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Gamma Irradiation Effect on the Callus Growth and Shoot Regeneration of *Pogostemon cablin* Benth.

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Abstract. Patchouli is an essential producer of patchouli oil in the perfume industry, and its production needs to be increased to meet market demand. In vitro mutagenesis using gamma irradiation has the potential to produce superior patchouli plants. This study aimed to evaluate the effect of gamma irradiation on callus growth and shoot formation on patchouli callus. Calli were gamma-irradiated at doses of 0, 15, 30, 45, 60, and 75 Gy, then cultured on Murashige and Skoog (MS) medium with the addition of 0.1 mg.L^{-1} NAA and 0.3 mg.L^{-1} BA for four weeks. The results showed that gamma irradiation inhibited callus growth and shoot formation. Gamma irradiation increased the percentage of explant browning and decreased explant survival, explant formation of shoots, the total number of shoots, number of shoots/explants, fresh weight of callus and shoots, growth index, and growth rate. The higher the dose of gamma irradiation, the smaller the percentage of explant survival and the lower the ability to form shoots with an average number of shoots formed less. The percentage of explants that survived on callus without irradiation and at a low dose of 15 Gy reached 100%, with an average number of shoots formed between 4.34-5.63 shoots/explant. Meanwhile, the percentage of explants that survived at high doses of 60-75 Gy was 76-82%, with an average number of shoots formed about two shoots/explant. The lethal dose (LD_{20}) for explant survival was 67.98 Gy.

INTRODUCTION

Patchouli oil produced from patchouli (*Pogostemon cablin* Benth.) is one of the essential oils that have high ranks high among essential oils and the only essential oil that contains patchouli alcohol and is a fixative capable of binding alcohol [1]. This oil has a persistent and tenacious aroma, which is why it is used in the perfumery and high-end cosmetics industries [2]. There are no other volatiles or synthetic substitutes that can replace patchouli oil, increasing the value [3].

The report on world patchouli oil production shows that Indonesia is the largest supplier of patchouli oil in the international market at around 90%, with an average production exceeding 2000 tons per year, and world patchouli oil demand is estimated to increase 5% every year [4]. In order to increase production and meet current needs, it is necessary to improve the genetic improvement of patchouli. Patchouli propagation was carried out asexually through shoot cuttings because patchouli rarely flowered and failed to form seeds; therefore, conventional breeding techniques

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could not be carried out [5]. One of the efforts to overcome these problems is the development of improved varieties of patchouli through biotechnology approaches to increase genetic diversity, assembling varieties, and plant propagation.

In vitro mutagenesis is a very effective technique for plant breeding because it is beneficial for modifying desired traits without changing other characters. Mutations can be carried out using physical mutagens such as irradiation which are preferred over chemical mutagens because they are easier to use. Physical mutagens widely used are gamma rays that induce high plant mutations [6]. Gamma rays are electromagnetic radiation that initiates or inhibits the growth and differentiation of plant cells and organs [7]. Gamma rays can modify plant physiological characteristics to produce new mutants [8]. Depending on the dose given, gamma rays interact with the internal components of cells and create free radicals that alter or harm differentiation processes, morphology, physiology, and bioactive components [9]. In vitro mutagenesis using gamma irradiation has been used to induce mutations, among others, in sugarcane [10], groundnut [11], *Rosmarinus officinalis* L. [12], and *Artemisia annua* [13]. The objective of this study was to evaluate the effect of gamma irradiation on the callus growth and shoot regeneration of patchouli.

MATERIAL AND METHOD

Callus Induction and Multiplication

Patchouli plant cv. Lhokseumawe comes from the laboratory collection. Young patchouli leaves were washed under running tap water for 30 minutes. The leaves were then transferred to 70% ethanol under a laminar air flow cabinet for one minute. After that, the leaves were disinfected with 5.25% sodium hypochlorite for 10 minutes and then rinsed with sterile distilled water three times (5 minutes each time).

The sterilized leaves were then cut to 1 x 1 cm and grown on MS media added with 3 $mg.L^{-1}$ NAA combined with 0.5 $mg.L^{-1}$ BA. The culture was then incubated at a temperature of 25±1°C and a photoperiod of 16/8 hours (light/dark) at a light intensity of 600 lux for four weeks. The cultured calli were then multiplied by subculture on the same medium every three weeks until the third subculture.

Gamma irradiation of Callus Culture

Clump calli resulting from multiplication weighing 0.05 g each were precultured on MS + NAA 0.1 $mg.L^{-1}$ and BA 0.3 $mg.L^{-1}$ media [6] for one week. Clump calli patchouli from subcultures were gamma-irradiated at 15, 30, 45, 60 and 75 Gy doses. Gamma irradiation was carried out using a ^{60}Co Gammacell 220 radiation source with a dose rate of 3400.3 Gy/h at the Isotope and Radiation Application Center-National Nuclear Energy Agency (PAIR-BATAN), Indonesia. The irradiated calli were subcultured on new media; each treatment was repeated ten times (10 bottles), each bottle was filled with five callus clumps. The cultures were incubated at 25±1°C, photoperiod 16/8 hours (light/dark), and light intensity 600 lux for four weeks. Callus growth and shoot regeneration were observed at week 4, which included a percentage of explant browning, percentage of explant survival, percentage of explant formed shoots, total number of shoots, number of shoots per explant, fresh weight of callus and shoots, growth index and growth rate. The growth index (Gi) is determined based on the equation described by [14], while the growth rate is calculated based on [15].

$$\text{Growth index (\%)} = \frac{\text{Final fresh weight} - \text{Initial fresh weight}}{\text{Initial fresh weight}} \text{ x } 100 \quad (1)$$

$$\text{Growth rate (g/day)} = \frac{\text{Final fresh weight} - \text{Initial fresh weight}}{\text{culture periode}} \quad (2)$$

Statistical Analysis

Data were analyzed using SPSS 20 with Analysis of Variance (ANOVA) and continued with the Duncan test if there was a significant difference between the gamma irradiation doses. The lethal dose of 20 (LD_{20}) and 50 (LD_{50}) was determined using Curve Expert Professional 2.7.

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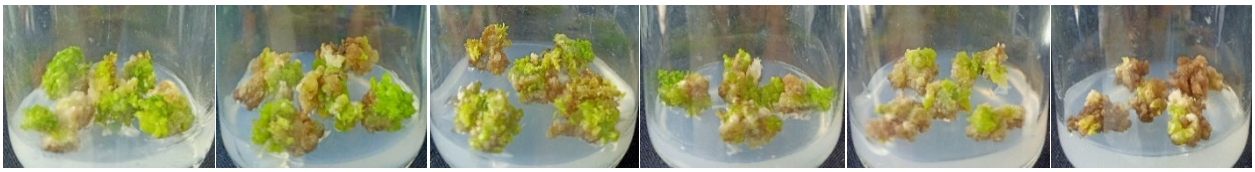
RESULT AND DISCUSSION

Gamma irradiation on callus affected callus growth and shoot formation (Fig. 1). In callus without gamma irradiation, all callus can grow and form shoots, while callus irradiated by gamma rays causes brown and death in some callus and inhibits shoot formation. The higher the gamma irradiation dose, the more explants died and the higher the inhibition of shoot formation. Furthermore, the higher the gamma irradiation dose, the lower the ability to form buds in the culture.



(a) (b) (c) (d) (e) (f)

FIGURE 1. *Pogostemon cablin* Benth. callus explant growth response with gamma irradiation treatment at week 4 of culture. (a) Without gamma irradiation (control), (b) 15 Gy, (c) 30 Gy, (d) 45 Gy, (e) 60 Gy, (f) 75 Gy

All callus explants cultured on MS media with the addition of growth regulator 0.1 mg.L-1 NAA combined with 0.3 mg.L-1 BA without gamma irradiation were able to survive and form shoots. Gamma irradiation on callus significantly affected the percentage of browning explants, the percentage of explants that survived, the percentage of explants that could form shoots, the total number of shoots, the number of shoots per explant, fresh weight of callus and shoots, growth index (Gi%) and growth rate (Fig. 2).

Gamma irradiation on leaf explants significantly increased the percentage of browning explants (Fig. 2a). The percentage of browning explants was markedly seen in gamma-irradiated explants at a dose of 30 Gy at four weeks of culture. The higher the irradiation dose, the more explants that experienced browning. Calli that were not gamma-irradiated and callus irradiated with a dose of 15 Gy experienced the lowest browning at 14-16%. Gamma irradiation at a dose of 30-45 Gy in explants caused about 42-66% of explants, while high doses of gamma irradiation of 60-75 Gy caused browning of about 92-96 Gy in four weeks of culture.

The dose of gamma irradiation influenced the percentage of explants that could survive. Callus explants that were not irradiated and callus irradiated with low doses of 15 Gy were able to survive 100%, while the percentage of explants that survived at moderate doses of 30 and 45 Gy was 90-94%. The percentage of surviving explants decreased significantly with explant irradiation at a dose of 45-75 Gy. The percentage of explants that survived with high gamma irradiation, 60 and 75 Gy, ranged from 76-82% (Fig. 2a).

The percentage of explants that could form shoots decreased in line with the decline in explants that could survive. The ability of explants to form shoots declined in explants given 15 Gy gamma irradiation, although not significantly. A significant decrease in the ability to form shoots at the age of 4 weeks of culture began to occur in explants treated with 30 Gy gamma irradiation. The percentage of explants capable of forming shoots at irradiation doses of 30 and 45 Gy was 64% and 58%, while at high doses of 60 and 75 Gy, only 42 and 26% (Fig. 2b).

In addition to affecting the ability of explants to form shoots, gamma irradiation on the explants also affected the number of shoots formed. The total number of shoots formed decreased with an increasing dose of gamma irradiation. Significant differences in the total number of shoots began to be seen at the 15 Gy gamma irradiation dose. At four weeks after culture, the total number of shoots formed on callus without gamma irradiation treatment was 23.80, while the total number in callus with 15-45 Gy gamma treatment ranged from 9.70-18.70 shoots. The total number of shoots at a 60-75 Gy dose was between 2.80-5.10 (Fig. 2b).

Callus explant irradiation with gamma rays greatly affected the average number of shoots per explant. The average number of shoots per explant decreased with increasing gamma irradiation dose. In the fourth week of culture, the average number of shoots per explant on callus that was not gamma irradiated was 5.63 shoots, while the average number per explant at a dose of 15-30 Gy was 4.28-4.34 shoots. A sharp decrease in the average number of shoots per explant occurred at doses of 45-60 Gy, namely 2.43-2.97 shoots and a dose of 75 Gy only 1.85 shoots (Fig. 2c).

Gamma irradiation was also able to inhibit callus growth. The average fresh weight of calli and shoots without gamma irradiation was 0.63 g, while explants with 15-45 Gy gamma irradiation ranged from 0.52-0.60 g. Calli irradiated with higher gamma irradiation of 60-75 Gy had a fresh weight of 0.45-0.48 g or 25% lower than the control (Fig. 2c).

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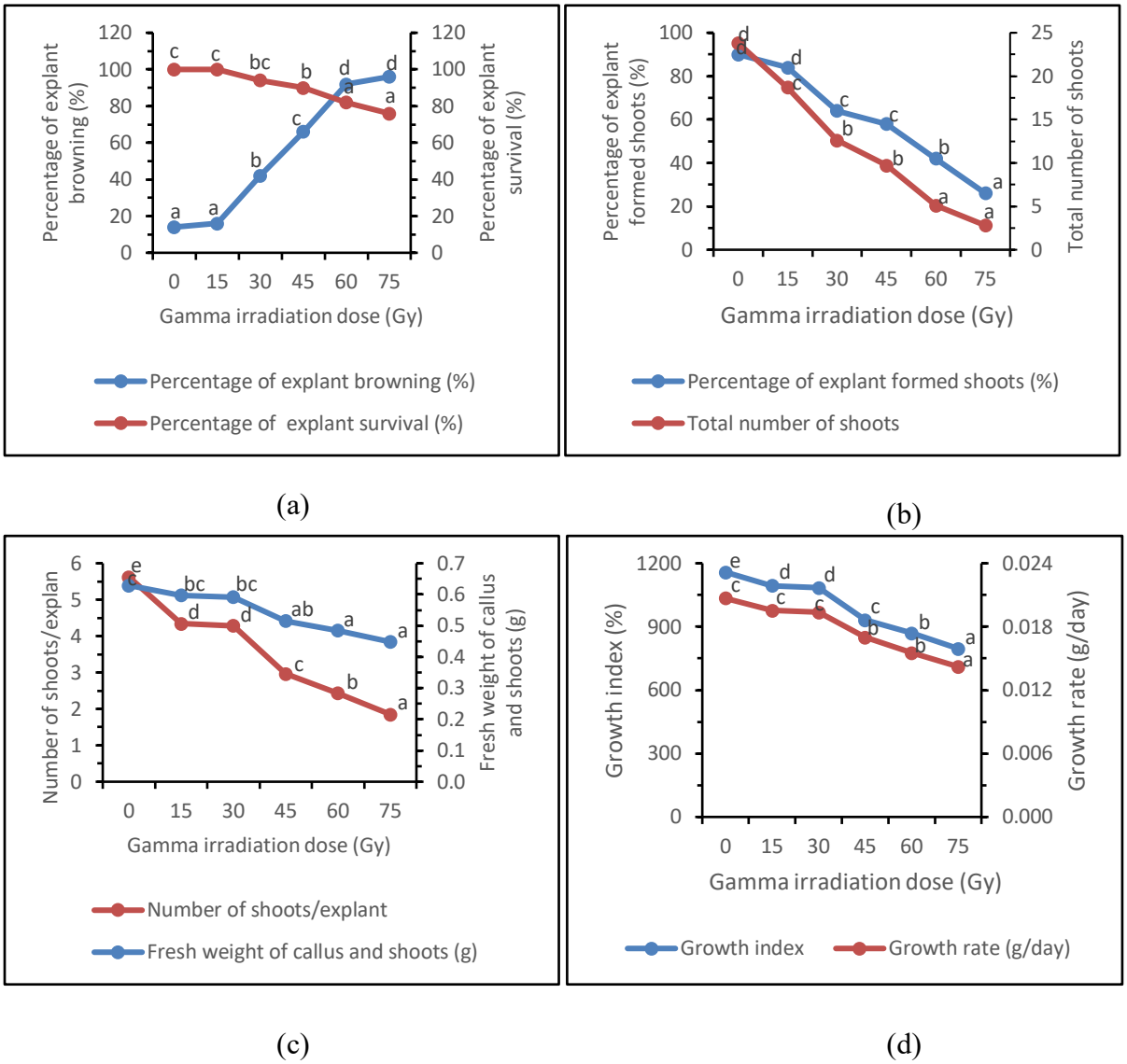

FIGURE 2. Effect of gamma irradiation on callus of *Pogostemon cablin* Benth. (a) Percentage of browning explants and percentage of explants surviving, (b) Percentage of explants forming shoots and the total number of shoots, (c) Number of shoots/explants, and average fresh weight of callus and shoots, (d) Growth index and growth rate

Besides the growth index, gamma irradiation in explants also affected the growth rate. The growth rate at a dose of 0-30 Gy did not differ significantly, with the growth rate ranging from 0.019-0.021 g/day. Significant differences in growth rates began to be seen at doses higher than 30-45 Gy, where the growth rate was 0.016-0.017 g/day. The smallest growth rate value was found at a dose of 75 Gy with a growth rate of 0.014 g/day, where the value was reduced by 31% compared to the growth rate in control (Fig. 2d).

The lethal dose (LD) is the amount of irradiation that causes a particular percentage of the plant population or explants to die. Explants that continue to grow after irradiation have the potential as mutants [8]. Exposure to gamma irradiation at doses below the lethal dose caused damage but did not result in explant death. *Pogostemon cablin* Benth callus irradiation using gamma irradiation doses of 0-75 Gy showed a decrease in the percentage of surviving explants. The analysis of callus irradiation using Curve Expert Professional 2.7 showed that the LD_{20}, which is 20% mortality of explants, was at a dose of 67.98 Gy, and the LD_{50} was at a dose of 156.46 Gy. The gamma irradiation dose for LD_{50} was not included in the dose range used in this study. The correlation coefficient value r = -0.9758 shows a strong

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negative relationship; the higher the dose of gamma irradiation given, the lower the explant's ability to survive (Fig. 3).

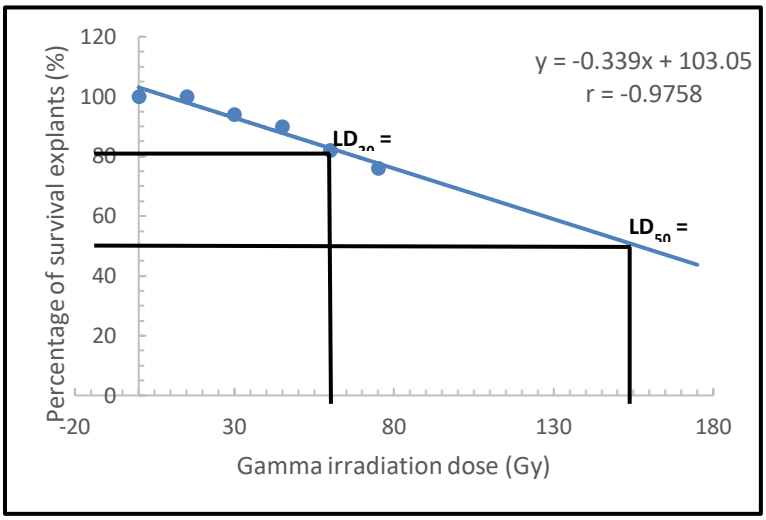

FIGURE 3. Radiosensitivity curve based on the percentage of explants surviving from *Pogostemon cablin* Benth. callus at the fourth week of culture

An LD_{50} at the fourth week of culture based on the percentage of explant survival has not been obtained. The observations during the study showed that the longer the culture period, the higher the explants that died, where the number of browning explants that would die was more than the browning explants that were able to experience recovery so they could survive. Therefore, we estimated that at week 8 of culture, LD_{50} would be obtained in the range of doses used in this study. A radiosensitivity curve analysis was carried out based on the percentage of browning explants to further investigate tissue damage due to gamma irradiation from patchouli callus and determine LD_{20} and LD_{50}. The percentage of browning explants increased with an increasing dose of gamma irradiation. The radiosensitivity curve of patchouli callus has the equation y = 1.261x + 7.0476 with LD_{20} = 10.27 Gy and LD_{50} = 34.06 Gy, and the correlation value close to 1 indicates a strong positive relationship, the higher the dose of gamma irradiation given, the higher the browning explant.

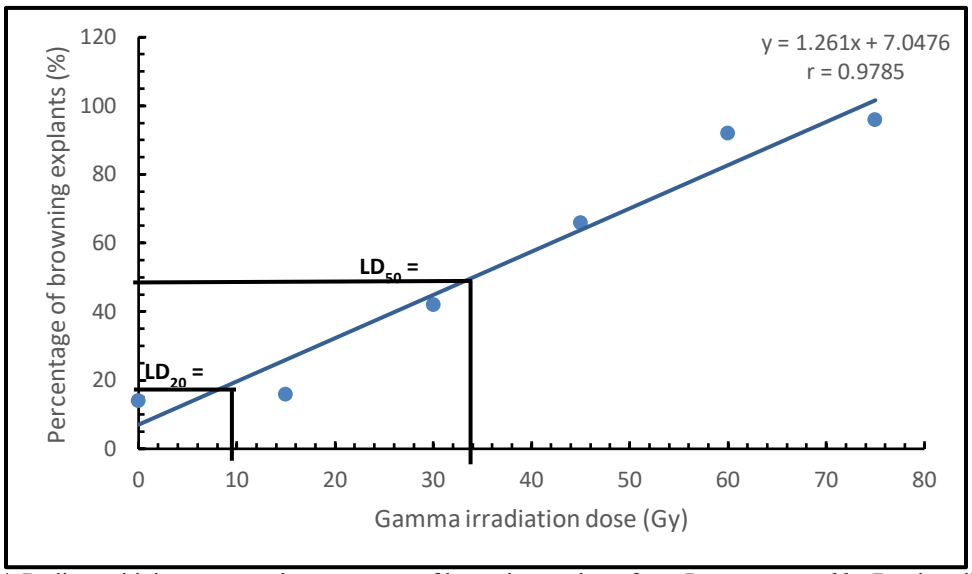

FIGURE 4. Radiosensitivity curve on the percentage of browning explants from *Pogostemon cablin* Benth. callus at the fourth week of culture

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Gamma irradiation can cause death, decreased cell viability, poor growth, and even death of callus cells that begin with a browning reaction of callus. Gamma irradiation increased the number of browning explants, reduced explant survival and regeneration ability, and inhibited callus and shoot growth, along with the increase in gamma irradiation dose. These results align with research that states that high irradiation doses can cause callus color changes from dark brown to black, and the callus ability to regenerate tends to decrease [10]. Callus browning is formed due to the presence of phenolic compounds. The phenolic is oxidized after the cell membrane is degraded or the cells are organized, followed by chlorophyll degradation [16]. The browning process is increasing due to the influence of gamma irradiation. Another study found that the enzyme indole acetate plays a role in synthesizing indole acetic acid, which is degraded due to gamma irradiation, causing the browning of explants [17]. The effect of gamma irradiation on callus browning was also found in the callus of *Ferula gummosa* [18], sugarcane [10] and rice [19].

Explants capable of forming shoots and the number of shoots produced were affected by gamma irradiation. Less number of shoots was found at higher doses of gamma irradiation. Gamma irradiation will interfere with hormone activity, especially cytokinins which affect shoot regeneration. In addition, irradiation treatment can suppress cell division, elongation, and proliferation of explants [20], inhibiting callus and shoot growth. This inhibition will affect the fresh weight of callus and shoots, growth index, and growth rate. Inhibition of shoot growth at higher doses of gamma irradiation was caused by the inability of cells to absorb nutrients, reduced mitotic activity in meristematic tissue, disruption of hormonal balance and enzymatic activity, and reduced water content in explants [21][22][23]. A previous study on callus irradiated by gamma irradiation was at high doses, resulting in fewer shoots in *Cucumis melo* cv. Bathasa [24], *Anthurium andreanum* [25] and *Capparis spinosa* L. [26].

Gamma irradiated explants gave different responses. The dose of irradiation and type of culture can affect the response shown by the explants. These results align with research that states that the sensitivity level of explants is influenced by the type of plant, growth phase, size and material to be mutated, the media used and will have various effects on different plant genotypes [27]. The success of irradiation was also determined from the radiosensitivity level of the plant genotype. Radiosensitivity refers to the genetic material's susceptibility to irradiation and can be quantified using the lethal dose value, the dose at which a particular percentage of the irradiated plant population dies. A low radiation dose permits the mutant to return to its source, whereas a high radiation exposure can kill it. The optimal dose that produces the most significant number of mutants is typically found near the lethal level. High variability is expected in irradiation treatment between the LD20 and LD50 levels, and these doses provide a higher possibility of acquiring specific properties for plant breeding purposes [28]. Gamma irradiation research on sugarcane callus found that the LD_{20} and LD_{50} were at a dose of 12.57 Gy and 28.88 Gy [29]. This dose is different from this study, where LD_{20} was obtained at a high dose of gamma irradiation and LD_{50} beyond the doses used. We reasoned that the doses applied may not be optimal for causing lethality or callus death, or additional time was required to determine the effect of gamma irradiation on the callus. The results of this study are in line with studies on *Ficus carica,* where the dose of gamma irradiation given did not cause death in explants [30]. Research [31] assumed that callus *I. zollingeriana* had thick cell walls so that gamma-irradiated callus did not show any difference with the control and LD_{50} could not be calculated.

CONCLUSION

Gamma irradiation on callus affects callus growth and shoot regeneration. Gamma rays as physical mutagens decreased the survival of explants and explants capable of forming shoots, number of shoots, fresh weight of callus and shoots, and index and growth rate. The decrease was in line with the increase in the gamma irradiation dose. The lethal dose of LD_{20} gamma irradiation on callus at the fourth week of culture was 67.98 Gy.

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