Agriculture and Food

AIMS Agriculture and Food, 7(3): 536–552.
DOI: 10.3934/agrfood.2022033
Received: 22 February 2022
Revised: 08 May 2022
Accepted: 10 May 2022
Published: 14 July 2022

http://www.aimspress.com/journal/agriculture

Research article

Callogenesis and somatic embryogenesis of *Oryza sativa* L. (cv. MARDI Siraj 297) under the influence of 2,4-dichlorophenoxyacetic acid and kinetin

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Abstract: Callogenesis and embryogenesis are integral parts of many tissue culture procedures for genetic manipulation in rice. However, the efficiency of both processes is largely dependent on the media constituent especially the plant growth regulators (PGRs) due to the genotype-dependent nature of *in vitro* culture protocols. Therefore, this study investigates the effect of two PGRs; 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kin) on callus growth and somatic embryogenesis of an important Malaysian rice cultivar (*Oryza sativa* L. cv. MARDI Siraj 297). Mature rice seeds explants were inoculated in Murashige & Skoog (MS) medium supplemented with different combinations of 2,4-D (0 to 3.5 mg/L) and Kin (0 to 0.5 mg/L) to induce callogenesis. Parameters for callus growth such as fresh weight (FW), callus induction frequency (CIF), embryogenic callus frequency (ECF), regeneration frequency (RF), number of plantlets per callus (PPC), callus texture and callus color were observed after 35 days of inoculation. The results show that the maximum callus growth was achieved in MS medium supplemented with combination of 2.0 mg/L 2,4-D and 0.2 mg/L Kin, represented by the highest FW (211 mg), CIF (95%), ECF (90%), RF

(100%) and PPC (22 plantlets); along with friable callus texture. Low concentration of 2,4-D (0 to 0.5 mg/L) in the presence or absence of Kin promotes root growth instead of callus, while high concentrations (above 3.0 mg/L) retard the callus formation. The embryogenic calli from this optimized PGRs combination were successfully formed shoots in MS medium supplemented with 2 mg/L BAP and 1 mg/L NAA, followed by rooting in PGRs-free MS medium. This finding provides an efficient protocol for callogenesis and somatic embryogenesis of MARDI Siraj 297, since this is the first published report regarding somatic embryogenesis induction of this cultivar.

Keywords: embryogenic callus; plant growth regulators; *in vitro*; regeneration; optimization; tissue culture

1. Introduction

Rice (*Oryza sativa* L.) is consumed as a staple food in Malaysia and it has become a central part of the nation's economy and culture. According to the current analysis, Malaysians consume about 200,000 metric tonnes of rice a month which is equivalent to approximately 2.5 million metric tonnes a year [1,2]. Despite high dependence of the population on rice consumption, yearly decreasing trend of rice production in Malaysia has been observed from 2018 to 2021 [3]. This insufficiency necessitates 33% of outsourced supply from other countries such as Thailand, Vietnam and Pakistan to supplement the 67% of local rice produce [4].

Rice breeding programs in Malaysia had been initiated since 1964 with 52 cultivars released until year 2021 by the Malaysian Agriculture Research and Development Institute (MARDI) in order to increase the nation's rice production capacity [5]. This figure is far behind other Asia's rice producers such as India, Philippines and Thailand which had established about 1900, 200 and 80 varieties respectively, over the same period [4]. Nevertheless, continuous efforts have been carried out by local research institutions to develop high yielding varieties with resilience to biotic and abiotic stress [6], especially through biotechnological intervention that speeds up the crop improvement programs. However, many of these genetic manipulations employ tissue culture technique that requires undifferentiated cells or embryogenic callus as an intermediary phase at which the manipulations will be applied [7,8]. Therefore, vigorously growing cell mass which is able to regenerate is needed as starting material for this manipulation purpose.

For this purpose, optimization of callogenesis and somatic embryogenesis has been routinely conducted to customize the need of every single species or variety [9,10]. To date, growth medium optimization for plant tissue culture remains as an intricate part of *in vitro* plant establishment due to various endogenous and exogenous interactions [11]. Among these influencing factors, nutrient medium composition is the most important and easily manipulated [12]. Although Murashige & Skoog (1962) [13] (MS) basal medium has been widely used in many plant tissue culture development protocols, it often requires optimization of other constituents such as plant growth regulators (PGRs) especially if a particular response is desired [14,15].

Callus formation or callogenesis is possible under *in vitro* condition when plant tissues were exposed to auxin and cytokinin, whereby the ratio between these plant hormones determines the extent of differentiation and dedifferentiation [16–18]. These two classes of PGRs also imposed synergistic or antagonistic effects during somatic embryogenesis induction depending on plant

species [19]. Numerous findings had demonstrated the effects of auxins and cytokinins on callus growth of various plants including rice [20–23].

Meanwhile, somatic embryogenesis is cellular processes that involves morphogenetic changes in which somatic cell acquire ability to produce plants without fusion of gametes [24]. Its generation in plants happens when somatic cells acquire ability to regenerate bipolar structures during cell differentiation [19]. During *in vitro* culture, the embryogenic potential is achieved through manipulation of different elements including explant sources, medium composition, genotype of the mother plant and incubation condition [25–28]. In general, 2,4-dichlorophenoxyacetic acid (2,4-D) had been widely used for embryogenic callus induction [18]. The auxins mainly 2,4-D was known for its function in somatic embryogenesis induction and initial embryo proliferation [29–31]. Although its exact role in acquiring cells' embryogenic competency is still unclear [29], 2,4-D was proved to influence the metabolism of endogenous IAA in carrot cells, which was proposed to have significant roles during formation of somatic embryo [32].

There were studies reported the sole use of 2,4-D as hormone for somatic embryo induction. For example, a study conducted on four Malaysian rice cultivars namely MR220-CL2, MR232, MR220 and Bario found out that 3 mg/L 2,4-D resulted in the highest percentage of embryogenic callus formation [33], while another studies on cultivar MR219 showed the optimum somatic embryogenesis was achieved in medium with 2 mg/L 2,4-D [34]. Meanwhile, many others reported positive effect of combining 2,4-D and auxins such as kinetin or BAP in callogenesis medium [35–39]. Kinetin which is known to promote cell division [40] was also found to be the sole source of PGR in somatic embryogenesis of certain plant such as *Corydalis yanhusuo* [41].

Since the plant cells response are genotype-specific, optimization of PGR requirement of each species and varieties are important to ensure satisfactory callus growth and somatic embryogenesis that enables the subsequent plant regeneration [42–45]. Besides, due to the fact that this cultivar belongs to subspecies *indica*, a robust and widely used protocol has not been established because of its genotype-dependent nature [46]. Hence in this study, medium optimization was aimed at obtaining a suitable PGR combination for efficient callogenesis and somatic embryogenesis of MARDI Siraj 297 since there was no reports available on the optimized medium for this cultivar to date. At present, majority of rice farmers in Malaysia are using MARDI Siraj 297 (47.9%) for cultivation [47] to replace the former MR 219 variety that was reported to show reduced resistance towards blast disease [48]. The establishment of callogenesis and embryogenic callus induction medium provides an efficient protocol for vigorous growth of MARDI Siraj 297 rice callus with high regenerability for further crop improvement via *in vitro* manipulation.

2. Materials and methods

2.1. Plant material

The mature seeds of *Oryza sativa* L. cv. MARDI Siraj 297 (Accession No. MRGB13019) were obtained from the Genebank & Seed Centre of Malaysian Agricultural Research and Development Institute (MARDI) at Seberang Perai, Malaysia. This study was conducted at the Cell Biology Laboratory, Faculty of Science, Universiti Putra Malaysia between February to May 2019.

2.2. Seed sterilization

Intact rice seeds were hulled and washed under running tap water for 3 minutes. The seeds were then completely immersed in 70% (v/v) ethanol for 3 minutes, followed by 5% (v/v) sodium hypochlorite and Tween-20 (10:1) for 20 minutes [34]. Finally, the seeds were rinsed 5 times with sterile distilled water and blotted dry on autoclaved paper towels.

2.3. Callus induction and regeneration

Sterilized seeds were used as explant and inoculated on Murashige and Skoog's Gamborg Modified Basal Media (Sigma-Aldrich®, USA) supplemented with different combinations of 2,4-dichlorophenoxyacetic acid (PhytoTech, USA) ranging from 0 to 3.5 mg/L and Kinetin (Sigma-Aldrich®, USA) from 0 to 0.5 mg/L. The media was added with 30% (w/v) sucrose (Chemiz®, Malaysia) and 0.3% (w/v) Gelrite® (Sigma-Aldrich®, USA) at pH 5.8. The culture flasks were kept under dark condition in controlled growth room at 25 ± 1 ℃ at relative humidity of 50 to 60% [49]. The experiment was arranged in completely randomized design (CRD) with 5 replicates for each treatment. The morphology and growth of the explants were observed after 35 days of inoculation based on the following parameters:

i. Callus induction frequency, CIF [50]

$$\text{CIF (\%)} = \frac{\text{Number of calli}}{\text{Total number of inoculated seed}} \times 100 \quad (1)$$

ii. Embryogenic callus frequency, ECF [50]

$$\text{ECF (\%)} = \frac{\text{Number of embryogenic calli}}{\text{Total number of calli}} \times 100 \quad (2)$$

Embryogenic calli obtained from different PGR treatments were transferred shoot induction which consists of MS salts supplemented with 2 mg/L BAP, 1 mg/L NAA, 100 mg/L myo-inositol, 3% (w/v) sucrose and 0.4% (w/v) Gelrite® at pH 5.8 [22]. The cultures were maintained in a controlled growth room at 25 ± 1 ℃ with 16 hours photoperiod using fluorescent lamps. The shoot-producing calli were then transferred into MS medium without plant growth regulator for 2 weeks for root induction. Callus regeneration frequency (Eq 3) and number of plantlet per callus of each treatment were recorded.

iii. Regeneration frequency, RF [22]

$$\text{RF (\%)} = \frac{\text{Number of calli with shoots and roots}}{\text{Total number of calli inoculated in regeneration medium}} \times 100 \quad (3)$$

iv. Callus fresh weight, FW

The shriveled endosperms were removed and fresh weight of each callus clumps were measured [51] using an analytical balance.

v. Calli texture was classified into 2 subgroups (friable or compact) based on their macroscopic characteristics according to [17].

vi. Calli were observed for identification of somatic embryogenesis using stereo microscope with 45 times magnification. Embryogenic calli were recognized by their distinctive somatic embryo

development that involves the formation of proembryo, globular, scutellar and coleoptilar [18,52].

2.4. Statistical analysis

The data were analyzed using one-way ANOVA using SAS software for Windows version 9.4, followed by Duncan's Multiple Range Test (DMRT) at $p < 0.05$ for mean comparison.

3. Results

3.1. The effect of 2,4-D and kinetin concentrations on callogenesis and regeneration

In this study, the maximum CIF ($p < 0.05$) were achieved at all kinetin levels: 0 mg/L Kin (T3), 0.2 mg/L (T10, T11, T12) and 0.5 mg/L (T19) as presented in Table 1. All of the PGR treatments were able to induce callus, except for the treatments with the lowest concentration of 2,4-D between 0.5 to 1.0 mg/L (T1, T2, T8, T15, T16). The maximum CIF was attained in treatment with 2,4-D ranging between 1.5 to 2.5 mg/L, while concentrations lower or higher than this range reduced the frequency of callus induction.

Despite the ability to induce callus, not all callus produced in the treatments were embryogenic (Figure 1). For instance, in treatments without kinetin (T1 to T7), concentration of 1.5 to 3.5 mg/L 2,4-D produced callus, but only callus in 2.5 mg/L was embryogenic. Similarly in treatments with 0.2 mg/L and 0.5 mg/L kinetin, the embryogenic calli were only formed in combination of 2,4-D concentrations between 1.5 to 2.5 mg/L and 1.5 to 3.5 mg/L, respectively. Meanwhile, the highest ($p < 0.05$) ECF of 95% was observed in combination of 2.0 mg/L 2,4-D and 0.2 mg/L kinetin (T11). The ECF increased with the increment of 2,4-D concentration, but significantly declined at concentration above 3.0 mg/L (Table 1).

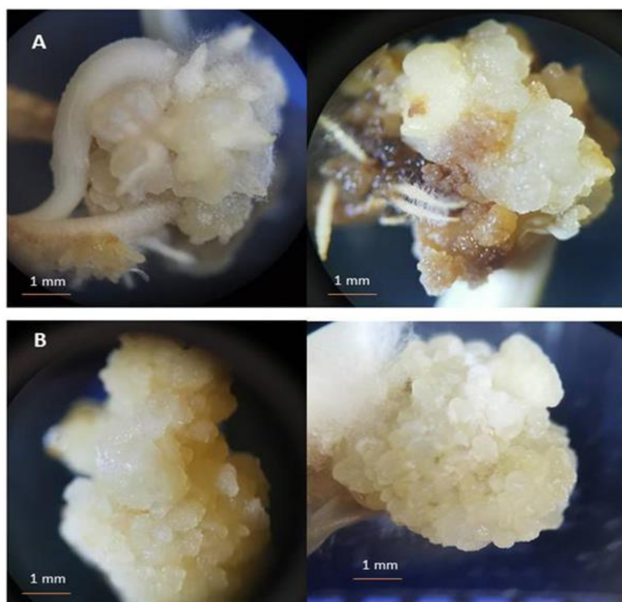

Figure 1. Non-embryogenic (A) and embryogenic (B) calli of MARDI Siraj 297. The non-embryogenic calli mostly develop root-like structure with no appearance of globular embryogenic stages as observed in the embryogenic calli.

Table 1. The effect of different 2,4-D and Kin concentrations on callus growth of MARDI Siraj 297.

TRT	PGR combination (mg/L)		CIF (%)	ECF (%)	RF (%)	PPC	FW (mg)	Callus texture	Callus color
	2,4-D	Kin							
T1	0.5	0	-	-	-	-	-	-	-
T2	1	0	-	-	-	-	-	-	-
T3	1.5	0	90.0 ± 6.1[a]	0 ± 0[f]	0 ± 0[d]	0 ± 0[h]	42.8 ± 4.1[h]	F	LY
T4	2	0	85.0 ± 6.1[ab]	0 ± 0[f]	0 ± 0[d]	0 ± 0[h]	61.7 ± 3.3[g]	F	LY
T5	2.5	0	65.0 ± 10.1[bc]	20.0 ± 5.0[e]	100.0 ± 0[a]	9.4 ± 0.5[d]	85.2 ± 3.7[f]	F	LY
T6	3	0	85.0 ± 6.1[ab]	0 ± 0[f]	0 ± 0[d]	0 ± 0[h]	60.4 ± 2.1[g]	F	LB
T7	3.5	0	45.0 ± 9.4[d]	0 ± 0[f]	0 ± 0[d]	0 ± 0[h]	3.9 ± 1.6[i]	F	B
T8	0.5	0.2	-	-	-	-	-	-	-
T9	1	0.2	80.0 ± 9.4[ab]	0 ± 0[f]	0 ± 0[d]	0 ± 0[h]	109.4 ± 6.5[e]	F	CR
T10	1.5	0.2	90.0 ± 6.1[a]	60.0 ± 6.1[c]	93.3 ± 6.7[ab]	15.8 ± 1.2[b]	146.4 ± 5.4[d]	F	CR
T11	2	0.2	95.0 ± 5.0[a]	90.0 ± 6.1[a]	100.0 ± 0[a]	22.0 ± 0.9[a]	221.9 ± 8.6[a]	F	CR
T12	2.5	0.2	90.0 ± 6.1[a]	55.0 ± 5.0[c]	53.3 ± 3.3[c]	10.6 ± 0.7[c]	176.2 ± 5.9[c]	F	CR
T13	3	0.2	85.0 ± 6.1[ab]	0 ± 0[f]	0 ± 0[d]	0 ± 0[h]	91.0 ± 5.1[f]	F	CR
T14	3.5	0.2	80.0 ± 12.3[ab]	0 ± 0[f]	0 ± 0[d]	0 ± 0[h]	40.4 ± 5.6[h]	F	B
T15	0.5	0.5	-	-	-	-	-	-	-
T16	1	0.5	-	-	-	-	-	-	-
T17	1.5	0.5	65.0 ± 6.1[bc]	60.0 ± 6.1[c]	56.6 ± 4.1[c]	6.8 ± 0.4[e]	138.7 ± 4.1[d]	C	CR
T18	2	0.5	80.0 ± 5.0[ab]	60.0 ± 6.1[c]	86.6 ± 8.2[b]	7.4 ± 0.4[e]	202.4 ± 4.5[b]	C	CR
T19	2.5	0.5	90.0 ± 6.1[a]	50.0 ± 0.0[c]	50.0 ± 0[c]	5.4 ± 0.4[f]	116.1 ± 5.5[e]	C	CR
T20	3	0.5	80.0 ± 5.0[ab]	75.0 ± 0.0[b]	53.3 ± 8.2[c]	3.0 ± 0.3[g]	107.3 ± 7.4[e]	C	CR
T21	3.5	0.5	55.0 ± 5.0[cd]	40.0 ± 6.1[d]	0 ± 0[d]	0 ± 0[h]	64.7 ± 6.9[g]	F	T

Note: Values represent the mean ± standard error. Means within a column denoted by different letters are significantly different at $p < 0.05$ using DMRT. The FW = fresh weight; CIF = callus induction frequency; ECF = embryogenic callus frequency; RF = regeneration frequency; PPC = number of plantlets per callus; F = friable; C = compact; LY = light yellow; LB = light brown; B = brown; CR = cream; T = translucent. Symbol - indicates no callus was formed.

Among the treatments, T5 (2.5 mg/L 2,4-D) and T11 (2.0 mg/L 2,4-D and 0.2 mg/L Kin) were found to be the most responsive for regeneration, by showing the significantly highest ($p < 0.05$) RF of 100% (Table 1). It was observed that plant regeneration occurred in certain range of 2,4-D concentrations, which was 2.5 mg/L (in treatment without kinetin), 1.5 to 2.5 mg/L (in treatment with 0.2 mg/L Kin) and 1.5 to 3.0 mg/L (in treatment with 0.5 mg/L Kin). Treatments with very low or high 2,4-D concentration reduced the capacity for regeneration. Meanwhile, the highest PPC (22 plantlets) was also observed in T11 (Table 1). Although the calli in T5 showed the similar regeneration frequency as T11, the number of plantlets produced per callus was lower. In general, calli supplemented with lower kinetin (0.2 mg/L) produced more plantlets than those with higher kinetin concentration (0.5 mg/L).

The stages of calli regeneration are shown in Figure 2. The cream colored calli formed green pigments upon 7 days of exposure to light (Figure 2A). After 3 weeks, coleoptilar structures

developed into greenish shoot buds (Figure 2B). Shoots were then proliferate and elongate in shoot regeneration medium (Figure 2C) and finally produced roots in rooting medium (Figure 2D).

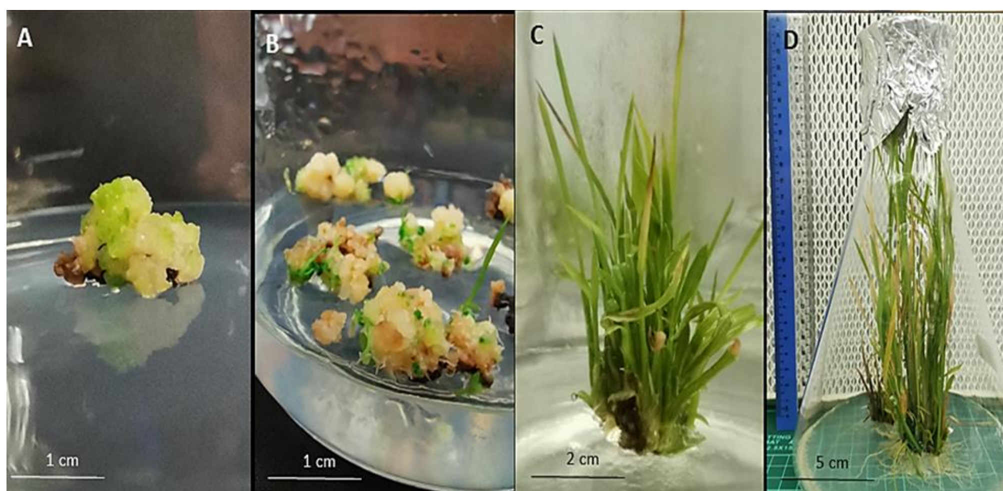

Figure 2. Regeneration of MARDI Siraj 297 embryogenic calli obtained from the optimized callus induction medium (MS + 2.0 mg/L 2,4-D and 0.2 mg/L Kin). (A) Green pigment appeared on cream colored calli (B) Shoot buds grow from scutellum-like structure (C) Shoots proliferate (D) Complete plantlet at 7 weeks old after transferred to regeneration medium.

The highest ($p < 0.05$) FW was observed in MS medium supplemented with 2.0 mg/L 2,4-D and 0.2 mg/L kinetin (T11). Meanwhile, the calli produced in treatments containing both 2,4-D and kinetin (T8 to T21) had significantly higher FW compared to the treatments without kinetin (T1 to T7). Irrespective of the kinetin concentrations, seed explants inoculated in very low 2,4-D concentration (0.5 mg/L) did not form any callus. The FW increased with the increasing 2,4-D concentration but declined after reaching concentration of 2.5 mg/L (in medium without kinetin) and 2.0 mg/L (in medium with kinetin).

The calli texture produced was friable except for those treated in 0.5 mg/L Kin (T17, T18, T19 and T20). There was no tissue browning observed except for calli treated in the highest 2,4-D concentration (T17 and T14), whereas calli in other concentrations were either cream or light yellow in color.

Considering all the parameters assessed for calls growth, the optimum responses were achieved in MS medium supplemented with 2.0 mg/L 2,4-D and 0.2 mg/L Kin (T11), represented by the highest CIF (95%), ECF (90%), RF (100%), PPC (22 plantlets) and FW (211 mg), with friable callus texture and cream colored callus.

3.2. The effect of 2,4-D and kinetin concentrations on callus morphology and somatic embryogenesis of MARDI Siraj 297

MARDI Siraj 297 seeds cultured on different combinations of 2,4-D and Kin treatments exhibited different morphological responses after 35 days of culture as shown in Figure 3. The callus formation started with the coleoptile emergence 2 days after inoculation on the respective growth

media. This was followed by swelling of scutellum tissue and formation of undifferentiated cell mass in the scutellum region on day 7 to 9. The calli were noticeable by day 13 in treatments that produced callus. While most treatments gave rise to callus without organ regeneration, certain treatments such as T1, T2, T8, T15 and T16 showed different response, whereby partial organ regeneration was produced in the form of rhizogenic callus. Calli formed in 0 mg/L Kin (T3 to T7) and 0.2 mg/L Kin (T9 to T14) were friable and light yellow or cream in color, except for calli in the highest 2,4-D concentration (T7 and T14) with brown colored calli. Meanwhile, calli derived in 0.5 mg/L Kin (T17 to T21) were compact and cream in color, except for T21 that were friable and translucent.

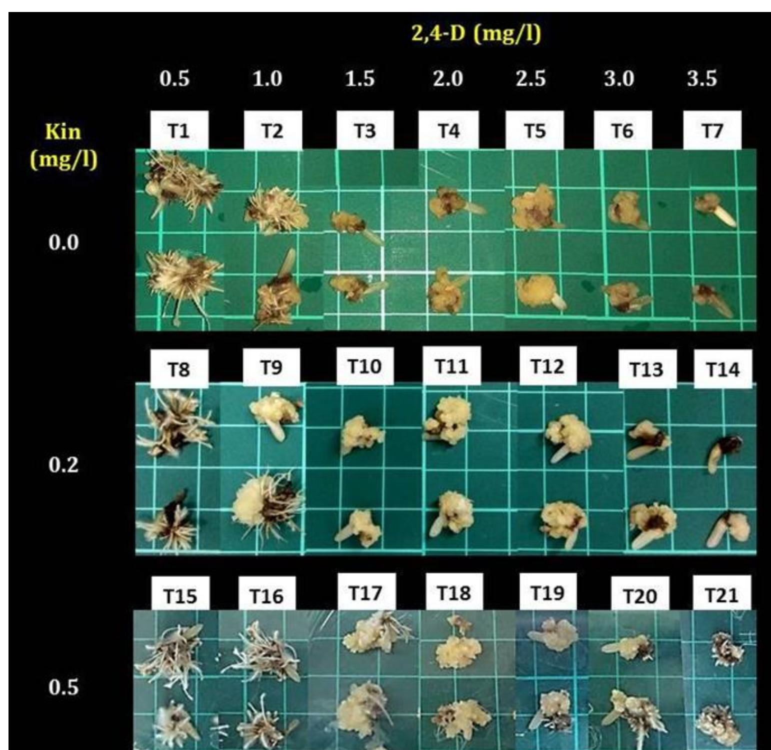

Figure 3. Morphogenic response of rice calli at 35 days on MS medium supplemented with different concentrations of 2,4-D and Kin (Grid = 1 cm × 1 cm).

The distinctive developmental stages of somatic embryo in monocots which involves the formation of proembryo, globular, scutellar and coleoptilar stages were observed in treatments that produced embryogenic calli (T5, T10, T11, T12, T17, T18, T19, T20 and T21). The stages of somatic embryogenesis in MARDI Siraj 297 rice callus are shown in Figure 4. The somatic embryogenesis begins with the formation of callus at scutellum region (Figure 4A), followed by development of proembryogenic cells cluster in the periphery of calli after 20 days of culture (Figure 4B). The size of cells in proembryogenic region increased afterwards, indicating distinct differentiation of embryogenesis stages. Subsequently, the globular structure was observed on day 23 to 24 (Figure 4C) before turning into coleoptilar stage (Figure 4D).

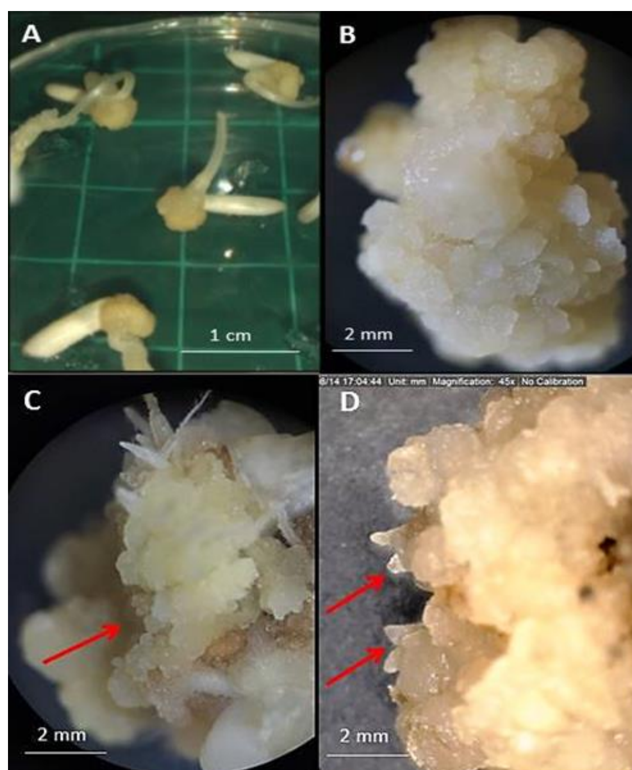

Figure 4. Progressive development of somatic embryogenesis in MARDI Siraj 297 rice callus. The somatic embryogenesis begins with the (A) formation of callus at scutellum region, followed by (B) proliferation of proembryogenic cells, (C) globular stage (arrow) and (D) coleoptilar stage (arrow) embryos.

4. Discussion

The CIF is influenced by PGR constituents [22]. A comparative study on callogenesis potential of 15 Indonesian *indica* rice genotypes showed significant difference in callus induction ability, although most of them shared similar genetic background [53]. In this study, all treatments containing 2,4-D were able to induce callus formation except for those with low 2,4-D concentration of 0.5 and 1.0 mg/L (T1, T2, T8, T15, T16). This was attributable to the function of 2,4-D in inducing and regulating callus formation [54], initiating callus growth and reverting differentiated explant cells to dedifferentiated (callus) state in order to start proliferation [55]. Studies shown that *in vitro* callus formation is possible when plant tissues were exposed to auxin and cytokinin, whereby the ratio between these plant hormones determines the extent of differentiation and dedifferentiation [16,18]. However in this cultivar, induction of callus happened either in the presence or absence of cytokinin (kinetin), although the growth (represented by the FW) was significantly enhanced by cytokinin presence. This suggested that cytokinin might be unessential for callus initiation of MARDI Siraj 297 cultivar, but important to enhance its subsequent proliferation.

Although most PGR combinations used in this study were able to induce callus, many of those callus were non-embryogenic (Figure 1). Therefore, determination of ECF is required to ensure high regeneration potential [37,56,57]. It was obvious that the number of treatments producing embryogenic callus (9 treatments) was much reduced from the total number of treatments producing

callus (16 treatments). This is because somatic embryogenesis requires the presence of specific PGR treatments in order to achieve embryogenic competence through cells dedifferentiation, chromatin restoration, gene expression programming and stressful events [58], which could be highly dependent on the PGR composition of medium [18,59]. In this study, auxin level between 2.0 to 3.0 mg/L 2,4-D triggered the initiation of embryonic response, thereby allowing somatic cells to acquire embryogenic competence [19,24]. However, exposure of explants to high auxins concentration such as 2,4-D hinders normal embryo development [30,60], which explains the reason of reduced ECF in treatment with the highest 2,4-D concentration (3.5 mg/L) in this study.

The RF and PPC are two important determinants in plant regeneration establishment protocol [21,61–63]. Optimization of synthetic auxin such as 2,4-D during callus induction were previously done to achieve high frequency plant regeneration from embryogenic callus of rice [49,64]. The stimulatory effect of auxin and cytokinin has been reported to facilitate plant regeneration in various rice callus cultures [38,50,65]. The present study revealed that the sole use of 2.5 mg/L and combination of 2.0 mg/L 2,4-D with 0.2 mg/L Kin were found to be optimum for complete plant regeneration from embryogenic callus while the presence of higher kinetin concentration (0.5 mg/L) somewhat reduced the RF. Because the calli in this experiment were regenerated in the same regeneration medium, their response during regeneration was mostly dependent on the PGR treatments during callus induction. In plant regeneration aspect, somatic embryos are intermediate stage between undifferentiated callus and differentiated seedlings from embryogenic calli [64]. A study on another monocot plant; buffel grass (*Cenchrus ciliaris* L.) demonstrated a significant positive correlation between the number of embryogenic calli per cultivated explant and the number of regenerated plants, whereby cultivars exhibiting high embryogenic callus formation also promote an increased number of regenerated plantlets [66]. The almost similar trend was also observed in this study, in which treatment that produced high ECF also exhibited higher PPC.

In callus induction, fresh weight is an important parameter that represents the extent of callus growth. In this study, the use of 2,4-D alone (T1 to T7) resulted in lower FW compared to those with kinetin (T8 to T21). It was clear that the combination of kinetin with 2,4-D enhanced callus growth of MARDI Siraj 297 cultivar. Similar to other plant species, the presence of low kinetin concentration below 0.5 mg/L was also reported to increase callogenesis while higher concentrations at 1.0 mg/L were inhibitory towards callus growth [36]. In association with cytokinins (kinetin), auxins (2,4-D) are able to stimulate cell proliferation and enhance cell differentiation [40].

Variation in 2,4-D concentrations in this study has resulted in different morphological response on seed explants. Adventitious roots were formed at 0.5 to 1.0 mg/L 2,4-D while higher concentrations induced formation of callus, regardless of the kinetin concentration. During tissue culture, adventitious roots or shoots can be induced by transferring the callus to medium containing different ratios of auxin and cytokinin [67]. The role of auxin in adventitious root development was proved to be associated with its uneven distribution within the callus mass, thereby causing partial differentiation of callus which leads to formation of root apical meristem [68].

In this study, embryogenic calli were derived from scutellum of mature seeds, which was previously demonstrated to be the best source of explant for *in vitro* callus induction and regeneration due to its high embryogenic potentiality and availability throughout the year [69–71]. In MARDI Siraj 297, embryogenic calli were produced at 2,4-D concentration of 1.5 to 3.5 mg/L 2,4-D, although the frequency was much reduced or completely omitted at 3.5 mg/L. It was evident that the exposure of explants to high auxins concentration especially 2,4-D hindered normal embryo

development [30,60], decreased callus induction and caused callus browning [21]. The 2,4-D is the most frequently used PGR for *in vitro* somatic embryogenesis induction due to its strong auxinic activity by influencing endogenous activity of other phytohormones such as IAA [72]. Although its exact role in acquiring cells embryogenic competency is still unclear [29], 2,4-D was proved to influence the metabolism of endogenous IAA, which was proposed to have significant roles in formation of somatic embryo [32,73]. While in other study on *Arabidopsis* embryo culture, the presence of 2,4-D had initiated the procambial cell division at the cotyledons base, which eventually expand to the whole cotyledon to form callus on which globular and heart-shaped embryos emerged [64].

The typical developmental stages of somatic embryo in monocots as described by [18,74] were also observed in this rice cultivar, which involves the formation of proembryo, followed by globular, scutellar and coleoptilar stages. Somatic embryogenesis is a distinctive physiological, morphological, and molecular changes that involve six phases; induction, expression, multiplication, development, maturation, and germination [25]. The somatic embryos derived in the optimized callus induction medium in this study had successfully undergone maturation and germination stage of somatic embryogenesis that gave rise to the regeneration of whole plantlets.

Despite previous studies conducted on callus formation and somatic embryogenesis of *indica* rice varieties, there was no single protocol widely applicable for all genotypes since each of them responds differently towards PGRs [39,75–77], growth condition [78,79] and other medium constituents [80–83]. In fact, studies revealed that some *indica* varieties are recalcitrant and therefore failed to produce embryogenic calli [84]. Due to these shortcomings, PGRs optimization for each variety is an important determinant in successful establishment of rice culture.

5. Conclusions

In this study, supplementation of 2,4-D and kinetin into callus induction medium at different ratio significantly affects callogenesis and embryogenesis of MARDI Siraj 297 rice cultivar. The complimentary effect of both PGRs enhanced the callus and somatic embryo formation as compared to single 2,4-D application. The maximum growth response was achieved in medium supplemented with combination of 2.0 mg/L 2,4-D and 0.2 mg/L Kin, represented by the highest CIF (95%), ECF (90%), RF (100%), number of plantlets per callus (22 plantlets) and FW (211 mg), which was accompanied by formation of friable embryogenic cells. The optimization of callogenesis and somatic embryogenesis medium for MARDI Siraj 297 cultivar is crucial to ensure an efficient establishment of embryogenic calli as starting materials for further crop improvement via *in vitro* genetic manipulation.

Acknowledgments

This work was funded by the Ministry of Higher Education Malaysia under Fundamental Research Grant Scheme, FRGS (Grant number: FRGS/1/2020/STG03/UPM/02/1).

Conflict of interest

The authors declare no conflict of interest.

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