The effect of 2,4-D, BA and Thidiazuron on somatic embryo induction of liberica coffee of Tungkal Composite from Jambi

L. Lizawati¹²*, Z. Zulkarnain¹³, D. Antony¹, and R. Purnamaningsih⁴

¹University of Jambi, Department of Agroecotechnology, Faculty of Agriculture, Jambi Indonesia
²University of Jambi, Center of Excellence for E2-KOLIM, Jambi, Indonesia
³University of Jambi, Center of Excellence for Land-use Transformation System, Jambi, Indonesia
⁴National Research and Innovation Agency, Research Center for Horticultural and Estate Crops, Bogor, Indonesia

Abstract. The Tungkal Composite of liberica coffee rejuvenation program at Jambi Province is urgently needed due to the latest status of the plants are old and showing diseased attacked. To support the rejuvenation, the provision of seedlings could be done by tissue culture approach through somatic embryogenesis. This research aimed to study the effect of 2,4-D, BA and TDZ on callus formation and embryo induction from liberica coffee leaf explants. Explants used were leaf sections with or without veins. Explants were grown on Murashige Skoog (MS) basal medium with 2,4-D + BA and 2,4-D + TDZ. A completely randomized design was employed in the trial, and each experimental unit was replicated three times. Results showed that callus could be formed on both types of explants, but leaf explants with veins could produce callus faster. The use of BA in medium could induce callus formation faster than TDZ. The higher the concentration of 2,4-D, BA and TDZ, the slower callus initiation. The addition of TDZ in the medium resulted in mostly callus, while callus from medium with BA showed a friable structure. Globular somatic embryos were formed in all treatments with varying amounts. TDZ can induce more globular somatic embryo than BA.

1 Introduction

Indonesia is one of great coffee producing country along with Brazil, Vietnam, and Colombia [1]. In world coffee trade, the arabica coffee (C. arabica) and robusta coffee (C. canephora) are two important types. In Indonesia, about 85% of national coffee production is dominated by robusta, and only 15% occupied by arabica coffee [2].

In addition to arabica and robusta coffee, there is another type of coffee, namely liberica [3]. Liberica is a coffee plant that can be cultivated on marginal lands, such as peatlands, and it has a unique taste and distinctive aroma. It is easy to grow in lowlands area (< 800 m above sea level). Tungkal Composite of Liberica is a type of liberica coffee from Tanjung Jabung

* Corresponding author: liza_wati@unja.ac.id
Timur Regency, Jambi Province [4] which has been released as a national superior variety. Currently the demand for this coffee tends to increase so that it is necessary to expand the planting area to increased production.

The problems faced in the development of Tungkal Composite of Liberica coffee are the availability of old trees (35-50 years) and many plants are suffering from pests and diseases attack. Therefore, a method of rapid propagation is needed to produce seeds that are free from pests and diseases.

One method of rapid clonal propagation to produce quality coffee seeds on a large scale is tissue culture techniques through somatic embryogenesis. This technique can create uniform true-to-type seeds in large numbers in short time [5, 6]. Somatic embryogenesis successfully regenerated in robusta and arabica coffee and has been widely reported, but not much research has been conducted for liberica type.

Somatic embryos in coffee can be formed directly through pro-embryogenic leaf cells without callus proliferation or indirectly through embryogenic callus formation [7]. The regeneration method used is strongly affected by plant genotype, source of explant, and the use of growth regulators [5]. A study by Ibrahim et al. [7] revealed that each coffee species required different and specific composition of growing medium. Dichlorophenoxyacetic Acid (2,4-D), Benzyl Adenine (BA) and Thidiazuron (TDZ) were reported to be widely used in coffee propagation through tissue culture. Wattimena [8] claimed that 2,4-D was the most common auxin used to induce somatic embryogenesis. Besides auxin, the cytokinin BA also affected cell differentiation during somatic embryogenesis. TDZ at low concentrations could induce direct somatic embryo formation in Phalaenopsis amabilis [9] and Eurycoma longifolia [10]. Riyadi and Tirtoboma [11] reported direct formation of somatic embryos from immature leaves of arabica coffee cv. Kartika-1 on MS medium added with 4 mg L\(^{-1}\) 2,4-D and 0.1 mg L\(^{-1}\) kinetin. Successful somatic embryogenesis using TDZ was reported in Coffea canephora [12, 13].

Coffee propagation through somatic embryogenesis has been successfully made for C. arabica and C. canephora [3, 14–19]. In C. liberica, however, this is among few reports on tissue culture induction according to our knowledge. This study aimed to evaluate the effect of growth regulators 2,4-D, BA and TDZ on embryogenic callus induction and embryo formation on Liberica Coffee of Tungkal Composite immature leaf explants.

## 2 Materials and methods

### 2.1 Explants source

The explants used were young leaves of Liberica Coffee of Tungkal Composite clone from Jambi, Indonesia. The explants were washed with liquid detergent, rinsed with tap water, soaked in 2 g L\(^{-1}\) Benlate solution for 1 hour, then rinsed in 70% ethanol for 10 minutes. Further, the explants were shaken in a commercial bleach solution (NaOCl 5.25%) with a concentration of 30% and 20% for 30 minutes, respectively, and rinsed three times with sterile water.

### 2.2 Medium preparation

The experiment consisted of 2 trials, namely 1) effect of 2,4-D and BA on callus induction and somatic embryogenesis, and 2) effect of 2,4-D and TDZ on induction of somatic. In the first trial, the culture medium used was MS basal medium [20] supplemented with 2,4-D at 1, 2, 3, and 4 mg L\(^{-1}\) combined with BA at 1, 2, 3, and 4 mg L\(^{-1}\). Therefore, there were 16 combinations of growth regulators (D1BA1 = 2,4-D 1 mg L\(^{-1}\) + BA 1 mg L\(^{-1}\), D1BA2 = 2,4-
D 1 mg L^{-1} + BA 2 mg L^{-1}, D1BA3 = 2,4-D 1 mg L^{-1} + BA 3 mg L^{-1}, D1BA4 = 2,4-D 1 mg L^{-1} + BA 4 mg L^{-1}, D2BA1 = 2,4-D 2 mg L^{-1} + BA 1 mg L^{-1}, D2BA2 = 2,4-D 2 mg L^{-1} + BA 2 mg L^{-1}, D2BA3 = 2,4-D 3 mg L^{-1} + BA 3 mg L^{-1}, D2BA4 = 2,4-D 3 mg L^{-1} + BA 4 mg L^{-1}, D3BA1 = 2,4-D 3 mg L^{-1} + BA 1 mg L^{-1}, D3BA2 = 2,4-D 3 mg L^{-1} + BA 2 mg L^{-1}, D3BA3 = 2,4-D 4 mg L^{-1} + BA 2 mg L^{-1}, D3BA4 = 2,4-D 4 mg L^{-1} + BA 3 mg L^{-1}, D4BA1 = 2,4-D 4 mg L^{-1} + BA 1 mg L^{-1}, D4BA2 = 2,4-D 4 mg L^{-1} + BA 2 mg L^{-1}, D4BA3 = 2,4-D 4 mg L^{-1} + BA 3 mg L^{-1}, D4BA4 = 2,4-D 4 mg L^{-1} + BA 4 mg L^{-1}).

In the second trial, the same medium composition was used with the addition of 2,4-D at 1, 2, 3, and 4 mg L^{-1} combined with TDZ at 1 and 2 mg L^{-1}. There were 8 combinations of growth regulators (D1TDZ1 = 2,4-D 1 mg L^{-1} + TDZ 1 mg L^{-1}, D2TDZ1 = 2,4-D 3 mg L^{-1} + TDZ 1 mg L^{-1}, D1TDZ2 = 2,4-D 1 mg L^{-1} + TDZ 2 mg L^{-1}, D2TDZ2 = 2,4-D 3 mg L^{-1} + TDZ 2 mg L^{-1}, D1TDZ3 = 2,4-D 1 mg L^{-1} + TDZ 3 mg L^{-1}, D2TDZ3 = 2,4-D 3 mg L^{-1} + TDZ 3 mg L^{-1}, D1TDZ4 = 2,4-D 1 mg L^{-1} + TDZ 4 mg L^{-1}, D2TDZ4 = 2,4-D 3 mg L^{-1} + TDZ 4 mg L^{-1}).

The acidity of the medium was determined at pH 5.8 by the addition of 1N KOH or 1N HCl. Bacto Agar (7 g·L^{-1}) was used to solidify the medium. The medium was boiled, then poured into culture flasks, and sterilized in an autoclave at a temperature of 121°C and 1.2 kg.cm^{-2} pressure for 15 minutes.

2.3 Explants inoculation and subcultures

The sterilized leaves were cut with a size of 5 x 5 mm where there were 2 types of explants used: 1) leaf section with veins, and 2) leaf section without veins. Five explants in each flask with the adaxial surface attached to medium. Cultures were incubated in dark condition in culture room at 25 ± 1°C for 4 weeks to initiate callus formation. The callus was subcultured to new medium with the same composition to stimulate embryogenic callus formation.

2.4 Experimental design

A completely randomized design with 3 replications was used in the trial. Each experimental unit consisted of 3 culture flasks. The observed parameters were time for callus formation, percentage of explants forming callus, time of somatic embryo formation, and number of somatic embryos obtained.

3 Results and discussions

3.1 Callus induction

All tested media could induce callus formation, but the rate of callus growth was different in each treatment. Responses began to occur when the explants were 15 days after culture, which was characterized by swelling of the leaves. Callus formation started from the wounded parts of leaf that was in contact with medium in leaf explants without veins, whereas in leaf explants with veins, callus first formed at the tip of the veins, followed by other parts of the leaf (Figure 1).

The use of 2,4-D + BA at different concentrations resulted in different effects on callus formation on explants with or without veins. In general, 1 mg L^{-1} 2,4-D + BA (1 - 4 mg L^{-1}) could induce faster callus formation compared to 2,4-D and BA at higher concentrations (Figure 2).

Leaf explants with veins was able to form callus faster on all treatment compared to leaf explants without veins. The fastest callus formation was obtained 1 mg L^{-1} 2,4-D + BA (1, 2
or 3 mg L$^{-1}$), while the longest callus formation time was found on 2,4-D (3 and 4 mg L$^{-1}$) + 4 mg L$^{-1}$ BA. The combination of 2,4-D + BA might induce cell growth and division as reported by Mayerni [21]. The explant type, plant genotype, nutrition status, and interactions between endogenous and exogenous hormones could affect the development of somatic embryogenesis [22].

**Fig. 1.** Callus formation on explants (A = leaf explants with veins, B = leaf explants without veins).

The percentage of callus formation indicated that the use of 2,4-D (1 and 2 mg L$^{-1}$) with the addition of BA (1, 2, 3 mg L$^{-1}$) could induce callus formation by 100%. The results were different from those of 2,4-D (3 and 4 mg L$^{-1}$) in combination with BA (1, 2, 3, 4 mg L$^{-1}$), where callus was only formed by 40 - 75%. It was assumed that the use of 2,4-D + BA at a concentration of more than 2 mg L$^{-1}$ inhibited callus formation (Figure 3). Our results were different from those reported by Riyadi and Tirtoboma [11] on arabica coffee. They found that increasing the concentration of 2,4-D to 8 mg L$^{-1}$ could increase the number of callus and globular somatic embryo. Another study by Hapsoro et al. [5] indicated that callus formation and somatic embryogenesis was strongly influenced by plant genotype.

**Fig. 2.** Time to callus initiation on media containing 2,4-D + BA (B = leaf explants with veins, WB = leaf explants without veins).
Fig. 3. Percentage of callus formation on media containing 2,4-D + BA (B = leaf explants with veins, WB = leaf explants without veins).

The time to callus formation on explants with leaf veins was faster than without leaf veins. Leaf veins are transport tissues consisting of xylem and phloem. One of auxin transport mechanisms in plants is passive transport through the phloem vessels from the source to the sink, so that the phloem vessels always contain auxin [23].

The use of 2,4-D in combination with TDZ gave different results. In general, the use of 2,4-D + TDZ at all concentrations could induce callus formation faster than 2,4-D + BA, with 75 - 100% explants forming callus. Callus initiation was slower with 2 mg L\(^{-1}\) 2,4-D + TDZ (3 and 4 mg L\(^{-1}\)), as well as the percentage of explants forming callus (Figures 4 and 5).

Fig. 4. Callus initiation time on medium containing 2,4-D + TDZ (B = leaf explants with veins, WB = leaf explants without veins).

Fig. 5. Percentage of callus formation on media containing 2,4-D + TDZ (B = leaf explants with veins, WB = leaf explants without veins).
Study by Ardiyani [24] found that the best medium composition for callus induction of *C. liberica* Arruminensis clone was 1.08 mg L\(^{-1}\) 2,4-D. However, in our study the use of auxin along with cytokinins was able to increase the effectiveness of callus formation, which indicated that different plant genotypes require different methods. In this case the callus induction medium containing 1 mg L\(^{-1}\) 2,4-D + BA (1 and 2) mg L\(^{-1}\) was the most effective among the treatments tested.

The same finding occurs in robusta coffee embryogenesis [6, 25]. It was found that exogenous auxin was not required for embryogenic callus formation and its multiplication during in vitro embryogenesis in most of robusta genotypes. Whereas a study reported that the use of single cytokinin (BA) was beneficial to produce robusta coffee seedlings through somatic embryogenesis [26].

### 3.2 Callus growth and somatic embryo formation

The combination of 2,4-D (1, 2, 3, 4 mg L\(^{-1}\)) + BA (1, 2, 3, 4 mg L\(^{-1}\)) produced callus that was friable and clear in color, although several treatments produced compact callus. Table 1 indicated that 1 mg L\(^{-1}\) 2,4-D at all concentrations of BA induces cell division that can be seen from increased callus size. In contrast, in 2,4-D treatments (3 and 4 mg L\(^{-1}\)), where decreasing in callus size occurred when BA was applied at more than 1 mg L\(^{-1}\). Moreover, with 2 mg L\(^{-1}\) 2,4-D, a decrease in callus size occurred in the addition of 3 mg L\(^{-1}\) and 4 mg L\(^{-1}\) BA. This is probably due to the high concentration of 2,4-D causes the formation of non-embryogenic callus with lack ability to regenerate. Thus, the higher the concentration of 2,4-D and BA, the smaller the amount of globular somatic embryos produced.

#### Table 1. Effect of 2,4-D and BA on callus diameter and number of somatic embryos in globular stage.

<table>
<thead>
<tr>
<th>Plant growth regulators (mg L(^{-1}))</th>
<th>Callus diameter (cm)</th>
<th>Number somatic embryos in globular stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA 1</td>
<td>2.13 bcd</td>
<td>11.40 fg</td>
</tr>
<tr>
<td>BA 2</td>
<td>2.21 abcd</td>
<td>28.60 a</td>
</tr>
<tr>
<td>BA 3</td>
<td>2.86 a</td>
<td>26.60 ab</td>
</tr>
<tr>
<td>BA 4</td>
<td>2.20 abcd</td>
<td>14.60 def</td>
</tr>
<tr>
<td>BA 1</td>
<td>2.38 abc</td>
<td>26.60 ab</td>
</tr>
<tr>
<td>BA 2</td>
<td>2.86 a</td>
<td>22.50 bc</td>
</tr>
<tr>
<td>BA 3</td>
<td>1.70 cde</td>
<td>18.00 cd</td>
</tr>
<tr>
<td>BA 4</td>
<td>1.57 de</td>
<td>14.00 def</td>
</tr>
<tr>
<td>BA 1</td>
<td>2.50 ab</td>
<td>18.0 cd</td>
</tr>
<tr>
<td>BA 2</td>
<td>1.93 bcd</td>
<td>16.50 de</td>
</tr>
<tr>
<td>BA 3</td>
<td>1.90 bcd</td>
<td>14.60 def</td>
</tr>
<tr>
<td>BA 4</td>
<td>1.19 ef</td>
<td>14.50 def</td>
</tr>
<tr>
<td>BA 1</td>
<td>2.40 abc</td>
<td>12.32 ef</td>
</tr>
<tr>
<td>BA 2</td>
<td>1.93 bcd</td>
<td>12.32 ef</td>
</tr>
<tr>
<td>BA 3</td>
<td>1.10 ef</td>
<td>7.00 g</td>
</tr>
<tr>
<td>BA 4</td>
<td>0.71 f</td>
<td>7.00 g</td>
</tr>
</tbody>
</table>

The highest number of globular somatic embryo (28.60) produced on 1 mg L\(^{-1}\) 2,4-D + 2 mg L\(^{-1}\) BA. This indicated that the combination of 1 mg L\(^{-1}\) 2,4-D + BA (2 and 3 mg L\(^{-1}\)) and 2 mg L\(^{-1}\) 2,4-D + BA (1 and 2 mg L\(^{-1}\)) are the best treatment for the induction of somatic embryos.

Callus regenerated on medium containing TDZ was mostly compact (Figure 6). The application of 1 mg L\(^{-1}\) 2,4-D + TDZ (1, 2, 3, and 4 mg L\(^{-1}\)) could increase cell division as indicated in increase in callus size. However, when the concentration of 2,4-D raised to 2 mg
L\(^{-1}\), the use of TDZ at a concentration of more than 1 mg L\(^{-1}\) caused the decreasing on callus size.

The results indicated an inhibition of cell division which decreasing callus size (Table 2). The largest callus diameter was obtained from 1 mg L\(^{-1}\) 2,4-D + 4 mg L\(^{-1}\) TDZ which was significantly different from other treatments, while the smallest diameter was obtained on 2 mg L\(^{-1}\) 2,4-D + 2 mg L\(^{-1}\) TDZ, which was not significantly different from 2 mg L\(^{-1}\) 2,4-D + TDZ (2, 3, 4 mg L\(^{-1}\)).

Table 2. Effect of 2,4-D and TDZ on callus diameter and number of somatic embryos in globular stage.

<table>
<thead>
<tr>
<th>Plant growth regulators (mg L(^{-1}))</th>
<th>Callus diameter (cm)</th>
<th>Number somatic embryos in globular stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDZ 1</td>
<td>1.20 e</td>
<td>22.30 b</td>
</tr>
<tr>
<td>TDZ 2</td>
<td>2.40 b</td>
<td>28.50 ab</td>
</tr>
<tr>
<td>TDZ 3</td>
<td>2.18 bc</td>
<td>27.33 ab</td>
</tr>
<tr>
<td>TDZ 4</td>
<td>3.50 a</td>
<td>31.00 a</td>
</tr>
<tr>
<td>2,4-D 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDZ 1</td>
<td>2.50 b</td>
<td>24.50 ab</td>
</tr>
<tr>
<td>TDZ 2</td>
<td>1.43 de</td>
<td>24.75 ab</td>
</tr>
<tr>
<td>TDZ 3</td>
<td>1.60 cde</td>
<td>22.60 b</td>
</tr>
<tr>
<td>TDZ 4</td>
<td>1.93 bcd</td>
<td>30.00 ab</td>
</tr>
</tbody>
</table>

The somatic embryos at globular stage were formed on the upper and lower surfaces of the leaves and on wounded parts that were in direct contact with the medium. This was in accordance with result obtained by Priyono [27] on robusta coffee. However, the results of Hatanaka et al. [25] showed that in robusta coffee, somatic embryos were only formed on the sides of the leaves, which indicated that different responses occurred because of different genotypes and medium used.

The highest amount of globular somatic embryos was resulted on 1 mg L\(^{-1}\) 2,4-D + 4 mg L\(^{-1}\) TDZ, although it was not significantly different among all treatments. It was assumed that 1 mg L\(^{-1}\) 2,4-D + TDZ (1-4 mg L\(^{-1}\)) was able to induce cell division, in addition to induce callus development to form embryos. The combination of 2 mg L\(^{-1}\) 2,4-D + TDZ (2, 3, 4 mg L\(^{-1}\)) decreased callus growth but increased callus development into embryos.

MS medium containing 1 mg L\(^{-1}\) 2,4-D + 1 mg L\(^{-1}\) BA was the most effective to induce the formation of globular somatic embryos in liberica coffee tissue culture [28], while callus induction in robusta coffee could be induced using 2-ip growth regulators [25]. The combination of 2-ip and 2,4-D was the best growth regulator for callus induction in Komari clone of robusta coffee [5]. These results provide information that there is a genetic influence of the genotype used. According to Santana-Buzzy et al. [18], the combination of growth regulators and their concentrations is specific for each plant species.
4 Conclusion

Callus could be formed on both types of explants, but faster callus production was from leaf explants with veins. The use of BA in medium could induce callus formation faster than TDZ. The higher the concentration of 2,4-D, BA, and TDZ, the slower callus initiation. The addition of TDZ in the medium resulted in the mostly compact callus, while callus from medium with BA had a friable structure. Globular stage embryos were formed in all treatments with varying amounts. TDZ can induce more globular somatic embryos than BA.

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