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Research Article
Optimization of Rice (*Oryza sativa* L.) 'Tubtim Chumphae', for Callus Induction, Proliferation and Plantlet Regeneration

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Abstract

Background and Objective: Tissue culture techniques are now used to increase rice yield and quality. This technology offers wide-ranging plant improvements including development, preservation and genetic variability. A rapid and reliable protocol for callus production and plantlet regeneration of Thai rice (*Oryza sativa* L.) cultivar Tubtim Chumphae was investigated. **Materials and Methods:** Murashige and skoog (MS) medium supplemented with various concentrations of 2,4-D (0, 1, 1.5, 2 and 2.5 mg L^{-1}) and BAP (0, 0.1 and 0.5 mg L^{-1}) was used for callus induction from mature seed. High callus formation was recorded in MS medium containing 2.5 mg L^{-1} 2,4-D alone and medium with 2 mg L^{-1} 2,4-D in combination with 0.5 mg L^{-1} BAP, giving callus induction at 96.42 and 92.86%, respectively. The effect of chitosan on callus growth was determined using an MS medium supplemented with 2 mg L^{-1}2,4-D and 0.5 mg L^{-1} BAP, combined with chitosan concentrations of 0, 25, 50 and 100 ppm. **Results:** Results indicated that 50 ppm chitosan treatment was the most suitable for callus growth. Three weeks old calli were cultured on MS medium comprising 0.5 mg L^{-1} NAA with various concentrations of BAP (0, 1, 2, 3, 4 and 5 mg L^{-1}). The highest regeneration performance was obtained in 0.5 mg L^{-1} NAA with 3 mg L^{-1} BAP, with new plantlets completely regenerated within 4 weeks. **Conclusion:** These research results can be used as a starting point for *in vitro* rice propagation based on demand and provide valuable important information that can be used in combination with other agronomic traits in rice improvement or breeding programs to increase the adaptability of tolerant cultivars under abiotic stress.

Key words: Callus, chitosan, growth performance, MS medium, multiple shoot, regeneration, rice

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Rice (*Oryza sativa* L.) is the world's most widely farmed cereal crop and an economically significant staple food for more than 80% of Asians[1]. Various abiotic and biotic stresses negatively impact rice yield and quality[2]. Utilizing natural varieties through traditional breeding has shown significant progress but rice production is still unable to meet rising demand. The success of biotechnological techniques, especially tissue culture, is now critical to achieving increased rice productivity and quality. Tissue culture systems offer plant improvements through development, preservation and genetic variability[3]. Callus induction and plantlet regeneration are important prerequisites for other advanced biotechnological techniques such as genetic transformation and *in vitro* and *in situ* hybridization[4]. However, the lack of an effective method for regenerating new plantlets derived from callus is a major impediment to crop improvements by genetic modification for many plant species[5]. Various explant rice varieties have been used for callus development and plantlet regeneration including panicle[6], leaf[7], root[8] and anther[9]. Seeds are the most popular explants for rice callus induction[10-13]. Auxins and cytokinins are key hormones that assist rice- development from callus to plantlet[12,14-15], while chitosan is a chemical compound that is frequently used in *in vitro* breeding, with several advantages in plant development[16] including promoting plant growth. Chitosan has low cost[17] as a non-toxic substance that is safe for both humans and the environment[18].

Tubtim Chumphae rice (SRN06008-18-1-5-7-CPA-20) is a hybrid cultivar between KDML105 (Mother)×Sangyod Phatthalung (Father). Tubtim Chumphae rice is a photoperiod insensitive cultivar with upright clumps. The brown rice becomes soft after cooking and develops a clear red ruby colour with high antioxidant, phenolic, flavonoid and vitamin E contents, other important substances including gallic acid, myricetin, luteolin, kaempferol, apigenin and cyanidin-3-glucoside[19]. Amylose content was low compared with other commercial Thai rice cultivars[19] and beneficial for diabetics and people wishing to control their sugar intake. Contained hydrolysate substances such as Tubtim Chumphae Rice Bran Hydrolysates (TCRH) reduce oxidative stress and improve blood vessel relaxation. This rice cultivar is considered tolerant to salinity stress, drought stress and some biotic stresses[20]. Here, a modified murashige and skoog (MS) medium was used to regulate callus formation, proliferation and multiple shoot regeneration of Tubtim Chumphae rice by plant growth regulators and chitosan. The findings of this study can be utilized as the first step of *in vitro* propagation to improve rice production and satisfy demand.

MATERIALS AND METHODS

Study area: This study project was performed from September, 2021 to February, 2022.

Callus induction: Tubtim Chumphae rice seeds were kindly provided by Ms. Chanyapat Visaetjirakul, an organic farmer from Bang Pahan District, Ayutthaya Province, Thailand. Mature Tubtim Chumphae rice seeds were used as explants for the callus induction experiment. Dehusked seeds were sterilized for 20 min, with shaking in a solution of 20% (v/v) sodium hypochlorite (Clorox) mixed with 2-3 drops of Tween 20, before washing with sterile distilled water three times.

The sterilized seeds were cultivated on MS medium containing 3% (w/v) sucrose with various concentrations of 2,4-D (2,4-Dichlorophenoxyacetic acid) (0 ,1, 1.5, 2 and 2.5 mg L^{-1}) and BAP (6-Benzyl Aminopurine) (0, 0.1 and 0.5 mg L^{-1}) for 3 weeks. All cultures were subjected to a light flux density condition of 40 µmol m^{-2} sec^{-1} at 25±2°C (16/8 hrs light/dark) for 4 weeks. Each treatment had seven replicates, with four seeds cultivated in each. There were 28 samples in each treatment and 420 total samples across all treatments. Survival percentage, callus induction percentage, callus width and length and fresh and dry weight of callus were measured at 21 days.

Chitosan treatment: Calli at 0.4 cm were used as explants and cultured on MS medium supplemented with 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP, combined with various concentrations of chitosan (0, 25, 50 and 100 ppm) for 4 weeks. All cultures were grown at 25±2°C with a 16/8 hrs (light/dark) cycle and 40 µmol m^{-2} sec^{-1} flux density for 4 weeks. The survival rate, response percentage and growth performance of the calli were recorded. Callus viability was tested using the tetrazolium (2,3,5-triphenyl tetrazolium chloride, TTC) assay[21]. Explants that were alive and growing in response to the culture medium were characterized as response samples. Percentages of survival and response were determined[22] as follows:

$$\text{Survival (\%)} = \frac{\text{Final number of survival calli}}{\text{Initial number of calli}} \times 100$$

$$\text{Response (\%)} = \frac{\text{Final number of response calli}}{\text{Initial number of calli}} \times 100$$

Plantlet regeneration: Three weeks old calli were used as explants. The calli were cultured on MS medium comprising 0.5 mg L^{-1} NAA (Naphthylacetic acid) with various concentrations of BAP (0, 1, 2, 3, 4 and 5 mg L^{-1}) for 4 weeks. Six different treatments were used, each with seven replicates and four calli per replicate. All treatments were cultured at 25±2°C with a 16/8 hrs (light/dark) cycle and 40 µmol m^{-2} sec^{-1} flux density. Shoot number per callus, green spot number per callus, plant height and root length were measured.

Statistical analysis: All experiments were conducted with seven replications in a completely randomized design (CRD). One-way Analysis of Variance (ANOVA) was used to test the data, with Duncan's Multiple Range Test ($p<0.05$) used to differentiate differences between means. Results were presented as Mean±Standard error of the mean (SE).

RESULTS AND DISCUSSION

Callus induction: All plant samples exhibited survival at 100%, while only seedlings from dehusked Tubtim Chumphae seeds germinated on media without 2,4-D. The MS medium containing every concentration of 2,4-D promoted callus formation (Table 1). Appropriate media for callus induction were MS medium supplemented with 2.5 mg L^{-1} 2,4-D alone and MS medium with 2 mg L^{-1} 2,4-D in combination with 0.5 mg L^{-1} BAP, giving callus induction at 96.42 and 92.86%, respectively. The fresh and dry weights of both treatments were not significantly different. For callus formation, the highest callus size (0.48×0.66 cm) was found in the treatment of 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP (Table 1). Tubtim Chumphae calli were generally creamy or yellow, compact and granular in shape (Fig. 1).

Plant growth regulators (PGRs) are important factors in callus formation. The ratio of auxins and cytokinins in medium cultivation determines the efficacy of callus induction. The 2,4-D is the most common auxin utilized in callus induction, while BAP is the most common cytokinin. Considering callus formation performance, callus induction of Tubtim Chumphae rice was optimized using 2 mg L^{-1} 2,4-D without additional PGRs or with 0.5 mg L^{-1} BAP. Similar results were recorded in other rice cultivars including Jow Haw rice[11], Panderas rice[10] and Pakaumpuel rice[13].

The auxin 2,4-D was most effective at suppressing organogenesis and triggering cell proliferation in the scutellum and mesocotyl to generate callus[23]. Rice callus induction was achieved with only 2 mg L^{-1} 2,4-D[24,25] but the addition of more cytokinin such as kinetin or BAP[26] or auxin such as NAA[10] was suitable for callus induction of some plant species. Callus was successfully formed in some plant species by culture with relatively high auxin and cytokinin[27-29].

Chitosan treatment: Growth performances of calli after culture on MS medium containing 2 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BAP in combination with various concentrations of chitosan for 4 weeks were recorded. Results showed that survival rates and response percentages of treated calli were 100% in all treatments including the control group (Fig. 2a). The highest growth performance was presented in the 50 ppm treatment (Fig. 2b, c), while all parameters were significantly higher than other treatments including the control group. Higher concentrations of chitosan resulted in a high green spot number per callus in this study (data not shown) (Fig. 3). This result was inconsistent with a previous study that chitosan impacted plant growth by reducing protocorm proliferation and plantlet regeneration of *Phalaenopsis* orchid[30].

Table 1: Characteristics of calli derived from Tubtim Chumphae rice seed after cultured on MS medium containing various concentrations of 2,4-D and BAP for 21 days

PGRs (mg L^{-1})							
BAP	2,4-D	Survival (%)	Callus formation (%)	Width (cm)	Length (cm)	FW (mg)	DW (mg)
0	0.0	100.00±0.00^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
0	1.0	100.00±0.00^{a}	35.71±12.02^{b}	0.23±0.06^{b}	0.33±0.09^{ab}	18.90±0.00^{ab}	3.57±0.00^{ab}
0	1.5	100.00±0.00^{a}	75.00±9.44^{ab}	0.25±0.04^{b}	0.41±0.04^{ab}	100.00±0.03^{a}	4.29±0.00^{ab}
0	2.0	92.85±7.14^{b}	75.00±7.71^{ab}	0.21±0.05^{b}	0.32±0.08^{ab}	21.40±0.00^{ab}	2.71±0.00^{ab}
0	2.5	100.00±0.0^{a}	96.42±3.57^{a}	0.22±0.03^{b}	0.45±0.02^{ab}	15.70±0.00^{b}	3.34±0.00^{b}
0.1	0.0	100.00±0.00^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
0.1	1.0	100.00±0.00^{a}	82.14±7.14^{ab}	0.31±0.02^{b}	0.47±0.04^{ab}	28.60±0.00^{ab}	5.43±0.00^{ab}
0.1	1.5	96.42±3.57^{ab}	78.57±6.52^{ab}	0.49±0.05^{a}	0.59±0.11^{ab}	104.80±0.03^{a}	1.10±0.00^{ab}
0.1	2.0	92.85±4.61^{b}	82.14±7.14^{ab}	0.30±0.01^{b}	0.51±0.02^{ab}	71.40±0.04^{ab}	5.57±0.00^{ab}
0.1	2.5	96.42±3.57^{ab}	82.14±7.00^{ab}	0.24±0.04^{b}	0.43±0.07^{ab}	25.70±0.00^{ab}	13.29±0.00^{a}
0.5	0.0	100.00±0.00^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
0.5	1.0	100.00±0.00^{a}	78.57±8.50^{ab}	0.48±0.07^{ab}	0.66±0.09^{a}	92.90±0.01^{a}	12.49±0.00^{a}
0.5	1.5	96.42±3.57^{ab}	89.28±5.05^{ab}	0.31±0.03^{b}	0.50±0.02^{ab}	35.70±0.00^{ab}	7.00±0.00^{ab}
0.5	2.0	100.00±0.00^{a}	92.85±4.61^{a}	0.43±0.03^{b}	0.59±0.04^{ab}	45.70±0.00^{ab}	10.00±0.00^{a}
0.5	2.5	100.00±0.00^{a}	89.28±5.05^{ab}	0.20±0.04^{b}	0.29±0.07^{b}	20.00±0.00^{ab}	4.14±0.00^{ab}

Mean±SE values followed by different superscripts in the same column are significantly different according to ANOVA and Duncan's Multiple Range Test ($p<0.05$)

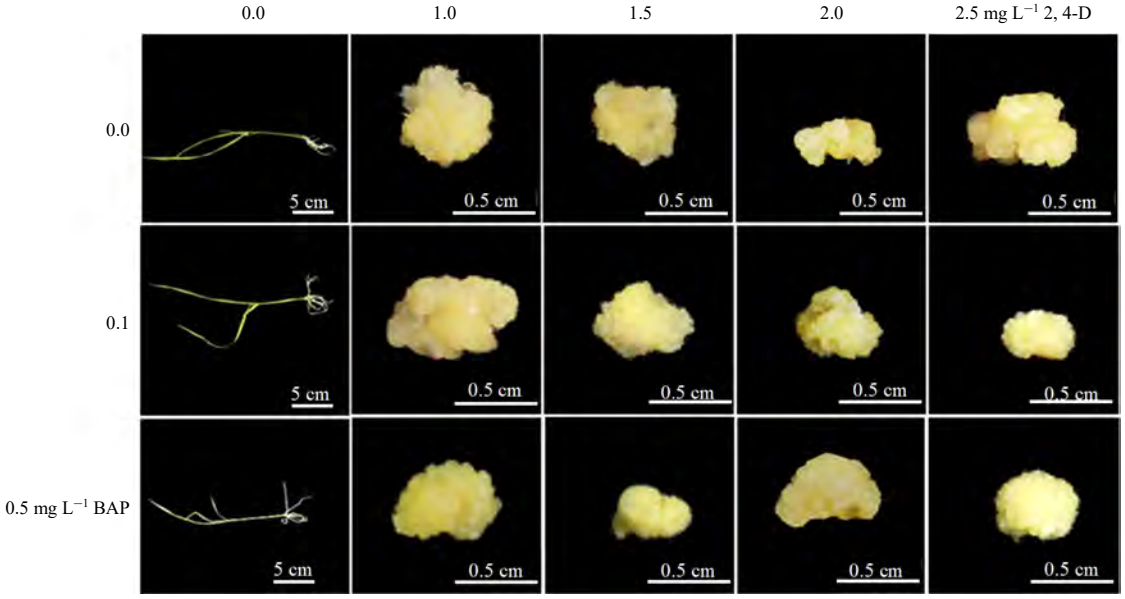

Fig. 1: Calli derived from Tubtim Chumphae rice seed after cultured on MS medium containing various concentrations of 2,4-D and BAP for 21 days

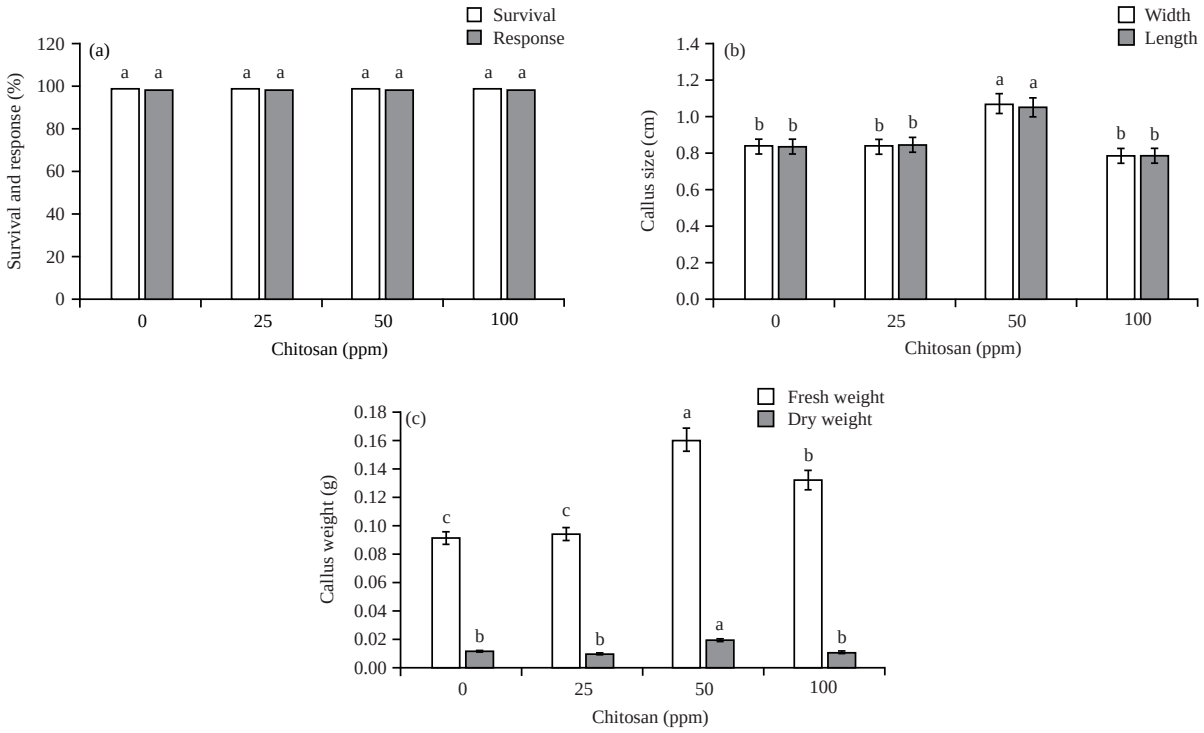

Fig. 2(a-c): Callus growth of Tubtim Chumphae rice after being treated with various concentrations of chitosan for 4 weeks, (a) Survival and response percentage, (b) Callus size and (c) Callus weight

Chitosan was effective against the most common pathogenic fungi and bacteria in rice, acting both directly on the pathogen and indirectly on resistance induction[31,32]. Moreover, chitosan treatment effectively reduced the severity of drought damage[33] and salinity stress[34] in rice plants but suitable application methods are required[35]. Different concentrations of chitosan also impacted the explants and developmental stages of different plant species. An increase

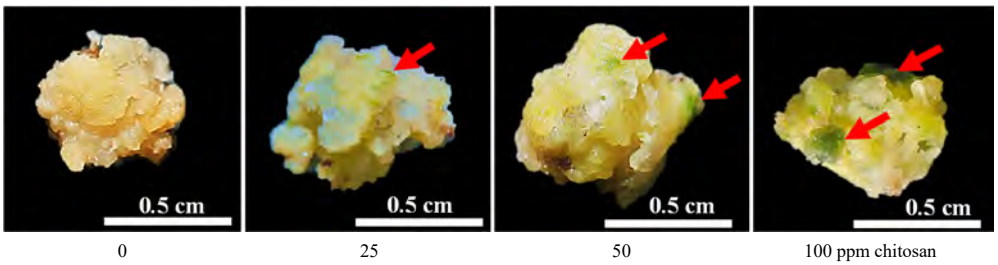

Fig. 3: Treated callus with various concentrations of chitosan presented green spot (arrows)

Fig. 4(a-f): Plantlets of Tubtim Chumphae rice after cultured on regeneration media, 0.5 mg L^{-1} NAA with various concentrations of BAP for 4 weeks, (a) 0 mg L^{-1} BAP treatment, (b) 1 mg L^{-1} BAP treatment, (c) 2 mg L^{-1} BAP treatment, (d) 3 mg L^{-1} BAP treatment, (e) 4 mg L^{-1} BAP treatment and (f) 5 mg L^{-1} BAP treatment

in callus size and fresh and dry weight by chitosan encouraged the production of hormones such as gibberellins[36]. In this study, 50 ppm chitosan was appropriate for callus growth of Tubtim Chumphae rice but a proper application technique for plantlets or *ex vitro* propagation is still required.

Plantlet regeneration: After culture on a medium supplemented with 0.5 mg L^{-1} NAA and various concentrations of BAP for 4 weeks, the calli were enlarged with a green spot. The MS medium supplemented with 0.5 mg L^{-1} NAA and 4 mg L^{-1} BAP exhibited the highest survival percentage. The 1, 4 and 5 mg L^{-1} BAP treatment presented a high green spot number, while the 5 mg L^{-1} BAP treatment presented the highest shoot number per callus. The highest root number per callus was recorded in 0.5 mg L^{-1} NAA alone (control group) (Table 2, Fig. 4a). The 1, 3, 4 and

Table 2: Survival percentage and regeneration performance of Tubtim Chumphae rice callus after 4 weeks of treatment

Plant growth regulators (mg L^{-1})					
NAA	BAP	Survival percentage	Root number	Shoot number	Green spot number
0.5	0	32.14±4.04^{ab}	11.14±2.57^{a}	1.29±0.44^{b}	1.29±0.26^{b}
0.5	1	53.57±10.10^{a}	4.08±0.92^{b}	2.57±1.03^{ab}	10.86±3.07^{a}
0.5	2	10.71±3.05^{b}	1.86±0.69^{b}	0.00±0.00^{c}	0.86±1.55^{b}
0.5	3	32.14±8.98^{ab}	4.57±1.70^{b}	3.86±1.48^{a}	7.57±2.11^{ab}
0.5	4	57.14±4.61^{a}	1.71±0.71^{b}	3.14±1.05^{ab}	11.71±1.79^{a}
0.5	5	28.57±3.57^{ab}	0.82±0.34^{b}	8.57±0.18^{a}	10.85±1.43^{a}

Mean±SE values followed by different superscripts in the same column are significantly different according to ANOVA and Duncan's Multiple Range Test ($p<0.05$)

5 mg L^{-1} BAP treatments resulted in new plantlet regeneration. Although green spots were found from the calli of the 2 mg L^{-1} BAP treatment after 4 weeks of culture, these spots cannot be successfully regenerated into a new plantlet (Fig. 4b-f), especially multiple shoots were obtained from the 3, 4 and 5 mg L^{-1} BAP treatments (Fig. 4d-f).

Plant growth regulators play a crucial role in plantlet regeneration, especially cytokinin. Medium containing 0.5 mg L^{-1} NAA and 3 mg L^{-1} BAP was optimal for multiple shoot development of new plantlets derived from callus, demonstrating that the appropriate type and concentration of cytokinin promoted shoot proliferation. This finding concurred with observations from many plant species such as rice KDML 105 and glutinous rice RD 6[26], sugarcane[37], *Chlorophytum borivilianum* Sant. and Fernandez[38] because cytokinin enhanced cell division, shoot meristem maintenance and antioxidant activity[39-40]. Nevertheless, new plantlets regenerated from callus can be successfully induced using 10% coconut water alone without other plant growth regulators to influence somatic embryogenesis formation in some *Indica* and *Japonica* rice[41], sugarcane 'KK3'[42]. This may be caused by numerous compounds in coconut water such as some vitamins, amino acids, minerals and plant hormones (cytokinins, auxins) that impact plant growth under *in vitro* propagation[22]. However, other factors that affected plantlet regeneration were concerned such as type and concentration of plant growth regulators or adjuvants, explant size and developmental stage and cultural conditions (photoperiods, media properties, temperature and abiotic stress).

CONCLUSION

Tubtim Chumphae rice calli were produced within 3 weeks using an MS medium containing 2.5 mg L^{-1} 2,4-D alone or a medium with 2 mg L^{-1} 2,4-D in combination with 0.5 mg L^{-1} BAP. The addition of 50 ppm chitosan enhanced callus growth. MS medium supplemented with 0.5 mg L^{-1} NAA and 3 mg L^{-1} BAP was effective for callus regeneration. This simple, rapid, efficient and generic approach showed potential as a significant tool for rice production improvement.

SIGNIFICANCE STATEMENT

This study exposed an effective protocol for callus induction, plantlet regeneration and chitosan treatment in Tubtim Chumphae rice. This study will assist researchers in uncovering important sections of the Tubtim Chumphae rice *in vitro* propagation system that many researchers have yet to investigate. As a result, a new theory on Tubtim Chumphae rice *in vitro* callus induction, plantlet regeneration and chitosan could be developed.

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REFERENCES

1. Pawar, B., P. Kale, J. Bahurupe, A. Jadhav, A. Kale and S. Pawar, 2015. Proline and glutamine improve *in vitro* callus induction and subsequent shooting in rice. Rice Sci., 22: 283-289.
2. Poeaim, A., S. Poeaim, R. Poraha, S. Pongjaroenkit and P. Pongthongkam, 2016. Optimization for callus induction and plant regeneration from mature seeds of Thai rice variety: Nam Roo (*Oryza sativa* L.) Bioeng. Biosci., 4: 95-99.
3. Gosal, S.S. and M.S. Kang, 2012. Plant Tissue Culture and Genetic Transformation for Crop Improvement. In: Improving Crop Resistance to Abiotic Stress, Tuteja, N., S.S. Gill, A.F. Tiburcio and R. Tuteja (Eds.), Wiley-VCH Verlag GmbH & Co. KGaA, Germany, ISBN-13: 9783527632930, Pages: 357-397.
4. Vennapusa, A.R., R.S. Vemanna, B. H.R. Reddy, K.C. Babitha, K. Kiranmai, A. Nareshkumar and C. Sudhakar, 2015. An efficient callus induction and regeneration protocol for a drought tolerant rice *Indica* genotype AC39020. J. Plant Sci., 3: 248-254.

5. Azizi, P., M.Y. Rafii, M. Mahmood, M.M. Hanafi, S.N.A. Abdullah, R. Abiri and M. Sahebi, 2015. Highly efficient protocol for callogenesis, somagenesis and regeneration of *Indica* rice plants. C. R. Biol., 338: 463-470.
6. Lal, D., H.E. Shashidhar, P.H.R. Godwa and T.H. Ashok, 2014. Callus induction and regeneration from *in vitro* anther culture of rice (*Oryza sativa* L.). Int. J. Agric. Environ. Biotech., 7: 213-218.
7. Ramesh, M., V. Murugiah and A.K. Gupta, 2009. Efficient *in vitro* plant regeneration via leaf base segments of *Indica* rice (*Oryza sativa* L.). Indian J. Exp. Biol., 47: 68-74.
8. Guo, F., H. Zhang, W. Liu, X. Hu and N. Han *et al*., 2018. Callus initiation from root explants employs different strategies in rice and Arabidopsis. Plant Cell Physiol., 59: 1782-1789.
9. Sah, B.P. and R.K. Niroula, 2007. Successful regeneration and characterization of anther derived rice hybrid plants from *O. sativa* L. X *O. rufipogon* Griff. Sci. World, 5: 14-18.
10. Din, A.R.J.M., F.I. Ahmad, A. Wagiran, A.A. Samad, Z. Rahmat and M.R. Sarmidi, 2016. Improvement of efficient *in vitro* regeneration potential of mature callus induced from Malaysian upland rice seed (*Oryza sativa* cv. Panderas). Saudi J. Biol. Sci., 23: S69-S77.
11. Poraha, R., A. Poeaim, S. Pongjaroenkit and P. Pongthongkam, 2016. Callus induction and plant regeneration on optimization of the culture conditions in Jow Haw rice (*Oryza sativa* L.). Int. J. Agric. Technol., 12: 241-248.
12. Mostafiz, S.B. and A. Wagiran, 2018. Efficient callus induction and regeneration in selected *Indica* rice. Agronomy, Vol. 8. 10.3390/agronomy8050077.
13. Trunjaruen, A., S. Raso, P. Maneerattanarungroj and W. Taratima, 2020. Effects of cultivation media on *in vitro* callus induction and regeneration capabilities of pakaumpuel rice (*Oryza sativa* L.), Thai rice landrace. Walailak J. Sci. Technol., 17: 37-46.
14. Karthikeyan, A., S.T.K. Pandian and M. Ramesh, 2009. High frequency plant regeneration from embryogenic callus of a popular *indica* rice (*Oryza sativa* L.). Physiol. Mol. Biol. Plants, 15: 371-375.
15. Ho, T.L., S. Te-Chato and S. Yenchon, 2018. Callus induction and plantlet regeneration systems in *Indica* rice (*Oryza sativa* L.) cultivar sangyod. Walailak J. Sci. Tech., 15: 753-763.
16. Pornpienpakdee, P., R. Singhasurasak, P. Chaiyasap, R. Pichyangkura, R. Bunjongrat, S. Chadchawan and P. Limpanavech, 2010. Improving the micropropagation efficiency of hybrid *Dendrobium* orchids with chitosan. Sci. Hortic., 124: 490-499.
17. Uthairatanakij, A., J.A.T. da Silva and K. Obsuwan, 2007. Chitosan for improving orchid production and quality. Orchid Sci. Biotech., 1: 1-5.
18. Kean, T. and M. Thanou, 2010. Biodegradation, biodistribution and toxicity of chitosan. Adv. Drug Delivery Rev., 62: 3-11.
19. Rungruang, R., J. Modsuwan, N. Sae-Lee, N. Kaisangsri, O. Kerdchoechuen and N. Laohakunjit, 2018. Effect of pH on the extraction of anthocyanin and antioxidants of Tubtim Chumphae rice. Agric. Sci. J., 49: 225-228.
20. Sangartit, W., U. Kukongviriyapan, P. Pakdeechote, V. Kukongviriyapan and S. Thawornchinsombat, 2017. Effects of Tubtim Chum Phae rice bran hydrolysates on blood pressure and oxidative stress in L-NAME-induced hypertensive rats. KKU Res. J. Graduate Stud., 17: 19-29.
21. Mikuła, A., M. Niedzielski and J.J. Rybczyski, 2006. The use of TTC reduction assay for assessment of *Gentiana* spp. cell suspension viability after cryopreservation. Acta Physiol. Plant, 28: 315-324.
22. Rohmah, K.N. and W. Taratima, 2022. Effect of chitosan, coconut water and potato extract on protocorm growth and plantlet regeneration of *Cymbidium aloifolium* (L.) Sw. Curr. Appl. Sci. Technol., Vol. 22.
23. Thadavong, S., P. Sripichitt, W. Wongyai and P. Jompuk, 2002. Callus induction and plant regeneration from mature embryos of glutinous rice (*Oryza sativa* L.) cultivar TDK1. Agric. Nat. Resour., 36: 334-344.
24. Sompornpailin, K. and S. Chutipaijit, 2012. Enhancement of plant regeneration efficiency from mature grains of Thai *Indica* rice (*Oryza sativa* L. cv. KDML 105). Pak. J. Bot., 44: 1385-1390.
25. Buddharaksa, P., W. U-kong, R. Chundet, P. Wongsawad and M. Sanguansermsri, 2013. Callus induction from seeds *in vitro* culture of rice KDML 105 and glutinous rice RD 6. Naresuan Phayao J., 6: 100-105.
26. Osman, N.I., N.J. Sidik and A. Awal, 2016. Effects of variations in culture media and hormonal treatments upon callus induction potential in endosperm explant of *Barringtonia racemosa* L. Asian Pacific J. Trop. Biomed., 6: 143-147.
27. Beyl, C.A., 2011. PGRs and Their Use in Micropropagation. In: Plant Tissue Culture, Development, and Biotechnology Trigiano, R.N. and D.J. Gray (Eds.), CRC Press, Boca Raton, Florida, ISBN-13: 9780429111891, pp: 36-56.
28. Ying-Hua, S., L. Yu-Bo and Z. Xian-Sheng, 2011. Auxin-cytokinin interaction regulates meristem development. Mol. Plant, 4: 616-625.
29. Cheng, Z.J., L. Wang, W. Sun, Y. Zhang and C. Zhou *et al*., 2013. Pattern of auxin and cytokinin responses for shoot meristem induction results from the regulation of cytokinin biosynthesis by AUXIN RESPONSE FACTOR$_3$. Plant Physiol., 161: 240-251.
30. Sukma, D., S.A. Aziz, N. Andini, H.A. Putri and S. Sudarsono, 2019. Medium composition, coconut water, and chitosan effects on *Phalaenopsis* protocorm growth and development. Acta Hortic., 1262: 133-140.

31. Ramírez-Arrebato, M.Á., A.T. Rodríguez-Pedroso, S. Bautista-Baños and E. Ventura-Zapata, 2016. Chitosan Protection From Rice Diseases. In: Chitosan in the Preservation of Agricultural Commodities, Bautista-Baños, S., G. Romanazzi and A. Jiménez-Aparicio (Eds.), Academic Press Elsevier Inc., Cambridge, United States, ISBN-13: 978-0-12-802735-6, Pages: 115-125.
32. Stanley-Raja, V., S. Senthil-Nathan, K.M.P. Chanthini, H. Sivanesh and R. Ramasubramanian *et al*., 2021. Biological activity of chitosan inducing resistance efficiency of rice (*Oryza sativa* L.) after treatment with fungal based chitosan. Sci. Rep., Vol. 11. 10.1038/s41598-021-99391-w.
33. Boonlertnirun, S., E. Sarobol, S. Meechoui and I. Sooksathan, 2007. Drought recovery and grain yield potential of rice after chitosan application. Kasetsart J. Nat. Sci., 41: 1-6.
34. González, L.M., Y.R. Guerrero, A.F. Rodríguez and M.N. Vázquez, 2015. Effect of seed treatment with chitosan on the growth of rice (*Oryza sativa* L.) seedlings cv. Inca LP-5 in saline medium. Cultivos Tropicales, 36: 136-142.
35. Boonlertnirun, S., E. Sarobol, S. Meechoui and I. Sooksathan, 2006. Effects of molecular weight of chitosan on yield potential of rice cultivar Suphan Buri 1. Kasetsart J.: Nat. Sci., 40: 854-861.
36. Restanto, D.P., B. Santoso, B. Kriswanto and S. Supardjono, 2016. The application of chitosan for protocorm like bodies (PLB) induction of orchid (*Dendrobium* sp.) *in vitro*. Agric. Agric. Sci. Procedia, 9: 462-468.
37. Gopitha, K., A.L. Bhavani and J. Senthilmanickam, 2010. Effect of the different auxines and cytokinins in callus induction, shoot, root regeneration in sugarcane. Int. J. Pharm. Bio. Sci., Vol. 1.
38. Ashraf, M.F., M.A. Aziz, N. Kemat and I. Ismail, 2014. Effect of cytokinin types, concentrations and their interactions on *in vitro* shoot regeneration of *Chlorophytum borivilianum* Sant. & Fernandez. Electron. J. Biotechnol., 17: 275-279.
39. Kyozuka, J., 2007. Control of shoot and root meristem function by cytokinin. Curr. Opin. Plant Biol., 10: 442-446.
40. Grzegorczyk-Karolak, I., Ł. Kuźma and H. Wysokińska, 2015. The effect of cytokinins on shoot proliferation, secondary metabolite production and antioxidant potential in shoot cultures of *Scutellaria alpina*. Plant Cell Tiss. Organ Cult., 122: 699-708.
41. Biswas, A. and A.B. Mandal, 2007. Plant regeneration in different genotypes of *Indica* rice. Indian J. Biotechnol., 6: 532-540.
42. Phamontree, S., W. Taratima , P. Songsri and N. Jongrungklang, 2022. Development of a highly efficient callus induction and regeneration protocol for sugarcane using apical meristem. Asian J. Plant Sci., 21: 78-87.