
Callus induction and plantlet regeneration from mature embryos of *indica* rice (*Oryza Sativa* L.) cultivar *Kra Dang Ngah*

Zhang Yinxia^{1,2} and Te-chato, S.^{2*}

¹School of Agriculture, Ningxia University, Ningxia, YinChuan, 750021 China, ²Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand

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Hom Kra Dang Ngah is a kind of favorable *indica* rice in southern Thailand. It was selected for the present study because it is local variety and no regeneration system has been reported earlier. In order to improve its quality by the genetic transformation, the establishment of the suitable plantlet regeneration system is prerequisite. In this way, mature embryos from seeds were used to induce callus and establish the high efficiency plantlet regeneration system. The optimum medium was obtained by manipulating different concentrations various plant growth regulators (PGR). Experimental results showed that addition of 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 1.0 mg/L of α -naphthalene acetic acid (NAA), 1.0 mg/L of 6-benzyladenine (6-BA) and 0.5 mg/L of kinetin (Kn) to callus induction medium which was MS medium gave the most suitable for embryogenic callus induction at 56.3 %. The calluses derived from seeds were well proliferated on MS medium supplemented with 1.0 mg/L 2, 4-D, 0.5mg/L indole-3-acetic acid (IAA), 0.25 mg/L 6-BA, 0.25 mg/L Kn without browning. It also indicated the highest capability of plantlet regeneration. At the same time, 1.5 mg/L thidiazuron (TDZ) in combination with 1.0mg/L 2,4-D showed the most efficient in plantlet regeneration.

Key words: *Indica* rice, Hom KraDangNgah, callus induction, mature embryos, plantlet regeneration.

Introduction

Rice (*Oryza sativa* L.) is one of the most important crops and a major staple food in the worldwide. More than half of the world's population depends on rice for its major daily source of energy and protein. Moreover, in the world 80% rice production based on *indica* (Wanichananan *et al.*, 2010). With the increasing of population and decreasing of arable land, the biotic and abiotic stress is more and more serious. Rice production is under the heavy pressure.

* Corresponding author: Sompong Te-chato; e-mail: stechato@yahoo.com

Therefore, breeding new rice varieties using genetic transformation is one of the available ways to increase rice yield and improve its quality.

In recent years, the genetic transformation as a key technology of genetic engineering of rice has made a great progress. At the same time, the rapid, efficient, stable and large-scale genetic transformation system in most japonica rice was established. On this basis, a lot of important foreign gene, such as insect and disease resistance, stress tolerance, quality improvement, and nutritional gene were transferred into japonica rice (Chen *et al.*, 2009; Zhou, 2010).

However, the transformation of indica rice is still difficulty due to the low induction rate of embryogenic calli and plantlet regeneration. Many indica rices are particular genotype-dependent (Lin *et al.*, 2005 Hiei and Komari, 2006; 2008). Although a few successful genetic transformation in indica rice was reported (Chan. *et al.*, 1992; Xiao *et al.*, 2008; Lin *et al.*, 2009; Wang *et al.*, 2010), generally, it is still lack of commonly used genetic transformation technologies for indica rice and there are many problems which challenge in the practical application.

The basic prerequisite for the potential use of genetic transformation in indica rice improvement is the establishment of the efficient plantlet regeneration system. Successful regeneration system mainly depends on genotype, explant type, medium composition, plant growth regulator and culture environment. Therefore, the objectives of this study were to find a suitable culture condition for callus induction and plantlet regeneration from mature zygotic embryos of indica rice variety Hom Kra Dang Ngah and established the plantlet regeneration system further gene transformation.

Materials and methods

Plant material and sterilization

Mature seeds of *Oryza sativa* L. cultivar Hom Kra Dang Ngah were used as explant source. Mature and healthy seeds were selected and dehusked manually. The surface disinfestation was carried out by immersing in 70 % ethanol for 1 min, followed by 20 % Clorox in the presence of a wetting agent “Tween 20” for 5 min. Finally, the explants were rinsed with sterile distilled water for four to five times in laminar air flow bench.

Callus induction

The surface sterilized seeds were cultured on solidified MS medium (Murashige and Skoog, 1962), containing 3% sucrose, 0.75 % agar-agar powder and different concentration of 2,4-D ranging from 1 to 4mg/L. The pH

of the medium was adjusted to 5.7 prior to autoclaving at 121°C, 1.07 kg/cm for 15 min. All cultures were placed in the culture room at 25-27°C, under 16 hours photoperiod. After being cultured for one month the frequency of callus induction was recorded.

Enhancing callus formation

In order to enhance the frequency of callus induction, the surface aseptic seeds were cultured on MS medium supplemented with 2,4-D at the optimum concentration (result from trial 1) in combination with various kinds and concentrations of phytohormone, which was 0.5 mg/L or 1mg/L 6-BA together with 1mg/L NAA or 1mg/L IAA with Kn 0.5 mg/L. The treatments were followed: (1) 1mg/L 2,4-D and 1.5mg/L TDZ, (2) 2 mg/L 2,4-D, 1.0mg/L NAA and 1.0mg/L 6-BA, (3) 2 mg/L 2,4-D, 1.0mg/L NAA, 1.0mg/L 6-BA and 0.5mg/L Kn, (4) 2 mg/L 2,4-D, 1.0mg/L IAA, 0.5mg/L 6-BA and 0.5mg/L Kn, (5) 4 mg/L 2,4-D, 1.0mg/L NAA, 1.0mg/L 6-BA and 0.5mg/L Kn, (6) 4 mg/L 2,4-D, 1.0mg/L IAA, 0.5mg/L 6-BA and 0.5mg/L Kn. All culture media were supplemented with 1g/L casein hydrolysate (CH), 3% sucrose and solidified with 0.75 % agar. Seed cultures were incubated at the same conditions as previously mentioned for one month. After one month of culture, the percentage of seed forming callus and the size of callus were recorded.

Callus proliferation

The calluses produced on above medium were transferred to different proliferation medium. The treatments were (1) 1mg/L 2,4-D, (2) 1mg/L 2,4-D and 1.5mg/L TDZ, (3) 1 mg/L 2,4-D and 0.5mg/L NAA, 0.5mg/L 6-BA, (4) 1 mg/L 2,4-D and 0.5mg/L NAA, 0.5mg/L 6-BA, 0.25mg/L Kn, (5) 1mg/L 2,4-D and 0.5mg/L IAA, 0.25mg/L 6-BA, 0.25mg/L Kn, (6) 2mg/L 2,4-D, (7) 2mg/L 2,4-D and 0.5mg/L NAA, 0.5mg/L 6-BA, 0.25mg/L Kn, (8) 2 mg/L 2,4-D and 0.5mg/L IAA, 0.25mg/L 6-BA, 0.25mg/L Kn, (9) 3mg/L 2,4-D, (10) 4mg/L 2,4-D. All culture MS media were supplemented with 1g/L casein hydrolysate (CH), 3% sucrose and solidified with 0.75 % agar. After being cultured for 4 weeks the percentage of browning response and frequency of embryogenic callus were recorded. Embryogenic and non-embryogenic callus were determined under the stereo microscope.

Plantlet regeneration

In order to optimize plantlet regeneration through somatic embryogenesis, calluses were transferred from proliferation medium to the regeneration

medium. MS medium supplemented with difference concentrations of various phytohormone at the optimum concentration (result from above). CH at 1g/L was also added to this medium. Within 4 weeks, growth and development of the callus were recorded and compared among those kinds and concentrations of phytohormones.

Statistical data analysis

The experiment was arranged as a randomized complete block design (RCBD) with 3 replicates per treatment. Analysis of variance (ANOVA) was applied to indicate which variety or combination of which concentration of growth regulators was the best for callus induction. Data were analyzed using the SAS program Version 6.11 (SAS Institute, Cary, NC). A probability level of 5% ($p=0.05$) was chosen for all statistical inferences.

Results and discussions

Callus induction

The scutellum region of the seeds swelled in 7 days. However, a few calluses formation took place after being cultured for 4 weeks. Increase in concentration of 2,4-D to 3 mg/L promoted the increment of callus formation from 20 to 33%. However, higher concentration than this inhibited callus induction frequency. Without the addition of 2,4-D in the medium callus formation was not obtained (Table 1). Similar result was also reported by Zuraida Abdul *et al.* (2010). An optimum concentration of 2,4-D for callus induction varied depending on the explant source and genotype of rice (Raina, 1987). Our result revealed that 2,4-D at concentration of 1-4 mg/L can produce callus from mature seeds of rice cultivar Hom Kra Dong Ngah, while the percentage of callus induction is still low. Ge *et al.* (2006) reported that 2,4-D alone in culture medium induced a low frequency of callus induction in rice. In the presence study, it concluded that 2,4-D alone was not the best treatment to induce embryogenic callus in Hom Kra Dong Ngah rice. The characters of four types of the callus were classified in different concentrations of 2,4-D containing medium. The first type was yellowish-brown compact obtained from 1.0 mg/L 2,4-D containing medium. The second type was yellow compact obtained from 2.0 mg/L 2,4-D containing medium. The third type was yellowish-white granular obtained from 3.0 mg/L 2,4-D containing medium. The last type was yellow friable one obtained from 4.0 mg/L 2,4-D containing medium. All characters of calluses were shown in Fig.1.

Table 1. The frequency of callus induction from mature seed cultured on MS medium supplemented with different concentrations of 2,4-D after being cultured for one month

2,4-D (mg/L)	Callus induction frequency (%)	Character of callus
0	0b	No callus formation
1.0	20.0a	Yellowish-brown compact
2.0	25.0a	Yellow compact
3.0	33.9a	Yellowish-white granular
4.0	31.2a	Yellow friable
F-test	*	
C.V.(%)	61.3%	

*Significant difference at 0.05 level

Means of callus induction frequency with the same letter are not significantly different using least significant different (LSD).

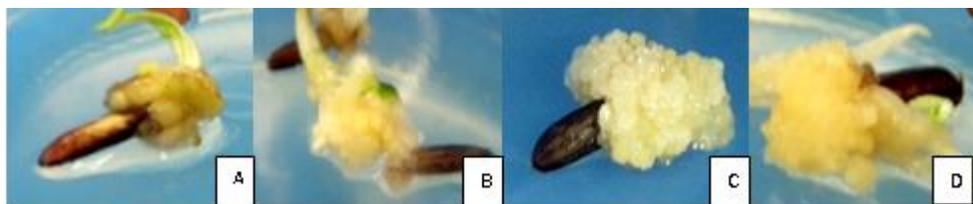


Fig. 1. Callus induction from rice caryopsis on MS medium supplemented with different concentrations of 2,4-D after being cultured for one month.

A: 1mg/L 2,4-D, B: 2mg/L 2,4-D, C: 3mg/L 2,4-D and D: 4mg/L 2,4-D .

Enhancing callus formation

When the seeds were inoculated on the basal medium containing different auxin and cytokinin, the mature seeds swelled within 3-4 days and after being cultured for 7 days callus formation occurred. These calluses proliferated with the passage of time and an excellent callus growth was obtained after 4 weeks of culture. The results were shown in Table 2. The frequency of callus induction was significantly enhanced and the quality improved when the seeds were cultured on the MS medium supplemented with 2 mg/L of 2,4-D, 1.0 mg/L of NAA, 1.0 mg/L of 6-BA and 0.5 mg/L of Kn at the 56.3%. The analysis of variance showed significant different effect of phytohormones on callus induction rate. This suggested that different concentrations of various phytohormones added to the MS medium were effective in callus induction of indica rice like those reported by Chowdhry *et al.* (1993) and Shazia *et al.* (2005).

Most of these calluses were friable embryogenic, hard and yellowish-white in colour (Fig. 2). The callus induced on MS medium containing 2.0

mg/L,4-D, together with different auxin and cytokinin gave creamy white, compact, and some green spots usually which also were classified as embryogenic type. The green spots had further developed into shoots. Very few calluses were induced on MS containing 1.0 mg/L 2,4-D and 1.5 mg/L TDZ. These callused were compact, hard, white in color and has slower growth rate than those obtained in another culture media. Some of which were embryogenesis. The callus initiated on MS medium containing 4.0 mg/L 2,4-D and another different auxin and cytokinin was somewhat compact and yellow to white in color. The size of those calluses was small and some were brown in color (Table 3). Only a small percentage of this callus turned into be embryogenic in nature (Fig. 3).

Table 2. The frequency (%) of callus induction from mature seeds cultured on MS medium supplemented with different phytohormone

Treatment	2,4-D (mg/L)	NAA (mg/L)	6-BA (mg/L)	IAA (mg/L)	Kn (mg/L)	TDZ (mg/L)	Callus induction frequency(%)
1	1	0	0	0	0	1.5	23.3c
2	2	1	1	0	0	0	28.9b
3	2	1	1	0	0.5	0	56.3a
4	2	0	0.5	1	0.5	0	35.3b
5	4	1	1	0	0.5	0	31.6b
6	4	0	0.5	1	0.5	0	38.9b
F-test							*
C.V.(%)							33.2%

*Significant difference at 0.05 level.

Means of callus induction frequency with the same letter are not significantly different using least significant different (LSD).

Table 3. The growth and morphological characters of callus cultured on MS medium supplemented with different combinations of auxin and cytokinin.

Treatment	Callus growth	Morphology of callus
1	+	white, compact
2	+	Yellowish-white, compact, no browning, seeding
3	++++	Whitish-green, friable and somewhat compact, no browning, seeding
4	+++	Yellowish-white, friable and somewhat compact, seeding
5	++	Whitish-green, compact, a little browning, no seeding
6	++	Whitish-green, compact, a little browning, no seeding

Sign for callus growth: + = Small size; ++ = Medium size; +++ = Large size; ++++ = Very large size

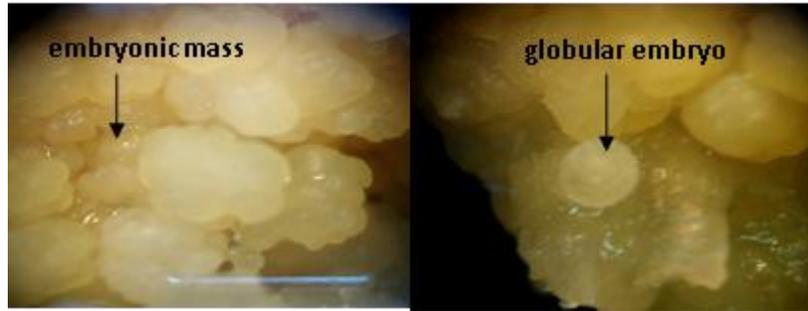


Fig. 2. Morphological characters of embryogenic callus under the observation of stereo microscopy. The callus were cultured on MS medium containing 2mg/L 2,4-D , 1.0mg/L NAA, 1.0mg/L6-BA and 0.5mg/L Kn.

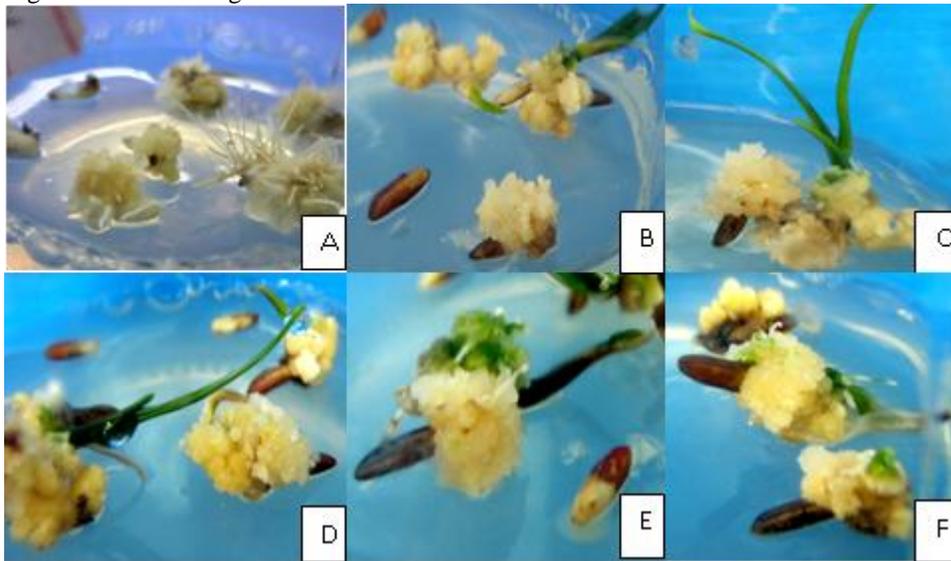


Fig. 3. Morphological characters of callus on MS medium supplemented with different types and concentrations of phytohormones after being cultured for 4 weeks.

- A: 1mg/L 2,4-D and 1.5mg/L TDZ.
- B: 2 mg/L 2,4-D and 1.0mg/L NAA, 1.0mg/L6-BA
- C: 2 mg/L 2,4-D and 1.0mg/L NAA, 1.0mg/L6-BA ,0.5mg/L Kn
- D: 2 mg/L 2,4-D and 1.0mg/LIAA, 0.5mg/L6-BA, 0.5mg/L Kn
- E: 4 mg/L 2,4-D and 1.0mg/L NAA, 1.0mg/L6-BA ,0.5mg/L Kn
- F: 4 mg/L 2,4-D and 1.0mg/LIAA, 0.5mg/L6-BA, 0.5mg/L Kn

In this experiment, combination of two auxins, 2,4-D and NAA and two cytokinins, BA and Kn gave the best result in embryogenic callus formation. Addition of 6-BA, Kn and NAA to the callus induction media in the presence of 2,4-D was more effective for the production of embryogenic calluses than using 2,4-D alone. The results were in agreement with Ge *et al.* (2006).

Moreover, addition of Kn and NAA to the callus induction media can improve the quality of calluses (Fig. 3C) (Tian *et al.*, 1994). The calluses obtained from those PGR containing medium were friable, hard, some compact, more granular structure and green spots, while the calluses on the medium containing 2,4-D alone were mucilaginous, smooth and hardly to regenerate into plantlet (Fig.1). So the culture medium containing 2 mg/L of 2,4-D, 1.0 mg/L of NAA, 1.0mg/L of 6-BA and 0.5mg/L of Kn was the most effective for callus induction.

Callus proliferation and plantlet regeneration

Calluses obtained from the above trial were sub-cultured onto the fresh media supplemented with the same kinds and concentrations of phytohormone or half concentration of hormone in order to induce further development of embryogenic calli. During 3 weeks of the sub-cultured, some treatments showed more callus browning. The calluses which were cultured on medium containing 2,4-D alone at the same concentration with previous study produced higher percentage of browning ranging from 13.6 to 100% (Table 4). Browning of the calluses decreased when 2,4-D in culture medium increased from 1 to 4 mg/L. However, plantlet regeneration was more difficult. Similar results were reported in Malaysian indica rice and *jaumala* indica rice. Maintenance of those calluses on high concentration of 2,4-D for a longer period played inhibitory effect on plantlet regeneration (Aparna, 2004; Zuraida *et al.*, 2010). Therefore, using 2,4-D alone was not the suitable treatment to induce embryogenic callus in indica rice variety Hom Kra Dang Ngah and also due to a higher browning response after sub-culture.

When the calluses were sub-cultured on MS medium supplemented with 1 mg/L or 2 mg/L 2,4-D in combination with 1.5 mg/L TDZ or 0.5 mg/L NAA, 0.5 mg/L 6-BA, 0.25 mg/L Kn or 0.5 mg/L IAA, 0.25 mg/L 6-BA, 0.25mg/L Kn percentage of browning produced after sub-culture was 0-27.2%, lower than the medium containing 2,4-D alone. Moreover, the combination of 1.0 mg/L 2,4-D with 0.5mg/L NAA or 0.5 mg/L IAA, 0.25-0.5mg/L 6-BA and 0.25mg/L Kn resulted in the most effective for embryogenic callus induction without browning. The calluses from these treatments were yellow in color and produced green buds (Fig. 4). It indicated that these calluses were easy to regenerate. Furthermore, the calluses grown on 1.0 mg/L 2,4-D, 0.5mg/L IAA, 0.25 mg/L 6-BA and 0.25mg/L Kn produced shoot buds within 3 weeks after sub-culture (Fig. 4A).

In this present study, the somatic embryos derived from previous experiments were transferred onto MS medium with 1.5 mg/L TDZ and 1.0 mg/L 2,4-D in order to induce plantlet regeneration. TDZ showed a remarkable

regenerative ability in tissue culture of indica rice variety Hom Kra Dang Nga (Fig. 4 D). It was not only shortened the time of plantlet regeneration but also enhanced number of shoot formation. Similar results were obtained from Tian *et al.* (1994) and Jutta (2007) who reported that TDZ was more efficient on improving regeneration from mature seeds of rice than the combination of 2,4-D with NAA. However, TDZ did not promote callus induction in *jaumala* rice (Aparna, 2004). So, in this study, the combination of 2,4-D with TDZ was not the effective treatment for callus induction but the most suitable for plantlet regeneration.

In summary, the results from this study showed that concentrations and combinations of phytohormones had greatly influence on the proliferation of calluses. Combination of 1.0 mg/L 2, 4-D with 0.5mg/L IAA, 0.25mg/L 6-BA and 0.25mg/L Kn was the most suitable for callus proliferation. Similar findings were reported in MR 219 rice (Syaiful *et al.*, 2009; Zuraida *et al.*, 2010). But different point of view also reported. Such as the opinion that the incorporation of NAA and Kn in the callus induction medium supplemented with 2 mg/L 2,4-D did not significantly improve the callus induction frequency (Shahsavari *et al.*, 2010). It evidenced that the indica rice has recalcitrant in nature and genotypes (Rashid *et al.*, 2001). In addition, 1.5 mg/L TDZ was the most suitable for plantlet regeneration.

Table 4. The percentage of browning callus after sub-culture on medium supplemented with various phytohormones for 3 weeks

Treatment	2,4-D (mg/L)	NAA (mg/L)	6-BA (mg/L)	IAA (mg/L)	Kn (mg/L)	TDZ (mg/L)	Callus browning (%)
1	1	0	0	0	0	1.5	100
2	1	0	0	0	0	0	26.7
3	1	0.5	0.5	0	0	0	3.6
4	1	0.5	0.5	0	0.25	0	0
5	1	0	0.25	0.5	0.25	0	0
6	2	0	0	0	0	0	20.3
7	2	0.5	0.5	0	0.25	0	16.0
8	2	0	0.25	0.5	0.25	0	27.2
9	3	0	0	0	0	0	13.6
10	4	0	0	0	0	0	0

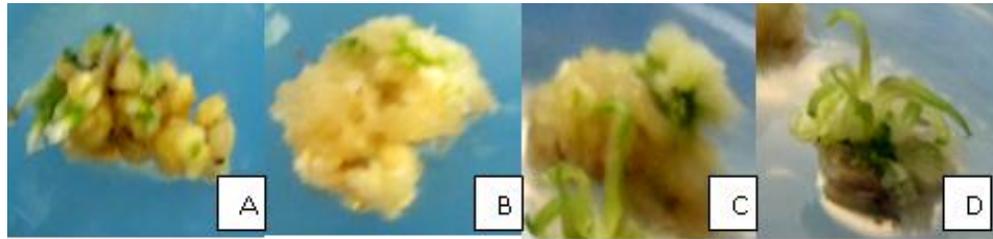


Fig. 4. Somatic embryo initiation, turning into green bud and starting to regenerate into plantlets after 3 weeks of sub-culture on MS medium supplement with different phytohormones.

(A) 1.0 mg/L 2, 4-D, 0.5mg/L IAA, 0.25 mg/L 6-BA, 0.25mg/L Kn

(B) 1.0 mg/L 2, 4-D, 0.5mg/L NAA, 0.5 mg/L 6-BA, 0.25mg/L Kn

(C) 1.0 mg/L 2, 4-D, 1.5mg/L TDZ

(D) Plantlets elongation on medium containing 1.0 mg/L 2, 4-D and 1.5mg/L TDZ and more plantlets regeneration after being sub-cultured to PGR-free MS medium.

Conclusion

The present study confirmed the production of embryogenic calluses, somatic embryos as well as plantlet regeneration of indica rice cultivar Hom Kra Dang Ngah. The somatic embryogenesis system was preliminarily established and can be used for genetic engineering purposes to produce high yield and quality of new indica rice variety through genetic transformation.

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