Determination of growth and antioxidant activity assay of \textit{in vitro} gamma-irradiated \textit{Tacca leontopetaloides} shoots

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Abstract. \textit{Tacca leontopetaloides} (L.) is a tuberous plant with high starch content similar to those found in potatoes. However, despite its potential as a functional crop, the plant is still underutilized due to limited cultivation by farmers. Besides, a class of specific metabolites found in this plant has the potential as an anticancer drug. In this study, we sought genetic improvement of \textit{T. leontopetaloides} using Gamma-ray irradiation. The aim of the research was to observe growth, antioxidant activities, and phytochemical properties after Gamma-ray irradiation treatment at 20 Gy dose. We grew the irradiated culture on MS medium supplemented with 0.5 mg/L BAP or 0.5 mg/L Kinetin. We recorded the growth every week for eight weeks. After eight weeks of culture, the total fresh biomass, antioxidant activities, and phytochemical properties were calculated. The results showed that a modest growth reduction was found on irradiated plant culture compared with the control culture. Antioxidant IC50 on irradiated culture was lower than the control, which was indicating higher antioxidant activities on irradiated culture. However, no change of phytochemical properties was observed on both irradiated and control culture measured qualitatively. This study reveals a new candidate of \textit{T. leontopetaloides} mutant induced by gamma-ray irradiation with higher antioxidant activities than its wild type.

Keywords: antioxidant activity, gamma-ray, growth, mutant, phytochemical properties, \textit{Tacca leontopetaloides}

1. Introduction
\textit{Tacca leontopetaloides}, also known as Polynesian arrowroot or kecondang (Indonesian local name) is a tuberous flowering plant species. Previous classification of the Tacca genera was assemble in uniquely \textit{Taccaceae} family, whereas recent study using morphological and molecular technique revealed that \textit{Tacca} genera was taxonomically related to \textit{Dioscoreaceae} family \cite{1, 2}. The tubers of \textit{T. leontopetaloides} has high content of starch, in which the composition is comparable with the starch of potatoes and corn. \textit{Tacca} starch also has unique properties, i.e., resistant to compression, making it suitable for excipient in tablet formulation \cite{3, 4}. However, despite its potential, \textit{Tacca} is still under-utilized due to limited cultivation by farmers.

Equally important, many plants from family \textit{Taccaceae} are also known for producing unique secondary metabolite, known as taccalonolide which has potential as new anticancer drugs \cite{5-9}, and recently some taccalonolides were successfully isolated from tuber of \textit{T. leontopetaloides} \cite{10}. Recent epidemiological studies proved that natural antioxidant could reduce risk cardiovascular and cancer, therefore, the consumption of natural antioxidant is increasing \cite{11}. Major natural antioxidant metabolite in plant are flavones, iso flavones, coumarins, catechins, carotenoids, and anthocyanins \cite{12}.
These metabolites production could be influenced by ploidy level [13] also mutation using gamma ray irradiation [14; 15]. In Indonesia, since 2011, research on *T. leontopetaloides* has been carried out including polyploid induction [16] and inducing mutation using Gamma ray irradiation [17]. Therefore, we aim to scrutinize the effect of gamma ray irradiation at 20 gy dose on growth and antioxidant properties, as well as qualitative phytochemical properties of *T. leontopetaloides* *in vitro* culture.

2. Materials and methods

2.1. Plant materials

The plant materials used in this study were Gamma-irradiated clones stock culture obtained from previous study [17; 18]. The stock culture was maintained on MS [19] medium containing 30 g/L sucrose and 4 g/L agar (Gelzan ™, Caisson). The stock culture was placed in a culture room at 25 ± 2 ºC under continuous light regime with 1000 lux intensity.

2.2. Treatment medium

Approximately four-months-old clonal shoot culture obtained from previous study were selected. We used five clones of gamma-irradiated stock culture, i.e., 20.6.4.3.1; 20.10.2.3.1; 20.11.1.1.1; 20.11.2.3.1; 20.13.1.3.1 and control (wild type). The leaves were excised, leaving corms-like mass, the corms were planted on either control or treatment medium. The treatment medium was MS [19] medium containing 30 g/L sucrose and 4 g/L agar (Gelzan ™, Caisson) supplemented with 0.5 mg/L Benzylyaminopurine (BAP) or MS medium supplemented with 0.5 mg/L kinetin. The hormone-free MS medium was used as control medium. The pH media were adjusted to 5.7 – 5.8 and sterilized by autoclaving at 121 ºC, 1 atm, for 15 min. The media were dispensed in 25 mL aliquot into culture bottles with plastic caps. There was a total of twelve replicates for each clones and treatment medium in three culture bottles. The cultures were incubated in a culture room at 25 ± 2 ºC under continuous light regime with 1000 lux intensity. The growth of the culture was determined by measuring the shoot height, leaves count, shoots count, and roots count. The observation was conducted every week for eight weeks after subculture. Subsequently, after eight weeks of culture, fresh weight, antioxidant activity, stomata density, and phytochemical properties were determined. The data was analyzed by anova with Duncan’s post hoc test using IBM SPSS ver. 25.

2.3. Antioxidant activity, Stomata measurement, and Phytochemical analysis

The antioxidant activity was determined as previously reported by Blois [20] with slight modification [21]. Stomata measurement was determined as previously described by Wu and Zhao [22], while phytochemical assay were carried out as previously described by Harborne [23] with slight modification [24].

3. Results and discussion

The growth performance assay of mutant clones was compared to its wild type as shown in Figure 1. There were many reports on the effect of Gamma-ray irradiation on growth of the plant’s physiology [25-30]. Experiment on *in vitro* condition is beneficial to assure the controllable growth condition and measurement preciseness. The results showed that the culture growth of wild type (WT) was similar to mutant culture in hormone-free MS medium (Figure 1). Although, a slight reduction of shoot height also observed on mutant culture in MS medium supplemented with BAP or kinetin (Table 1). In addition, the shoot height of mutant culture on MS medium supplemented with 0.5 mg/L kinetin was higher than the mutant culture on MS medium supplemented with 0.5 mg/L BAP (Table 1). The highest shoot height on wild type plant was recorded on MS medium supplemented with 0.5 mg/L kinetin, while the highest shoot height on Gamma-irradiated mutant plant (20.13.1.3.1) was recorded on hormone-free MS medium.
Figure 1. Shoots height of *T. leontopetaloides* during eight weeks of culture on MS medium supplemented with hormone-free (A), 0.5 mg/L BAP (B), and 0.5 mg/L kinetin (C).

The observation of leaves count revealed similar trend with the increasing of shoot height. Apparently, the leaves count on hormone-free MS medium was the highest. (Figure 2). Whereas leaves count on MS medium supplementoed with 0.5 mg/L BAP is the lowest. Mutant clone of 20.13.1.3.1 showed the highest leaves count on MS medium supplemented with 0.5 mg/L kinetin. Overall, it was observed that Gamma-irradiated mutant plants had higher leaves count on MS medium supplemented with 0.5 mg/L kinetin. (Table 1).

The formation of new shoots and shoots count development from 0 to 8 weeks after culture was observed on the fifth week in hormone-free MS medium, whereas in MS medium supplemented with plant growth regulator, we observed new shoots increment start from second weeks of culture (Figure 3). The shoots count was higher on MS medium supplemented with 0.5 mg/L BAP in wild type plant. On the other hand, MS medium supplemented with 0.5 mg/L kinetin has lower shoots count. Interestingly, gamma-irradiated mutant plant has higher shoots count than its wild type (Table 1). The highest shoots count was recorded on wild type in MS medium supplemented with 0.5 mg/L BAP, while the lowest shoots count was recorded on gamma-irradiated mutant plant (20.6.4.3.1) in hormone-free MS medium.

The maximum roots count was observed in hormone-free MS medium (Figure 4). The Gamma irradiated clones (20.6.4.3.1) cultured onto hormone-free MS medium was the highest roots count, while the gamma-irradiated clones (20.10.2.3.1) cultured onto MS medium supplemented with kinetin was the lowest roots count.
Table 1. The growth of *T. leontopetaloides* after eight weeks of culture on MS medium supplemented with hormone-free, 0.5 mg/L BAP and 0.5 mg/L kinetin.

| Clones | Wild type | 20.6.4.3.1 | 20.10.2.3.1 | 20.11.1.1.1 | 20.11.2.3.1 | 20.13.1.3.1 |
|--------|-----------|------------|------------|-------------|-------------|-------------|
| **Hormone-free MS medium** | | | | | | |
| Leaves count | 5.50 ± 0.48<sup>a</sup> | 4.33 ± 0.50<sup>b</sup> | 4.33 ± 0.50<sup>b</sup> | 4.08 ± 0.23<sup>b</sup> | 3.17 ± 0.30<sup>b</sup> | 4.25 ± 0.18<sup>b</sup> |
| Roots count | 1.33 ± 0.19<sup>ab</sup> | 1.58 ± 0.15<sup>a</sup> | 1.42 ± 0.14<sup>ab</sup> | 1.42 ± 0.15<sup>ab</sup> | 1.33 ± 0.14<sup>ab</sup> | 1.00 ± 0.17<sup>b</sup> |
| Shoots Height | 3.80 ± 0.21<sup>a</sup> | 3.54 ± 0.13<sup>ab</sup> | 3.28 ± 0.09<sup>b</sup> | 3.72 ± 0.13<sup>ab</sup> | 3.60 ± 0.09<sup>ab</sup> | 3.68 ± 0.16<sup>ab</sup> |
| Shoots count | 2.67 ± 0.28<sup>bc</sup> | 1.67 ± 0.28<sup>d</sup> | 2.08 ± 0.31<sup>cd</sup> | 3.50 ± 0.29<sup>a</sup> | 3.08 ± 0.23<sup>ab</sup> | 2.50 ± 0.19<sup>bc</sup> |
| **MS medium supplemented with 0.5 mg/L BAP** | | | | | | |
| Leaves count | 3.83 ± 0.30<sup>a</sup> | 3.17 ± 0.44<sup>a</sup> | 3.17 ± 0.32<sup>a</sup> | 3.33 ± 0.28<sup>a</sup> | 3.08 ± 0.26<sup>a</sup> | 3.67 ± 0.38<sup>a</sup> |
| Roots count | 1.08 ± 0.36<sup>a</sup> | 0.75 ± 0.22<sup>a</sup> | 0.67 ± 0.22<sup>a</sup> | 0.83 ± 0.27<sup>a</sup> | 0.50 ± 0.19<sup>a</sup> | 0.67 ± 0.19<sup>a</sup> |
| Shoots Height | 2.91 ± 0.43<sup>c</sup> | 3.54 ± 0.09<sup>a</sup> | 3.01 ± 0.07<sup>c</sup> | 2.81 ± 0.10<sup>c</sup> | 3.24 ± 0.06<sup>b</sup> | 2.96 ± 0.05<sup>c</sup> |
| Shoots count | 4.42 ± 0.23<sup>c</sup> | 3.58 ± 0.26<sup>c</sup> | 3.25 ± 0.25<sup>b</sup> | 3.17 ± 0.17<sup>b</sup> | 3.17 ± 0.17<sup>b</sup> | 3.08 ± 0.29<sup>b</sup> |
| **MS medium supplemented with 0.5 mg/L kinetin** | | | | | | |
| Leaves count | 2.42 ± 0.67<sup>d</sup> | 2.83 ± 0.37<sup>c</sup> | 4.08 ± 0.45<sup>abc</sup> | 4.33 ± 0.50<sup>b</sup> | 3.25 ± 0.39<sup>bcd</sup> | 5.00 ± 0.30<sup>a</sup> |
| Roots count | 0.92 ± 0.31<sup>a</sup> | 0.58 ± 0.23<sup>a</sup> | 0.42 ± 0.19<sup>a</sup> | 0.92 ± 0.34<sup>b</sup> | 0.92 ± 0.23<sup>a</sup> | 0.92 ± 0.23<sup>a</sup> |
| Shoots Height | 3.86 ± 0.09<sup>a</sup> | 3.67 ± 0.23<sup>c</sup> | 3.71 ± 0.05<sup>c</sup> | 3.11 ± 0.15<sup>b</sup> | 3.26 ± 0.03<sup>b</sup> | 3.16 ± 0.06<sup>b</sup> |
| Shoots count | 2.50 ± 0.15<sup>bc</sup> | 2.33 ± 0.19<sup>c</sup> | 3.08 ± 0.19<sup>a</sup> | 2.75 ± 0.18<sup>abc</sup> | 3.17 ± 0.17<sup>a</sup> | 3.00 ± 0.17<sup>bc</sup> |

Mean ± s.e. with different letter(s) in the same row are significantly different according to Duncan’s at 95% confidence interval.

**Figure 2.** The leaves count of *T. leontopetaloides* during eight weeks of culture on MS medium supplemented with hormone-free (A), 0.5 mg/L BAP (B) and 0.5 mg/L kinetin (C).
Figure 3. The shoots count of *T. leontopetaloides* during eight weeks of culture on MS medium supplemented with hormone-free (A), 0.5 mg/L BAP (B), and 0.5 mg/L kinetin (C).

Figure 4. The roots count of *T. leontopetaloides* during eight weeks of culture on MS medium supplemented with free-hormone (A), 0.5 mg/L BAP (B), and 0.5 mg/L kinetin (C).
The growth observation of the fresh weight (FW) of leaves, petioles, and roots were observed after eight weeks of culture (Table 2). All of the gamma-irradiated mutant clones had less FW than wild type plants. Nevertheless, the FW was higher in hormone-free MS medium than MS medium supplemented with BAP or Kinetin (Table 2). In studies using Gamma ray irradiation on cowpeas [30], the plant height and FW of shoots were decreased along with the increase of Gamma ray dose. Several studies also show that various growth parameter in crops also decrease regardless the source of radiation [31; 32]. These decreases of growth were mainly due to the stopping of cell division during G2/M phase, damage of enzyme, increased peroxide, and genome damage because of irradiation [30]. Here, we also demonstrate that Gamma radiation decreases the growth T. leontopetaloides plant. Our results also indicated that not all gamma-irradiated mutant clones were suppressed growth like showed by 20.11.2.3.1 clones and 20.13.1.3.1 clones that have more biomass of FW than the wild type, which is also similarly reported in studies using Arachis hypogea [33].

### Table 2. Fresh weight (FW) of T. leontopetaloides after eight weeks of culture

| Clones | Hormone-free MS medium | MS medium supplemented with 0.5 mg/L BAP | MS medium supplemented with 0.5 mg/L Kinetin |
|--------|-------------------------|------------------------------------------|-------------------------------------------|
|        | Wild type | 20.6.4.3.1 | 20.10.2.3.1 | 20.11.1.1.1 | 20.11.2.3.1 | 20.13.1.3.1 |
| Leaves | 0.22 ± 0.05a | 0.09 ± 0.01b | 0.09 ± 0.02b | 0.08 ± 0.01b | 0.13 ± 0.03ab | 0.07 ± 0.01b |
| Petioles | 0.31 ± 0.07a | 0.07 ± 0.01b | 0.10 ± 0.05b | 0.06 ± 0.01b | 0.13 ± 0.03b | 0.06 ± 0.01b |
| Roots | 0.33 ± 0.05a | 0.20 ± 0.02b | 0.14 ± 0.05b | 0.13 ± 0.02b | 0.20 ± 0.03b | 0.18 ± 0.02b |
| Total | 0.86 ± 0.13a | 0.36 ± 0.03b | 0.34 ± 0.09b | 0.28 ± 0.03b | 0.46 ± 0.06b | 0.31 ± 0.04b |
|        |            |                          |                      |                          |                      |
| Leaves | 0.12 ± 0.02a | 0.09 ± 0.01ab | 0.09 ± 0.01ab | 0.08 ± 0.01abc | 0.06 ± 0.02bc | 0.04 ± 0.00c |
| Petioles | 0.17 ± 0.04a | 0.05 ± 0.01b | 0.07 ± 0.01b | 0.05 ± 0.01b | 0.06 ± 0.02b | 0.06 ± 0.01b |
| Roots | 0.33 ± 0.07b | 0.25 ± 0.05b | 0.28 ± 0.06b | 0.24 ± 0.04b | 0.42 ± 0.08ab | 0.59 ± 0.08a |
| Total | 0.61 ± 0.10ab | 0.38 ± 0.05b | 0.44 ± 0.06b | 0.37 ± 0.05b | 0.54 ± 0.09ab | 0.69 ± 0.08a |
|        |            |                          |                      |                          |                      |
| Leaves | 0.11 ± 0.02a | 0.08 ± 0.01a | 0.10 ± 0.01a | 0.07 ± 0.01a | 0.11 ± 0.02a | 0.10 ± 0.01a |
| Petioles | 0.11 ± 0.01a | 0.05 ± 0.01b | 0.05 ± 0.01b | 0.40 ± 0.01b | 0.06 ± 0.01b | 0.08 ± 0.01ab |
| Roots | 0.17 ± 0.03ab | 0.17 ± 0.02ab | 0.06 ± 0.01c | 0.10 ± 0.02bc | 0.18 ± 0.03a | 0.21 ± 0.02a |
| Total | 0.40 ± 0.05a | 0.30 ± 0.03b | 0.21 ± 0.02b | 0.20 ± 0.04b | 0.35 ± 0.02a | 0.39 ± 0.02a |

Mean ± s.e. with different letter(s) in the same row are significantly different according to Duncan’s at 95% confidence interval.

The results of stomatal density measurement were the lowest in in vitro condition whereas plants grown in ex vitro condition was the highest (Table 3). This also indicated that stomata density was heavily affected by environmental condition, in this case in vitro condition.

Antioxidant activity was measured by means of DPPH (2,2-diphenyl-1-picrylhydrazlyl) activity. DPPH interaction with antioxidant causing the purple color of DPPH changed to α, α-diphenyl-β-picrylhydrazyl which gave rise to yellowish color [20]. These changes were measured by UV-vis to get IC50 value, i.e., concentration of the extract that can capture 50% or free radicals. Several studies also mentioned the power classification of antioxidant measured by IC50 [20; 34; 35]. The antioxidant considered very strong if the IC50 is lower than 50 ppm, strong if IC50 between 50 to 100 ppm, medium if IC50 between 100 to 150, weak if IC50 between 150 to 200 ppm, and very weak if IC50 is bigger than 200 ppm. Our results revealed that mutant clones had lower IC50 value than wild type plants (Table 4). The 20.6.4.3.1 clones of T. leontopetaloides showed the lowest IC50 value in MS medium supplemented with kinetin. The enhancement of antioxidant properties, caused by Gamma ray irradiation also mentioned in several studies, for example in Ocimum basilicum M2 mutant generated by Gamma ray irradiation has higher antioxidant activity than its wild type [36]. Similar results also reported in Ziziphus mauritiana that has augmented biological activity e.g., antimicrobial and
antioxidant after Gamma ray irradiation [37], although the underlying mechanism of the increasing biological activity is not fully understood due to random mutation caused by Gamma ray irradiation.

### Table 3. Stomata properties of *T. leontopetaloides* leaves

| Clones                        | Length (µm)  | Width (µm)  | Density (/mm²) |
|-------------------------------|--------------|-------------|----------------|
| Wild type (*in vitro*)        | 28.90 ± 6.51a| 21.70 ± 3.69a| 122.72 ± 14.72c|
| 20.6.4.3.1                    | 25.51 ± 0.16a| 20.48 ± 0.25abc| 127.94 ± 10.80c|
| 20.10.2.3.1                   | 25.83 ± 0.24a| 22.30 ± 0.32a | 124.02 ± 9.78c |
| 20.11.1.1.1                   | 25.66 ± 0.35a| 20.35 ± 0.44abc| 133.16 ± 8.21c |
| 20.11.2.3.1                   | 24.62 ± 0.34a| 20.98 ± 0.43abc| 168.26 ± 6.32b |
| 20.13.1.3.1                   | 26.05 ± 0.54a| 21.46 ± 0.53a | 138.38 ± 9.77bc|
| Wild type (*ex vitro*)        | 22.11 ± 1.52a| 16.58 ± 1.12abc| 205.98 ± 8.36a |
| Wild type (Acclimation plant) | 22.53 ± 1.54a| 16.19 ± 0.99abc| 156.66 ± 16.51bc|

Mean ± s.e. with different letter(s) in the same column are significantly different according to Duncan’s at 95% confidence interval.

### Table 4. IC50 value from the various *T. leontopetaloides* plantlet

| Sample                                                    | IC50  |
|-----------------------------------------------------------|-------|
| Quercetin (control)                                       | 1.35  |
| Wild type *in vitro* (MS medium)                          | 332.28|
| 20.6.4.3.1. (MS medium)                                   | 108.20|
| 20.6.4.3.1. (MS + 0.5 mg/L BAP)                           | 78.69 |
| 20.6.4.3.1. (MS + 0.5 mg/L Kinetin)                       | 62.98 |
| Wild type (Acclimation plant)                             | 192.55|
| Wild type (*ex vitro*)                                    | 316.19|

Phytochemical analysis on several gamma-irradiated clones and its wild type was presented in Table 5. The result showed that both gamma-irradiated clones and wild type plants had the same phytochemical characteristics. Our previous studies did not detect the alkaloid and flavonoid on *in vitro* *T. leontopetaloides* plant [38]. This might happen due to different age sample. In this research, the analysis of phytochemical was conducted as qualitative analysis. Thus, it is important to measure the intensity of phytochemical properties because flavonoid and saponin are known as major antioxidant in plant [12; 39].

### Table 5. Phytochemical properties of *T. leontopetaloides*

| Number | Material samples                        | Alkaloid | Flavonoid | Steroid | Tannin | Saponin |
|--------|----------------------------------------|----------|-----------|---------|--------|---------|
| 1      | Wild type (Seed-derived plant)          | +        | +         | +       | -      | -       |
| 2      | Wild type (Acclimation plant)           | +        | +         | +       | -      | -       |
| 3      | *In vitro* wild type plant (control)    | +        | +         | +       | -      | -       |
| 4      | 20.6.4.3.1. (*in vitro*)                | +        | +         | +       | -      | -       |
4. Conclusion
Gamma-irradiated of *T. leontopetaloides* culture has lower growth than the wild type plant. On the contrary, antioxidant activity of gamma-irradiated culture was higher than the wild type plant. This study revealed Gamma ray irradiation could induce new *T. leontopetaloides* variety with higher antioxidant properties.

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