Histological and SEM Studies on Organogenesis in Endosperm-Derived Callus of Kiwifruit (*Actinidia deliciosa* cv. Hayward)

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Regeneration in endosperm-derived callus of *Actinidia deliciosa* cv. Hayward was documented by SEM combined with histology. Two kinds of callus, morphogenic and nonmorphogenic, were observed. Morphogenic callus consisted of compact cell clusters with epidermis-like tissue covered with a mucilaginous or continuous membranous layer, which partially disappeared, turned into fibrils, or became damaged. Regenerating shoots consisted of the apex and primordial leaves. Abnormal structures were also formed, frequently arrested in development. PAS reactions indicated that the mucilaginous layer and network present in intercellular spaces contains polysaccharides. Nonmorphogenic callus consisted of weakly attached cells without a covering membranous layer.

Key words: *Actinidia*, endosperm culture, regeneration, SEM, histological analysis.

INTRODUCTION

Endosperm in angiosperms is a unique tissue in its origin, development and ploidy level. It is a product of double fertilization, but unlike the embryo it is usually triploid and it develops into a tissue finally consumed by the embryo. Because of its unique ploidy level, endosperm is an interesting model for in vitro experiments, including regeneration. Traditionally, triploid plants are produced by crossing tetraploid and diploid parent plants. Regeneration of plants from endosperm offers a direct, single-step approach to triploid production (for review see: Bhojwani and Razdan, 1996).

Endosperm cell totipotency was first demonstrated in endosperm culture of *Exocarpus cupressiformis* by Johri and Bhojwani (1965). Since then, induction of organogenesis in endosperm culture has been achieved in many taxa (Seghal and Khurana, 1985; Thomas et al., 2000; Chaturvedi et al., 2003; Góralski et al., 2005).

Kiwifruit (*Actinidia deliciosa*) is a dioecious woody species originating from China. Nowadays it is an important crop plant around the world. Endosperm culture followed by organogenesis has been achieved in several *Actinidia* taxa (Kin et al., 1990; Gui et al., 1993; Góralski et al., 2005). Regeneration processes in culture in vitro are often observed by SEM technique for more detailed analysis of morphogenic events (e.g., Rodríguez et al., 1995; Ovečka and Bobák, 1999; Chandra and Bhanja, 2002; Konieczny et al., 2005; Namasivayam et al., 2006).

In several plants cultured in vitro, SEM analysis revealed that induction of morphogenesis is linked to the appearance of a fibrillar network referred to as the extracellular matrix surface network (ECMSN) (Šamaj et al., 1995). According to Bobák et al. (2003/4), the chemical composition and structural arrangement of the ECMSN on the cell surface indicate that it may play a fundamental role in cell-to-cell recognition and interaction, cell division and differentiation, and also in generation and maintenance of some traits in plant cell populations. Šamaj et al. (1999a, 2006) reported that the ECMSN plays an important morphoregulatory role during somatic embryogenesis and organogenesis, implying an active role in plant morphogenesis.

The present paper reports a continuation of our previous studies on callus induction and regeneration in mature endosperm culture of *Actinidia deliciosa* (Góralski et al., 2005). Long-term callus culture was used to study maintenance of the capacity for proliferation and regeneration. The experiments also undertook to improve regeneration efficiency. In the current study we focused mainly on regeneration process

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morphology, documented by scanning electron microscopy and histology. Special attention was paid to the presence of ECMSN in both long- and short-term endosperm-derived callus culture. Previously, ECMSN was noted in callus derived from somatic (2n) tissues (Šamaj et al., 1995, 1999a,b; Namasivayam et al., 2006) and in androgenic (1n) callus (Konieczny et al., 2005) but not in 3n tissue culture.

MATERIALS AND METHODS

PLANT MATERIAL AND CULTURE CONDITIONS

Long-term culture of endosperm-derived callus of *Actinidia deliciosa* cv. Hayward was initiated and maintained as described previously (Góralski et al., 2005). Briefly, commercial fruits of *Actinidia deliciosa* cv. Hayward were kept at room temperature to allow softening. Pieces of fruit (3 × 3 cm) were surface-sterilized for 12 min in Ace commercial bleach diluted 1:1 with distilled water and rinsed three times in sterile distilled water. Endosperm was isolated from seeds and cultured on MS medium (Murashige and Skoog, 1962) supplied with kinetin (5 mg/l) and 2,4-D (2 mg/l) (*Actinidia* endosperm medium – AEM) to induce callus. Endosperm-derived callus obtained on AEM was maintained for long-term proliferation on MS medium supplied with TDZ (0.5 mg/l) (regeneration medium – RM). To shorten the time needed to obtain callus, proliferation and shoot regeneration, new induction medium (IM) was used. Isolated endosperm (as described above) was treated by auxin shock (MS supplied with 10, 20 or 40 mg/l 2,4-D) for 24 or 48 h and then transferred on RM medium.

Regenerative cultures were incubated at 26±3°C under a 16 h photoperiod provided by cool-white fluorescent tubes (60–90 µmol photons m^{-2}s^{-1}).

SCANNING ELECTRON MICROSCOPY

Material for SEM was prefixed in 5% buffered glutaraldehyde (0.1 M phosphate buffer, pH 7.2) for 2 h at room temperature. After dehydration through a graded ethanol series, samples were dried with a CO_2 critical-point drying system (EMITECH K850 critical-point dryer), sputtered with gold (SPI SUPPLIES ion-sputtering system) and observed with a scanning electron microscope (PHILIPS XL 30).

HISTOLOGICAL ANALYSIS

Small clumps of calli were excised and prepared using a method of embedding tissues in Technovit 7100 (2-hydroxyethyl-methacrylate) (Heraeus Kulzer). Explants were fixed in 10% glutaraldehyde for 24 h, washed four times in phosphate buffer (PBS; pH = 7.2) followed by dehydration in a graded ethanol series (10%, 30%, 50%, 70%, and 96%) for 15 min, and then kept overnight in absolute ethanol. Later, samples were infiltrated in a mixture of absolute ethanol and Technovit (3:1, 1:1, 1:3 v/v; 1 h in each mixture) and stored for 12 h in pure Technovit. The resin was polymerized with the addition of hardener. The material was sectioned 5 µm thick with a rotary microtome (Microm, Adamas Instrumenten), stained with toluidine blue and/or PAS reaction and mounted in Entellan (Merck).

Microscopic sections were photographed with a Zeiss Axio Cam MRe digital camera with Zeiss Axio Vision 3.0 software.

RESULTS

CALLUS INDUCTION AND ORGANOGENESIS

When auxin shock was applied, callus induction was observed after 2–3 weeks of the culture. At the beginning of the culture the calli were white or cream-colored (Fig. 1). In the following weeks the firm, nodular callus largely proliferated and turned dark green (Fig. 2). Initiation of organogenesis was observed after 6–7 weeks, mostly in culture treated by 10 mg/l 2,4-D for 24 h and 20 mg/l for 48 h. Green bulges of shoot primordia grew separately or in clusters. Morphogenic response appeared only in certain sectors of the compact callus. Shoots successively emerged from the masses (Fig. 3). Shoots with a well-developed apex and primordial leaves were observed, but cylindrical or conical structures also appeared. Rhizogenesis occurred sporadically (Fig. 4). Shoot growth was accompanied by development of normal leaves, although abnormal leaves also formed. Some cells of the callus and shoots turned purplish (Fig. 5). Shoots with leaves developed from bunches of primordia (Fig. 6). In some cases the growth of shoots, especially those atypically organized, was inhibited (Fig. 7).

Figs. 1–7. Observations of callus and organogenesis induction. **Figs. 1, 2**. Callus after 3 weeks of culture (IM 20 mg/l, 24 h). **Fig. 1**. Cream-colored callus. **Fig. 2**. Green callus. Bars = 500 µm. **Fig. 3**. Bunch of regenerating shoot buds on callus surface (7 weeks of culture, IM 40 mg/l, 24 h); marked area enlarged (3a). Bars = 1 mm. **Figs. 4, 5**. Organogenesis after 9 weeks of culture. **Fig. 4**. Shoot (S) and root (R) derived from callus (CAL); visible are trichomes on leaf (IM 20 mg/l, 24 h). **Fig. 5**. Callus and shoots with cells containing anthocyans (IM 40 mg/l). Bars = 1 mm. **Fig. 6, 7**. Shoots developing after 11 weeks of culture (IM 20 mg/l, 24 h). **Fig. 6**. Multiple shoot proliferation. Bar = 1 mm. **Fig. 7**. Inhibited growth of shoot buds. Bars = 1 mm.
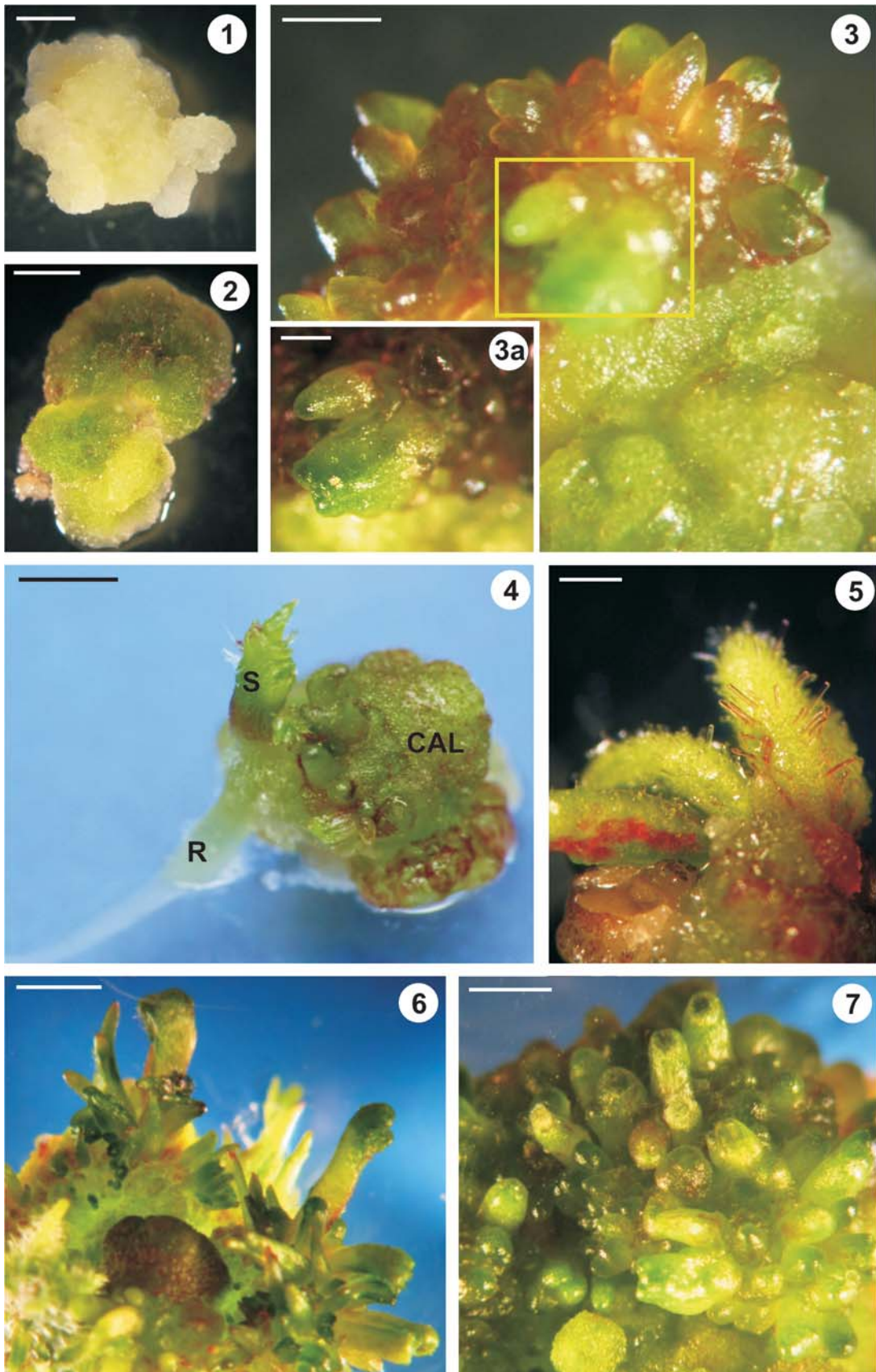
Long-term endosperm-derived callus culture (Góralski et al., 2005) still displayed callus proliferation and shoot regeneration potential after being maintained more than 2 years. The differences in regeneration efficiency (number of shoots per explant) between our previous studies (Góralski et al., 2005) and the new culture were not statistically significant (data not shown).

SEM OBSERVATIONS

SEM study of shoot bud differentiation from long- and short-term callus cultures showed that some cells of callus were aggregated into segments (Figs. 8–11). We observed several groups of structures representing bulges of potential shoot primordia growing over the surface of these segments. Each shoot primordium was covered by epidermis built of more or less equal-size cells. SEM observations revealed typical shoot formation with an apex and leaf primordia (Figs. 8–10), but also abnormal structures (Fig. 11).

Regenerated leaves were covered by epidermis with visible stomatal guard cells and long few-celled trichomes (Fig. 12). The trichomes were not associated with the shoot primordia at the initial stage, but developed in later stages.

There were two kinds of cells on the callus surface: potential morphogenic and nonmorphogenic. The first type was represented by large, rounded cells forming clusters of different sizes (Figs. 13, 14). The surface of these cell walls was covered by a continuous membranous layer, which partially disappeared, turned into fibrils, or became damaged. More or less equal-size, tightly connected cells on the surface of calli segments were also noted. The compact cell arrangement resembled that of epidermal tissue. The cell walls were covered by a discrete, more or less coherent membranous layer with fibrillar structures.

Nonmorphogenic callus was composed of elongated and highly dissociated cells. The surface of those cells was smooth (Fig. 15) and free of fibrillar and membranous structures.

HISTOLOGICAL STUDIES

Histological sections of 5-week-old morphogenic callus showed bulges of developing shoots (Fig. 16) connected with callus tissue by a protodermal layer and vascular strands (Fig. 17). Other differentiated structures were covered by epidermis-like tissue (Fig. 18) composed of small, tightly connected cells. That layer was discontinuous, with gaps covered by amorphous material.

PAS reaction-positive material was identified on the cell wall surface and in intercellular spaces. An amorphous mucilaginous layer covered the callus surface with loosely attached cells (Fig. 19), whereas the intercellular spaces were filled with a network of fibrillar structures connecting callus cells (Figs. 20, 21).

DISCUSSION

The present work depicts the process of organogenesis observed in culture in vitro of endosperm derived from *Actinidia deliciosa* cv. Hayward seeds. In our experiment, plant regeneration occurred only by indirect organogenesis: the emergence of shoots or roots was preceded by the formation of callus from cultured endosperm. Despite the well-known tendency for degeneration and disturbances of callus tissue, the kiwifruit culture maintained its proliferation and organogenic ability more than 2 years. Although the previously used protocol gave good regeneration efficiency, it was time-consuming. Our new protocol with auxin shock treatment shortened the time for organogenesis initiation from several months to 6–7 weeks.

We distinguished two types of callus present in cultures: morphogenic and nonmorphogenic. They differed not only in their capacity for organ formation but also in their structure, appearance and morphology.

Callus that displayed the ability to form shoots or occasionally roots was generally compact and smooth. Nonmorphogenic callus, on the other hand, was usually friable and soft. SEM observations of callus structures revealed that morphogenic callus formed compact clusters with organogenic potential. The surface of the callus was covered by a smooth cellular layer resembling epidermis. Nonmorphogenic callus formed groups of highly dissociated and elongated cells. Similar types of callus tissue and similar results on morphogenic capacity were found

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Figs. 8–15. SEM observations of callus surface after long-term culture and organogenesis. **Fig. 8**. Leaf primordia (LP) and clusters of incipient shoot primordia (arrowheads) growing over callus surface; visible remnants of membranous layer (arrow). Bar = 500 μm. **Fig. 9**. Leaf primordia and partially damaged membranous layer (arrows) on callus surface. Bar = 200 μm. **Fig. 10**. Shoot formation with apex (A) and two leaf primordia (LP); visible remnants of membranous layer (arrow). Bar = 200 μm. **Fig. 11**. Differentiation of leaf primordia (LP) on morphogenic callus segment covered with remnants of membranous layer (arrows). Bar = 500 μm. **Fig. 12**. Guard cells (arrows) and few-celled trichomes (arrowheads) visible in epidermis covering regenerated leaf. Bar = 200 μm. **Fig. 13**. Cell surface of morphogenic callus; note fibrillar and membranous structure. Bar = 20 μm. **Fig. 14**. Nodular morphogenic callus composed of round cells covered with membranous layer (partially damaged). Bar = 200 μm. **Fig. 15**. Elongated and disorganized cells in nonmorphogenic callus; note smooth cell surface. Bar = 200 μm.
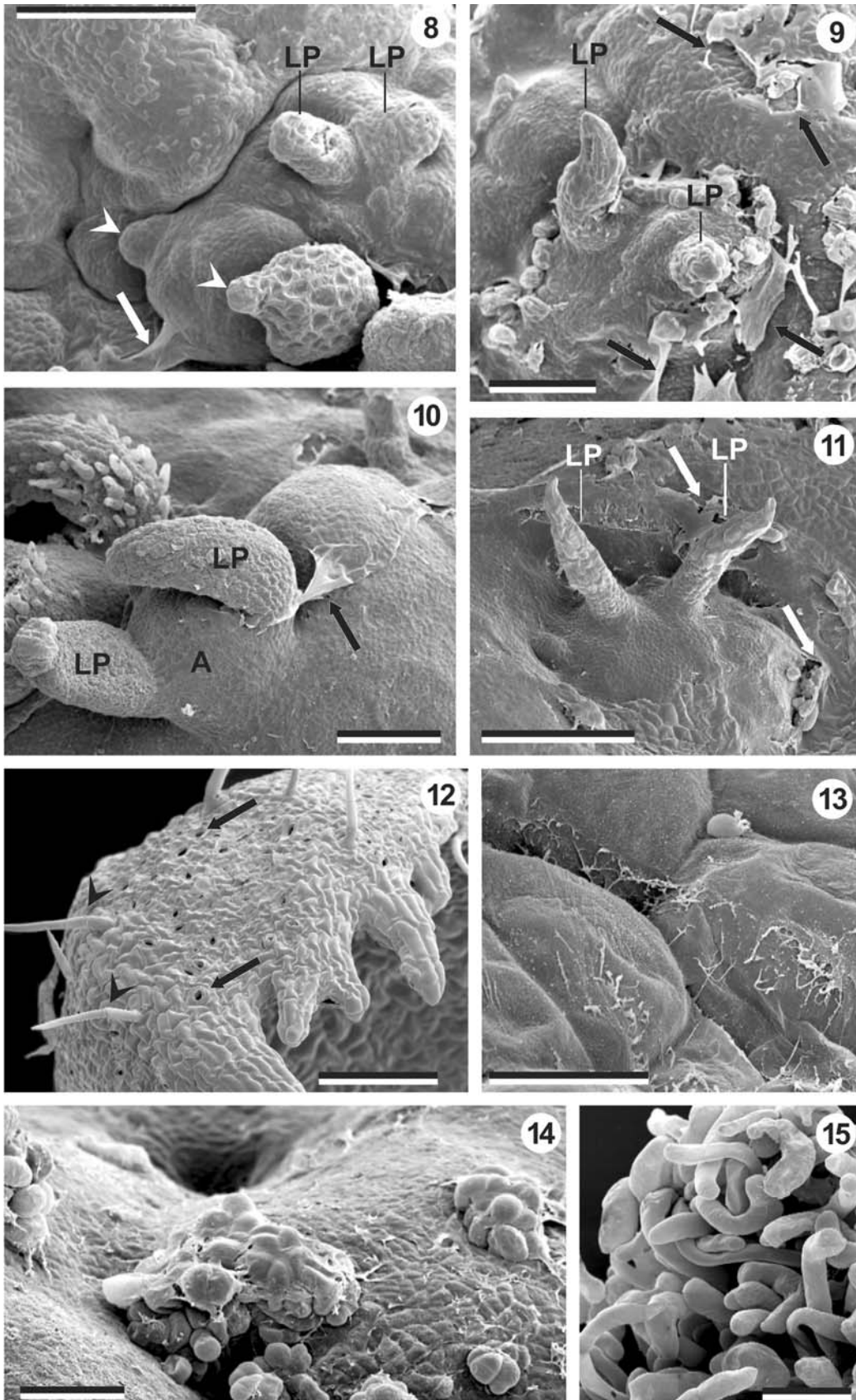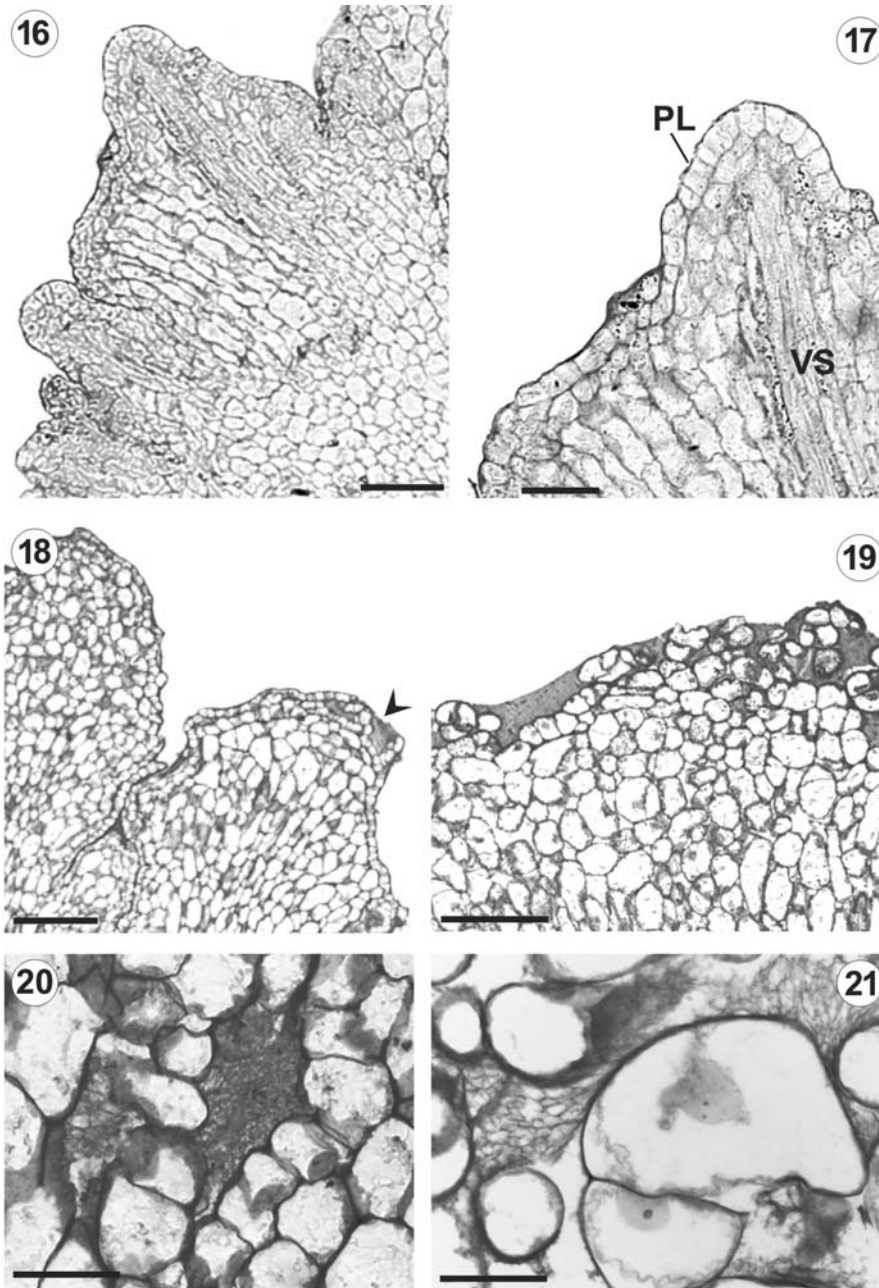
Figs. 16–21. Sections through morphogenic callus tissues and differentiation regions (IM 20 mg/l, 24 h) after 5 weeks of culture. **Fig. 16**. Bulges of regenerated shoot buds. Bar = 200 μm. **Fig. 17**. Vascular strand (VS) and protodermal layer (PL) in regenerated shoot bud. Bar = 100 μm. **Fig. 18**. Differentiation region; segments composed of compact tissue covered with epidermis-like layer; small area covered with amorphous material is included (arrowhead). Bar = 100 μm. **Fig. 19**. Distinct layer of amorphous material on callus surface. Bar = 100 μm. **Figs. 20, 21**. Fibrillar and reticular filling of intercellular spaces within callus tissue. Bars = 200 μm.

in callus obtained from sugarcane spindles of the apical verticil (Rodríguez et al.,1995) and in plantlet-derived callus of *Citrus* hybrid (Chapman et al., 2000). This suggests that the features of morphogenic and nonmorphogenic calli are independent of the species and the origin of the callus. We often observed red zones of callus and regenerated structures, a phenomenon previously described in *A. deliciosa* callus by Oliveira and Pais (1992), who reported similar purplish spots of cells accumulating anthocyanins.

In our culture the organogenic sectors and shoot primordia were covered by a layer with the appearance of epidermis. These observations are similar to results obtained in *Passiflora* species (Guzzo et al., 2004), where green clusters of compact embryo-derived callus gradually developed an epidermis-like tissue.

At early stages of kiwifruit shoot formation, structures differing in appearance were formed. Jeannin et al. (1998) described a variety of structures differentiated in culture of isolated zygotic embryos of sunflower. Sunflower tissue chimeras obtained in the experiment had a wide array of shapes (cylinder, crown, thick blade) and did not develop into functional embryos or shoots, but these types of structures occurred only on explants having somatic embryos and shoots present simultaneously.

The presence of shoots at different developmental stages at the same time indicates asynchrony of that regeneration process, for example incipient bulges and clearly recognized primordia with an apex occurring at the same time. This phenomenon is often reported. Asynchrony is especially visible in somatic embryogenesis (Rodríguez et al., 1995; Namasivayam et al., 2006).

SEM observations under higher magnification revealed that smooth-surfaced nodular callus segments of *A. deliciosa* were covered by a membranous layer. The layer completely covered some parts of the callus but in other regions was torn or partly damaged and had the appearance of a network. These observations correspond with those in a SEM study of *Papaver* (Ovečka and Bobák, 1999) and rice (Basu et al., 1997), describing the presence of dense amorphous mucilage on the surface of meristemoids. Those authors reported that the layer was built of protein or mucilaginous secretions that dried on the callus surface. They suggested that rupturing at various sectors of the membrane was caused by cell enlargement and multiplication on the surface of the callus clusters. On the other hand, during preparation of material for SEM observations, critical-point drying may cause shrinkage and hole formation in the extracellular surface layer covering morphogenic cells (Šamaj et al., 1999b).

The presence of an extracellular layer covering morphogenic regions of callus culture raises important questions about its nature, components, and possible role in organogenesis. Among the potential components of such a matrix are pectins, polysaccharides present in higher plant cell walls and secretions. They play an important role in cell-to-cell adhesion, and in control of primary cell wall ionic status and wall porosity. Moreover, pectin oligosaccharide fragments can be released from the cell wall and function as signalling molecules directly involved in regulating developmental processes (Baluška et al., 2003).

Our histological studies showed that in the intercellular spaces of callus tissue there was a more or less dense fibrillar or reticular network. Ovečka and Bobák (1999) reported a dense reticular network filling large intercellular "caves" formed during somatic embryogenesis. Plant mucilages are complexes of polysaccharide polymers having in particular cases a microfibrillar network texture (Mariani et al., 1988; Sawidis, 1991). The PAS reaction indicated that materials observed on the callus surface and in the intercellular spaces were rich in polysaccharides. It might be suggested that the fibrillar and reticular network mediates intercellular contacts among cells (Šamaj et al., 1999a).

The surface coat of mucilage could protect the surface of callus segments before epidermis-like tissue develops. Since pectins play a function in cell-to-cell adhesion, the possible involvement of the surface layer in integration and recognition of morphogenic cells within the multicellular nodular structure may be hypothesized. In several plants cultured in vitro, induction of morphogenesis is linked to the appearance of fibrillar material covering the surface cells of induced explants. The surface network, referred to as the extracellular matrix surface network (ECMSN), has been reported during somatic embryogenesis in several plants including *Coffea arabica* (Sondahl et al., 1979), *Cocos nucifera* (Verdeil et al., 2001) and *Drosera spathulata* (Bobák et al., 2003/4). The surface network has also been noted during androgenic plant development of wheat (Konieczny et al., 2005), direct organogenesis of flax (Šamaj et al., 1997, cit. after: Šamaj et al., 1999a) and also in maize roots, where it was described as an outer pellicle of young meristematic epidermal cells (Abeysekera and McCully, 1993). Bobák et al. (2003/4) reported that the formation of ECMSN can be a stress response of explants imposed by specific in vitro conditions.

The presence of a well-developed ECMSN in endosperm-derived callus of kiwifruit indicates that it could be a useful system for studying the extracellular matrix. Experiments addressing the chemical composition and possible function of this layer in plant morphogenesis are in progress.

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