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# 籼稻体细胞胚胎发生及器官发生的比较研究

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## 摘 要

在以幼穗为外植体的诱导培养及其以后的继代培养与悬浮培养中，从以下几个方面对3个籼稻品种的体细胞胚胎发生（E）与器官发生（O）的特点作了比较研究。在形态上，（E）型愈伤由胚状体组成，具有层次分明的结构。胚状体由胚芽、胚根及盾片组成。而（O）型的愈伤没有上述组织的分化，没有层次结构。植株再生能力方面，与器官发生途径相比较，体细胞胚胎发生途径之分化频率高，保持再生能力持续的时间长，从每个愈伤上发生的再生植株数多。（O）型愈伤结构较疏松，建立结构良好的、细胞团较小的悬浮细胞系容易，但这种细胞系在液体条件下几乎丧失再生能力。而（E）型愈伤则相反，建立好的悬浮细胞系，需要时间较长，但在悬浮条件下仍然具有再生植株的能力。在诱导条件方面，2，4-D及激动素分别作用时可诱导器官发生，共同作用时则诱导体细胞胚胎发生。再生植株途径方面，由于水稻同时具有器官发生及体细胞胚胎发生的特点，无论在诱导条件上还是在（E）与（O）本身的特点上均不似萝卜或烟草那样专一，并且有（E）与（O）相互转化和混合发生的复杂情况。

关键词：体细胞胚胎发生；器官发生；植株再生；体细胞培养；籼稻

# SOME REGULATIONS OF SOMATIC EMBRYOGENESIS AND ORGANOGENESIS IN INDICA RICE

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## Abstract

Different concentrations (0, 0.2, 0.5, 1.0 and 2.0 mg/L) of 2,4-D and kinetin (25

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combinations of them) in the media were studied for inducing embryogenesis and organogenesis from young panicle of rice. When the medium contained both 2,4-D and kinetin, the plantlets were regenerated via somatic embryogenesis. When the medium contained 2,4-D only or kinetin only, plantlets were regenerated via organogenesis but the former was adventitious bud from organogenic callus and the later, from the explant directly. In subculture condition, the medium containing both 2,4-D and kinetin could maintain the culture keeping a high frequency for embryogenesis and the medium with 2,4-D only, for organogenesis. Frequency of plant regeneration, duration of keeping the capacity for plant regeneration and number of regenerated plant per callus in embryogenesis were higher, longer and more than those in organogenesis respectively. Embryogenic callus consisted of embryoids, which was made up coleoptile, coleorhiza and scutellum, possessed the structure of distinct and polylayers. The organogenic callus did not possess this organlets and friable in structure.

**Key words**: Somatic embryogenesis; Organogenesis; Plant regeneration; Somatic cell culture; Indica rice

## Introduction

Somatic embryogenesis (E) and organogenesis (O) are the two major pathways of plant regeneration in *in vitro* culture[20]. Somatic embryogenesis mimics zygotic embryogenesis in origin, structure and developmental process. Like zygote embryo, the origin of somatic embryos derives from single cells[7,12,22] and develops through the regular stages: globular, heart shape, torpedo shape in dicotyl and globular, scutellum and mature in monocotyl respectively. For organogenesis, buds or roots are regenerated neither regular stage nor usually synchronous by adventitious way.

These two pathways of plant regeneration of *in vitro* culture were demonstrated in many species[14,20] but it seems to be special relationship to some species. It is well known for example, that embryogenesis occurs easily in carrot, while organogenesis occurs preferentially in tobacco. In *in vitro* culture of rice, plantlets can be regenerated via both ways, O and E, although each way, of O or E, was not so typical and easy to be induced as tobacco and carrot. Researches of E and O were reported numerously in many species, however, few investigations on comparison of these two pathways from the same explant were reported and none in rice. Here, we report the results obtained from comparative study between somatic embryogenesis and organogenesis in *Indica* rice.

## Materials and Methods

### Plant Materials

Young panicles from three varieties of *Indica* rice, Hunan Zhao, Qin Er-ai and Gui Chao, were used as explants. The developmental stage of young panicles used for inoculation was the initial of the 2nd branch to be initiated (1—2 cm in length of the inflorescence).

**Media**

Media for primary culture were MS basic medium supplement with 3% sucrose, 3% mannitol and 300 mg/L casein hydrolysate (CH), and one of 25 combinations of different concentrations (0, 0.2, 0.5, 1.0 and 2.0 mg/L) of 2,4-D and kinetin (Kt). Media for subculture were selected 2 from the 25 media mentioned above. One was MS supplemented with only 2mg/L 2,4-D (Medium No. 21) which used for subculture of organogenic cell line. The other was MS supplemented with 1mg/L Kt and 1mg/L 2,4-D (Medium No. 19) which used for the subculture of embryogenic cell line. Media were solidified with 0.8% agar. The cultures were inocubated at 27℃ in the dark subcultured every four weeks.

**Criteria of embryogenesis and organogenesis**

To identify the culture belonging to embryogenic or organogenic, the criteria is as follows.

Embryogenesis: embryogenic callus is made of embryoids which consist of scutellum, coleoptile and coleorhiza. Thus, embryogenic callus is compact and granulate with polylayers in structure.

Organogenesis: adventitious buds and roots form directly from explants or indirectly from organogenic callus which is friable and has no embryogenic structure. To calculate the frequency of E and O in the cultures, stereomicroscop was used or sometimes naked eye only.

**Plant regeneration**

MS basic medium supplemented with BAP 1mg/L, Kt 0.1mg/L, NAA 2mg/L and solidified with 0.8% agar. Fifteen pieces of calli per petri dish (9 cm) were used and at least three dishes were repeated for each experiment. Plant regeneration frequency (%) =No. of regenerated plant/No. of calli transferred X 100.

**Plant regeneration in liquid condition from suspension culture**

For direct regeneration in liquid condition, the cell lines, which were well established in suspension culture for 4 months, were first passed through nylon cell sieve with pore size of 290 μm and washing medium (the regeneration medium or basic medium without hormone) by Komagome pipe. And then, the cell clusters in the washing medium were collected on the nylon sieve with pore size of 80 μm. One drop of the cell cluster from the 80 μm cell sieve was transferred to the liquid regenerated medium in a 6 cm petri dish. The cultures for plant regeneration were kept under the light condition in a growth chamber.

## The Experimental Results

**1. The relationship between E/O and phytohormones in primary culture**

In order to understand the relationship between E/O and hormones in the medium, five concentrations (0, 0.2, 0.5, 1.0 and 2.0mg/L) of both 2,4-D and Kt (all together 25 different combination

media) were studied. From Fig. 1 (only 15 of them were showed), some regulations on E or O in the primary cultures could be observed.

(1) When the medium was 2, 4-D free and contained Kt only (A1, B1 and E1 in Plate Ⅰ), no E was observed. In this case, plantlets were directly regenerated from the explants via O by adventitious buds. The effect of concentration of Kt in the medium on formation of adventitious bud was obvious. When no Kt was contained (without any phytohormone) in the medium, no response of the explants was observed (A1 in Plate Ⅰ). When 1 mg/L Kt was used, few adventitious buds (O) were observed but the growth speed of the buds was slow (D1 in Plate Ⅰ). When 2 mg/L Kt, many adventitious buds were formed from young panicles and the speed of the bud shooting was fast.

(2) When the medium was Kt free and 2, 4-D contained only (A1—A5 in Plate Ⅰ), non-embryogenic callus was formed and the way of plant regeneration was organogenesis. Different from the case (1) mentioned above, the adventitious buds were formed from organogenic callus and not from the explant directly (B and D in Plate Ⅱ). The photos A1—A5 in Plate 1 also showed the effects of diferent concentration of 2, 4-D in the medium on organogenesis. When 2, 4-D was O in the medium, no response of the explants was observed (A1 in Plate Ⅰ). When the 2, 4-D concentration was higher (1. 0 and 2. 0mg/L), the friable non embryogenic callus was formed (A4 and A5 in Plate I). When the concentration of 2, 4-D was lower (0. 2 and 0. 5 mg/L), the intermediate case between adventitious bud and callus could be observed (A2 and A3 in Plate Ⅰ).

(3) When both 2, 4-D and Kt were contained, E-callus was formed from which plantlets were regenerated via somatic embryogenesis. The effect of the ratio of Kt: 2, 4-D in the medium on the quality and frequency of E-callus was very notable. When the concentration of them was low (0. 2mg/L) and the ratio was larger than 1 the explants developed both adventitious bud and E-callus. Although this kind of callus could regenerate into plant when they were transferred to regenerated medium, they lost their capacity of plant regeneration very fast during the subculture passages. In other words, the callus from the medium, in which the ratio of Kt: 2, 4-D was larger than 1 and the content of the hormones was low, was very difficult to keep the capacity of plant regeneration for a long duration even though they had embryogenic structure. When the ratio of Kt: 2, 4-D was balance and their content was appropriate (1 or 2 mg/L) in the medium, the callus would possess good embryogenic structure (D4 and E5 in Plate Ⅰ and 2A in Plate Ⅱ) and could keep the capacity of plant regeneration for a long duration (Table 1).

From this results, we concluded that E - callus was developed when the media contained both 2, 4-D and Kt and in this case, plants were regenerated via somatic embryogenesis. When the media contained 2, 4-D or Kt only, plants were regenerated via organogenesis, but the former was adventitious bud from organogenic callus and the later, from explants directly (Plate Ⅱ).

Table 1 Comparison of capacity for plant regeneration during subculture passages in agar/liquid medium between embryogenesis and organogenesis *

| Subculture passages | | Medium | 1st | 2nd | 3rd | 4th | 5th | 6th | 7th |
|---|---|---|---|---|---|---|---|---|---|
| Embryogenesis | % of regeneration | Agar | 45.8±1.5 | 67.5±6.8 | 82.1±7.9 | 85.0±9.4 | 80.0±10.5 | 83.6±11.1 | 86.5±12.8 |
| | No. of plant/callus | medium | 12.8±2.3 | 13.6±3.5 | 10.5±3.4 | 11.8±4.2 | 13.2±2.6 | 1.6±0.4 | 9.4±1.5 |
| | % of regeneration | Liquid | 21.4±3.5 | 18.3±2.9 | 4.5±1.3 | 0 | 0 | — | — |
| | No. of plant/callus | medium | 8.5±2.6 | 11.6±3.3 | 2.8±0.8 | 0 | 0 | — | — |
| Organogenesis | % of regeneration | Agar | — | — | — | 55.0±6.5 | 2.7±0.4 | 3.3±0.8 | 0 |
| | No. of plant/callus | medium | — | — | — | 6.5±1.8 | 3.5±0.3 | 2.5±0.2 | 0 |
| | % of regeneration | Liquid | 25.6±4.1 | 13.5±2.5 | 0 | 0 | — | — | — |
| | No. of plant/callus | medium | 4.5±0.5 | 3.6±0.4 | 0 | 0 | — | — | — |

* Each data in the table is the average of three repeat experiments and each experiment from more than 75 calli in 5 petri dishies. Passage duration: For each passage, one month for solid culture in agar medium and one week for suspension culture in liquid medium.

## 2. The subculture condition

In order to keep the plant regeneration through the pathways of O or E in the condition of subculture, and according to the results from the experiment of 25 different media for the primary culture mentioned above, two media, No. 21 (MS with 2mg/L 2,4-D) and No. 19 (MS with 1mg/L each of 2,4-D and Kt), were selected and used as subculture medium for O and E respectively. According to the criteria of O and E, calli were strictly selected before they were transferred to a new medium for each subculture passage. The capacity for plant regeneration of O and E callus during the subculture passages was shown in Table 1. In order to test the effect of the two media on maintaining ability of plant regeneration from O and E callus in each subculture passage, the callus formed in each medium was transferred to the two different media (No. 19 and 21) and at the same time the frequency of O and E callus was respectively calculated. The results in Table 2 showed the maintenance of ability of embryogenesis and organogenesis from E and O callus of different cell lines in the two media. The formation and maintenance of E and O callus in some callus lines was mainly affected by the medium. For example, the callus lines No. 2 and 3 formed O callus completely of mainly when they were subcultured in medium 21 whereas, they formed E callus when they were subcultured in medium 19. The frequencies of E-and O-callus from cell line No. 2 in the medium 21 were O and 100% respectively and the ones in medium 19 were 46.7% and 5.6% respectively. The three E callus lines in Table 2 also showed the similar relationship between the callus type and medium, namely, the frequency of E-callus was higher in medium 19 (containing 2,4-D and Kt) and the frequency of O-callus was higher in the medium 21 (containing 2,4-D only). But in the condition of subculture, E-callus formed not only E but also O in medium 19 and the O callus formed not only O but also E or mixture one in the medium 21. Besides, among 8 callus lines tested (Table 2), three of them (No. 4, 5 and 8) did not form embryogenic callus in any medium.

Table 2　The capacity of maintaining for embryogenesis and organogenesis of E and O callus in subculture condition

| Variety | No. and type of callus line * | Medium | The frequency of E/O callus making up the total no. of callus | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | The 4th passage | | | The 5th passage | | |
| | | | E-type callus line | O-type callus line | E+O | E-type callus line | O-type callus line | E+O |
| Qin Erai | 1 E-type | 19 | 65.2±13.5 | 0 | 34.8±8.0 | 8.6±12.9 | 0 | 0 |
| | | 21 | 5.6±1.8 | 64.7±15.0 | 29.4±7.2 | 7.1±2.3 | 10.7±2.1 | 46.0±15.1 |
| | 2 O-type | 19 | 46.7±9.8 | 5.6±1.5 | 46.7±6.5 | 25.5±8.7 | 70.0±8.3 | 0 |
| | | 21 | 0 | 100 | 0 | 0 | 100 | 0 |
| | 3 O-type | 19 | 39.3±4.5 | 3.6±0.8 | 57.1±11 | 14.3±5.8 | 28.0±6.9 | 42.9±13.4 |
| | | 21 | 3.6±0.6 | 42.8±7.5 | 53.1±6.8 | 0 | 100 | 0 |
| Hunan Zhao | 4 O-type | 19 | 0 | 100 | 0 | 0 | 100 | 0 |
| | | 21 | 0 | 100 | 0 | 0 | 100 | 0 |
| | 5 O-type | 19 | 0 | 100 | 0 | 0 | 100 | 0 |
| | | 21 | 0 | 100 | 0 | 0 | 100 | 0 |
| | 6 E-type | 19 | 29.2±4.7 | 0 | 70.8±15.6 | 0 | 0 | 0 |
| | | 21 | 0 | 100 | 0 | 0 | 100 | 0 |
| Gui Chao | 7 E-type | 19 | 100 | 0 | 0 | 24.0±8.3 | 0 | 64.0±12.3 |
| | | 21 | 12.5±2.1 | 25.3±3.6 | 62.5±18.2 | 5.6±1.5 | 55.6±11.2 | 0 |
| | 8 O-type | 19 | 0 | 100 | 0 | 0 | 100 | 0 |
| | | 21 | 0 | 100 | 0 | 0 | 0 | 0 |

Medium: 19=MS + BAP 1mg/L + 2,4-D 1mg/L; 21=MS + 2,4-D 2mg/L

* The original callus line which was used for subculture.

E and O show callus ines in which embryogenesis or organogenesis occurs when transferred to differentiation medium respectively.

E + O shows that callus line in which both embryogenesis and organogenesis occur.

### 3. The comparison of morphology and the capacity for plant regeneration between embryogenic and organogenic callus

From a embryogenic callus, the differentiation of scutellum, coleoptile and coleorhiza were clearly observed (2A and 2C in Plate Ⅱ), indicating the distinctly polylayer and polarity structure. On the other hand, the organogenic callus did not show the structure of differentiated polarity and polylayer (2B and 2D in Plate Ⅱ). In regenerated medium, E was progressed and the structure of embryoid became much clear. Plate Ⅱ (2 C) shows a developing embryo at 7 days after the callus was transferred to regeneration medium. From the photo, coleoptile and coleorhiza were observed on the same embryo-axile. In the case of O, buds and roots were separately regenerated neither at the same time nor at the same axile (2D in Plate Ⅱ). In this case, neither embryogenic organs nor suspensor-like structure were observed.

More than 10 plantlets per callus could be regenerated via E in solid medium, the frequency of plant regeneration was more than 80% (2E in Plate Ⅱ and Table 1) and the capacity for plant regeneration was maintained in subculture condition for seven months (Table 1). But for O, number of plantlet per callus was less than six (2E in Plate Ⅱ and Table 1), the frequency of plant regeneration

was less than 20% and the duration of keeping the capacity for plant regeneration was only two months in liquid condition (Table 1).

## DISCUSSION

### 1. The factors influencing embryogenesis and organogenesis in rice

Phytohormones are important factors in somatic embryogenesis of plant tissue culture[2,17]. But different species has quite different requirement to phytohormone for their developing of E[5,20]. In some species, the requirement was simple, for example, in carrot, only 2,4-D could induce E[13]. In other species, the condition for E was rather strict, for example, in *Pennisetum americanum* L., the E required the definite concentrations of 2,4-D and Kt[21]. In *Cucumis melo* L., low concentration of auxin (2,4-D,NAA and IAA) induced O and high concentration of them, induced E[19]. In rice, the relationship between phytohormones and E/O was more complex. As we reported here that both E and O could be induced by different condition. O could be induced by kinetin (adventitious bud from explant directly) and 2,4-D (adventitious bud from callus) respectively and E was induced by definite concentration of both 2,4-D and Kt. Thus, 2,4-D and Kt are the essential factors for inducing somatic embryogenesis. The same results were also reported in rice and Pennisetum[1,6,21,22].

Among the factors for inducing E/O, 2,4-D was thought to be the most important one. Evans et al.[5] calculated the conditions for inducing E in literatures and pointed out that among all of the factors induced E, 2,4-D as an essential one made up 57.1%. The result of our experiment demonstrated this point as well.

### 2. The pathway of plant regeneration *in vitro* culture

Somatic embryogenesis and organogenesis are two pathways of regeneration in plant tissue culture[2,20]. Most of plants take one of them and the famous example is carrot and tobacco. Carrot takes the pathway of embryogenesis and tobacco does the one of organogenesis preferably. But some species possess both E and O as the pathway of regeneration, for example, *Asparagus officinalis* L.[4], *Solanum melongena* L.[10], *Antirrhinum majus* L.[15], *Medicago sativa* L.[16] and so on. In rice, the plantlets not only can regenerate via both ways, the two pathways can be induced from the same explant as well. From young panicle of rice, E or/and O could be controlled to develop by adjusting different kind and concentration of plant hormones (Plate I). So rice might be a good material for studying the differences, gene expression between E and O through phytohormone regulation.

### 3. The capacity for plant regeneration of embryogenesis and organogenesis

In the normal condition, the capacity for plant regeneration in the way of E *in vitro* culture could be kept in a high frequency for a long duration because somatic embryo had the similar structure to zygotic embryo. This character was demonstrated in rice[6,8] and in other crops[9,11]. Although Table 1 showed that the yield of plantlets (No. of regenerated plant per callus) was higher in E than that in O,

it was also observed in both solid and liquid culture conditions that plenty of embryoids could not developed into whole plants. During the subculture passages, the capacity for plant regeneration was gradually decreased. This was because some somatic embryos were abnormal in structure (lack some embryo organ) or in developmental stage (immature, over-mature and pre-mature) and so on[2,18,20]. On the other hand, it was shown in suspension cultures of *Cucumis sativa* L. that the capacity for plant regeneration in O was higher than that in E. By controlling the culture condition, Bergervoet et al. [3] obtained the regenerated plants through the two regenerated ways of E and O. They found that not only the frequency of plant regeneration was higher but also the duration of keeping the capacity for plant regeneration was longer in O than that in E. They found that in the culture of E, there were plenty of abnormal structure and immature embryoids. In rice, Abe and Futsuhara[1] established a system of keeping high frequency of plant regeneration for a long duration by *in vitro* screening using 2,4-D. This was a typical example of organogenesis. From these examples and results obtained here, it could be concluded that if the culture conditions were well established, high frequency and long duration of plant regeneration could be obtained via any of the regeneration way, embryogenesis or organogenesis.

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## Explanation of plates

### Plate I

The effect of different concentrations (0, 0.2, 0.5, 1.0 and 2.0mg/L) of 2, 4-D and kinetin on inducing E and O in the young panicle culture of rice. In the photoes. 1. 2. 3. 4 and 5 represent the five concentrations of 2. 4-D and Λ, B, C. D and E, kinetin. The two concentration. 0.2 and 0.5 (B and C) of kinetin were not showed.

### Plate II

2Λ. Variety: Qin Erai. Embryogenic callus consists of many embryoids. The embryogenic callus possessed distinct layer structure derived from differentiation of embryoids; × 35

2B. Variety: Qin Erai. The organogenic callus does not possess the differentiation in tissue, like E, and has no layer structure; × 35

2C. Variety: Qin Erai. Λ mature embryoid is germinating which possessed the typical structure of the zygote embryo in rice; × 100

S: scutellum; cl: coleoptile; cr: coleorhiza; coleoptile and coleorhiza are on the same embryo axis.

2D. Variety: QinErai. The plantlet is regenerating by the pass way of organogenesis. The adventitious bud and roots were derived from different space of the O callus and not on the same axis; × 35

2E. Comparison of the capacity for plant regeneration between E—type(left)and O—type (right)callus showing that the regenerated plants from each E-callus were much more than that from O type of callus.