

HISTO – ANATOMICAL ASPECTS OF AERIAL VEGETATIVE ORGANS OF *STEVIA REBAUDIANA* BERTONI CULTIVATED *IN VITRO* (II)

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Abstract: Shoot apex, nodal, and leaf explants of *Stevia rebaudiana* Bertoni can be cultivated on Murashige and Skoog (MS) medium supplemented with auxins (NAA naphthyl acetic acid, IAA indole-3-butyric acid and 2,4-D) and cytokinins (BAP benzylamino purine and kinetin). The histo-anatomical features of the callus and regenerated plantlets were underlined. The structural modifications were interpreted in correlation with the presence and combinations of growth regulators in the culture medium.

Key words: anatomy, callus, explant, shoots

Introduction

Stevia (Stevia rebaudiana Bertoni) is a perennial plant belonging to the *Asteraceae* family. This species is characterized by a very limited range of natural habitats and is an endemic plant originating from Paraguay. *Stevia* leaves contain a number of diterpenoid steviol-glycosides (SGs) that are about 300 times sweeter than sucrose. These glycosides are non-toxic, non-mutagenic and low-caloric compounds, and, unlike traditional sugar substitutes such as xylitol or sorbitol, acquired tolerance to them does not occur [1].

The *in vitro* culture of *Stevia rebaudiana* makes the object of some studies from the scientific literature [10, 5, 9, 11, 2, 3]. Histo-anatomical data concerning *in vitro* cultivated plants was not found in consulted literature.

In a previous work [8], the structural modifications occur during *in vitro* culture of *Stevia rebaudiana* on MS medium without growth regulators were underlined.

Material and methods

The nutrient basal medium used in all experiments consisted of the inorganic salts of Murashige & Skoog (1962) [4]. The pH was adjusted to 5.7 prior to autoclaving at 121 °C during 15 min. Plants of *Stevia rebaudiana* used on the experiment were kindly given by Botanical Garden of Chișinău (Moldavia Republic) and were grown in a greenhouse of Botanical Garden of Iassy. Nodal stem segments with 2 cm were excised from these plants, disinfected in a solution of sodium hypochlorite (1.5%) for 15 min and then rinsed for three times with sterilized water. These nodal segments were cultivated on basal medium and after 15 days lateral buds with 3 to 4 pairs of leaves developed. When the plantlets attained 5-7 cm in length the vegetal material was used for testing the morphogenetic reaction on MS medium supplemented with different concentrations of growth regulators (table 1).

The method used for obtaining permanent samples (sections) was classical: fixed samples were sectioned along a transversal line on Minot microtome, after paraffin including.

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The obtained sections were colored either with fastgreen + saphranine or with red-ruthen and methyl-blue. After that, the sections were fixed in Canada balsam.

Variants	Explant type	REGULATORI DE CREȘTERE (mg/L)				
		K	BAP	NAA	IAA	2,4-D
KN	Nodal explants (basis)	2	-	2	-	-
	Nodal explants (middle)					
BI	Nodal explants (basis)	-	2	-	2	-
	Nodal explants (middle)					
KI	Nodal explants (basis)	2	-	-	2	-
	Nodal explants (middle)					
B ₂	Nodal explants (basis)	-	2	-	-	-
	Nodal explants (middle)					
	Apical explants					

Table 1 – Experimental variants used for testing the in vitro morphogenetic reaction of some explants from *Stevia rebaudiana Bertoni*

Results and discussions

On **KN** medium the morphogenetic reaction of nodal explants was represented by callus proliferation. This has different consistencies and colors: fragments of compact green callus alternate with portions of light-yellow friable callus; is lately, a brownish friable callus could be observed.

The cross sections through the explant basis show the origin of this callus in the cortical parenchyma of the stem. The general contour of the cross section is profoundly affected by the callus proliferation. The epidermis, when is still present, have transversal division walls.

The areas with friable callus are formed by large cells, with very thin walls, with large aeriferous spaces between them. In the external part these cells are elongated, and they loose the contact one with another. In the areas with compact callus numerousness meristematic nodules could be observed. They are formed from small, isodiametric cells, with large nuclei. In the majority of the nodules short traheids, with reticulate thickness and moderately lignified was differentiating.

The central cylinder structure of the explant basis is almost intact. The xylem, predominately by secondary origin has a ring shape. It is composed of vessels and xylemic fibers with thick and moderate lignified walls. In some areas a proliferation of the phloem zone could be observed. The pith is made up of isodiametric parenchymatic cells.

The formation of new shoots is based on the presence of the axilar buds; their growth is stimulated by the presence of the kinetine in the culture medium. The presence of the callus in the basal part of the explant blocks the rhizogenesis process.

The stem structure is quite normal [8]; in the cortical and in the pith parenchyma division walls are visible. The vascular tissues have primary structure and are grouped in collateral vascular bundles (photo 1). The epidermic cells are small, with thin walls and

without a cuticle visible at the optic microscope. The axilar buds have a normal structure (photo 2); the foliar primordia have a opposite disposition like at *in vivo* cultivated plants.

On **BI** medium a small amount of callus is visible at the basis of the explant. This is green, compact and homogenous.

The presence of BAP in the culture medium stimulates the axilar buds growth (photo 3); in the same time, adventitious buds are formed from the basal callus. Like on KN medium, the presence of the callus on the explant basis block the roots formations. Sivaram at al., (2003) [7] demonstrate that different explants (shoot apex, nodal explants, leaf fragments) could produce shoots on a medium wit citoknins and auxins and the root formation is induced in next subculture on a medium without auxins.

The stem and leaf structure present small modifications. Isolately “twin apexes” could appear because an anomalous ramification (photo 6). In a first stage, they have foliar primordia only on external part, but subsequently their development is normal. The anomalies of apex function could be the reason for the presence of a lot a shots on the explant basis.

The foliar primordia have normal structure, with a compact meristematic tissue; on the epidermis tector and glandular hairs, in different stages of development could be observed. The mature leaves are a quite normal structure, with no significant vitrifications features (photo. 5). The mesophyll is differentiated into a unilayered palisade parenchyma and a multilayered spongy one. The vascular bundle from the midvein is not prominent at the abaxial side; it is formed by few xylem vessels, some phloemic elements and a sclerenchyma girdle composed by cells with thick and lignified walls. The stomata are numerousness, placed over the epidermis level. Most of them are unfunctional and have the ostiole wide oped. However, the cuticle is thinner than that of the normal leaves.

The structure of the new formed shoots is normal; in the central cylinder a procambial ring could be observed (photo. 4). The vascular tissue will develop from it.

On **B2** medium a small callus could be observed at the basis of the initial explant. Cross sections from this callus shows meristematic centers which will develop in shoots apexes and then in stems. In the middle part of the explant, from the external part of the cortex and from epidermic a friable callus appears (photo 7). It consists in large cells, have no organization or meristemaric centers. The vascular bundles have secondary structure (photo 8); the xylem is well developed, formed by vessels with thick and lignified walls, xylem fibers and parenchyma cells. The phloem has a normal structure and is formed by sieve tubes and companion cells.

The mature leaves have a homogenous mesophyll, consist only in spongy parenchyma. On **KI** medium the morphogenetic reaction was similar with that from BI mediu. The process of shoots formation from axilar buds is intense. The contour of the stem cross-section is circular; the epidermis persists on the explant exterior part, although it is interrupted here and there, as a result of intense proliferation of the cortical parenchyma’s cells (photo. 9). In some epidermal cells, division walls with different orientation may be observed. Internal proliferation is more reduced than in the previously described situation. The procambium suffers some additional divisions, yet the central cylinder maintains its individuality (photo 10).

The cross sections from the new formed shoots show the proliferation of the cortical parenchyma in a callus without any organization. The vascular tissues are well developed, they have rings shapes: the internal one, of the xylem has vessels with thick and lignified walls. At the phloem periphery several sclerenchyma fibers could be observed.

At the basis of the explant, adventitious roots could be observed. They provided from the stem pericycle and have a simple, diarch structure. The cortical parenchyma is thick, with large aeriferous spaces between cells.

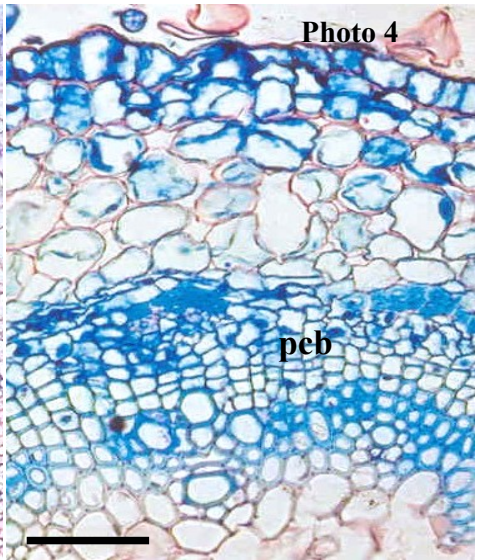
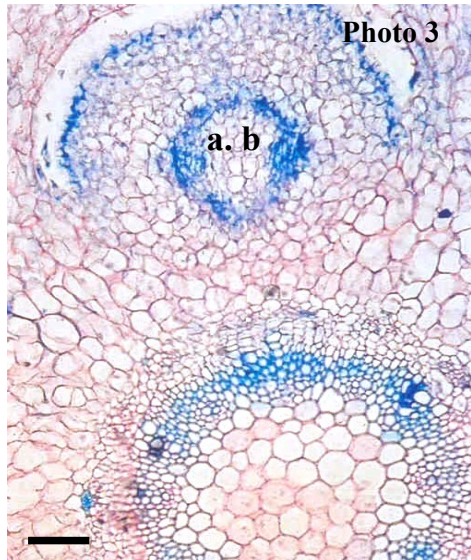
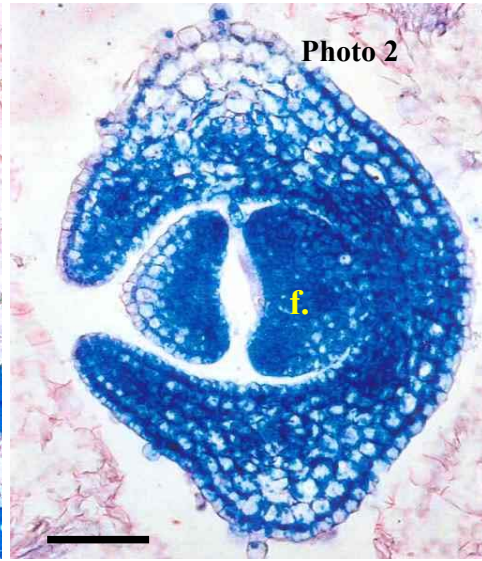
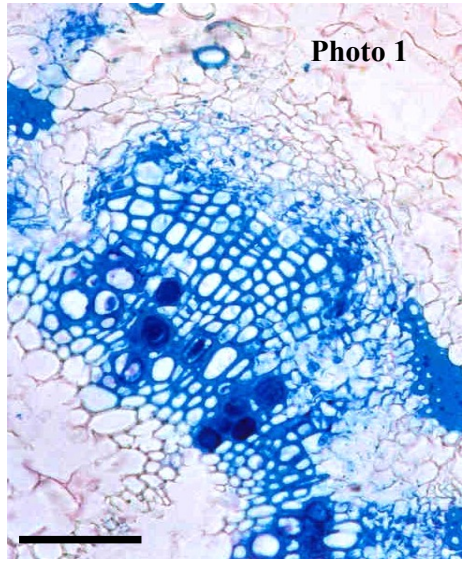
The leaves structure is not constant. Some of them have a well developed mesophyll, with one layer of elongated palisadic cells, without aeriferous spaces, and 4-5 layers of spongy parenchyma. The vascular bundles are small; the stomata are numerous, especially in the lower epidermis. Another has thick and undifferentiated mesophyll consist only from spongy parenchyma.

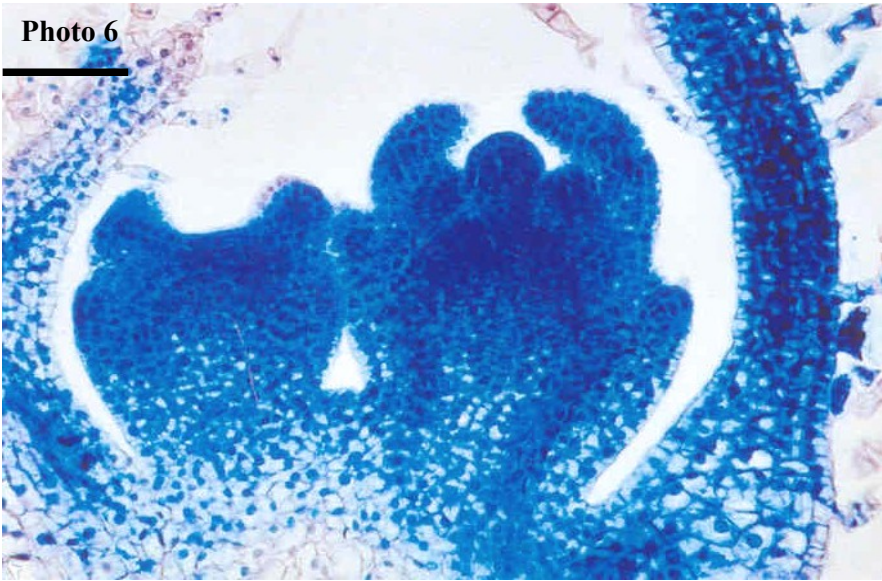
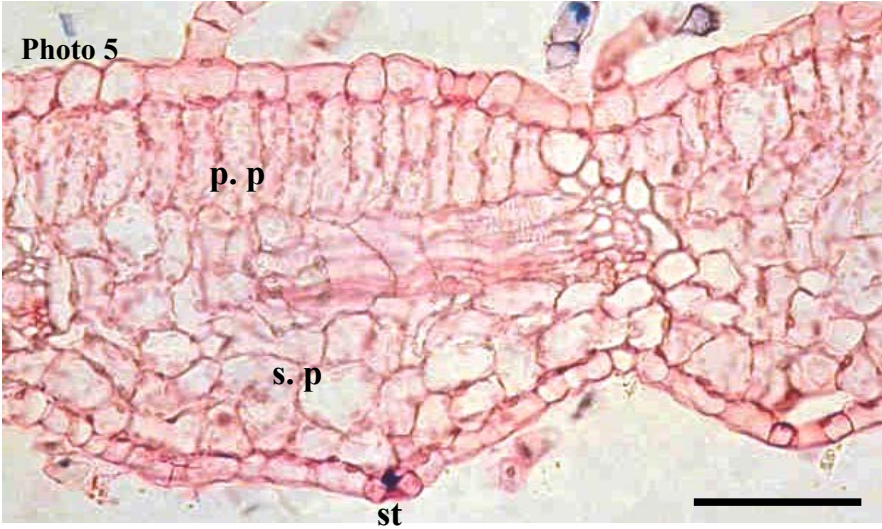
Conclusions

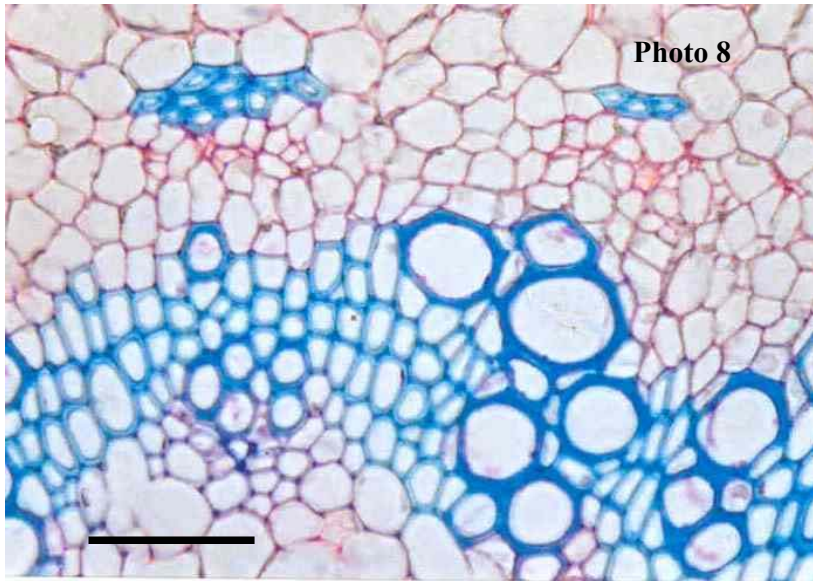
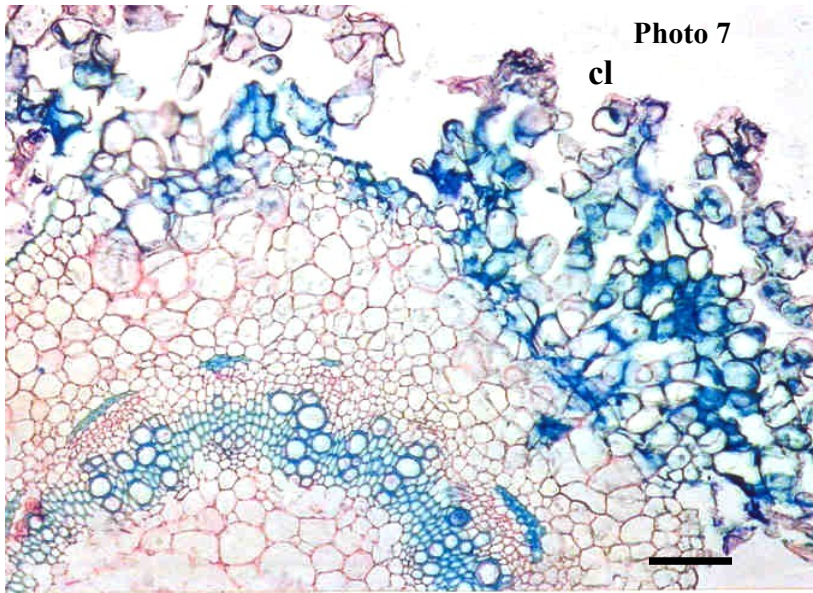
In all experimental variants, at the basis of the explant a mass of callus appear. That is more or less developed in relation with the hormonal balance. The largest callus was formed on KN medium but only with histogenic potential. On B2 medium the callus has organogenic capacity and was capable to regenerating shoots. On KN, BI and B2 medium the direct caulogenesis was stimulate. Adventitious roots appear only on KI medium.

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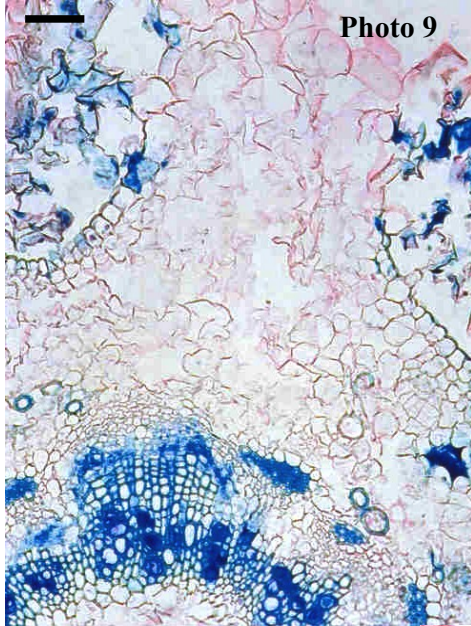


Photo 9

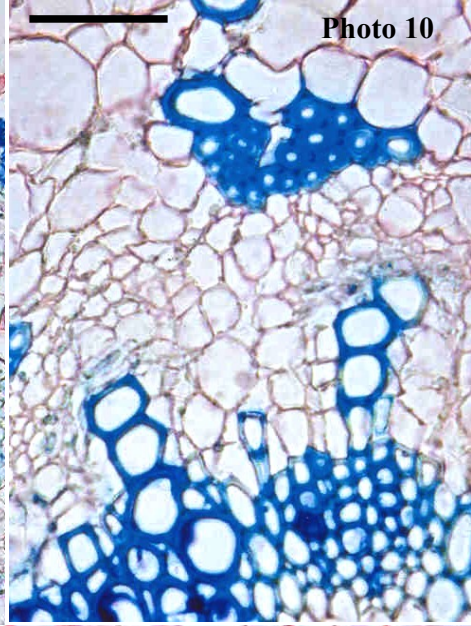


Photo 10

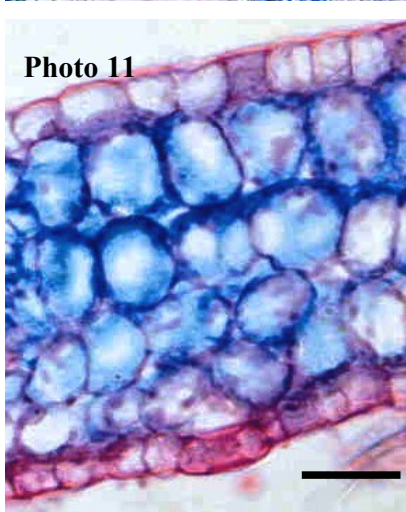


Photo 11

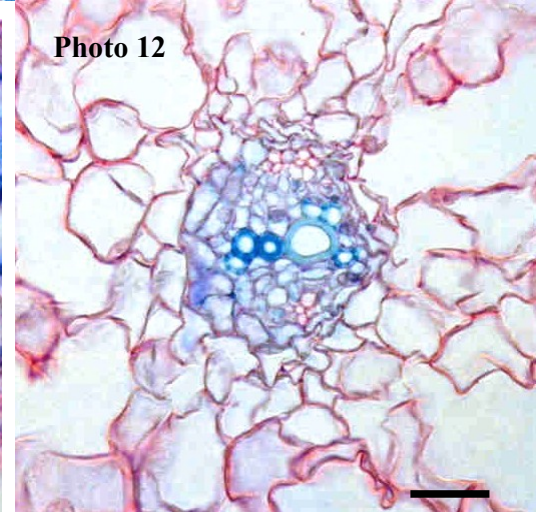
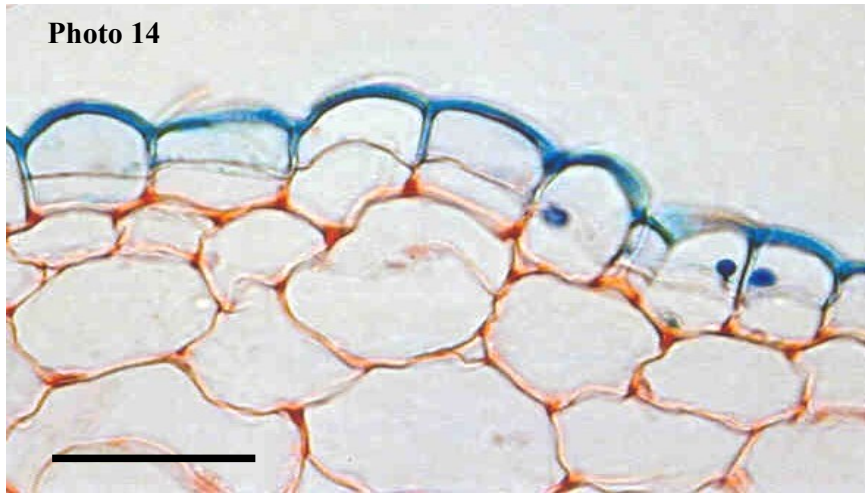
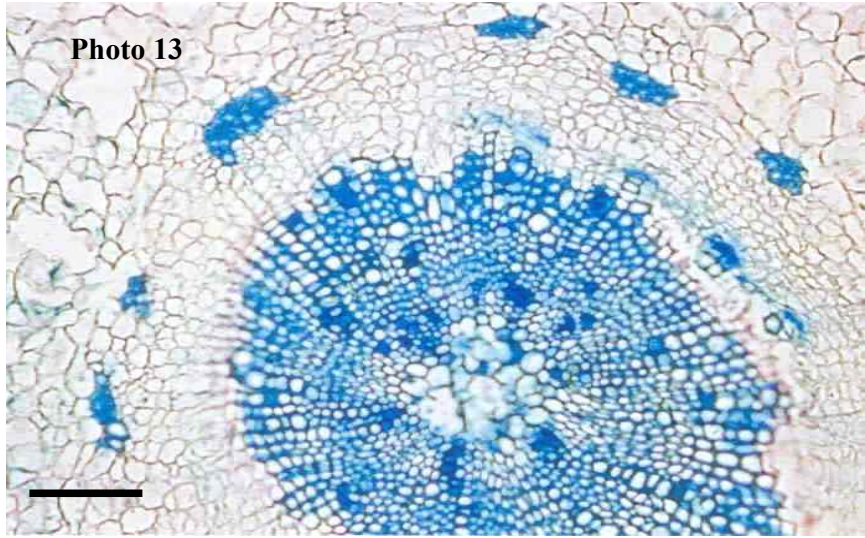


Photo 12



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Explications of photos

Photo 1- Crossection through the shoot regenerated on KN medium

Photo 2 – Foliar primordia from a axilar bud (on KN medium)

Photo 3 - Crossection through the shoot and an axilar bud from BI medium

Photo 4 - Crossection through the shoot from BI medium

Photo 5 - Crossection through the leaf lamina (BI medium)

Photo 6 - Isolately “twin apexes” at the basis of the stem (longitudinal section)

Photo 7 – Crossection through the shoot from B2 medium

Photo 8 – Crossection through the shoot from B2 medium (detail from the vascular tissues)

Photo 9 - Crossection through the basal part of the shoot from KI medium

Photo 10 - Crossection through the basal part of the shoot from KI medium (detail from the vascular tissues)

Photo 11 - Crossection through the leaf lamina (KI medium)

Photo 12 - Crossection through an adventitious root (KI medium)

Photo 13 -Crossection through the basal part of the initial explant from KI medium

Photo 14 – Crossection through the shoot from B2 medium (detail from epidermis – transversals division walls could be observed)

Abreviations: a. b – axillary bud, c. c – central cylinder, cl – callus, f. p – foliar primordia, p. p – palisade parenchyma, pcb - procambium, s.p – spongy parenchyma, x. vs – xylem vessels (barr = 50 μ m)