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Patterns of regenerating structures in beet calli

by

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Summary

Regenerating calli of haploid and diploid genotypes of *Beta vulgaris* L., of an allotriploid interspecific hybrid of *Beta vulgaris* L. and *Beta procumbens* CHR. SM., and of an allotetraploid interspecific hybrid of *B. vulgaris* and *Beta patellaris* MOQ. were observed by phase contrast microscopy and scanning electron microscopy. The patterns of regenerating structures proved to be different for the four types tested. Embryo-like structures observed on the regenerating calli of an haploid genotype are discussed in terms of genesis and morphology.

Keywords : regenerating calli, somatic embryogenesis, beet.

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1. Introduction

Many attempts were made, in sugar beet, to domesticate the *in vitro* production of shoots [DE GREEF and JACOBS, 1979 ; SAUNDERS and MAHONEY, 1982 ; SAUNDERS and DAUB, 1984 ; SAUNDERS and SHIN, 1986 ; SAUNDERS and DOLEY, 1986 ; FREYTAG *et al.*, 1988 ; DETREZ *et al.*, 1988 ; RITCHIE *et al.*, 1989 ; DETREZ *et al.*, 1989 ; KRENS and JAMAR, 1989] and somatic embryos [TETU *et al.*, 1987 ; FREYTAG *et al.*, 1988]. However, shoot regeneration from completely dedifferentiated calli (TETU, personal communication) or single cells (KRENS, personal communication) remains problematic. The genotype is determinant for organ specificity, efficiency, and type of regeneration obtained (CARELS, unpublished results). Indeed, buds may be formed on midrib or blade of leaves in culture ; the regeneration efficiency by means of direct bud formation on leaves may vary from a few to 100 % of the leaves in culture, according to the interactions between genotypes, medium composition and culture conditions. Regeneration may arise by typical bud or by embryo formation. Cautious scoring of the regenerating behaviour in the existing gene pool is a prerequisite to the tagging of the determinants of types and efficiency of shoot regeneration from dedifferentiated tissues. In this paper, we describe the results of histological investigations on regenerating calli of beets with various genotypic structures.

2. Materials and methods

2.1. PLANT MATERIALS

The sugar beet clones Pr3 (2n=18) and HAP (n=9) were kindly provided by SES (Société Européenne de Semences, Tienen, Belgium) and NICKERSON-ZWAANESE (Stompijk, The Netherlands), respectively. Allotriploid (VVPro ; 2nV+nPro=27) hybrid of *Beta vulgaris* L. (4n=36) and *Beta procumbens* CHR. SM. (2n=18), and an allotetraploid (VVPatPat ; 2nV+nPat=36) hybrid of *B. vulgaris* (4n=36) and *Beta patellaris* MOQ. (2n=36) were gratefully received from SVP (Stichting voor Plantenveredeling, Wageningen, The Netherlands). The crossing scheme and characteristics of these hybrids were described by SPECKMANN *et al.* [1985].

2.2. CULTURE CONDITIONS

Plant cultures were kept in a growth chamber at a temperature of 20 ± 1°C. Light was provided by Sylvania Gro-Lux and Philips TL65 fluorescent tubes (300 µE $m^{-2}sec^{-1}$) with a light/dark cycle of 16/8 hr. Shoots were placed in glass pots containing 80 ml of solidified culture medium (Merck agar-agar ; 5.5 g/l). Regenerating calli were grown in Petri dishes. All media were autoclaved prior to use for tissue culture.

2.3. INDUCTION AND VEGETATIVE PROPAGATION OF REGENERATING CALLI

Pr3, HAP and VVPatPat shoots were subcultured on PGo supplemented with Kin (0.3 mg/l) while VVPro shoots were subcultured on PGo supplemented with BAP (0.3 mg/l), GA_3 (0.2 mg/l) and NAA (0.1 mg/l). GA_3, sterilized by filtration, was added to the autoclaved culture medium. Leaves from these shoot cultures were used for the induction of primary regenerants, following the procedure described by TETU *et al.* [1987]. Primary regenerants evolving spontaneously into regenerating calli were subcultured on propagation medium : MS supplemented with BAP (0.3 mg/l) and NAA (0.1 mg/l) (1).

2.4. HISTOLOGICAL PROCEDURES

Histological investigations were performed with samples of regenerating calli grown on MS medium. After fixation for 48 hr in 2.5 % glutaraldehyde (in 100 mM cacodylate buffer pH 7), explants were post-fixed for 2 hr with a solution containing 1 % osmium tetroxide, 1 % potassium dichromate and 10 mM calcium chloride. Samples were dehydrated through a graded series of ethanol and propylene oxide and inbedded in Epon Spurr mixture. Semi-thin sections (2.5 µm) were cut with a glass knife.

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(1) PGo = PGo medium [DE GREEF and JACOBS, 1979].
Kin = Kinetin.
BAP = 6–benzylaminopurine.
GA_3 = Gibberellic acid.
NAA = Naphtalenacetic acid.
MS = MURASHIGE and SKOOG [1962] medium.

2.5. SAMPLE OBSERVATIONS

A stereo microscope ZEISS was used to look for regenerating structures on regenerating calli prior to histological investigations. HAP calli were further observed by scanning electron microscopy (SEM) using an OMNISCAN D511-2300 apparatus from ETEC Corporation. The samples for scanning electron microscopy were fixed and dehydrated as described in histological procedures, prior to be fixed on stubs with silver glue and coated with gold in a sputter coater from LEYBOLD for 90 sec. Semi-thin sections were observed by phase contrast microscopy using an ANOPTRAL-REICHERT microscope.

3. Results

Regenerating calli of *B. vulgaris* were obtained as well with haploids as with hybrids containing chromosomes of wild species of the *Patellares* section. Such calli proved to be stable in terms of efficiency and expression of bud regeneration, and were grown in our laboratory up to 3 years.

Pr3 calli grew as amorphous cellular corpuses with inbedded buds (Plate 1A, 1C). Meristematic zones appeared in the epidermal cell layer over large parenchymatous cells (Plate 1B). Buds normally differentiated from these superficial meristematic zones (Plate 1C). Differentiated buds evolved into shoots when subcultured on PGo without hormones.

The HAP calli grew as multiglobular corpuses (Plate 2A) with a zone of meristematic cells in the epidermic layer on top of globules (Plate 2B). When such calli were maintained on PGo without phytohormones during 4 weeks, scattered embryo-like structures were observed (Plate 2D). These embryo-like structures spontaneously pulled away of the callus corpus ; their center appeared devoid of cells and vascular tissues (Plate 2E). Small non meristematic cells were observed at the bottom of these structures (Plate 2E). The attachment point to the callus corpus was still observable and was made of a few cells only. No vascular tissues linking head and bottom of the embryo-like structures could be seen. Moreover, in semi-thin sections, we observed that some of these globules were attached to the callus corpus by a few cells (Plate 2C) as for embryo-like structures (Plate 2E). In other situations, globules evolved into typical buds which looked like Pr3 buds.

The VVPro calli grew as aggregates of compact tissues and leaflets (Plate 3A). Extended meristematic zones were observed in semi-thin sections in compact tissues (Plate 3C). Limited meristematic zones were

observed at the basis of leaflets (Plate 3B). Organization of both types of meristematic tissues (Plate 3D) led to bud differentiation (Plate 3E) and eventually shoot formation, depending on the culture procedure.

The VVPatPat calli grew as green corpuses on which red clusters formed (Plate 4A). Histological observations of semi-thin sections showed that these clusters were made of meristematic cells (Plate 4B) ; they were observed only in connection with the epidermic cell layer. When calli were subcultured 15-20 days on a propagation medium, the red clusters evolved into buds (Plate 4C). If subcultures were not made at the right moment, vacuoles of meristematic cells enlarged and clusters evolved into calli.

4. Discussion

The plant material studied covered haploid, diploid, allotriploid, and allotetraploid genotypes. In all cases, we obtained the type of regenerating calli described by DE GREEF and JACOBS [1979]. Even with interspecific hybrids including *B. patellaris* or *B. procumbens* chromosomes, regenerating calli were obtained. However, bud regeneration of leaf explants of *B. patellaris* or *B. procumbens* seems to be ineffective under culture conditions where this phenomenon exists on the *B. vulgaris* leaf explants [YU, 1989]. As bud regeneration in tissue culture of interspecific hybrids between *B. vulgaris* and those species is possible, somatic hybridization with *B. patellaris* or *B. procumbens* followed by subsequent bud regeneration should be feasible [KRENS *et al.*, 1990].

The regenerating calli described here were stable in terms of type of morphogenesis and regeneration ability through the years. Shape patterns of meristematic zones and buds differed considerably from one genotype to the other, but all regenerating structures arose from the epidermic cell layer. The presence of an epidermic cell layer on regenerating calli, revealed that such autonomous growing structures were not typically dedifferentiated calli, but rather more complex structures including epidermic cells and parenchymatous cells. These parenchymatous cells were still able to grow for several subcultures, but lost the ability to regenerate buds.

Culture sequence for the obtention of somatic embryos in sugar beet with diploid, triploid and tetraploid accessions was first reported by TETU *et al.* [1987]. In our experiments, we observed embryo-like structures spontaneously arising on the HAP calli, in long term cultures. Embryo-like structures were embryo shaped but were devoid of root meristem. The attachment point to the callus corpus was of at least 30 cells (as its diameter was of 6 cells and assuming that its section

was circular). Large spaces devoid of cells were observed in the center of the embryo-like structures, suggesting that peripheral cells had sustained divisions while internal cells had not, or that peripheral cells had elongated while internal cells had not. These gaps showed a lack of harmony in cell growth during the formation of these embryo-like structures. It is suggested that such structures arose by bud elongation rather than by a typical embryogenesis process [KAMEYA and UCHIMIYA, 1972]. However, in contrast to the typical buds observed on the Pr3, VVPro and VVPatPat regenerating calli, no vascular tissues could be observed between the callus corpus and the embryo-like structures.

Analysis of the sections showed that the multiglobular calli from HAP arose from two regenerating processes. The first process, implied the division of one or a few epidermic cells, which formed an independent globular corpus attached by a few cells to the callus corpus. The globular corpuses were named proembryogenic-like structures. These proembryogenic-like structures might evolve into embryo-like structures in long term cultures. The second regenerating process, which formed buds on the callus corpus or on proembryogenic-like structures might continue to extend and give rise to a multiglobular callus. It was impossible to distinguish the primary apical meristem from secondary arising meristems in sections in proembryogenic-like structures, but root meristems were never observed. Secondary regenerations are probably due to an excess of cytokinin. This hypothesis is strengthened by the fact that embryo-like structures arose in long term cultures on hormonefree medium.

From the above considerations, one should consider such embryo-like structures as atypical embryos. Further work on screening of haploids for regeneration types and ability should help to clarify the situation.

Résumé

Profils de différenciation de structures régénérantes de cals de betterave

La morphogenèse de cals régénérants de génotypes haploïdes et diploïdes de *Beta vulgaris* L., d'hybrides interspécifiques allotriploïdes de *B. vulgaris* et *Beta procumbens* CHR. SM., et allotétraploïdes de *B. vulgaris* et *Beta patellaris* MOQ. est analysée par microscopie en contraste de phase sur coupes semi-fines et par microscopie électronique à balayage. Nous constatons que les cals régénérants ne sont pas constitués de cellules dédifférenciées, mais plutôt de tissus comportant une assise de cellules de type parenchymateux couverte

d'une couche de petites cellules de type épidermique. Le tissu composé de l'association de ces types cellulaires est stable du point de vue de l'augmentation de sa biomasse et de sa capacité de régénération de bourgeons quel que soit le nombre de repiquages. Les bourgeons se différencient à partir de cellules méristématiques dans la couche épidermique, quel que soit le type de cal. Par contre, le profil de régénération de bourgeons est différent d'un cal à l'autre et est apparu dépendant du génotype. Nous observons que le cal haploïde donne lieu à des structures analogues aux embryons somatiques. Toutefois, nous concluons à de l'embryogenèse somatique atypique en raison de l'absence de certaines caractéristiques associées à la définition de l'embryogenèse somatique chez les espèces modèles.

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Legends to plates

Plate 1 : Pr3 callus. (A) Stereo microscope photograph of a regenerating callus grown on propagation medium ; arrows indicate buds – scale bar : 2.5 mm. (B) Semi-thin section in a callus showing a zone of meristematic cells formed at the callus surface prior to bud differentiation – scale bar : 60 µm. (C) Semi-thin section in a callus showing the differentiation of a bud from a zone of meristematic cells – scale bar : 240 µm.

Plate 2 : HAP callus. (A) SEM micrograph of a regenerating callus grown on propagation medium, showing its multiglobular shape ; arrow shows bud differentiation from a zone of meristematic cells, on top of a globule – scale bar : 2 mm. (B) SEM micrograph of an embryo-like structure – scale bar : 800 µm. (C) Semi-thin section into a multiglobular aggregate, showing buds or globules differentiation – scale bar : 1 mm. (D) Semi-thin section in multiglobular aggregate originating from a few epidermic cells (an area of maximum 25 cells) as it appeared from the narrow attachment point (arrows) to the callus corpus – scale bar : 1 mm. (E) Semi-thin section of an embryo-like structure showing the top meristem (TM), the bottom part (B) free of root meristem, the attachment point to the callus corpus (AP), and the embryo-like center (C) with large spaces devoid of cells – scale bar : 400 µm.

Plate 3 : VVPro callus. (A) Stereo microscope photograph of a regenerating callus on propagation medium, showing compact tissues (CT) and leaflets (LF) – scale bar : 2.5 mm. (B) Semi-thin section in a callus showing a zone of meristematic cells as it appeared at the base of leaflets – scale bar : 60 µm. (C) Semi-thin section in the compact tissue of a callus showing a zone of meristematic cells – scale bar : 120 µm. (D) Meristematic cells showing the dense cytoplasm and the large nuclei (arrows) – scale bar : 10 µm. (E) Semi-thin section of a callus showing the differentiation of a bud with its obvious vascular tissues (LT) – scale bar : 240 µm.

Plate 4 : VVPatPat callus. (A) Stereo microscope photograph of a regenerating callus grown on propagation medium, showing a meristematic cluster (CL) and a bud (BD) – scale bar : 2 mm. (B) Semi-thin section of a cluster, showing its epidermic origin and its meristematic nature – scale bar : 60 µm. (C) Semi-thin section of a callus showing a bud as it appeared after the differentiation of a cluster – scale bar : 240 µm.

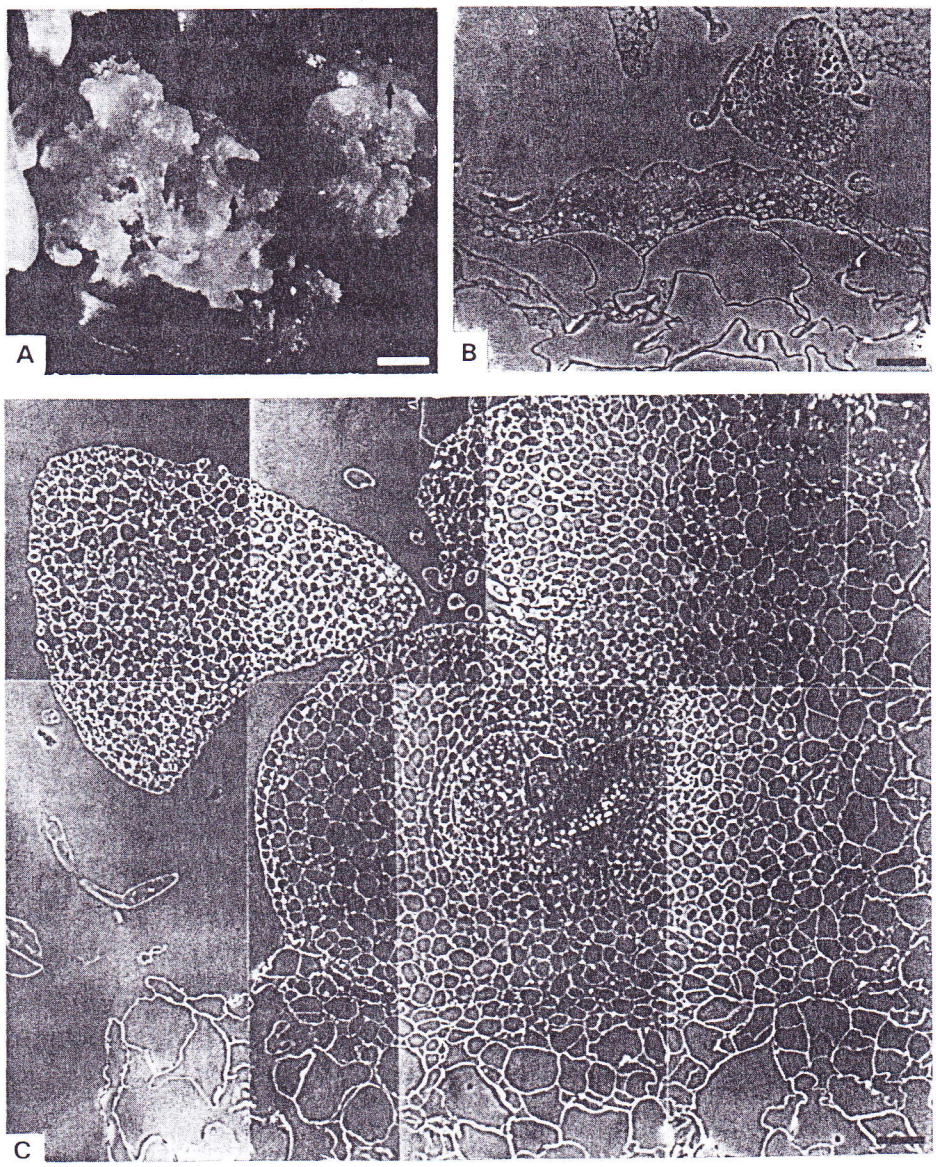
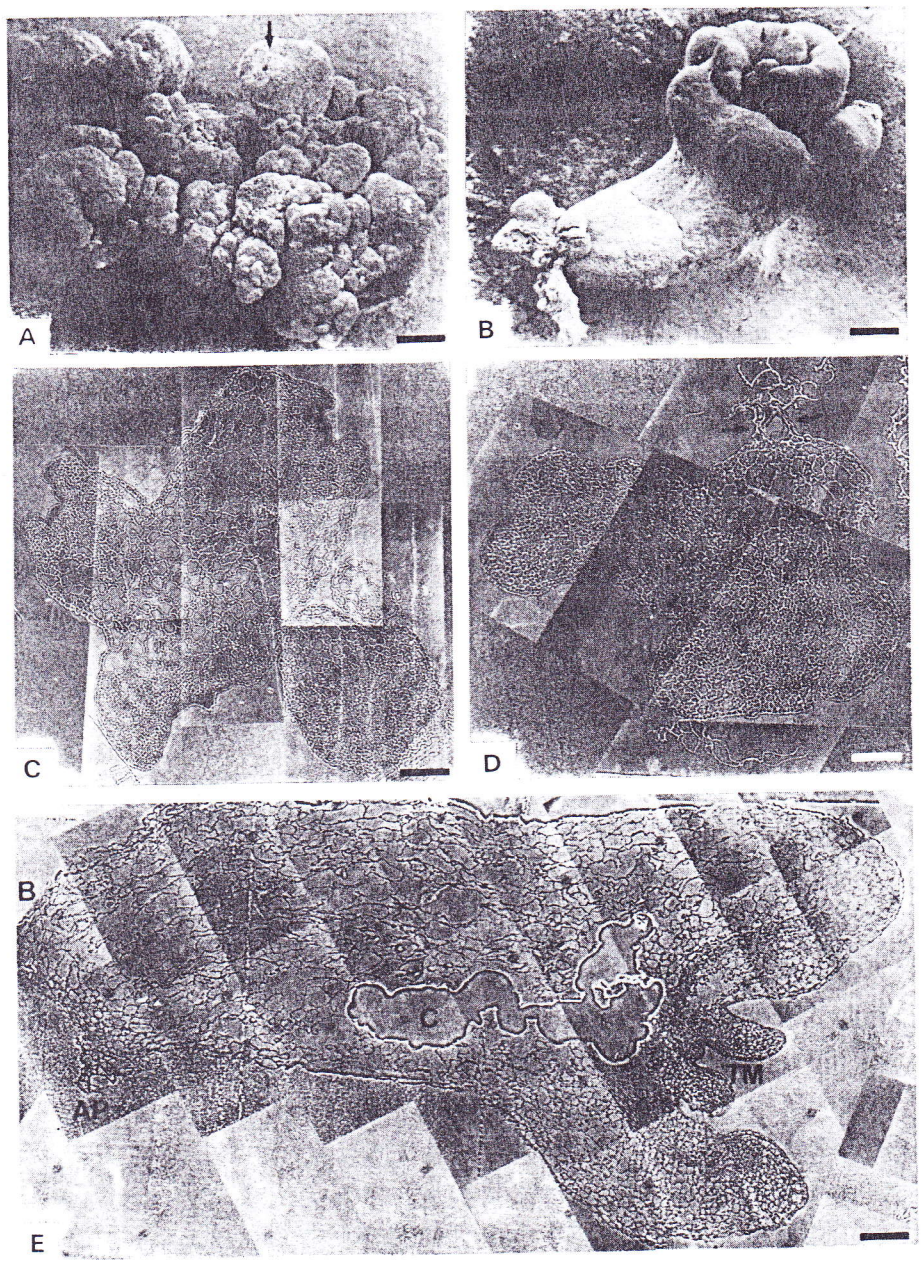


Plate 1 : Legend, see p. 467.



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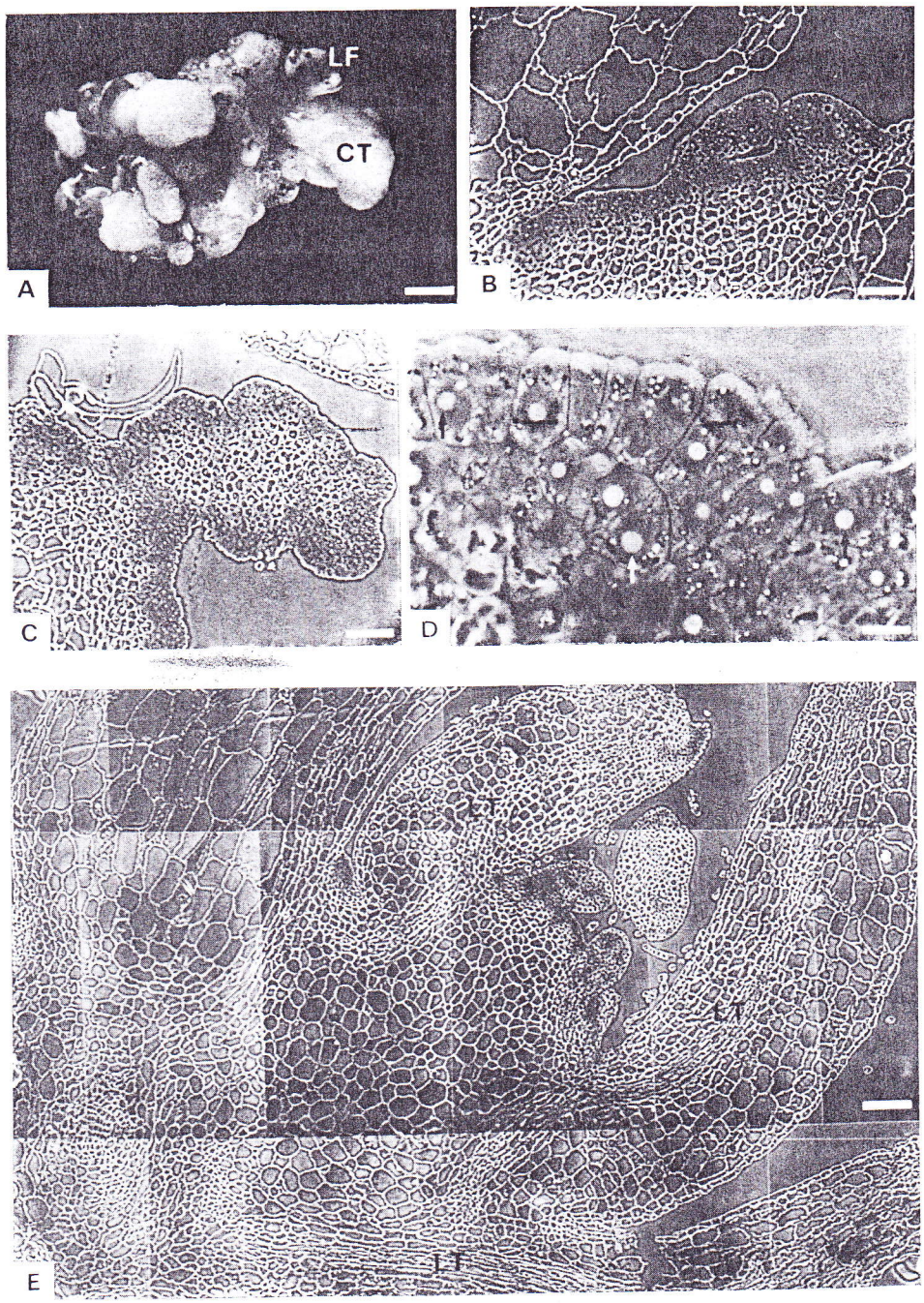

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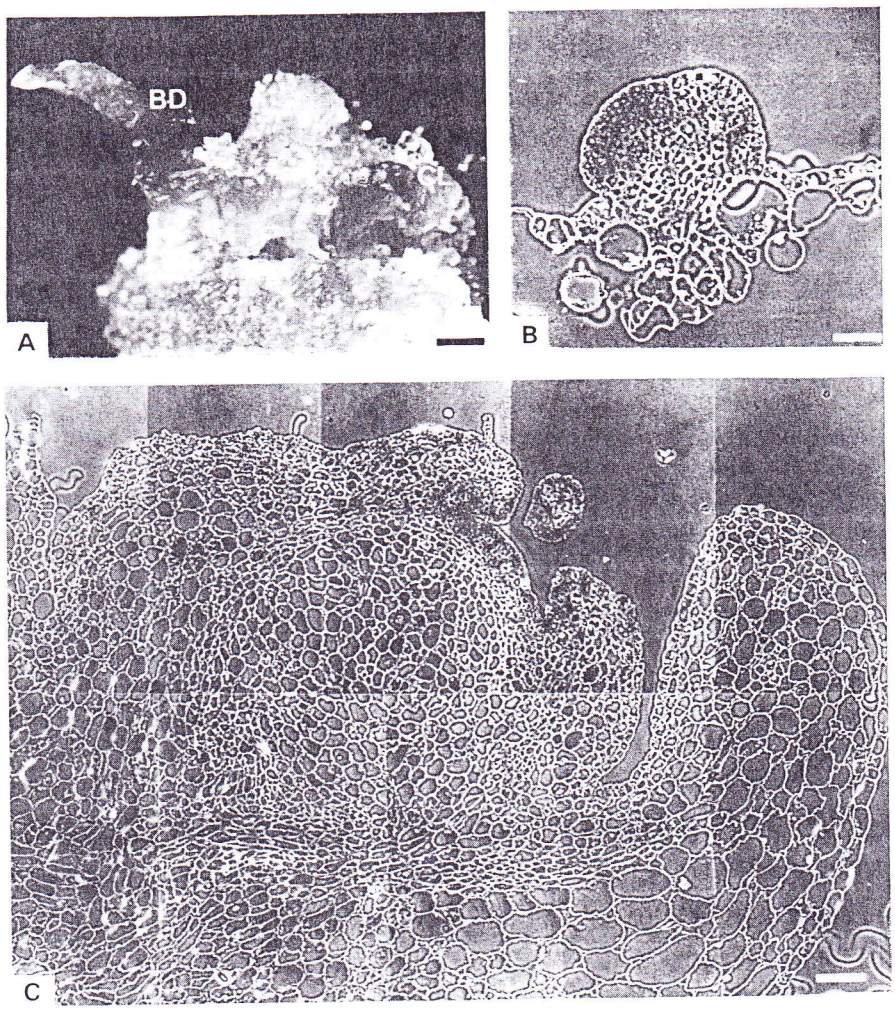

Plate 4 : Legend, see p. 467.