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RESEARCH ARTICLE

THE EFFECTS OF *IN VITRO* CULTURE ON THE LEAF ANATOMY OF POTATO (*SOLANUM TUBEROSUM* L. CV. ARIZAONA)

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ABSTRACT

The leaves of *Solanum tuberosum* has been studied in plants cultivated both *in vitro* and ex vitro under illuminated growth chamber conditions during acclamation period, as well as conventionally plants in natural conditions. These conditions induce anatomical alterations, the results showed that number of stomata in the acclimatization plants greater than in the conventionally plants. The results showed that similarity plantlets producing from tuber *in vitro* with field plants in some anatomical characteristic. There are two types of stomatal types in plantlets producing from explant, while found five types in plantlets produced from tuber and field plants. As well as the results showed that palisade and spongy layers had greater air space within it and the cells of these leaves were irregular shape. In contrast, the anatomy of leaves under plantlets produced from tuber and conventionally plants were noticed reduce air space between cells and the cells more arrangement. In plantlets produced from explantmidrib contains continuous layer of palisade layer which losing in other treatments.

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INTRODUCTION

Potato (*Solanum tuberosum* L.) belongs to the family Solanaceae, it is an economically important crop of the world (Heywood, 1979; Gebre and Sathyanarayana, 2001). Potato represent one of main cultivated plants of the temperate zone, and extensively cultivated from the cool hills to the warm regions, moist and humid plains (Ciobanu, 2013). Potato is highly nutritious and contains carbohydrates (22%), proteins (2%), fats (0.1%) and water (74%).It is also rich in minerals and trace elements potassium, sodium, iodine and magnesium, folic acid, pyridoxine, vitamin C, ascorbic acid, Iron (8-10 %), thiamine and riboflavin (Gebre and Sathyanarayana, 2001; Pandey and Chadha, 2009; Ashrafzadeh *et al*., 2015). Anatomical studies assume greater importance when associated to ecological, physiological and comparative aspects (Metcalfe and Chalk, 1979; Da Costa *et al* 2004).Anatomical abnormalities are common on plants grown *in vitro* (Ehrendorfer, 1971; Reeve; Sherman, 1993; Righetti *et al*., 1993). After transfer of plantlets from *in vitro* cultures to the greenhouse or field, substantial changes on the leaves anatomy and morphology are observed, especially on the size, shape and distribution of epidermal cells, thickness and differentiation of leaf mesophyll, and number and structure of chloroplasts (Pospisilova *et al*., 1999).

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During acclimatization, most plants lose during this stage, or reduce survival rates of plantlets during the period of acclimatization (Marin *et al*., 1988). The reasons for this difficulty include improperly functioning stomata reduced amounts of epicuticular wax, poor cuticle development, which result in excessive water loss and poor photosynthetic capacity and anatomical abnormalities (Grout, 1975; Brainerd and Fuchigami, 1981; Sutter and Langhans, 1982; Wetzstein and Sommer, 1982; Donnelly *et al*., 1984; Grout and Millam, 1985; Van Huylenbroeck and Debergh 1996; Hazarika, 2006). The aim of this work is thus to acclimatization potato plants by using plantlets produced from callus and tuber tissue culture in addition field plants understand the effects of change conditions on the growth and anatomy of tissue cultured potato plantlets (*Solanum tuberosum* L.) both in and ex vitro and compared with field plants, as well as this review is described some ways of improving plant survival and resistance the conditions during acclimatization.

MATERIAL AND METHODS

Source of Explants

Potato (*Solanum tuberosum* L. CV. Arizaona) tubers plants were certified cv. Arizaona were bringing from Basra directorate culture, the tubers were washed thoroughly and

incubated in 20 –22°c at 16 hours' photoperiod.in illuminated growth chamber.

Preparation of media

Potato plants were cultivated in growth chamber (*ex vitro* conditions) on College of Science, Basrah University, for*ex vitro* analyses were conducted with 45 days old plants, 40 cm tall, and *in vitro* analyses were conducted with 30 days old shoots regenerated from potato cultured *in vitro* on Murashige and Skoog (1962) medium from Caisson American company which consisted of meso-inositol (4.43 gm L^{-1}), thiamine HCl, adenin sulphate (0.04 gm L^{-1}) nicotinic acid (0.005 gm L^{-1}) and pyridoxine (0.005 mg L^{-1}). Sucrose was provided at 30 g L^{-1}. The pH of the medium was adjusted to 5.7, a semi-solid medium was desirable agar. The medium was dispensed in to tubes (2x 20 cm) were used for growing cultures. Flasks were stoppered with nonabsorbent cotton plugs, and then covered with aluminum foil. The media-containing vessels were then autoclaved for 20 minutes at 121°C.

Media for microtubrization: Liter of MS basal salts in addition to BA (3mg/L) sucrose (60gm/L) was prepared and dispensed in 8 parts followed by the addition of salicylic acid 100 mg/L.

Acclimatization of Potato Plantlets

Acclimatization studies were carried out during winter and summer. Plants used for adaptation were previously proliferated *in vitro* in 2% PEG. Before transplantation, agar was gently washed off the roots with tap water. Plants were transplanted into 10 cm plastic pots composed of Sand: Petmose (coco compact Potting) at a ratio of 1:2 by volume. Caps were loosely opened for the first two days, and then totally removed for the remaining three days of acclimatization to expose the plants to the natural environment while in the test tubes. After five days of acclimatization in the test tubes, the plants were taken out, washed and transplanted into the same soilless mix. All plants were transferred to an open bench and grown under illuminated growth chamber conditions. The number of stomata and cells in tissue culture-derived and conventionally-propagated plants was examined to ascertain stomatal function and influence relative to plant survival upon transfer from *in vitro* to natural conditions.

Anatomical study

For anatomical analyses, samples were fixed in F.A.A. fixative, dehydrated in ethanolic series, and embedded in paraffin. Transverse sections 17-20 µm thick, were made in rotary microtome, and stained with Safranin and Fast grain. Epidermal fragments were obtained with Jeffrey's mixture (Johansen, 1940), and were stained with 0.5% Safranin. All samples were measured on photos from transmission light microscope**.**

RESULTS AND DISCUSSION

Stomata density: The results showed that all leaves cultivated *in vitro* (explant and tuber) and conventionally (field plants) are amphistomatic, by having stomata on the adaxial (upper epidermis) and abaxial surface (lower epidermis). The stomata on abaxial surface more than adaxial surface. There are different between plantlets producing from cultured explant and tuber *in vitro* compared with conventionally plant, the results showed that the conventionally plant and plantlets acclimatization producing from tuber *in vitro* more developed by having five major types of stomata are recognized types (Figure 1).

- **Anomocytic type:** The stomata are anomocytic in which the guard cells are not surrounded with any subsidiary cell.
- **Anisocytic type:** The guard cells are surrounded by three unequal sized subsidiary cells, the common wall of which is at right angle to the longitudinal axis of stoma**.**
- **hemidiacytic type:** one of subsidiary cells enclose the stoma.
- **Paracytic type:** In the paracytic type guard cells are accompanied by two subsidiary cells, the longitudinal axis of which are parallel to that of the guard cells and aperture.
- Hemiparacytic type:one of subsidiary cells parallel the stoma from one side.

In contrast, the plantlets produced from explant contain two types of stomata anomocytic and hemiparacytic type (Figure 1).

Commonly stomata anomocyticfound in all treatments, anaisocytic, hemiparacytic and hemidiacytic stomata are randomly distributed on both surface of plants. More rarely, diacyticstomata are observed on the regions of the epidermal surfaces of the leaves of field plants and plantlets producing from tuber tissue culture.Guard cells in stomata are considered to be one of the most highly differentiated living cell types in plants (Zeiger, 1983), It is having one of the highest degrees of morphological adaptation and unique metabolic organization. As well as, guard cells can be induced to divide in situ and that regenerable callus is produced, and possible to induce a reexpression of full embryogenic in these cells (Sack, 1987; Assmann, 1993; Hall *et al*., 1996).

Measurement of stomata are summarized in Table (1) and Figure (1). Number of stomata greater in the field plants than in the acclimatization plants, on the other hand, the stomata on abaxial surface greater than adaxial plant. There are differences in stomata number between treatments, there was a significant an explants of adaxial and abaxial surfaces were 21 and 112.5 stoma/mm difference in the average number of stomata of conventionally plants was82 and 117.42stoma/mm^2 respectively. Many researchers reported that the decreased density of stomatal and epidermal cell number may affect plant growth physiology, which caused altered leaf structures and increased disease susceptibility (Zhao *et al*., 2003). The reduced stomata in leaves of acclimatized plants may have been due to cell enlargement of leaves. The similar results found strawberry plants (Fabbri *et al* 1986), apple plants Blanke and Belcher (1989). They reported that main the result of cell enlargement of leaves was increased in size, as well as the increase in cell number. Many factors have been found to cause anatomical changes in modified plants, such as differences in moisture levels, concentrations of ethylene, oxygen and carbon dioxide. (Mohamed and Alsadon, 2009). Stomatal density decreased after transplantation (Wetzstein and Sommer 1983, Noé and Bonini 1996, Tichá *et al*. 1999).

Table 1. Measurement of stomata and epidermis cells of Potato plantlet

Treatment	Upper epidermis (um)		Lower epidermis(um)		Upper stomata (um)		Lower stomata (um)		No. of cells mm^2		No. of stomata mm^2	
	length	width	length	width	length	width	length	width	upper	Lower	upper	Lower
conventionally plants	(22.5-125) 70.55	(32.5-50) 39.16	(30-130) 80.5	(30-52.5) 41.66	(35-50) 42.77	(25-37.5) 33.33	(30-45) 39.64	(22.5-35) 25	(312-474) 399.12	(301-432) 384.10	(78-84) 82	(48-192) 117.42
Tuber plantlets	(42.5-100) 76.38	(25-57.5) 40.25	(25-85) 55.62	(27.5-40) 31.07	(30-50) 34.43	(22.4-29.34) 25.53	(22.5- 37.5) 31.87	(20-37.5) 23.61	(330-390) 355.5	(570-900) 675.20	(54-84) 64.5	(210-276) 261.6
Explant plantlets	(32.5-120) 78.63	(42.5-100) 70.35	(50-125) 91.78	(25-67.5) 50.41	(30-47.5) 36.87	(24-30) 27.5	(30-42.5) 35.83	(20-30) 24.64	(138-210) 177	(372-492) 448	(12-30) 21	(102-126) 112.5

Table 2. Measurement of Leaves of Potato plantlet

Treatment	Cuticle thichness (um)	Leaf blade thichness (um)	Upper epidermis (um)	Lower epidermis (um)	Balisade layer thichness (um)	Spongy layer thichness (um)	Bundle sheath thichness (um)	Mid rib thichness (um)	No. of Xylem
Conventionally plants	(12.5-25 8.22	(285- 300) 295.10	(7.5- 52.5) 28.37	(12.5- 27.5) 19.52	(125- 162.5) 149.2	(75- 100) 90	(175- 200) 184.3	(475-562.5) 510.4	9-11
Tuber plantlets	(7.5- 25) 16.5.	(375- 450) 405.10	(12.5- 37.5) 27.9	(12.5- 30) 23.05	(162.5- 187.5) 175.21	(150- 162.5) 155.8	(355 -300) 300.20	(132- 305) 200	20-22
Explant plantlets	(15 – 2.5) 7.60	(250– 300) 277.5	(22.5- 42.5) 24.5	(10.5- 37.5) 18.32	(75- 100) 86.10	(25- 112.5) 62.5	(125- 152.5) 139.40	(287.5-330) 312.5	6-8

The total numbers of stomata per leaf were more than doubled in ex vitro plants (Pospíšilová *et al.* 1998; Pospíšilová *et al.* 1998). Some researcher showed that *in vitro* grown plantlets had ring-shaped stomata, but in leaves of ex vitro transferred plants stomata were elliptical (Marín *et al.* 1988, Noé and Bonini 1996, Tichá *et al*. 1999). In field plants and plantlets produced from tuber tissue culture stomata are rounded or elliptic shaped present on either side (Amphistomatic leaves), and often more numerous on the abaxial epidermis is the remaining species. This characteristic difference in stomata produced from explant, it was elliptic elongated with pore of stomata closed, in stomata of field plants were elliptical or rounded with a narrow opening pore, the average long/ width in plantlets produced from tuber was 34.43 x 25.53- 31.87 x 23.61 um on upper surface and lower surface respectively, while those of *in vitro* plants were more spherical. Some researchers note that the stomatal complexes are elliptical in the field plants while circular in plants grown *in vitro* (Sha Valli *et al*., 2003). The average length of stomata in the adaxial surface and abaxial surface reneged between tuber tissue culture 42.77x 33.35um and 39.64x25um in field plants. In adaxial surface showed that epidermal cells of acclimatization potato leaves varied from the conventionally plants were irregular and strongly undulate anticlinal walls (Fig. 3). However, the anticlinal walls of epidermal cells sinuate in the field and plantlets produced from tuber culture, in addition the anticlinal wall cells more thickness, but it was thinner and straight to curved plantlets produced from callus, while the lower epidermis was strongly undulating of all treatments.

The cells were also varied in size and shape, it was in conventionally plants 76.38 x 40.25 and 55.6 x 31.07 on adaxial and abaxial respectively, the cell size bigger in culture plantlets especially on lower surface 78.63 x 70.35 and 91.78 x 50.41 um (Table1).

Transverse sections of leaves

The leaves of *Solanum tuberosum* are dorsiventral, with a prominent midrib vein which very clearly in plants grown in field (Figure 2). There was some different between field plants and plantlets produced from *in vitro* (explant and tuber). Plantlets produced *in vitro* from tuber was nearly similar in level rates compared with field plants. The results in Figure (2) and Table (2) showed that reduced cuticle layer in plantlet produced *in vitro* from explant, but observed high levels of epicuticular wax found plantlets producing from tuber tissue culture was (average 16.5 um), the reduced amounts of cuticle layer on acclimatized plants could be attributed to the rapid cell enlargement in expanding leaves, more rapid than the rate of wax formation. These results similar with (Sama *et al*., 2014). Transverse sections of leaves showed that the average of upper epidermis on the acclimization plants longer compared with field plants and plantlets producing from tubes, it was 28.37 and 27.9 respectively, while the average of lower epidermis of plantlets producing from explant was 24.5 um.

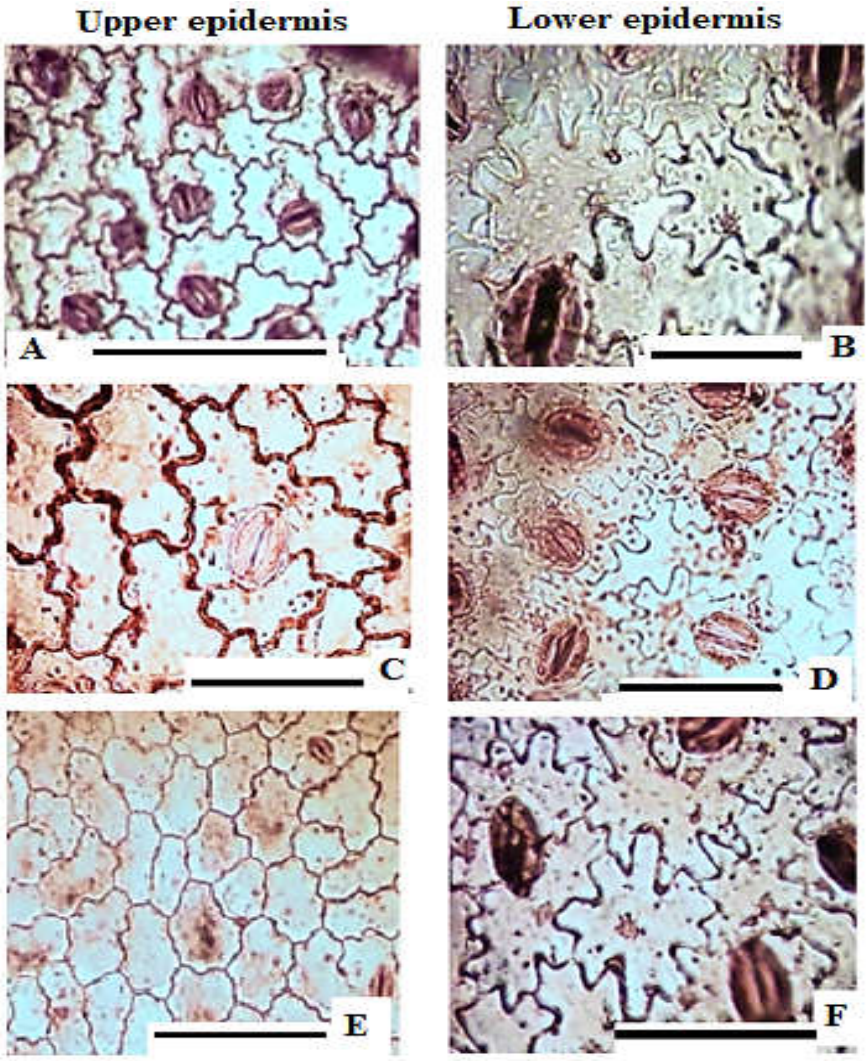

Figure 1. Plantlets of potato A, B conventionally plants C, D epidermis of plantlets producing from tuber tissue culture; E,F epidermis of of plantlets producing fromex plant tissue culture

The average of leaf blade more thickness of plantlets produced from tuber*in vitro* (405.10 um), while it was thinner in plantlets produced from explant (Table 2). The thickness of palisade layer was reduced in plantlets *in vitro* from explant tissue culture, it was 86.10 um. Palisade and spongy layers of culture plants from explant had greater air space within it (Figure 2 E, F). The cells of these leaves were irregular shape. In contrast, the sections of leaves of plantlets produced from tuber and field plants. Mesophyll consist of one layer of palisade layer, it was differed from field plants and plantlets produced *in vitro* (Table 2). Transverse sections in Figure (2) showed that conventionally plants and plants development from tuber tissue culture not contain palisade layer on the midrib between upper epidermis and vascular bundle, but can observed one layer of epidermis rounded parenchyma cells and followed by 1-4 layers' collenchyma cells, while plants culture *in vitro* from explant palisade layer is observed continuous layer occurred over the vascular bundles and represented continuous layer with mesophyll (Fugure 2 F). Leaves of these plantlets grown *in vitro* especially plantlets producing from explant consist of poorly developed mesophyll with large intercellular spaces and low number of inadequately functioning stomata (Brainerd *et al.* 1981, Donnelly and Vidaver 1984, Johansson *et al.* 1992, Tichá and Kutík 1992; Dami and Hughes 1995; Noé and Bonini 1996; Van Huylenbroeck and Debergh, 1996; Hazarika, 2006).

Some researcher reported that underdeveloped palisade tissues is characteristic of *in vitro* cultivated leaves, indicating a pattern of reduced cell division, development, or differentiation for *in vitro* grown plants. Reduced of cell divisions in plants cultivated *in vitro* causes low gas exchanges and ethylene production Fidelis *et al*. (2000) Hazarika (2006). The mesophyll cells of *in vitro* grown plantlets had limited cytoplasmic content, and flattened chloroplasts with irregularly arranged internal membrane systems (Wetzstein and Sommer 1982), as well as, increase air spaces in mesophyll compared to leaves of plants grown under field conditions (Hazarika, 2006). In addition, the plantlets produced from explant influence the internal conditions in flask and these results agree with (Zobayed *et al*., 2001a; Marino; Berardi, 2004; Goncalves *et al*., 2008; Ribeiro *et al.,* 2009). Spongy tissue followed the palisade layer, it consists of several layers (5-8), lobed, semicircular and rounded cells, and contains air space between cells. In plantlets in vitro contains more air space (Figure 2 E,F),this result agree with (Brainerd; Fuchigami, 1981; Fidelis *et al*., 2000). The results showed that reduced vascular bundles in the middle of the mesophyll in *in vitro* culture, and sand crystal observed throughout the mesophyll and midrib of all treatments, In vitro tissue cultured are maintained in small sealed flasks, which have high relative humidity, high sugar, artificial temperature, there is also a low level of air exchange between the external and internal environments, low active photon flux density, elevated CO2 fluctuation, salt and plant

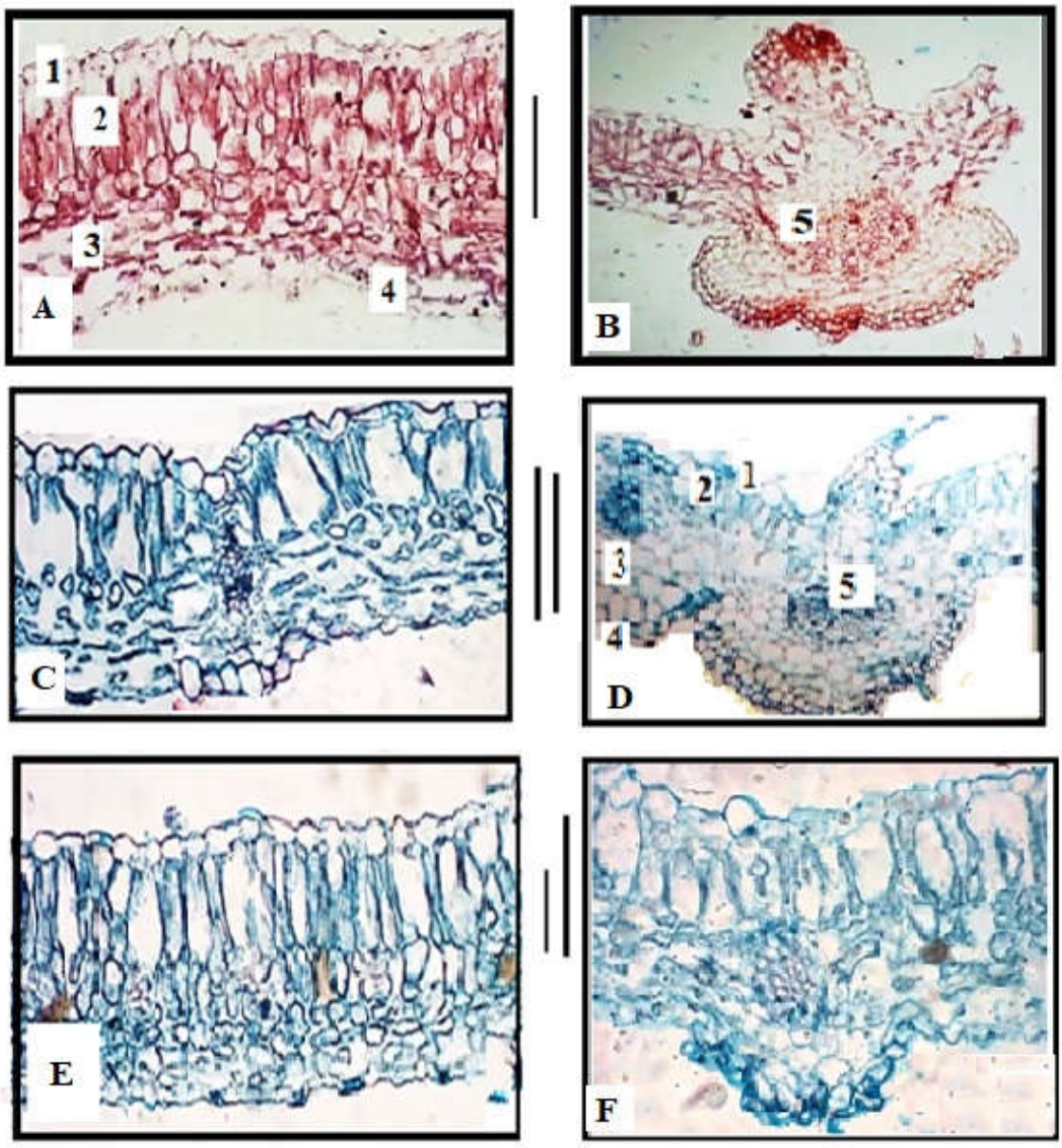

Figure 2. Leaves pf potato plants cultivated in vitro and ex vitro. A, B –leaves of field plants with reduced intercellular spaces ; C, D – Ex vitro cultured leaf of tuber with contains little intercellular spaces; E, F - In vitro cultured leaf with more air spaces. A, C, E- leaves; B, D, F- midrib. Upper epidermis, 2- Palisade layer3- spongy layer 4- Lower epidermis 5- vascular bundle

hormone contents in the medium (Aitken-Christie *et al*., 1995; Chen, 2004). These conditions may induce anatomical and physiological alterations in cultivated plants (Rodrigues *et al*., 2014).

Conclusion

The diagnostic features, such as stomatal density, air space in palisade and spongy layers and reduced the vascular bundle of the plants cultivated in vitro indicate possible problems during the acclimatization process. But when culture tubers produced from tissue culture should provide better characteristic *in vitro*, developmental conditions for the plants, and pear characteristic similar with field plants and improving the morphogenesis, and anatomical structure of the plantlets produced from callus influence the internal conditions in flask. Current results reinforced the proposal of using anatomical diagnosis to help adjusting culture conditions aiming to improve survival rate and quality of micro propagated plants.

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