

CALLUS FORMATION AND PLANT REGENERATION FROM IMMATURE EMBRYOS OF PIGMENTED UPLAND RICE (*Oryza sativa* cv. Tadong)

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Graphical abstract



Abstract

Application of biotechnology in upland rice improvement programmes depends on the availability of efficient regeneration protocols. Although protocols for shoot regeneration of upland rice are available, none has been reported for pigmented cultivars. This study reports on a protocol for callus induction and regeneration of Tadong, a pigmented upland rice cultivar from Sabah. For callus induction, immature embryos were cultured on media containing 2,4-Dichlorophenoxyacetic (2,4-D) at various concentrations (0 – 2.5 mg/L) and on different types of media (MS; MSB5; N6B5; N6). To induce shoot regeneration, callus explants were cultured on MS medium supplemented with combinations of 6-Benzylaminopurine (BAP) at various concentrations (0 – 3.0 mg/L) and 1-Naphthaleneacetic acid (NAA) at 1.0 mg/L. To induce shoot development, callus explants were pre-treated with Thidiazuron (TDZ) at various concentrations (0-1.0 mg/l) and exposed to different desiccation period (0 – 72 hours). 2,4-Dichlorophenoxyacetic at 2.5 mg/L and N6B5 medium resulted in the highest percentages of explant forming callus which were 60.3 ± 17.0 % and 58.7 ± 9.8 % respectively. The regeneration media failed to induce shoot on callus explants, instead green spots were formed on the surface of the callus. The green spots were stimulated to develop into shoots when the callus explants were pre-treated with 0.5 mg/L TDZ or exposed to partial desiccation for 24 h, the percentages of explant forming shoot were 35.7 ± 4.8 % and 47.7 ± 6.8 % respectively. Shoots developed into complete plants on hormone free MS medium and acclimatised. The protocol developed can facilitate the efforts to improve the cultivar through genetic modification approach.

Keywords: immature embryos, pigmented upland rice, shoot regeneration, TDZ pre-treatment, partial desiccation

Abstrak

Aplikasi bioteknologi dalam program penambahbaikan padi bukit bergantung kepada adanya protokol pertumbuhan pucuk yang cekap. Walaupun terdapat protokol pertumbuhan pucuk untuk padi bukit, namun tidak satu pun pernah dilaporkan bagi kultivar berpigmen. Kajian ini melaporkan protokol untuk aruhan kalus dan pertumbuhan pucuk untuk Tadong, sejenis padi bukit berpigmen dari Sabah. Untuk aruhan kalus, embrio pra-matang dikultur pada media yang mengandungi 2,4-Dichlorophenoxyacetic (2,4-D) pada pelbagai kepekatan (0 – 2.5 mg/L) dan pada pelbagai jenis media (MS; MSB5; N6B5; N6). Untuk mengaruh pertumbuhan semula pucuk, eksplan kalus dikultur pada media MS ditambah dengan kombinasi 6-benzylaminopurine (BAP) pada pelbagai kepekatan (0 – 3.0 mg/L) dan 1-Naphthaleneacetic acid (NAA) pada kepekatan 1.0 mg/L. Untuk mendorong tunas berkembang, eksplan kalus dirawat terlebih dahulu dengan Thidiazuron (TDZ) pada pelbagai kepekatan (0 - 1.0 mg/L) atau didedahkan kepada keadaan kering pada tempoh yang berlainan (0 - 72 jam). 2,4-Dichlorophenoxyacetic pada kepekatan 2.5

mg/L dan medium N6B5 memberikan peratus eksplan menghasilkan kalus tertinggi iaitu masing-masing dengan 60.3 ± 17.0 % dan 58.7 ± 9.8 %. Media pertumbuhan pucuk gagal mengaruh tunas pada eksplan kalus, sebaliknya hanya bintik-bintik hijau yang terbentuk pada permukaan kalus. Bintik-bintik hijau dirangsangkan untuk berkembang menjadi tunas setelah eksplan kalus didedahkan kepada pra-rawatan TDZ pada kepekatan 0.5 mg/L atau mendapat rawatan separa dehidrasi selama 24 jam, peratus pembentukan tunas masing-masing adalah 35.7 ± 4.8 % dan 47.7 ± 6.8 %. Pucuk berkembang menjadi tumbuhan lengkap pada medium MS tanpa hormone dan diaklimatiasi. Protokol yang dibangunkan boleh membantu usaha untuk memperbaiki kultivar tersebut melalui pendekatan transformasi genetik.

Kata kunci: Embrio pra-matang, padi bukit berpigmen, pertumbuhan pucuk, pra-rawatan TDZ, dehidrasi.

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1.0 INTRODUCTION

Rice is one of the most important staple foods that feeds half of the world's population (IRRI, 2006). About 90% of rice is cultivated in Asia, rice production is also one of the most important economic activities in the region as it provides employment and income for rural people (GRiSP, 2013). There are two types of rice being cultivated around the globe, which are the lowland and upland rice. The upland rice is described as one that grows on higher ground where the field does not require any irrigation system (Pandey *et al.*, 2006). Usually, this type of area is located in the rural districts. The grains of upland rice are of various characteristics, which include aromatic smell and unique shapes. Cultivars producing pigmented grains are the most popular among the rural population in Borneo as the grains can be sold at higher price. Tadong is an example of such cultivars. Tadong produces dark coloured grains that look almost black in colour (Figure 1). The colour is due to the formation of anthocyanins on the pericarp layer, which can be intense red, purple and black in appearance (Abdel-Aal *et al.*, 2006). A research on the chemical content of Tadong revealed that the grains have high antioxidant properties, which might be due to their high total anthocyanin and phenolic contents (Jong *et al.*, 2016). Unfortunately, the cultivar is very susceptible to diseases such as blast and sheath blight, hence resulted in unstable and low grain yield. One of the possible approaches to improve this cultivar is by employing recombinant DNA technology to produce transgenic rice resistant to fungal diseases. However, before such technology can be used, an efficient *in-vitro* shoot regeneration protocol needs to be developed for the cultivar. Factors such as rice genotype (Mostafiz and Wagiran, 2018), type of explants (Maharani *et al.*, 2020), plant growth regulators (Shukla *et al.*, 2018), the nutrient compositions and sugar can affect the regeneration potential of a plant (Feng *et al.*, 2011). To date, calli derived from matured seeds are commonly used explants for shoot regeneration (Wani, *et al.*, 2011; Yingxia and Te-Chato, 2012; Mohd Din *et al.*, 2016) and *Agrobacterium*-mediated transformation (Saika and

Toki, 2010). However, immature embryos have been reported to be excellent explants to produce embryogenic callus in rice, including *indica* rice varieties, which are known to be recalcitrant to tissue culture and transformation (Hiei and Komari, 2008). Freshly isolated immature embryos were reported to be highly competent for *Agrobacterium* infection in other monocotyledon species such as maize and sorghum (Zhang *et al.*, 2003; Zhao *et al.*, 2000). Immature embryos respond better in culture because they are made of young cells that are highly totipotent (Gasparis, *et al.*, 2008). Hence, they have much higher regeneration competency than matured embryos (Ward and Jordan, 2001; Wang, *et al.*, 2014). Plant growth regulators (PGRs) also play an important role in callus induction, proliferation and regeneration (Shahsavari *et al.*, 2010). Callus induction media containing 2,4-D and amino acid are reported to increase the frequency of callus formation in rice (Pravin *et al.*, 2011). According to Ge *et al.* (2006), a regeneration medium with a high ratio of cytokinins to auxins is good for the regeneration of plantlets for *indica* rice. Thidiazuron (TDZ), a synthetic cytokinin-like PGR, also has been reported to be effective in inducing somatic embryogenesis and organogenesis in monocots (Cheruvathur *et al.*, 2010; Deroles *et al.*, 2010). To date there is no report on a plant tissue culture protocol for pigmented upland rice like Tadong cultivar. Therefore, the purpose of this study is to establish an effective regeneration protocol for this cultivar.



Figure 1 Tadong grains (Scale bar = 0.7cm)

2.0 METHODOLOGY

2.1 Plant Material Preparation

The seeds of Tadong were collected from a village in the area near to Mount Kinabalu and sown in pots with a mixture of black soil, compost and fertilizer using the ratio of 5:3:1. The seeds were allowed to germinate and developed into mature plants that subsequently produced seeds after 4 months. The seeds from these plants were collected 14-25 days after anthesis, at this age the embryos were fully formed having rigid texture surrounded by undeveloped endosperm with a creamy consistency while the husks were still soft. The immature embryos were separated from the endosperm manually and used as explants.

2.2 Surface Sterilization

The embryos were washed a few times using sterilised distilled water and then soaked in 20% (v/v) Chlorox with Tween 20 (1 drop) for 5 min. The embryos were then transferred into 70% (v/v) ethanol for 30 s before a final rinse with sterilised distilled water. The sterilised explants were dried on an autoclaved filter papers for 15 min.

2.3 Effect of 2,4-dichlorophenoxyacetic Acid (2,4-D) and Media on Callus Formation

The immature embryos were cultured on callus induction media based on MS basal medium supplemented with various concentrations of 2,4-D (0, 1.0, 1.5, 2.0, 2.5 mg/L) and containing 5 g/L casein hydrolysate, 0.5 g/L L-proline, 10% (w/v) glucose, 20% (w/v) sucrose, 1 ml/L plant preservatives mixture (PPM) and 2.2 g/L gelrite. The medium was adjusted to pH 5.7 and autoclaved for 20 minutes at 121°C. Except for PGRs, all these components and pH were used in all subsequent media. Next, to evaluate the effect of media on callus formation, the immature embryos were cultured on 4 different media containing Murashige and Skoog (1962) salt (MS); media containing MS salts + Gamborg, (1968) vitamin (B5) (MSB5); media containing Chu, (1975) salts (N6) + Gamborg, (1968) vitamin (N6B5) and N6 salt medium (N6). The media were supplemented with 1.5 mg/L 2,4-D based on previous work by other researchers (Mohd Din et al., 2016; Zhao et al., 2011; Shamsavari et al., 2010) and the components as described before. The cultures were incubated in the dark at 25 ± 2 °C for four weeks. Exactly 20 immature embryos were cultured in each Petri dish and each treatment was repeated for 3 times. Observations were made once a week and the percentage of immature embryos forming callus was calculated as below and recorded. The diameter of the callus was also measured and recorded.

$$\text{Percentage of explant forming callus} = \frac{\text{Number of immature embryos formed callus}}{\text{Number of immature embryos cultured}} \times 100\%$$

2.4 Effect of 6-Benzylaminopurine (BAP) with Naphthalene Acetic Acid (NAA) on Shoot Regeneration

Four-week old callus explants were cultured on shoot regeneration media based on MS basal medium supplemented with different concentrations of BAP (0, 1.0, 2.0 & 3.0 mg/L) with NAA concentration was constant at 1.0 mg/L and containing components and pH as described before. The cultures were exposed to 16 h photoperiod at 25 ± 2 °C. About 10 callus explants were cultured on each Petri dish and each treatment was repeated for 3 times. Observation was made once a week.

2.5 Effect of Pre-Treatment of Calli with Thidiazuron (Tdz) on Shoot Regeneration

Four-week old callus explants were pre-treated on media based on MS basal medium supplemented with 0, 0.5 or 1.0 mg/L TDZ, the components and pH were as described before. The cultures were exposed to 16 h photoperiod at 25 ± 2 °C for 2 weeks before transferred onto MS shoot regeneration medium containing 1.0 mg/L NAA + 3.0 mg/L BAP. The number of shoots formed per explant and number of callus forming shoots were calculated as below:

$$\text{Percentage of explant forming shoots} = \frac{\text{Number of explant forming shoots}}{\text{Number of explants cultured}} \times 100\%$$

2.6 Effect of Partial Desiccation on Shoot Regeneration

Four-week old callus explants were placed on Whatman No.1 filter disc inside Petri dishes and sealed with parafilm. The calli were kept inside a laminar flow cabinet for 0, 24, 48 and 72 h to dehydrate. Next, the desiccated calli were cultured on the shoot regeneration medium as previously used and were exposed to 16 h photoperiod at 25 ± 2 °C. Ten callus explants were cultured in each Petri dish and each treatment was repeated for 3 times. The percentage of desiccation (%) of the explants, the number of shoots formed per explant and the percentage of callus explants forming shoots were calculated as below:

$$\text{Percentage of desiccation} = \frac{\text{Fresh weight of callus explants} - \text{Weight of callus explants after desiccation}}{\text{Fresh weight of callus explants}} \times 100\%$$

$$\text{Percentage of callus explant forming shoots} = \frac{\text{Number of callus explant forming shoots}}{\text{Number of callus explants cultured}} \times 100\%$$

2.7 Statistical Analysis

Each experiment was conducted in three replicates in completely randomised block design. Data were

analysed using analysis of variance (ANOVA) and differences of means were evaluated by Duncan's multiple range test (DMRT) of SPSS statistical package.

3.0 RESULTS AND DISCUSSION

3.1 Effect of 2,4-dichlorophenoxyacetic Acid (2,4-D) and Media on Callus Formation

Since immature embryos were used as the explants, their viability was evaluated by scoring the germination. Germination started after one week of culture and was recorded to be above 80 % for all the treatments. These results proved that majority of the immature embryos were viable. On the callus induction media, two types of responses were observed; the capacity of the explants to form primary callus which was represented as the percentage of explant forming callus; and the capability of the primary callus to proliferate on the explants as reflected by callus growth. Table 1 shows the result of callus formation on MS medium with various concentrations of 2,4-D. Callus started to form on the germinated embryos after two weeks, the medium supplemented with 2.5 mg/L 2,4-D, resulted in the highest number of the immature embryos to form callus where $60.3\% \pm 17.0$ of them formed callus, this was followed by the medium containing 1.0 mg/L 2,4-D ($59 \pm 7.4\%$), while the lowest was that supplemented with 2.0 mg/L 2,4-D ($52 \pm 18.5\%$). The primary callus proliferated on the explants at different rate into creamy white and attained nodular morphology after 4 weeks. Further observation on the growth of the primary callus showed that callus proliferation reduced as the concentration of 2,4-D increases from 1.0 to 2.5 mg/L (Table 1). No significant difference in callus induction was observed ($P < 0.05$) at various concentrations of 2,4-D.

Table 1 Effect of different concentrations of 2,4-D (mg/L) on germination and callus formation of immature embryos

Conc. of 2,4-D (mg/L)	Germination (% \pm SD)	Explant forming callus (% \pm SD)	Callus Morphology	Callus Colour	Callus Growth
0	87.7 \pm 6.8 ^a	0.0 \pm 0.0 ^b	-	-	-
1.0	82.0 \pm 15.6 ^a	59.0 \pm 7.4 ^a	Friable Nodular	Creamy White	++++
1.5	84.7 \pm 6.8 ^a	52.0 \pm 18.5 ^a	Friable Nodular	Creamy White	+++
2.0	83.3 \pm 12.3 ^a	49.0 \pm 13.8 ^a	Friable Nodular	Creamy White	+++
2.5	83.3 \pm 15.3 ^a	60.3 \pm 17.0 ^a	Friable Nodular	Creamy White	+++

Values represent mean \pm standard deviation. Means sharing the same letters are not significantly different ($p < 0.05$) using Duncan's Multiple Range Test. + = 25% explant surface covered by callus; ++ = 50% explant surface covered by callus; +++ = 75% explant surface covered by callus; ++++ = 100% explant surface covered by callus.

This study proved that the presence of 2,4-D was necessary for the immature embryos to form calli. The presence of synthetic auxin 2,4-D ranged between 1 - 4 mg/L was reported to be very effective in inducing embryogenic callus in rice (Gari and Rashid, 2004;

Shaukat et al., 2004; Lee et al., 2002). Our work showed that 2,4-D at 2.5 mg/L induced the highest number of the explant to form primary callus (Figure 2E), this concentration was slightly higher compared to that reported by Shahsavari et al. (2010) where matured embryos of 4 upland rice cultivars produced the highest callus formation at 2.0 mg/L 2,4-D. In a shoot regeneration protocol that involved callus as an intervening stage, it is important that the primary callus on the explants continue to proliferate to support the subsequent shoot regeneration phase. But, in this present work the growth of the primary callus was reduced at high concentration of 2,4-D (Table 1). According to Rueb et al. (1994), high dosage of 2,4-D suppresses not only the formation of callus, but also shoot regeneration on calli induced from mature embryos. The auxin 2,4-D is an auxinic herbicide, as an herbicide its mode of action is by inducing uncontrolled and unsustainable growth that leads to plant death (Song, 2014). A functional analysis carried out by Pan et al. (2010) on *Citrus sinensis* Osbeck showed that high concentration of 2,4-D induced various stress on the callus such as oxidative stress and osmotic stress. Hence, in our study, the inability of the callus explants to form shoot when they were cultured on the media supplemented with BAP and NAA might have due to high concentration of 2,4-D in the callus induction media.

Table 2 shows the results of immature embryos germinated and formed callus on different types of media supplemented with a constant concentration of 2,4-D at 1.5 mg/L. The embryos germinated at consistently high percentages with the values ranges from 84.7 % to 94.7 %. The calli were creamy white in colour and nodular in shape. The medium based on N6B5 (Figure 2D), gave the highest percentage of explant forming callus ($58.7 \pm 9.8\%$), followed by MSB5 medium ($57.7 \pm 19.7\%$) (Figure 2B) while N6 salts medium resulted in the lowest percentage ($47.7 \pm 5.0\%$) (Figure 2C). No significant difference ($P < 0.05$) in callus formation for types of media evaluated. However, the size of the callus formed on N6B5 medium was only 4.8 ± 1.9 mm in diameter, while that formed on MS medium was the biggest with the diameter measurement of 6.9 ± 1.3 mm. This indicated that N6B5 medium was less effective in inducing cell division which resulting in small callus mass growing around the explants (Figure 2D). On the other hand, MS medium produced better quality of callus as the callus biomass was bigger in sizes (Table 2) and had a creamy appearance which was described by Sharma et al. (2017) to be embryogenic.

In this present finding, N6 medium was not suitable for callus induction of this rice. This result is consistence with the report by Kaushal et al. (2014) who stated that N6 medium produced the lowest frequency of callus induction and green plant regeneration on the anther culture of *indica* rice. In contrast, a report by Niroula et al. (2005) showed that N6 medium was the best for callus induction, cell proliferation and plant regeneration from seeds of rice. A report by Koetje et al. (1989) showed that immature

embryos of IR54 grown on N6 medium exhibited a linear dose response to 2,4-D supplemented in the callus induction medium, while regeneration from cells grown on MS medium was practically unconnected to the concentration of 2,4-D. Plant responded differently on MS and N6 medium although there were only slight differences in the components of these media. The major difference existed between these media is the absolute and relative amounts of inorganic nitrogen (Grimes and Hodges, 1990). One possible theory is that the $\text{NO}_3^-:\text{NH}_4^+$ ratio is altering cell sensitivity to auxin hence regulating the uptake or metabolism of hormones (Davies, 1987; Grimes and Hodges, 1990).

Table 2 Effect of different types of media (MS;MSB5; N6;N6B5) on germination and callus formation of immature embryos

Type of Media	Germination (%)	Explant forming callus (%)	Size of callus in Diameter (mm)	Callus Morphology	Callus Colour
MS	84.7 ± 6.8 ^a	54.3 ± 18.6 ^a	6.9 ± 1.3 ^a	Friable nodular	Creamy White
MSB5	85.3 ± 0.4 ^a	57.7 ± 19.7 ^a	5.0 ± 1.5 ^b	Friable nodular	White
N6	89.7 ± 5.8 ^a	47.7 ± 0.5 ^a	4.7 ± 0.8 ^b	Friable nodular	White
N6B5	94.7 ± 0.4 ^a	58.7 ± 9.8 ^a	4.8 ± 1.9 ^b	Compact nodular	White

Values represent mean ± standard deviation. Means sharing the same letters are not significantly different ($p < 0.05$) using Duncan's Multiple Range Test.

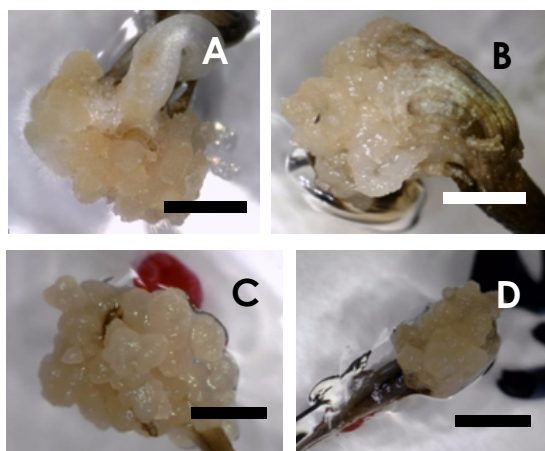


Figure 2 Formation of callus by immature embryos on different types of media. A) MS with 1.5 mg/L 2,4-D (Scale bar = 3.0 mm); B) MSB5 with 1.5 mg/L 2,4-D (Scale bar = 2.5 mm); C) N6 with 1.5 mg/L (Scale bar = 2.0 mm); D) N6B5 with 1.5 mg/L 2,4-D. (Scale bar = 4.5mm)

Callus formation from rice has been shown to be highly affected by media and genotypes (Ng et al., 2019). N6 medium is more suitable for *japonica* rice, as it has high content of KNO_3 and $(\text{NH}_4)\text{SO}_4$ compared to MS. On the other hand, *indica* rice requires a lower level of NH_4^+ (Kaushal et al., 2014). Nevertheless, variation of callus growth could also be seen within the rice races. For instance, in the *indica* rice, 2 wetland cultivars namely Sangyod (Ho et al., 2018) and

AC39020 (Vennapusa et al., 2015), thrived well in MS and LS medium respectively. Similar situations could be seen in *japonica* rice, in producing embryogenic calli, the cultivar Kitaake responded better on MS medium (Sah et al., 2014), while cultivars Dong-Jin, Hwa-Chung and Nak-Dong performed better on N6 medium (Lee et al., 2002).

Inconsistency in callus growth and sizes as shown in Table 1 and 2 suggests that Tadong's immature embryos are sensitive towards the concentrations of PGR and types of media. This could be due to the property of the immature embryos which might have been at different developmental stages when they were harvested for the experiments. Compared to mature embryos, the developmental stage of immature embryos is much difficult to control (Han et al., 2011). According to Matthys-Rochon et al. (1998), the hormone requirement of immature embryos is variable according to the developmental stage; immature embryos removed from a mother plant at more advance developmental stage contained higher endogenous growth factors as compared to those harvested at less advance developmental stage. These variation in endogenous PGR could have explained the inconsistency of callus growth at different concentrations of exogenous 2,4-D and types of media for Tadong cultivar.

3.2 Effect of Hormone Benzylaminopurine (BAP) and Naphthalene Acetic Acid (NAA) on Shoot Regeneration

Callus explants cultured on different concentrations of BAP and NAA continued to proliferate resulted in an increase of callus mass. Low level of differentiation was observed on the proliferating calli, only the top region of the callus mass differentiated into bud-like structure with green colours or green spots within two weeks of culture (Figure 3A), but these structures failed to regenerate into shoots. The medium with 3.0 mg/L BAP + 1.0 mg/L NAA had induced the highest number of explants forming the green spots (43.3 ± 5.8 %). Unfortunately, the green spots turned brown and died after 4 weeks of cultures. The control medium (hormone free) failed to induce any differentiation. Table 3 shows the responses of callus explants on shoot regeneration media.

The present results (Table 3) are in contradiction with the report by Sharma et al. (2012) where BAP in combination with NAA was able to facilitate shoot regeneration in rice. Auxin and cytokinin have been shown to trigger somatic embryogenesis and organogenesis (Chaudhury and Qu, 2000; Jia et al., 2008; Liu et al., 2008). According to Jones et al. (2010), both cytokinins and auxins influence the cell cycle and morphogenic competence in plant growth, hence a balance between the PGRs must be reached to achieve optimum shoot regeneration. Negative effect occurs if unbalanced concentration between auxin and cytokinin is applied, it may result in the decrease of regeneration rate and suppression of multiple shoot proliferation.

In our study, although the media containing BAP and NAA tested for shoot regeneration failed to produce shoots, they had shown potential in capacity to induce shoot as green spots were formed on the callus. Formation of green spots on callus prior to development into shoots have been reported in other rice regeneration studies (Ng et al., 2019; Ho et al., 2018; Shahsavari et al., 2010; Wagiran et al., 2008). According to Nabors et al., (1982), green spots are predictors for shoot formation capacity in oat, hence, in our study, attempts were made to induce the green spots to develop into shoots by pre-treating the callus explants with TDZ and desiccation.

Table 3 Percentages (%) of callus formed green spots on shoot regeneration media supplemented with different combinations of BAP (mg/L) + NAA (mg/L)

Conc. of BAP (mg/L)	Conc. of NAA (mg/L)	Explant Forming green spots (% ± S.D.)	Number of shoots formed/callus (No ± S.D.)
0	0	0.0 ± 0.0 ^c	0
0	1.0	20 ± 10 ^b	0
1.0	1.0	16.7 ± 5.8 ^b	0
2.0	1.0	26.7 ± 4.7 ^b	0
3.0	1.0	43.3 ± 5.8 ^a	0

Values represent mean ± standard deviation. Means sharing the different letters are significantly different ($p < 0.05$) using Duncan's Multiple Range Test.

3.3 Effect of Pre-treatment with Thidiazuron (TDZ) on Shoot Regeneration

The callus explants produced green spots within a week on the media containing TDZ. In contrast, the green spots were not formed on the controlled medium. Following transfer to the regeneration medium, pre-culturing on TDZ at 0.5 mg/L induced 35.7 ± 4.8 % of the callus explants to form shoot, while those pre-cultured on 1.0 mg/L TDZ only resulted in 27.6 ± 5.3 % explants to regenerate shoots (Table 4) with about 3 - 4 shoots were formed on each explant.

Table 4 Shoot regeneration by callus explants pre-treated with TDZ (mg/L)

Conc. of TDZ (mg/L)	Explant forming shoot (% ± S.D.)	Number of shoots formed/callus (No ± S.D.)
0	0 ± 0 ^b	0 ± 0 ^b
0.5	35.7 ± 4.8 ^a	4 ± 1.6 ^a
1.0	27.6 ± 5.3 ^a	3 ± 1.4 ^a

Values represent mean ± standard deviation. Means sharing the same letters are not significantly different ($p < 0.05$) using Duncan's Multiple Range Test.

Pre-treatment with TDZ did influence the regeneration capability of Tadong. Our result is similar to that of Zhao et al. (2011) where the number of

regenerated plantlets were higher when the explants were pre-cultured on 0.5 mg/L TDZ. According to Guo et al. (2011), pre-treatment with TDZ induces regeneration either by influencing a tissue to accept other inductive stimuli or it can make a tissue to commit to a regenerative route that is expressed even after TDZ is removed. TDZ is able to play the role of both cytokinin and auxin for regenerative response in various plant species (Li et al., 2003; Chauhan et al., 2007; Ma et al., 2011). Murch and Saxena (2001) reported that exposure to TDZ enhances the accumulation and translocation of auxin within the tissues. However, if TDZ is present in high concentration, it can result in the decrease of regeneration frequency as proven in this research. Similar result was reported by Zhao et al. (2010) on Chinese upland rice of Handao 297 cultivar, where TDZ at a low concentration promoted morphogenesis while at high concentration it was toxic to calli and inhibited morphogenesis. This present work also discovered that pre-treatment with TDZ shortened the time for the formation of the green spots on the callus from two weeks in the previous finding using BAP and NAA to one week.

3.4 Effect of Partial Desiccation on Regeneration

Desiccating the explants for 24 h proved to be optimal for shoot regeneration, as shown in Table 5, up to 47.7 ± 6.8 % callus explants produced shoots. However, the regeneration capacity dropped to 22.0 ± 1.0 % and 11.3 ± 1.2 % when the desiccation period was prolonged to 48 and 72 h respectively. Results of the present study are consistent with many previous findings where the highest shoot regeneration frequency was obtained in the moderate desiccation period. The average number of shoots formed on each callus was 2 - 3 shoots. There was significant difference ($P < 0.05$) in shoot regeneration for all desiccation treatments. When plantlets were subcultured onto hormone free medium, shoots developed, and roots were produced (Figure 3C).

In this study, treatment of the explants with partial desiccation is giving better results in inducing shoots from callus of Tadong as compared to pre-treatment with TDZ. With 24 h desiccation, shoot formation by explants is 1.3 fold higher as compared to pre-treatment with 0.5 mg/L TDZ. This is beneficial for the regeneration protocol as by not using TDZ it can reduce the cost of the regeneration system.

Desiccation had induced the formation of shoots from callus explants of Tadong without the need of altering the composition of the PGR content of the regeneration medium which is based on MS basal medium containing 3.0 mg/L BAP and 1.0 mg/L NAA, 5 g/L Casein hydrolysate, 0.5 g/L L-proline, 10 % (w/v) glucose, 20 % (w/v) sucrose, 1 ml/L plant preservatives mixture (PPM) and 2.2 g/L gelrite. The benefit of partial desiccation had also been reported to other *indica* rice regeneration (Haq et al., 2009; Saharan et al., 2004; Diah and Bhalla, 2000; Chand

and Sahrawat, 2001). The present study shows that Tadong cultivar requires a shorter desiccation time (24 h) as compared to lowland rice of MR220 (48 h) and MR232 (72 h) cultivars as reported by Makerly *et al.*, (2012). Compared to *japonica* rice of Nipponbare, Hayahishiki and Fujisaka varieties, where 48 h desiccation was optimum (Wagiran, 2008), Tadong requires a shorter desiccation period for maximum shoot regeneration.

Table 5 Shoot regeneration by callus explants partially desiccated

Desiccation Period (Hours)	Degree of Desiccation (%)	Explant forming shoot (% \pm S.D.)	Number of shoots formed/callus (No \pm S.D.)
0	0.0 \pm 0.0 ^c	0.0 \pm 0.0 ^c	0.0 \pm 0.0 ^b
24	69.9 \pm 8.9 ^b	47.7 \pm 6.8 ^a	3 \pm 1.2 ^a
48	70.3 \pm 10.1 ^b	22.0 \pm 1.0 ^b	3 \pm 1.4 ^a
72	78.6 \pm 7.2 ^a	11.3 \pm 1.2 ^c	2 \pm 1.0 ^a

Values represent mean \pm standard deviation. Means sharing the same letters are not significantly different ($p < 0.05$) using Duncan's Multiple Range Test.

Desiccation treatment reduces water availability but enhances the uptake of nutrients (Vennapusa *et al.*, 2015). According to Datta and Datta, (1999), uptake of nitrate and ammonium was enhanced by more than 1 fold when desiccated *Catenella repens* was returned to its normal growth condition. Increase in nitrogen uptake has been reported to aid growth in plant, this could have induced shoot regeneration in this present study. Desiccation also activates genes of late embryogenesis abundant (LEA) protein (Bartels, 2005). Plants LEA proteins have a protective role in embryo tissue during dehydrating of cells, the proteins protect enzyme activities from inactivation caused by water depletion (Reyes *et al.*, 2005; Goyal *et al.*, 2005). Desiccation induces higher production of abscisic acid (ABA) (Yang *et al.*, 1999), in turn ABA has been reported to promote regeneration in *Panax ginseng* somatic embryos (Langhansova *et al.*, 2004). However longer desiccation treatment is detrimental to plant as it causes damage on callus (Makerly *et al.*, 2012). The higher the degree of desiccation, the least number of shoots regenerate as shown in the result in Table 5. Prolonged desiccation resulted in loss of more than 20 – 50 % water content of the cells; this level of desiccation is lethal to higher plants (Kranmer *et al.*, 2002). When plant cell losses water, reduction in cell volume or plasmolysis occurs (Moore *et al.*, 2006), this causes the withdrawal of the plasma membrane from the cell wall. Separation of the plasma membrane from the rigid cell wall can tear the membrane which cause damage to the cell (Shivaraj *et al.*, 2018). In addition, production of reactive oxygen species (ROS) is elevated under stress condition such as desiccation (Pammenter and Berjak, 2014), when the level of ROS is high in a cell, it enhances lipid peroxidation which is oxidative decomposition of polyunsaturated lipid in

plasma membrane causing damage to cell membrane (Sharma *et al.*, 2012; Xie *et al.*, 2019).

Our work shows that Tadong immature embryos can produce embryogenic callus and regenerate shoots. However, the growth of callus is variable and pre-treatment of the explants with TDZ or partial desiccation is mandatory for a successful shoot regeneration.

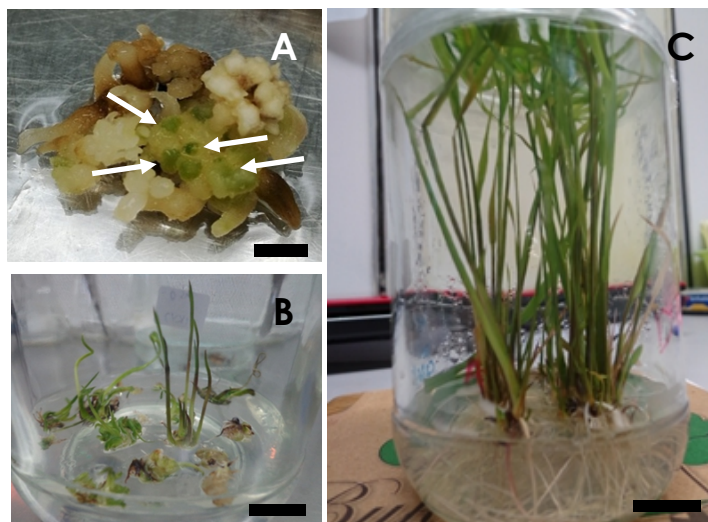


Figure 3 The effect of partial desiccation on shoot regeneration. A) Desiccated callus formed green spots. B) Explants formed shoots. C) Shoot developed and root elongated on hormone free medium. Arrows indicate green spots

4.0 CONCLUSION

The present study reports an effective callus induction and shoots regeneration protocol from immature embryos of Tadong cultivar. Since immature embryos have been reported to be much more responsive towards *Agrobacterium* infection, this finding can be used for planning of transformation systems for upland rice to obtain a disease-resistant transgenic cultivar, e.g. blast disease, and other beneficial traits that can contribute to increase the yield.

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