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Short Communications

Scanning Electron Microscopy Analysis of Tea's Embryo Axis Explant Cultured on Murashige and Skoog Medium Containing 2,4-Dichlorophenoxyacetic acid

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ABSTRACT
Camellia sinensis L. is an important crop in Indonesia as healthy beverage that contains several secondary metabolism compounds, such as polyphenols and catechins. Tissue culture including somatic embryogenesis and organogenesis has been used for propagating plant for various needs. In this present short-communication, scanning electron microscopic (SEM) analysis of tea was conducted and discussed. This study aimed to investigate surface ultrastructure of TRI2025 embryo axis tea clone cultured on Murashige and Skoog (MS) medium containing 2,4-Dichlorophenoxyacetic acid (2,4-D). The results revealed two different forms of explant's development, i.e. somatic embryo and transitional form between somatic embryogenesis and organogenesis; or called by "Globular-like Structure" (GLS). Surface ultrastructure analysis of somatic embryo and GLS revealed respectively many stages of somatic embryo development i.e. globular, torpedo, and cotyledon stage, and leaf development form GLS regeneration.

Tea is perennial and cross-pollinated plant (Mondal et al. 2004) with long life span and cultivated for its leaves. Tea's antioxidant contents such as polyphenols and catechins were reported as agent of anti-aging, anticancer, and antitumor (Lambert & Yang 2003; Zaveri 2006; Khan & Mukhtar 2019). According to the above studies, it is well known that tea's leaf can be formulated as healthy drink.

According to those facts, it is important to increase tea production for following the increasing of world population growth. For its propagation, it is well known that generative propagation of tea is by seed. However, in tea, seed propagation has disadvantages such as self-sterility (Chen et al. 2012) and adversity of manual pollination (Mondal et al. 2004) so that they made vegetative propagation became one of alternative choice for its clonal propagation. Furthermore, promising vegetative propagation methods such as cutting or grafting has many disadvantages such as limitation of source plant and lack of tap-root system (Mondal et al. 2004) so it brings up micropropagation or tissue culture being an al-

ternative choice for propagating tea within short-time with high yielding of propagated-plants.

Recently, micropropagation can be done by two common regeneration pathways, i.e. somatic embryogenesis and organogenesis. In somatic embryogenesis, totipotent ability of plant somatic cell is induced to develop a whole plant. During this process, biochemical changes occur resulting in growth and development of explant into a whole plant. In somatic embryogenesis, explant progresses through successive morphogenetic stages termed globular, heart-shaped, torpedo, and cotyledonary stage for dicots and conifers, or globular, scutellar, and coleoptilar stage for monocots. In tea, successful somatic embryogenesis regeneration has been reported with several types of clone and specific growth mediums (Akula & Dodd 1998; Tahardi et al. 2000; Seran et al. 2006; Kaviani 2013; Eskundari et al. 2018).

Another regeneration pathway in micropropagation is organogenesis. It also uses somatic cells as explants and goes through two stages of propagation, called by shoot and root induction (Duclercq et al. 2011). There are several successful reports of tea's organogenesis using specific mediums and plant growth regulations (PGRs) (Mondal et al. 1998; Gunasekare & Evans 2000; Gunasekare & Evans 2000; Gonbad et al. 2014).

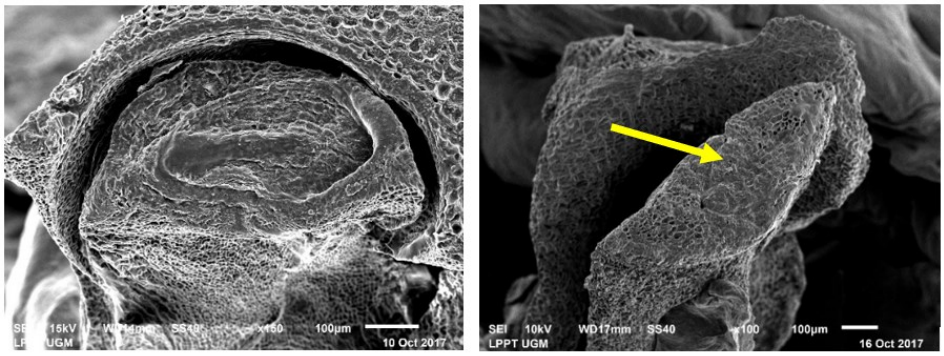
Surface ultrastructure analysis gives detailed information about surface condition an object(s), and this analysis has been conducted for tissue culture of many commercial plants. Steinmacher et al. (2011) reported surface ultrastructure analysis of peach's globular stage with small groups of somatic embryos until its further development. Kumar et al. (2015) reported surface ultrastructure analysis of indirect somatic embryogenesis of *Pelargonium sidoides*. Mandal & Datta (2005) also reported asynchronous developmental stages of direct somatic embryogenesis from ray floret explant of *Chrysanthemum* using scanning electron microscopy.

Recently, surface ultrastructure analysis of tea's leaf powder showed the presence of many leaf's fragment and layer quiet lit of fine hairs (Ekayanti et al. 2017). Particularly, embryo axis of TRI2025 tea clone cultured on MS medium containing 2,4-D has not been observed yet by scanning electron microscopy to confirm and to reveal the detailed development of *in vitro* regeneration. The important significance of this study relies on the necessity of surface ultrastructure analysis for characterizing the process of tea's *in vitro* regeneration. The objective of the present study is to use scanning electron microscopy to obtain information regarding *in vitro* regeneration of TRI2025 tea clone cultured on MS medium containing 2,4-D.

Seed of TRI2025 tea clone were sterilized with antibacterial (Agrept 20 WP; Streptomicin sulphate 20%) and antifungal (Dithane M-45; Mankozeb 80%) then washed with running water. After that, those that were sterilized with 96% ethanol, burned, and cut their shell off to get uncovered seed aseptically. The embryonic axes then were taken, removed their growth points and then cultured on MS medium containing 2,4-D with concentration 0; 1; 2; and 5 mg. L^{-1}. Induction of somatic embryogenesis and GLS regeneration were conducted following Eskundari et al. (2018).

Somatic embryos and GLS with many stages were vacuumed for several minutes then were coated with platinum particles JEC-3000FC (JEOL, Tokyo, Japan). After that, somatic embryos and GLS were analysed to get surface ultrastructure information using scanning electron microscopy JSM-6510LA (JEOL, Tokyo, Japan).

Surface ultrastructure analysis revealed smooth surface at former incision of shoot apical meristem (SAM) at 0-DAC (Figure 1A). These explants then cultured on MS medium containing 2,4-D 2 mg.L^{-1} for inducing somatic embryogenesis. The selection of 2,4-D as PGR for inducing somatic embryogenesis was based on consideration that 2,4-D is a powerful embryogenesis inducer and has succeeded in triggering explant response to embryogenesis (Caeiro et al. 2022; Gustian et al. 2022) although it was also reported can result in abnormalities related to epigenetic and genetic changes (Fraga et al. 2016; de Morais Oliveira et al. 2023). All of explants showed the mountain-like structure exactly at centre of former incision of SAM after 7-DAC (Figure 1B).



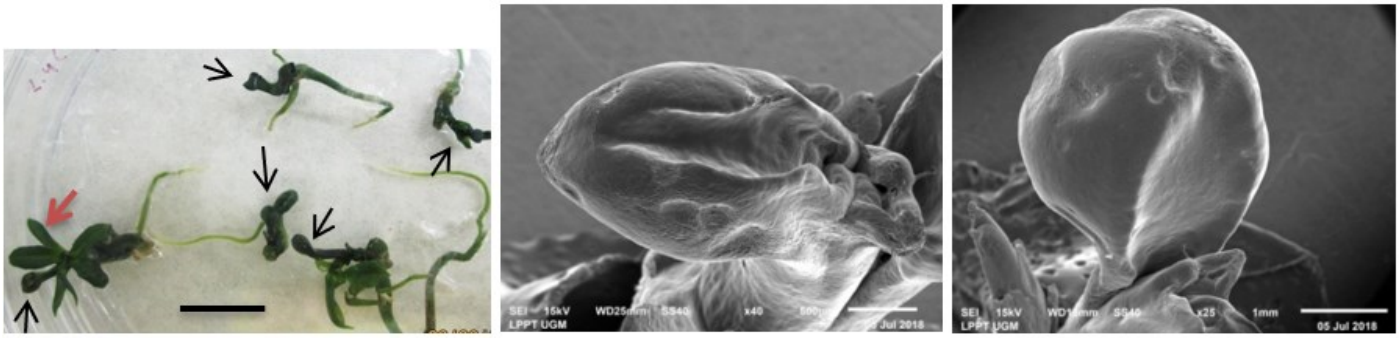
A B

Figure 1. Surface ultrastructure analysis of embryonic axis of TRI2025 tea clone. Removed growth point of embryonic axis at SAM at 0-DAC (A); Explant cultured on induction medium at 7-DAC (B). Yellow arrow showed mountain-like structure. Bars: 100 µm.

This mountain-like structure later called hereinafter referred to as GLS, viz., "transition" phenomenon between somatic embryogenesis and organogenesis; such as reported in *Camellia* genus (Lu et al. 2013). Seran et al. 2006 named this structure by nodular embryogenic structure or small succulent leaves. This structure can be induced by culturing explant on different medium either PGR(s) such as MS or Woody Plant Medium (WPM) using Kinetin, 6-Benzylaminopurine (BAP), and 1-Naphtaleneacetic Acid (NAA) as PGR(s) (Seran et al. 2006; Lu et al. 2013). In this study, this unique structure was almost similar to globular stage in somatic embryogenesis at its first occurrence, then it developed to be leaf with increasing culturing time.

This GLS formed in almost all of explants cultured on induction medium at 30-DAC. This GLS later developed to be leaf (Figure 2A) following its own mechanism that differ with common leaf development pathway. At first initial appearance, GLS structure was similar with early globular stage at somatic embryogenesis then it developed to be heart-like structure with indentation at its centre (Figure 2B). Later, this structure developed to "blooming-like" phenomenon with wider indentation (Figure 2C) compared to previous stage (see Figure 2B) and finally developed to be leaf.

In this study, a mountain-like structure at former incision of SAM was initial response of explant cultured on MS medium containing 2,4-D. This structure might be a response through reconstruction meristem by peripheral region cells, such as reported on tomato (Reinhardt et al. 2003). Auxin might be important for this process due to auxin's role at peripheral zone. Two most important genes related to auxin, PIN-FORMED1 (PIN1) (Reinhardt et al. 2000) and AUX1 (Reinhardt et al. 2003), were reported mainly worked at peripheral zone of SAM.



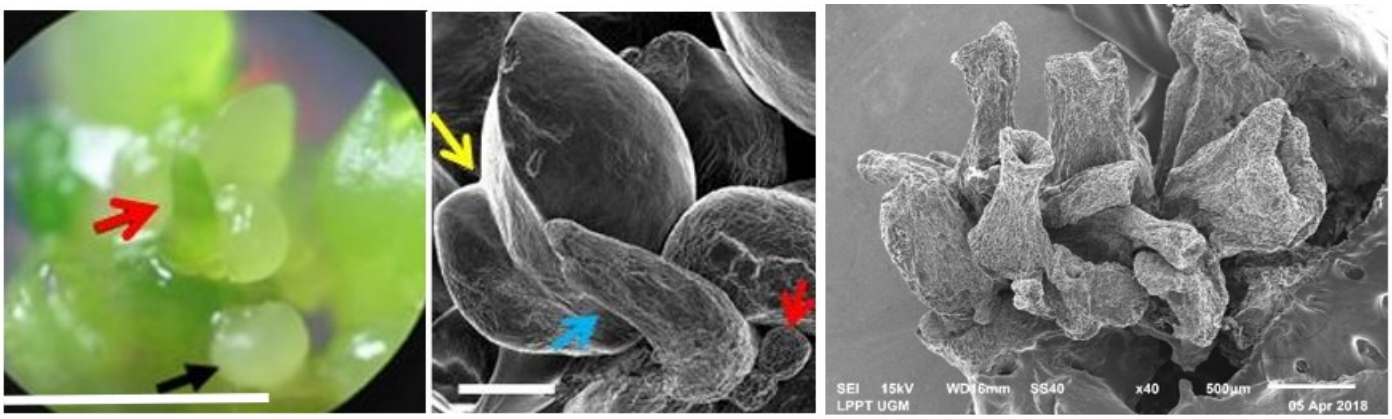
A B C

Figure 2. Morphological and surface ultrastructure analysis of GLS of TRI2025 tea clone. Morphological analysis of GLS-derived leaf (A); surface ultrastructure analysis at early stage of GLS (B); surface ultrastructure analysis at late stage of GLS (C). Black arrows indicated GLS, red arrows indicated GLS developing into leaves. Bars: 100 mm (A); 500 μm (B); 1 mm (C).

In this study, we proved that GLS was another pathway for leaf development. This unique structure was always seen at the initial stage of this process. Later, a heart-like structure with thick form confirmed to be further stage of this process and it might be a sponge-like structure inside. Eskundari et al. (2019) reported approximately 55,76 KDa of protein band found only at GLS and it might relate to stress-induced or storage protein.

Somatic embryogenesis was also occurred when explant cultured on induction medium, but occurrence of somatic embryo was fewer than that of GLS. Somatic embryos were unsynchronised form in morphology; one of them was at globular stage and the others were at further ones (Figure 3A). Surface ultrastructure analysis confirmed that unsynchronised form of somatic embryo i.e. the globular, heart, and torpedo stage occurred at an explant (Figure 3B).

Globular stage of somatic embryo was densely cytoplasmic structure and transparent in colour. Surface ultrastructure analysis revealed that globular embryo has globular shape and small in size, but surface ultrastructure of torpedo stage showed bigger size compared with globular, and has stem-like morphology with long pipe and pointed end. This globular embryo was similar with *Swertia chirayita* globular embryo from leaf explants cultured on MS medium containing 2,4-D and kinetin



A B C

Figure 3. Morphological and surface ultrastructure analysis of somatic embryos of TRI2025 tea clone. Morphological analysis of somatic embryo at globular (black arrow) and torpedo stage (red arrow) (A); surface ultrastructure analysis of somatic embryo at globular (red arrow), heart (yellow arrow), and torpedo stage (blue arrows) (B); Abnormal of somatic embryo at cotyledon stage (C). Bars: 10mm (A); 500μm (B-C).

(Kumar & Chandra 2014) and in *Dendrobium* from leaf explants cultured on MS medium containing TDZ (Chung et al. 2007) .

Surface ultrastructure analysis revealed elongated structure at torpedo stage and this was distinctive shape with other stages. This morphology was similar with globular embryo of *Catharanthus roseus*, with specific elongated structure without any groove at apex region (Aslam et al. 2014). In this study, we also confirmed abnormality in somatic embryo at cotyledon stage marked by abnormal cup-shaped structure (Figure 3C). This abnormality was probably caused by usage of 2,4-D. Hadfi et al. (1998) reported many abnormalities in cotyledon stage of *Brassica juncea* and this abnormality might be caused by auxin and its inhibitors. In contrast with this study, Aslam et al. (2014) reported normal cotyledon stage of *Catharanthus roseus* characterized by the presence of two cotyledons that later developed to be leaf primordial from hypocotyl plant cultured on MS medium containing 2,4-D. Therefore, surface ultrastructure analysis using SEM along with others analysis such as morphology and histology are very useful for better understanding related to plant development.

Induction medium containing 2,4-D was powerful PGR for inducing GLS and somatic embryogenesis. This phenomenon could be associated with strong capability of 2,4-D for inducing somatic embryogenesis, as reported in many plant tissue culture (Raghavan 2004; Kaviani 2013; Aslam et al. 2014; Eskundari et al. 2018). Early response of explant cultured on growth medium containing 2,4-D in this study was relatively fast i.e. 7-DAC. This result was similar with inducing indirect somatic embryogenesis in *Arabidopsis* using 2,4-D that only needed 10-DAC for callusing (Raghavan 2004) and direct organogenesis in *Passiflora* that only needed few days (Fernando et al. 2007). In sugarcane, callus as first response of explants cultured on induction medium (containing 2,4-D, kinetin, and IAA) could be seen at 3-DAC (Rodríguez et al. 1996).

Surface ultrastructure analysis on tissue culture has been reported in many commercial plants. Surface ultrastructure analysis in maize revealed callus occurrence when shoot tips explant cultured on MS medium containing 2,4-D, benzyladenine, and adenin (Marín-Méndez et al. 2009). Rodríguez et al. (1996) revealed that sugarcane's callus was also as first response when spindle explants cultured on MS medium containing 2,4-D, kinetin, and IAA using SEM.

In this study, we did not prepare samples using a fixation solution. Dehydration step was done by vacuuming samples for a few moments. In our opinion, this unusual sample preparation technique still produced good SEM images because the vacuum technique was carried out only a few moments and the platinum are immediately coated. This is intended to reduce sample's damage due to fragility of the sample's structure and presence of a large number of air voids. It can be seen at SEM image that the sample remained in good condition, no imbalance of light-dark distribution was detected on sample surface. Simplification of sample preparation process for SEM analysis has been widely reported, such as in blood samples using tetramethylsilane (TMS) (Ting-Beall et al. 1995) and in cell culture using carbon tape continued air-drying (Ali et al. 2021).

This study showed the stages of somatic embryogenesis and GLS regeneration of tea using SEM so that might be useful for increasing the knowledge on tea's tissue culture. Globular, heart, and torpedo stages could be seen clearly but cotyledonary stage was in abnormality. GLS regeneration might be the other pathway of leaf development on tissue culture-derived plant.

AUTHOR CONTRIBUTION

R.D.E, T.T., D.I., and Y.A.P. designed the research. R.D.E. collected and analysed data and wrote the manuscript. T.T., D.I., and Y.A.P. supervised all the processes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in preparing this research article.

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