen storage pool and source of NADP + necessary for rapidly growing embryos. The tissues grown in controlled condition in artificial nutrient media undergo a kind of *in vitro* stress simulating to drought and/or cold stress. The growth of calli will naturally be hampered and in some recalcitrant species, callus induction, its growth, and nature of calli become extremely affected. Proline accumulation is a common phenomenon in response to abiotic stresses. Proline acts as osmotic stabilizer. Plant species sensitive to abiotic stresses, accumulate lower level of proline under stressful condition and these species need extraneous supplementation of proline to the medium to sustain normal growth and development of calli.

In the present investigation, addition of proline with increased concentration elicited marginal increase in callus induction frequency and it was highest (82.5±0.15%) at 150 mg/l with optimum growth of calli [Table 1]. Organogenic response remained more or less unaltered (66.5–66.8%) with increased concentration up to 100 mg/l and it was suddenly increased to 68.8 + 0.63% at 150 mg/l, but it marginally improved somatic embryogenic response (76.8 + 0.65%) [Supple. Table 3]. Somatic embryogenesis and regeneration was reported to be enhanced when proline was added to the medium along with 2,4-D [11]. Saharan *et al.* [12] and Pawar *et al.* [13] successfully induced somatic embryogenic calli in MS basal medium containing elevated level of proline (500 mg/l) and 2.0–2.5 mg/l 2, 4-D. However, Abiri *et al*. [9] reported much lower concentration of proline (50mg/l) to stimulate somatic embryogenesis and plant regeneration in Malaysian rice cv. MR 219.

3.1.3. Carbon source

Sucrose – a disaccharide of glucose and fructose serves as the chief source of carbon and energy. Besides, it has role in cellular osmotic adjustment by altering cell wall properties [14] and modulation of

gene expression by acting as chemical signal in plants [15]. It remains metabolically stable at pH 5.5–5.8 and even at autoclave conditions while sterilization of the media. It is accumulated in the cell as starch which gets converted to simple sugars by sucrolytic enzymes and acid invertase in the cell to meet heavy demand of energy during callus growth and morphogenetic differentiation [16].

In the present study, glucose, sucrose, and maltose at varying concentrations (2.0–4.0%) were tried [Supple. Table 4]. Glucose at 2.5% induced callus as early as 8 days of primary culture, but sub-culturing was needed at short intervals (12–15 days) to maintain growth of calli. In this context, organogenesis induced favorably by 3% sucrose whereas, still lower concentration (2.5%) of it proved to be better for somatic embryogenic plant regeneration [Table 1]. However, increased sucrose content resulted decline in callus induction frequency and morphogenetic response possibly due to decrease in the cellular water content. Thus, sucrose seems to be the best source of carbon for plant regeneration, followed by glucose and maltose [17] and it is an absolute requirement for embryogenic callus formation [18] in Japonica rice.

3.1.4. Gelling agents

A solidifying agent is universally added to the medium to support (or hold) the explants and calli at a stationary state on the medium. Agar is widely used for *in vitro* culture of mature seeds in rice and other crops though other gelling agents, for example, gelrite and phytagel are used either singly or both or in combination with agar in certain cases to standardize the medium [19,20].

In the present investigation, 0.3% agar + 0.2% phytagel revealed rapid callus growth and highest callusing response (85.2 + 0.51%) [Table 1]. Using such a combination and concentration of gelling agents, organogenic and somatic embryogenic regeneration response (78.2 ± 0.45% and 82.0 ± 1.02%, respectively) was also appreciably increased over 6% agar used alone [Supple. Table 5]. This is in agreement with Sahoo *et al.* [19].

3.2. Optimization of Culture Conditions

3.2.1 Photoperiod

In general, callus cultures from caryopsis of rice are incubated under dark until onset of shoot morphogenesis [20,21]. However, Luo *et al*. [22] observed slightly better calli that grew well in the light than in the dark condition. Revathi and Pillai [23] and Roy *et al.* [24] observed satisfactory callus induction and plant regeneration in dark but, Wani *et al.* [25] obtained similar result under continuous fluorescent light in growth camber at an ambient temperature of 25 ± 2°C. In the present investigation, light intensity of 2500 lux was maintained for different photoperiod treatments at 25 ± 1°C and 68% RH. There was a progressive increase in callus induction frequency and callus growth with reduction of 4 h photoperiod per day. Complete dark was shown to be conducive for higher frequency of callus induction (89.6 ± 0.91%) as well as callus growth in the callus induction medium [Table 1]. However, 12 h photo period was optimum for organogenic response (80.2 ± 0.88%) and for maturation of somatic embryos and their follow-up plant regeneration in the regeneration medium [Supple. Table 6]. In contrast, Verma *et al*. [26] and Vikrant *et al.* [27] reported 16 h photoperiod at 25°C to be optimum for both callus induction and plant regeneration.

3.2.2. Extent of desiccation

Desiccation due to partial dehydration of regenerative calli for 48 h was found to yield positive response on both organogenic and somatic embryogenic regeneration. Comparatively, somatic embryogenic regeneration frequency increased over the organogenic response [Table 1, Supple. Table 7]. Partial air desiccation pre-treatment of calli for 45 h gave maximum green plant regeneration (76.19%) in cv. BRRI Dhan 32 and it was 2-3 fold increase than the control [28]. Similarly, Saharan *et al.* [12] recorded maximum shoot regeneration frequency (63%) in partially desiccated calli and it significant differed from non-desiccated calli. Further, transgenic shoots *in vitro* culture regenerated much faster on desiccation of calli and as such improved transformation efficiency by 77% [29]. Desiccation can also induce plant regeneration even in non-regenerative calli which might be due to elicitation of genes related to morphogenetic potential of plants. Desiccation resulting 20% loss of fresh weight of callus was reported to increase the regeneration frequency significantly in four Australian rice varieties [30]. Similar simple dehydration treatment was reported to promote somatic embryogenic plant regeneration in indica [31] and japonica [32] rice. Besides, dehydration coupled with starvation (without medium) and higher level of ABA biosynthesis might have provoked the cellular biochemical and physiological change, which is necessary for efficient plant regeneration.

3.2.3. Age of calli

Repeated sub-culturing at high concentration of 2,4-D (2.0–2.5 mg/l) in the sub-culture medium for callus proliferation may lead to increased chromosomal instability which otherwise hinders plant regeneration and plant survival. Therefore, information relating to extent of regenerability of callus cultures at different ages is a priori for recovery of higher frequency of plantlet regeneration. Sustenance of regeneration capacity until 9–10 weeks is essential to recover plants from transformed sectors after allowing two or three cycles of selection [7]. In the present investigation, about 85% of the calli showed organogenic plant regeneration and more than 88% of calli induced somatic embryogenic plant regeneration after 4 weeks of culture [Table 1]. Such calli produced profuse microtillers [Figure 3a] in regeneration medium (MS + 2 mg/l BAP + 0.5 mg/l NAA) added with 500mg/l adenine sulfate and traces of thidiazuron (TDZ) (0.01 mg/l), but microtillering capacity decreased with the age of the calli beyond 4 weeks of culture [Supple. Table 8]. In fact, the calli sustained regeneration capacity even after 16 weeks (four passages of subculture, each with 4 week duration) though there was a slow and gradual decline in regenerability and survival of plants. This may be due to *in vitro* induced genome stress leading to transposable element-mediated chromosomal repatterning and altered gene regulation. Further, it envisaged that the occurrence of somaclonal variation is more likely among the regenerants from long term callus cultures than direct regeneration or early generation calli.

3.3. Optimization of Hormonal Concentrations for Rhizogenesis

In contrast to somatic embryogenic plantlets, organogenic calli-derived plants usually devoided of roots and hence, required an additional step to induce rooting. Auxin alone or with very low concentrations of cytokinin is important for induction of root primordia [33]. However, it is not always true. Excised shoots when transferred to hormone-free MS [31] or half-strength MS basal medium either liquid [26] or in solid form [25] induced rooting.

In the present investigation, full strength MS basal medium failed to develop roots [Table 2]. However, half – strength MS medium was shown to initiate healthy rooting with few laterals, although % – response of rooting from the excised shoots was poor (45.08 + 0.72%). This is because low salt levels and more specifically a lower nitrogen level is usually favorable for root initiation. Therefore, an attempt was taken to optimize the hormonal combination at varying concentrations in half strength MS basal medium. NAA at 1.0mg/l with increased

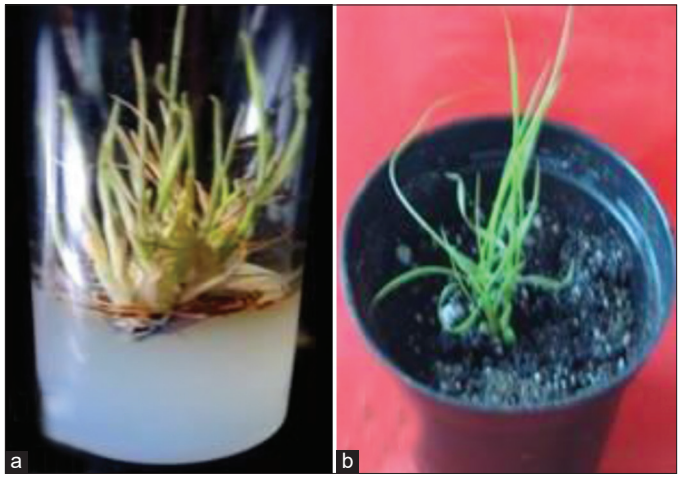

Figure 3: *In vitro* micro tiller formation (a) using MS + 2 mg/l BAP + 0.5 mg/l NAA + 500 mg/l adenine sulfate + 0.01 mg/l thidiazuron in cv. "Chittimutyalu." Plantlet establishment in pot mixture (b) of cv. "Chittimutyalu."

Table 2: Effect of different hormonal concentrations on rhizogenesis of plantlets of cv. "Chittimutyalu".

Hormone recipe (mg/l)	% response	Remark (s)
MS basal medium	0.0	No response
½ MS basal medium	45.08±0.72*	Healthy roots with few laterals
½ MS basal+NAA+BAP		
½ MS basal+1.0+0.1	54.8±1.08	Weak roots without laterals
½ MS basal+1.0+0.2	86.6±0.85	Profuse normal rooting within a week
½ MS basal+1.0+0.3	52.6±0.94	Rooting delayed, short and fibrous
½ MS basal+1.0+0.4	0.0	No response, shoots remain fresh for a few days
½ MS basal+1.0+0.5	0.0	No response, shoots remain fresh for 1 week

*Values are mean+S.E

BAP up to 0.2 mg/l gave highest rhizogenetic response (86.6 + 0.85 %) and the excised shoots developed profuse normal rooting within a week. Further, increase in BAP (0.3 mg/l) at 1.0 mg/l NAA had shown delayed rooting with short fibrous roots, and even no rooting response at concentrations beyond 0.3 mg/l BAP. In contrast, Bano *et al.* [11] reported that 0.5 mg/l BAP with 0.3 mg/l IAA was sufficient for induction of roots in the regenerated plantlets.

The plantlets with healthy roots were transferred to pot mixture (peat moss: perlite 2:1), and successfully acclimatized in glasshouse under partial shade [Figure 3b]. The plants regenerated from first few callus cultures were phenotypically normal and fertile. The *in vitro* protocol formulated in this study may be suitably used for *in vitro* mutagenesis and Agrobacterium mediated genetic transformation in zinc rich rice.

4. CONCLUSION

High throughput somatic embryogenic callus induction, proliferation, and follow-up rapid plant regeneration are in fact needed ready-in-hand for success in genetic transformation. A number of media supplements including CH, proline, various sources of carbon and gelling agents; and culture conditions, for example, photoperiod, desiccation pre-treatment, and age of calli have been verified for maximum callusing response and morphogenetic potential in a zinc rich rice cv. "Chittimuthyalu." Besides, the role of TDZ over traditionally used cytokinins, for example, BAP and Kinetin for huge number (microtillering) of plant regeneration has been demonstrated. Supplementation of 500 mg/l CH, 150 mg/l proline, 3% sucrose, 0.3% agar + 0.2% phytagel revealed rapid callus induction and highest callusing response under dark condition. Somatic embryogenic plant regeneration was improved at 2.5% sucrose under 12-h photoperiod using 4 weeks old partially desiccated calli. The high throughput rapid somatic embryogenic regeneration system developed in this study can be amenable for genetic transformation for biotic and abiotic stress tolerance and improvement in quality traits in zinc rich rice.

5. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

6. FUNDING

There is no funding to report.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

REFERENCES

1. Roohani N, Hurrell R, Kelishadi R, Schulin R. Zinc and its importance for human health: An integrative review. Journal of Research in Medical Science. 2013; 18: 144-157.
2. Wang LC, Busbey S. Images in clinical medicine acquired Acrodermatitis enteropathica. The New England Journal of Medicine. 2005; 352: 1121.
3. Sadeghzadeh B. A review of zinc nutrition and plant breeding. Journal of Soil Science and Plant Nutrition. 2013; 13 (4): 905-927.
4. Tripathy Swapan K., Maharana M, Panda S, Sahoo B, Sahoo DB, Behera SK, *et al*. Exploring efficient callusing and rapid regeneration system in upland rice (*Oryza sativa* L.). Rice Genomics and Genetics. 2018; 9(2): 7-14.
5. Indoliya Y, Tiwari P, Chauhan AS, Goel R, Manju Shri, Bag SK, Chakrabarty D. Decoding regulatory landscape of somatic embryogenesis reveals differential regulatory networks between japonica and indica rice subspecies. Scientific Reports. 2016; 6: 23050. https://doi.org/10.1038/srep23050

6. Dafaallah AB. 13 Design and Analysis of Factorial Experiments using Completely Randomized Design (CRD). 2019; University of Gezira, Sudan. doi: 10.13140/RG.2.2.29684.71045
7. Thi Linh H, Yenchon S and Te-chato S. Effects of culture media and plant growth regulators on callus induction and regeneration of indica rice (*Oryza sativa* L. cv. Sangyod). Songklanakarin Journal of Plant Science. 2019; 6(1): 48-58.
8. Khaleda L, Al-Forkan M. Stimulatory effect of casein hydrolysate and proline in *in vitro* callus induction and plant regeneration from five deep water rice (*O. sativa* L.). Biotechnology. 2006; 5(3):379-384.
9. Abiri R, Maziah M, Valdiani A. Enhancing somatic embryogenesis of Malaysian rice cultivar MR 219 using adjuvant materials in a high efficiency protocol. International Journal of Environmental Science &Technology. 2017; 14: 1091-1108.
10. Das NK, Hoque H, Hasan MN, Prodhan SH. Effect of plant growth regulators and proline in efficient regeneration of recalcitrant indica rice (*O. sativa* L.). Journal of Biological Science. 2019; 19(4):290-299.
11. Bano S, Jabeen M, Rahim F, Ilahi I. Callus induction and regeneration in seed explants of rice (*Oryza sativa* cv. Swat-ii). Pakistan Journal of Botany. 2005; 37(3): 829-836.
12. Saharan V, Yadav RC, Yadav NR, Chapagain BP. High frequency plant regeneration from desiccated calli of indica rice (*Oryza sativa* L.). African Journal of Biotechnology. 2004;3(5): 256-259.
13. Pawar BD, Kale PB, Bahurupe J, Jadhav A, Kale A, Pawar S. Proline and glutamine improve *in vitro* callus induction and subsequent shooting in rice. Rice Science. 2015; 22(6): 283-289.
14. Yaseen M, Ahmad T, Sablok G, Standardi A, Hafiz IA. Review: role of carbon sources for in vitro plant growth and development. Molecular Biology Reports. 2013; 40: 2837-2849. https://doi.org/10.1007/s11033-012-2299-z
15. Tognetti JA, Pontis HG, Martínez-Noël GM. Sucrose signaling in plants: a world yet to be explored. Plant Signal Behaviour. 2013; 8(3):e23316. doi:10.4161/psb.23316
16. Yadav SK, Singh V, Narayana JL, Vanaja M, Tiwari Y, Patil A, Nagendram E, Venkateswarlu B. Carbohydrates and sucrose metabolizing enzymes in the leaves of *Vigna mungo* genotypes as influenced by elevated CO_2 concentration. Journal of Agricultural Science and Technology. 2013; 15(6):1107-1120.
17. Annonymous. The components of plant tissue culture media ll:organic additions, osmotic and pH effects and support systems. In: E. F. George *et al.* (eds.), Plant Propagation by Tissue Culture 3rd Edition, © 2008 Springer,2008; p.115-173.
18. Lee K, Jeon H, Kim M. Optimization of a mature embryo-based *in vitro* culture system for high-frequency somatic embryogenic callus induction and plant regeneration from japonica rice cultivars. Plant Cell, Tissue and Organ Culture. 2002; 71(3): 237-244.
19. Sahoo KK, Tripathi AK, Pareek A, Sopory SK and Singla-Pareek SL. An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars. Plant Methods. 2011; 7: 49.
20. Khatun R, Islam Shahinul SM, Bari Miah MA. Studies on plant regeneration efficiency through *in vitro* micropropagation and anther culture of twenty five rice cultivars in Bangladesh. Journal of Applied Sciences Research. 2010; 6(11): 1705-1711.
21. Evangelista FC, Aldemita RR, Ungson LB. Callusing and regeneration potential of rice(*Oryza sativa* L.) genotypes towards the development for salt tolerance. Philippine Journal of Science.2009; 138: 169-176.
22. Luo TK, Zhang XL, Zhang XY, Liu Q, Zhu XF, Lin GS. Effects of the proportion of NH_4^+ and NO_3- on callus formation and plant regeneration of embryo of indica rice(Zhenshan 97). Journal of Yunnan Agriculture University. 2004; 19(2): 131-134.
23. Revathi S, Pillai AM. *In vitro* callus induction in rice (*Oryza sativa* L.). Research in Plant Biology. 2011; 1(5): 13-15.
24. Roy A, Aich SS, Mukherjee S. Differential responses to indirect organogenesis in rice cultivars. International Journal of Science and Research Publication. 2012; 2(9): 1-5.
25. Wani SH, Sofi Parvez A, Gosal Satbir S, Singh Naorem B. *In vitro* screening of rice (*Oryza sativa* L) callus for drought tolerance. Communications in Biometry and Crop Science. 2010; 5(2): 108-115.
26. Verma D, Joshi R, Shukla A, Kumar P. Protocol for *in vitro* somatic embryogenesis and regeneration of rice (*O. sativa* L). Indian Journal of Experimental Biology. 2011; 49(12): 958-963.
27. Vikrant MR, Khurana P . Somatic embryogenesis from mature caryopsis culture under abiotic stress and optimization of Agrobacterium-mediated transient GUS gene expression in embryogenic callus of rice (*Oryza sativa* L.). Journal of Phytology. 2012; 4(5):16-25.
28. Siddique AB, Ara I, Sahinul I, Tuteja N. Effect of air desiccation and salt stress factors on *in vitro* regeneration of rice (*Oryza sativa* L.). Plant Signalling & Behaviour. 2014; 9(12):e977209.
29. Basu D, Veluthambi K. Partial desiccation of scutellum derived rice callus improves Agrobacterium mediated transformation. Journal of Bio Innovation. 2016; 5(5):632-642.
30. Pilahome W, Bunnag S and Suwanagul A. Development of a Plant Regeneration System from Seed-derived Shoot Segments of Rice (*Oryza sativa* L.). Asian Journal of Crop Science. 2014; 6: 305-319. doi:10.3923/ajcs.2014.305.319
31. Ikram-Ul-Haq, Chang-Xing Z, Mukhtar Z, Jaleel CA, Azooz MM. Effect of physical desiccation on plant regeneration efficiency in rice (*Oryza sativa* L.) variety super basmati. Journal of Plant Physiology. 2009; 166(14):1568-75.
32. Wagiran A, Ismail I, Radziah C, Zain CM, Abdullah R. Improvement of plant regeneration from embryogenic suspension cell culture of japonica rice. Journal of Biological Sciences. 2008; 8(3): 570-576.
33. Su YH, Liu YB, and Zhang XS. Auxin–Cytokinin Interaction Regulates Meristem Development. Molecular Plant. 2011; 4(4): 616-625. doi: 10.1093/mp/ssr007

How to cite this article:
Tripathy SK. Optimization of culture variables for efficient callus induction and rapid plant regeneration in zinc rich rice (*Oryza sativa* L.) cv. "Chittimuthyalu." J App Biol Biotech. 2021;9(4):1-9.
DOI: 10.7324/JABB.2021.9401

SUPPLEMENTARY TABLES

Supple. Table 1: Characteristic features of zinc rich rice cv. "Chittimatyalu" pooled over 2 years in advance varietal trial -2 under AICRP on rice, India in wet season.

Trait	2016	2017	Mean
1. Iron content (ppm)*	5.1	3	4.05
2. Zinc content (ppm)*	23.01	23.9	23.45
3. Protein content (%)*	9.02	9.6	9.31
4. Amylose content (AC%)	24.11	23.52	23.81
5. Head rice recovery (HRR %)	66.5	65.7	66.1
6. Days to 50% flowering	104	106	105
7. Plant height (cm.)	89	94	91.5
8. Panicles/m^2	277	283	280
9. Grain yield (kg/ha)	4005	4597	4301

*Data of trait Sl. No. 1–3 are based on polished rice samples

Supple. Table 2: Effect of different concentrations of casein hydrolysate on callus induction and plantlet regeneration of cv. "Chittimatyalu".

Concentration (mg/l)	Callus induction			Plant Regeneration	
	Days first callus observed	CIF (%)	Callus growth	Organogenic response (%)	Somatic embryogenic response (%)
0	11	50.4±0.85*	+++	36.2±0.32*	68.0±0.62*
100	12	68.0±0.75	+++	45.2±0.90	69.5±0.82
200	12	70.9±1.03	+++	50.6±0.52	70.5±0.70
300	10	72.4±1.25	++++	55.8±0.47	72.0±0.58
400	11	75.4±1.08	+++	58.0±0.83	73.8±0.68
500	10	79.9±0.42	+++++	62.8±0.55	74.4±0.35
600	11	74.8±0.47	+++	52.2±1.05	66.2±1.06
800	12	71.2±0.23	+	40.2±0.82	60.2±0.82
1000	14	65.8±1.25	+	32.6±0.72	68.6±0.92
$C.D_{0.05}$		4.40		4.70	3.80

*Values are mean+S.E, [a]MS media with 2.5 mg/l 2,4-D+0.5 mg/l Kn, [b]MS media with 2.0 mg/l BAP+0.5 mg/l NAA

Supple. Table 3: Effect of different concentrations of proline on callus induction and regeneration frequencies of cv. "Chittimutyalu".

Concentration (mg/l)	Callus induction			Plant regeneration	
	Days first callus observed	CIF (%)	Callus growth	Organogenic response (%)	Somatic embryogenic response (%)
25	15	78.8±1.01*	+++	66.5±1.12*	70.0±0.50*
50	14	80.2±1.05	+++	66.9±0.62	71.2±0.60
100	12	81.0±0.63	++++	66.8±0.57	75.5±0.75
150	**10**	**82.5±0.05**	**++++**	**68.8±0.63**	**76.8±0.63**
200	14	77.8±0.13	++	46.8±1.25	66.0±1.20
300	18	75.2±0.62	+	56.2±1.01	60.2±1.05
400	20	75.8±1.08	+	50.2±0.32	56.8±0.62
500	21	74.4±1.15	+	52.6±0.62	50.6±0.82
$CD_{0.05}$		4.62		5.41	5.10

*Values are mean+S.E, [a]MS media with 2.5 mg/l 2,4-D+0.5 mg/l Kn, [b]MS media with 2.0 mg/l BAP+0.5 mg/l NAA

Supple. Table 4: Effect of different levels of carbon source (glucose, sucrose and maltose) on callus induction and regeneration frequencies of cv. "Chittimutyalu".

Carbon source	Concentration (%)	Callus induction			Plant regeneration	
		Days first callus observed	CIF (%)	Callus growth	Organogenic response (%)	Somatic embryogenic response (%)
Glucose	2.0	9	76.2±0.28*	+++	77.2±0.22*	47.2±0.32*
	2.5	8	80.0±0.22	+++	80.6±0.62	50.6±0.42
	3.0	10	83.8±0.62	++++	83.8±0.27	54.8±0.37
	3.5	12	80.4±0.62	+++	78.0±0.37	55.0±0.27
	4.0	14	75.9±0.27	+++	76.8±0.43	45.8±0.53
Sucrose	2.0	14	67.8±0.53	++	66.2±1.08	65.2±1.20
	2.5	12	75.2±1.05	++	**80.2±1.00**	**76.2±1.02**
	3.0	**10**	**80.6±0.35**	++++	75.5±0.47	71.6±0.28
	3.5	11	75.0±0.42	+++	76.0±1.08	70.8±0.46
	4.0	16	63.0±0.25	++	71.6±0.72	65.8±0.52
Maltose	2.0	18	47.0±0.29	+++	29.8±0.47	22.9±0.47
	2.5	17	53.8±0.33	+	34.2±0.43	26.2±0.33
	3.0	15	57.0±0.36	+	42.0±1.05	36.0±1.05
	3.5	12	60.5±0.25	+	43.8±0.45	38.0±0.35
	4.0	15	56.2±1.02	+	40.2±1.0	32.2±1.01
$CD_{0.05}$			5.31		4.16	4.58

*Values are mean+S.E, [a]MS media with 2.5 mg/l 2,4-D+0.5 mg/l Kn, [b]MS media with 2.0 mg/l BAP+0.5 mg/l NAA

Supple. Table 5: Effect of different concentrations of gelling agents (Agar, gelrite, and phytagel) on callus induction and plantlet regeneration of cv. "Chittimutyalu".

Gelling agent (s)	Concentration (%)	Callus induction			Plant Regeneration	
		Days first callus observed	CIF (%)	Callus growth	Organogenic response (%)	Somatic embryogenic response (%)
Agar	0.6	18	74.8±0.85d*	++	62±0.52*	61.8±0.50*
	0.7	15	75.0±0.90	+++	75.6±0.72	67.6±1.02
	0.8	13	81.5±1.00	++++	76.0±1.10	70.6±1.01
Gelrite	0.3	14	79.2±1.00	+++	57.1±1.00	55.6±0.48
	0.4	12	76.6±0.93	+	58.8±1.03	55.1±0.90
	0.5	15	46.0±0.55	+	56.7±1.00	52.6±0.58
Phytagel	0.3	12	77.6±1.03	+	59.2±0.42	57.6±0.68
	0.4	12	79.0±0.72	+++	62.8±0.52	63.6±0.60
	0.5	15	75.6±0.82	++	59.2±0.62	62.9±0.91
Agar+Gelrite	0.2+0.2	14	75.5±0.72	++	48.6±0.60	43.6±0.42
	0.3+0.2	14	77.4±0.90	+++	52.7±0.47	52.8±0.57
	0.4+0.2	12	72.5±1.00	++	50.4±0.43	54.8±0.73
Agar+Phytagel	0.2+0.2	12	81.8±1.05	+++	73.8±0.93	70.8±1.05
	0.3+0.2	**10**	**84.2±0.61**	+++++	**80.2±0.45**	**76.2±1.08**
	0.4+0.2	16	75.0±1.01	++	76.5±0.72	68.2±1.00
Gelrite+Phytagel	1.5+1.5	15	54.5±0.52	+	63.6±0.82	56.4±0.60
	0.2+0.2	13	69.0±1.03	++	61.8±0.62	61.0±0.72
	0.25+0.25	17	65.5±0.42	+	62.0±0.60	52.0±0.42
$CD_{0.05}$			3.88		3.69	5.23

*Values are mean+S.E, [a]MS media with 2.5 mg/l 2,4-D+0.5 mg/l Kn, [b]MS media with 2.0 mg/l BAP+0.5 mg/l NAA

Supple. Table 6: Effect of photoperiod on callus induction and plantlet regeneration of cv. "Chittimutyalu ".

Photo period (Light/dark in hours)	Light intensity (Lux)	Callus induction		Plant Regeneration	
		CIF (%)	Callus growth	Organogenic response (%)	Somatic embryogenic response (%)
24/0	2500	52.2±0.65*	+	60.0±0.85*	62.0±0.92*
20/4	2500	61.5±1.02	++	68.0±0.70	63.0±0.70
16/8	2500	66.0±0.01	++	72.0±0.29	68.6±1.00
12/12	2500	72.4±0.90	+++	76.5±0.88	74.0±0.85
8/16	2500	74.0±0.65	+++	63.0±1.05	65.0±1.00
4/20	2500	76.0±0.65	++++	52.0±1.00	58.0±0.85
0/24	2500	80.2±0.91	+++++	25.8±1.05	25.0±1.00
$CD_{0.05}$		4.53		5.81	5.33

*Values are mean+S.E, [a]MS media with 2.5 mg/l 2,4-D+0.5 mg/l Kn, [b]MS media with 2.0 mg/l BAP+0.5 mg/l NAA

Supple. Table 7: Effect of partial desiccation (48 h) on organogenic and somatic embryogenic plant regeneration in cv. "Chittimutyalu".

Desiccation	Organogenic response	Somatic embryogenic response
No desiccation	80.5±0.65*	82.3±0.45
Partial desiccation	82.7±1.02	85.7±0.87

*Values are mean+S.E, [a]MS media with 2.5 mg/l 2,4-D+0.5 mg/l Kn, [b]MS media with 2.0 mg/l BAP+0.5 mg/l NAA

Supple. Table 8: Organogenic and somatic embryogenic regeneration frequency of calli at different ages in cv. "Chittimutyalu".

Age of calli	Organogenic response (%)	Somatic embryogenic response (%)
After 4 weeks	85.3±0.65*	88.0±0.45*
After 8 weeks	67.6±0.70	74.8±0.70
After 12 weeks	40.2±0.56	45.0±0.91
After 16 weeks	21.0±0.83	28.8±1.02

*Values are mean+S.E, [a]MS media with 2.5 mg/l 2,4-D+0.5 mg/l Kn, [b]MS media with 2.0 mg/l BAP+0.5 mg/l NAA