J. Phytol. Res. **35**(2): 95-103, 2022 **ISSN 0970-5767**

HISTOLOGICAL ANALYSIS OF SOMATIC EMBRYOGENESIS IN RICE (*ORYZA SATIVA* L.)

MRANALI CHAUHAN
Department of Botany, Government College, Barmer, India 344001
Corresponding Author's Email: chauhan.mranali@gmail.com

Rice (*Oryza sativa* L. cultivar Pusa Basmati-1) cultures initiated from mature caryopses were used to study the pathway of morphogenesis. Histological analysis revealed almost all the stage of somatic embryogenesis. In the initial stage of development, scutellum swelled and callus was formed that contained embryogenic cells on the periphery and parenchymatous cells in the internal region. Embryogenic cells underwent a series of organised divisions and formed proembryos that further developed into globular somatic embryos with suspensor. There was no apparent vascular connection between the developing embryos and mother tissue. The embryos further developed into heart-shaped and ultimately into bipolar embryos. Fully developed somatic embryos resembled zygotic embryos with coleoptile and scutellum. Development of secondary somatic embryos from the scutellum of primary somatic embryos was also observed.

Keywords: Histology, Indica rice, *In vitro* culture, Morphogenesis, *Oryza sativa*, Somatic embryogenesis.

Introduction

Rice is the most widely consumed staple food for over half of the world's human population especially in Asia and Africa. It is the agricultural commodity with the third-highest worldwide production. Besides its economic significance, rice is considered as an important plant for genetic transformation and genomic studies. However, genetic modification via transformation is largely dependent on the ability of the explant to produce embryogenic calli and regenerate into whole plants. The availability of an efficient and reproducible plant regeneration protocol is a pre-requisite for the application of tissue culture technology for plant breeding and genetic transformation studies[1]. In tissue culture the efficiency of production of regenerable cultures and pathway of morphogenesis depend on several factors including the composition of culture medium, plant growth regulators, genotype and explant used[2-6]. Pescador et al. [7] reported that the *in vitro* plant manipulation system that is used to obtain embryogenic and morphogenic responses are dependent on morphological, genetic, biochemical, cytological and physiological factors.

In rice regenerable callus has been induced from various explants[8-15] but mature embryos are the most preferred because of their availability in large number throughout the year[16]. Most of the studies on rice tissue culture report the development of somatic embryos[17-20] but these reports have evaluated the callus on morphological basis. The present study was, therefore, undertaken to determine the pathway of morphogenesis in rice through histological evidences.

Material and Methods

Mature seeds of indica rice (*Oryza sativa* L. cultivar Pusa Basmati-1) were used as explant. The caryopses were procured from GB Pant University of Agriculture and Technology, Pant Nagar, Uttarakhand, dehusked and surface sterilized with 0.1% aqueous solution of mercuric chloride for

eight min followed by five rinses with sterile distilled water. Callus was induced and plants were regenerated according to the protocol proposed by[21]. The cultures were used for histological analysis at different intervals. The material was fixed by immersion in Formalin: Acetic acid: Ethanol (FAA, 5:5:90) for 24 hours and preserved in 70% ethanol. After dehydration in a tertiary butyl alcohol (TBA)-xylene series, the material was infiltrated with liquid paraffin followed by three changes of solid wax. It was embedded in solid wax. Sections (10 µM in thickness) were obtained with a rotary microtome. The sections were affixed to slide by Haupts adhesive. The slides were kept overnight in xylene to remove the wax, passed through an alcohol series, stained with 1% safranine and 0.5% fast green and again passed through an alcohol series. The slides were mounted in DPX and analyzed under a microscope.

Results and Discussion

The first discernible change after the culture of seed explants was the swelling of the embryo. The embryonal axis showed little germination that stopped after 4-5 days while the scutellum convoluted with the appearance of ridges and grooves. The scutellum proliferated and by 10 d formed a creamish- yellow mass of firm, opaque callus designated as embryogenic callus. It was covered with small translucent nodules (Fig 1A). After 15 d approximately half of the nodules enlarged and were organized into structures resembling young zygotic embryos. By this time, somatic embryos that progressed beyond the globular stage were apparent. Clusters of 10-15 embryos were often found on scutellum surface. After 28 d mature somatic embryos nearly identical to mature zygotic embryos were present. The endosperm of the cultured seeds was consumed by the developing embryogenic masses and embryoids and only the hollow pericarp was left (Fig 1B). Mature embryos were not as numerous as immature embryos at 15 d and were often in isolated cluster of 4-6 embryos. The embryos turned green within 2-3 d (Fig 1C) when transferred to regeneration medium and ultimately germinated.

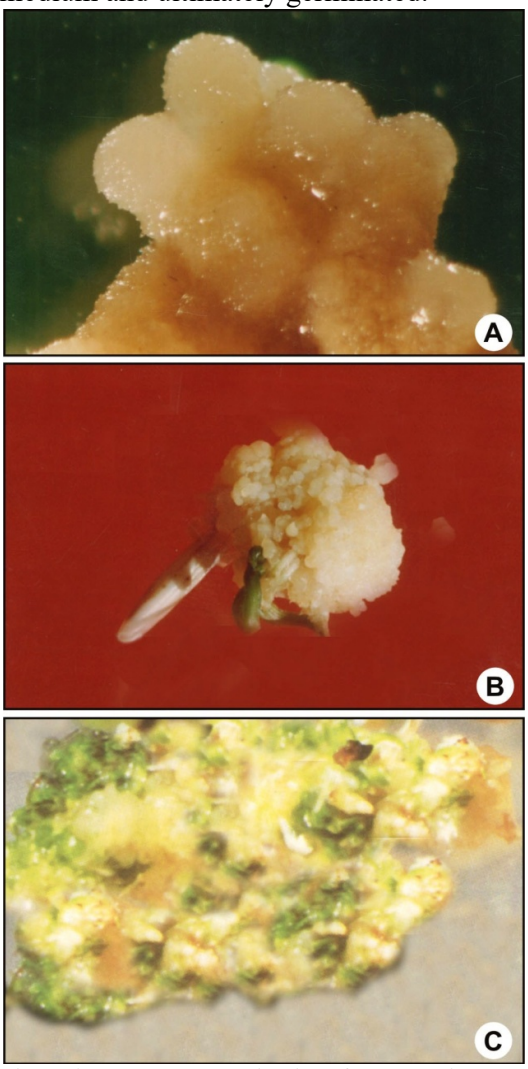

Figure1. Morphogenesis in *Oryza sativa* L. cultures

(A) Translucent nodules on 10 day old callus
(B) Embryogenic callus after 28 days
(C) Germination of embryoids

In the initial stages of culture, the cells of the peripheral region of the scutellum became richly cytoplasmic and divided to form small ridges and grooves (Fig 2A) that gave the scutellum convoluted appearance. In the interior region developed parenchymatous cells. The scutellar epidermis of the mature zygotic embryos, that is a layer of columnar cells at the interface of embryo with the scutellum, along with the sub-

epidermal cells underwent initial periclinal and later both periclinal and anticlinal divisions to give rise to masses of embryogenic pre-determined cells (Fig 2B).

At the periphery the rate of divisions was faster and the divisions were highly organized to form several layers. The intensive cell division activity in the outer layers of the scutellum resulted in the considerable folding of the upper surface of the scutellum and in substantial break-up of the original scutellum giving rise to the large internal cavities. Further divisions in the superficial scutellar cells led to the formation of the zones of embryogenic cells. In cross-section, these appeared as continuous band of cells that increase the girth of scutellum (Fig 2C). Rapid localized proliferation in the initial evenly distributed areas of embryogenic cells gave rise to discrete groups of 2-8 cells (Fig 2D). These groups were distinct and separate from each other. Continued division and organization in some of these discrete groups formed rounded or elongated proembryos. Protoderm was not differentiated in the early stages of proembryos. Further cell division and differentiation of proembryos led to the development of globular somatic embryos with well-developed protoderm. The globular embryoids were formed in large groups (Fig 2E) and appeared macroscopically as nodules on the surface of 10 d old cultures. These embryoids had a distinct suspensor (Fig 2F) and grew by apparently random or rarely anticlinal and periclinal divisions in the internal cells but only anticlinal divisions of the protoderm. Subsequently the embryoids flattened and a scutellar notch was formed laterally on the developing somatic embryo, delineating the outer scutellar region from the inner margin where the shoot meristem was formed (Fig 2G). The rim of the tissue grew into a cone with a pole and formed coleoptile. Polarity was established by the differentiation of a band of prominently cytoplasmic cells at one end that later formed the root meristem (Fig 2H). The tissue of the embryoid surrounding the radicle developed into coleorhiza. The portions of the embryos that did not contribute to the formation of the embryonal axis continued to enlarge and became the scutellum. The development of embryoids was not synchronous and various stages could be found adjacent to each other. The embryoids finally appeared on the surface of the callus. The embryoids were attached loosely to the callus tissue and could be separated easily (Fig 3A). The peripheral cells of the scutellum proliferated in many embryoids to give rise to secondary embryogenic callus tissue (Fig 3B). Continued development of the embryoids gave rise to the formation of clearly identifiable mature embryoids (Fig 3C-F). Well-differentiated somatic embryoids had almost all the features similar to a zygotic embryo. The embryonal axis was a bipolar structure subtended by a scutellum. The scutellum was composed of several layers of parenchymatous cells with a well-developed abaxial epidermis and procambial strands (Fig 3C). The shoot pole was enclosed in a coleoptile and consisted of shoot meristem with primary leaf primordia (Fig 3G,H). The root was bent substantively relative to the embryonal axis and was covered by the coleorhiza. Cells of the root tip were smaller and cytoplasmically rich. There was also development of secondary embryoids from the scutella of primary embryoids.

Embryogenic callus produced in the present study was yellowish color and had similar morphological characteristics as reported by[22-23]. The pathway of morphogenesis determined by histological analysis revealed that rice explants regenerated through somatic embryogenesis. Primary response to culture in the form of faster rate of cell division on the abaxial surface of the scutellum, was similar to the previous reports for wheat, maize, pearl millet and

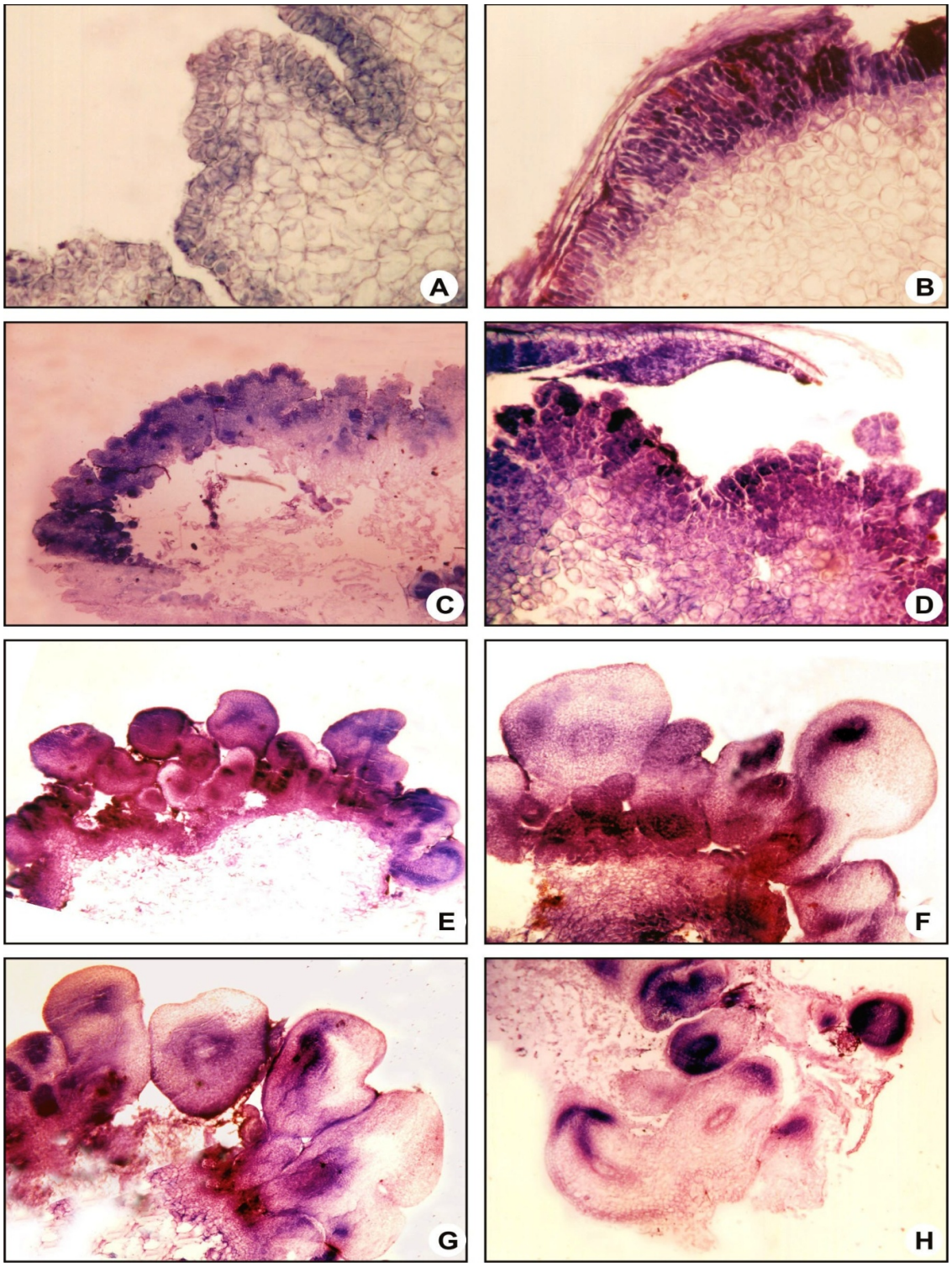

Figure 2: Histological details of *Oryza sativa* L. Cultures (A) Formation of ridges and grooves on the scutellar surface. (B) Embryogenic predetermined cells formed by periclinal and anticlinal divisions in epidermal and sub epidermal cells. (C) Formation of continuous band of embryogenic cells and breakup of internal region to form cavities. (D) Proembryonal masses on scutellar surface. (E) Several embryoids present in groups. (F) Globular embryoids with and without suspensor. (G) Formation of scutellar notch in embryoids. (H) Development of bipolar structures.

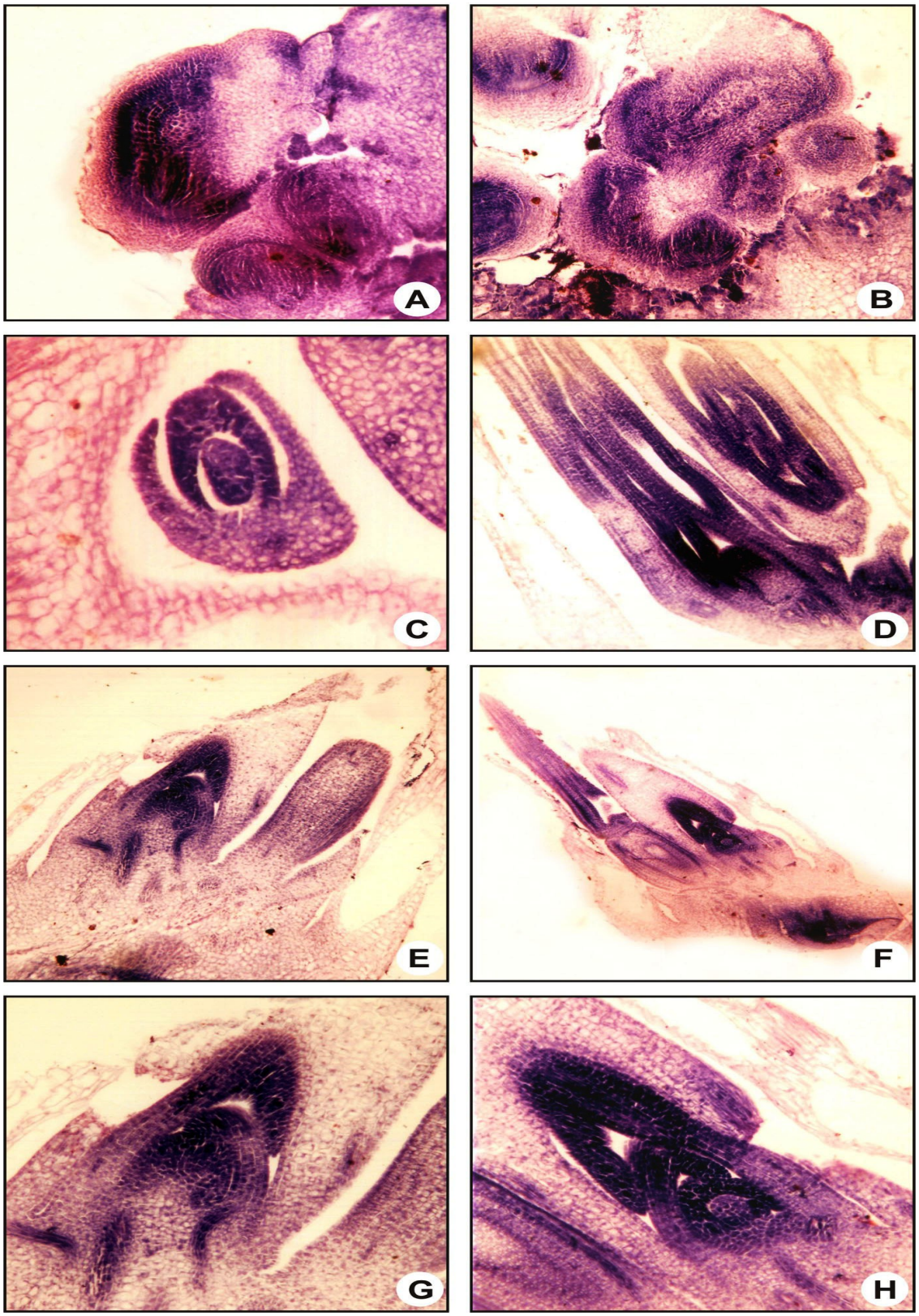

Figure 3: Histological details of *Oryza sativa* L. Cultures (A) Distinct embryoids separated from the callus. (B) Formation of secondary embryoids from primary embryoids. (C) A mature embryoids in transverse section. (D-F) Mature embryoids in longitudinal section. (G&H) Shoot pole of mature embryoids under higher magnification.

sorghum[24-30]. Parenchymatic cells in the interior part and meristematic cells in the peripheral part of rice callus is similar to that reported by[31-33]. The development of somatic embryos from embryogenic calli derived from the rice scutellum has also been reported by[34, 35].

The initial divisions were periclinal as has been reported for rice[36]. In our study these cells divided both periclinally and anticlinally to form zones of pre-embryogenic determined cells and did not directly form proembryoids. Proembryoids developed from these embryogenic cells by first periclinal division supporting their unicellular origin. The later divisions were under less restrictive control. Even in study by[36] somatic embryogenesis that clearly began with a single scutellar epidermal cell, involved random cell divisions. The unicellular origin of embryoids in cereals has been reported in pearl millet and sugarcane[25,37-38] though other reports[26,27] supported multicellular origin of embryoids in maize and sorghum. According to[24,39] the somatic embryoids may have both unicellular and multicellular origin. According to[35] when somatic embryos have unicellular origin, coordinated cell divisions are observed and the embryos are connected to the maternal tissue by a suspensor-like. In contrast, multicellular origin is characterized by no coordinated cell divisions and somatic embryos are observed as a protuberance and fused to the maternal tissue. Our histological observations showed co-ordinated cell division and formation of suspensor indicating that somatic embryos had unicellular origin.

The proembryoids as in previous studies of maize and pearl millet callus[25,38,40] formed discrete groups of cells. Later they formed globular structures with distinct protoderm. The development of somatic embryos from these structures was similar to somatic embryo formation in rice[32,36,41,42], wheat[43], barley[39], maize[27,28], sorghum[24,26], pearl millet[25,30], sugarcane[37] and guinea grass[44]. In each case formation of a scutellar notch marked the development of shoot apex. Several other features of somatic embryo formation in rice were similar to findings of previous reports of somatic embryogenesis in graminaceous plants. This includes the appearance of densely stained meristematic cells at root and shoot apex initiation sites[27,28,30,40,45,46]. It is also interesting to note that many more somatic embryos were initiated than actually completed development, the fact also reported[36]. This reduction in the number of mature embryos was probably due to the competition for resources. Formation of secondary embryoids was also observed which is a common phenomenon in cereal tissue culture [25,30,38]. We could trace almost all the stages of somatic embryo development. There was no indication of organogenesis as reported by [47].

References

1. Schulze J 2007, Improvements in Cereal Tissue Culture by Thidiazuron: A Review. *Fruit, Vegetable and Cereal Science and Biotechnology.* **1**(2) 64-79.
2. Chauhan M and Kothari SL 2004, Influence of potassium dihydrogen phosphate on callus induction and plant regeneration in rice (*Oryza sativa* L.) *Cereal. Res. Comm.* **33**(2-3) 553- 560.
3. Chauhan M and Kothari SK 2005, Optimization of ionic and chelated iron and its interaction with disodium ethylenediaminetetraacetic acid for enhancement of plant regeneration in rice (*Oryza sativa* L). *J Plant Biochem Biotech* **13** 33-37.
4. Tara N, Meena and Kaur 2017, Problems and progress in Indica rice tissue culture techniques. *Ann Biol* **33**(2) 191-198.
5. Ming NJ, Mostafiz SB, Johon NS, Zulkifli NSA and Wagiran A 2019, Combination of plant gowth regulators, maltose and partial desiccation treatment enhance somatic embryogenesis in selected Malaysian

rice cultivar. *Plants* **144**(8) doi:10.3390/plants8060144

6. Sundararajan S, Sivakumar HP, Nayeem S, Rajendran V, Subiramani S and Ramalingam SK 2021, Influence of exogenous polyamines on somatic embryogenesis and regeneration of fresh and long-term cultures of three elite indica rice cultivars. *Cereal. Res. Comm.* **49** 245–253.
7. Pescador R, Araujo PS, Maas CH, Rebelo RA, GIOTO CR, Wendhausen Jr R, Largura G and Tavares LBB 2000, Biotecnologia de Piper hispidinervium—pimenta longa. *Biotecnol Cienc Desenvolv* **3** 19–23.
8. Oinam GS and Kothari SL 1995, Totipotency of coleoptile tissue in indica rice (*Oryza sativa* L. cv. CH-1039). *Plant Cell Rep* **14** 245-248.
9. Karthikeyan A, Pandian STK and Ramesh M 2009, High frequency plant regeneration from embryogenic callus of a popular indica rice (*Oryza sativa* L.). *Physiol Mol Biol Plants* **15**(4) 371-375.
10. Verma D, Joshi R, Shukla A and Kumar P 2011, Protocol for in vitro somatic embryogenesis and regeneration of rice (*Oryza sativa* L.). *Indian J Exp Bio* **49** 958-963.
11. Chutipaijit S and Sutjaritvorakul T 2018, Titanium dioxide (TiO_2) nanoparticles induced callus induction and plant regeneration of indica rice cultivars (suphanburi1 and suphanburi 90). Dig *J Nanomater Bios* **13**(4) 1003-1010.
12. Guo F, Zhang H, Liu, Hu X, Han N, Qian Q, Xu L and Bian H 2018, Callus initiation from root explants employs different strategies in Rice and Arabidopsis. *Plant Cell Physiol* **59**(9) 1782–1789.
13. Paul S and Roychoudhury A 2019, Comparative Analyses of Regeneration Potentiality of Eight Indigenous Aromatic Indica rice (*Oryza sativa* L.) Varieties. *Biol Sci* **6**(1) 55-64.
14. Lee HJ, Seebauer JR and Below FE 2000, An improved technique for culture of rice panicles. *Plant Cell Tiss Org Cult* **60** 55-60.
15. AbdRahman Z, AhmadSeman Z, NazreenaOthman A, BahagiaAb Ghaffa M, AbRazak S, Mohd Yusof MF, HisamNasir K, Ahmad K, LitChow Y and Subramaniam S 2021, Efficient callus induction and plant regeneration of Malaysian indica rice MR219 using anther culture. *Biocat and Agri Biotech* **31** 101865.
16. Hiei Y and Komari T 2008, Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nat Protoc* **3** 824–834.
17. Abe T and Futsuhara Y 1985, Efficient plant regeneration from protoplast through somatic embryogenesis. *Biol Technol* **4** 1087–1090.
18. Chen TH, Lam L and Chen SC 1985, Somatic embryogenesis and plant regeneration from cultured young inflorescences of Oryza sativa L. (rice). *Plant Cell Tissue Org Cult* **4** 51–54.
19. Mendoza AB, Hattori K and Nishimura T and Futsuhara Y 1993, Histological and scanning electron microscopic observations on plant regeneration in mungbean cotyledon (*Vigna radiata* (L.) Wilczek) cultured in vitro. *Plant Cell Tiss Org Cult* **32** 137–143.
20. Muktirianur M, Supriyanto B, Sunaryo W and Nurhasanah N 2022, Somatic embryos induction of east kalimantan local rice (*Oryza sativa* L.) cultivars and i*n vitro* selection against salinity. *Agrivita, J Agri Sci* **44**(2) 207–215.
21. Chauhan M 2021, Influence of calcium chloride and it's optimization for high frequency in vitro plant regeneration in barley (*Hordeum*

vulgare L.) and rice (*Oryza sativa* L.) *J Phytol Res* **34**(1) 1-11.
22. Sahoo KK, Tripathi AK, Pareek A, Sopory SK, Singla-Pareek SL 2011, An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars. *Plant Methods* **7** 49–59.
23. Din ARJM, Ahmad FI, Wagiran A, Samad AA, Rahmat Z and Sarmidi MR 2016, Improvement of efficient in vitro regeneration potential of mature callus induced from Malaysian upland rice seed (*Oryza sativa* cv. Panderas). *Saudi J Bio Sci* **23** S69–S77.
24. Dunstan DI, Short KC and Thomas E 1978, The anatomy of secondary morphogenesis in cultured scutellum tissues of *Sorghum bicolor*. *Protoplasma* **7** 251-260.
25. Vasil V and Vasil IK 1982, The ontogeny of somatic embryos *on Pennisetum americanum* (L.) K. Schum. I. in cultured immature embryos. *Bot Gaz* **143** 454-465.
26. Wernicke W, Potrykus I and Thomas E 1982, Morphogenesis from cultured leaf tissue of *Sorghum bicolor*-the morphogenetic pathways. *Protoplasma* **111** 53-62.
27. Vasil V, Lu C and Vasil IK 1985, Histology of somatic embryogenesis in cultured immature embryos of maize (*Zea mays* L.). *Protoplasma* **127** 1-8.
28. Mc Cain JW and Hodges TK 1986, Anatomy of somatic embryos from maize embryo cultures. *Bot Gaz* **147** 453-460.
29. He DG, Yang YM, Bertram J and Scott KJ 1990, The histological development of the regenerative tissue derived from cultured immature embryos of wheat (*Triticum aestivum* L.). *Plant Sci* **68** 103-111.
30. Taylor MG and Vasil IK 1996, The ultrastructure of somatic embryo development in pearl millet (*Pennisetum glaucum*, Poaceae). *Amm J Bot* **83** 28-44.
31. Alfonso-Rub' J, Carbonero P and D′ıaz I 1999, Parameters influencing the regeneration capacity of calluses derived from mature indica and japonica rice seeds after microprojectile bombardment *Euphytica* **107** 115–122.
32. Vega R, Vásquez N, Espinoza AM, Gatica AM and Valdez-Melara M 2009, Histology of somatic embryogenesis in rice (*Oryza sativa* cv. 5272). *Rev Biol Trop* **57** 141–150.
33. Narciso JO and Hattori K 2010, Genotypic differences in morphology and ultrastructures of callus derived from selected rice varieties. *Philipp Sci Lett* **3** 59–65.
34. Molina D, Aponte M and Cortina H 2002, The effect of genotype and explant age on somatic embryogenesis of coffee. *Plant Cell Tiss Org Cult* **71** 117–123.
35. Quiroz-Figueroa FR, Rojas-Herrera R, Galaz-Avalos RM and Loyola-Vargas VM 2006, Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. *Plant Cell Tiss Org Cult* **86** 285–301.
36. Jones TJ and Rost TL 1989, The developmental anatomy and ultrastructural of somatic embryos from rice (*Oryza sativa* L.) scutellum epithelial cells. *Bot Gaz* **150** 41–49.
37. Ho W and Vasil IK 1983, Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.). I. The morphology and physiology of callus formation and the ontogeny of somatic embryos. *Protoplasma*. **118** 169-180.
38. Botti C and Vasil IK 1984, Ontogeny of somatic embryos of *Pennisetum americanum*. II. In cultured immature inflorescences. *Can J Bot* **62** 1629-1635.
39. Nonohay JS, Mariath JEA and Winge H 1999, Histological analysis of somatic embryogenesis in Brazilian cultivars of barley, *Hordeum vulgare vulgare*, Poaceae. *Plant Cell Rep* **18** 929-934.

40. Fransz PF and Schel JHN 1991, An ultrastructural study on the early development of *Zea mays* somatic embryos. *Can J Bot* **69** 858-865.
41. Bevitori R, Popielarska-Konieczna M, dos Santos EM, Grossi-de-Sá MF and Petrofeza S 2014, Morpho-anatomical characterization of mature embryo-derived callus of rice (*Oryza sativa* L.) suitable for transformation. *Protoplasma* **251** 545–554.
42. Mesbah HA, Nassar AE-SM and El-Shekh MA 2021, Histological studies on somatic embryogenesis in rice (*Oryza sativa* L.) *African J. Biol. Sci.*, **17** (1) 73-81.
43. Kothari SL and Varshney A (1998) Morphogenesis in long-term maintained immature embryo-derived callus of wheat (*Triticum aestivum* L.)- Histological evidence for both somatic embryogenesis and organogenesis. *J Plant Biochem Biotech* **7** 93-98.
44. Lu C-Y and Vasil IK 1985, Histology of somatic embryogenesis in *Panicum maximum* (guinea grass). *Amer J Bot* **72** 1908-1913.
45. Gray DJ, Conger BV and Hanning GE 1984, Somatic embryogenesis in suspension and suspension-derived callus cultures of *Dactylis glomerata*. *Protoplasma* **122** 196-202.
46. Brisibe EA, Nishioka D, Miyake H, Taniguchi T and Maeda E 1993, Developmental electron microscopy and histochemistry of somatic embryo differentiation in sugarcane. *Plant Sci* **89** 85-92.
47. Yoshida K 1995, Evidence for the involvement of glycanase activities in the dissociation of cortical cell walls during the emergence of callus from rice root tissues in the presence of 2,4-D. *Plant Cell Rep* **15** 43-50.