Histological investigation of the secondary somatic embryogenesis of *Alyssum borzaeanum* (*Brassicaceae*)

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Abstract. For the first time, secondary somatic embryogenesis was investigated as an induced process for increasing the efficiency of plantlet regeneration in *Alyssum borzaeanum*, an endangered endemic in the flora of SE Europe. The competence of somatic embryos for secondary embryogenesis is discussed, together with the histological origin of neoformations and the early developmental stages of the resultant embryos. An efficient and reproducible protocol is described for establishing embryogenic lines as the preliminary stage for *ex-situ* conservation of this species.

Key words: *Alyssum borzaeanum*, *ex-situ* conservation, somatic embryogenesis

Introduction

The ultimate long-term objective of the Global Strategy for Plant Conservation is to halt the continuing loss of plant diversity in the world (http://www.bgci.org/worldwide/gspc/). Global concern about the loss of valuable genetic resources has stimulated many new programs for the conservation of plant genetic resources. Advances in biotechnology, especially the *in vitro* culture techniques and molecular biology, provide some important tools for conservation and management of plant genetic resources. *In vitro* techniques have increasingly been used in plant conservation, with recent establishment of extensive germplasm collections (Sarasan & al. 2006). It is of paramount importance to optimize the multiplication stage by controlling the somatic embryogenesis process (Williams & Maheswaran 1986). Many research papers have been published on the initial establishment of embryogenic lines (Tisserat & al. 1979; Sharp & al. 1980; Wann 1988) but the multiplication stage has been less investigated. Multiplication is one of the most important stages directly influencing the normal development of embryos and maturation of plantlets. In angiosperm species, somatic embryos and viable plantlets can be obtained directly by subculturing embryogenic calli (Tisserat & al. 1979), or indirectly by dedifferentiation of the immature embryonic tissues, a process referred to as secondary or adventive embryogenesis (Raemakers & al. 1995).

Alyssum borzaeanum Nyár. is an endangered endemic with a limited distribution along the coast of the Black Sea. It was first mentioned in Romania by Nyárády (1926). It is also recorded for the Ukraine (Kotov 1979), Turkey, Northern Greece (Dudley 1965), and Bulgaria, where it is classified as *A. tortuosum* Willd. subsp. *borzaeanum* (Nyár.) Stoj. & Stef. (Stojanov 1970). In Romania it is found only in the Constanta district, between Tuzla and the seaside. In the *Red List of Higher Plants from Romania* (Oltean

& al. 1994), *A. borzaeanum* is classified in the *endangered* sozologic category, and since 1998 it has been included in *Annex* I of the *Bern Convention*.

In order to improve the successful establishment of more productive embryogenic lines within *in vitro* conservation of *A. borzaeanum,* secondary somatic embryogenesis was induced and histological monitoring of the process was performed.

Material and methods

Plant material

Mature seeds of *A. borzaeanum* were collected in late August 2006 from the population naturally growing in the Agigea Sand Reserve, Constanta district. The seeds were surface disinfected by immersing in 70 % ethanol for 10 minutes, followed by three washings with double-distilled water. After the preliminary disinfection, seeds were incubated in a 5 % hypochlorite solution for 24 hours inside a laminar hood, and finally rinsed with sterile double-distilled water.

In vitro germination of seeds

Disinfected seeds were placed in Petri dishes containing basal medium (Murashige & Skoog 1962) with regular organic compounds, but without growth regulators. Germinating seedlings were grown until they reached 0.5 cm in length, when they were aseptically removed from the culture vessels, and the cotyledons were excised.

Embryogenic lines induction

In order to induce the development of embryogenic callus, excised cotyledons were cultured on basal medium supplemented with auxin 2.4– D (1.0 mg l^{-1}) and cytokinin kinetin (0.1 mg l^{-1}). After 3 weeks of culture at 25 °C under a 12 hour photoperiod, the embryogenic callus arises with embryonic structures in globular, heart and cotyledonary stages.

Somatic embryos in early cotyledonary stage were excised from the established embryogenic callus and placed on basal medium supplemented with growth regulators as shown in Table 1.

The cultures were maintained in an 8 hour photoperiod (2000 lux), at 25 °C and regularly transferred onto fresh medium.

Newly-formed secondary somatic embryos were transferred for germination and plantlet regeneration onto a medium without growth regulators (referred to as germination medium) but supplemented with 0.001 mg l^{-1} GA_3 (Gibberellic acid).

Table 1. Culture media variant and explant response type.

Hormone (mg l-1) / Media variant	NAA	IBA	NOA	IAA	2,4 D	2,4,5 T	BAP	Response type
M0	–	–	–	–	–	–	–	DSSE
M1	2	–	–	–	–	–	–	DSSE
M2	1	–	–	–	–	–	–	DSSE
M3	–	2	–	–	–	–	2	ISSE
M4	–	1	–	–	–	–	1	ISSE
M5	–	0.2	–	–	–	–	0.2	ISSE
M6	–	–	2	–	–	–	2	ISSE&DO
M7	–	–	1	–	–	–	1	ISSE&DO
M8	–	–	0.2	–	–	–	0.2	ISSE
M9	–	–	–	1.7	–	–	2	ISSE
M10	–	–	–	0.85	–	–	1	ISSE
M11	–	–	–	0.17	–	–	0.2	ISSE
M12	–	–	–	–	2	–	2	ISSE
M13	–	–	–	–	1	–	1	ISSE
M14	–	–	–	–	0.2	–	0.2	ISSE
M15	–	–	–	–	–	2.5	2	ISSE
M16	–	–	–	–	–	1.25	1	ISSE
M17	–	–	–	–	–	0.25	0.2	ISSE

NAA – 1-aphthylacetic acid; **IBA –** Indole 3-butyric acid; **NOA –** 1-naphthyloxyacetic acid; **IAA –** Indole 3-acetic acid; **2,4 D –** 2, 4-dichlorophenoxyacetic acid; **2,4,5 T –** 2,4,5-trichlorophenoxyacetic acid; **BAP –** 6-benzylaminopurine; **DSSE –** Direct Secondary Somatic Embryogenesis; **ISSE –** Indirect Secondary Somatic Embryogenesis; **DO –** Direct Organogenesis.

Histological studies

For the histological investigation, fresh tissue samples in different developmental stages were fixed for two weeks in a formalin buffered solution [15 % (v/v) formalin, 2 % (w/v) sodium bicarbonate]. Dehydration was conducted at room temperature in graded ethanol series (5 to 100 %), followed by clearing with xylene and embedding in paraffin according to usual procedures (Dashek 2000). Serial sections (7–10 μm thick) were cut using a rotary microtome and transferred onto glass slides. The paraffin was removed and the sections were stained with 0.12 % (w/v) toluidine blue, in a water solution containing 5 % (w/v) sodium tetraborate decahydrate and 0.1 % (w/v) basic fuchsin (Junqueira 1990) and finally mounted in synthetic resin (Entallan). Histological observations were made using bright-field light microscopy under a Nikon Eclipse E200 microscope and micrographs were recorded using a Nikon Coolpix 5400 digital camera.

Results

Histological analysis shows that the process of dedifferentiation of somatic embryos became visible after 25 days, mainly in cotyledonary tissues. Two distinct patterns of secondary embryogenic response were observed: an indirect one (via callus) and direct formation of somatic embryos (without the callus stage).

The first pattern was characterised by processes of dedifferentiation of ground parenchymatous cotyledonary tissue and intense cell division forming an internal callus. Consequently, the explants became swollen; the newly formed tissues pressed against the epidermis causing ruptures and emergence of callus (Plate I, Fig. 2). The neoformated tissue (callus) had two structural zones: an inner one made up of large vacuolated cells and a fragmented cortical-peripheral zone with small, actively dividing cells (meristemoids), which formed proembryogenic structures (Plate I, Fig. 3). Initially, these structures were globular (globular embryoids) but then elongated and evolved into bipolar structures (bipolar embryos) with evident polarization of tissues (hypocotyl-radicle axis) and tracheary elements (Plate I, Fig. 4). Bipolar embryos underwent consecutive embryonic stages, developing mature embryos (cotyledonary stage) which became viable plantlets on germination media. This pattern of forming somatic embryos corresponds to the indirect somatic secondary embryogenesis pathway described by Sharp & al. (1980).

The second pattern of embryogenic response was observed mainly in the epidermal cells. A few epidermal cells and probably some competent cells beneath the epidermis became mitotically active and started to divide periclinally (not anticlinally as is normal for epidermal cells). This pattern of division was not followed throughout the entire process of cell proliferation; asynchronous divisions also occurred and generated meristemoids with visible protoderm (Plate I, Fig. 5). The newly-formed meristemoids first developed embryogenic globular structures (Plate II, Figs 1, 2) and, subsequently, all the normal embryonic stages and finally plantlets, when transferred on media containing GA_3. The pattern of embryos formation followed the pathway described by Sharp & al. (1980) for direct somatic secondary embryogenesis.

Culture media effects became visible after 25 days of culture. Three types of response occurred, depending on the media growth regulators content:

- On media without growth regulators (M_0 variant), and corresponding to direct somatic secondary embryogenesis process. The same type of response was observed on media supplemented with NAA (M1 and M2 variant), where the embryogenic response (as quantified by the rate of embryo formation) was *ca* 10–30 per embryogenic explant. When transferred onto germination media, regeneration rate and yield of plantlets were optimal.
- Indirect somatic secondary embryogenesis occurring with high efficiency (10–40 embryos per embryogenic explant) on media containing 1–2.5 $mg\,l^{-1}$ auxins (IBA, NOA, IAA, 2,4D, 2,4,5T), and poor efficiency (1–3 embryos per embryogenic explant) on media with low auxin content.
- Direct organogenesis (shoot formation) was observed on media supplemented with NOA and BAP only (M6 and M7 variant), with satisfactory efficiency (8–15 shoots per explant).

Discussion

The multiplication stage of *A. borzaeanum* depends on the physiological state of the explant (degree of tissue differentiation) and the hormone balance of the culture media. The optimum type of explant comprises cotyledon fragments excised from the somatic embryo in early cotyledonary stage (Plate I, Fig. 1) (Păunescu unpubl.). Two pathways of secondary embryogenesis were described, separated by the occurrence of callogenesis: a direct pathway (no callus formation) and an indirect pathway (via callus).

In the indirect pathway the early sign of cell dedifferentiation was observed in the ground parenchyma where competent cells become mitotically active, generating callus and embryogenic structures (Plate II, Fig. 3). Some of the resultant embryos seem to be connected to the originating tissue, suggesting multiple cell origins as previously reported in *Daucus* (Haccius & Bhandari 1975).This type of embryogenic process was defined by Sharp

& al. (1980) as indirect secondary somatic embryogenesis and occurred on media supplemented with BAP and different auxins (except NAA). BAP concentrations ranging from 1–2 mg l^{-1} reduce the occurrence of secondary embryogenesis (Cruz & al. 1990) by an inhibitory effect upon the dedifferentiation of pre-embriogenically competent cells. Because of the internal location of the ground parenchyma and the better permeability for auxin of the component cells, the BAP inhibitory effect was substantially reduced and the stimulatory effect of auxins sustained subsequent dedifferentiation of competent cells. As a consequence, optimal embryogenic response was obtained on the variants with combined auxin/BAP and auxin concentrations ranging from 1–2.5 mg l^{-1} (M3, M4, M6, M7, M9, M10, M12, M13, M15 and M16 variant). The response was almost indistinguishable on variants with low auxin contents ranging from 0.17–0.25 mg l^{-1} (M5, M8, M11, M14 and M17).

All embryogenic globular structures (developed by the indirect pathway on media containing auxin) show differentiation of tracheid cells in the inner zone of their structure (Plate II, Fig. 4). The presence of vascular tissue primordia at the very early stages of embryo development is not a common event and suggests that the auxin content of culture media may be responsible. Auxins are known to induce linear vascular strands when applied externally and auxin transport inhibition interacts with vascular strand formation (Scheres 2000).

Direct secondary somatic embryogenesis is the only response on a culture medium that is free from growth regulators and on media containing the auxin NAA only (M0, M1 and M2 variant). This pathway originated mainly from epidermal cells which divided periclinally, instead of normal anticlinal orientation. After several divisions a meristem-like structure (meristemoid) is formed, suggesting that some epidermal cells are preembrionically determinate cells, being embriogenically competent (or preembryogenic determined cells, as defined by Konar & al. 1972). The embryogenic process then follows the classical pattern resulting in spherical globules which develop further into typical embryos. The determinism of embryogenic competence of the epidermal cells is very complex, the most important feature being the differentiation state of explant tissue. The presence of some auxins (NAA) in the culture media seems to have no direct causal significance, because the direct pathway of response also occurs with good efficiency in the absence of any growth regulators (M0 variant).

A different development pathway is observed on media containing NOA and BAP (M6 and M7 variant), simultaneously with the indirect secondary somatic embryogenesis involving the epidermal cells. Some epidermal cells, after several periclinally divisions, form an apical meristem-like structure, which develops shoots directly, without undergoing the embryonic stages, along a developmental route corresponding to a direct organogenesis pathway (Plate II, Fig. 5). The different morphogenetic response shown by the competent epidermal cells under apparently identical external conditions suggests that the endogenous regulation of organogenesis and embryogenesis pathways is interrelated.

Epidermal cells have multiple roles including crucial events such as dedifferentiation and embryogenic competence, growth and development. It has recently been reported that the epidermis controls shoot growth by providing a non-autonomous signal to the ground tissues (Savaldi-Goldstein 2007). Pien & al. (2001) also report that mechanical wall relaxation applied locally to the epidermis of shoot apex induces growth of a leaf primordium, demonstrating the organogenetic competence of epidermal cells.

Both types of response (direct and indirect secondary somatic embryogenesis) are effective in multiplication for *in vitro* conservation of *A. borzaeanum*. Formation of adventitious embryos contributes to an increase in plantlet regeneration, when compared with the regeneration via somatic embryogenesis or organogenesis only. From a single somatic embryo, more than 40 plantlets can regenerate via somatic secondary embryogenesis. The developmental stage of the explant (somatic embryos in early cotyledonary stage) and the formulation of the culture media are both extremely important in monitoring the somatic embryogenesis process for an efficient regeneration rate and yield of the plantlets.

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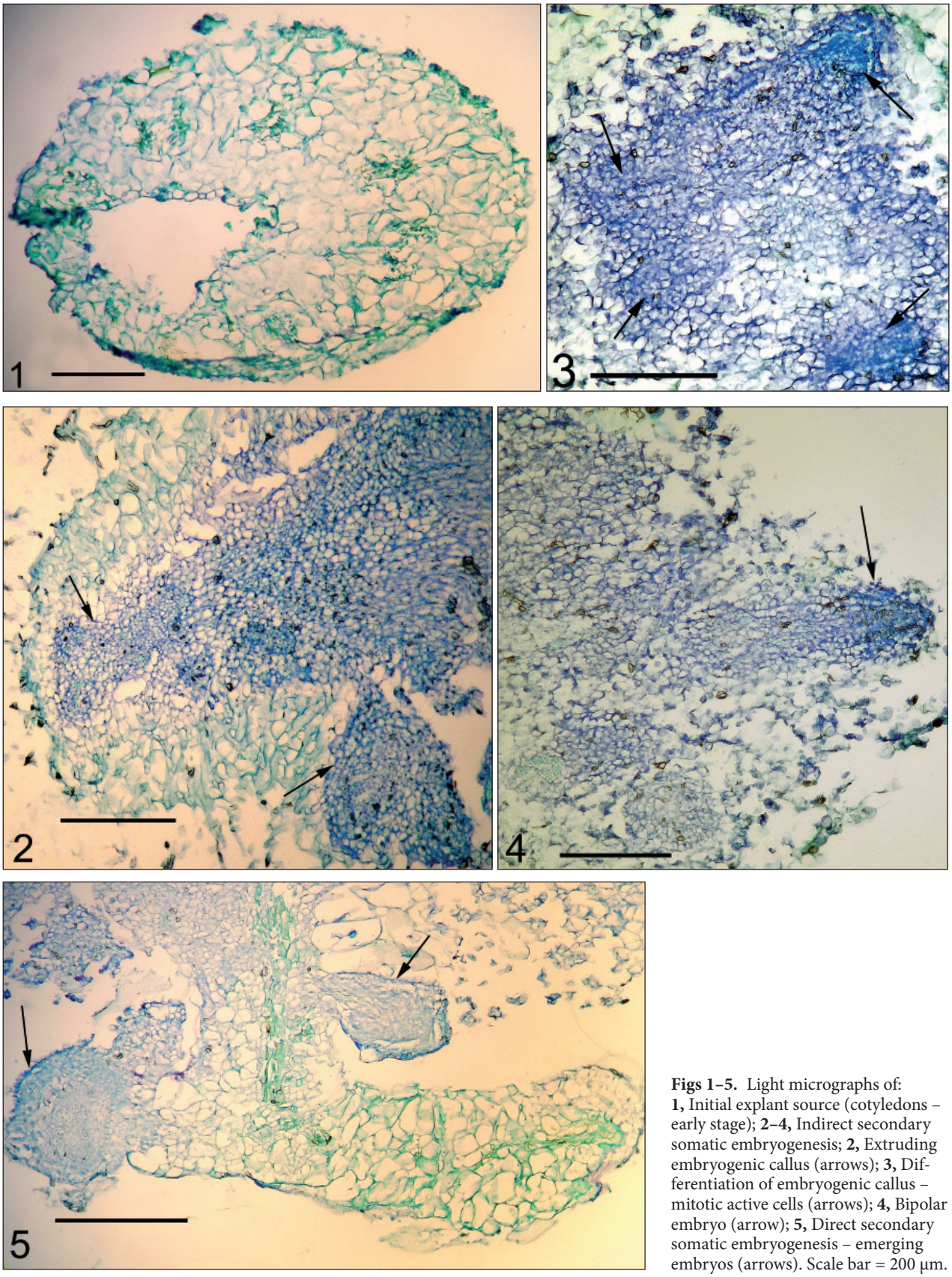
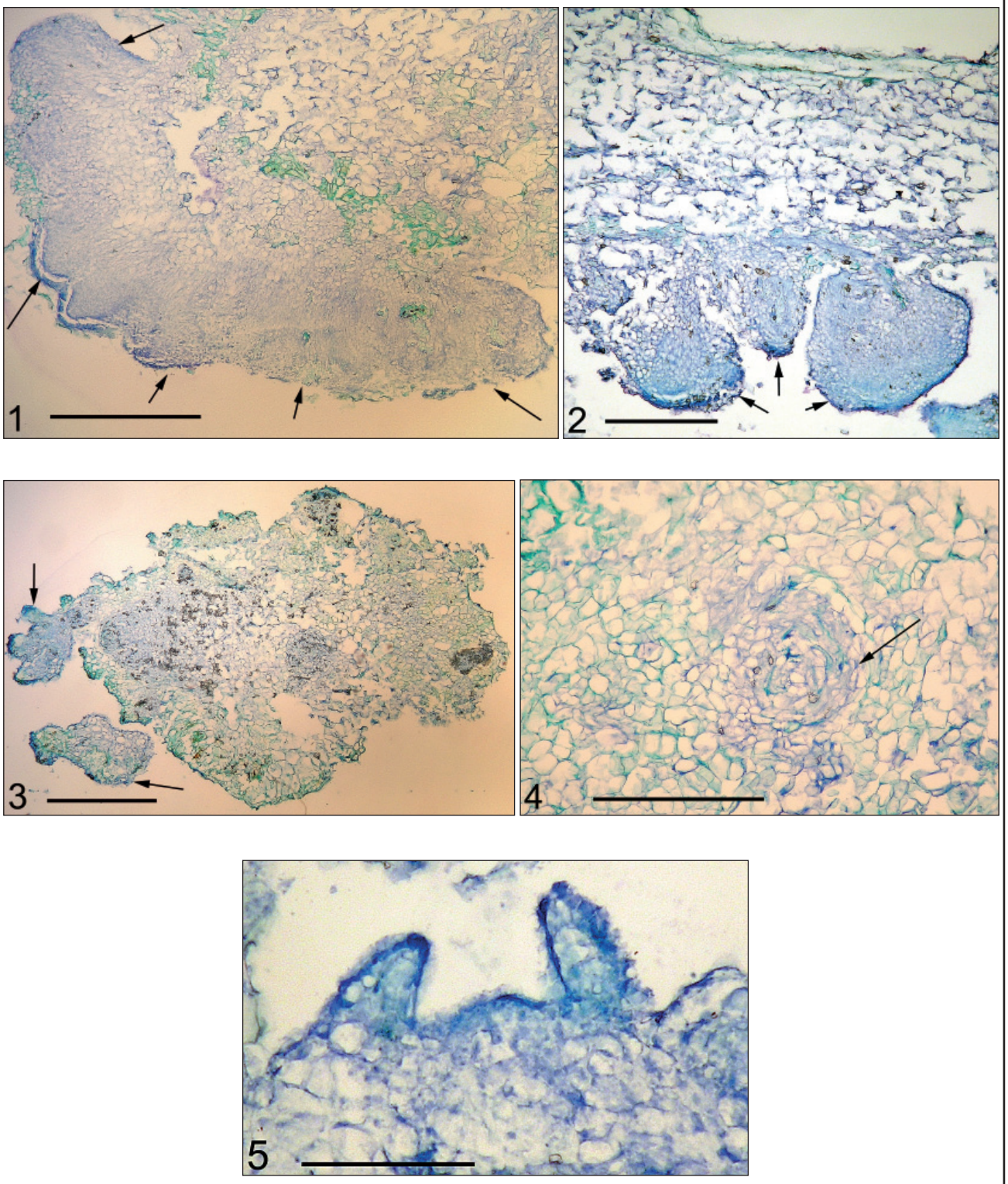
Plate I



Figs 1–5. Light micrographs of: **1,** Initial explant source (cotyledons – early stage); **2–4,** Indirect secondary somatic embryogenesis; **2,** Extruding embryogenic callus (arrows); **3,** Differentiation of embryogenic callus – mitotic active cells (arrows); **4,** Bipolar embryo (arrow); **5,** Direct secondary somatic embryogenesis – emerging embryos (arrows). Scale bar = 200 μm.

Plate II



Figs 1–5. Light micrographs of:
1–2, Direct secondary somatic embryogenesis; **1,** Compact meristematic masses (arrows); **2,** Emerging embryos (arrows); **3–4,** Indirect secondary somatic embryogenesis; **3**, Dedifferentiated cotyledonar tissue with emerging embryos (arrows); **4,** Embryogenic globular structure with tracheid cells (arrow); **5,** Apical meristem-like structure showing direct organogenesis. Scale bar = 200 µm.

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