Adaptation of acid-tolerant Leucaena leucocephala cv Tarramba Calluses after gamma irradiation on regenerative media

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Abstract. The aim of this study to measure adaptation of callus Lamtoro (Leucaena leucocephala cv. Tarramba) after gamma irradiation on regenerative media. Lamtoro plant breeding in the form of callus with gamma ray irradiation is required for the development of a new plant-tolerant strain line with a gene mutation approach in tissue culture. The experimental design in this study was a complete randomized design (CRD). There were 13 treatments consisting of control (M0), 6-benzyl amino purine (BAP) media (M1-M4), kinetin media (M5-M8), and combination of BAP and kinetin media (M9-M12) during 8 weeks. The measured variables were viability of callus, the addition of diameter and height callus, colour and texture callus. Data were tested using analysis of variance (ANOVA) and continued with Duncan test if there were a significant difference among treatment. Data of viability colour and texture used descriptive analysis. The data showed that formulation media M12 (combination 1 mg L⁻¹ BAP and 1 mg L⁻¹ kinetin) was significantly different (P<0.05) in 8 week after plant (WAP). It was concluded that the best regenerative media for adaptation of callus lamtoro was combination of BAP and kinetin media.

1. Introduction
Lamtoro commonly used for forage that can be referred as multifunction plant. Lamtoro which have infected root nodules of nitrogen-fixing bacteria can act as soil fertilizers [1]. Other benefits of lamtoro can be bioremediation to agricultural land with unbounded copper contamination and can be used for silvopastura or the integration of ruminants with forests [2,3].

Lamtoro is multipurpose because all parts of the plant can be beneficially utilized both for humans and animals. Lamtoro in ruminant rations reaches 24-30% in each variety [4]. In general, the use of lamtoro as a supplement to agriculture in tropical countries has been widely applied. Lamtoro is one type of tree leguminosa with a high protein quality, which is about 15% to 38% [5] depending on the age of the plant. In addition lamtoro also as a forage source of calcium and phosphorus minerals.

Lamtoro grow well in Indonesia, especially in eastern Indonesia. One of lamtoro plants that exist in that area is lamtoro cv. Tarramba. Tarramba are tolerant of pest attack and drought, but there is no reports of tolerance to acid conditions. Indonesia has the potential land with extensive dry soil properties. Acidic soil characterized by low pH can be caused by a fairly high aluminum content [6,7].
Aluminium excess can be toxic to plants growth. Therefore lamtoro plants are tolerant to low pH conditions. They can exploit the potential of marginal landscapes in Indonesia especially with dry conditions. This problem can be solved by applying of plant biotechnology through tissue culture that can select acid-tolerant lamtoro plant.

Tissue culture is a technique to isolate part of plants such as protoplasm, cells, a group of cells, tissues, and organs, and grow them in aseptic conditions [8]. In vitro cultures, genetic diversity can be enhanced by somaclonal diversity.

Such diversity can be enhanced by various treatments such as the provision of physical mutagen (gamma rays) on embryogenic somatic cell groups. Mutations can cause genetic change due to change in the nucleotide base sequence of DNA causing a change in the amino acid sequence so that a protein is formed as well. Mutation induction can increase the frequency of somatic variation of the plant which is expected to be formed by several variations of plants with various superior properties [9]. The most appropriate mutation induction for genetic engineering is radiation of callus cells. Callus is a collection of amorphous cells that occur from tissue cells that divide itself continuously. The gamma-irradiated spheres acidic stress through the aluminium media. The tolerance level of L. leucocephala cv. Tarramba optimal at 100 ppm aluminium level (pH 5.5) based on plant morphological characteristics [10]. Sources of calluses that have been adapted acid has unknown on growth media after the aluminium was covered. Cultivated or subcultured on the media contained growth promoters such as auxin hormone. According to Lestari [11] for callus formation using auxin was 2,4-D (*dichlorophenoxyacetic acid*). Putative mutant lamtoro calluses of cytokinin media is furthermore potential to be regenerated on regenerative media. For regeneration through the path of somatic embryogenesis requires maturation using cytokines such as *6-benzyl amino purine* (BAP) or kinetin [12].

The aim of this study to measure adaptation of callus Lamtoro (*Leucaena leucocephala* cv. Tarramba) after gamma irradiation on regenerative media.

2. Materials and methods

Calluses stock of lamtoro (*L. leucocephala* cv. Tarramba) after gamma irradiation of 40 gray which have been selected on AlCl$_3$ acid media was propagated on 2 mg L$^{-1}$ 2,4-D media, basal media that contained 1 L of aquades, 4.43 gram L$^{-1}$ media Murashige and Skoog (MS) [13], and 30 gram L$^{-1}$ sugar for 8 weeks with temperature 25°C under florence lamp lighting 800-1000 lux 16 hours day$^{-1}$.

### Table 1. Media compound of MS.

|                    | Macronutrient | Vitamin          |
|--------------------|---------------|------------------|
| KMNO$_3$          | 1900          | Glycine 2        |
| NH$_4$NO$_3$      | 1650          | Myo Inositol 100 |
| MgSO$_4$          | 180.5         | Nicotinic acid 0.5 |
| KH$_2$PO$_4$      | 170           | Pyrodoxin HCl 0.5 |
| CaCl$_2$          | 332.02        | Thiamine HCl 0.1 |

| Micronutrient          |                |
|------------------------|-----------------|
| FeSO$_4$.7H$_2$O       | 27.8            |
| Na$_2$EDTA             | 37.3            |
| CoCl$_2$.6H$_2$O       | 0.025           |
| CuSO$_4$.4H$_2$O       | 0.025           |
| H$_3$BO$_3$            | 6.2             |
| FeSO$_4$.7H$_2$O       | 27.8            |
| MnSO$_4$.H$_2$O        | 16.9            |
| Na$_2$MoO$_4$.2H$_2$O  | 0.25            |
| ZnSO$_4$.7H$_2$O       | 8.6             |
Experimental design was a complete randomized design (CRD) with 13 treatments based on. The treatment were M0= MS 0 (Control). M1= 0.5 ml BAP. M2= 1 ml BAP. M3= 1.5 ml BAP. M4= 2 ml BAP. M5= 0.5 ml KIN. M6= 1 ml KIN. M7=1.5 ml KIN. M8= 2 ml KIN. M9= 0.25 ml BAP + 0.25 ml KIN. M10= 0.5 ml BAP + 0.5 KIN. M11= 0.75 ml BAP+ 0.75 ml KIN. and M12= 1 ml BAP + 1 ml. Measured variables include viability (%) diameter and callus height (mm). the addition in diameter and callus height (mm week-1). The diameter and height of the callus were measured by measuring the canopy portion of the callus (the maximum growth point of the callus) to the part of the plant still appearing on top of the media by using a 150 mm slotted KANON range. The addition in diameter and height of callus was measured from the result of week x minus week x-1. Viability of the plant was done by calculating the percentage of live callus (cells still differentiate to form new callus and shoots) each treatment that grows. Data of viability was analyzed using descriptive analysis. The response to the mutant callus was analyzed using analysis of variance (ANOVA). and continued with Duncan test if there was a significant difference among treatment [14].

3. Results and discussion

3.1. The viability of callus (L. leucocephala cv. Tarramba) in the regenerative media.

Viability of calluses describes the growing power of callus that have been exacerbated by acid media on the regenerative media. The results showed that there were varied differences on value of mutant calluses with different formulation in regenerative media (Figure 1).

![Figure 1. Viability of callus in regenerative media.](image)

The results of the highest viability were M2 and M3 (96%) and the lowest in M5 (62.5%). The high viability capabilities of M2 and M3 showed that the cell ability of the callus to develop. moreover potential of cell damage was low. Decrease in the percentage of life due to reduced callus ability to grow [10].

3.2. The addition in diameter and height callus (L. Leucocephala cv. Tarramba)

The increasing in the diameter of the mutant calluses represent a good growth by calluses in the regenerative media. The increasing in diameter was indicated by the explant swelling of the calluses.

Explant swelling is characterized by changes in the thickened scarring caused by the increasing of the size and number of explant cells in the area [15].

Initial response of the injury tissue that begins to change shape into calluses mass in line with increasing age of culture [16] The increasing in diameter and height callus (L. leucocephala cv. Tarramba) can be seen on table 2.
Table 2. The addition in diameter and height (mm/week) of the putative mutant callus (L. leucocephala cv. Tarramba) selected in the regenerative media at 8 WAP.

| Treatment | Height | Diameter |
|-----------|--------|----------|
|           | mm week|          |
| M0        | 0.76±0.51 | 0.56±0.41d |
| M1        | 0.97±0.94 | 1.15±0.85abcd |
| M2        | 0.52±0.74 | 0.74±0.86cd |
| M3        | 0.63±0.76 | 0.62±0.64cd |
| M4        | 0.95±0.94 | 1.32±1.39abc |
| M5        | 0.91±0.59 | 0.92±0.90bcd |
| M6        | 0.97±0.86 | 0.79±0.49bcd |
| M7        | 0.75±0.72 | 0.91±0.04bcd |
| M8        | 0.71±0.67 | 0.86±0.97bcd |
| M9        | 0.89±0.71 | 1.68±1.35a  |
| M10       | 0.67±1.02 | 0.47±0.78d |
| M11       | 0.37±0.67 | 0.79±1.11ed |
| M12       | 0.99±1.00 | 1.49±0.91ab |

M0= MS 0 (Control). M1= 0.5 ml BAP. M2= 1 ml BAP. M3= 1.5 ml BAP. M4= 2 ml BAP. M5= 0.5 ml KIN. M6= 1 ml KIN. M7=1.5 ml KIN. M8= 2 ml KIN. M9= 0.25 ml BAP + 0.25 ml KIN. M10= 0.5 ml BAP + 0.5 KIN. M11= 0.75 ml BAP + 0.75 ml KIN. dan M12= 1 ml BAP + 1 ml. Different superscript letters on the same line showed significantly different test at the 5% level.

There was no difference of callus in the eighth week (P <0.05) for each treatment but the highest addition of callus was on M12 with value 0.99 ± 1.00 mm. while at the highest diameter addition was on M9 and M12 with values of 1.68 ± 1.35 mm and 1.49 ± 0.91 mm there was a significant difference (P> 0.05) when compared with other treatments. This proves that the use of combination BAP and kinetin media can provide effective growth for callus diameter.

Cells in tissue explant there is a competent nature. It is the ability of the cell or tissue to respond to signals from growth promotor regulators are added so that the cell could grow [17].

3.3. Characteristics of colour and texture callus (L. Leucocephala cv. Tarramba)

The morphology of color and callus was the most easily visible variable of callus morphology. in contrast to callus texture indicating the ability of callus to regenerate later. Callus texture was measured by reference to the Hopkins method which consists of a compact texture and crumb texture [18].

The compact texture have a compact. tightly bound. and grombolic cell structure. whereas the crumb texture has a rare. hollow. scattered. and sparse cellular arrangement marked by a slight white spot color.

Table 3. Characteristic of colour and texture callus the putative mutant callus (L. leucocephala cv. Tarramba) selected in the regenerative media at 8 WAP.

| T  | Colour | Texture |
|----|--------|---------|
|    | Green  | Brown   | Black  | Crumb | Compact |
|    | %      | %       | %      |       |         |
| M0 | 100    | 0       | 0      | 0     | 100     |
| M1 | 82     | 0       | 18     | 11    | 89      |
| M2 | 40     | 0       | 60     | 13    | 88      |
| M3 | 36     | 0       | 64     | 44    | 56      |
| M4 | 55     | 0       | 45     | 36    | 64      |
| M5 | 80     | 20      | 0      | 0     | 100     |
| M6 | 85     | 15      | 0      | 9     | 91      |
| M7 | 69     | 19      | 13     | 0     | 100     |
| M8 | 69     | 0       | 0      | 31    | 100     |
| M9 | 100    | 0       | 0      | 6     | 94      |
| M10| 41     | 0       | 59     | 43    | 57      |
| M11| 30     | 0       | 70     | 3      | 100     |
| M12| 88     | 0       | 13     | 57    | 43      |

T= Treatment. M0= MS 0 (Control). M1= 0.5 ml BAP. M2= 1 ml BAP. M3= 1.5 ml BAP. M4= 2 ml BAP. M5= 0.5 ml KIN. M6= 1 ml KIN. M7=1.5 ml KIN. M8= 2 ml KIN. M9= 0.25 ml BAP + 0.25 ml KIN. M10= 0.5 ml BAP + 0.5 KIN. M11= 0.75 ml BAP + 0.75 ml KIN. dan M12= 1 ml BAP + 1 ml.

Green calluses with the highest presentation on M0. M9. and M12 (> 88%) and the crumbling calluses with the highest value was M12 with value 57%. The most ideal callus color is green calluses colour.
because there is still chlorophyll [19]. The colour brown callus caused by browning is considered to be a breed due to decreasing the quality of a callus which will result in cell death marked with calluses cell necrosis [20].

Figure 2. a. Green. b. Brown. c. Black. d. Crumb. e. Compact.

4. Conclusion
Value of callus viability on regenerative media with BAP formulation was higher > 88% than kinetin media and BAP + Kinetin combination.

The addition diameter on media M9 and M12 with values of $1.68 \pm 1.35$ mm week$^{-1}$ and $1.49 \pm 0.91$ mm week$^{-1}$. This proves that the use of combination BAP and kinetin media can provide effective growth for callus diameter. the crumbling callus with the highest value was M12 (57%).

The best regenerative media formulation for adaptation of callus lamtoro was combination BAP and kinetin media.

Acknowledgement
We acknowledgement to School of Postgraduate in Bogor Agricultural University Indonesia.

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