Effect of Gamma-Ray Irradiation on Vetiver Grass (Vetiveria Zizanioides (L.) Nash.) in Vitro Shoots Growth and Multiplication

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Abstract. The vetiver plant, Vetiveria zizanioides (L.) Nash, yields vetiver oil, which is a fixative for perfumes. It is vital to create improved vetiver plants by in vitro mutagenesis because the low quality of vetiver in Indonesia causes vetiver oil productivity to decline year after year. This study sought to determine how gamma radiation affected the development and reproduction of vetiver in vitro shoots. Gamma radiation was used to irradiate the in vitro vetiver shoots at doses of 0; 15; 30; 45; 60; and 75 Gy. The irradiated shoot explants were subsequently cultivated on medium containing MS + NAA 0.1 mg/L + BAP 1 mg/L during eight weeks. The outcomes demonstrated that in vitro shoot growth and multiplication were reduced by gamma radiation. With 50 shoots per explant, the percentage of explants generating shoots without radiation and with a low dose of 15 Gy was 100%. When exposed to moderate doses of 30–45 Gy, 60–68 percent of the explants survived and produced 13–25 shoots per explant. Less than 50% of explants survived high dosages of 60–75%, and less than 30% of explants developed shoots with fewer than 10 shoots per explant. The gamma-ray irradiation lethal dosage (LD50) for the survival rate of the shoots was 61 Gy.

Keywords: Gamma Irradiation · In Vitro Culture · Shoot Multiplication · Vetiveria zizanioides

1 Introduction

The plant known as vetiver grass (Vetiveria zizanioides (L.) Nash) yields vetiver essential oil (VEO), which is used in aromatherapy and as a fixative for perfumes. Around 300–350 tons of vetiver oil are required annually by the world [1], but Indonesia is only able to produce about 25–30 tons per year [2]. Due to Indonesia's poor vetiver grass quality, both production and consumption of VEO are declining year over year. The low quality is due to the most vetiver plants produce flowers but do not form seeds (sterile) and are propagated vegetatively through tillers [3], making it difficult to produce variants through conventional sexual hybridization.

The development of superior vetiver plants can be done by somatic hybridization, genetic transformation, and in vitro mutagenesis. In vitro mutagenesis is a method that can be used to increase somaclonal variation, improve quality, increase production and

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plant bioactive compounds. The advantages of in vitro mutagenesis are high mutation frequency, uniform mutagen treatment, time and space efficiency, and a controlled environment, to increase genetic diversity [4].

Increasing the success of in vitro mutagenesis can be done by selecting species and explants, determining the appropriate doses of mutagen, selecting in vitro mutants, and analyzing the genome of mutant plants [4]. The mutagens used can be chemical and physical. The chemical mutagens are the mutagens caused by chemical substances, such as; colchicine and ethyl methanesulfonate (EMS), while physical mutagens are the mutagens caused by ionizing and non-ionizing radiation. Physical mutagens that can be given include; X-rays, ultraviolet (UV) rays, alpha, beta, and gamma rays. Gamma rays are physical mutagen that is often used compared to other radiation rays. Gamma-ray mutagens can produce 1604 mutants more than X-rays which can produce 561 mutants [5].

Gamma rays have two paths, direct and indirect effects [6]. The direct effect will affect the DNA molecule directly and cause changes in the DNA structure of the cell, while the indirect effect, gamma rays will cause the exposed biological material to produce free radicals known as reactive oxygen species (ROS), the direct interaction of radiation with target macromolecules through water hydrolysis products that will cause damage to the cells [5, 6]. The double strand of DNA will break and cause changes in the chemical structure, resulting in mutations or the expression of new genes. This will affect the morphology, physiology, anatomy, and biochemistry of plants [5].

Adventitious ginseng (Panax ginseng Meyer) in vitro roots irradiated at a dose of 50 Gy had genetic diversity (larger and longer roots) and increased secondary metabolite compounds (saponins and ginsenosides) [7]. Contrary to the control, which had a flavonoid content of 8.940.04 mg/g, the node segment explants of Centella asiatica accession CA23 exposed to gamma radiation at a dose of 30 Gy had a flavonoid content of 16.8370.008 mg/g [8]. In vitro shoot growth and regeneration are also impacted by the gamma radiation exposure. Gamma-ray irradiation of Gerbera jamesonii petiole and in vitro plantlets at a dose of 10 to 60 Gy revealed that the higher the irradiation dose, the fewer shoots were formed [9]. In vitro shoot regeneration of Etlingera elatior was hindered by high doses of gamma radiation at a dose of 140 Gy [10]. A dose of 15–45 Gy of gamma radiation on orchid shoot cultures of Dendrobium sonia dramatically decreased shoot length, leaf area, and fresh weight. The percentage of survival shoots that were irradiated also decreased with the high dose of gamma rays [11]. This study aimed to evaluate the effect of gamma-ray irradiation on the growth and multiplication of vetiver in vitro shoots.

2 Materials and Method

2.1 Plant Material

The plants material used was vetiver grass derived from Sengklek, Pamalayan Village, Bayongbong District, Garut Regency, West Java, Indonesia.

2.2 Shoots Induction and Multiplication

The vetiver in vitro shoots was collected from the plant tissue culture laboratory's collection, Biology department, Mathematics and Natural Sciences Faculty, Brawijaya University, Malang. The in vitro shoots were induced from crown explants and cultured on Murashige and Skoog (MS) medium supplemented with NAA 0.1 mg.L-1 and BAP 1 mg.L-1 [12]. The in vitro shoots were multiplied on MS medium + NAA 0.1 mg.L-1 + BAP 1 mg.L-1 for 4 weeks to form small shoots. The cultures were incubated at 25 ± 1 °C, photoperiod 16/8 h (day/night), and light intensity 600 lx.

2.3 Irradiating in Vitro Shoot Cultures with Gamma Rays

An experimental design utilizing a completely randomized design was used for the research. The small clump of vetiver shoots resulting from multiplication was sub-cultured on the same medium for a week. The small clump of vetiver shoots from sub-cultures a week of culture was irradiated with gamma rays at doses of 15, 30, 45, 60, and 75 Gy. Gamma-ray irradiation was using a 60Co Gammacell 220 radiation source with a dose rate of 4115.5 Gy/h at the Isotope and Radiation Application Center-National Nuclear Energy Agency, South Jakarta.

The irradiated shoots were sub-cultured on a fresh medium, each treatment repeated ten times (10 bottles), each bottle filled with five clumps of small shoots, each clump weighing 0.01 g. The cultures were incubated at 25 ± 1 °C, photoperiod 16/8 h (day/night), and light intensity 600 lx for 8 weeks. The growth and development of shoots were observed every 4 weeks for 8 weeks, including the percentage of survival explants, percentage of explants formed shoots, the average total number of shoots, and the number of shoots per explant.

2.4 Statistical Analysis

Data were analyzed using SPSS 25 with Analysis of variance (ANOVA) and continued with Duncan's test if there was a significant difference ($P < 0.05$) in the dose of gamma-ray irradiation. The lethal dose 50 (LD50) was determined using CurveExpert Professional 2.7.

3 Result and discussion

The growth and reproduction of vetiver in vitro shoots were hindered by gamma radiation. The growth of shoot explants is more inhibited and the capacity to create and multiply new shoots is decreased with increasing irradiation exposure (Fig. 1). At the 4 weeks of culture, shoot explants that were not irradiated with gamma rays (control) and exposed with gamma rays at a level of 15 Gy were green in color and shown better growth (Fig. 1 A & B). Shoot explants exposed to gamma radiation at dosages of 30 Gy and 45 Gy displayed a yellowish-green color and mildly inhibited shoot growth (Fig. 1 C & D), while shoot explants at high gamma-ray irradiation of 60 and 75 Gy were browning with very obstructed growth (Fig. 1 E & F). All explants exposed to gamma radiation at

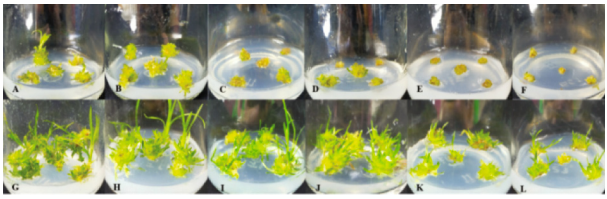

Fig. 1. The growth of shoots at 4 and 8 weeks of culture after gamma-ray irradiation at various doses. The top figure is 4 weeks of culture, the bottom figure is 8 weeks of culture. A, G: 0 Gy (control); B, H: 15 Gy; C, I: 30 Gy; D, J: 45 Gy; E, K: 60 Gy; and F, L: 75 Gy.

doses ranging from 15 to 75 Gy at the end of the 8-week culture period were capable of developing new shoots and were green in color, but shoot formation and multiplication in explants irradiated with high doses of 60 and 75 Gy were more inhibited (Fig. 1 K & L), whereas shoot formation and multiplication at 8 weeks of culture between control and 15–45 Gy irradiated looked different (Fig. 1 G–J).

The percentage of explants that survive, the capacity of explants to produce shoots, and the quantity of shoots produced can all be impacted (P0.05) by gamma radiation to the shoot (Fig. 2). Gamma radiation exposure can kill explants and prevent them from developing into shoots. The more gamma radiation is exposed to, the more explants died and the fewer explants were able to form shoots. All explants that were not irradiated or irradiated with the low dose of 15 Gy gamma rays were able to survive and form shoots in culture. At the fourth and eighth weeks, explants exposed to low dosage irradiation of 15 Gy both survived and had 100 percent of their ability to generate shoots. At the 4 weeks of culture, the percentage of survival explants at irradiation doses of 30 and 45 Gy was 88% and 82%, while at high doses of 60 and 75 Gy it was only 82% and 74%. After 8 weeks of culture, there was a decrease in the percentage of survival explants in explants treated with gamma irradiation. In 8 weeks of culture, the percentage of survival explants at 30 and 45 Gy irradiation was 68% and 66%, there was a decrease of 16–20% compared to 4 weeks of culture, while at high doses of 60 and 75 Gy of 44%, there was a decrease of about 30–38% compared to 4 weeks of culture (Fig. 2 A).

The ability of explants to form shoots also began to decrease in explants given 30 Gy gamma-ray irradiation although not significantly. A significant decrease in the ability to form shoots at 4 weeks and 8 weeks of culture began to occur in explants treated with 45 and 60 Gy irradiation. At 4 weeks of culture, 88 percent and 82 percent of the explants exposed to radiation at doses of 30 and 45 Gy went on to develop shoots, whereas only 82 percent and 74 percent of the explants exposed to high doses of 60 and 75 Gy did. In line with the decrease in the percentage of survival explants from explants treated with gamma-ray irradiation at the fourth week, there was also a decrease in the percentage of explants forming shoots. A dose of 30–75 Gy of gamma-ray irradiated explants were capable of forming shoots in about 75–90 percent of cases, but 8 weeks of culture, the dose of 30–75 Gy was progressively reduced by less than 70% (Fig. 2 B).

However, explants irradiated with a low dose of 15 Gy of gamma rays produced shoot numbers that were not significantly different from shoots that were not irradiated. In the 4 weeks of culture, the average total number of shoots and the number of shoots formed per explant on the in vitro shoots that were not irradiated and irradiated with

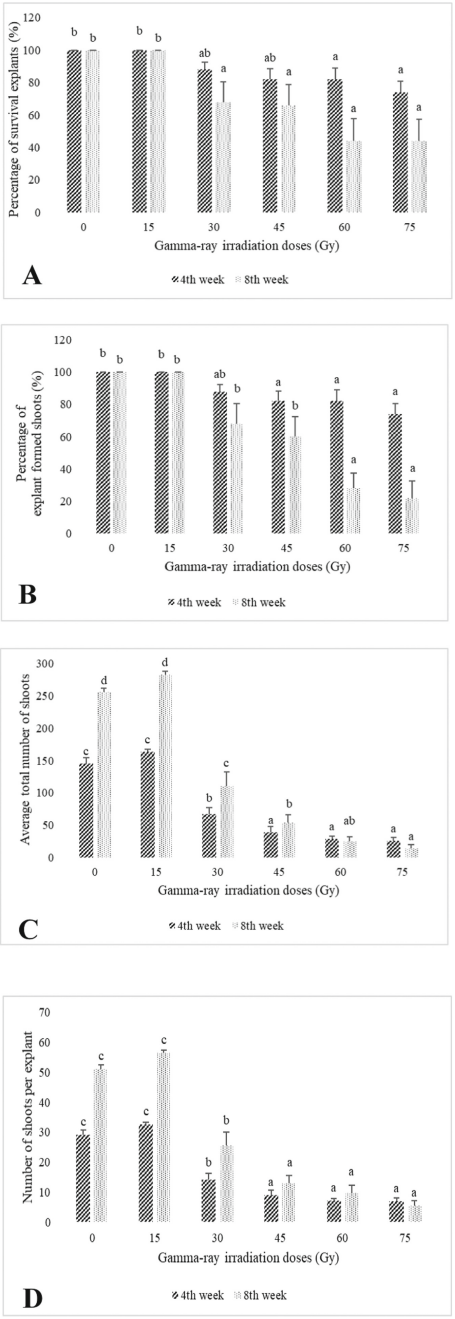

Fig. 2. The percentage of explants that survive, the percentage of explants that generate shoots, the average number of shoots overall, and the number of shoots per explant at the fourth and eighth weeks after gamma-ray irradiation. Note: the same letter in the same week of culture indicated no significant difference according to the Duncan test ($P < 0.05$).

the low dose of 15 Gy gamma rays were not different, which ranged from 146–163 shoots and 29–33 shoots/explant, in contrast to the average total number of shoots and the average number of shoots per explant at a dose of 30 Gy, which were 67 shoots and 12 shoots/explant. The average total number of shoots and the number of shoots per explant formed from gamma-ray irradiated explants at doses of 45, 60, and 75 Gy were not different significantly, which was around 26–39 shoots and 5–8 shoots/explant (Fig. 2 C & D).

At 8 weeks of culture, there was a significant increase in the number of shoots formed by almost 50% of explants that were not irradiated and gamma-irradiated at a low dose of 15–30 Gy compared to 4 weeks of culture, while the number of shoots formed in explants irradiated with 45 Gy gamma rays only increased slightly, even explants with high dose 75 Gy gamma-ray irradiation produced fewer shoots than those at 4 weeks of culture (Fig. 2 C). This is because at 60 and 75 Gy irradiation doses, there was no increase in the number of shoots due to the shoot explants in several duplicates browning and dying (static).

One of the crucial elements in the production of superior mutants is the application of the proper dose of gamma radiation [5]. At low doses of irradiation, vetiver in vitro shoot explants was able to survive (high survival rate) and produced new shoots. Low doses of gamma rays were able to stimulate cell division, growth and development in various plant species [13]. Low dose irradiation on banana cv. Tanduk shoots had a positive effect on increasing chlorophyll content [14] and on Pterocarpus santalinus seeds could increase growth and germination of endangered Pterocarpus santalinus [15].

In contrast to high-dose irradiation, browning of shoot explants and a smaller proportion of explants surviving were caused by higher radiation doses. Gamma-ray irradiation on Etlingera elatior at a dose of 10–140 Gy showed a decrease in survival rate with increasing irradiation dose [10]. Research on nucellus of *Citrus reticulata* cv. Limau Madu irradiated at a dose of 100–120 Gy became brown in the second week and died on the 35 days of culture [13]. The browning reaction occurs due to the presence of oxygen, phenol content, and polyphenol oxidase (PPO). This process occurs due to two reactions: hydroxylation of o-monophenol to o-diphenol and oxidation of o-diphenol to o-quinone [16]. Polyphenol oxidase (PPO) catalyzes the oxidation of monophenols and/or o-diphenols to o-quinones by reducing oxygen to water resulting in protein complexes and the formation of brown melanin pigment [17].

Gamma-ray irradiation causes the production of free radicals known as reactive oxygen species (ROS), such as superoxide radicals (O_2•–), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and alkoxyl radicals (RO) within cells. The accumulation of ROS can cause damage to lipid membranes, chloroplasts, pigments, enzymes, nucleic acids, and cause cell death. Polyphenol oxidase (PPO) can be associated with ROS. Hydrogen peroxide (H_2O_2) is the predominant ROS that mediates changes in PPO activity, causing high PPO activity [18].

The dose of gamma-ray irradiation that is too high also causes the ability of shoot formation and multiplication to be lower. High doses of irradiation affect water intake into cells and endogenous hormone synthesis. Reactive oxygen species (ROS) change the ratio of endogenous hormones (phytohormones) auxins and cytokinins, resulting in

changes in the pattern of cell differentiation [14]. Increased H_2O_2 accumulated in the elongation zone causes cell differentiation and stem cell termination [19, 20].

Gamma-ray irradiation reduces mitotic division activity in meristematic tissue [14] and can damage meristem cells. The shoot apical meristem (SAM) is aberrant or dysfunctional and WUSCHEL (WUS) expression is abnormal [21]. Mutations in ATP-dependent mitochondrial protease, cause accumulation of oxidative stress in SAM, thereby affecting meristematic cell death [22]. The direct interaction of radiation with target macromolecules through water hydrolysis products also causes progressive oxidative and cellular homeostatic damage, resulting in cell death [5]. This caused the ability of explants to form new shoots to be obstructed so that the number of shoots formed is low.

At a low dose of 10 Gy, the percentage of survival explants for banana cv. Tanduk (Musa spp.) in vitro shoots was 71 percent, but at a high dose of 70 Gy, it was only 15 percent [14]. Micro shoot chrysanthemum cv. "Candid" that had been exposed to 10–40 Gy of gamma radiation showed reduced growth in the number of survival explants, the number of leaves, and the number of leaves per explant along with the high dose of irradiation [23]. Pseudostem Banana (*Musa acuminata* cv. Chestnut) which was irradiated by gamma rays at a dose of 20–80 Gy also decreased the percentage of survival explants along with the high dose of gamma-ray irradiation, nonetheless, with a modest dose of 10 Gy, there were 23.33 percent more surviving explants than there were at higher doses.[24].

The success of irradiation was being also determined from the level of sensitivity or radiosensitivity of the plant genotype. Lethal dosage 50 can be used to gauge radiosensitivity (LD50). The optimal dose for producing mutants with the most diversity is known as lethal dose 50, which kills 50% of the plant population [25]. Analysis of LD50 on vetiver in vitro shoots based on the percentage of survival shoots at 8 weeks of culture, it was found that a lethal dose 50 (LD50) occurred at a dose of 61 Gy. These results indicate that the dose of 61 Gy is the dose of irradiation that can kill 50% of vetiver in vitro shoot explants so that the gamma-ray irradiation on the vetiver shoots should not be more than 61 Gy and is the optimal dose to produce mutants with the highest diversity (Fig. 3). Compared with other researches, a lethal dose of 50 in banana cv. Tanduk (Musa spp.) in vitro shoots occurred at 33 Gy [14], callus of sugarcane (Saccharum officinarum L.)

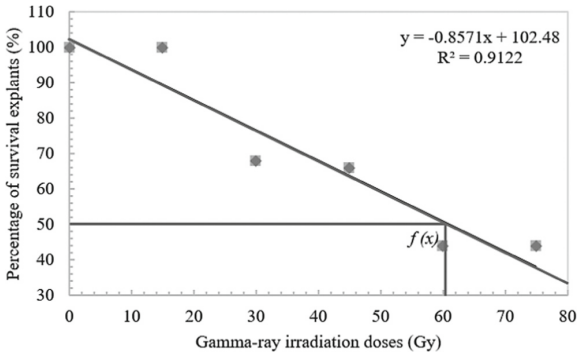

Fig. 3. Radiosensitivity curve and determination of lethal dose (LD_{50}) on the survival rate of vetiver in vitro shoot at 8 weeks of culture.

happened with a dosage of 28.8 Gy [26], With an 18 Gy dose, grape variety "Red Globe" in vitro shoots appeared [27], and callus Citrus reticulata cv. Limau Madu occurred at a dose of 30 Gy [13]. This shows that the level of radiosensitivity in each species was different.

4 Conclusion

Gamma-ray irradiation with the dose of ≥ 30 Gy caused the death of explants and inhibited the formation and multiplication of shoots. The higher the irradiation dose up to 75 Gy, the ability of explants to survive and form shoots decreased. The lethal dose (LD50) of gamma irradiation on the survival rate of vetiver shoots was at 61 Gy.

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