The radio sensitivity of mature callus and selection of irradiated 40 Gy lamtoro (*Leucaena leucocephala*) callus on acid stress through tissue culture

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**Abstract.** Lamtoro (*Leucaena leucocephala*) cv Tarramba is a good forage has a high crude protein, flea tolerance and drought resistant but only grow well at pH >5.5 and have high mimosine. The aim of this study was to determine radio sensitivity value of mature callus and the optimal tolerance level of callus that has been irradiated by gamma rays (40 Gy) on acid stress. The first phase of research was conducted by Completely Randomized Design (CRD) with 5 radiation treatments used gamma rays including 0, 30, 40, 50, and 60 Gy. Second phase of the research used a Completely Randomized Design (CRD) with treatment of using the different level of Al$^{3+}$ with different acidity. The treatment consisted of 6 levels Al$^{3+}$, including 0 ppm (pH = 6.5), 100 ppm (pH = 5.5), 200 ppm (pH = 4.4), 300 ppm (pH = 3.4), 400 ppm (pH = 3.0) and 500 ppm (pH = 2.6). The radio sensitivity value of the mature callus on post–irradiated gamma-ray LD$_{50}$ was 56.227 Gy. Based on acid selection (AlCl$_3$), the best performance up to 300 ppm Al$^{3+}$ (pH 3.4), although the callus was able to grow and tolerant up to 500 ppm Al$^{3+}$ (pH 2.6).

1. Introduction

Legumes have high nutritional value used to compliance protein and fibre needs for livestock. Lamtoro (*Leucaena leucocephala*) is a good forage that has a high crude protein and digestibility of 70% [7]. Lamtoro has a chemical composition such as dry weight 34.5%, crude protein 21.5%, non-nitrogen extract 49.5%, crude fibre 14.3%, crude fat 6.5%, ash 6.28%, Ca 2.7%, and Phosphor 0.17% [14]. Lamtoro grows well in Indonesia, especially in eastern Indonesia. One of lamtoro plants that exist in this area is lamtoro cv. Tarramba, that is tolerant of insect attack and drought, but there is not tolerant to acidic conditions [9]. Indonesia has the potential land with extensive dry soil with acidic condition about 99.6 million Ha. Acidic soil characterized by low pH can be caused by a fairly high aluminum content [10]. Aluminum excess can be toxic to plants grow, therefore lamtoro plants tolerant to low pH conditions can exploit the potential of marginal landscapes in Indonesia especially with acidic conditions. According to [5] that Lamtoro cv. Taramba callus is tolerance in > pH 5.5.

This problem can be solved by applying of plant biotechnology through tissue culture that can select acid-tolerant lamtoro plant. In vitro cultures, genetic diversity can be enhanced by somaclonal diversity.
Genetic diversity can be enhanced by various treatments such as the provision of physical mutagen (gamma rays) on embryogenic callus [1].

2. Materials and Methods

2.1 Materials
This research was conducted in Forage Tissue Culture Laboratory, Division of Forage Technology and Pasture Science, Faculty of Animal Science, Bogor Agricultural University. The equipment used in this study were laminar air flow, tissue culture bottles, callipers, magnetic stirrer, pH meter, balance, scissors, scalpel, tweezers, autoclave. Materials used in the study were callus before and after irradiated with 40 Gy was obtained from the collection of Plant Tissue Culture Laboratory, 70% alcohol, laundry soap, Clorox 10% - 20%, distilled water, plant growth regulators 2,4D (dichlorophenoxyacetic acid), sugar, agarose, MS (Murashige Skoog) medium, AlCl3.

2.2 Methods
The first experiment, the explant was obtained from the mature phase of the seed and planted on media with 2.4 D addition for callus growing. Callus was irradiated with Gy, after that the callus planted on 1 ml 2.4 D/liter of media. 40 Gy Callus were grown on MS media with addition of 1 ml 2.4 D until enough for replication for the second experiment and the callus were treated on addition of AlCl3.

2.3 Experimental design and data analysis
The first experiment was conducted by Complete Randomized Design (CRD) design with 5 radiation treatment used gamma rays including 0, 30, 40, 50, and 60 Gy with 6 replications. Second experiment used a completely randomized design (CRD) with treatment using the level of Al3+ with different acidity of media consisted of 6 levels with 10 replications. The treatments of AlCl3 addition were 0 ppm (pH = 6.5), 100 ppm (pH = 5.5), 200 ppm (pH = 4.4), 300 ppm (pH = 3.4), 400 ppm (pH = 3.0) and 500 ppm (pH = 2.6). The data was analysed by multi-ANOVA using SPSS software, then further by Duncan test [4].

2.4 Parameters observation
Parameters observation were callus viability, percentage of lethal dose (LD)50, height and diameter of callus (cm), texture callus, pH alteration.

3. Results and Discussion

3.1. Callus viability and radiosensitivity
The result of variance analysis showed callus irradiated with Gy was significantly decreasing of viability (Table 1). The lowest of viability was on 60 Gy about 42.85 %. The decreasing of viability occurred in this treatment with increasing with Gy irradiated.

| Species                  | Dose (Gy) | Viability (%) |
|--------------------------|-----------|---------------|
| Callus of *Leucaena leucocephala* var. Tarramba | 0         | 100.0 a       |
|                          | 30        | 71.42b        |
|                          | 40        | 71.42b        |
|                          | 50        | 57.14c        |
|                          | 60        | 42.85d        |

Note: Different superscript on different column showed significantly on 5 % (P<0.05)

The decrease of viability percentage was due to reducing of plant growth. Based on the research of [15] the plant cells death and organelles was caused by the interaction factor of irradiation rays with...
molecules in cells that are dominated by water and produce free radicals H⁺ and OH⁻. Another factor presented by [6] was the tendency of dead callus due to deterministic effects, namely the effect of cell death due to the irradiation rays exposure.

The treatment of gamma ray irradiation doses in determining LD₅₀ was analysed using polynomial equations (Table 1) in the Curve Expert 1.3 application to get the best curves (Figure 1). Based on the analysis, LD₅₀ value for callus L. leucocephala cv. Tarramba was produced is 56.227 Gy through the equation \( y = 99.933 - 2.074 x + 0.06 x^2 - 0.00068 x^3 \). According to [11] plant radio sensitivity depended on genetic constitution, irradiation dose, amount of DNA, water content, phase of genotype development from callus. The irradiation factor has an important role because irradiation is random so that at certain times it can cause different mortality rates in each type of plant. There is a pattern of dosage ranges that can be used to increase genetic diversity of a plant. The dosage ranges for obtaining optimum genetic diversity is in the range of LD₂₀ - LD₅₀ [13].

![Figure 1. Callus viability of L. leucocephala cv. Tarramba with different Gy radiation](image)

### 3.2. Selection of Callus on Acid Media

The result of variance analysis showed that the addition of AlCl₃ influenced the response of plant height (Table 2). The higher AlCl₃ addition causing Aluminium poisoning of callus. The best effect was on the addition of AlCl₃ 300 ppm and 200 ppm. Aluminium causes a decrease in plant growth by inhibiting meristematic zone extension, irregular cell structure and defective cell shape [17].

#### Table 2. The effect of acid treatments on callus height, callus diameter, callus texture and pH alteration (irradiated 40 Gy callus Lamtoro)

| Parameters       | 0 ppm AlCl₃ (pH = 6.50) | 100 ppm AlCl₃ (pH = 5.50) | 200 ppm AlCl₃ (pH = 4.40) | 300 ppm AlCl₃ (pH = 3.40) | 400 ppm AlCl₃ (pH = 3.00) | 500 ppm AlCl₃ (pH = 2.60) |
|------------------|-------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| Callus height    | 0.23±0.04b              | 0.25±0.05b                | 0.26±0.05ab               | 0.30±0.08a                | 0.13±0.04c                | 0.10±0.00c                |
| Callus width     | 0.33±0.06a              | 0.32±0.09a                | 0.27±0.04a                | 0.35±0.05a                | 0.20±0.06ab               | 0.10±0.05b                |
| Callus Texture   | Compact                 | Compact                   | Compact                   | Compact                   | crumble                   | Crumble                   |
| pH alteration    | -0.56±0.06a             | 0.23±0.08b                | 1.17±0.08d                | 2.28±0.09e                | 1.06±0.08c                | 1.02±0.09c                |

Note: Different superscript on different row showed significantly on 5 % (P<0.05)
The result of this study showed that the addition of AlCl₃ significantly affected the callus width. The callus width tolerance until the addition of AlCl₃ 400 ppm. Aluminium accumulated in cell walls and cell membranes will bind phospholipid compounds which disrupted cell permeability and nutrient uptake into cells and resulting in growth inhibition [12]. The Aluminium tolerance plant mechanism was to secretion of acid organic such as oxalate, citrate and malate [3].

The result of this study indicated that calluses texture were compact until the addition of AlCl₃ 300 ppm, but the addition of AlCl₃ 400 ppm and 500 ppm were crumble. According to [8] the rapid entry of external fluid into the cell caused rapidly increasing the formation of cell walls and the calluses becomes compact.

pH alteration ranged for -0.56-2.28 (Table 2, Figure 2). The result of variance analysis showed that the addition of AlCl₃ influenced pH alteration. The highest of pH alteration was found on the addition of AlCl₃ 300 ppm. pH alteration showed the capacity of callus to recovery from the pH decreasing. According to [2] if cation absorbed more than anion will cause the excretion of OH⁻ so that the pH of media will increase. The increasing of pH is one of acid tolerance plant through Aluminium exclusion [16].

![Figure 2. The addition of AlCl₃ on pH alteration](image)

4. Conclusions
The value of mature callus radiosensitivity at LD₅₀ after gamma ray irradiation was 56.227 Gy with the polynomial regression model with equation: \( Y = 99.933 - 2.074 x + 0.06 x^2-0.00068 x^3 \). The results of AlCl₃ addition in acid media selection for mature callus showed the best growth performance and tolerance up to 300 ppm AlCl₃ (pH 3.4). The research will be continuing to obtain embryogenic somatic cells from callus which are tolerant at 300 ppm AlCl₃

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