

A Light and Scanning Electron Microscopic Study of the Initiation and Development of Sugarcane Callus

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Abstract : Initial callus was primarily formed on the cut ends and occasionally on the abaxial surface of sugarcane immature leaf explants. The growing callus became nodular and purple in colour. Some specimens became white. Subsequent growth of callus led to the breaking through callus surface. The newly formed cells emerged from the breakages and grew into oval and hairy, elongated shapes; this led to the formation of soft callus masses on the explants where spherical structures developed consisting of meristematic cells.

Key words : Callus pigmentation, Callus surface, Fibrillar structure, Membranous layer, Scanning electron microscope, Sugarcane, Ultrastructure.

サトウキビカルスの発生と発育に関する光学顕微鏡及び走査型電子顕微鏡による研究：スマルディ・イシ
レップ・ナカムラ・チエミ・谷口 武・前田英三（ガジャマダ大学理学部・名古屋大学農学部）

要 旨：サトウキビカルスは、主に葉片の切断面及び背軸側表層から生じる。カルスは塊状となり、ときに白色となる。カルスが生長すると、カルス表面に裂け目が生じ、この裂け目の内部から新しい細胞が形成される。この細胞は卵型となるか、または長く伸長し、柔らかいカルスを形成する。このカルス内に、分裂細胞からなる球状構造が生じる。

キーワード：カルスの着色、カルス表面、サトウキビ、繊維構造、走査型電子顕微鏡、薄膜層、微細構造。

The successful tissue cultures in monocotyledonous plant species have been reported using seeds and seedlings of rice^{5,6,13}, immature embryos of wheat¹⁶, embryos and mesocotyl explants of barley^{7,8}, leaf explants of rye⁹, immature embryos of *Panicum maximum*¹², immature inflorescences of *Penisetum americanum*², and stems and young inflorescences of *Cocos nucifera*³. Numerous similar reports have been made during the last decade. Especially, those describing callus formation, organogenesis and somatic embryogenesis in sugarcane have been published in recent years^{1,4,10,11}.

However, detailed information regarding the ultrastructure of callus initiation and its development remains to be gathered. Since 1965, rice callus cultures and the ultrastructural examination have been extensively studied in our laboratory^{13,14}. We now report the initiation and its development of callus on sugarcane leaf explants examined by a light stereomicroscope and a scanning electron microscope (SEM) which may represent progress toward the high frequency of plantlet production.

Materials and Methods

Young leaves of sugarcane (*Saccharum officinarum* L., cv. NCo310) were excised as described by Liu et al.¹¹ and cultured in Murashige and Skoog's basal medium, supplemented with 10^{-5} M 2,4-D (2,4-dichlorophenoxyacetic acid), 78.4 mg/l FeEDTA, 200 mg/l myo-inositol, 1 mg/l thiamine, 3 g/l casein hydrolysate, 3% sucrose and 0.8% agar.

All cultures were kept in a culture room at 24—25°C under continuous light. After 1 week and 2 weeks in culture medium, callus induction and its development were observed. From the observed specimens, their photographs were taken using a light stereomicroscope of OLYMPUS SZH model.

For electron microscopic observation using SEM, the materials were fixed with a mixture of 3% glutaraldehyde, 1.5% paraformaldehyde and 0.1M cacodylate buffer as a fixative solution, by gently shaking at room temperature for 5 hours. Refrigerated overnight, the materials were rinsed repeatedly with distilled water, and dehydrated with ethanol series (30%, 50%, 70%, 80%, 90%, 95%, 100%)

respectively, for 30 min each. However, dehydration with absolute ethanol was repeated twice more. The dehydrated materials were treated with isoamyl acetate and dried using a critical point dryer apparatus of HITACHI HCP-1 model.

After drying the materials were fastened to metal holders with double sticky tape. The surfaces of the holders with the material on were smeared with electroconductive silver paste. The materials were coated with gold using an ion sputtering equipment of EIKO IB-3 model and examined by a scanning electron microscope of HITACHI S-415 model.

Experimental Results

After one week in the culture medium, callus initiation was already seen with the naked eye. Usually callus was easily formed on the upper cut surface of leaf explants. Sometimes callus masses rose abruptly from the epidermal layer and also from the lower cut

surface that had direct contact with the culture medium.

(1) Observation using a light stereomicroscope

A number of small protuberances, as initial callus, were found on the abaxial surface that touched the upper cut edge of the leaf explants. They grew into linear rows on vascular strands and were conspicuous by their purple colour. Occasionally the rows lacked pigmentation (Figs. 1, 2 and 3). Longitudinal files of the protuberances continued their growth during the experimental period. Some files were pigmented with purple and the others with purplish green. White protuberances arose from some portions of the cut ends too. Finally, all the initial callus tissues grew bigger, keeping their smooth epidermal surfaces and formed nodule-like purple, white, or whitish-purple structures (Figs. 2 and 3). As a result, the upper cut ends of outer aged and inner young leaves were entirely covered by the developing callus masses (Fig. 4). Hairy, elon-

Explanation of figures

Figs. 1 to 6. Light micrographs of callus structure occurred on sugarcane explants (bar : 2 mm).

Fig. 1. Small protuberances as callus initials appeared along vascular strands.

Fig. 2. Occurrence of each callus initial over vascular strands.

Fig. 3. Magnification of callus initials, showing surface cells arranged longitudinally.

Fig. 4. Callus initials on the cut ends of growing young leaf segments and advanced growth of callus masses on an aged leaf segment surrounding young leaves.

Fig. 5. Magnification of a callus masses, showing its rough surface.

Fig. 6. Hairy long cells on a callus mass.

Figs. 7 to 22. Scanning electron micrographs of callus initiation and its growth on sugarcane explants, and its newly cut surface of a cultured explant.

Fig. 7. Swelling callus initials at the cut end of an explant (bar : 300 μ m).

Fig. 8. A small breakage of surface cell wall on a callus initial (bar : 100 μ m).

Fig. 9. Magnification of a breakage occurred on a bulge of callus surface (bar : 30 μ m).

Fig. 10. Growth of both oval and elongated cells from a breakage of callus surface (bar : 200 μ m).

Fig. 11. A transverse cut surface of a leaf explant after 1 week in culture (bar : 500 μ m).

Fig. 12. Magnification of transverse cut surface, showing vascular bundles and mesophyll cells (bar : 500 μ m).

Fig. 13. Cell growth at the cut ends of a cultured leaf explant during 1 week (bar : 500 μ m).

Fig. 14. Cell elongation at the cut end (bar : 300 μ m).

Fig. 15. Cell elongation and membranous layer over cells at the cut end (bar : 500 μ m).

Fig. 16. Membranous layer developed at the cut end (bar : 500 μ m).

Fig. 17. Fibrillar structure connecting between elongated cells (bar : 150 μ m).

Fig. 18. Magnification of fibrillar structure (bar : 60 μ m).

Fig. 19. Spherical structures in the masses of elongated cells (bar : 100 μ m).

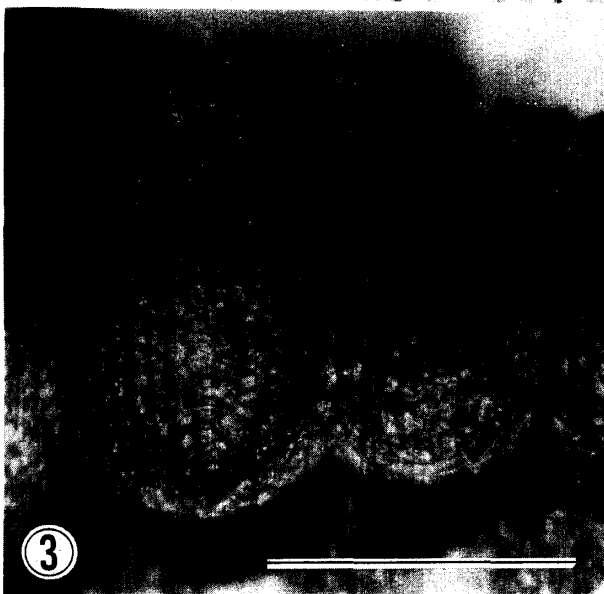
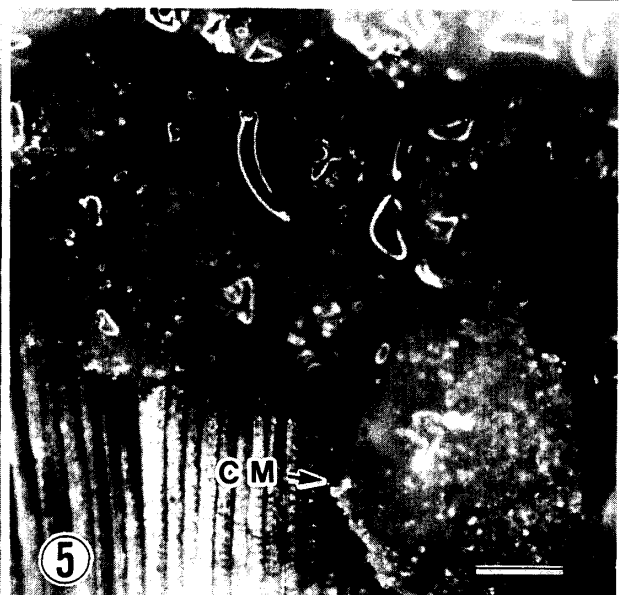
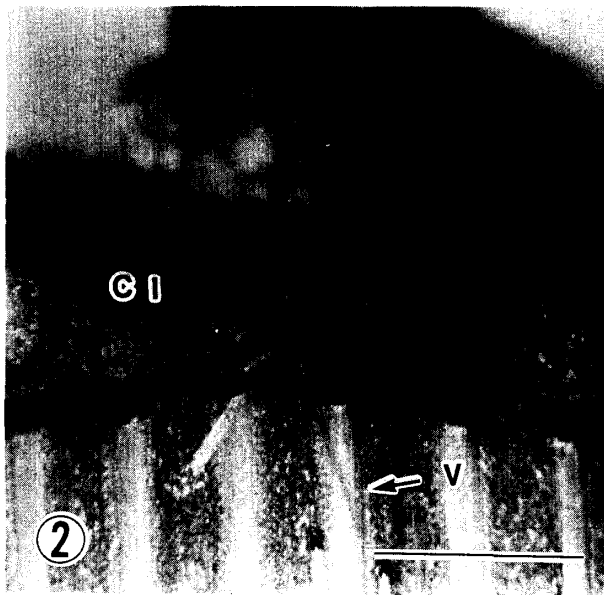
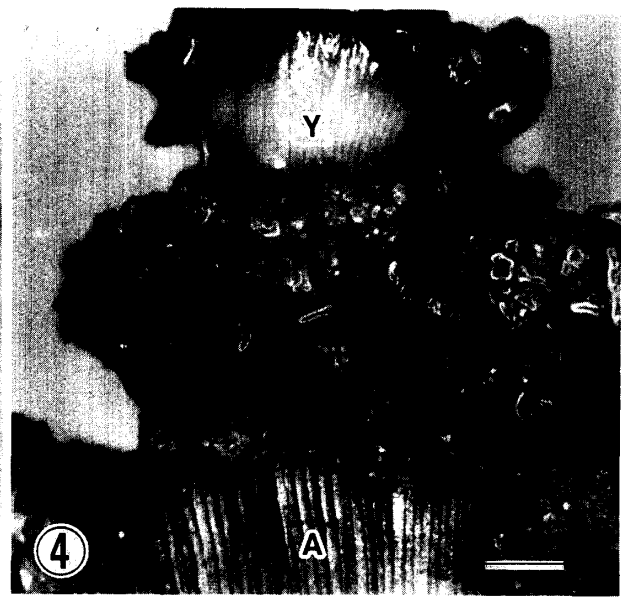
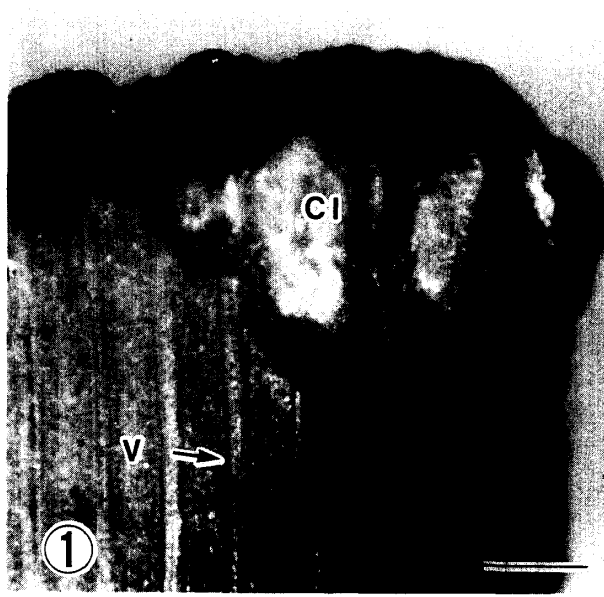
Fig. 20. Magnification of spherical structures (bar : 30 μ m).

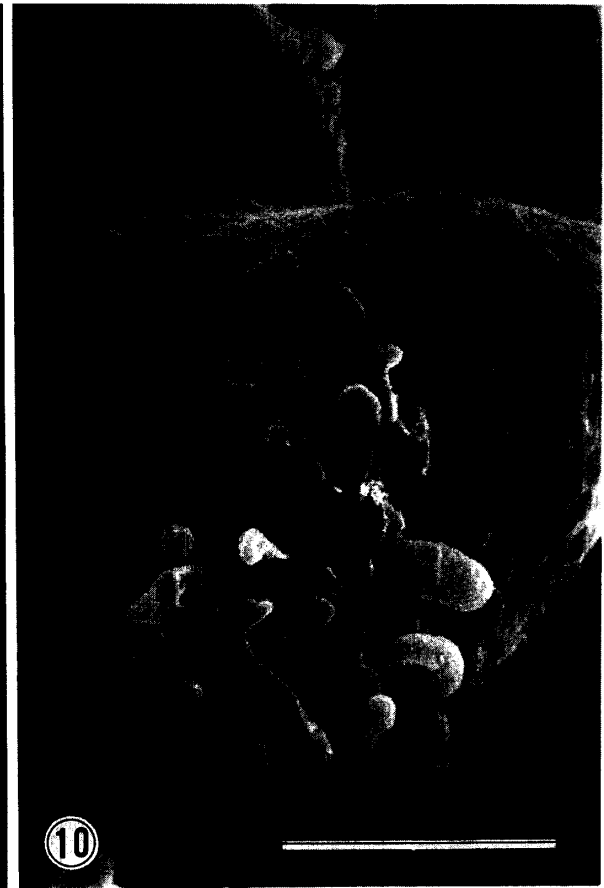
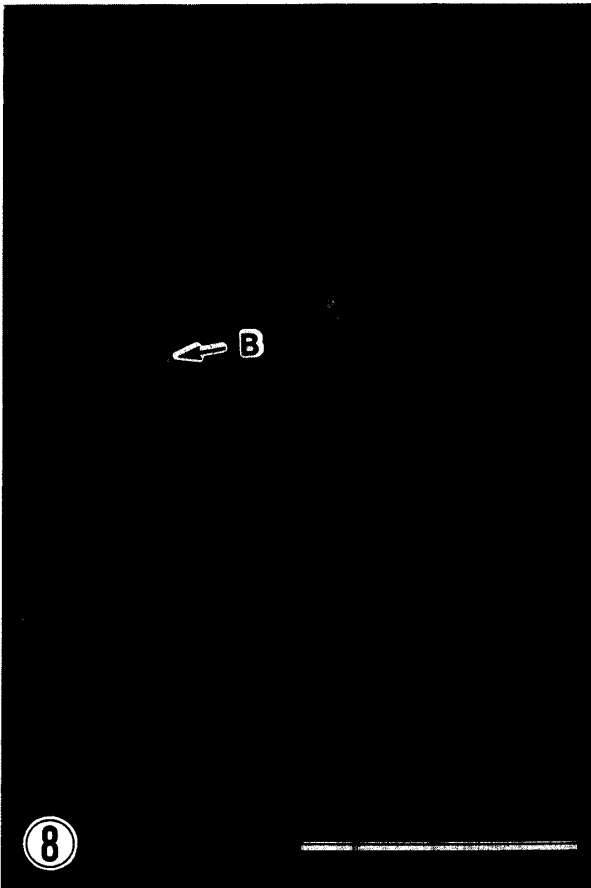
Fig. 21. Elongated cells and the connection with membranous layer (bar : 150 μ m).

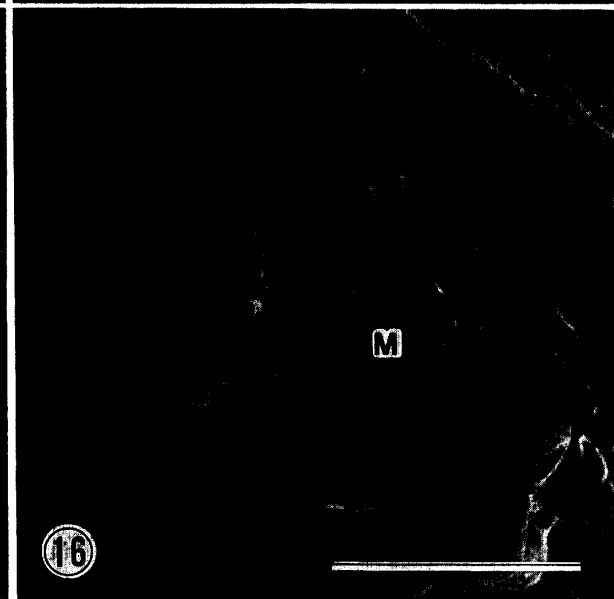
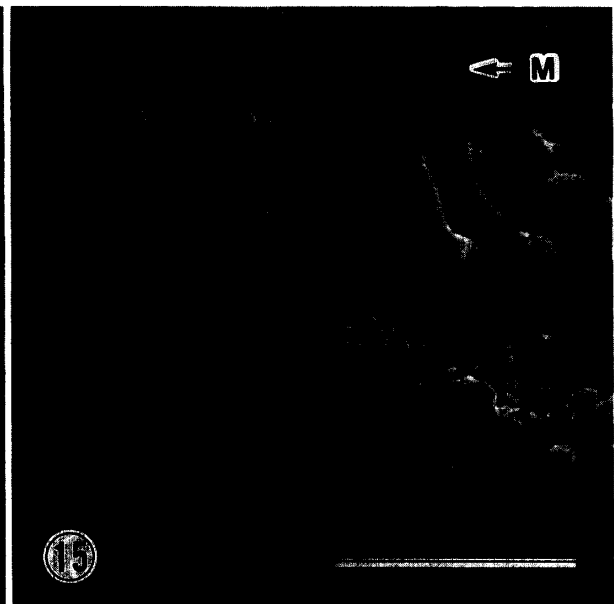
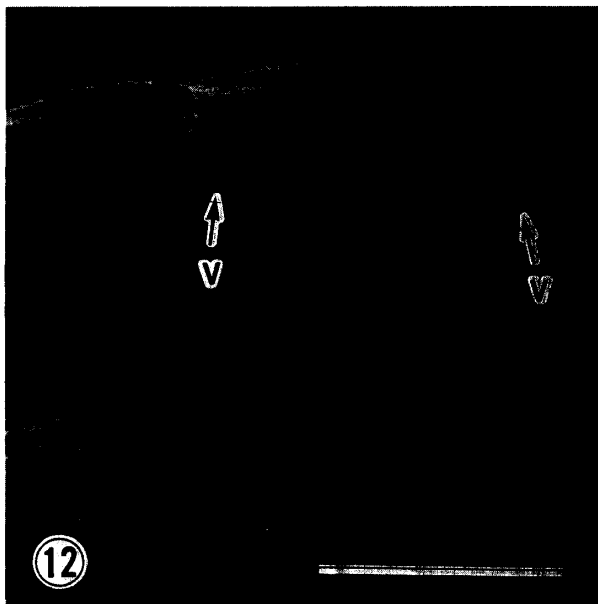
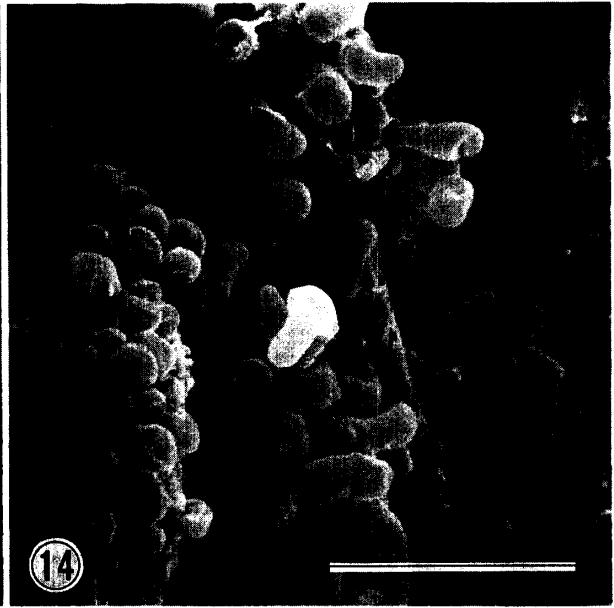
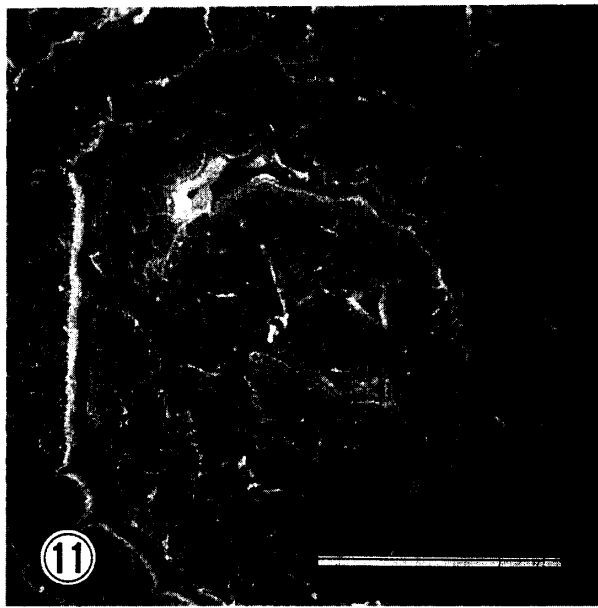
Fig. 22. Growth of elongated cells to various shapes (bar : 200 μ m).

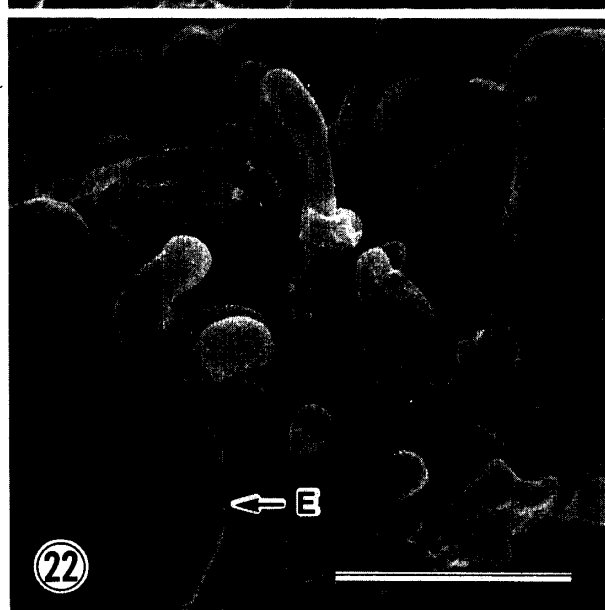
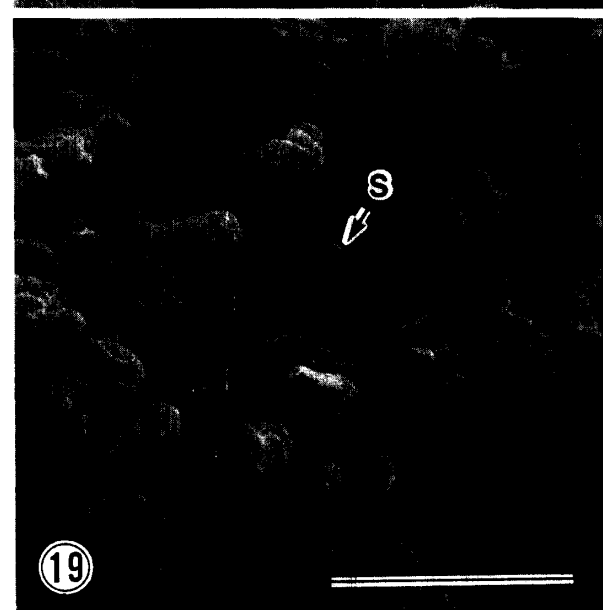
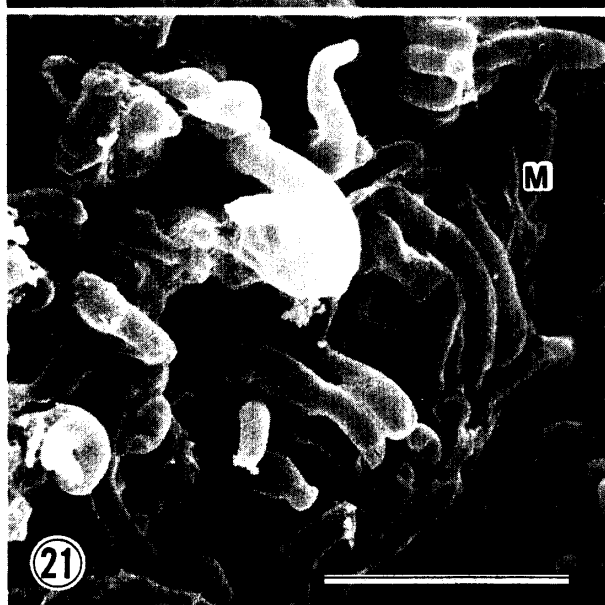
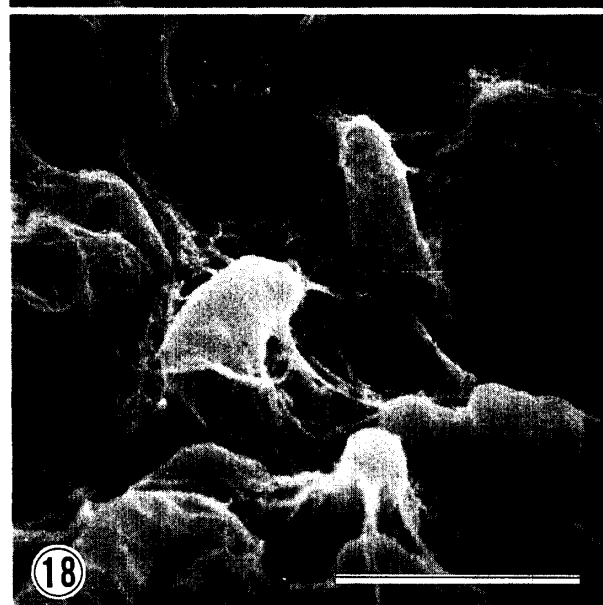
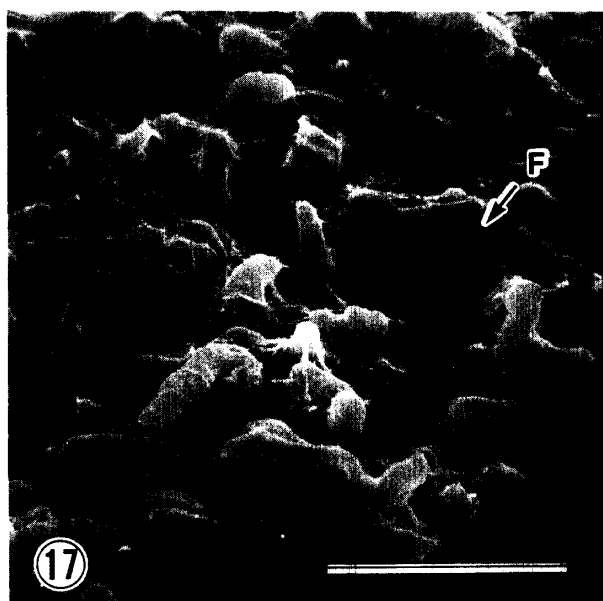
Abbreviations

A : aged leaf segment, B : breakage, CI : callus initial, CM : callus mass, E : enlarged cell, F : fibrillar structure, H : hairy long cell, M : membranous layer, S : spherical structure, V : vascular strand, Y : young leaf segment.









gated cells occasionally appeared on the surface of large white callus tissues where the smooth epidermal surface was markedly disturbed (Figs. 5 and 6).

Besides the cut ends of leaf explants, the pigmented and unpigmented callus masses rarely grew bigger at some portions on the abaxial side of epidermal layer near the cut ends. Sometimes, large masses of callus developed at both the adaxial and abaxial sides on the midrib that was in contact with the medium.

(2) *Observation using a scanning electron microscope (SEM)*

One week after explantation, numerous swelling tissues of different shapes and sizes were found on the abaxial side of the upper cut ends, suggesting the formation of callus initials (Fig. 7). The swelling structure usually grew downward direction from the cut surface to form linear arrangement. Sometimes they developed in some portions on the abaxial surface where slightly apart from the cut ends. Stomata and trichomes were clearly present between these swelling linear structures (Fig. 7). Under high magnification, the surface of the swelling epidermis appeared undulated and the arrangement of epidermal cells was disturbed. Furthermore, small-sized breakage appeared on the swelling surface (Fig. 8). Further magnification clearly illustrated that a smooth surface was exposed inside the breakage area (Fig. 9). These structures may suggest that the breakages originate from stomatal apertures.

After the swelling structure grew bigger into the callus of various shapes, a cluster of both oval and elongated cells extruded from these breakages of the swelling surface (Fig. 10). Further a number of the extruded cells grew in different sizes, and vigorously continued to grow, finally forming masses of long cells.

After one week in culture, the inoculated leaf explant was transversely cut at the middle part and their newly cut surface was exposed. By observing the exposed cut surface, we found ground parenchyma cells of a large size and vascular bundles appeared in rows within the ground tissues (Figs. 11 and 12). On the other hand, new cell groups were numerous formed within one week culture in both the central part and the peripheral region on the upper cut ends of explants. They were spheri-

cal and developed on almost the entire cut surface (Fig. 13). In the peripheral region, their structures resembled bird's nets at a side view. Cell proliferation occurred from the ground parenchyma around or between vascular bundles.

Emergent cells elongated and some cells were interconnected together with membranous coverings (Figs. 14, 15 and 16). On the other parts of the cut surface, several stages of developing callus masses were found. In the more developed callus, the cells were elongated, but undeveloped cells were small and short and some of them were partially covered with a thin membranous layer. The epidermal layer near this structure consisted of unarranged cells.

After 2 weeks in culture, the remaining of membranous layer appeared like fibrillous covering the developing cell masses (Figs. 17 and 18). Spherical structure appeared among the elongated cells (Figs. 19 and 20). As a result, superficial layer of the callus derived from the sugarcane leaf explants consisted of oval, long and enlarged cells except in the areas with a membranous covering (Figs. 21 and 22).

Discussion

To understand the morphological character involved in the growth and development of plant tissue cultures is of critical importance for the agricultural application of plant biotechnology related to clonal propagation. The purpose of this study was to examine the surface ultrastructure of callus induction in sugarcane leaf explants by aid of light and scanning electron microscopy.

Developing callus tissues of sugarcane leaf explants were purple, whitish purple and white in colour. At first, the purple colour were clearly found after 1 week in culture medium. Nozue and Yasuda¹⁵⁾ found such pigmented vesicles in the anthocyanin-containing cells in sweet potato suspension culture. They have suggested that anthocyanin synthesis initially began to occur 24—48 hours after exposure to light. Rye callus was yellow in colour and embryogenic aspects were found in the MS medium supplemented with 2 mg/l 2,4-D⁹⁾. If the basal medium had been supplemented with combination of 2,4-D (0.5 mg/l), naphthaleneacetic acid (1 mg/l), and 6-

benzylamino purine (0.5 mg/l), callus became dark in colour and root growth was highly promoted. When rice callus cultures were kept in the medium with thiamine (T) or without it (WT) for prolonged periods, and were transferred in WT medium added with oxythiamine, T callus became necrotic and turned dark brown, while WT callus was watery and soft in its character⁵⁾. Callus cultures derived from scutellum of immature wheat embryos were yellow to yellow white, while cultures derived from roots of the immature embryos were white and had no embryogenic aspects¹⁶⁾. Therefore, callus pigmentation is remarkable for rapid formation of shoot buds.

Liu et al.¹¹⁾ have reported that callus proliferation initially starts from the parenchyma cells near the primary phloem. In our experiment, however, it appears that proliferation apparently occurs from mesophyll parenchyma cells between vascular bundles because the vascular bundles in the explants has not yet dedifferentiated during the experimental periods. The origin of proliferated callus must be studied in more detail through histological research of the dedifferentiation process.

In conclusion, this paper suggests that newly initiated callus occurs from both the cut ends and the epidermal surface as described by Ho and Vasil⁴⁾. However, it reveals that initial callus is covered with an epidermis-like smooth surface. Subsequent callus growth resulted in small breakages on the smooth surface. Thereafter, numerous cells extruded from the breakages and vigorously developed to form soft callus masses. Within the soft callus masses, spherical structures appeared as newly formed meristematic masses. An ultrastructural study of shoot-bud formation from sugarcane callus masses is now in progress.

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