I. INTRODUCTION

Developmental biology actually focuses on the quantification of cellular properties during development and experimental data are useful to analyze the plant (Kiss et al., 2017; Prusinkiewicz & Rolland-Lagan, 2006) development of their cellular structures, tissues and organs. A variety of computational techniques have been proposed to improve the visualization of data by the particular biological components like individual cells and cell walls, establishing their geometry and connectivity, through the extraction of important information that includes image processing, digital morphology and computational geometry (Haseloff, 2003; de Reuille et al., 2005; Jönsson et al., 2005). This requires the analysis of cells or tissues by segmentation to generate its computerized versions useful to be easily analyzed. Authors note that quantitative assessment of shape changes in growing tissues requires precise identification of its geometry at different scales taking high-quality images and the conversion to a digitized format by process known as segmentation (Kiss et al., 2017). It is known that in vitro massive growth of disorganized plant cells named "callus" or "calli" derived from a single differentiated cell with totipotential ability; regenerate a whole plant considering their high plasticity as the principal characteristic for their differentiation. Ikeuchi et al. (2013) also established that calli are morphological diverse and may be classified based on their macroscopic characteristics. Zimmerman, (1993) and Frank et al. (2000) established two principal kinds of them; one with no apparent organ regeneration called as friable or compact callus and the other that shows some degrees of organ regeneration called rooty, shooty, or embryonic callus. Iwase et al. (2011) established that this diversity is a consequence of their cellular gene expression profiles giving some degrees of differentiation when the balance between auxins and cytokinins that determines the state of differentiation and dedifferentiation. CellSet software can perform quantitative analysis of the resulting cellular geometry data quickly, converting the two-dimensional positions of all cell walls into biologically relevant measurements, including cell area and wall length (Pound et al., 2012). This work analyzed the application of CellSet software to analyze the morphogenetic development of Fouquieria splendens callus by segmentation analysis of images.

II. MATERIALS AND METHODS

A. Fouquieria splendens Callus Culture and Development

Branches of the desert plant F. splendens, were collected from the Botanical Garden of the Facultad de Estudios Superiores, Iztacala (FES)-UNAM and maintained in water 15 days for the development of leaves. Leaves were surface-sterilized with sodium hypochlorite solution (10%) for 90 seconds, rinsed three times in sterile distilled water and leaf explants were obtained aseptically cutting fractions of 1cm². Five explants were placed separately in baby food flasks with Magenta SIGMA caps containing 25 mL of Murashige and Skoog (MS) ¼ salts “MSA” medium with 42.5 mg/L
NaH₂PO₄ + 10mg/L BAP (Bencil Amino Purine) + 1mg/L NAA (Naphthalene Acetic Acid) and supplemented with 30 g/L of sucrose and 3 g/L phytagel. Flasks were incubated at 28 °C under photoperiod of 16h light/8h dark with a fluorescent Phillips T8 32 Watts 5000 °K lamp, finally, all the experiments were performed by 3 replicates and growth was analyzed at 21 days.

B. Analysis of Growth by Image Morphological Changes of Fouquieria Splendens Callus

Selected fractions of F. splendens callus were prepared for scanning electron microscopy (SEM) for their analysis of morphological changes, according to Corona-Álvarez et al. (2018) as follows: samples were cut into 4 or 5 mm pieces, fixed in 2.5% glutaraldehyde for 1h at room temperature (27 °C), then, washed three times in a phosphate buffer, postfixed in 1% OsO₄ for 1h at room temperature, washed and dipped into distilled water for three times. The samples were undergone a series of dehydration processes: 30 % ethanol for 10 min; 40 % ethanol for 10 min; 50 % ethanol for 10 min; 60 % ethanol for 10 min; 70 % ethanol for 10 min; 80 % ethanol for 10 min; 90 % ethanol for 10 min and finally 100 % ethanol for 10 min (three times). The material was dried in critical point dryer apparatus, mounted and sputter-coated with gold in an ion coater for 60 seconds. Finally, the samples were ready for viewing under field scanning electron microscope JEOL-JSM 5800 LV for their examination and photography.

C. CellSet Analysis

A series of callus SEM images were obtained between 40X to 45X augments, selected and adjusted to analyze by CellSet software that includes image segmentation to extract and identify zones to be analyzed by the perforation of a number of direct measurements on the cell network delimited and related with cell areas, circularity and the mean of total integrated intensities of each individual wall segment and for each cell. These geometry and numerical data considered as shape descriptors were exported as text and excel files and finally a statistical analysis of individual cells or groups of cells was done.

D. Statistical Analysis

All data obtained from the CellSet software applied to F. splendens callus selected SEM images were analyzed at first by frequency histograms and selected intervals were represented by matrix plots and finally, Principal Component Analysis (PCA) was done considering the shape descriptors: area, circumference and intensity compared with the selected SEM images; measuring its variance-covariance correlation by the employ of PAST software (Paleontological Statistics Software Package) Ver. 4.09.

III. RESULTS

A. Image Analysis of F. splendens Callus Induction

At first, is important to note that the in vitro response of plants induce the cell proliferation producing a tissue named “callus” formed by an amorphous cell mass (Dods & Robert, 1985; Aitchison et al., 1978) and the phytohormonal composition of the medium is the most important factor for

in vitro regeneration; considering also that the morphogenetic capacity of explant tissues can produce organogenic callus and differentiation via organogenesis or embryoidogenesis (Jéhan et al., 1994; Shimizu et al., 1996; Wang et al., 1999; Boltenkov et al., 2007). In this study, the medium employed (MSA) has a particularly phytohormones concentration plus phosphate salt and both induced the production of abundant calli. CellSet software applied to the F. splendens SEM callus images by serial steps determined the characteristics of them. Fig. 1 shows the CellSet software sequence of selected images (Ia.1, Ib.1, Ila.1, Iib.1, IIIa.1 and IIIb.1) from different sides of F. splendens callus: first, the cells segmentation by the employ of a line-drawing algorithm, selecting cell walls contours represented by a series of interconnected node points, with wall end points connected to others via junction nodes ( Ia.2, Ib.2, Ila.2, Iib.2, IIIa.2 and IIIb.2). Second, as Pound et al. (2012) this algorithm calculate the mean and total cell areas (Ia.3, Ib.3, Ila.3, Iib.3, IIIa.3 and IIIb.3) and third, a heat maps showing the average intensity for highlighted cell walls (Ia.4, Ib.4, Ila.4, Iib.4, IIIa.4 and IIIb.4).

![Fig. 1. Scanning electronic micrographs of selected Fouquieria splendens callus (Ia, Ib, Ila, Iib, IIIa and IIIb) analyzed by CellSet software, where sequence shows: 1) original images of non-morphogenic callus; 2) segmented SEM images; 3) contoured SEM images and 4) intensity (heat map) SEM images.](image-url)
indicates a particular relationship between I and III images with intervals: 15, 17, 18, 21, 23, 24, 25 and 28 with the following area dimensions (AU): 700-750, 800-850, 850-900, 1,000-1,050, 1,100-1,150, 1,150-1,200, 1,200-1,250 and 1,350-1,400, respectively. Fig. 3b analyzed the callus classification according to the selected circumference intervals and their relationship only between images I and II; where Component 1 values (99.09%) with intervals: 10, 11, 12, 13 and 14 with the following circumference dimensions (AU): 140-156, 156-171, 171-187, 187-203 and 203-218, respectively. Finally, Fig. 3c shows the analyzed callus classification according to the heat map (cell walls intensity) and their relationship between images I and II, where Component 1 values (97.51%) with intervals: 3, 12, 14 and 15 with the following intensity dimensions (AU): 3,622-5,433; 19,924-21,735; 23,546-25,357 and 25,357-27,169, respectively. Component 2 (1.83%) with the association between image III only with intervals: 16 and 17, with the following intensity dimensions (AU): 27,619-28,980 and 28,980-30,791, respectively.

IV. DISCUSSION

From all these data, F. splendens selected callus image grown in MSA medium showed a characteristic shape descriptor obtained with CellSet software, where the most important data obtained to characterize the development of calli was the area descriptor. PCA analysis allows the comparison between SEM images I and III, showing that the size of cells was diverse; 700 to 1,150 AU with 100 to 84% of the cells measured and for Image II area size between 600 to 800 AU in 100 to 78% of the cells measured. Circumference descriptor was not adequate because it only could be compared between images I and II where the data showed only one highest data (140 to 171 AU) that was unable to analyze and determinate regarding to the morphogenesis process in F. splendens calli development as intensity descriptor (heat map) with values that also does not reflect any important information. Finally, in this study regarding to the morphometric analysis of F. splendens callus development appearance and growth was according to Meftahizade et al. (2000) and Gopi & Vatsala, (2006) whose mention that the high concentration of cytokinins like BAP instead of auxins, induced calli with lighter green and translucent appearance and as Smolenskaya et al. (2007) reported, the maintaining of callus lines in cultures showing also a diverse area size with the CellSet software related to the morphogenesis state of them to induce callus formation.

V. CONCLUSION

Image analysis that involves the employment of cell segmentation’s software allows the tissue geometries description and become important strategies to the plant in vitro developmental research. In this work, the morphological changes of Fouquieria splendens callus were analyzed by CellSet software, showing that the area shape descriptor was useful to describe the cell size and distribution as product of the induced regeneration of plants grown under in vitro conditions; even this software applied to scanning electron images does not cover all the details, it gives an initial approximation and view of how this interesting desert plant species begin to regenerate at 21 days.
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