Research Article

Morphological, Histological, and Protein Profiling of Tea Embryo Axis at Early Stage of Culture

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ABSTRACT
Tissue culture is an alternative choice of plant propagation either through somatic embryogenesis or *in vitro* organogenesis techniques. TRI2025 tea clone has been cultured successfully, however, the scientific information related to morphology, histology, and protein profile at an early event of culturing time has not been reported yet. This study aimed to determine the differences between those pathways, in the context of morphology, histology, and protein profile. The explants were the embryo axis of TRI2025 tea clone cultured on two different induction mediums; somatic embryogenesis and *in vitro* organogenesis induction medium. The results showed that most of the explants cultured on A medium developed to be a globular-like structure at 11-day after culture (DAC), while all explants cultured on B medium showed the initiation stage of *in vitro* organogenesis. Histological analysis showed meristem reconstruction at shoot apical meristem (SAM) and root apical meristem (RAM) at 11-DAC at explants cultured on B medium, while explants cultured on A medium showed callusing at 21-DAC. Protein profile analysis using SDS-PAGE showed protein bands of 54 and 81 KDa that only appeared at explants cultured on A medium start from 14-DAC, and those two protein bands thought to be a differentiator at the early stages of the two tissue culture techniques. Thus, these parameters can be used as early detection for plant tissue culture, especially in tea.

Keywords: *auxin, Camellia sinensis*, cytokinin, micropropagation, tissue culture

INTRODUCTION

Tea is known as one of the major healthy crops cultivated in the world and its propagation can be through generative or vegetative techniques. One alternative choice of its vegetative propagation is through tissue culture, both somatic embryogenesis or *in vitro* organogenesis. There have been many reports that provided information about the success of tea propagation, with different culture conditions, such as differences in plant growth regulators (PGRs), incubation time, and other tissue culture conditions (Seran et al. 2006; Kaviani 2013; Gonbad et al. 2014; Eskundari et al. 2018).

TRI2025 tea clone belongs to the Cambod type (Kumar et al. 2014) and resistant to drought (Munivenkatappa et al. 2018). This clone is also suitable to be planted in various elevations but has high catechin content at higher altitudes of tea plantations (Mitrowihardjo et al. 2012). Those reasons make it to be one of the commonly cultivated tea clones in many plantations in the world, including Indonesia (Indonesian Tea and Quinine Research Center 2006; Haq & Mastur 2018).

Somatic embryogenesis is known as tissue culture technique that requires somatic cells as explants that then be induced its further development as the embryogenesis development pathway. On the other hand, *in vitro* organogenesis is likely the previous one, but its further development is different. One crucial point is the root-apical axis development that only occurs at explants induced through somatic embryogenesis (Bassuner et al. 2006). In tea, there were many reports about somatic embryogenesis and *in vitro* organogenesis using different clones (Gunasekare & Evans 2000; Sandal et al. 2005; Seran et al. 2006; Kaviani 2013; Gonbad et al. 2014), and TRI2025 tea clone has been reported for source clone of various explants; through both techniques (Akula & Dodd 1998; Seran et al. 2006; Eskundari et al. 2018). Besides specific clone requirements, successful of somatic embryogenesis and *in vitro* organogenesis also require specific PGRs to promote the explant's growth and development. Several studies *in vitro* related to somatic embryogenesis and *in vitro* organogenesis of tea plants were reported using PGR(s), such as auxin and cytokinin (Mondal et al. 1998; Gunasekare & Evans 2000; Sandal et al. 2005; Seran et al. 2006; Kaviani 2013; Gonbad et al. 2014).

Auxin is known as PGR that acts as an inducer of embryogenesis, exactly on apical-basal axes formation through dynamic gradient concentration (Bowman & Floyd 2008). Many transcription factors and hormonally related genes to auxin, such as *Agamous-15* (*AGL15*), *Leafy Cotyledone1* and *2* (*LEC1*; *LEC2*), and *Yucca* (*YUC*) gene are related to the somatic embryogenesis signal transduction through somatic embryogenesis pathway (Horstman et al. 2016). The LEC2 protein was reported to have a role in activating the gene encoding the MADS-box transcription factor called AGL15 (Braybrook et al. 2006) and vice versa (Zheng et al. 2009). In addition, the LEC2 protein is associated with *YUC* gene activation (Wojcikowska et al. 2013). One kind of auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), is known well as a herbicide but it can be useful for many plant tissue cultures for its low concentration addition on a culture medium (Mahalakshmi et al. 2003; Aslam et al. 2011; Kaviani 2013). Its role related to somatic embryogenesis (also to zygotic embryogenesis) is well correlated with its capability to induce cell expansion, cell cycle, and morphogenesis in plant tissue culture (Raghavan 2004). Especially in tea's tissue culture, 2,4-D is usually added to the culture medium to induce somatic embryogenesis, either by callusing pathway or directly (Kaviani 2013; Eskundari et al. 2018).

Cytokinin is known to have a role in initiating adventitious shoots (Raven & Johnson 2009). At shoot initiation, it plays an important role in proliferation and differentiation of cells around the shoot meristem through the encoding shoot meristem-related gene; *Shoot Meristemless* (*STM*) gene (Dello Ioio et al. 2008) via KNOX protein pathway (Scofield et al. 2013). Cytokinin is in relation to root initiation, particularly in meristem cell differentiation (Kyozuka 2007) in terms of cell growth initiation from differentiation zone (differentiation zone; DZ) towards the elongation and differentiation area (elongation and differentiation zone; EDZ) (Dello Ioio et al. 2008). Benzylaminopurine (BAP) is one kind of cytokinin, belongs to the purine class, and has an adenine-like configuration (Raven & Johnson 2009). BAP deficiency was reported to be associated with a decline in the ability to

initiate shooting (Werner et al. 2008). In tea's tissue culture, the use of BAP has been widely reported, such as using shoot tips and axillary buds of TRI2025 from the field as explants (Widhianata & Taryono 2019); with leaf explants of TRI2024 and TRI2025 (Farida & Muslihatin 2017); also cotyledon explants of TRI2025, Malabar2, Cinyuruan 143, and Kiara8 clone (Putra et al. 2015).

Morphology analysis is often used for knowing spatio-temporal information of explant development, while histological one is usually used to determine the process of cell development (Ogita et al. 2004; Qosim et al. 2013). These have also been used in tea's research using embryo axes of TRI2025 tea clones without removal of their growth points as explants and lead to both somatic embryogenesis and *in vitro* organogenesis (Seran et al. 2006). The surface ultrastructure of many plants derived tissue cultured has been reported widely, such as in *Passiflora* spp. (Carvalho et al. 2014), *Zea mays* (Mendez et al. 2009), and *Canna indica* L. (Wafa et al. 2016). In tea, but not derived from tissue culture, there were many reports about surface ultrastructure analysis such as white tea leaves (Ekayanti et al. 2017), trichomes of green tea (Liang et al. 1993), and polysaccharides of green tea (Yi et al. 2011).

Protein profiling has a relation with spatio-temporal aspects. In tissue culture, there were many reports about protein profiling, such as in *Medicago truncatula* (Imin et al. 2005), *Coffe spp.* (Lopez et al. 2012), and hybrid larch (Han et al. 2014). There were several reports related to tea's proteomics from the field, such as proteomics of albino tea leaves (Li et al. 2011), proteomics of tea leaf related to abscisic acid application and drought stress (Zhou et al. 2014), proteomics of tea shoot and leaves from the field Li et al. (2015), and proteomics of purple shoots tea leaves (Zhou et al. 2016). Eskundari et al. (2019) reported protein profiling of tea's tissue culture at callus, somatic embryo, globular-like structure (GLS), and initiation of leaves stages.

Tissue culture of TRI2025 tea clone using embryo axis as explant has been successfully done but the scientific information about its early event of somatic embryogenesis and *in vitro* organogenesis has been not reported yet. This research aims to analyze morphology, histology, and protein profile at an early stage of somatic embryogenesis and *in vitro* organogenesis of embryo axis of TRI2025 tea clone.

MATERIALS AND METHODS

Materials
Tea seeds of TRI2025 clone with an age average of eight months after seed-set forming were collected from the polyclonal garden of PT Pagilaran (Batang, Central Java-Indonesia) and used for initial explants. These were later sterilized using an antimicrobe agent (Agrept 20WP; Streptomycin sulphate 20%) and antifungal agent (Dithane M-45; Mankozeb 80%) then soaked into 96% alcohol for about 10 minutes. These sterilized seeds later were burned just a little moment and their seed coat was crushed using a walnut breaker.

Methods
Culture condition
The embryo axis was taken from the cotyledonary region of uncoated seeds then its two growth points at SAM and RAM were cut using a scalpel. This removed growth points of axis embryo later to be cultured on two different induction media; somatic embryogenesis and *in vitro* organogenesis induction medium (or called by A and B medium, respectively). The A medium was MS

medium added with 2 mg L^{-1} of 2,4-D (Eskundari et al. 2018) and the other was ½ MS media added with 2 mg L^{-1} of BAP (according to our best result of preliminary study). Both of these media were adjusted to 5.6 of pH prior to autoclaving. The culture condition was at light condition (with light intensity at 3250 lm, 90 lm/W (Philips TLD 36/865 cool daylight)) and with the temperature at 23ºC. This experiment has been repeated three times, with details that the experiment consisting of 20 explants to be cultured on each induction medium. The cultured explants were taken for morphological and histological analysis at 0, 11, and 21-DAC, as well as for protein profiling at 0, 3, 7, and 14-DAC.

Morphological analysis

Morphological analysis was conducted using a light microscope and scanning electron microscope (SEM). For morphological analysis using a light microscope was conducted by observing the samples under a light microscope and documented by a digital camera (Canon PowerShot A2500). For morphological analysis using SEM microscope, the prepared samples for SEM analysis were carried out following Fernando et al. (2007) with some modifications. The water content of the first explants was reduced by a vacuum dryer (PELCO 813-600), later coated the vacuumed explants with platinum (JEOL JEC-3000FC), and analyzed the explants with SEM (SEM JLM-6510LA). Time of morphological observation was at 0, 11, 21- DAC both by light and SEM microscope. Forty times (500 µm) and 100x (100 µm) at magnification were used at 11-DAC, while 22x (1 mm) at magnification was used at 21-DAC of this SEM microscope analysis.

Histological analysis

Histological analysis was carried out following Jensen (1962) at 0, 11, and 21-DAC. Explants fixed in a formaldehyde solution: glacial acetic acid: 70% ethanol at 5: 5: 9 for 24 h and dealcoholisation were done by soaking explants in alcohol-xylol mixture successively (3: 1; 1: 1; 1: 3) as well put in xylol (change twice, 30 minutes for each change). The xylol was then removed and replaced with a xylol: paraffin mixture 1: 9 at 57˚C for 24 h. The explants were added to paraffin pure at a temperature of 57˚C for 24 hours and cloaked by replacing it with new paraffin, and finally, after an hour a block was made. In the last stage, explants were sliced with rotary microtome with a thickness of 10-20 µm and stained with Safranin 1% and Fast Green 0,1% (Sass 1958). The slices obtained were observed under a light microscope and documented using a digital camera (Canon PowerShot A2500).

Protein extraction and profiling analysis

Protein extraction was carried out by grinding explants added with 300 µL of phosphate saline buffer (PBS). The scour was centrifuged at 9,000 rpm for 5 min at 4ºC. The supernatant was taken to obtain crude protein extract, the concentration was measured using the Biorad method (Bradford 1976). The next step was protein separation which done using the SDS-PAGE method. SDS-PAGE electrophoresis was performed according to Maniatis et al. (1982) with modification; that began with the making of resolving gel followed by stacking gel formation. The well was made by placing a comb at the corner of one side of the stacking gel. A protein mixture with a buffer with a total volume of about 25 µL was injected into the well, which was previously treated by heating followed by a short cooling. For the standard marker, 5 µL marker (Fermentas) was used. The process of separating proteins using this method was done for about 2 hours with a voltage of about 100 Volts. Staining of the electrophoresis results was done by

immersion in 0.1% brilliant blue Coomasie solution overnight and continued with de-coloration through instant heating in the microwave. The protein bands formed were then analyzed using ImageJ software (www.imageJ.com).

RESULTS AND DISCUSSION

Morphological and Histological Analysis

Both growth points were removed partially but the corpus region of the SAM was still a little left (Figure 1A). It was done in order to determine the pattern of explant development. It can be shown that the procambium and ground meristem layers were still intact as seen at Figure 1B and histological analysis showed that the cutting of the lower growth point still left the root meristem part of the quiescent center (QC) area (Figure 1B). Maybe it was the cause of reconstruction meristem took place.

The unique thing that only happened to almost explants cultured on A medium was the visible globular-like structure; a slightly transparent white near the shoot meristem (Figure 1C, white cycle). This transparent globular-like structure was the main differentiator of explants cultured on A medium and this phenomenon had been reported (Eskundari et al. 2018). It was likely that this globular-like structure was the result of the presence of 54 kDa and 81 kDa protein. On the other hand, it did not occur in explants cultured on B medium but all explants cultured on B medium then showed the development of *in vitro* organogenesis (Figure 1D; the area that delimited by black dotted lines) started at 7-DAC.
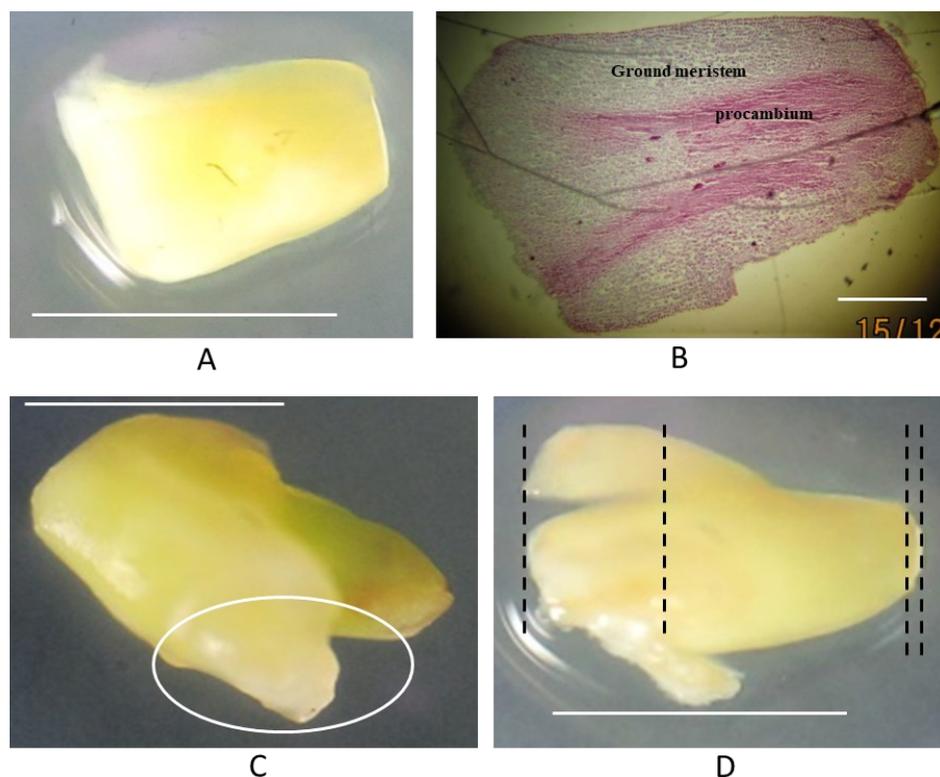

Figure 1. Morphological analysis using light microscope. Explant at 0-DAC (A); histological analysis at 0-DAC (B); at 7-HST on A media (C); at 7-HST on B media (D). Bars: A,C, D = 2.5mm; B = 0.5mm.

In this study, microscopic observations showed callusing at explants cultured on A medium especially started at 21-DAC, and this callus occurred at the surrounding root regions (Figure 2A; white dotted line) and was never found at surrounding apical regions. On the other hand, there was no callus formation that preceded shoot or root *in vitro* organogenesis. This was

probably caused by not adding auxin to B media so that did not make the accumulation of auxin-related proteins such as *PLT3*, *PLT5*, *PLT7*, and *CUP -SHAPED COTYLEDONE1* (*CUC1*) also *CUC2* (Gordon et al. 2007) and in the end, it would not eventually activate meristem-related genes such as *STM* genes and *PIN-FORMED1* (*PIN1*) (Ikeuchi et al. 2016). On the contrary, the direct *in vitro* organogenesis that occurred in this study was probably due to the accumulation of the expression of wound-encoding genes (*WOUND INDUCED DEDIFFERENTIATION*; *WIND*) that will activate cytokinin-related genes; *ARR1* and *ARR2*, and their downstream genes such as *ESR1*, then will eventually activate genes related to shoot meristems (Iwase et al. 2011). Another possible cause was an accumulation of endogenous cytokinins induced by injury (Crane & Ross 1986) so that initiated development of plants *in vitro* organogenesis. At 120-DAC, although not all explants can be induced their somatic embryos capability, some lead to the development of somatic embryogenesis (had been reported, please see Eskundari et al. 2018). The globular stage can be seen histologically with the presence of a globular structure that was initially formed through embryogenic callus (Figure 2B). The surface ultrastructural analysis also clearly demonstrated the globular embryo stage with the spherical structure (Figure 2C).
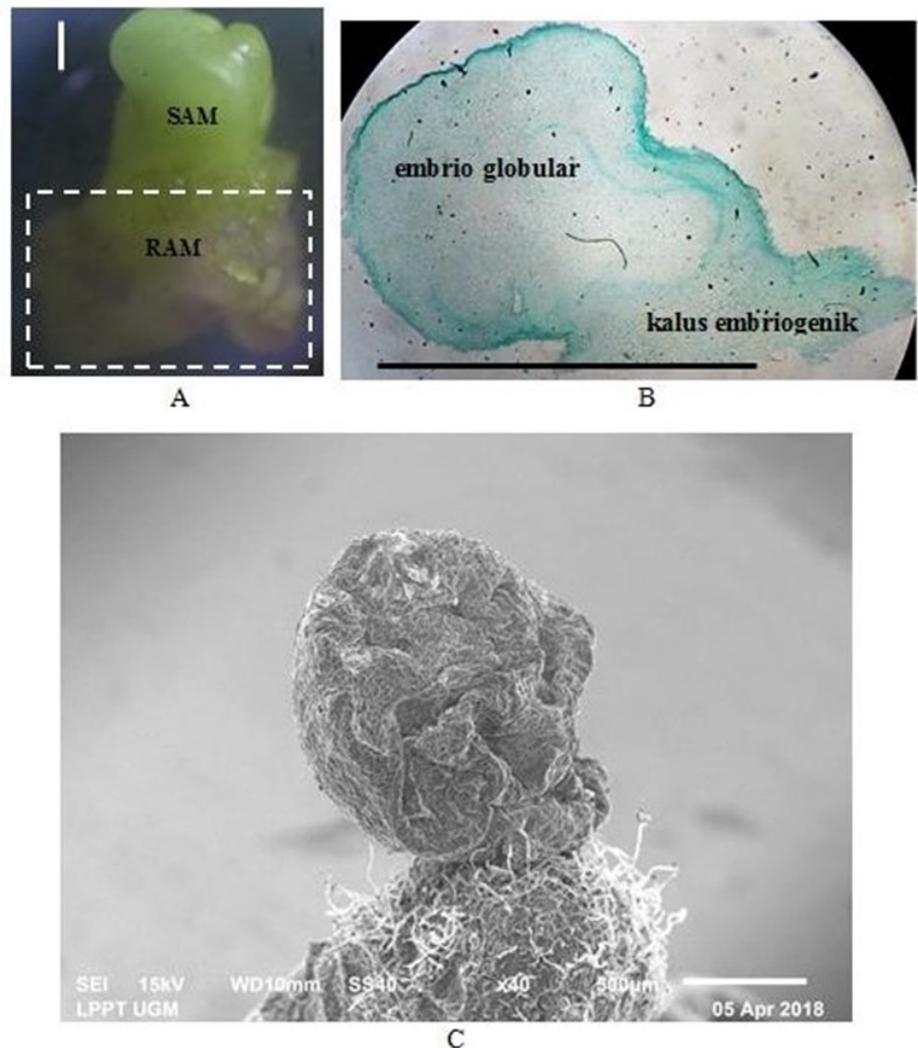

Figure 2. Morphological and histological analyses of tea's embryo axis explant cultured on A medium. Analysis using a light microscope; explant cultured on A medium at 21-DAC with callusing marked by white dotted lines (A); histological analysis of globular embryo at 120-DAC (B); surface ultrastructure of a globular embryo at 120-DAC (C). Bars: A: 0.5mm; B: 10 mm; C: 500 μm.

The development stage of *in vitro* organogenesis was seen at 11-DAC which was characterized by the development of shoots and roots on explants cultured on B medium, especially marked by arising both of leaf primordial and root elongation (Figure 3A). Furthermore, shoot and root *in vitro* organogenesis were seen at 11-DAC through meristem reconstruction; one mechanism of *in vitro* organogenesis was caused by removing some of the functional meristem areas (Reinhardt et al. 2003; Sena et al. 2009). Histological analysis showed the development of both SAM and RAM, and leaf primordial as one characteristic of plant development (Figure 3B). *In vitro* shoot, organogenesis was seen with the emergence of leaf primordial, preceded by reconstruction. In this research, SAM reconstruction was seen as the result of rearranging the missing part of the meristem. It later resulted in the initiation of leaf development by invading the adjacent empty area that was preceded by cell division. Furthermore, the reconstruction of the meristem also occurred in the RAM region. This can be proven by the development of RAM that comes from a division of surrounding cells then resulted in re-formed of the RAM region. This phenomenon was also reported by Sena et al. (2009) in *Arabidopsis* with the removal of the QC region (one of the parts included in RAM). Surface ultrastructure analysis at 11-DAC confirmed the occurrence of plant development especially at the initiation stage of *in vitro* organogenesis. At SAM region, visible developing regions at the former incision were marked by a rising plateau-like structure that later developed to be leaf primordial (Figure 3C). On the other hand, at RAM region, visible developing regions were also seen that marked by root lengthening at elongation zone (white arc; Figure 3D).
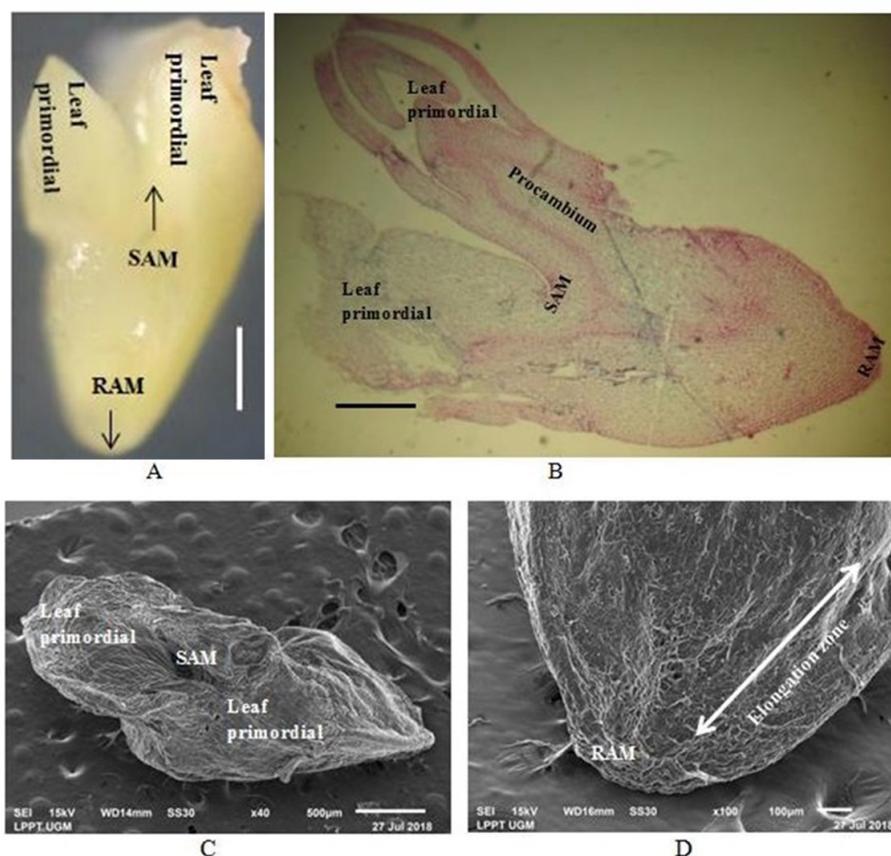

Figure 3. Morphological and histological analyses of tea's embryo axis explant cultured B medium at 11-DAC. Morphological analysis of explants cultured on B medium (A); histological analysis of explant cultured on B medium (B); SAM region morphology was analyzed using SEM (C); RAM region morphology was analyzed using SEM (D). Bars: A-C: 0.5mm; D: 500 μm; E: 100 μm.
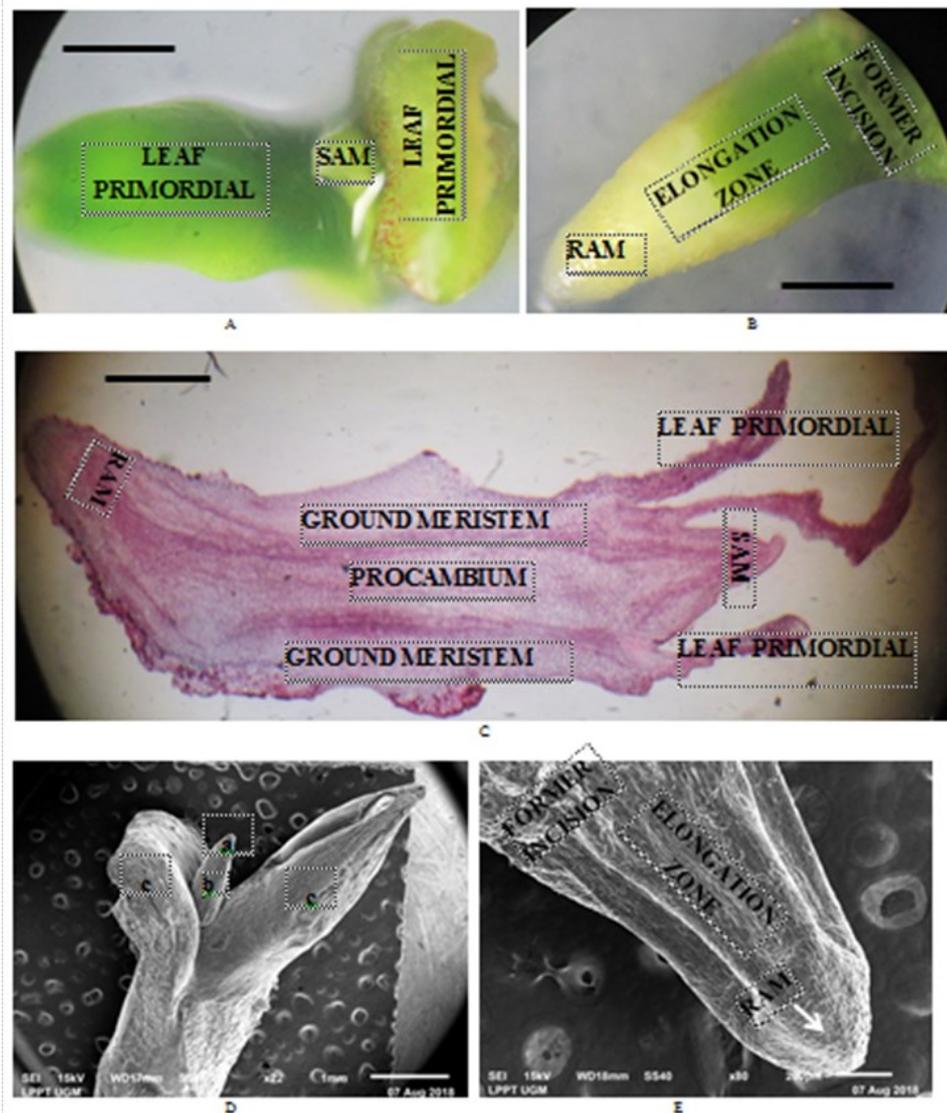
Figure 4. Morphological and histological analysis of planlet cultured on B medium at 21-DAC. The Upper region of planlet (A); lower one (B); histological analysis (C); upper region using SEM (D); lower one (E). a: former incision; b: SAM; c: leaf promordial. Bars: A-C: 0.5mm; D: 1mm; E: 200μm.

The plantlets that formed at 21-DAC on B medium were marked by the root and shoot formation, then followed by the initiation of leaf development (Figure 4A-B). The histological analysis showed that leaf development started with initiation of one leaf primordial with three rosette leaves (Figure 4C). Histological analysis also showed that the roots formed were the main roots, not the lateral roots. This was probably due to cutting in some regions of the root meristem so that the root meristem was still be maintained. Next, the explant development can be seen by increasing at volume and size of the explants, also the development of shoot and root that started from the former incision region (Figure 4D-E). It was also seen that there was root development at the elongation stage.

Morphological analysis using SEM was often used to examine the development of plant derived tissue cultures, such as in passion fruit (Biasi et al. 2000), *Lolium perrene* L. (Bradley et al. 2001), and *Passiflora* (Fernando et al. 2007). In this study, we proved the successful method without standard preparation, just directly to be vacuumed and coated with platinum then finally were observed using SEM. The results of SEM images were not so bad, so that, this would be an alternative method for SEM analysis, especially for limited laboratory equipment.

The time for initiation of somatic embryogenesis and *in vitro* organogenesis was vary in many plants derived from tissue culture. Kaviani (2013) reported that somatic embryo occurred directly at embryonic axes of tea after two months cultured on MS medium containing 1 µM 2,4-D. Tahardi et al. (2003) also reported that cotyledon explants of Yabukita tea's variety can be induced in its somatic embryo at four weeks after culturing on MS medium supplemented with BAP. Ali et al. (2016) reported that indirect *in vitro* organogenesis of ginger was at 15-20 -DAC for root initiation and at 30-40 -DAC for shoot initiation. Initiation of *in vitro* organogenesis of soybean occurred at four weeks after culture (Joyner et al. 2010) while petiole of *Gerbera jamesonii* Bolus ex. Hook f. needed 28-DAC to induce its shoot *in vitro* organogenesis (Hasbullah et al. 2008). In this study, some explants showed globular embryo at 120-DAC for explants cultured on A medium and initiation of *in vitro* organogenesis at 11-DAC followed by planlet formation at 21-DAC for explants cultured on B medium.

The ability of this PGR; 2,4-D, for induction somatic embryogenesis might be due to its role in plant development that irregular distribution of auxin must be established first in order to initiate embryo formation. This irregular (or asymmetrical) auxin distribution results from differential transport (Márquez-López et al. 2018). Next, at *in vitro* organogenesis, it has been proposed that at shoot initiation, cytokinin plays a important role in proliferating and differentiating cells within SAM through influencing the expression of key regulator gene at SAM; *SHOOT MERISTEMLESS* (*STM*) gene (Dello Ioio et al. 2008). Its deficiency revealed the reduction of shoot initiation that can be seen by the difference of nuclear DNA (Werner et al. 2008). Then, cytokinin also has an important role in initiating differentiation at RAM (Kyozuka 2007) especially to promote cells moving from differentiation zone (DZ) to elongation and differentiation zone (EDZ) (Dello Ioio et al. 2008).

Many functions of auxin were in line with the results of this study that can be proven by the presence of somatic embryos at some explants cultured on A medium. Later, two functions of cytokinin both at SAM and RAM were also in line with the results of this study. *In vitro* organogenesis, both shooting and rooting can be achieved by culturing the explants just into MS medium supplemented with BAP alone. These results suggested that the capability of cytokinin alone for promoting two kinds of *in vitro* organogenesis simultaneously. *In vitro* organogenesis is one way for propagating any part of a plant to be a partial or whole planlet, including for tea plant. This way was easy for rapid propagation of tea but with some limitations, such as hyperhydricity (Gonbad et al. 2014). In tea *in vitro* organogenesis, a shoot regeneration commonly to be induced first, and then root organogenesis was done subsequently (Ranaweera et al. 2013; Gonbad et al. 2014).

Protein Profiling
It was clear that there were at least two different protein bands in explants cultured on both mediums, namely protein bands with MW of 54 and 81 KDa (Figure 5). Both of these protein bands were visible vaguely at 7-DAC and were seen clearly at 14-DAC at explants cultured on A medium. This was likely to provide information that at beginning of the explants cultured on both mediums would induce the similar proteins up to 7-DAC and later started from 7-DAC there were different proteins involved in this different medium culture resulted in different developmental pathways further.

During 7 until 14-DAC, these two 54 and 81 KDa protein bands were only appeared at explants cultured on A medium, but these were not found at explants cultured on B medium. These two differentiating protein bands

were probably related to the key proteins that direct the explants to the pathway of somatic embryogenesis development. Probably, that somatic embryogenesis and *in vitro* organogenesis have different developmental pathways and those two protein bands were probably protein candidates for somatic embryo direction pathway. These results were correlated to PGRs that are believed to affect the development of explants in tissue culture by influencing the levels of auxin and cytokinin of the explants so that they can cause differences in gene expression that in turn produce different proteins (Mendez-Hernandez et al. 2019). The differences in the proteins that are formed are what will lead the explants to their development, whether through somatic embryogenesis or *in vitro* organogenesis.

Besides the differences, there were similarities in protein profile between explants cultured on A and B medium. One similarity between them was the protein bands of 69 KDa that appeared at 0, 3, 7, and 14 DAC. This similarity probably showed the similarity between the two pathways within two weeks of culturing. This protein band was predicted as HSP70 protein that has a function against abiotic stress (Usman et al. 2017) and this protein has also been reported in rice (Sarkar et al. 2013).

2,4-D is known as herbicide and strong synthetic auxin; has an important role in the induction of somatic embryogenesis. This 2,4-D is correlated with the expression genes related to somatic embryogenesis, such as *YUC* and *LEC2* genes (Wojcikowska et al. 2013). Several types of *YUC* genes were reported to be one of the genes expressed within seconds to 12 h after wounding to both light and dark conditions and induce auxin biogenesis in mesophyll regions and competent cells (Chen et al. 2016). Ledwon & Gaj (2009) reported that expression of the *LEC2* gene at *Arabidopsis thaliana* was increased between 1 until 15-DAC but reached the maximal level of its expression at 5-DAC. Related to this case, it probably related to protein bands with MW of 54 KDa that only found at explants cultured on A medium but need further analysis; that was corroborated by the report of Qin et al. (2020) that the presence of YUC protein in *Isatis indigotica* was with a MW of 38.64-49.77 KDa.

Protein bands around 81 KDa were also reported to appear in zygotic immature peanut embryos cultured on MS medium supplemented with 2,4-D at 0 to 15 DAC with fluctuating band thickness (Rani et al. 2005). This showed that there was a similarity in the protein bands obtained in this study with that, namely the protein band of 81 KDa, and this protein was thought to be a protein involved in the somatic embryogenesis process. Probably, this 81 KDa protein was the same protein as the protein that Sung & Okimoto (1981) reported in carrot somatic embryo culture as a somatic embryo marker protein with a MW of 77 KDa. In addition, Hakan-Mo et al. (1996) reported that in *Picea abies* there was a somatic embryogenesis marker protein with a MW of 85 KDa from embryogenic cells with high density and secreted as extracellular proteins.

Unlike 2,4-D as auxin that induces somatic embryo initiation, BAP is an example of exogenous cytokinins that is widely used in tissue culture, especially to induce *in vitro* organogenesis. BAP has the role as an activator that produces proteins for the development of *in vitro* organogenesis, such as *Wuschel* (*WUS*), *Enhancer of Shoot Regeneration* (*ESR1*), and *ESR2* genes, that in turn leads to shoot development (Ikeuchi et al. 2016). Gallois et al. (2004) reported collaboration between the key meristem gene, *SHOOTMERISTEMLESS* (*STM*), and *WUS* that would eventually lead to the induction of *in vitro* organogenesis. The *ESR1* and *ESR2* genes were reported to be responsible for shoot organogenesis but the expression patterns of the two are different. The *ESR1* gene was expressed when the hypocotyl was cultured on shoot inducing medium (SIM) media until the 4th
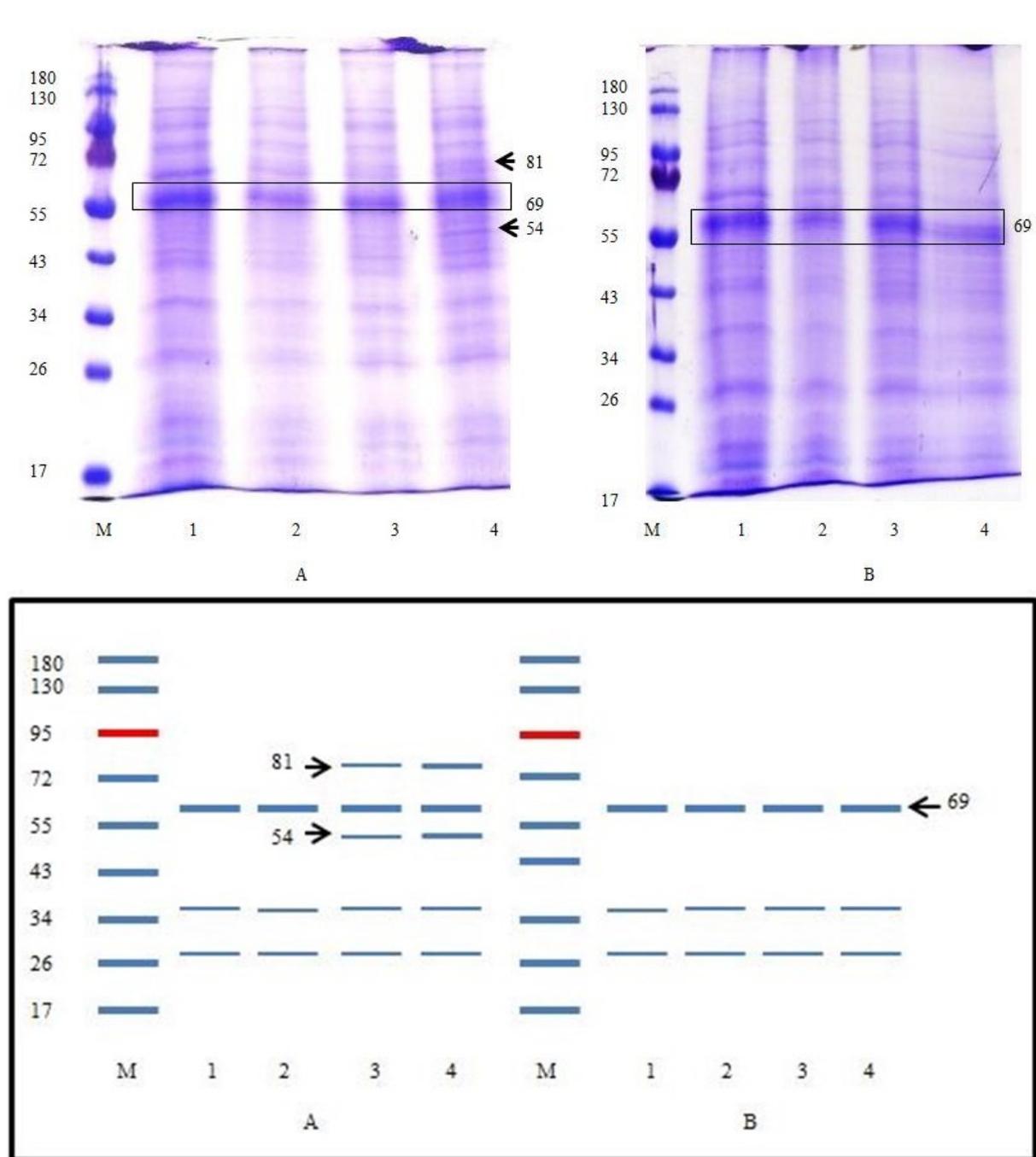
Figure 5. Protein profiling of explants on 0, 3, 7, and 14-DAC (up) and its representative (down). Cultured on somatic embryogenesis induction media (A); cultured on organogenesis induction media (B). M= marker; 1= 0-DAC; 2= 3-DAC; 3= 7-DAC; 4= 14-DAC. Left = explants cultured on MS media supplemented with 2,4-D; right = supplemented with BAP. MW of protein bands was in KDa.

day, while the *ESR2* gene was expressed after the 4th day of culturing on SIM medium (Matsuo et al. 2011).

Protein profiling can be used as an initial screening for the process of growth and development of plant tissue culture (Utami et al. 2007) as well as morphological and histological analysis that can be used for explaining any microscopic changes in detail (Koyuncu & Balta 2004). This was in line with the results of this study that there were different protein bands between those two pathways regeneration started from 7-DAC and this can be proven by morphological observations using light microscopy and SEM as well by histological analysis.

CONCLUSION

This research showed that the two tissue culture techniques have different aspects at morphological, histological, and protein profile. At the early event of culturing, explants cultured on A medium showed the presence of a globular-like structure, while the explants cultured on B medium showed the initiation stage of *in vitro* organogenesis. The histological analysis showed that some of the explants cultured on A medium lead to the development of globular stage, while at explants cultured on B medium showed the meristem reconstruction at SAM and RAM. Protein profile analysis using SDS-PAGE showed protein bands of 54 and 81 KDa only appeared at explants cultured on A medium started from 7-DAC, and those two protein bands thought to be a candidate of different key protein at the early stages of those two tissue culture processes.

AUTHORS CONTRIBUTION

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

CONFLICT OF INTEREST

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

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