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Research Article

Early Event at *In Vitro* Propagation of PGL-15 Tea Clone

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ABSTRACT
In vitro propagation is one solution to maintain or even to increase tea's productivity in Indonesia. This study aimed to obtain early-event information related to *in vitro* tea propagation using embryo axis and cotyledon of PGL-15 clone. This research was carried out by culturing the explants on MS medium with half strength (½ MS) (A medium); ½ MS + BAP 2 mg L^{-1} (B medium); ½ MS + BAP 2 mg L^{-1} + GA3 0.5 mg L^{-1} (C medium); and ½ MS + BAP 2 mg L^{-1} + GA3 0.5 mg L^{-1} + eco enzyme 0.1 % v v^{-1} (D medium), with five explants per bottle and each treatment was repeated three times. The result showed that the D medium was the best medium in inducing further development of embryo axis and cotyledon explants. Morphological analysis showed that embryo axis explants cultured on the B, C, and D medium seemed better vigour, indicated by the increasing size and blooming of leaf primordial(s). Surface ultrastructure analysis showed that speed development of leaf primordial(s) on embryo axis explant varied among tested explants, depending on the type of the culture medium. For cotyledon *in vitro* propagation, it showed that at 7 days after culture (7-DACs), the D medium gave the best results in inducing explants for further development. This successful induction of further development of these explants is expected to open possibility of its multiplication in large quantity and its application for further research on tea plant.

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INTRODUCTION

Tea is known as the most widely drink consumed by people, especially in Indonesia. Apart from being known as a thirst-quenching drink, tea is also known as a healthy drink many benefits because of its content, i.e. active compounds such as polyphenol Gramza et al. (2005). This polyphenol compounds and other antioxidant substances are one reason for almost people in the world consumed it as healthy drinks.

The uniqueness of tea makes it to be one of the most widely consumed healthy drinks in Indonesia. This is very important for Indonesia to ensure the availability of tea plant for domestic consumption. Apart from that, Indonesia is known as one of the largest tea exporting countries in the world, next to Kenya, Sri Lanka, India, and Vietnam (Bolton 2015). These two things must be maintained and even increased by Indonesia for tea production both in quantity and quality.

Tea is an annual plant that is usually propagated vegetative through cuttings. However, in the field, obstacles are often found in efforts to increase tea production, for example lack of cuttings as planting material. Difficulty of rooting and obtaining the materials are the main problems for the use of cutting to be used as planting materials (Liu et al. 2019). The solution to this problem is through in-vitro propagation using seeds. One of the advantages of propagation through seeds is that the resulting plants have strong roots in addition to normal shoots because of the completeness of the two axis poles in the seed (El-Maarouf-bouteau 2022). Several research reports have shown the success of *in-vitro* tea propagation with specific clones, culture medium, or explant types (Gonbad et al. 2014; Eskundari et al. 2018; Widhianata & Taryono 2019).

PGL-15 tea clone is one of the superior tea clones in Indonesia. This clone is known for its high yield and pest resistance (Wagiman & Triman 2011). However, no method for *in vitro* propagation of PGL-15 clones has been reported so far. Thus, researchers consider that it is necessary to conduct research regarding the method of propagating PGL-15 clones, especially using seeds as explants. Thus, it is hoped that the current research results will be useful in efforts to increase tea planting materials to meet tea needs both domestic and foreign demands.

MATERIALS AND METHODS

Materials

The PGL-15 clone tea seeds used as explants were obtained from Kayulandak Garden-PT. Pagilaran, Batang, Central Java, Indonesia (109°40'19"-110° 03'06" E and 6°51'46"-7°11'47" S) at 1000-1500 m above sea level during September 2023. For culture media, the used materials include MS medium (Phytotech), benzylaminopurin (BAP; Himedia), gibberellic acid (or GA3; Himedia), and gelzan (Phytotech). The eco-enzyme solution which will then be added to the culture media was made by fermenting molasses, fruit skins (melon, mango, and banana), and water with comparison 1:3:10 for about 6 months. These fruits were collected from Gunung Kidul Region, Yogyakarta, Indonesia (110°21'-110°50' E and 7°46'-8°09' S) at altitude 206 m above mean sea level during January 2023 and these fruits were verified by botanist from Universitas Veteran Bangun Nusantara, Indonesia.

Methods

Preparation for culture medium

Culture medium that used in this study were MS medium with half strength (½ MS) (A medium); ½ MS + BAP 2 mg L^{-1} (B medium); ½ MS + BAP 2 mg L^{-1} + GA3 0.5 mg L^{-1} (C medium); and ½ MS + BAP 2 mg L^{-1} + GA3 0.5 mg L^{-1} + eco-enzyme 0.1 % v v^{-1} (D media). To all medium, 3 % (w v^{-1}) sucrose

and 0.8 % (w v^{-1}) gelzan were added. All medium was adjusted to 5.6-5.8 for its pH before autoclaving at 121 °C for 15 minutes.

Preparation seed of PGL-15 as explant

PGL-15 tea seeds with age of around 8 months after seed-set formation were sterilized using antibacterial (Agrept 20WP, Streptomycin sulphate 20 %) for about 10 minutes, followed by rinsing using running water three times, sterilized using antifungal (Antracol 70WP; Propineb 70 % and Sulticob 93WP; copper oxysulphate) for 5-10 minutes, and rinsed using running water. The sterilisation process was continued in laminar air flow by soaking the seeds in a 96 % alcohol solution for 1 minute, then the seeds were burned and the seed shell was opened. Axis and cotyledon embryos were separated and then cultured on tested medium respectively.

Seed *in-vitro* propagation

Embryo axis and cotyledon were then cultured on tested medium with five explants on a bottle with three replications. Culture conditions were set at temperature of 22 ± 2 °C and light illumination intensity of 3250 lm, 90 lm W^{-1} (Philips TLD 3684 cool daylight).

Morphological analysis

Explant was observed at the morphological indicators that occur (i.e. the emergence of germination) and captured by digital camera (Canon™).

Data Analysis

This research was conducted in a completely randomised design with three repetitions and five explants in each bottle. The data was analysed statistically using F-test and the means among each treatment were analysed separately using Tukey test and $P<0.05$ was considered as statistically significant.

Surface ultrastructure analysis

Explant at 21-DAC was treated using low vacuum at 70 KPa for about 20 seconds until stability was achieved, and then the explant was observed its morphology using scanning electron microscope (SEM Quanta 250).

RESULTS AND DISCUSSION

Embryo Axis Culture

The observation results showed that almost all embryo axis explants cultured on tested medium had grown that characterised by development of root and shoot pole at 14-DAC (Table 1). The A medium provided the least explant that responded to lead further development compared to other three culture mediums. The D medium, which was half strength of MS medium with the addition of BAP, GA3, and eco-enzyme, was succeeded in promoting further development of axis embryo.

The success of D medium in inducing further development may be due to the PGR's content on D medium. PGRs on D medium consist of BAP, GA3, and eco-enzymes and they were possible reasons for axis embryo to develop further. Cytokinin is known as a PGR which plays a role in plant development, such as the initiation and development of the shoot apical meristem (SAM) (Hnatuszko-Konka et al. 2021). Cytokinin are reported to not only play role in rating cell division but also in regulating the transition from undifferentiated cells to primordial differentiated cells, via one of the regulations of *Knotted-like homeobox1* (*Knotted 1*) gene so it can be said that these two things are related to each other (Uzelac et al. 2012). Furthermore, cytokinin will attach to the Arabidopsis Histidine Kinase (AHK) receptor which will then result in phosphorylation cascade marked by the activation of Arabidop-

sis Histidine Phosphotransfer Proteins (AHPs). This protein will then activate the Arabidopsis Cytokinin Response Regulators gene (type-A and -B ARRs) so that the ARR protein will activate genes related to SAM formation such as *WUSCHEL* (*WUS*) and inactivate the *YUCCA* gene (*YUC*) (Pokimica et al. 2024).

Cytokinin is known as one of the PGRs that plays role in shoot germination. According to Villalobos and Martin (1992), cytokinin is found at embryo axis, whereas conjugated cytokinin is found at epicotyl and hypocotyl with its highest concentration is in the apical area. Therefore, the D medium provided further developmental response for embryogenic axis explants, and this matter might be due to the presence of cytokinin added to the D medium.

The D medium also contained gibberellic acids in the form of GA3. Several studies reported that GA3 plays a role in seed germination, such as on coffee's embryo axis (Bojórquez-Quintal et al. 2011). GA3 actually plays a role in germination because of the degradation of mannose in the endosperm cell walls so that it will have an impact on the breakdown of food reserves and finally it can support germination (Subandi et al. 2015).

The success of germination may also be influenced by addition of fruit-based eco-enzyme solution on culture medium. Eco-enzyme is reported to contain phytochemical compounds such as alkaloids (Eskundari et al. 2022) so it is suspected that these chemical compounds can induce the embryonic axis response to germination. Alkaloids are compounds that contain nitrogen with one or more nitrogen atoms (Debnath et al. 2018) so they are beneficial for plant growth (Bakri 2020). Thus, once again, the addition of fruit-derived eco-enzyme was thought to stimulate further development of tea embryonic axis explants.

Morphological analysis showed that at 0-DAC, the embryo axis was yellowish and no germination was visible (Figure 1). After 21-DAC on tested medium, almost of the explants progressed to further development, i.e. germination (Figure 2). The cultured explants seemed fresh, indicated by the change in colour of the explants from greenish to dark green. Apart from changing colour, explants also experienced germination which is characterized by the development of shoots (leaves) and roots.

After 35 days of culture, most of the explants proceed to further development (Figure 3.). Explants cultured on the B, C, and D medium grew well that can be seen in increasing of size and blooming of leaf primordial(s). Explants cultured on the A medium also experienced further development although the development pattern was not similar to explants cultured on the B, C and D medium. Root development on explants cultured on the A medium appeared to be more developed than shoot development. This phenomenon was possibly due to the absence of PGR added to the A medium so that the A medium is more suitable for root development. In contrast, shoot development in explants cultured on the B, C, and D medium was more dominant than root development. This phenomenon was most likely due to the addition of cytokinin to culture medium. Cytokinin plays a role in shoot organogenesis (Šmeringai et al. 2023), and there are many reports regarding the *in-vitro* propagation of tea that used cytokinin on culture medium to induce shoots, such as clones TRI2025 (Eskundari et al. 2018), Kiara-8 (Widhianata & Taryono 2019), and Iran-100 (Gonbad et al. 2014).

Table 1. Mean number of embryonic axis explants that experienced further development at14-DAC

Type of medium	A	B	C	D
Mean of developing explants	3.35[a]	4[a]	4.35[a]	4.65[a]

* Means followed by the same letter are not significantly different based on Tukey's test ($p > 0.05$

Figure 1. Axis embryos a moment after culture (0-DAC). Black arrow showed shoot apical meristem; blue arrow showed root apical meristem.

Figure 2. Morphological analysis of embryo axis explant at 21-DAC. Various explants were cultured on: A medium (a); B medium (b); C medium (c); and D medium (d).

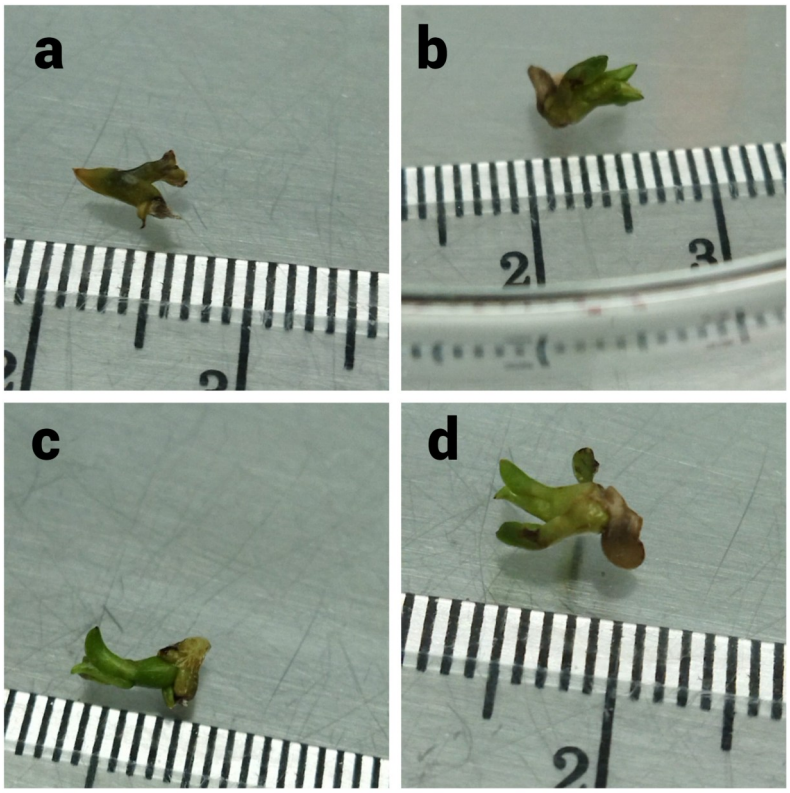

Figure 3. Morphological analysis of embryo axis explant at 35-DAC. Various explants were cultured on: A medium (a); B medium (b); C medium (c); and D medium (d).

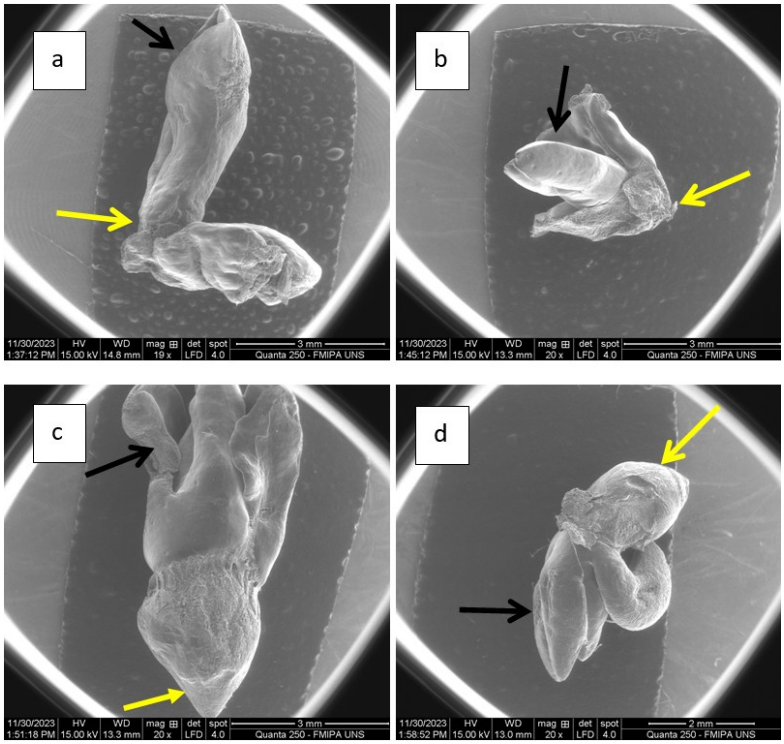

Figure 4. Surface ultrastructure analysis of embryo axis explant at 21-DAC cultured on: A medium (a); B medium (b); C medium (c); and D medium (d). Black arrows showed leaf primordial development; yellow arrow showed root development.

Surface ultrastructure analysis showed that at 21-DAC explants have had grown to germination phase, one of which was marked by the development of shoots (leaves). The speed of development of leaf primordial(s) varied at tested explants, depending on the culture medium type (Figure 4). It can be seen that explants cultured on B, C and D medium, the leaf primordial(s) has

further developed than explants cultured on A medium. This matter was most likely due to the suitability of the PGR(s) added to the medium so that the explants responded faster and finally the leaf primordial(s) developed.

Surface ultrastructure analysis showed the response of root organogenesis occured at upper basal end of embryo axis. This phenomenon was indicated by changes in root size and the development of taproot. According to Zobel and Waisel (2010), taproot develops from a central root pole called the radicle, forming a central root axis system and Mackay et al. (2020) reported that this type of root allows plants to more easily absorb water found at the deep of the soil, and finally this is one way for plants to deal with drought stress.

Cotyledon culture

Tea propagation in this study was also carried out using cotyledons as explants. Almost all of the cotyledon explants responded when cultured on tested medium and observation at 7-DAC showed that the D medium gave the best results in inducing explants to further development (Table 2; Figure 5). On the other hand, the A medium which did not receive any additional PGR (s) has the lowest results in terms of the average number of explants that experienced for further development.

Table 2. Mean number of cotyledon explants that experienced to further development at14-DAC.

	A medium	B medium	C medium	D medium
Mean of developing explant	3[a]	4[a]	3.65[a]	4.34[a]

* Means followed by the same letter are not significantly different based on Tukey's test ($p > 0.05$

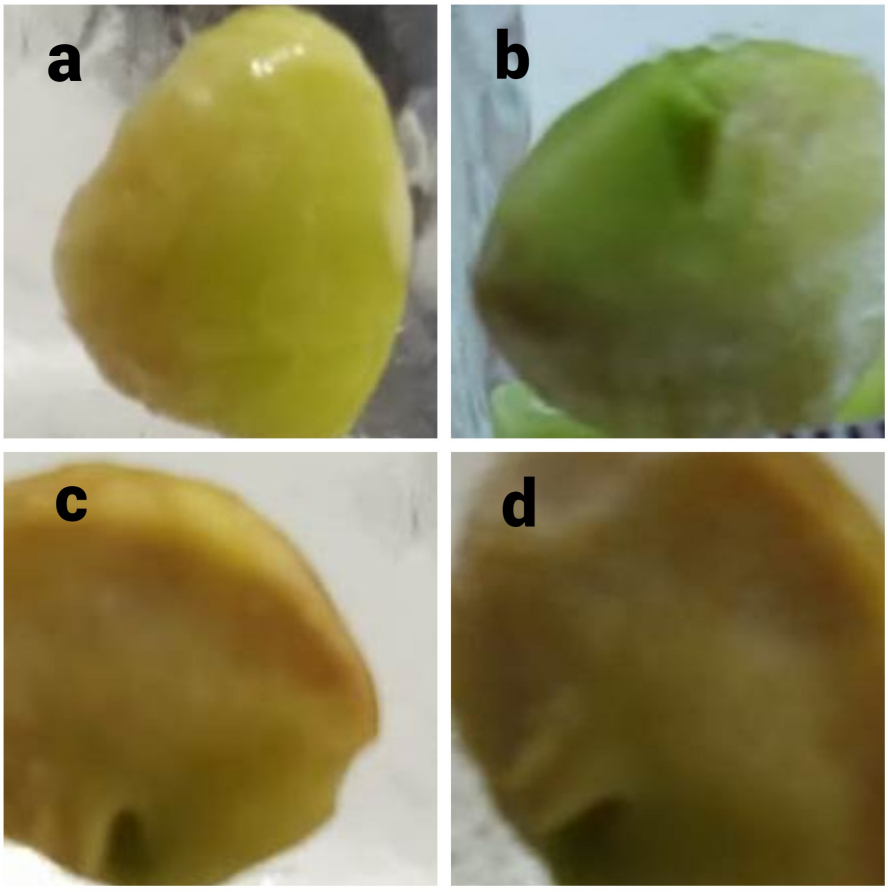

Figure 5. Morphological analysis of cotyledon explant on 7-DAC. Explants were cultured on: A medium (a); B medium (b); C medium (c); and D medium (d).

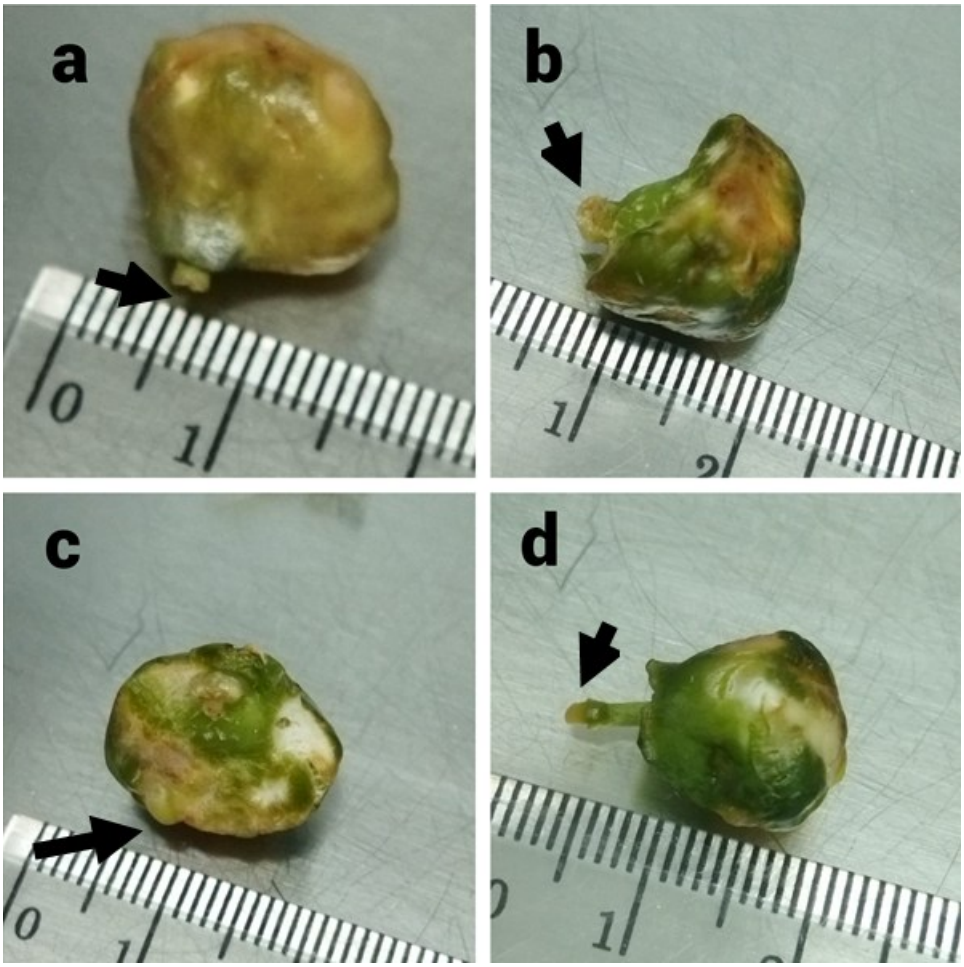

Figure 6. Morphological analysis of cotyledon explant on 35-DAC. Explants were cultured on: A medium (a); B medium (b); C medium (c); and D medium (d). Black arrow showed cotyledon petiole.

The D medium gave the best results in inducing organogenesis of PGL-15 tea clone in the form of cotyledon explants. This phenomenon was likely due to the suitability between the composition and ratio of PGR contained at culture medium and the explant's needs for further development. The D medium contained BAP 2 mg L^{-1}, GA3 0.5 mg L^{-1}, and eco-enzyme 0.1 % (v v^{-1}) and this composition was thought to be a reason of explants developed more quickly through the organogenesis pathway.

Explant that cultured on all tested medium showed the further development with different speed. The D medium was the fastest inducer for further development of explants and this medium successfully induced further explant development in the form of cotyledon petioles (Figure 6). This phenomenon was in line with the cotyledon petiole structure reported by Giovannelli et al. (2004) in chestnuts *in-vitro* propagation and Sari et al. (2021) in *ex-vitro* propagation of sugar palm. This structure had the unique characteristic on its first appearance in the area of embryo's axis former region.

Root development was seen at explants cultured on D medium in the form of cotyledon petiole. It was characterized by the presence of a long structure with a yellowish white tip. Later, this structure underwent to adventitious root. Similar thing has been reported in walnuts's histological analysis that showed cell division activity at the petiole's tip globular protein bodies in the central vacuole and starch grains in the cytoplasm (Gutmann et al. 1996).

This cotyledon petiole structure was a unique structure, and related to this structure, Giovannelli et al. (2004) stated that there were several chestnut explants that experienced to root organogenesis and the experienced to

shoot organogenesis. This phenomenon characterised by the presence of a nodular structure at the end of the petiole. Our previous study also showed the same phenomenon using TRI2025 cotyledon explants cultured on ½ MS media with the addition of BAP 2 mg L^{-1} (unpublished result; Figure 7).

Figure 7. Cotyledon of TRI2025 tea clone culture. Black arrow showed root development and yellow arrow showed shoot organogenesis.

The quantity of cotyledon explants that responded to culture medium were not as much as embryo axis explants that responded to further development after being cultured on tested medium. This matter was probably because the embryogenic axis has two growth poles, i.e. shoot and root pole, so that with just little induction, the explant would respond to grow. On the other hand, not all of explants experienced to further development and this phenomenon might be due to the presence of a small part of the embryonic axis remaining in the cotyledon area, resulting in response such as cotyledon petiole response in certain explants. This phenomenon was in line with the research results of Gutmann et al. (1996) that showed not all walnut cotyledon explants underwent to organogenesis initiation.

Cotyledon development occurred on all tested medium, with the D medium providing the best response for the development. The D medium contained cytokinin, gibberellic acid, and eco-enzyme on ½ MS media. Among cytokinin, gibberellic acid, and eco-enzyme are strongly suspected to play positive role in initiating further development of tea cotyledons of the PGL-15 tea clone.

In this study, the cotyledon explants especially that cultured on the D medium appeared to regenerate, i.e. in the form of cotyledon petiole. Although none of the tested media contained auxin, Plant Growth Regulator (PGR) known for regulating root development, the compatibility between the PGR and explants for further development may play a role. Additionally, there is the possibility that a small portion of the embryogenic axis remained in the cotyledon.

Since MS medium contains macronutrients, micronutrients, and vitamin, changing the strength or concentration of MS medium could change the

nutrients component. Therefore, the effect of different medium strength could also affect the growth and development of plant. The amount of nutrients in medium for successful *in-vitro propagation* might vary in genotype of plant (Rezali et al. 2017). In other word, the suitability of the culture medium might influence the success in inducing further development of cultured explant.

Murashige & Skoog (MS) medium at half strength was used in this study, and the use of it at this study referred to the successful of in-vitro propagation of TRI2025 tea clone with axis embryo explants (Eskundari et al. 2018, 2019, 2021). Apart from that, the use of ½ MS media was also reported to be successful in inducing an explant response in other tea clones such as the Tambi, Jingga, Cinyuruan-143, and Kiara-8 clones (Widhianata & Taryono 2019). The use of ½ MS was also reported to provide a higher germination success rate than the use of full strength MS as in *Nothapodytes foetida* in-vitro propagation (Kaveri & Rao 2015).

In addition, in this study, eco-enzyme that also be added on to the D medium might be has an effect to initiate further development of tested explant. This result might be correlated with the content of eco-enzyme solution such as alkaloid that has one or more nitrogen ring that useful for plant growth development (Ain et al. 2016). Alkaloids are known as one kind of antioxidant that will play role in controlling the amount of Reactive Oxygen Species (ROS) through the ascorbate-reduced glutathione (AsA-GSH) pathway (Hasanuzzaman et al. 2020), so that it can increase the plant defence to stress oxidation. Furthermore, one type of alkaloid; purine alkaloid (Dey et al. 2020), can be broken down to alantoin and this compound compensates for soil nitrogen deficiencies, thereby maintaining nitrogen homeostasis and promoting plant growth and development (Kaur et al. 2023). This organic solution is reported has powerful inducer to initiate plant growth via ex-vitro propagation, such as in cassava (Faoji et al. 2021), chili (Wahyuni et al. 2023), and *Impatiens balsamina* (Eskundari et al. 2023).

In this study, no segregation test and percentage of phenotype changes that occur due to the use of seeds as explants were carried out. This is because this study aims to obtain a method for propagating PGL-15 clone tea using seeds. The use of seeds as planting material can almost certainly cause segregation and phenotype changes. In addition, according to (Chen et al. 2012), tea is a plant that cross-pollinates due to self-incompatibility, so it is certain that segregation will definitely occur in its propagation through seeds.

Based on the results of this research, it can be concluded that embryo axis explants promise a higher percentage of growth response compared to cotyledon explants. Embryo axis explants offer perfect plant development with two growth poles, i.e. roots and shoot meristem, so that the embryo axis is suitable as an explant for genetic engineering (Paes de Melo et al. 2020) although its number is limited compared to cotyledon explants. From this, we know that there were differences in in-vitro propagation of PGL-15 clone tea using axis embryo and cotyledon explants, thus it can be said that the success of *in vitro* propagation is influenced by many factors, such as the specificity of explant's type or culture medium. Certainly, specific research in order to find established methods for in-vitro propagation for other types of plants needs to be carried out. With the discovery of an established in-vitro propagation method, it is hoped that it will facilitate further research such as its application for genetic engineering or genome editing in certain plants.

CONCLUSIONS

The D medium was the best in initiating further development for both embryonic axis and cotyledon explants. Morphological analysis showed that embryo axis cultured on the B, C, and D medium seemed fresher and healthier, as noted by the increasing size and blooming of leaf primordial(s). Surface ul-

trastructure analysis showed that development's leaf primordial(s) speed varied among tested embryo axis explants, depending on the kind of culture medium. For cotyledon in-vitro propagation, it showed that the D medium gave the best results in inducing explants to further development i.e. "cotyledon petiole". This successful development induction with embryo axis and cotyledon explants of PGL-15 tea clone is expected to open the possibility for large scale multiplication or for further tea's research such as genetic engineering or genome editing.

AUTHOR CONTRIBUTION

R.D.E, T.T., and T.A. designed the research, R.D.E., T.T., T.A., and D.O. collected and analysed the data and wrote the manuscript, R.D.E and T.T. 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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