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ORGANOGENESIS AND SOMATIC EMBRYOGENESIS INDUCED IN PETAL CULTURES OF *SEDUM* SPECIES

MARIA KATARZYNA WOJCIECHOWICZ*

Biology Department, Adam Mickiewicz University, ul. Umultowska 89, 61–614 Poznań, Poland

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Plant regeneration was studied in petal cultures of three *Sedum* species: *S. aizoon*, *S. spectabile* and *S. gracile*. The course of morphogenesis was examined by light and electron microscopy. Histological examination revealed that morphogenesis took place as direct organogenesis, indirect organogenesis or somatic embryogenesis, depending on the species and the concentrations of growth regulators in the medium. Initial petals and explants from cultures were studied to determine the origin of organogenesis. Petal histology showed that all cells at the time of culture initiation were differentiated. Epidermal and parenchymatous cells were highly vacuolated and the parenchyma contained chloroplasts with starch grains. TEM revealed that cell dedifferentiation occurred in culture under the influence of BAP and IBA. In petal culture the first cell division started subepidermally on day 2 of culture initiation. Epidermal cells underwent regular anticlinal divisions on day 3 of culture initiation, as confirmed by histology and SEM. Direct formation of adventitious buds in petals was observed in meristematic cells dedifferentiated from the epidermis and parenchyma. In indirect organogenesis, callus tissue resulted from division of dedifferentiated parenchyma cells. Somatic embryos were formed directly from subepidermal parenchymatous cells.

Key words: *Sedum*, adventitious buds, somatic embryos, callus, petal culture, light and electron microscopy.

INTRODUCTION

Regeneration of ornamental plants in vitro has been achieved in many species on culture medium containing auxins and cytokinins, and from various explant sources including tuber segments, shoot tips, stems (node and internode), leaf tissue, peduncles and floral parts (Teixeira da Silva, 2004; Rout et al., 2006). Plant regeneration from petals has been reported in several ornamental species: *Pelargonium* (Bennici,1974), *Chrysanthemum morifolium* (Bush et al., 1976; Mandal and Datta, 2005), *C. coccineum* (Fujii and Shimizu, 1990), *Hemerocallis* (Heuser and Apps, 1976), *Saintpaulia ionantha* (Vazques and Short, 1978), *Dianthus caryophyllus* (Kakehi 1979; Fisher et al., 1993; Miller et al., 1991; Nugent et al., 1991; Simard et al., 1992), *Rosa hybrida* (Noriega and Sondahl, 1991; Murali et al., 1996), *Cyclamen persicum* (Karam and Al-Matahoub, 2000), *Araujia sericifera* (Torne et al., 1997) and *Rhododendron simisii* (Schepper et al., 2004). It has also been induced in five *Sedum* species (Wojciechowicz, 2007). These studies show the high regeneration potential of petals, and focus on enhancement of the plants' ornamental value and genetic variation. In carnation, petal-derived regenerants were characterized by high variation of features including plant height and flower color (Biautti et al., 1986). In culture of *Rhododendron simisii*, tetraploid plants were regenerated from the margin of differently colored petals (Schepper et al., 2004).

Each year millions of ornamental plants are produced by in vitro culture (Rout et al., 2006). Only eight *Sedum* species have been propagated this way: *S. sieboldii* (Uhring, 1983), *S. telephium*, (Brandao and Salema, 1977), *S. erythrostichum* (Yoon et al., 2002), *S. acre, S. aizoon S. floriferum, S. gracile* and *S. spectabile* (Wojciechowicz, 2007).

Many *Sedum* species are ornamental plants which tolerate drought and poor, stony soils (Stephenson, 1994; Yoon et al., 2002; Andry et al., 2005), so they are often planted in gardens, especially rock gardens. Many highly decorative *Sedum* species are also cultivated as indoor plants (Gudrupa et al., 2002).

*e-mail: kaswoj@amu.edu.pl

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A new market for the horticulture field in Europe and the United States is presented by green roofs (van Woert et al., 2005; Getter and Rowe, 2006). Establishing plant material on rooftops provides many ecological and economic benefits, including stormwater management, energy conservation, mitigation of the urban heat island effect, and increased longevity of roofing materials, as well as providing a more aesthetically pleasing environment in which to work and live (Monterusso et al., 2005). Because environmental conditions are often more extreme on rooftops, many xerophytic plants, especially *Sedum*, are ideal for extensive green roofs because they are physiologically and morphologically adapted to withstand drought (Durhman and Rowe, 2006).

Elaborating a regeneration protocol and analyzing the course of morphogenesis are important steps toward using in vitro culture to obtain plants with novel, desirable traits, whether by exploiting somaclonal variation or by applying molecular techniques.

The ability to regenerate plants and the high regeneration potential of flower part explants in *Sedum* has been shown elsewhere (Wojciechowicz, 2007). Here I use light and electron microscopy to study the course of morphogenesis in cultures of petal explants from three *Sedum* species: *S. aizoon, S. gracile* and *S. spectabile*.

MATERIAL AND METHODS

EXPLANT AND IN VITRO CULTURE CONDITIONS

Plants of *S. aizoon, S. gracile* and *S. spectabile* were grown at the Botanical Garden of Adam Mickiewicz University in Poznań, Poland. Flower buds were harvested just before anthesis. The buds were surface-sterilized in 70% (v/v) ethanol for 30 s, washed with sterile deionized water, and immersed in sodium hypochlorite solution (0.15% available chlorine) for 8 min. The petals were then excised and placed horizontally with the abaxial side in contact with the culture medium.

All experiments were carried out on MS medium (Murashige and Skoog, 1962). The medium was supplemented with 30 g l^{-1} sucrose, solidified with 8.0 g l^{-1} agar, and pH was adjusted to 5.8. The plant growth regulators added were BAP (3.0 mg l^{-1}) and/or IBA (0.1, 0.5, 1.0 or 1.5 mg l^{-1}).

Cultures were grown with a 16 h photoperiod under cool white fluorescent lamps (35 μmol m^{-2} s^{-1}) at 24°C and 70–80% relative humidity.

MICROSCOPIC OBSERVATIONS

Explants were collected from the in vitro cultures at various time intervals. Initial petal analysis was done in parallel. Forty samples of each investigated species were examined.

The material was fixed for 2 h at room temperature in a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 6.8). Samples were then rinsed three times in the same buffer. Samples for SEM and TEM were postfixed for 1 h at 4°C in a solution containing 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 6.8).

Light microscopy

For histological analysis, both paraffin and thin sections were prepared.

For paraffin sections the fixed material was dehydrated in an ethanol series (5, 10, 30, 50, 70, 80, 90, 96, 100%). The samples were embedded in paraffin and cut into sections 10 μm thick. The sections were fixed on glass slides, double stained with safranin and fast green (Jensen, 1962), and mounted in entellan.

For thin sections the fixed petals were embedded in a mixture of low-viscosity epoxy resins (Spurr, 1969). The samples were cut into sections 1.5 μm thick, fixed on glass slides and stained with methylene blue and basic fuchsin (Humprey and Pittman, 1974).

The preparations were examined under a Zeiss Axioscop microscope. Specimens were photographed using an OPTON Axioscop microscope fitted with a Zeiss MC 80 DX camera, on Fujifilm 100.

Scanning electron microscopy (SEM)

After postfixing, the dehydrated petals were dried in liquid carbon dioxide. The samples were coated with gold and examined in a Phillips SEM-515 scanning electron microscope at 7.5 kV accelerating voltage. Images were stored digitally.

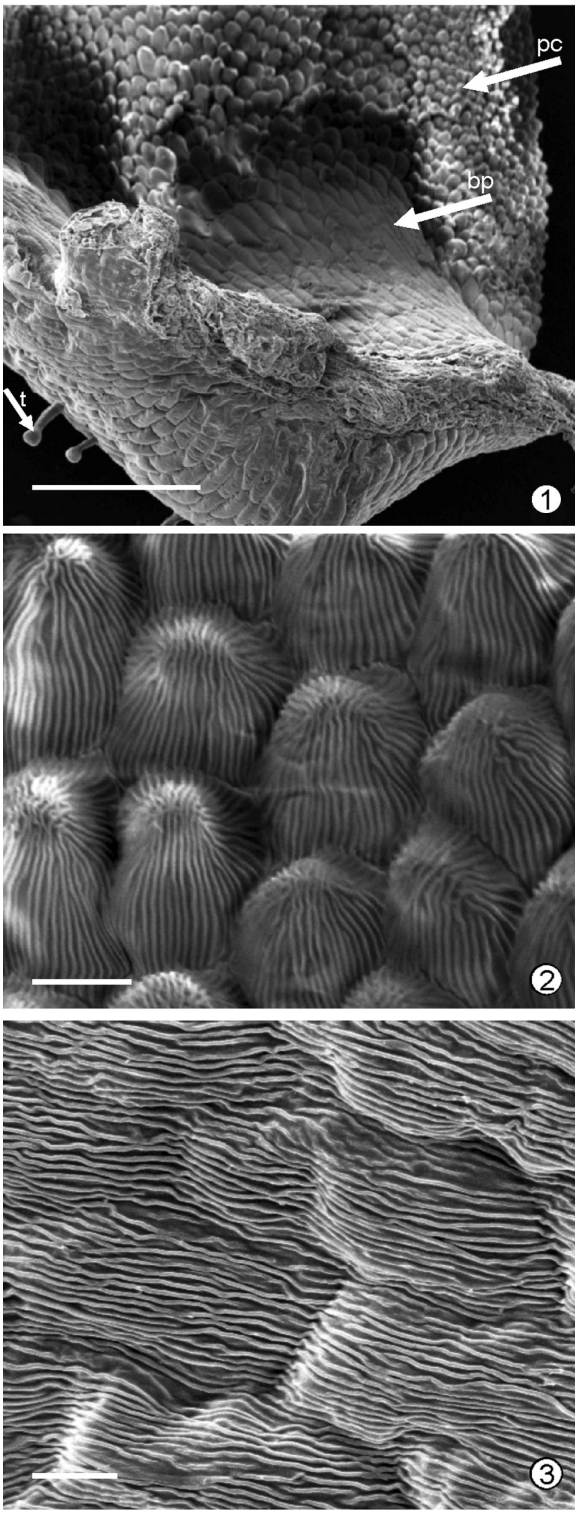
Transmission electron microscopy (TEM)

After postfixing, the petals were contrasted with 2% uranyl acetate (pH 5.0), embedded in a mixture of low-viscosity epoxy resins (Spurr, 1969) and cut into sections 90 nm thick. The sections were mounted on copper grids, counterstained in lead citrate, and examined under an EOL JEM 1200 EXII transmission electron microscope at 80 kV accelerating voltage.

RESULTS

INITIAL EXPLANTS

SEM of epidermis showed that the cells from the base of the petals were elongated along the petal axis. The external surface of the cells was smooth (Fig. 1). Cells from above the base of the petals had a folded cell surface. In *S. gracile* the cells were papil-



Figs. 1–3. SEM micrographs of initial petals of *Sedum* species. **Fig. 1.** Basal part (bp) and papilliform cells (pc) on the adaxial surface of *S. gracile*. Glandular trichomes (t) on the abaxial surface. Bar = 300 μm. **Fig. 2**. Papilliform adaxial epidermal cells of *S. gracile* with radial folds in the cell. Bar = 10 μm. **Fig. 3**. Adaxial epidermal cells of *S. aizoon* with parallel folds in the cell wall. Bar = 10 μm.

TABLE. 1. Effect of growth regulators BAP 3.0 mgl^{-1} and IBA (0.1–1.5 $mg \cdot l^{-1}$) on morphogenesis induction in petal cultures of *Sedum* spp.

Species	Concentration of IBA (mgl^{-1})	Type of regeneration	Initial tissue
S. aizoon	0.1	D	EP
	0.5	SE	P
	1.0	D	EP
	1.5	ID	P
S. gracile	0.1	D	EP
	0.5	SE	P
	1.0	D	EP
	1.5	ID	P
S. spectabile	0.1	D	EP
	0.5	SE	P
	1.0	D	EP
	1.5	ID	P

D – direct organogenesis; ID – indirect organogenesis; SE – somatic embryogenesis; EP – epidermis and parenchyma tissue; P – parenchyma tissue.

lose, and folds on the cell surface radiated from their center in a star-like pattern (Fig. 2). In *S. aizoon* and *S. spectabile* the cells were elongated, and the folds lay parallel to the long axis of the cell (Fig. 3).

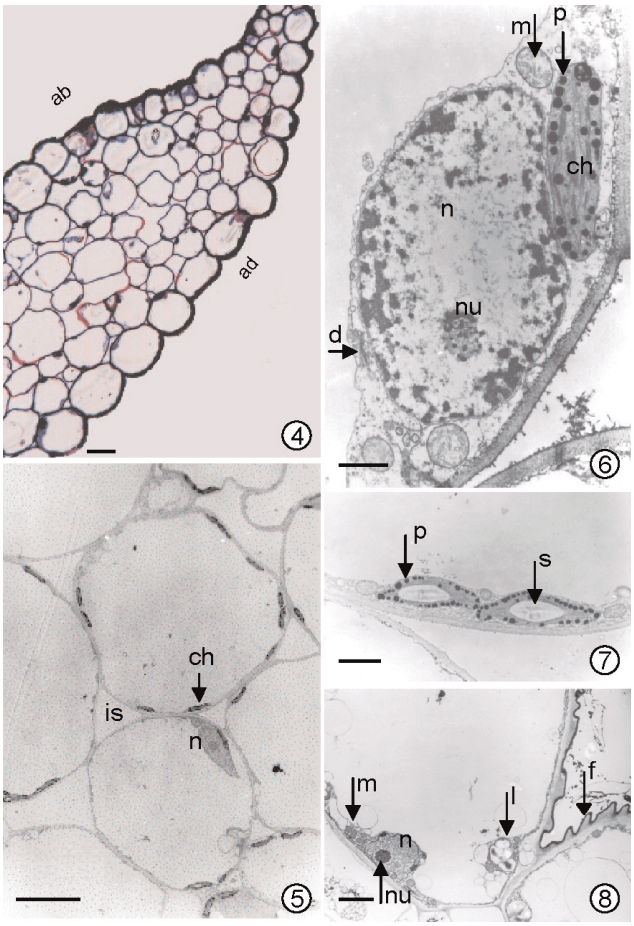
In petal cross section there was a single-layered epidermis surrounding the parenchymatous cells (Fig. 4). The vascular bundles in the parenchyma were visible. The parenchymatous cells were loosely arranged, highly vacuolated and thin-walled (Figs. 4, 5). Sparse chloroplasts, mitochondria, endoplasmic reticulum and dictyosomes of the Golgi apparatus and electron-dense granules were concentrated in the thin band of cytoplasm near the cell wall (Figs. 5–7). The oval nucleus contained one or two nucleoli (Fig. 6). The stroma of the chloroplasts included starch grains and numerous plastoglobuli (Fig. 7). The thylakoid system was poorly developed in both the stroma and grana (Figs. 6, 7). Nuclei, mitochondria and leucoplasts were visible in the vacuolated cells of the epidermis (Fig. 8).

ORGANOGENESIS IN CULTURE

In the species included in this study, IBA at a concentration of 1.5 mg l^{-1} in combination with BAP induced indirect organogenesis. Lower concentrations of IBA induced direct organogenesis (Tab. 1).

Direct organogenesis in culture

Cell division in the epidermal cells at the base of the *S. aizoon* petal was detected with SEM as early as day 3 of culture on medium containing BAP and 1.0 mg l^{-1} IBA. In *S. spectabile* and *S. gracile*, epidermal cell



Figs. 4–8. Histology and cytology of initial petals of *Sedum* species. **Fig. 4.** Cross section through petal of *S. gracile* – abaxial (ab) and adaxial (ad) epidermis and parenchymatous cells. Bar = 10 μm. **Fig. 5.** Parenchyma of *S. gracile* with intracellular spaces (is), nucleolus (n) and chloroplasts (ch). Bar = 2 μm. **Fig. 6.** Parenchymatous cell of *S. aizoon* with nucleus (n) and nucleolus (nu), chloroplast (ch) with plastoglobuli (p), mitochondria (m) and dictyosome (d). Bar = 0.2 μm. **Fig. 7.** Chloroplasts with starch grains (s) and plastoglobuli (p) in parenchymatous cell of *S. aizoon*. Bar = 0.5 μm. **Fig. 8.** Adaxial epidermal cell of *S. gracile* with nucleus (n), nucleolus (nu), leucoplast (l) and mitochondria (m) in cytoplasm. Folds (f) in cell wall. Bar = 0.05 μm.

division was detected on day 4 on the same medium (Fig. 9). Cross sections of the epidermis in *S. gracile* revealed cells that had undergone anticlinal division on day 6 on medium containing 0.5 mg l^{-1} IBA. The epidermal cells were still papillose (Fig. 10).

Histological sections showed differentiation and cell division starting earlier in parenchymatous cells than in epidermis. In both *S. aizoon* and *S. spectabile*, cell division was detected in the subepidermal layer of the parenchyma on day 2 of culture. In *S. gracile*, parenchymatous cells underwent divisions on day 3.

The effect of growth regulators on the dedifferentiation of epidermal and parenchymatous cells into meristematic cells was confirmed by TEM. The meristematic cells were small and contained minute vacuoles distributed throughout the dense cytoplasm. The nucleus was centrally located and contained a large, distinct nucleolus (Fig. 11). The meristematic cells contained abundant mitochondria and a well-developed endoplasmic reticulum and Golgi apparatus, which indicates that they were metabolically active (Fig. 11).

The first cell divisions were anticlinal in epidermal cells, and periclinal in parenchymatous cells (Fig. 12). Regular anticlinal divisions of the epidermal cells gave rise to the epidermis of the newly formed adventitious buds. Periclinal divisions in the parenchymatous cells gave rise to ground tissue of the differentiating buds. Within several days, meristematic strands began to form along the petal axis. These strands consisted of small cells regularly arranged in rows (Fig. 12).

In *S. aizoon* and *S. spectabile*, strands of meristematic cells in the petals were visible on day 3 of culture on media containing either 0.1 or 1.0 mg l^{-1} IBA (Fig. 12). On media containing 1.0 mg l^{-1} IBA, patches of intensively colored cells could be seen on the surface of the meristematic strands. These cells gave rise to primordia of adventitious buds (Fig. 13).

SEM showed that the developing leaf primordia of adventitious buds tended to retain the whorled, alternate leaf arrangement characteristic for the genus *Sedum* (Fig. 14).

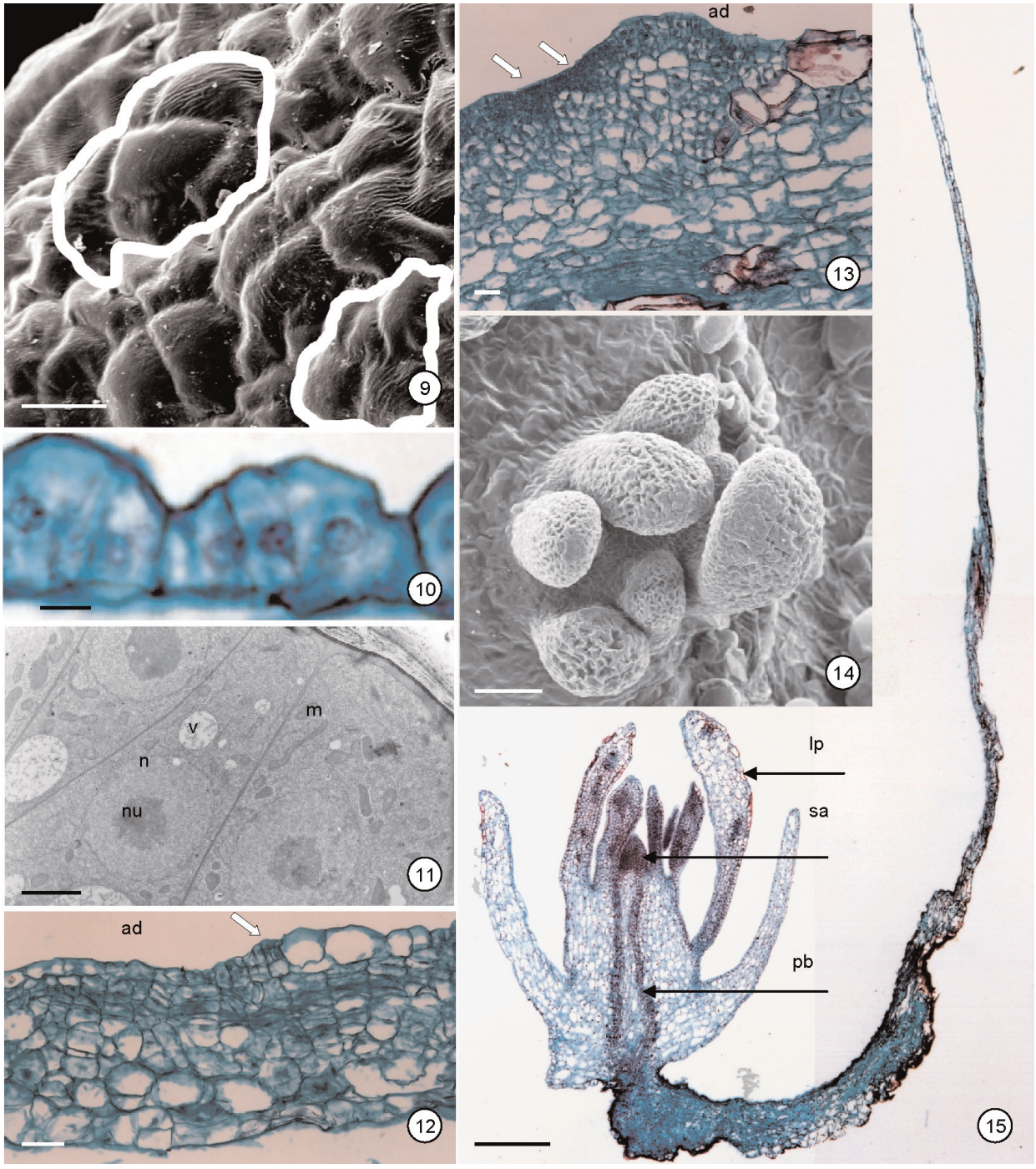
In the three *Sedum* species, adventitious buds with a well-defined shoot apex, leaf primordia and two procambial strands appeared, as visible on histological sections, between days 9 and 17 of culture. The exact time of their appearance depended on the species and on the composition of the medium (Fig. 15).

Indirect organogenesis in culture

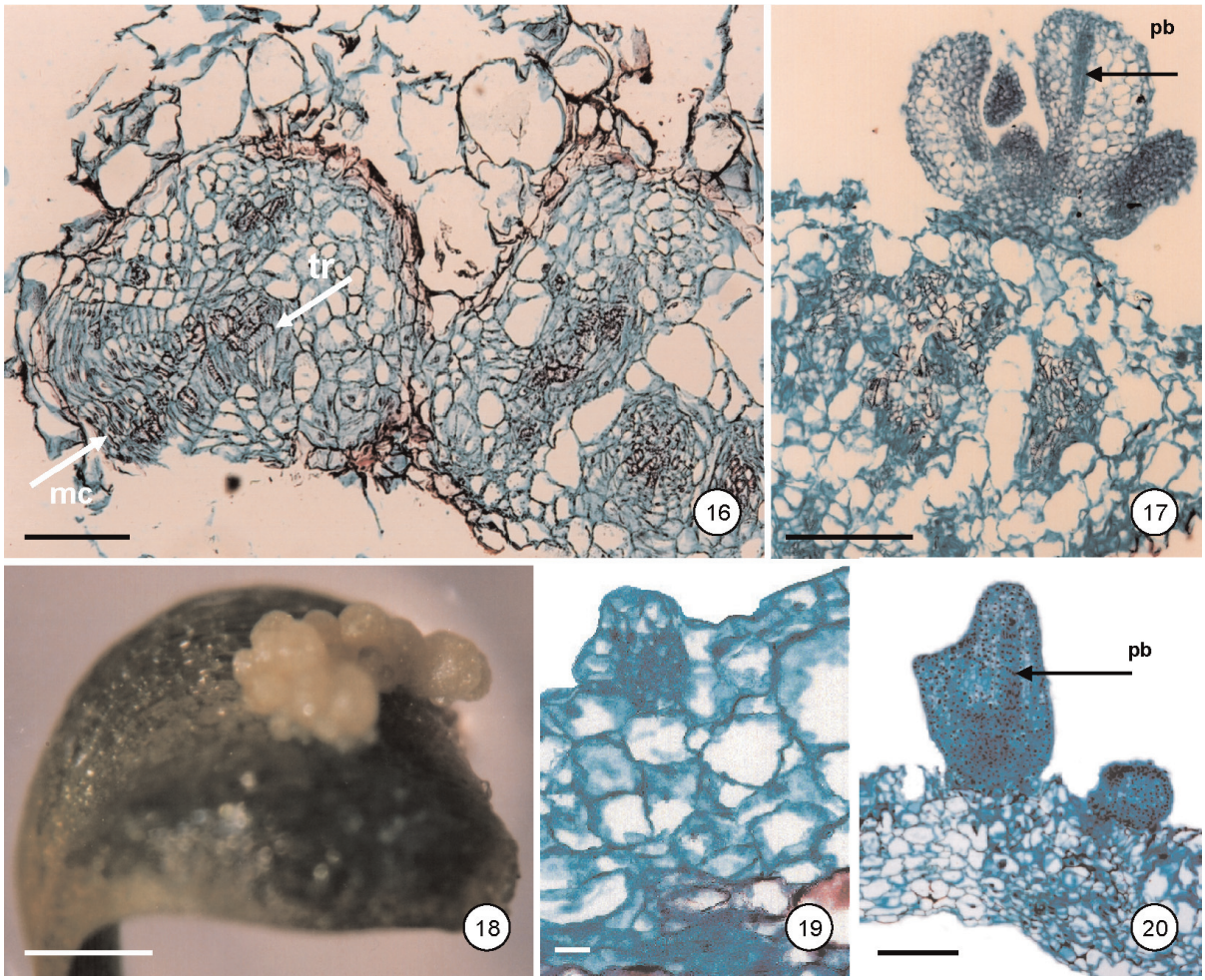
Callus development was preceded by divisions of the parenchymatous cells, which began on day 3 of culture in *S. spectabile* and on day 5 in *S. aizoon* and *S. gracile* (Tab. 1). In *S. gracile*, nests of callus tissue were distributed among the parenchymatous cells on day 8 (Fig. 16).

In all three species, vascularization occurred in the callus tissue. Callus also contained numerous small cells arranged in regular rows, and highly vacuolated cells with a peripherally located nucleus (Fig. 16).

At the periphery of the callus tissue there were groups of intensively stained cells which gave rise to adventitious buds. In *S. gracile*, these cells were seen as early as day 8 of culture. Tracheal cells present in the callus were probably the source of the vas-



Figs. 9–14. Direct organogenesis in petal explants of *Sedum* species. **Fig. 9.** Divisions of epidermal cells on adaxial surface of *S. spectabile* explant on day 4 of culture. White line indicates sectors of cells that have arisen by cell division. Bar = 30 μm. **Fig. 10.** Clones of epidermal cells formed after anticlinal divisions on adaxial side of *S. gracile* on day 6 of culture. Bar = 10 μm. **Fig. 11.** Meristematic cells with dense cytoplasm and small vacuoles (v) after dedifferentiation induced by growth regulators in *S. gracile* on day 7 of culture. n – nucleus; nu – nucleolus; m – mitochondrion. Bar = 0.5 μm. **Fig. 12.** Intensive subepidermal periclinal cell divisions and epidermal anticlinal cell divisions (→) in *S. spectabile* on day 3 of culture. Bar = 20 μm. **Fig. 13.** Intensive cell division and protruding bundles of two adventitious buds (→) on adaxial surface of *S. spectabile* on day 6 of culture. Bar = 20 μm. **Fig. 14.** Leaf development of two adventitious buds of *S. spectabile* on day 14 of culture. Bar = 300 μm. **Fig. 15.** Adventitious bud with shoot apex (sa), leaf primordia (lp), and two procambial bundles (pb) developed at basal part of *S. spectabile* on day 16 of culture. Bar = 200 μm.



Figs. 16–20. Indirect organogenesis and direct somatic embryogenesis in petal explants of *Sedum* species. **Fig. 16.** Transverse section through the basal part of *S. gracile* explant on day 8 of culture. Callus centers with tracheids (tr) and meristematic cells (mc). Bar = 100 μm. **Fig. 17**. Adventitious bud formed in callus at basal part of *S. spectabile* explant on day 16 of culture. pb – procambial bundle. Bar = 100 μm. **Fig. 18**. Petal of *S. aizoon* with somatic embryos developing near vascular bundles on day 9 of culture. Bar = 1 mm. **Fig. 19**. Proembryo developing in adaxial subepidermal parenchyma in longitudinal section of *S. spectabile* explant on day 5 of culture Bar = 25 μm. **Fig. 20.** Embryos at different stages of development in longitudinal section of *S. spectabile* explants on day 9 of culture. pb – procambial bundle. Bar = 100 μm.

cular tissue for developing adventitious buds. In *S. aizoon* the development of adventitious buds began on day 8 (Fig. 17). In *S. spectabile*, organogenesis proceeded with intense proliferation of callus tissue. In the studied *Sedum* species, SEM showed adventitious buds in the callus between days 12 and 20.

SOMATIC EMBRYOGENESIS IN CULTURE

In *S. aizoon* and *S. spectabile*, both adventitious buds and somatic embryos were formed when the culture medium contained 3.0 mg l^{-1} BAP and 0.5 mg l^{-1} IBA. The embryos formed at the explant base, near the vascular bundle on the adaxial side of the petal (Fig. 18). Mature embryos with well developed cotyledons protruded between days 13 and 16 culture on the adaxial surface of explants.

Histological analysis revealed that somatic embryos started to develop by direct embryogenesis at the adaxial subepidermal cells of the parenchyma on day 5 in *S. spectabile* and day 8 in *S. aizoon* (Tab. 1, Fig. 19). Somatic embryos at various stages of development were visible a few days later (Fig. 20).

DISCUSSION

In the *Sedum* species studied, plants were regenerated by direct organogenesis, indirect organogenesis or somatic embryogenesis, depending on the con-

centration of IBA in the culture medium. This is the first report of plant regeneration by somatic embryogenesis in *Sedum*. In earlier studies (Brandao and Salema, 1977; Yoon et al., 2002), *S. erytrostichum* plants were regenerated from leaf segments by direct organogenesis, and *S. telephium* plants were regenerated from leaf tissue cultures by indirect organogenesis.

In this study the anatomical and histological aspects of in vitro regeneration were analyzed by light microscopy, scanning and transmission electron microscopy. The initial petals of the studied species were anatomically similar to those of many other dicotyledonous plants (Esau, 1973). The cells of the petals were differentiated, and the cell walls were folded on the exterior layer of epidermis except at the base of the petal.

The parenchyma of the petals consisted of several layers of loosely arranged cells, which is typical for dicotyledons that do not have a fleshy corona (Esau, 1973). When examined with various types of microscopes, the cells of the initial explants were seen to be differentiated, and no meristematic activity was observed at the base of the petals. In culture in vitro on medium containing BAP and IBA, organogenesis began with the division and differentiation of cells in the epidermis and parenchyma. Petals placed on medium without growth factors did not respond (Wojciechowicz, 2007).

Induction of morphogenic events proceeded rather quickly in the petal cultures. In greenhouse carnations the cells at the base of the petal started to divide on day 6 of culture, and adventitious buds developed by direct organogenesis within two weeks of culture (Miller et al., 1991; Nugent et al., 1991). The nucleus and nucleoli of the epidermal and parenchymatous cells of *Dianthus caryophyllus* enlarged on day 3 of culture, and active cell division in the epidermis and subepidermal parenchyma was in progress on day 6. Leaf primordia and shoot apices formed on day 9, and adventitious buds were visible on day 14 (Simard et al., 1992). In *Chrysanthemum coccineum*, callus was formed one week after the petals were placed on culture medium (Fujii and Shimizu, 1990). In *Sedum spectabile*, callus was already developed on day 3 of culture.

Regeneration proceeded even faster in the *Sedum* species included in this study. In *S. spectabile*, for example, the parenchymatous cells at the base of the petal began to divide on day 2 of culture, and organogenesis began on day 3.

Fast regeneration in the *Sedum* species was confirmed by SEM. Active cell division was observed on day 3 of culture in *S. aizoon*, and on day 4 in *S. spectabile*. In petal cultures of *Dianthus caryophyllus*, meristematic activity started on day 8 of culture (Fisher et al., 1993).

During differentiation of the meristematic cells, the size of the progeny cells decreased because the rate of cell division exceeded the rate of cell growth. The progeny cells did not grow to the size of the parental cells, and this gave rise to sectors of meristematic cells. The presence of these sectors in the differentiating cellular clones indicated that many cells were in the initial phase of direct organogenesis in the *Sedum* petals. Periclinal divisions of cells led to adventitious bud differentiation. In tulip stem fragments, periclinal divisions in the parenchymatous cells also initiated direct organogenesis of shoots (Wilmink et.al., 1995). On the other hand, in cultures of leaflets derived from mature zygotic embryos of *Arachis hypogaea*, periclinal divisions in the parenchymatous cells gave rise to somatic embryos, in contrast to organogenesis which began with anticlinal divisions (Chengalrayan et al., 2001).

SEM of the petals showed the spatial distribution of the leaf primordia in the *Sedum* species: they were arranged cross-wise in the shoot buds; this matches the arrangement observed in nature.

TEM of the *Sedum* petals revealed starch grains in the plastids of the epidermal and parenchymatous cells. Plastids in petals often contain starch, which enables the petals to expand rapidly as the flower blossoms (Esau, 1973). Starch was present because the *Sedum* petals used in this study were collected immediately before corolla opening, which increased the probability of successfully inducing organogenesis.

The *Sedum* petals were capable of undergoing different morphogenetic processes, including direct organogenesis, indirect organogenesis and somatic embryogenesis. *Sedum* petals may prove useful in future studies on induction of genetic variability by mutation or transformation. They may also represent a valuable source of somaclonal variation. The petals of the *Sedum* species chosen for this study may also be used in research on how induction by growth regulators affects the capacity of the cells to undergo morphogenetic transformation toward a desired result.

These histological investigations indicated that desired morphogenic responses can be achieved in *Sedum* petal cultures in vitro by manipulation of IBA concentrations in the medium. The simple anatomical structure of petals and the fast response of petal cells to the applied growth regulator concentrations yield useful explants for basic developmental studies and horticultural breeding.

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