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REGENERASI TANAMAN IN VITRO DARI KALUS KRISAN SETELAH IRADIASI GAMMA DAN ANALISIS GENETIKA ISSR

IN VITRO PLANT REGENERATION FROM CHRYSANTHEMUM CALLUS INDUCED BY GAMMA IRRADIATION AND ISSR GENETIC ANALYSIS

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ABSTRAK

Metode pemuliaan konvensional tanaman krisan menghadapi beberapa tantangan untuk diatasi, dan mutagen fisik seperti sinar gamma telah terbukti efektif dalam menciptakan mutan krisan. Sinar gamma berperan dalam inovasi plasma nutfah bunga dan perbaikan varietas, sinar gamma memiliki daya tembus yang lebih besar dibandingkan dengan metode pemuliaan lainnya. Penelitian ini bertujuan untuk mengetahui pengaruh berbagai dosis iradiasi gamma terhadap pertumbuhan kalus krisan, regenerasi tanaman, dan morfologi akibat induksi serta mengetahui adanya variasi genetik menggunakan marka ISSR. Percobaan terdiri dari empat tahap yaitu, eksplan kalus krisan diiradiasi dengan dosis 0, 10, 20, dan 30 Gray menggunakan sinar gamma untuk menginduksi mutasi dan mengamati pengaruhnya terhadap pertumbuhan kalus. Hasil iradiasi ditanam dalam media BA 0,5, 1,5, dan 3,0 mg.L^{-1} untuk mengamati pertumbuhan tunas. Tunas ditanam di media perakaran yang mengandung NAA dengan dan tanpa arang aktif (AK) untuk regenerasi tanaman dan amplifikasi DNA genom krisan. Hasil penelitian menunjukkan bahwa dosis radiasi yang lebih rendah (10 Gray) memberikan pengaruh positif terhadap semua parameter pertumbuhan, sementara 30 Gray menyebabkan penurunan tinggi tanaman dan jumlah daun yang signifikan. Media NAA 1 mg L^{-1} AK 2 mg L^{-1} memberikan hasil terbaik dalam mendukung pertumbuhan tanaman krisan pada berbagai tingkat paparan radiasi. Analisis menggunakan penanda ISSR mengonfirmasi adanya variasi genetik pada tanaman yang diradiasi.

KEYWORDS:

Callus, genetic variation, plant regeneration, and tissue culture

ABSTRACT

Conventional breeding methods of chrysanthemum plants face several challenges to overcome, and physical mutagens such as gamma rays have proven effective in creating chrysanthemum mutants. As an important tool in flower germplasm innovation and variety improvement, gamma rays have greater penetrating power compared to other breeding methods. This research aims to determine the effect of various doses of gamma irradiation on chrysanthemum callus growth, plant regeneration, and morphology due to induction and to determine the existence of genetic variations using ISSR markers. The experiment consisted of four stages, namely, chrysanthemum callus explants were irradiated at doses of 0, 10, 20, and 30 Gray using gamma rays to induce mutations and observe the effect on callus growth. The irradiation results were planted in BA media 0.5, 1.5, and 3.0 mg L-1 to observe shoot growth. Next, the shoots were planted in rooting media containing NAA with and without activated charcoal (AC) for plant regeneration and amplification of chrysanthemum genomic DNA. The results showed that a lower radiation dose (10 Gray) had a positive influence on all growth parameters, while 30 Gray caused a significant reduction in plant height and number of leaves. NAA 1 mg L-1 AC 2 mg L-1 media gave the best results in supporting the growth of chrysanthemum plants at various levels of radiation exposure. Analysis using ISSR markers confirmed the presence of genetic variation in irradiated plants.

1. INTRODUCTION

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is an economically significant flowering plant widely used as a cut and potted in the international floriculture trade. Furthermore, it is characterized by culinary, medicinal, and ethnopharmacological properties (Shao, 2020). In modern floriculture and industry, new varieties and color are often in demand to meet consumer satisfaction (Khidirov *et al*., 2023). Among various species, Chrysanthemum, belonging to the Asteraceae family, is considered the second most important floricultural crop worldwide after roses (Spaargaren *et al*., 2018). Although there are many varieties, a significant market demand still exists for chrysanthemum with alternative colors, which are essential to increase economic value (Wang *et al*., 2020). The conventional breeding methods of this plant face several challenges, such as limited genetic diversity, differences in parental ploidy levels that can hinder crossbreeding, and self-incompatibility in certain types. To address these challenges, physical mutagens like gamma rays, X-rays, and ion irradiation have proven effective in creating chrysanthemum mutants (Miller *et al*., 2023; Haspolat, 2024).

Gamma-ray radiation can be used to induce new variants in cell groups like calluses or other plant tissues, including leaves and stems. Irradiating calluses tend to produce higher variant frequencies, as cells in callus form remain meristematic and sensitive to radioactivity, making them suitable for mutagenesis studies. Generally, irradiation at callus level produces variants with a higher frequency than using plant tissue due to its meristematic activity and increasing sensitivity to radioactivity. In in vitro culture studies, the choice of culture media is crucial to fulfilling the specific needs of chrysanthemum tissues, as nutrient requirements vary across species. In gamma-ray–induced mutagenesis, the success of callus explants and the production of high-quality callus as planting material depend not only on irradiation dose but also on culture conditions, particularly the type of medium used. The use of appropriate auxin concentrations in vitro culture can also slow down the morphogenic process and accelerate callus growth, thereby increasing the effectiveness of mutagenesis. Gamma-ray radiation is often used in in vitro culture at low doses and can potentially stimulate growth (Chowdhury *et al*., 2023; Murthy *et al*., 2024). When administered to callus, a significant improvement is expected in the quality and growth of chrysanthemum plantlets, creating new flower variants with more varieties.

To increase the success of adventitious shoots initiation directly from explants or indirectly by forming gamma-irradiated callus, cytokinins are needed in the culture medium. BA (Benzyladenine) is a cytokinin that is widely used to stimulate the multiplication of adventitious or axillary shoots in vitro in various plants (Pant *et al*., 2015; . The need for the type and concentration of auxin and cytokinin as stimuli in organ regeneration (shoots/roots) is species-specific based on the genotype of the cultured plant (Pandey and Chaundary, 2025). The rooting stage in plant is considered important, determining the success of development, growth, and acclimatization. The use of Naphthaleneacetic acid (NAA) has been proven to stimulate root formation in various types of plants, including chrysanthemums (Hussein *et al*., 2017). The healthier and stronger root can be produced by combining NAA and activated charcoal (AC) treatments for the success of plant acclimatization process after being transferred to conventional planting media (Martins *et al*., 2024).

In addition to the mutagenesis method, genetic analysis using Inter Simple Sequence Repeat (ISSR) is important to evaluate genetic variation resulting from irradiation. The ISSR technique identifies genetic changes induced by gamma radiation, helping to assess diversity and stability among resulting mutants. This analysis provides information on genetic diversity and kinship relationships between mutation varieties, which are crucial in breeding programs to obtain chrysanthemum cultivars with superior quality and higher adaptability. This study, therefore, aimed to investigate the effects of different gamma irradiation doses on chrysanthemum callus growth and plant regeneration, and the ISSR marker analysis.

2. MATERIALS AND METHOD

The plant material used was leaf explants of chrysanthemum (*Chrysanthemum morifolium* Ramat.) cultivar 'Armita', measuring 0.5 × 0.5 cm with the midrib included. The explants were obtained from aseptic in vitro shoot cultures maintained at Balithi. Explants were cultured on Murashige and Skoog (MS) medium supplemented with 30 g L^{-1} sucrose, 150 mL L^{-1} coconut water, and different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) 3.0 mg L^{-1}. The medium pH was adjusted to 5.8, solidified with 8 g L^{-1} agar, sterilised by autoclaving at 121 °C for 15 minutes, and dispensed into 30 mL culture bottles sealed with plastic film and rubber bands. Cultures were incubated for 30 days at 24 °C under fluorescent light with an intensity of 1000–2000 lux.

Compact green calli obtained from the 2 mg L^{-1} 2,4-D treatment were subsequently irradiated with gamma rays at doses of 0 (control), 10, 20, 30, and 40 Gray using a Cobalt-60 source in a Gamma Chamber 4000 A irradiator at the National Nuclear Energy Agency (PAIR-BATAN), Jakarta. Immediately after irradiation, the calli were transferred to regeneration medium supplemented with 2 mg L^{-1} benzyladenine (BA) and incubated for 60 days. Observations were recorded on the number of leaves, number of shoots, plant height, and fresh weight.

Plant regeneration was carried out through two experiments. The first experiment evaluated shoot proliferation of irradiated node explants (0, 10, 20, and 30 Gray) on MS medium containing 30 g L^{-1} sucrose with BA at concentrations of 0.5, 1.5, and 3.0 mg L^{-1}. The second experiment examined rooting of irradiated shoots on MS medium supplemented with combinations of naphthaleneacetic acid (NAA) (0, 1, and 2 mg L^{-1}) and activated charcoal (0 and 2 g L^{-1}). Both experiments followed a completely randomised design (CRD) with factorial arrangements and three replications. Each treatment unit consisted of three explants. Observations were carried out for 60 days after culture, recording the number of shoots, shoot height, number of leaves, number of nodes, number of roots, and root length. The experiment was arranged in a completely randomised design (CRD). Data were subjected to analysis of variance (ANOVA), and significant differences among treatments at the 5% level were further analysed using Duncan's Multiple Range Test (DMRT). Molecular data were analysed based on banding patterns to assess genetic diversity among irradiated treatments.

Genetic variation was further assessed using DNA amplification using the polymerase chain reaction (PCR) method with ISSR primers. DNA was extracted from irradiated calli using the GeneJET Genomic DNA Purification Kit, and amplification was performed with the KOD One Blue Mastermix PCR Kit (Toyobo, Japan). The ISSR primer used was $(AG)_8CT$: 5'-AGAGAGAGAGAGAGAGCT-3'. The PCR protocol consisted of an initial denaturation at 95 °C for 5 minutes, followed by 21 cycles of denaturation at 95 °C for 1 minute, annealing at 58 °C for 1 minute, and extension at 72 °C for 1 minute, with a final extension at 72 °C for 5 minutes. Amplified DNA fragments were visualised on 1% agarose gel using a Gel-Doc system.The size of the formula should fit one column or span across two columns.

3. RESULT AND DISCUSSION

Chrysanthemum leaf explants cultured on MS medium supplemented with 2 mg/L 2,4-D successfully formed compact green calli after four weeks of incubation. The formed calli were then treated with gamma ray irradiation at doses of 10, 20, 30, and 40 Gray. The impact of this radiation treatment can be observed through various growth parameters, one of which is plant height increase, which is an important indicator in assessing the morphological response to the influence of gamma radiation. Plant height measurements showed that gamma-ray radiation (x) correlated with chrysanthemum plant height (y). The regression equation $y = -0.0737x + 2.476$ ($R2 = 0.7537$) showed that 75.37% of chrysanthemum plant height was influenced by gamma-ray radiation and

24.63 was affected by external factors (Fig. 1A). Based on the results, a lower dose of 10 Gray correlated with an increase in plant height compared to higher doses (20-40 Gray). This suggested that gamma-ray irradiation interfered with vertical plant growth and was caused by damage to apical meristem tissue, affecting cell metabolism and protein synthesis. Radiation also damaged cell walls and inhibited elongation, which was essential for vertical shoot growth. Exposure to high doses of gamma-ray radiation can cause a decrease in the height of chrysanthemum (*Chrysanthemum morifolium*) plant through interference with the process of cell division and elongation. According to Chowdhury et al. (2023), higher radiation doses caused lower shoot regeneration rates and significant morphological changes in chrysanthemum plant. Miller et al. (2023) stated that radiation could cause genetic changes disrupting plant growth and development.

The results of measuring the number of shoots per callus showed that gamma-ray radiation (x) was related to the number of shoots (y). The regression equation y = −0.2096x + 7.736y (R2 = 0.9401) showed that 94.01% of the height of chrysanthemum plant was influenced by gamma-ray radiation and 5.99% was influenced by external factors, as presented in Fig. 1B. The decrease in the number of shoots was in line with the increasing dose of gamma-ray. The administration of 0 Gray treatment significantly affected the number of shoots but at 40 Gray no shoots appeared. Giving gamma-ray treatment can positively or negatively impact plant depending on the dose. Ali et al. (2016) stated that in Arabidopsis plant, growth increased with gamma-ray treatment at 1 - 2 Gray doses. When additional gamma-ray was higher than 50 Gray, a negative impact was observed on plant growth and development. The balance of plant hormones significantly influences the growth and development of shoots. This showed that giving gamma-ray radiation treatment could impact various physiological, biochemical, and growth processes and plant yields.. Gamma-ray radiation can affect essential hormones regulating plant growth, such as auxin, cytokinin, and gibberellin (Jain, 2010). These hormones help control several processes including shoot formation and leaf growth. When radiation dose increases, the balance of hormones is disrupted, causing plant growth to slow down and decrease in number of shoots. Radiation exposure can inhibit plant metabolic processes important for vegetative growth and shoots (Thenuja *et al*., 2024; Putra and Prasetya, 2024).

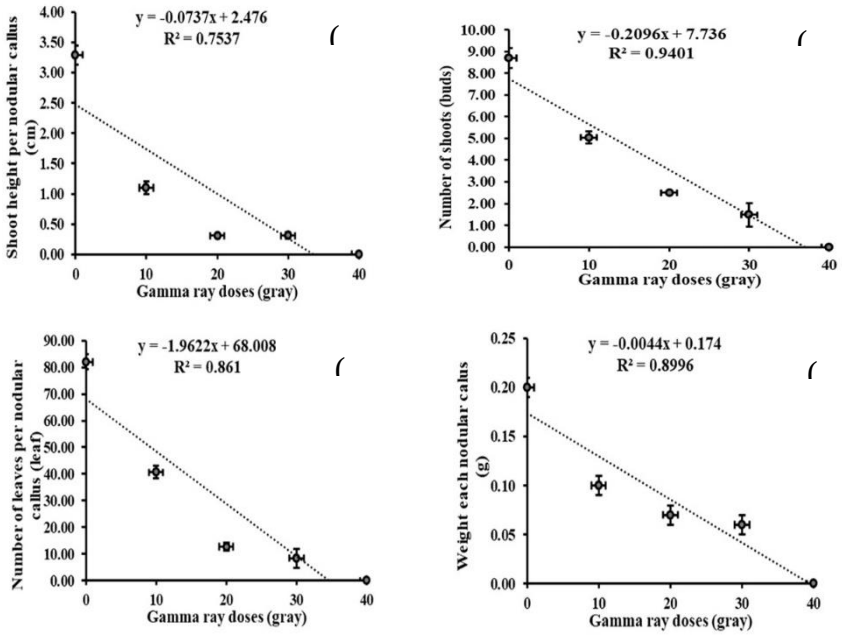

Figure 1. Effect of gamma-ray irradiation on chrysanthemum callus growth; A. Shoot height per nodular callus, B. Number of shoots per nodular callus, C. Number of leaves per nodular callus, D. Weight each nodular callus.

The regression equation y=−0.2097x+7.7378 (R2=0.9323) showed that 93.2% of the number of chrysanthemum leaf was affected by gamma-ray radiation and 7.8% was affected by external factors (Fig. 1C) The relationship pattern between radiation and the number of chrysanthemum leaf was described as a linear polynomial. This was shown by a decrease in the number of chrysanthemum leaf number and an increase in gamma-ray radiation (20-40 Gray). Gamma-ray irradiation can cause chromosome aberration (broken chromosome threads) and damage the DNA structure of plant cell, thereby disrupting cell division and tissue differentiation. In the context of leaf formation, high-dose radiation causes oxidative stress, which inhibits plant ability to produce new leaves. A significant decrease in the number of leaves due to gamma-ray radiation also occurred in the study of Thenuja *et al*., (2024) where increasing the dose caused a significant decrease in the rate of cell division, which affected plant growth. Similarly, Putra and Praseyta (2024) stated that plant exposed to high doses of gamma-ray radiation would have fewer leaves compared to the control.

The regression equation y = −0.044x + 0.174 (R2 = 0.8996) showed that 89.96% of the weight was influenced by gamma-ray radiation, and external factors affected 10.04% (Fig. 1D). The administration of many gamma-ray would produce low chlorophyll, plant root weight, and inhibited germination. This suggested that giving a high dose of gamma-ray affected essential processes in plant growth, protein synthesis, and enzyme activity in plant system (Mounir *et al*., 2022). Gamma-ray radiation at high doses can damage cell membranes, stop water absorption, and reduce the fresh weight of plant. The ability of plant to absorb water and nutrients also affects the fresh weight. Hong, *et al*., (2022) showed that gamma-ray radiation could disrupt the root system, and reduce the ability of plant to maintain water content and biomass accumulation.

The addition of BA to the culture medium increased plant height, number of leaves, and nodes in a 6-week irradiated chrysanthemum cuttings culture, as shown in Table 1. This response indicates that BA supplementation supported vegetative recovery and growth following gamma-ray irradiation, which is known to impose physiological stress on plant tissues. Meanwhile, the administration of BA 0.5-1.5 mg.l^{-1} to MS medium gave the highest plant height compared to BA 3 mg.l^{-1}. Using growth hormones at the correct dose can accelerate plant development but excessive application at specific doses, such as BA concentration of 3 mg.l^{-1} is capable of suppressing growth in chrysanthemum plants. Murthy *et al*., (2024) explained that cytokinins stimulate vegetative growth; at high concentrations, plant can experience growth inhibition caused by accumulating secondary compounds due to stress. The most significant number and several leaves were obtained on MS media with the addition of BA.0.5 mg.l^{-1}, while the 1.5 and 3 mg.l^{-1} BA treatments produced lower values and were more efficient in stimulating leaf formation alongside several nodes without causing adverse effects. Regarding the number of shoots, media variations, and gamma-ray irradiation did not have a significant effect. The average number of remaining shoots was approximately one per plant, showing that these factors did not directly affect shoot production. According to Kulpreechanan *et al*., (2023) the use of growth hormones such as BA could increase vegetative growth without a positive effect on shoot formation. High doses of growth hormone caused accumulation that inhibited cell division and affected other aspects of plant growth (Chougule and Rawat, 2024).

In this study, a decrease in plant height, number of leaves, and number of nodes occurred due to increased gamma-ray irradiation (30 Gray), as shown in Table 1. Callus that was not irradiated and irradiation at 10-20 Gray did not give a difference in the number of leaves and nodes. The decrease in chrysanthemum plant growth was observed at 30 Gray compared to other treatments. These conditions had the potential to reduce the number of leaves formed due to cell damage. Sushma Devi *et al*., (2023) showed that exposure to gamma-ray radiation could increase oxidative stress in plant, contributing to cell damage and reduced growth. This radiation-induced oxidative

stress damaged DNA, cell membranes, and essential proteins, inhibiting cell division activity and slowing plant growth processes (Armando *et al*., 2024).

Gamma-ray radiation causes physical damage to cells and can trigger genetic mutations. Gamma-ray radiation can be associated with cellular damage and the induction of genetic mutations in plant cells (Jain, 2010). At the molecular level, it has the potential to damage DNA by breaking double-strand breaks causing mutations when repaired properly. The mutations can lead to different phenotypes or increase genetic diversity of plant. This high-dose DNA damage tends to cause stunted growth or cell death (Hong *et al*., 2022; Murthy *et al*., 2024; Armando *et al*., 2024). At lower doses of 10 Gray, the resulting mutations may not be detrimental leading to more significant phenotypic variability, which could benefit plant breeding programs.

Table 1**.** Plant height, number of shoots, number of leaves, and number of nodes in chrysanthemum plant grown with doses of gamma-ray radiation and benzyl adenine (BA) for six weeks of culture.

Treatments	Shoot height (cm.)	Number of shoots (shoot)	Number of leaves (strands)	Number of nodes (node)
Media				
BA 0.5	6.38 ± 1.49 a	0.92 ± 0.33	12.00 ± 0.90 a	6.67 ± 0.57 a
BA 1.5	6.71 ± 1.64 a	1.00 ± 0.00	9.67 ± 0.65 b	5.67 ± 0.70 b
BA 3	5.19 ± 1.16 b	1.00 ± 0.00	8.67 ± 0.58 b	4.83 ± 0.89 b
Probability	***	ns	***	***
P-value	< 0.001	0.781	< 0.001	< 0.001
Gamma-ray				
0 gray	8.59 ± 1.09 a	1.00 ± 0.00	10.44 ± 1.19 a	6.00 ± 0.94 a
10 gray	5.67 ± 0.48 c	1.00 ± 0.00	10.56 ± 1.73 a	6.33 ± 0.87 a
20 gray	6.78 ± 1.44 b	0.89 ± 0.46	10.44 ± 1.28 a	6.11 ± 0.92 a
30 gray	3.33 ± 0.75 d	1.00 ± 0.00	9.00 ± 1.09 b	4.44 ± 0.87 b
Probability	***	ns	**	**
P-value	< 0.001	0.861	0.009	0.001
X-ray gamma media				
Probability	***	ns	ns	ns
P-value	< 0.001	0.955	0.505	0.157

Remark : The ns= non-significant difference at P<0.05; *= significant difference at P<0.05; **= significant difference at P<0.01; ***= significant difference at P<0.001.

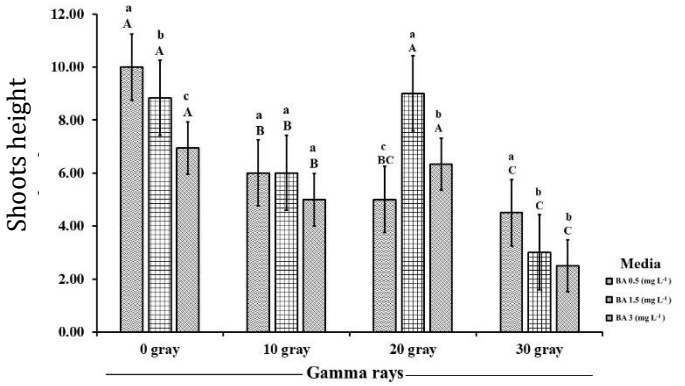

Figure 2**.** The average height of chrysanthemum shoots at various doses of gamma-ray and BAP during 6 weeks of culturing. Note: The average value followed by the same letter is not significantly different according to Tukey's Advanced Test (HSD) at a significance level of 0.05. Lowercase letters are read vertically, comparing two media at the same dose. Capital letters are read horizontally, comparing two doses on the same media.

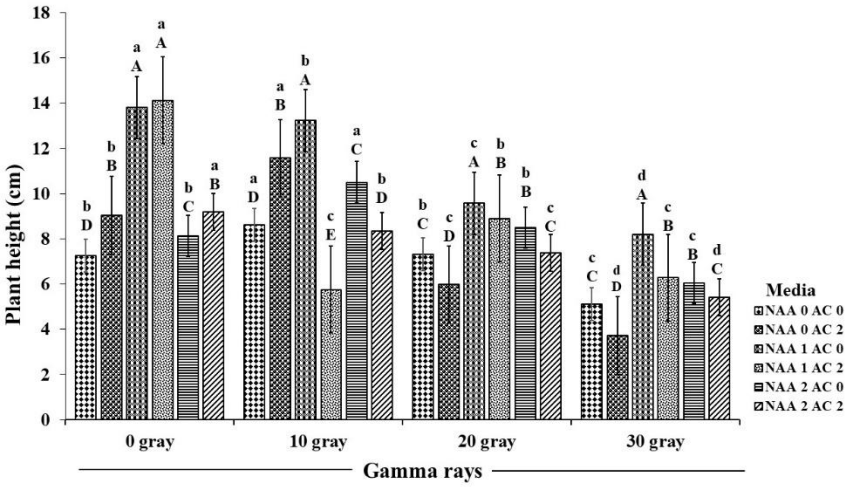

Figure 3. Height of chrysanthemum plant due to gamma-ray radiation using rooting media 60 days after planting. The average value followed by the same letter is not significantly different according to Tukey's Advanced Test (HSD) at a significance level of 0.05. Lowercase letters are read by comparing two media at the same dose. Capital letters are read by comparing two doses on the same media

Based on the data presented in Fig.2, the concentration ofBA and the dose of gamma-ray radiation significantly affectedshoots height. At radiation dose of 0 Gray, BA treatment of 0.5 mg L^{-1} produced the best plant height results compared to 1 3 mg L^{-1} With increasing gamma-ray dose, particularly at 10 and 20 Gray, BA at 1.5 mg L^{-1} resulted in higher shoot height than other BA concentrations, indicating its effectiveness in mitigating the inhibitory effects of moderate gamma irradiation. This response may be attributed to BA's optimal role as a cytokinin in maintaining hormonal balance, promoting cell division, and sustaining meristematic activity under radiation-induced stress (Davies, 2010). This showed the ability to help plant overcome the adverse effects of gamma-ray radiation. At 30 Gray, all BA concentrations, particularly 3 mg L^{-1}produced a significant decrease in shoots height. Radiation at high levels caused physiological stress that BA treatment could not fully overcome. BA is a member of the cytokinin group, stimulating cell division and slowing leaf senescence.

The results of Fig. 3 showed that gamma-ray radiation significantly affected the height of chrysanthemum plant, with effects varying depending on radiation dose and the type of growth medium used. At low to medium radiation doses (0 and 10 Gray), media containing AC, particularly NAA 1 mg.l^{-1} AC 2 g.l^{-1} and NAA 2 mg.l^{-1} AC 0 g.l^{-1}, could maintain and increase plant growth. This was because the combination supported the availability of water and nutrients, improving the physical condition of the growth medium. The hormesis effect that appeared at 10 Gray was caused by the physiological stimulation of plant due to exposure to light radiation, which triggered better growth on the support medium. Several studies have shown that low doses of ionizing radiation can produce positive changes in plant growth dynamics. Kazakova *et al*., (2024) stated that radiation affected gene expression stimulating plant growth.

Wang *et al*., (2020) exposure to glyphosate at low doses showed a hormesis effect, increasing plant tolerance to environmental stress and supporting better growth. This suggested that hormesis effect at 10 Gray occurred due to positive stimuli triggering physiological mechanisms enhancing plant growth on the support medium. At higher radiation doses of 20 and 30 Gray, the negative impact of radiation was more visible with a decrease in plant height in all media. High-dose radiation exposure damaged plant cell structure and photosynthesis processes, inhibiting growth. Media containing NAA and AC were still able to maintain relatively better growth than the control.

This showed the essential role of growth media in helping plant adapt to environmental stress, including radiation exposure. Ji *et al*., (2022) explained that DNA damage due to radiation could affect cell function, disrupt repair mechanisms, and cause abnormal cell division, reducing vegetative growth. Murthy *et al*., (2024) stated that low-dose radiation increases vegetative growth and stimulates secondary metabolism in plant, including chrysanthemums. Higher doses could cause significant cell damage, thereby inhibiting growth. Kazakova *et al*., (2024) found that ionizing radiation at high doses radiation plant growth by affecting the expression of genes essential for growth and development. Xu *et al*., (2023) showed that exposure to high doses of pesticides caused adverse effects on photosynthesis and growth. Nouman *et al*., (2023) suggested that the addition of plant growth regulators, such as NAA helped maintain better development under stress conditions, including high radiation exposure, due to the essential role of media in supporting plant adaptation.

These results showed that gamma-ray radiation significantly affected the root length of chrysanthemum plant, particularly at higher doses, as presented in Fig.4. High radiation doses such as 20 and 30 Gray tended to inhibit root growth, as observed from decreased root length in all media. Lower radiation doses, such as 10 Gray, NAA 0 mg L^{-1} AC 2 g L^{-1}, showed a better root growth stimulation response. Under non-irradiated conditions (0 Gy), the longest root length was observed in the medium containing NAA at 1 mg L^{-1} and activated charcoal at 2 g L^{-1}, and this value was significantly higher than those of the other media treatments at the same radiation dose. Lal *et al*. (2022) showed that exposureto UV-B radiation could change the biochemical composition of plant, impacting growth. Additionally, Ramamoorthy et al. (2022) showed that stress from radiation, both UV and others, inhibited overall plant growth and development. At 10 Gray, using media containing NAA and AC increased root growth. This showed the concept of hormesis, where low doses of stress stimulated positive responses in plant. Priatama *et al*., (2022) also reported that proper treatment increased plant resistance to stress, including radiation. NAA is essential in stimulating root formation and accelerating the vegetative phase. According to Kadhim et al. (2023), a concentration of NAA of 2 mg L^{-1} was effective in increasing the number of shoots and adventitious roots in chrysanthemums. At higher radiation doses (20–30 Gray), media with AC and NAA produced better results compared to the control. However, the adverse effects of high gamma-ray radiation were dominated, which inhibited overall plant growth. Papafotiou *et al*. (2023) showed that high doses of gamma-ray radiation could cause significant DNA damage, disrupt the cell division process, and inhibit growth even though the culture media was optimized.

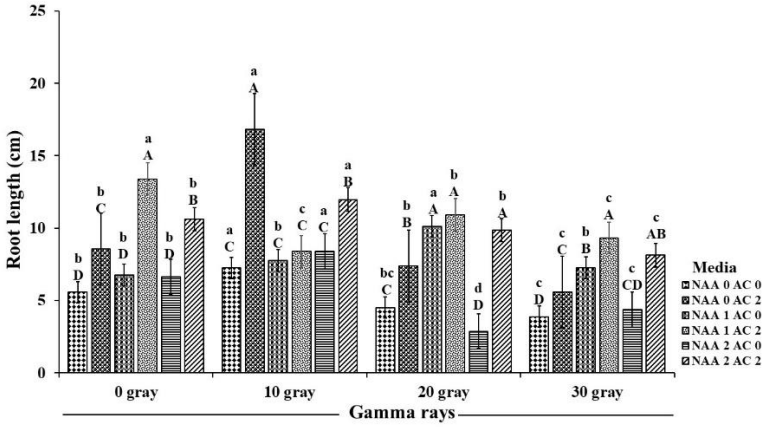

Figure 4. Chrysanthemum root length due to Gamma-ray radiation and NAA media with or without AC 60 days after planting. The average value followed by the same letter is not significantly different according to Tukey's Advanced Test (HSD) at a significance level of 0.05. Lowercase letters are read by comparing two media at the same dose. Capital letters are read by comparing two doses on the same media.

The results showed that gamma-ray radiation affected the number of roots in chrysanthemum plant. The significant effects were based on radiation dose and the growth media, as shown in Fig. 5. Increasing the dose of gamma-ray radiation tended to reduce the number of roots produced, particularly at the highest dose (30 Gray). Some growth media, such as NAA 1 mg.l^{-1} and AC 2 g.l^{-1} showed the ability to maintain and increase the number of roots despite exposure to gamma-ray radiation at a dose of 20 Gray. Another influencing factor is the combination of NAA and AC. Media with a combination of NAA 1 mg.l^{-1} and AC 2 g.l^{-1} consistently produced a significantly higher number of roots at all radiation levels. The addition of NAA in tissue culture media increased the formation of adventitious roots in chrysanthemum plant, which contributed to improving vegetative growth (Kadhim *et al*., 2023; Thamodharan *et al*., 2024).

The increase in root number at a specific radiation dose such as 20 Gray could be explained by the potential stimulatory effect of low-dose radiation (hormesis phenomenon), where exposure to moderate radiation levels triggered beneficial physiological responses in plant. At a higher radiation dose of 30 Gray, the adverse effects of radiation were more dominant, as shown by the decrease in root number in all media. This suggested that the combination of media containing NAA 1 mg.l^{-1} and AC 2 g.l^{-1} effectively increased root number and maintained growth in chrysanthemum plants exposed to radiation, specifically at low to moderate doses. Previous studies support these results, showing that high-dose radiation caused damage to cell structures and inhibited root growth. Hong *et al*., (2022) showed that gamma-ray radiation affected plant growth and germination based on the dose received. Appropriate growth media, such as a combination of NAA and AC, protected against the damaging effects of radiation. A study by Murthy *et al*., (2024) emphasized the importance of using plant growth regulators in increasing resistance to stress, including radiation exposure. The combination of NAA 1 mg.l^{-1} and AC 2 g.l^{-1} showed the ability to maintain and increase the number of roots. Similarly, Sushma Devi *et al*., (2023) reported that treatment with growth hormones increased plant response to environmental stress.

After being transferred to MS + NAA media with or without the addition of AC for 2 months, chrysanthemum seedlings grew properly, with most reaching a length of 5-14 cm, having 4-16 leaves, and a sound root system. These plantlets were hardened off at room temperature for one week before being removed from the culture bottle. After one week of cleaning from the remaining agar media, chrysanthemum plantlets were acclimatized using AC media. Approximately 92.33% of the plantlets were successfully acclimatized after two months in ex-vitro conditions. However, there were different morphological changes in chrysanthemums irradiated with doses of 0, 10, 20, and 30 Gray, as shown in Fig. 6.

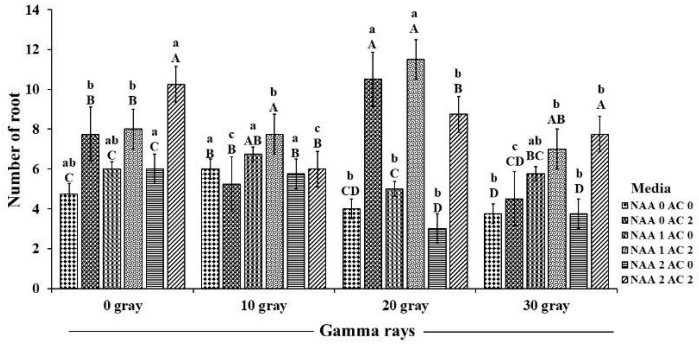

Figure 5. Number of chrysanthemum roots due to Gamma-ray radiation and NAA media with or without AC 60 days after planting. The average value followed by the same letter is not significantly different according to Tukey's Advanced Test (HSD) at a significance level of 0.05. Lowercase letters are read by comparing two media at the same dose. Capital letters are read by comparing two doses on the same media.

Figure 6. Chrysanthemum plant growth two months after acclimatization at gamma-ray radiation doses of 0, 10, 20, and 30 gray.

Table 2. Quantification of the extracted DNA results

No	Chrysanthemum Sample	Concentration of extracted DNA (ng/µl)	Absorbance A260/A230	Absorbance A260/A280
1	K0	107	1.372	1.726
2	K1	233	0.552	1.137
3	K2	428	0.389	0.881
4	K3	28	1.368	1.525

Chrysanthemums exposed to gamma-ray radiation produced variations in physiological and morphological responses based on doses. Fig. 6 shows a significant difference between the control treatment (0 gray) and radiation-exposed treatment (10, 20, and 30 gray). At 0 gray, the chrysanthemum plant showed average growth with a dark green leaf color without signs of physiological stress. Plants given a dose of 10 Gray started to show changes in leaf color, characterized by slight chlorosis or yellowing. Despite the changes, plants adapted to the conditions, with relatively low levels of damage. The high dose was assumed to trigger genetic changes without causing excessive damage to the plants metabolic system. This was similar to a previous study, showing that radiation low doses stimulated beneficial genetic variation without causing significant adverse effects (Wi *et al*., (2007).

At a higher dose of 20 Gray, the plant showed more apparent chlorosis symptoms. The leaves started to lose much of their green color, showing that the chloroplasts, the center of plant photosynthesis, had been destroyed. Previous studies have shown that gamma-ray radiation damaged the structure of chloroplasts, thereby causing the cessation of photosynthesis Kovács and Keresztes, 2002). More significant changes were observed at a dose of 30 Gray, where approximately all parts of the leaf experienced severe chlorosis. Due to the damage, plant showed that radiation exposure caused significant stress. Radiation can damage DNA and essential proteins that regulate important plant functions.

Table 2 shows that the concentration of three chrysanthemum isolates (K0, K1, and K2) ranged from 107 ng/µl to 428 ng/µl. This concentration was relatively high compared to the λ DNA of 100 ng/µl, which differed from K4. Meanwhile, the purity level of DNA extracted from the four chrysanthemum isolates at A260/A230 absorbance showed low purity due to contamination of EDTA, carbohydrates, and phenolic compounds during extraction. Isolates K1, K2, and K3 showed contamination in the form of protein based on the A260/A280 absorbance results, which was lower than 1.7.

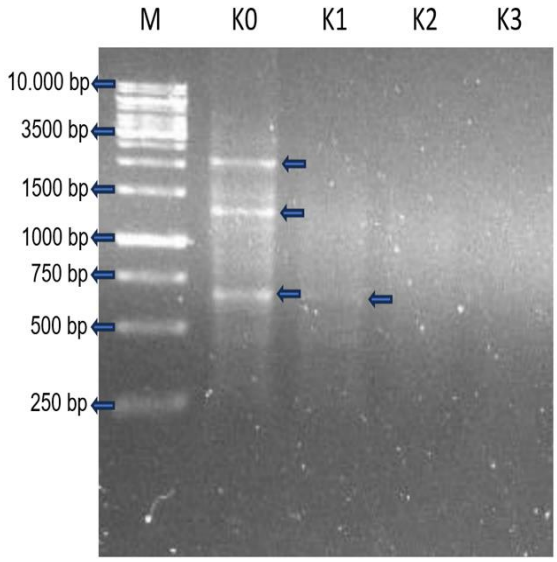

Figure 7. Chrysanthemum DNA amplification using ISSR (AG) 8CT primers (M is Marker 1 kb (kilobases) gene ruler by Thermo Scientific, USA. K0 is Chrysanthemum with no Gamma Irradiation. K1 is Chrysanthemum with 10 Gray dosse Gamma Irradiatoin. K2 is Chrysanthemum with 20 Gray dosse Gamma Irradioation. K3 is Chrysanthemum with 30 Gray dosse Gamma Irradiation).

Mutation analysis due to gamma-ray radiation was conducted using genetic analysis through PCR (DNA amplification). Visualization of DNA amplification results using ISSR primers (Fig. 7) showed changes in genetic material in chrysanthemum plant that received 10 Gray, 20 Gray, and 30 Gray compared to the control. As one of the negative impacts, the isolate of the chrysanthemum plants that was given 40 Gray died, limiting the continuation of molecular analysis. Physiological processes of plant controlled by genetic factors can experience changes or abnormalities due to genetic variations after irradiation.

Chrysanthemum isolates not given radiation treatment (K0) produced 3 DNA fragments after being amplified using the ISSR (AG) 8CT primer. The isolates that received 10 Gray (K1) produced 1 DNA fragment. However, K2 and K3 were not successfully amplified due to incompatibility or non-complementarity between the primer sequences and the genomic DNA (Carsono *et al*., 2014). Gamma-ray irradiation in ornamental plant is a form of physical mutagen that is carried out to increase genetic diversity. Radiation doses of 1 Gray to 10 Gray are categorized as moderate which significantly determines the success of the induction of genetic diversity produced (Damayanti *et al*., 2021).

4. CONCLUSIONS

This study shows that hormone balance, gamma radiation, and genetic analysis using ISSR markers significantly impact chrysanthemum growth and genetic variation. The response of chrysanthemum leaf explants to 2,4-D treatment at various concentrations was very different; basic media without 2,4-D growth regulators were less effective in stimulating callus growth. When explants were treated with 2,4-D, there was a significant increase in all measured parameters. The use of growth regulators BAP at the correct dose (0.5-1.5 $mg.l^{-1}$) can accelerate the growth of irradiated chrysanthemum plants (10, 20, 30 gary), but excessive use at specific doses, such as at BA concentrations 3 $mg.l^{-1}$, can suppress growth in chrysanthemum plants. High radiation doses above 20 tend to inhibit the growth of chrysanthemum plants; lower radiation doses, such as 10 Gray, show a better plant growth stimulation response. At 10 Gray, media containing Naphthalene acetic acid (1-2 $mg.l^{-1}$) and activated charcoal (2 $g.l^{-1}$) increased root growth. Exposure to high doses of radiation

inhibited growth; however, media containing naphthalene acetic acid (1-2 $mg.l^{-1}$) and activated charcoal (2 $g.l^{-1}$) were still able to maintain growth relatively better than the control. Gamma-ray radiation (10-30 gray) affects DNA fragmentation patterns, and the higher the radiation dose, the more significant the changes that occur in the DNA structure, which has implications for potential changes in genetic traits in plants exposed to this radiation.stand alone or form a subsection of a Discussion or Results and Discussion section. The entire paper must follow this pattern and not use bullet points or numbering systems. Important! Please encourage others to publish their works in the Jurnal Agrotek Tropika.

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