Organogenesis and Cell Culture in Rice Plants under Sterile Condition (Part II)

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In this article (Part II), histology of organ formation, androgenesis, suspension culture and protoplast isolation, etc. will be described.

Histogenesis in callus growth and shoot formation

Through observations of smear materials under a dissecting microscope, it was found that small smooth protuberances were produced in regions showing the dry appearance. Trichomes occurred in the regions showing the green color or the white and green color. There were tracheary elements having thickened wall in the inner part of callus, adjacent to which chloroplasts were observed¹¹⁾. Scanning electron microscopy was done to observe nuclear envelop, nucleoli and fibrillar structure in nuclei in the cultured cells¹⁾.

We thought, at first, a histological research of subcultured callus is an important prerequisite for evaluation of the inner structure of shoot-forming callus. As viewed by optical microscopy, there were several layers of densly stainable cells in the surface of callus and vascular-like elongated cells in the inner parts. From these results we called the callus growth with actively dividing cells in the surface as the first growth, and the growth with the meristematic cells in both the inner and the surface of callus, accompanied with differentiation and collapse in callus tissues, as the secondary growth. In addition, some root-like structure were also found in the callus tissues3).

From these results, further detailed observation was made during the growth process of Aichiasahi callus¹²⁾. The growth of compact



Fig. 2. A scheme representing development of rice callus subcultured in the medium containing 2,4-D 10-5M

IP: inner parenchyma, PM: peripheral meristem, PP: peripheral parenchyma, PSSM: parenchyma originated from subsurface meristem, SSM: subsurface meristem.

callus was divided into five phases (Fig. 2), namely:

- (1) initiation of small blocks of cells having cell dividing activity,
- (2) differentiation of the block cells into peripheral meristem and inner parenchyma,
- (3) initiation of subsurface meristem (or

subzonal meristem) just inside the peripheral meristem and development of parenchymatous cells from the subsurface meristem,

- (4) change of peripheral meristem to peripheral parenchyma by vacuolation of the former,
- (5) development of inner parenchyma by vacuolation of subsurface meristem.

The small blocks in phase 1 were often derived from the peripheral meristem in phase 2 and 3 or by reactivation in the peripheral parenchyma in phase 4. Thus, these growth phases were often recycled. In some cases, a root or a root apex-like structure was organized by an increased cell dividing activity localized in a part of subsurface meristem. From these observations, it was inferred that the growth of compact callus is the early process of forming an amorphous cell mass leading to the adventitious root. This speculation that the subculture callus has root-like nature is very interesting because callus induction occurred mainly from the outermost layer of vascular cylinders in the radicle, scutellum and mesocotyl in such a way as resembling normal root initiation. The friable callus grew through two stages. (1) initiation of small block in which meristematic cells and parenchymatous cells are mixed together, (2) differentiation of the cell blocks into inner parenchyma and peripheral meristem. The tracheary elements induced were found to be scattered or concentrated in a few loci near the meristematic cells. Wall thickening in parenchyma tissues and development of meristematic nodules were also seen in 9th generation of subculture⁶⁾.

When transferred from the subculture medium to shoot-forming medium, the compact callus behaved in a peculiar pattern in the growth, in which periclinal divisions were dominant except for the central part contacting the medium. In this way, the radial cell layer was formed in a direction from inner to outer parts of callus tissue. We called this layer the radial tissue. Periclinal divisions producing the radial tissue occurred at first in peripheral meristem of the callus after 3 days of transfer, and extended to almost all of the callus periphery after 6 days. Also they often occurred in reactivated cells in the peripheral parenchyma. Thus, the surface and subsurface cells in the shoot-forming callus usually became meristematic and rich in cytoplasm. Therefore, we assumed that the step by step progress of histo-morphological alteration from the radial tissue to leafy structure is an early structural process in the differentiation of adventitious shoots¹³.

Androgenesis in anther culture

Occurrence of adventitious embryos of monocot plants in the anther cultures has been already reported in several papers using rice and other plants. But detailed observation has not yet been obtained in respect to histological comparison with embryogenesis from fertilized eggs.

When rice anthers of 1.25 to 2.25 mm in length, taken at the stage from the first recovery stage on uninuclei to the early binuclear stage, were inoculated on Blaydes' medium with 2 ppm of 2,4-D, 2 ppm of IAA and 1 ppm of kinetin, globular bodies, hundreds of micron in diameter, were formed in the opened loculus of one theca. But the other theca of this anther remained closed and turned black in color. No globular bodies were obtained in shorter immature anthers in which any necrosis and dehiscence were not observed. Thus, the necrosis and dehiscence seem to be necessary for the formation of globular bodies. It is probable that these globular bodies were originated from pollen grains because of a presence of multicellular pollen in the loculus where these bodies developed. The globular bodies became a callus mass in the medium. When they alone (without theca), about 500 micron in diam., were transferred to the non-auxin-containing medium, they developed to plantlets. In this case, they grew at first to callus mass, then formed both adventitious shoots and adventitious roots, and finally became plantlet. Globular bodies had various shapes. However,



Plate 2. A globular body emerged from crack on pollen wall (×1200). b: globular body, d: degraded pollen, p: germ pore, w: pollen wall.

the subsurface meristem was observed at one pole. It was similar to that observed during root formation in subcultured callus. The radial tissue was observed at the other pole. It is thought to give rise to shoots in the organ forming callus. Therefore it can be assumed that they developed histologically to an organized system like an immature embryoid. Pollen grains, with an attached smooth surface cover in spots, were observed with a scanning electron microscope, and it was found that this type gave rise to the globular bodies. In such grains, a cell mass appeared from the crack which occurred on the pollen wall (Nakano and Maeda unpublished data).

Differentiation in endosperm callus

We have succeeded in inducing callus with

immature endosperms of rice plant, when cellular endosperms containing starch deposits were isolated from developing seeds 4 to 7 days after pollination and inoculated in Linsmaier-Skoog's medium with or without 10-5M 2.4-D. But the induction failed in endosperms isolated before 4 days. The success with the medium lacking growth substances suggests high level of endogenous auxin and cytokinin in the immature endosperms. The endosperm callus was subcultured in White's medium supplemented with 10-5M 2.4-D. Root differentiation from the subcultured callus was obtained in White's medium with or without 10-5M IAA. Roots were produced even after 10 months of the subculture. Plantlet formation was achieved in white's medium either with 10-5M IAA or 5×10^{-5} M kinetin. These results show that the rice endosperm cells have totipotency similar

to normal diploid cells¹⁴⁾.

Suspension culture of rice cells

In the liquid medium, single cells of various shape were derived from rice callus with rotary culture method²⁾. Size of these cells was about 41 micron in length and 19 micron in width. A cytochemical study was done on spherical granules which were often observed in the single cells using horizontal reciprocal culture method⁴⁾. These granules seemed to be acidic liquid lipids because they disappeared with treatment of formalin, ethanol, aceton or chloroform-methanol mixture and were stained by Nile blue. They were also extracted with such solvents of lipid-staining dyes as isopropanol, ethylene glycol and triethyl phosphate. The blue color by Nile blue were retained after decolorization of cytoplasm with 5% acetic acid or 0.5% hydrochloric acid.

The callus of Te-Tep and Tan-ginbozu produced single cells at the density over 106/ml after 40 days of culture with the reciprocal method. The freely separated cells, which contained protoplasm thread and were alive. increased in number with time in culture. These living cells often contain spherical lipid granules (Plate 3). The cells containing the granules accounted for over 50% in number when cell density was high. Large lipid granules over 8 micron in diameter were seen at the early stage of culture but no granules over 6 micron were found at the late stage. Thus, the appearance and size of the lipid granules seem to be related with the increase in cell density. It was further observed that Tan-ginbozu cells in the late stages in culture were ellipsoidal in shape with the length 64% of that of the early culture stage⁵⁾.

When the suspended cells at the cell density of $10^6/ml$ were transferred to the fresh medium and subcultured, the density fell at first and again reached at $10^6/ml$ after 60 days of culture. Population rate was computed against culture time for cells containing the lipid granules and for cells in which the nucleus can be seen under the non-stained condition. Both rates, which were 70% at the time of transfer reduced to 5% after 10 to 20 days, after then gradually increased and finally reached to 70% again. Feulgen stainability of nuclei was very pronounced in the inoculum at the early stage, and then decreased. But when the high cell density was reached, the pronounced stainability was recovered. In culture bottles, in which number of suspended cells increased greatly, the fresh weight of callus masses also increased. Therefore, increase in cell density in the liquid medium is closely correlated with 4 factors. (1) existence of lipid granules in cultured cells, (2) number of cells in which nuclei were able to be noticed under unstained condition, (3) Feulgen stainability of nucleus, and (4) callus growth⁹⁾.

Some roots and root-like structures also occurred on the callus tissues in the liquid medium⁹⁾. Nuclear DNA was estimated with 0.5 micron of line interval and 560 nm of wave length after Schiff's staining using scanning microspectrophotometer. The result indicated



Plate 3. Living (left) and dead (right) rice cells suspended in liquid medium (×625). g: lipid granule, n: nucleus, p: protoplasmic cyclosis of small particles.

Plant cells used	Mean diameter in µm±SE	Total absorbance			Percent of nuclei having		No. of
		$Mean \pm SE$	2C	4C	2C	4C	estimated
Suspended cells rice ¹⁾							
expt. 1. round nuclei	4.18±0.24	2.28 ± 0.22	2.0	—	95	5	19
elongated nuclei	6. $42 \pm 0.31^*$	2.87 ± 0.24			100	0	12
expt. 2.	5.05 \pm 0.15	2.15 ± 0.07	2.0		100	0	44
expt. 3.	5.13±0.13	2.70 ± 0.08	2.5		100	0	45
mulberry,	5. 29 ± 0.20	2.88 ± 0.27	2.0	5.5	84	16	31
Callus cells							7.27/23
rice ¹⁾	6.38 ± 0.23	4.77±0.34	4.0	9.0	87	13	39
mulberry,	5.75 \pm 0.24	4.82±0.44	4.0	9.0	87	13	30
cucumber,	7.24 \pm 0.27	6.58 ± 0.50	7.0	\rightarrow	100	0	33
morning glory	7.84 \pm 0.26	8.56 \pm 0.40	8.5		100	0	32
barley,	10.97±0.44	48.59±4.39	45.0	110.0	88	12	33
Root tip cells							
rice, seedling ²⁾	5.72 \pm 0.22	3.21 ± 0.18	3.0	6.0	95	5	43
adult plant ³⁾	6.01 ± 0.26	2.85 ± 0.15	2.5	7.0	98	2	44
mung bean, seedling	8.04 ± 0.23	7.21 ± 0.41	6.0	12.0	83	17	42
barley, seedling, round nuclei	10.93±0.33	41.83±1.57	40. 0	100. 0	98	2	41
elongated nuclei	18.38±1.02*	41.28 ± 4.10	40.0	90.0	90	10	21
corn seedling	11.04 ± 0.69	45, 45 ± 3 , 36	30.0	65.0	61	39	28

Table 2. DNA content per nucleus in rice cells and its comparison with other plant cells

that DNA content in rice cells cultured was about 80% of root tip cells. DNA amounts in rice nucleus, in general, was very low because mung bean showed 2.1 fold of rice and barley and corn 12 fold. Cell proliferation in suspended rice cells was not remarkable because 4 C nuclei in them were few in spite of 13% in callus cells and 5% in root tip cells (Table 2)⁸⁾.



Plate 4. Protoplasts isolated from rice root cells ($\times 600$).



Plate 5. Electron micrographs of protoplasts isolated from rice callus cells (A: \times 7,500; B: \times 12,000). In B, plasmodesma connecting between protoplasts can be seen even after cell wall digestion.

Isolation of rice protoplasts

Rice protoplasts were isolated from cells of leaves¹⁰⁾, roots⁷⁾ and callus tissues¹⁰⁾ with enzyme treatment (Plate 4). At first, the suitable concentrations of macerozyme, cellulase and mannitol, the suitable period of enzyme treatment and the suitable age of used materials were determind. Frequency distribution of size in isolated protoplasts was estimated with their diameter. The isolated protoplasts were embedded in epoxy resin, and semi-thin sections were cut from the resin blocks on an ultramicrotome. Lamellar structure in chloroplasts, nucleoli and small granules were observed with optical microscope. Polynuclear protoplasts were seen in the population isolated from leaves and callus tissues. They seem to be formed by the fusion of the protoplasts closely adjacent each other¹⁰⁾. At present, experiments are in progress with protoplast culture, cell wall regeneration, callus occurrence and cytology (by electron microscopy) of protoplasts (Plate 5 and 6).

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 Plate 6. Electron micrographs of protoplasts isolated from rice leaf cells (A:×40,000; B:×30,000). Cell wall has already been digested. Crystals of fraction 1 protein (f) can be seen in B.

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