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Ultrastructural Analysis of the Ontogenetic Development of Shoot Induced from Embryonic Axes of Costa Rican Bean Varieties (*Phaseolus vulgaris* L.) under *in Vitro* Conditions by Scanning Electronic Microscopy

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ABSTRACT

Common bean (*Phaseolus vulgaris* L.) is an economic important crop and one of the major grain legumes for human consumption in Latin America, Africa and Asia. A morphological study of shoot induced from embryonic axes development in four Costa Rican bean varieties (Brunca, Huetar, Guaymi and Bribri) cultivated on MS media with or without 5 mg·L^{-1} de N^6-benzylaminopurine (BAP) by scanning electron microscopy (SEM) was developed in the present work. Micrographs showed similarities and differences in the ultrastructure of the apical dome, epidermal surface, stomata and different types of trichomes in the varieties cultivated on organogenesis media. Genotypes with advantageous morphological characteristics for genetic transformation, in particular an exposed apical dome, were identified. This work will contribute to the optimization of the *in vitro* regeneration of four common bean varieties.

Keywords: *Phaseolus vulgaris* L.; Bean; *In Vitro* Organogenesis; Shoot; Scanning Electron Microscopy (SEM)

1. Introduction

The common bean (*Phaseolus vulgaris* L.) belongs to the legume family (Fabaceae), and represents 75% of the commercial legumes in the world [1].

Dry bean production is of great economic and social importance, as beans are grown mainly by small and medium-scale farmers. The common bean is a fundamental component of the protein diet of the population, especially in rural areas where consumption of animal protein is often reduced because of economic limitations [2]. It is estimated that beans represent the second most important source of daily protein and the third source of calories in Africa [1].

Species of *Phaseolus* are considered recalcitrant to *in vitro* culture and transformation [3,4]. Successful procedures for the *in vitro* culture and plant regeneration of bean varieties have been only developed in few laboratories around the world [1,5]. Reference [5] studied the effect of BAP and AS on the number of shoots induced from embryogenic axes of Costa Rican bean varieties (Bribrí, Brunca, Guaymí, Huetar and Telire) embryogenic axes were cultivated on induction medium supplemented using 5 mg·l^{-1} BAP combined with 20 mg·l^{-1} AS or 5 mg·l^{-1} BAP with 40 mg·l^{-1} AS.

Reference [5,6] the morphological characteristics of apical buds and the effects of BAP on multiple shoot induction is important for assessing important traits in the apical meristem. This information is very important because the response of common bean varieties grown in Costa Rica to tissue culture techniques is unknown. These techniques can be a useful complement to breeding programs. In addition, the study of the morphology of the apical meristems in Costa Rican genotypes, will allow the proper selection of bean varieties to use for genetic transformation and mutation induction.

Moreover, few morphological studies of bean have been reported. Reference [6] examined the morphology of the apical meristem of 17 "Carioca" and "Jalo" type Andean cultivars and found various anatomical aspects that affect the frequency of regeneration of transformed bean plants.

Reference [7] used transmission electron microscopy to observe changes at the cellular level in the epidermis, mesophyll and vascular tissue of bean (*P. vulgaris* L.) leaves inoculated with *Xanthomonas axonopodis* pv.

Phaseoli. Reference [8] studied the leaf anatomy of five genotypes of mung bean: var. "Criolla", "VC1973A", "VC2764B", "NM94" and "CHUN NAM 4", to provide information useful for genetic delimitation at the intraspecific level, and for a better interpretation of some aspects of agronomic performance. The results were homogeneous in the five genotypes studied, and differences were observed in the quantitative anatomic variables of stomata density, trichome density and leaf blade thickness.

The objective of the present study was to analyze the morphological characteristics of the ontogenetic development of shoot induced from embryonic axes of four Costa Rican varieties of common bean (*P. vulgaris* L.) under *in vitro* conditions.

2. Materials and Methods

2.1. Plant Material and *in Vitro* Culture Conditions

Zygotic embryos from the Costa Rican bean varieties Guaymí, Bribrí, Huetar, and Brunca were used because these varieties are the most consumed in our country.

These zygotic embryos were used, according with experimental protocols developed in Reference [5].

2.2. Scanning Electron Microscopy (SEM)

Shoot induced from embryonic axes were collected at 0, 1, 4, 8 and 12 days of culture under in vitro conditions. The samples were fixed in Karnovsky solution [9] for 2 h at 4ºC. Then, they were washed with sodium phosphate buffer, pH 7.4, and postfixed in 1% osmium tetroxide in phosphate buffer. Samples were then washed with distilled water and dehydrated through an ascending series of ethanols (30%, 50%, 70%, 80%, 90%, 95%, 100%), rinsed with terbutylic alcohol and dried by sublimation. Finally, the samples were mounted, coated with 40 nm of gold and observed with a Hitachi S-570 scanning electron microscope (SEM) operated at 15 KV.

Embryonic axes, fifty in total, were extracted at 0 days of culture under in vitro culture and prepared for SEM. The exposed portion of the apical dome was determined by tracing each of the apical domes projected on the screen of the SEM. Micrographs were taken using the same parameters of working distance, inclination and magnification. The micrographs were traced onto paper and the dome was measured using a ruler (mm) and the formula $A = \pi r^2$.

In order to do this, the percentage of Embryonic axes with the meristem completely exposed and the percentage of exposed meristematic area in each embryonic axis was determined. In addition, embryonic development over time was observed and characteristics such as trichomes, stomata and cuticle were evaluated and compared in embryonic axes cultured with or without BAP.

3. Results

3.1. Ultrastructural Analysis of Shoot Induced from Embryonic Axes without BAP

At day 0, the apical meristem of EA was completely exposed in 37% of the apices of the Brunca variety, followed by Guaymí (26%), Huetar (16%) and Bribrí (8%).

In the Guaymí variety, the percentage of exposed area was 79%, followed by Brunca (73%), Huetar (72%) and Bribrí (60%) (**Figures 1(a)-(d)**).

The epidermis was irregular, but towards the inferior portion of the dome the epidermal cells conserved a more regular structure (**Figure 1(e)**).

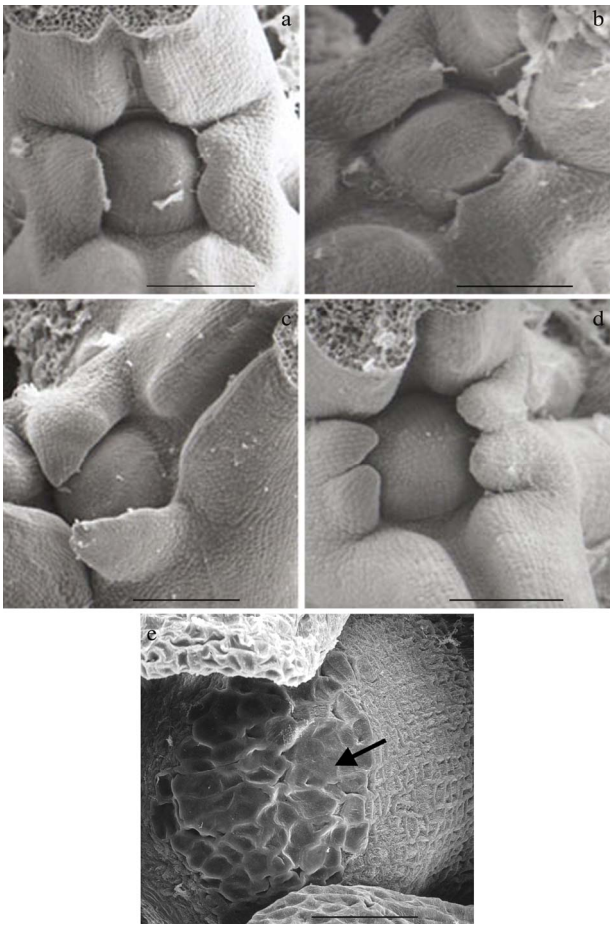

Figure 1. Scanning micrographs of apical meristems of Costa Rican common bean varieties at 0 days of culture without BAP, showing percentage of exposed area. (a) Brunca, scale 150 µm; (b) Guaymí, scale 150 µm; (c) Bribrí, scale 176 µm; (d) Huetar, scale 150 µm; (e) All varieties showing irregular cells at the dome tip (arrow), becoming more defined in the inferior portion, scale 40 µm.

The epidermis of the shoot presented depressions and protuberances corresponding to possible areas of origin of stomata and trichomes, respectively. Trichomes on the hypocotyl were fewer in number than on the epicotyl, and were situated in conical structures (data not shown).

In transverse sections, different layers could be observed: epidermis, parenchyma cells (cortex and pith), the vascular bundle and cuticle layer. In the variety Bribrí, intercellular spaces were observed between the parenchyma cells, and the subepidermal cells were more elongated than in other varieties (data not shown).

At growth day 1, the apical tip containing the stem apex was observed. Epidermal papillae covered the entire structure (**Figure 2(a)**). The epicotyl showed papillae covered with cuticular wax and structures that may be the origin of trichomes or stomata (data not shown).

At day 4, globose and/or elongated structures that covered the apex were observed. Epidermal papillae were conspicuous. Different levels of unicellular and pluricellular trichome development were observed in the older primordia (**Figure 2(b)**). The vascular cylinder was distinguished by a central ring showing two types of cells: medium cells of the cortex parenchyma and larger cells corresponding to the ground parenchyma. From day 4, phloem and xylem were more evident, and spiral ornamentation of the secondary side walls of the vessel elements was observed. Alternate pitting of the side walls was also observed (data not shown).

After 8 days of growth, the trichomes and stomata were more developed. Young globose structures and other more elongated structures with wide bases corresponding to young leaves beginning to take the form of the mature leaves were observed (**Figure 2(c)**). A greater number of stomata were present on the leaf surfaces.

At day 12 the apical shoot was formed. Growth and volume of young leaves was increased (**Figure 2(d)**), giving origin to mature leaves with a prominent central vein and lateral veins. A greater number of trichomes and stomata were evident on the surface of the leaves.

Anomocytic stomata were observed only in the Brunca variety (**Figure 3(a)**). The stomata in the four varieties were paracytic and were either level with the epidermis or raised (**Figures 3(b)** and **(c)**), with the exception of the Bribrí variety which showed sunken stomata (**Figure 3(d)**).

Several types of trichomes were found in the four varieties, including long simple unicellular trichomes, short pluricellular globose trichomes, short unicellular simple

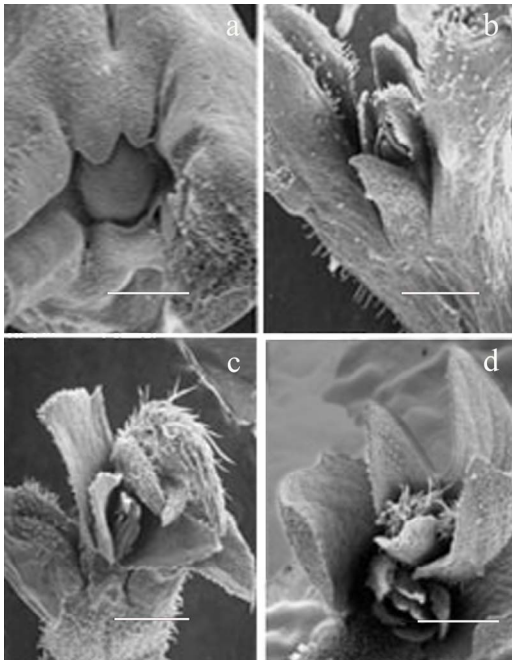

Figure 2. Scanning micrographs showing the apical surface of the dome for all common bean variety at 1 - 12 days of culture in MS media without BAP. (a) The first day, stem apex formed by small, isodiametric cells covered with epidermal papilla. The dome is surrounded by two very young primordial. Huetar, scale 150 µm; (b) 4 days, leaf primordia surrounding the apical meristem and young leaves with many trichomes. Guaymí, scale 0.38 mm; (c) 8 days, many young leaves with trichomes, young leaves beginning to mature. Brunca, scale 0.60 mm; (d) 12 days, leaf growth is more evident, long trichomes observed near the dome and on the abaxial leaf surface Bribrí, scale 0.75 mm.

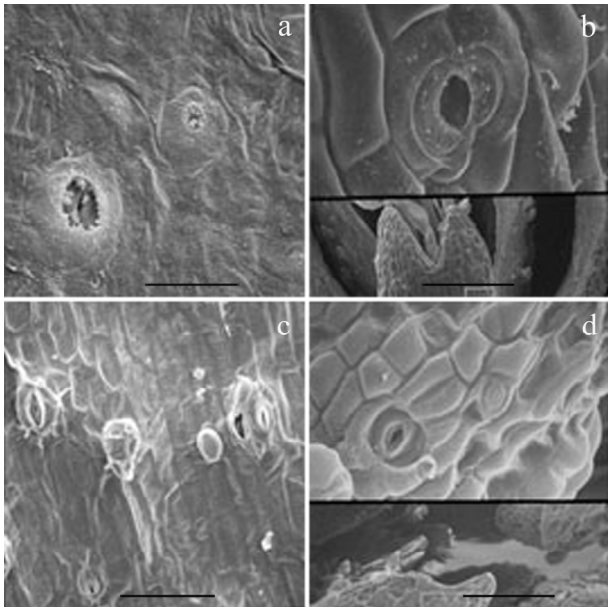

Figure 3. Stomata present in common beans at different days of culture *in vitro* on MS media without BAP. (a) Anomocytic stoma in Brunca variety. Scale 10 µm; (b) Paracytic stoma at the level of epidermal cells. Scale 200 µm; (c) Paracytic stoma raised above the epidermis scale 50 µm; (d) Paracytic stoma below the level of epidermal cells, scale 150 µm.

trichomes and pointed trichomes (data not shown). In the four varieties the trichomes are same.

3.2. Ultrastructural Analysis of Shoot Induced from Embryonic Axes with BAP Culture under *in Vitro* Conditions

The morphology of explants of the four bean varieties at day 1 showed the apical meristem with young primordia surrounding the dome (**Figure 4(a)**). After four days of culture, young leaves were beginning to form and cover the shoots (**Figure 4(b)**). On the eighth day of culture, the apical dome was covered by many young leaves with trichomes present on the abaxial surface (**Figure 4(c)**). At day 12, a greater number of leaves with many trichomes on the abaxial surface were observed. (**Figure 4(d)**). The trichomes on the upper leaf surface were more mature than those on the lower surface. Cuticular wax was observed in all of the varieties.

Morphological differences were observed in the epidermis of the epicotyl and hypocotyl. All of the varieties showed a papillate epidermis in the epicotyl. In the Guaymí variety, smooth cells were observed on the hypocotyl at day one; these were irregular in later observations. Papillae were present in the Brunca, Bribrí and Huetar varieties (data not shown).

Stomata were observed in the four varieties at day one, with the Bribrí variety showing the most immature stomata. At day 12, stomata were well formed, and the structures that form them (ostiole and guard cells) were well defined (data not shown).

Four types of trichomes were observed: two pluricellar (**Figures 5(a)**, **(b)**) and two unicellular (**Figures 5(c)**, **(d)**). The pluricellular trichomes were short, with globose structures of 3 - 5 cells, or simple and elongated with an enlarged base composed of two cells. The unicellular trichomes were long and simple or short and simple, both with an enlarged base.

These trichomes are agree on the classification of Reference [9]: long, thin, unicellular simple trichomes without ramifications; short unicellular simple trichomes without ramifications, present in leaf veins; short, pluricellular globose and long, pluricellular trichomes, found in the Bribrí variety at the third week of culture (**Figure 5(b)**).

In transverse stem sections, the vascular cylinder was observed on day 1 in the Bribrí variety. In contrast to the other three varieties, the ground parenchyma of the Bribrí variety was larger than the cortex parenchyma. In the

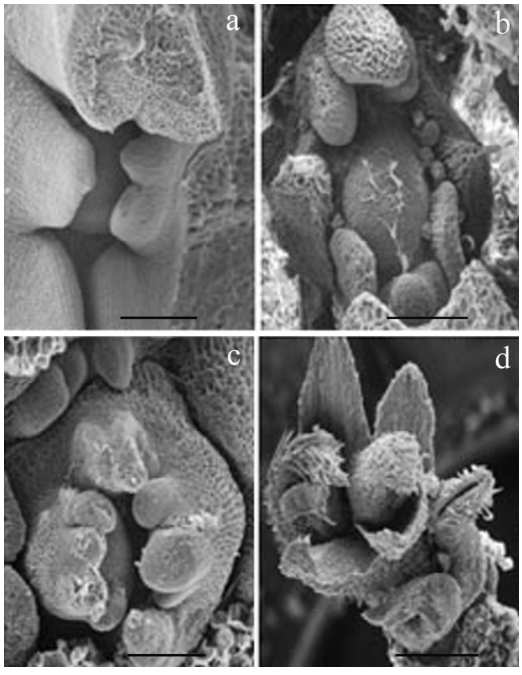

Figure 4. Scanning micrographs showing the apical dome surface for each of the four varieties of common beans at 1 - 12 days of culture in MS media supplemented with 5 mg·L^{-1} BAP. (a)-(c) formation of leaves surrounding the dome. (a) Bribrí at 1 day, scale 150 µm; (b) Brunca at 4 day, scale 100 µm; (c) Huetar at 8 day, scale 136 µm; (d) Formation of shoots covered by trichomes in the four varieties at 8 day. Brunca, scale 0.43 mm. Two shoots formed in the Brunca and Guaymí varieties, three shoots in Bribri and four shoots in Huetar.

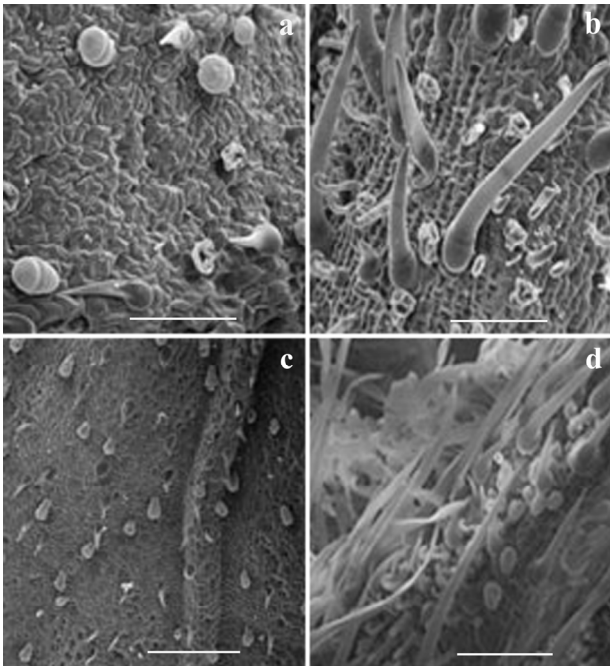

Figure 5. Trichomes present in common beans in MS media supplemented with 5 mg·L^{-1} BAP. (a) Globose short multicellular trichomes, scale 60 µm; (b) Long multicellular, formed by two cells, scale 75 µm; (c) Short unicellular trichomes in central vein and leaf lamina, scale 176 µm; (d) Long unicellular, scale 136 µm.

Brunca, Guaymí and Huetar varieties, the ground parenchyma was smaller than the cortex parenchyma. Two layers of epidermal cells were present in the four varieties. At day four the xylem tissue was well formed. Spiral and scalariform ornamentation was present on the secondary side walls of the vascular elements of the four varieties. In Brunca and Bribrí, opposite and scalariform pitting of lateral walls was also observed (data not shown).

This data shows that all varieties have a similar morphology. We couldn't observe differences in plants without BAP and plants with BAP. The only difference was the number of shoots developed in each variety. Varieties without BAP only developed one shoot.

4. Discussions

The use of the electron microscope facilitated the observation of differences and similarities in the development of the shoot induced from embryonic axis of the bean varieties studied, as these characteristics are very similar and observation with the light microscope offers limited resolution and magnification [10].

In the four bean varieties, the epidermis was more irregular at the dome tip, as the cells in this area correspond to cells of the central zone, a group of cells located in the apex tip that act as a reservoir of stem cells [11-13]. This is an area of young undifferentiated cells [14], which indicates that it is a zone of constant growth [15].

Cuticular waxes were observed over the epidermal layer in all four bean varieties with or without BAP in the culture media. These substances are present in early stages of development and serve to regulate plant transpiration and protect against dessication [14,16].

Stomata with surrounding auxiliary cells were observed. Reference [8] mentioned, in mung bean (*Vigna radiata* L.) stomata are mostly paracytic, although parallelocytic stomata with adjoining cells of unequal sizes have been observed. Reference [17] mentioned, paracytic stomata are frequent in Rubiaceae, Miosoideae and some genera of Papilionoideae.

It is important to note that although paracytic stomata are reported as the most common type in species of the subfamily Papilionoideae, parallelocytic stomata have also been reported in this subfamily [8]. In the present study, the majority of the stomata were paracytic, though some anomocytic stomata were observed in the Guaymí and Brunca varieties. Reference [16] mentioned, different types of stomata may be present in a single species.

Stomata on the surface of the embryonic axis were either sunken, at the same level as other epidermal cells, or raised above the level of the epidermis. This characteristic is variable on the plant surface [16]. Stomata were observed on both leaf surfaces (amphistomatic leaves) in the four varieties studied, a characteristic which is common in the genus *Vigna* [8].

A greater number of stomata were found on the abaxial surface. This is common in amphistomatic leaves [8], as the high density of stomata per unit of leaf area is correlated with a greater photosynthetic capacity.

Among the types of trichomes described for *P. vulgaris* L. on the adaxial and abaxial epidermis [8] are the unicellular tector trichome, in which the cells at the base have a polygonal arrangement in rosette form, and the glandular trichome with a short pedicel formed by three cells and a globose head with dense contents formed by four cells. The first type is more abundant on the adaxial surface, especially on the veins, and the second type is predominant on the abaxial epidermis. These trichomes were described by Dahlin and collaborators in three other cultivars of *P. vulgaris.*

With regard to the vascular tissues embedded in the parenchyma, xylem elements with spiral and scalariform ornamentation on the secondary side walls were observed. Reference [16] mentioned, more than one type of ornamentation may be present in vessel elements the same plant, being a characteristic of many species.

The area of exposure of the apical dome ranged from 60% and 79%. In a study with 17 Carioca and Jalo-type Brazilian bean cultivars, the apical meristem was observed to be partially (25% - 85%) exposed [6]. In some Brazilian bean cultivars, the apical meristem is partially or mostly covered by leaf primordia. In cultivars with the apical meristem 100% exposed, multiple shoots could be induced and the frequency of regeneration of transgenic plants from bombarded apical meristems was increased.

In this study, the apical dome had acquired a larger size and primordia had begun to develop by the fourth day of culture. Shoot formation was initiated on the eighth day and by day 12 shoots could be quantified by size (greater than 1 cm). In the Costa Rican varieties, the majority of the shoots developed in the peripheral zone of the meristem. Other studies have demonstrated that *de novo* shoot differentiation in bean embryos using cytokinins appears in the peripheral layer of the meristematic ring [6]. Using BAP, shoot formation was initiated on the second day of culture, with more than four shoots by day five. The majority of the shoots appeared in the peripheral zone of the meristem. A large number of shoots appeared between the seventh and tenth days of culture, but few reached more than 1 cm in size.

This is the first morphological study of shoot induced from embryonic axes development in Costa Rican bean varieties, using scanning electron microscopy. These observations indicate that the BAP stimulated shoot formation. This study is important for future research in

plant regeneration *in vitro*, in order to contribute to the optimization of the morphogenic process and *in vitro* regeneration of common bean varieties.

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Abbreviation

SEM: scanning-electron-microscopy
BAP: N^6-benzylaminopurine
MS: Murashige & Skoog
AS: Adenine sulphate.

