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# Histological and Scanning Electron Observations on Embryogenic and Non-embryogenic Calli of Aromatic Thai Rice (*Oryza sativa* L. cv. Khao Daw Mali 105)

**Nitsri Sangduen and Pranot Klamsomboon**


## ABSTRACT

Two types of callus proliferation namely, embryogenic (E) and non-embryogenic (NE) calli were obtained by culturing mature rice (*Oryza sativa* L. cv. Khao Daw Mali 105) seeds in modified MS medium supplemented with 2 mg/l 2,4-D, 10 mM proline and 3% sucrose. Both light and scanning electron micrographs were employed to distinguish the surface details as well as cell shape of E and NE calli. Light micrographs of E callus was compact, nodular, knobs, white or creamy. NE callus was soft, friable, unorganized light-stained cells, translucent, watery and light yellow. Scanning electron micrographs indicated distinct morphology of cells shape. NE cell shape was long-like tubular and loosely arranged cells. E callus comprised nodular or knobby, quite deep embedded and tightly packed cell was similar to typical E callus. Histological observations of 5 $\mu$m of callus paraffin sections revealed that E callus occurred on the surface as well as in the deeper regions of the callus. The abundance of vessel tracts in the callus which was consisted of a high content of vessel elements, rounded and cytoplasmically dense cells with totipotency. This may imply that nutrients are easily transported via these vessel tracts for the nourishment of growing somatic embryos or leafy structure. The number of vessel elements appeared to be a crucial factor to determine the E and NE calli development.


**Key word:** histological, scanning electron micrograph, *Oryza sativa* L. cv. Khao Daw Mali 105, callus

## INTRODUCTION

Calli from mature rice seeds (*Oryza sativa* L.) have been documented to develop mainly from the scutellar epithelial cells (Maeda *et al*., 1981; Maeda *et al*., 1988; Mendoza and Futsuhara, 1992). Many authors indicate the role of 2,4-D in proliferating rice callus mass (Nishi *et al*., 1968; Henke *et al*., 1978; Inoue and Maeda, 1979; Mendoza and Futsuhara, 1992) especially in Khao Daw Mali 105 (Klamsomboon, 1997). Visually distinct embryogenic callus may be a long term totipotent callus produced by many species of cereals and suitable for cell suspension culture, protoplast isolation and mutant selection.

Morphological evidence of rice somatic embryogenesis initiated by a bipolar structure was commonly observed (Abe and Futsuhara, 1985; Chen *et al*., 1985; Mendoza and Futsuhara, 1992). Somatic embryos arose from single cells located on the surface or in the deeper layers of the callus while shoots arose from meristematic cells that differentiated leafy structures in the callus (Mendoza and Futsuhara, 1992). Morphological variation between embryogenic and non-embryogenic calli have been also reported on certain rice varieties

Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

based on callus external morphology (Abe and Futsuhara, 1985) as well as the internal morphology of the callus (Mendoza and Futsuhara, 1992). Histological observations on plant regeneration in rice calli have been clarified (Mendoza and Futsuhara, 1992). We are unaware of any reports on histological and scanning electron observations of aromatic Thai rice. The variation in callus surface was thus observed following with the aid of light and scanning electron microscope.

The purpose of this study was to examine morphological and histological differences on embryogenic and non-embryogenic calli of aromatic Thai rice variety Khao Daw Mali 105.

## METERIALS AND METHODS

### Callus induction and plant regeneration

Mature rice (*Oryza sativa* L. cv. Khao Daw Mali 105) seeds were used for callus induction. Seed sterilization and callus initiation were done according to the method of Klamsomboon (1997). For plant regeneration, 4 week-calli consisting of embryogenic and non-embryogenic fractions were transferred to the regenerated medium (callus induction medium without growth regulator). After incubation under completely dark condition for a week, the cultures were transferred to a 2,000 lux light chamber with 16 hours photoperiod for another 4 weeks and used for scanning electron microscope and histological observations.

### Electron microscopic observation

Microcalli were prefixed in 25% butyl alcohol, rinsed 3-4 times in phosphate buffer, dehydrated in an ethanol series (30-100% V/V), and critical-point dried. Dried specimens were coated with gold and examined in a Joel JSM-35 of scanning electron microscope at 15 kV.

### Histological observation

Embryogenic and non-embryogenic calli were dissected and fixed in a fixative solution (5% formalin, 5% acetic acid and 90% of 70% ethanol, V/V) for 72 hours. Samples were dehydrated in a butyl alcohol series and embedded in paraffin blocks. Serial sections of 5 $\mu$m-thick were cut using microtome. The sections were stained with fast green and viewed under a light microscope.

## RESULTS AND DISCUSSIONS

Dehusked rice seeds cultured in the 2,4-D containing medium, growth of plumule and radical were suppressed and scutellum tissue abnormally proliferated to produce callus. The role of 2,4-D in the induction and early development of embryoids up to the globular stage in pearl millet (Vasil and Vasil, 1982) and in dehusked rice seeds (Mendoza and Futsuhara, 1992) were reported. Morita *et al*. (1999) revealed that the intracellular 2,4-D concentration should be controlled as low as $2.6 \times 10^{-2}$ $\mu$g/g fresh weight to reach the same synchronization in shoot regulation as seen with rice seed germination. Callus initiation occurred after plating seeds in the MS and $N_6$ media for 5-8 days, the scutellum appeared to be swollen (Figure 1A) and cells in the epithelial layer started to rapidly divide at 20 days after culture. Maeda *et al*. (1988) concluded that the epithelial cells of rice scutellum were obliged to diverge in their activity under different circumstances, for example, degenerate promptly, maintain their haustorial nature or actively proliferate. The cells have a characteristic structure and function in rice plants and probably also in other cereal species. Two types of rice callus were observed, they contained both embryogenic (E) and non-embryogenic (NE) regions. Embryogenic callus was characterized by nodular, compact, knobs and white or creamy (Figure 1B). Non-embryogenic callus was soft friable, loosely arranged cells, translucent watery and light yellow (Figure 1B). From scanning electron micrograph, we found that callus fraction with compact and nodular structures contained tightly packed cells was similar to typical embryogenic

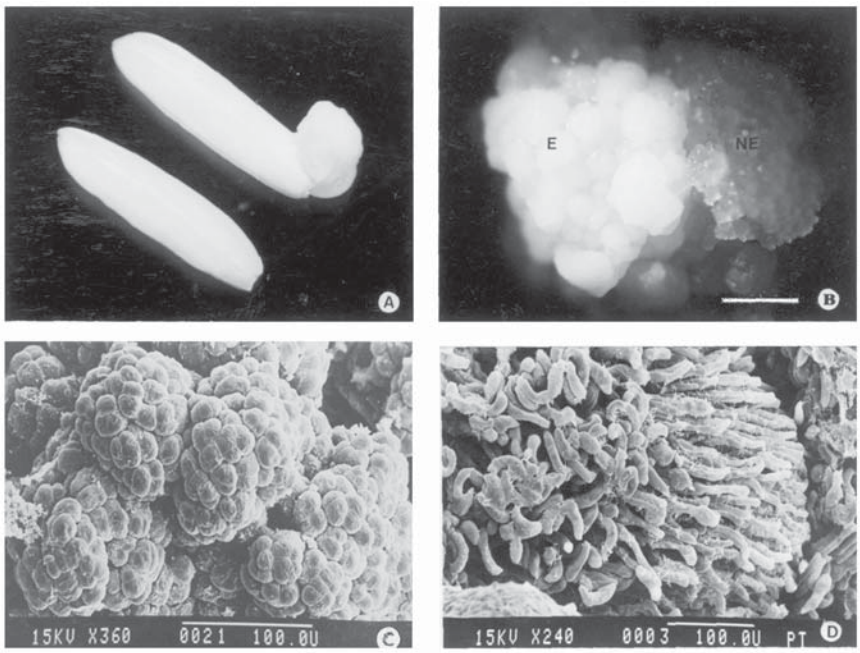


**Figure 1** Callus formation from mature seed of rice cv. Khao Daw Mali 105, scutellum appeared swelling with callus mass 5-8 days after plating on induction medium (bar = 2 mm) (A). After 4 weeks culture, two types of calli were distinguished, embryogenic (E) and non-embryogenic callus (NE) (bar = 2 mm) (B). Scanning electron micrographs of the tightly packed cells were formed in the embryogenic callus (C) and long-like tubular cells in non-embryogenic callus (D).

callus (Figure 1C). The other fraction that comprised long-like tubular and loosely arranged cells was characterized as non-embryogenic callus (Figure 1D) which described earlier for rice (Nabors *et al*., 1983) and in accordance with Wang *et al.* (1987). This finding gave the same microtopography of rice callus surface as well as in vetiver (Sangduen *et al.*, 2000).

Light microscopic observations of the paraffin-sectioned calli revealed the growing region of calli originated from either epidermal or subepidermal cells (Figure 2A), cells in the growing region were small in size and dense in cytoplasmic staining (Figure 2B). The embryogenic cells were elongated cytoplasmically dense cells with prominent nuclei located either on the surface of callus (Figure 2C) or inside flanked by parenchyma cells (Figure 2D). The results were similar to the report of Mendoza and Futsuhara (1992). The dominant type of differentiated tissue was parenchyma cell which frequently occurred with chlorenchyma located nearby callus surface (Figure 3A and 3B). Cell morphology was considerably more complicated than the homogenous parenchyma mass. Xylogenesis was also observed. At the early development, vessel elements were integrated in parenchyma cells (Figure 4A) and their orientations were diversified (Figure 4B). The vessel tracts were arranged in multiple and branched strands (Figure 4C). In adventitious root that frequently occurred with callus growing on medium containing NAA also formed certain amount of vessel elements in the central area (Figure 4D). A comparison of cell differentiation in embryogenic and non-

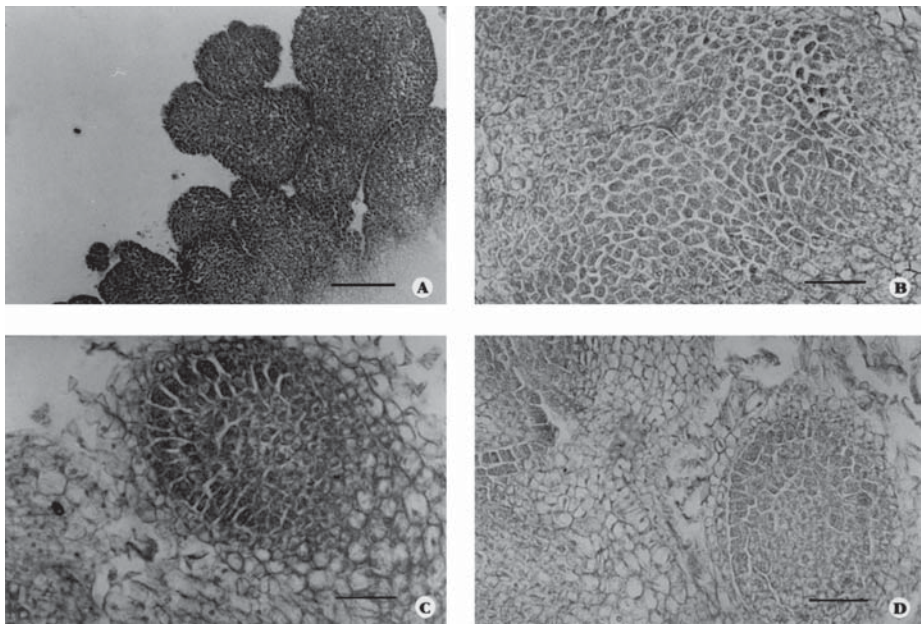


**Figure 2** Serial section of embryogenic callus of rice cv. Khao Daw Mali 105 revealed the growing region could be originated from epidermal cells (bar = 0.1 mm) (A). Cells in growing region were small in size and dense in cytoplasmic staining (bar = 0.05 mm) (B). The embryogenic cells were possibly elongated densely cytoplasmic cells with prominent nuclei located on the callus surface (bar = 0.05 mm) (C) or inside flanked by parenchymal cells (D). (bar = 0.05 mm)

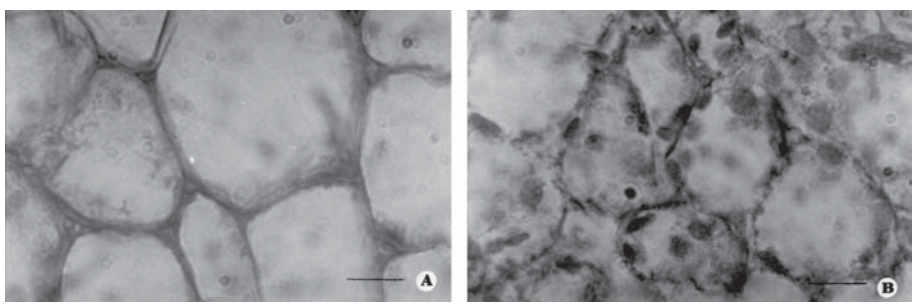


**Figure 3** The dominant type of differentiated tissue of rice cv. Khao Daw Mali 105 was parenchymal cells (A) which frequently occurred with chlorenchyma located nearby the callus surface (B). (all bars = 0.05 mm)

embryogenic calli type by the amount of vessel elements found that solid compact calli with nodular structures were supported by numerous vessel tracts consisting of a high content of vessel elements. The frequency of vessel elements suggested that the number of vascular tracts in callus and its abundance were associated with the compactness and embryogenic potential. The more compacted regenerable calli also gave a higher content of vessel elements. This may imply that nutrients are

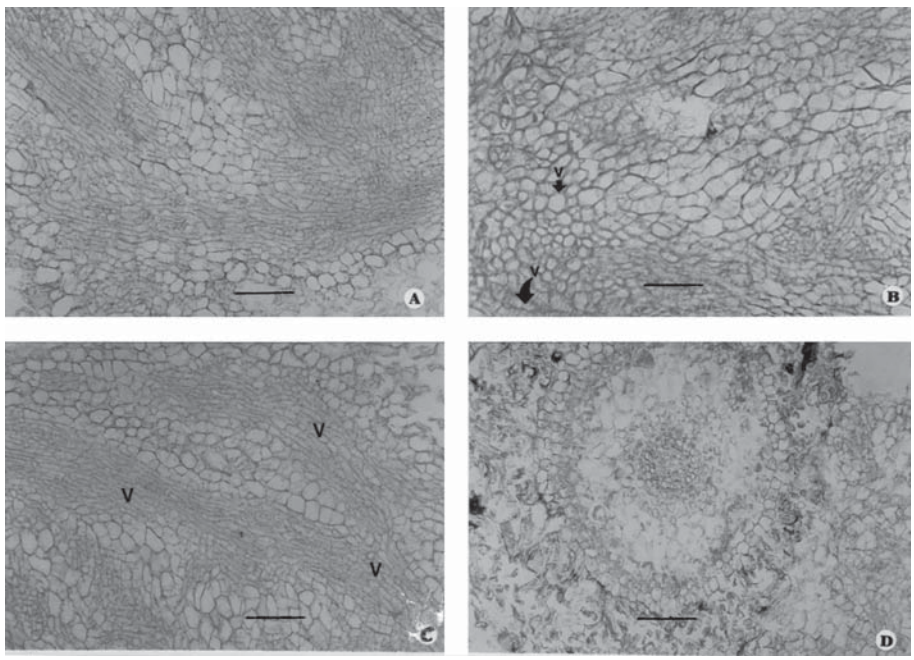


**Figure 4** In early xylogenesis of rice cv. Khao Daw Mali 105, vessel elements were integrated in parenchymal cells (A) and their orientations were diversified (v = vessel element) (B). The vessel element (v) array in multiple and branched strands (C). In adventitious root that frequently occurred with callus growing on medium containing NAA also formed certain amount of vessel elements in the central area (D). (all bars = 0.05 mm)

easily transported to these vascular systems for the nourishment of the growing somatic embryos and most indica rice had difficulty in obtaining a substantial number of protoplasts from embryogenic callus. The difficulty can be accounted for cell wall formation, limited area of embryogenic region/ fraction and abundance of vascular bundles in callus mass (Mendoza and Futsuhara, 1992). Thus the application of protoplast isolation from established cell lines is more practical. However, successful establishment of cell lines with the capacity of embryogenic potential depends on the ability to recognize and selectively proliferate a unique embryogenic cell type.

The results suggested that the number of vessel elements appear to be a crucial factor to determine the embryogenic and non-embryogenic calli development. Siriwardana and Nabors (1983) described an attempt to increase embryogenic callus formation and plant regeneration by using a medium containing tryptophan acting as an auxin precursor. Maeda *et al*. (1988) reported that at the early growth stage, the first signs of regeneration of an epidermis-like layer, consisting of isodiametric cells. After this early event, the cells became slightly elongated, and aligned in parallel, and appeared to be of a stabilized epicuticular nature. By the time the epidermal layer was produced by surface cells, the callus mass already had a vascular system inside. At this stage, the callus structure was covered partially or completely with epidermal cells which were coated with water-impermeable materials. Callus masses with epicuticular cells and a vascular system probably were in a favorable state for organizing a shoot apex, and finally were able to organize themselves into a miniature of a whole plant.

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