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

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Received April 28, 1988

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.

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%)

The successful tissue cultures in monocotyledonous plant species have been reported using seeds and seedlings of rice6,6,13, immature embryos of wheat14, embryos and mesocotyl explants of barley7,8, leaf explants of rye9, immature embryos of Panisum maximum12, immature inflorescences of Pennisetum americanum2, and stems and young inflorescences of Cocos nucifera3. 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 years4,10,11.

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 laboratory13,14. 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.

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respectively, for 30 min each. However, de-
hyration with absolute ethanol was repeated
twice more. The dehydrated materials were
treated with isooamyl 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 elec-
tron 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. Some-
times callus masses rose abruptly from the
epidermal layer and also from the lower cut
surface that had direct contact with the cul-
ture 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 pigmen-
tation (Figs. 1, 2 and 3). Longitudinal filles of
the protuberances continued their growth
during the experimental period. Some filles
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 μm).

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

**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.

Emerged 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 Yasuda 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-D. 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 character. 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 aspects. Therefore, callus pigmentation is remarkable for rapid formation of shoot buds.

Liu et al. 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 Vasil. 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 ultra-structural study of shoot-bud formation from sugarcane callus masses is now in progress.

Acknowledgment

We express our gratitude to Dr. Akihiro Nose, University of The Ryukyus, for his kind gift of sugarcane samples. One of our members (I.S.) wish to gratefully acknowledge the financial support of the Biotechnology Inter University Center, Gadjah Mada University used to fund her stay in Japan.

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