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Scanning electron microscopy and histological analyses of *Ficus carica* L. cv. Black Jack

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Abstract A protocol of micropropagation is established for *Ficus carica* L. cv. Black Jack with excised apical buds from one-year-old mother plant as *in vitro* explant materials. The response of explants on BAP and 2-iP at different concentrations (0, 10, 20, 30 and 40 µM) were investigated via histological analyses and scanning electron microscopy (SEM). Isodiametric cells and vascular tissues were observed from the cross-sections of apical buds under the treatments of BAP and 2-iP at all concentrations via SEM. Histological analysis confirmed that both cytokinins tested at different concentrations involved the formation of actively dividing meristematic cells prior to differentiate to parenchymatous tissues. It is also proven that more vascular tissues were found at lower epidermal layers. Histological analysis was proved that better response for callus induction was obtained by the adaxial surface on the leaf in contact with the media. SEM analysis confirmed that no structural distortion caused by cytokinins.

Keywords: Fig , BAP, 2-iP, Microscopy analysis

Introduction

Ficus carica L. is commonly known as Fig which is an ornamental plant under the Moraceae (Ronsted *et al.*, 2005; Somashekhar *et al.*, 2013). Presently, the micropropagation technique has been applied to propagate this plant which is difficult to be propagated through conventional practices such as grafting and air-layering leading to low survival rates. With plant tissue culture technology, *Ficus carica* L. could be propagated on a large scale with a high survival rate. Scanning electron microscopy (SEM) and histological study are vital to examine the anatomical, cellular structure and arrangement of the plant. SEM is commonly practiced in the three-dimensional structures of cells and plant surface structures, for instance, stomata, trichomes and waxy cuticles (Ross *et al.*, 2003; Saha *et al.*, 2011). Mamoucha *et al.* (2016) had studied the leaf trichomes and stomata micro-morphology of *Ficus carica* L., where two types of trichomes, as protective and capitate trichomes that found in the abaxial surface of the leaf. Another

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advancement from the SEM technology is the instalment of cryo-unit at SEM to view samples at subzero temperatures. This technique was used for water translocation in the plants with the application of freeze fixation with liquid nitrogen (Yazaki *et al.*, 2019). Histology is an important division of biology that practices an extensive array of methods to extend visual perception with the connection between microanatomy and the roles of cells and tissues in animals and plants (Yeung, 1999; Ross *et al.*, 2003). The histological technique provides additional insight into the cellular process as well as the clues leading to the hypotheses for further experiments. A study was conducted by Mamoucha *et al.* (2016) who concluded that histological study on leaves of *Ficus carica* L. was able to localize the presence of idioblasts and laticifers. They highlighted that condensed tannins were accumulated in the dilated lower trichomes as well as in the epidermal cells. In regard to the research conducted by Mamoucha *et al.* (2016), they pointed out that extraction could be based on the histological analysis of leaves of *F. carica* L. Besides, a comparative histological investigation was carried out by Chirinéa *et al*. (2012) on the leaves *in vitro* and acclimatized plantlets. They concluded that little differentiation and a higher number of stomata were shown *in vitro* leaf of *F. carica* L. cv. Roxo de Valinhos as compared to leaves of acclimatized plantlets. The study dealt with the comparative performances of cytokinins at various concentrations on the multiplication of shoots from apical buds.

The objectives were to carry out SEM and histology analyses to investigate the effects of cytokinins on the apical buds, and the anatomical structures of leaf were studied on a better clue for the proposed hypothesis for callus induction.

Materials and methods

Plant materials and culture conditions

The mother plants or source plants were one-year-old *Ficus carica* L. cv. Black Jack. The apical buds were surface sterilized and cultured in Woody Plant Medium (WPM) supplemented with 3.0 g/L of activated charcoal for one week, then cultured in WPM medium supplemented with 6-Benzylaminopurine (BAP) and 2-iP at concentrations of 0, 10, 20, 30 and 40 µM for each plant growth regulator for four weeks. The cultures were incubated in the Plant Tissue Culture laboratory under 16-hours photoperiod (16 hours of light and 8 hours of dark conditions) (Philips TLD, 36 W, 150 µmol m^{-2} s^{-1}) with maintained humidity at 55 % and temperature of ±25˚C.

Scanning electron microscopy analysis

The freeze-drying technique was employed in scanning electron microscopy (SEM) to view the samples. The used samples were 2 mm thin

transverse section of the basal part of five-weeks-old *in vitro* apical buds of *F. carica* L. cv. Black Jack above semi-solid media. A SEM planchette was prepared with double-sided tape (Tissue-Tek) which placed on a Whatman® No.1 filter paper (90 mm) in a Petri dish. The filter paper was dampened with a few drops of 2 % osmium tetraoxide (OsO_4) and the Petri dish was covered promptly. The sample was left in the fume hood for an hour to undergo vapour fixation. The planchette with vapour-fixed samples was plunged to slushy nitrogen at -210° C. The samples were placed on the Peltier-cooled copper stage of the freeze dryer (Emitech K750) and left to freeze-drying for 10 hours. If the samples are not being observed immediately after the process, the planchettes with the freeze-dried samples were carefully stored in a desiccator. Before viewing the samples in the SEM individually, the samples were sputter-coated with 5-10 nm of gold. The coated samples were viewed in Leo Supra VP-Ultra High-Resolution Analytical Field Emission Scanning Electron Microscope (Carl Zeiss SMT).

Histological analysis

Transverse sections *in vitro* apical buds and the first leaf from the shoot tip of *F. carica* L. cv. Black jack about 2 cm^2 for each treatment was analyzed histologically. The leaf was cut into two parts from the petiole section and leaf segment. Transverse section of the basal part of five-weeks-old *in vitro* apical buds 5 mm above semi-solid media was taken for the histological study. The leaf and transverse sections at the basal part of the apical buds were fixed in FAA solution (Formalin: Acetic acid: 95 % Alcohol in the ratio of 1: 1: 1) for a week at 4°C. The samples were undergone gradually dehydration using ethanol and tertiary-butyl alcohol (TBA) for increasing concentration. Dehydration series for apical buds required a longer duration than leaf sections. The samples were embedded in Paraffin wax and followed by incubation in an oven at 60°C (Memmert, Germany). The embedded samples were made into Paraffin wax blocks via an embedding machine (Leica EG 1160, Germany). The blocked samples were sectioned to the thickness of 6 µm for *in vitro* apical buds, while 11 µm for mother donor plant leaf sections. The sectioning was carried out by using 6 Micron Microtome (Leica RM 2135). The sectioned samples were held on glass slides and allowed to dry overnight in an Ecocell incubator at 40°C (MMM Medcenter Einrichtungen GmbH). The staining of samples took place with 0.1 % safranin red O and 0.05 % fast green and mounted on the glass slides using a drop of DPX mounting agent (AMRESCO H157-475ML) and covered with a coverslip. The samples were observed under a light microscope (Olympus BX41) in the Electron Microscopy Laboratory at the School of Biological Sciences, USM.

Results

The control treatments, which were basal WPM and WPM fortified with 2-iP at all concentrations, no multiple shoot formation was observed (Figure 1). On the other hand, treatments with BAP at different concentrations were found effectively to induce multiple shoots and leaves for *Ficus carica* L. cv. Black Jack. The highest rate of shoot induction and multiplication were observed *in vitro* shoot of *F. carica* L. cv. Black Jack treated with WPM added 20 µM BAP (Figure 1C). Therefore, the anatomical study on the cross-section of the basal shoots was carried out via SEM and histological studies.

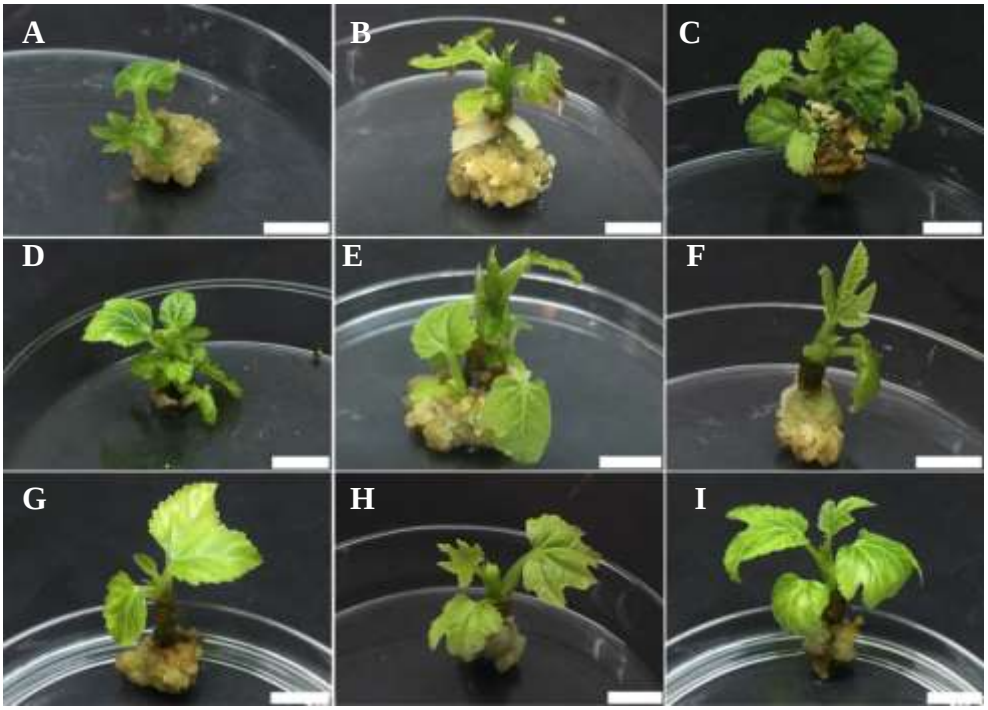

Figure 1. Shoot induction of *Ficus carica* L. cv. Black Jack on WPM supplemented with different concentrations of cytokinin after four weeks. (A) Control treatment, (B) 10 µM BAP, (C) 20 µM BAP, (D) 30 µM BAP, (E) 40 µM BAP, (F) 10 µM 2-iP, (G) 20 µM 2-iP, (H) 30 µM 2-iP, and (I) 40 µM 2-iP. (Scale bar = 1cm)

Through SEM analysis, it was confirmed that the arrangement of cells and tissues showed no differences *in vitro* apical buds treated with BAP and 2-iP at different concentrations. Latex produced by the plant during sectioning was observed (Figure 2B, 2G and 2H). Cracking of the cross-section of an apical bud during the freeze-drying process was shown in Figure 2D.

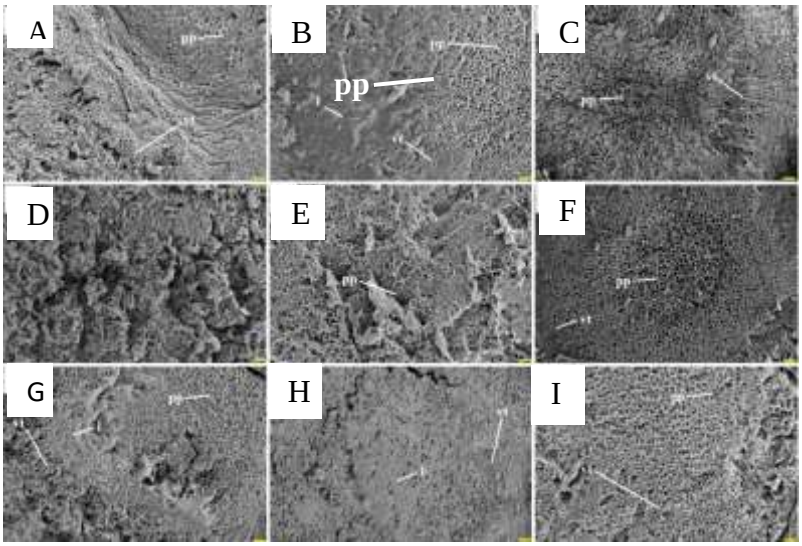

Figure 2. Scanning electron microscopy analysis of cross section *in vitro* apical buds of *Ficus carica* L. cv. Black Jack treated with different concentrations of cytokinins. (A) 0 µM (Control), (B) 10 µM BAP, (C) 20 µM BAP, (D) 30 µM BAP, (E) 40 µM BAP, (F) 10 µM 2-iP, (G) 20 µM 2-iP, (H) 30 µM 2-iP, and (I) 40 µM 2-iP. l, latex; pp, parenchymatous pith; vt, vascular tissue (Magnification = 50x, Scale bar = 100 µm)

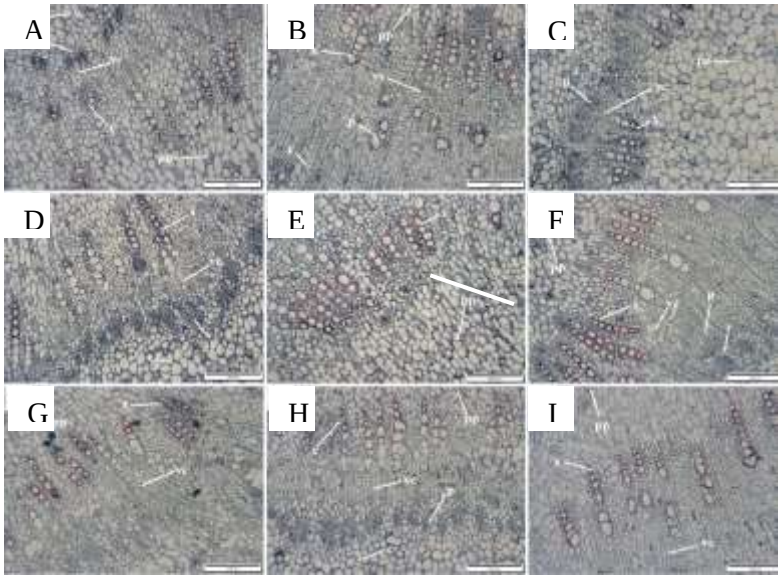

Figure 3. Histological analysis of cross-section *in vitro* basal part of apical buds for *Ficus carica* L. cv. Black Jack treated with different concentrations of cytokinins. (A) 0 µM (Control), (B) 10 µM BAP, (C) 20 µM BAP, (D) 30 µM BAP, (E) 40 µM BAP, (F) 10 µM 2-iP, (G) 20 µM 2-iP, (H) 30 µM 2-iP, and (I) 40 µM 2-iP. c, cortex; p, phloem; pp, parenchymatous pith; vc, vascular cambium; x, xylem. (Magnification: 10x x 10x, Scale bar = 200 µm)

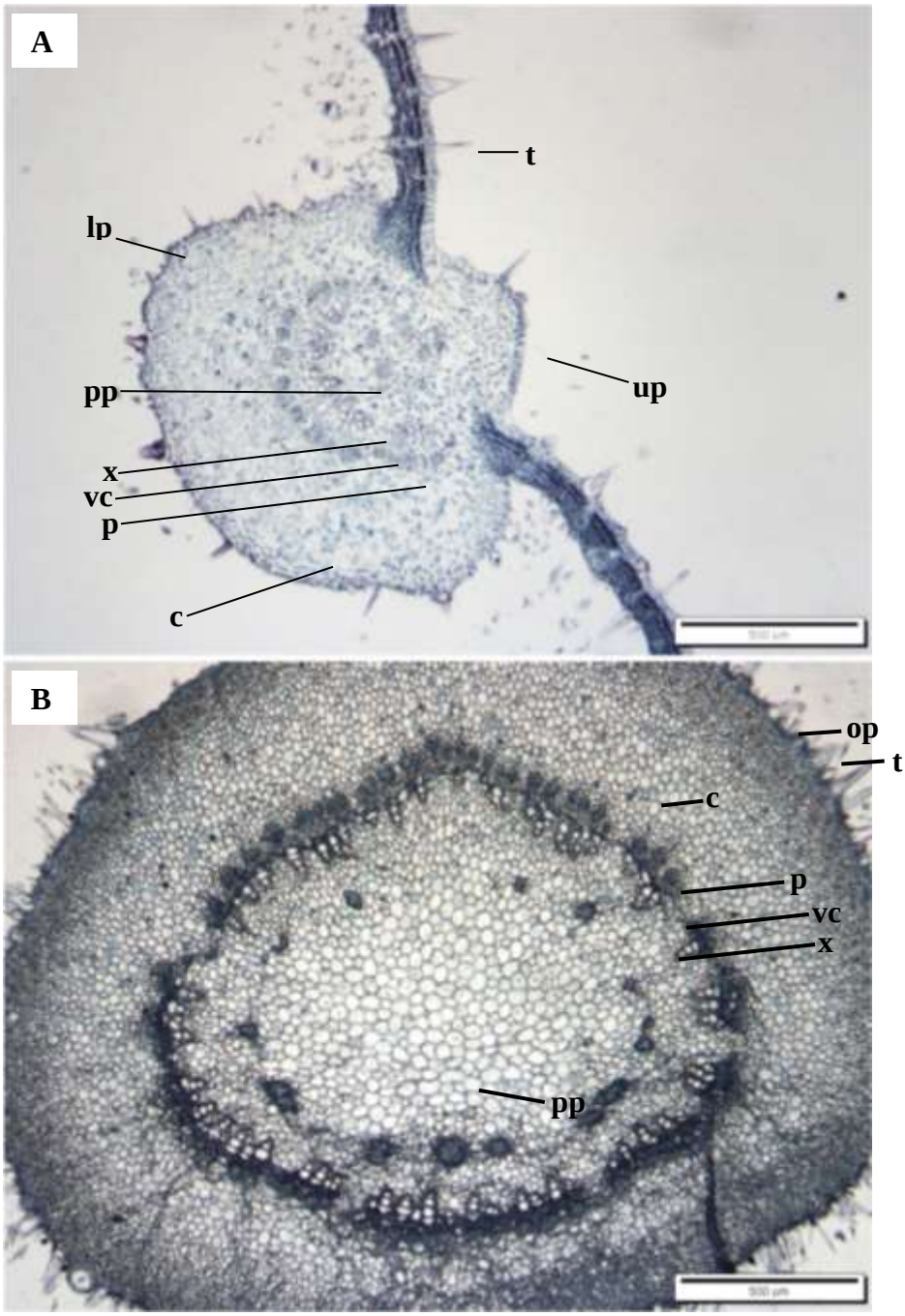

Figure 4. Histological analysis of cross-section for mother donor plant leaf of *Ficus carica* L. cv. Black Jack. (A) Leaf, and (B) Petiole. lp, lower epidermis; op, outer epidermis; p, phloem; pp, parenchymatous pith; t, trichome; up, upper epidermis; vc, vascular cambium; x, xylem. (Magnification: 10x x 4x, Scale bar = 500 µm)

Histological analysis indicated no differences in cells and tissue structures *in vitro* apical buds treated with BAP and 2-iP at different concentrations. Cells remained intact and vascular tissues can be clearly observed. Structures such as xylem and phloem as a vascular tissue remained intact to each other (Figure 3).

Histological analysis on the cross-section of leaf from mother donor plants showed that the petioles had more vascular tissues in a circular arrangement as compared to the leaf veins (Figure 4B). Cross-section of the mother donor plant leaf showed that lower epidermal layers and more vascular tissues than the upper epidermal layers (Figure 4A).

Discussion

In vitro cultures of *Ficus carica* L. cv. Black Jack was established using an aseptic technique. *In vitro* culture treated with chemicals such as plant growth regulators and different physical factors such as light and temperature eventually gave a wide variety of responses (Morini *et al.,* 2000). Each *in vitro* cultured plants responded differently. It was found that BAP at 20 µM displayed the best response in terms of leaves, shoots induction, and multiplication. The formation of chlorotic leaves was due to the high concentration of BAP in the research findings were in harmony with Nobre and Romano (1998) and Fráguas *et al*. (2004). It was found that 20 µM BAP was the best cytokinin treatment to produce multiple shoots and cost-effective. The apical buds of *F. carica* L. cv. Roxo De Valinhos, cv. Roxo De Valinhos (Fráguas *et al*., 2004), cv. Sultany, Aboodi, and White Adcy (Mustafa and Taha, 2012) gave a similar response of BAP effective to promote the multiple shoots formation.

Furthermore, the formation of friable callus was observed at the apical bud basement regardless of the types and concentrations of plant growth regulator. Similar observations were recorded *in vitro* cultures of *F. carica* L. cv. Zidi, Bither Abiadh and Assafri (Bayoudh *et al*., 2015). Fráguas *et al*. (2004) confirmed that medium supplemented with BAP promoted the calli induction of *F. carica* L. cv. Roxo de Valinhos and shoot vitrification. However, the morphology can be observed by naked eyes. Hence, it is important to carry out an anatomical study on the inner cellular arrangement for the effects of plant growth regulator at various concentrations on the cellular arrangement.

SEM analysis confirmed that there were no differences between the cells and tissue arrangement *in vitro* apical buds under BAP and 2-iP treatments at different concentrations (Figure 2). There was no abnormality observed in the samples. Furthermore, the mucus-like substance was observed after processing (Figure 2B, G and H), which proved that the samples secreted latex while acquiring transverse sections of apical buds, as

milky latex can be found in all parenchymatous tissues in Fig trees (Singh, 2010). It showed that the treatments of BAP and 2-iP maintained the characteristics of *F. carica* L. cv. Black Jack to produce latex under *in vitro* condition. A study conducted by Singh *et al.* (2003) on micro-morphology and protein characterization of rubber particles in *F. carica*, *F. benghalensis* and *Hevea brasiliensis* in the Moraceae showed that SEM analysis was functional in examining micro-morphology that aids the study of phylogenetics. Silva *et al.* (2019) identified a set of idiosyncratic leaves and stem characters of *Byrsonima sericea* DC that distinguished the species from others via SEM and light microscopy. Besides, SEM analysis enabled the study of micro-morphoanatomy to differentiate the plants of similar resemblance. The advantage was applied by Patil and Malpathak (2017) for an important medicinal plant in the ayurvedic medicine, *Tinospora cordifolia* (Wild.) Miers to differentiate the adulterant *Pergularia daemia* (Yue *et al*., 2019) used *in situ* SEM to observe the real-time modification in the natural plant fibres' structure with respect to stress during microtensile measurement, where associated microscopic slippage of microfibrils was observed. The high-resolution imaging by SEM can be utilized the plant-pathogen interactions. With slight modification on the protocol such as the application of paraffin in the embedding of samples, and region of pathogen colonization can be easily found (Caldwell and Iyer-Pascuzzi, 2019).

Cracks were observed in the cross-sections of apical buds during the freeze-drying process (Figure 2D). Parts of the explants cracked when the samples were being processed. This phenomenon might occur when the samples were plunged directly into slushy liquid nitrogen at -210˚C. The plant cellular contents tended to freeze and expand at higher rates at freezing point, which in turn punctured the cell walls due to the expansion of cellular content, hence cracks were observed in the samples (Hüner and Hopkins, 2008). This would be avoided if the smaller and thinner size of the sample is involved as a smaller sample in less water content.

A histology protocol is plant species-dependent, which varies from part to part. Histology protocol for leaves requires a shorter duration for serial dehydration, as the texture of the leaves is more fragile than apical buds. Hence infiltration can be accomplished within a shorter period (Ross *et al.*, 2003). Histology protocol for apical buds requires longer durations during dehydration processes. The rationale of the apical buds is harder than leaf samples. Therefore, dehydration took a long time for the infiltration process to take place. The cross-section of apical bud basal parts was compactly arranged and required a longer duration for efficient dehydration process via osmosis and infiltration without causing cellular damage (Hüner and Hopkins, 2008). Hence, histology protocol specifically modified for apical buds could prevent cellular damage to the cross-sections of the apical

bud basal parts during the serial dehydration processes (Images with cracked samples were not shown).

The histological analysis clearly showed that the cells were in contact and compactly arranged. The vascular tissues from the samples under different concentrations of BAP and 2-iP were observed anatomically stable. These observations inferred that both plant growth regulators are suitable to apply *in vitro* culture of *F. carica* L. cv. Black Jack. These analyses were done on micropropagation and callus induction of *F. carica* L. cv. Black Jack. Histological analyses did not differ in the structures of cell and tissue *in vitro* apical buds treated under different concentrations of BAP and 2-iP. Cells remained intact and vascular tissues can be clearly observed. Structures such as xylem and phloem as a vascular tissue remained intact to each other. The addition of BAP and 2-iP in the concentration tested did not cause any cellular to the explants. The plates showed that the cells were intact and compactly arranged.

Histological samples showed more vascular tissues at the lower epidermal layer of the leaves. Cross-section of the mother donor plant leaf showed that the lower epidermal layer had more vascular tissue as compared to the upper epidermal layer. For the histological examination on the cross-section of the petiole, it had more vascular tissues in a circular arrangement as compared to the leaf veins (Figure 4B). A leaf has two sides, namely abaxial and adaxial sides. Callus was induced from phloem-derived meristematic tissues like vascular cells and other different cell types such as cortical and pith cells (Ikeuchi *et al.,* 2013). During callus induction, the lower epidermal layer of the leaf contains more vascular tissues produce a better response. A palisade parenchyma cell that had better transportation which aided in callus induction at upper epidermal layer (Welander, 1998; Kim *et al.,* 2007). Hence, the wounded leaves incubated with the abaxial surface gave a better response on callus induction. Kim *et al.* (2007) reviewed that the response for callus induction was better when the adaxial surface was in contact with the medium because abaxial surface (lower epidermal layer) has more stomata as compared to the adaxial surface (upper epidermal layer) which indirectly leads to increased oxygen exchange. They pointed out that palisade parenchyma tissue is better in transporting plant growth regulators and nutrients from the medium to the explants (Kim *et al.,* 2007). The findings were inharmonious with those statements. Successful callus induction was induced on MS media supplemented with 2,4-D at different concentrations.

Post-analysis is vital in plant tissue culture. It aids double confirmation on the effects of treatments on the explants. Histological and SEM analyses proved that there is no cellular distortion or damage to cellular arrangement induced by the treatments under various concentrations of BAP and 2-iP. Through, histological analysis showed the effective callus

induction by facing wounded abaxial surface which more vascular tissues at the lower epidermal layer compared to the upper epidermal layer to facilitate the absorption of the supplements to the explants. The mother donor plant leaves in MS supplemented with the abaxial surface facing produced the best percentage of callus induction.

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