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Research Article
In vitro Culture and Field Evaluation of Two Generations of Sorghum Mutants Induced by Gamma Ray Irradiation

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Abstract

Background and Objective: Sorghum is one of the important cereal crops worldwide, with a limited option of varieties to culture in Indonesia. This study aimed to evaluate the *in vitro* culture and field evaluation of two generations of Suri 3 Agritan sorghum mutants induced by gamma-ray irradiation to obtain a new high yielding variety with an improvement of nutritional value. **Materials and Methods:** This study has consisted of two experiments, i.e., *in vitro* culture of sorghum mutant irradiated by gamma rays and field evaluation of genetic diversity and nutritional content of gamma-ray irradiated sorghum in two generations. A hundred sprouts with the size of about 2 mm was transferred into each petri dish that filled with pure Murashige and Skoog® (MS0) growing media with Various gamma-ray irradiations are (0, 40, 50, 60 and 70 Gy). **Results:** The results showed that *in vitro* growing media and gamma-ray irradiation doses significantly affected the plantlet mutant growth performance, where the increase of irradiation dose impeded the mutant growth and the addition of BA was significantly reduced shoot growth but favoured for the root part. Field evaluation revealed significant variance on agronomic characters on different mutant generations and irradiation doses. A broad diversity was found in the 40 and 70 Gy irradiation dose in the 2nd generation. The 40 Gy mutants also displayed a clear qualitative variation of leaf colour, seed and panicle form. **Conclusion:** Nutrition profiling analysis concluded that the increase of irradiation dose was associated with the increase of fat, protein, ash, amylopectin and dietary fibre content; and the decrease of carbohydrate and amylose levels.

Key words: Amylose, amylopectin, leaf colour, nutritional value, panicle form

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sorghum (*Sorghum bicolour* L. Moench) is an important cereal crop that is widely cultivated worldwide, either for food, feed, or fuel[1-4]. Wide cultivation of sorghum is supported by its competitive agronomical characters, such as short lifespan (harvest at 100-110 days after planting, DAP), better adaptability in suboptimal lands, such as acid and saline soil and relatively low production costs due to low input required[5,6]. Drought tolerance and pest-disease resistance are also comparative advantages of this plant[7] that associated with its ability to absorb water from deep soil solution[8] and to use water efficiently[9]. Not only the grain but also the entire biomass of this plant has economic value[10]. Bioethanol production of sorghum (per unit area) is also higher than other crops, even though it is cultivated in low input agroecosystems[5]. Sorghum showed a maximum bioethanol yield under full irrigation for about 2085 L ha^{-1} [11]. Although so many comparative advantages are reported, the recent status of this plant in Indonesia is still less popular[7], followed by the limitation in the choice of sorghum variety in Indonesia. Therefore, efforts to breed sorghum varieties are still continuously going and required for the future[12].

As introductory varieties, the genetic resource of sorghum in Indonesia is still limited. There is an urgency to improve the genetic diversity of sorghum through breeding activities[12,13-15]. The breeding technique combines science and art to design and obtain plant varieties equipped with desirable characters[16]. Plant breeding recently relies on various approaches, not only conventional but also modern methods. Conventional breeding by manual crossing techniques has been reported to obtain the desired sorghum varieties by designing the best cross combination, such as B-69× Numbu[17]. Modern methods relying on biotechnology has been reported to be useful tools to enhance sorghum genetic diversity[18]. Plant mutations can be induced either by chemical or physical mutagens[19]. Gamma-ray is one of the physical mutagens that is frequently used to induce plant mutation[15] as the effect of random mutations at various levels, i.e., cytoplasm, chromosome and genome. Previous studies reported the success of mutation breeding in sweet potato[20], soybean[21,22] and Namibia local sorghum[17].

In Indonesia, sorghum breeding programs have been also conducted to continuously obtain new desired sorghum varieties. As a part of the sorghum breeding program, the genetic diversity of local sorghum has been previously reported[23]. Additionally, the physicochemical content of local sorghum accession has described[24]. Suri 3 sorghum is a promising sorghum variety that still needs improvement through mutation breeding, both in terms of production quantity and nutritional quality. Earlier study[25] reported the promising sorghum mutant lines with an improvement in characters of drought tolerance and yield. The success of mutation breeding was also reported to be improved by having a combination with *in vitro* culture[26,27], however, there is still limited information specifically for Suri 3 Agritan sorghum. Therefore, this study aimed to evaluate the *in vitro* culture and field evaluation of two generations of Suri 3 Agritan sorghum mutants induced by gamma-ray irradiation.

MATERIALS AND METHODS

Place and time research: This study was conducted from January, 2020 to February, 2021 at the tissue culture laboratory and greenhouse of the Indonesian Center for Agricultural Biotechnology and Genetic Resource Research and Development (ICABIOGRAD) and Cikeumeuh experimental garden, Bogor, Indonesia. This study has consisted of two experiments, i.e., *in vitro* culture of sorghum mutant irradiated by gamma rays and field evaluation of genetic diversity and nutritional content of gamma-ray irradiated sorghum in two generations.

Research methods

***In vitro* mutation:** Explant in the form of Suri 3 Agritan seeds obtained from ICERI was sterilized in a laminar airflow by using alcohol 70% for 5 min and Clorox disinfectant 20% for 10 min, before planting in germination media. Before use, all growing media was sterilized using an autoclave (120°C, 121 Psi) for about 15 min. A hundred sprouts with the size of about 2 mm were transferred into each petri dish that filled with pure Murashige and Skoog® (MS0) growing media. Various gamma-ray irradiations (0, 40, 50, 60 and 70 Gy) were conducted in the National Nuclear Energy Agency of Indonesia, with 10 Petri dishes for each gamma-ray dosage. Post treated explant was transferred into a growing bottle, filled with two types of media, i.e., MS0 and modified MS with the addition of 0.1 mg l-1 benzyl adenine (MSBA). There were 10 explants planted in every bottle and there were 10 bottles for each gamma-ray dosage. The bottle was then arranged in a completely randomized design, under artificial light with intensity 1500 lux, photoperiod 16 hrs and ambient temperature 25°C. The height and root length of the plantlet were measured 5 days after transplanting. Afterwards, the plantlet was ready to acclimatization step in the greenhouse. All obtained data were tested by analysis of variance (ANOVA) and then continued by the Least Significant Difference (LSD) at a 5%. The interactions between *in vitro* growing media and gamma-ray irradiation doses were further processed by orthogonal polynomial test.

Evaluation of mutant among generation: The plantlet was acclimated for a month in the greenhouse before the field. The plantlet was planted into a small polybag (5 cm in diameter, 10 cm in height) filled with a mix of soil and organic fertilizer (1:1). The plantlet was covered by a plastic glass for the first two weeks. The plastic cover was removed and allowed the plant seedlings to grow up to 30 cm in height. During the greenhouse maintenance period, the plant was frequently exposed to mist-spray watering. A month-old seedling was replanted in a bigger polybag (25 cm in diameter, 50 cm in height) with the same growing media and then moved to an outside greenhouse. Approximately 20 genotypes for every gamma-ray dosage were raised until the mutant plants have been produced seeds (M1). Approximately 50 M1 genotypes from each irradiation dosage were germinated and planted in the field as the M2 plant, with a planting distance of 75×25 cm. The applied culture practice in the present study was followed a previous study[18]. On harvesting day (95 days after planting), the measured variables were plant height, stem diameter, panicle length, brix, panicle weight, leaf colour and the form of seed and panicle. Plant height (cm) was measured by using a rolling meter from the stem base to the tip of the panicle exit. The length of the panicle was also measured by using a rolling meter from the base to the tip of the panicle. Stem diameter (mm) was measured by using a digital calliper at the stem middle part (100 cm above the soil surface). A hand refractometer was used to determine the sugar brix content in the stem middle part. The weight of the panicle was measured in the form of fresh weight. Colour in leaves was visually observed with a reference to the RHS color chart. The form of seed was classified into moderate and big circular, while the form of panicle was categorized as moderate open type and moderate compact type. Additional nutritional compound analysis was also conducted, i.e., ash by gravimetric method, protein by Kjeldahl method, fat by Soxhlet method, carbohydrate and amylopectin by by-difference method, amylose by iodo colorimetry method.

Data analysis: Data obtained from observation were analyzed by analysis of variance (ANOVA) and Least-Squares Distance (LSD) test at 5% error and biplot of principal component analysis. The ANOVA and LSD test used STAR 2.1 software. Meanwhile, the principal component analysis used the RStudio 3.6.1 software.

RESULTS

The result of ANOVA on the height and root length of plantlets was displayed in Table 1. The different *in vitro* growing media, irradiation doses and interaction of both factors significantly affected the height of the plantlet. In contrast, the different *in vitro* growing media did not show a significant effect on the root length of the plantlet in Table 1. *Post hoc* LSD tests revealed that either *in vitro* media or irradiation dose showed a significant effect on plantlets. *In vitro*, growing media in the form of MS0 produced a significantly higher plantlet rather than MSBA (MS media enriched with BA), with an increase of about 0.60 cm in Table 2. Although there was an increase of plantlet root length by about 0.08 cm in MSBA than MS0, that difference was not significant.

The increase of irradiation dose significantly decreased the height and root length of the plantlet in Table 3. A higher reduction of plantlet height for about 62%, was found in the higher irradiation dosage (70 Gy gamma-ray), compared to control (0 Gy gamma-ray). In a similar, a higher reduction of root length variable, for about 60%, was also observed in the 70 Gy mutants. In opposite, the lowest reduction was measured in the 40 Gy gamma-ray irradiated mutants, with a reduction of about 51 and 54% on both the height and root length of the plantlet, respectively (Table 3). Based on the orthogonal polynomial test, the MS0 growing media showed a quadratic curve and allowed the decline of plantlet height ($R^2 = 0.9982$) in Fig. 1a and root length ($R^2 = 0.9971$) in Fig. 1b as the increase of gamma-ray irradiation dose. On the other

Table 1: Analysis of variance (ANOVA) of the height and root length of plantlets in different *in vitro* growing media and gamma-ray irradiation

Variables (cm)	M	G	M×D	SE	CV
Plantlet height	1.0182**	3.3675**	1.1061**	0.0412	12.38
Plantlet root length	0.076	2.8951**	0.3667**	0.0646	17.56

M: *In vitro* growing media, G: Gamma-ray irradiation dosage, M×D: Interaction of two factors, i.e., M and D, SE: Standard error, CV: Coefficient of variance, **Significant effect at a 1%

Table 2: Height and root length of plantlets at 5 days after transplanting in different *in vitro* growing media

In vitro growing media	Plantlet height (cm)	Plantlet root length (cm)
MS0	2.62[a]	1.68
MSBA	2.02[b]	1.76
LSD	**	ns

MS0: Pure Murashige and Skoog® growing media, MSBA: Modified MS growing media with the addition of 0.1 mg L^{-1} benzyl adenine, **Significantly different based on LSD at a 5%, ns: Not significantly different based on LSD at a 5%

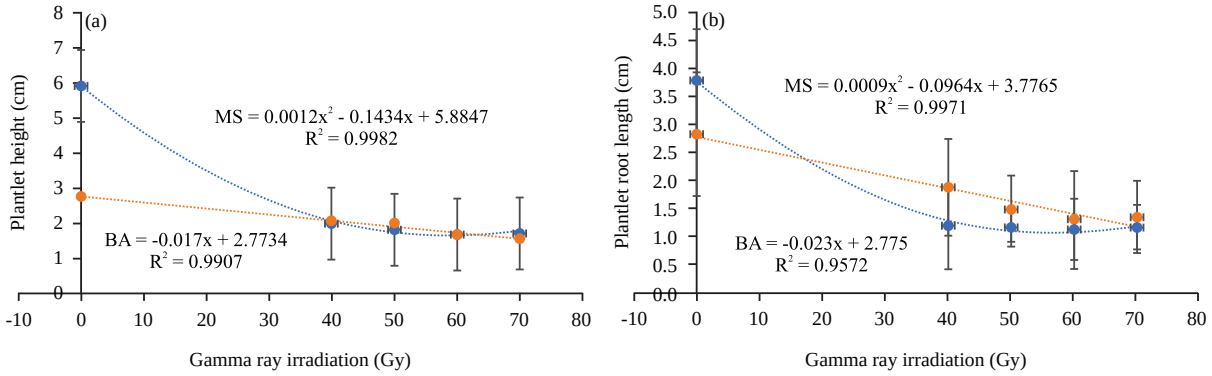

Fig. 1(a-b): Response curve of interaction between *in vitro* growing media and gamma-ray irradiation doses
(a) Height of plant and (b) Root length of plantlets

Table 3: Height and root length of plantlets at 5 days after transplanting in different doses of gamma-ray irradiation under *in vitro* conditions

Gamma ray irradiation doses	Plantlet height (cm)	Plantlet root length (cm)
0 Gy	4.33[a]	3.31[a]
40 Gy	2.04[b]	1.54[b]
50 Gy	1.90[c]	1.32[c]
60 Gy	1.70[d]	1.26[d]
70 Gy	1.66[e]	1.21[e]
LSD	**	**

Mean followed by different words is significantly different based on LSD at a 5%. Gy: Grey, **Significantly different based on LSD at a 5%

Table 4: Analysis of variance (ANOVA) of agronomical characters of sorghum mutants irradiated with gamma rays in different generations

Agronomical characters	G	D	M×D	CV
Plant height (cm)	42756.65**	3875.57**	4739.91**	8.87
Panicle length (cm)	227.39**	38.99**	3.03	14.30
Stem diameter (mm)	1.79	21.11**	23.28**	13.97
Sugar brix (%)	6.24	41.95**	29.53**	19.50
Panicle weight (g)	815.16**	18.52**	33.40**	21.90[tr]

G: Generation, D: Gamma ray irradiation dosage, G×D: Interaction of two factors, i.e., G and D, CV: Coefficient of variance, **Significant effect at 1% level, tr: Transformation by

hand, the MSBA growing media displayed a linear curve and a similar decreasing pattern on plantlet height (R^2 = 0.9907, Fig. 1a) and root length (R^2 = 0.9572, Fig. 1b). The plantlet height had a lower standard deviation than the root length character.

The ANOVA of several agronomic characters as the effect of different generation and gamma ray irradiation doses was displayed in Table 4. All variable showed lower value than 20%. The variance of generation caused the significant effect on plant height (Pr>F = 0.000), panicle length(Pr>F = 0.000) and panicle weight (Pr>F = 0.000). In contrast, the variance of irradiation produced a significant effect on all observed characters (plant height (Pr>F = 0.000), panicle length (Pr>F = 0.002), stem diameter (Pr>F = 0.000), sugar brix (Pr>F = 0.0001) and panicle weight (Pr>F = 0.000)). The interaction of both factors affected significantly to plant height (Pr>F = 0.000), stem diameter (Pr>F = 0.000), sugar brix (Pr>F = 0.0024) and panicle weight (Pr>F = 0.000). Meanwhile, the characters significantly affected by all variance sources were plant height and panicle weight (Table 4).

Four characters, namely, plant height, stem diameter, sugar, brix and panicle weight, were then processed by Principal Component Analysis (PCA). In the first PCA in Fig. 2a, there was a different diversity pattern between the two mutant generations (M1 and M2); however, both generations shared certain similarities as indicated by the overlapping area. In Fig. 2a, M2 showed the highest diversity in dimension 1 (Dim 1) as the main diversity dimension, while M1 showed the highest diversity in dimension 2 (Dim 2). In the PCA of the interaction between M1 and irradiation dosage in Fig. 2b, all irradiation doses displayed an overlap and centred diversity. Meanwhile, in the PCA of the interaction between M2 and irradiation dosage in Fig. 2c, 40 Gy seemed to produce the largest diversity, followed by 70 Gy, while the smallest diversity was found at 60 Gy irradiation dose.

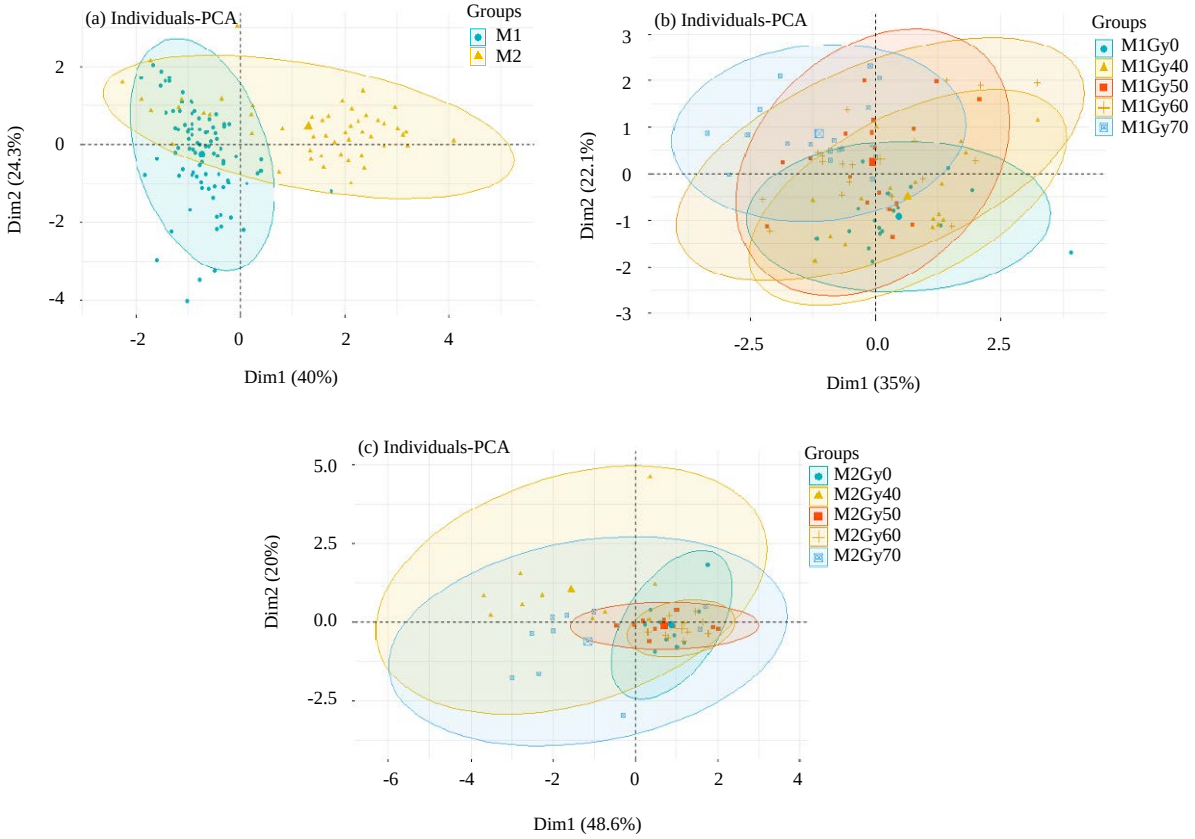

Fig. 2(a-c): Principal component analysis of the interaction
(a) M1 and M2, (b) M1 and irradiation dosage and (c) M2 and irradiation dosage

Table 5: Percentage of genetic diversity in terms of seed form, panicle form and leaf colour in the 2nd generation (M2) of sorghum mutants treated with various gamma-ray irradiation doses

Irradiation doses	Seed form (%)		Panicle form (%)		Leaves colour (%)	
	Moderate circular	Big circular	Moderate open	Moderate compact	Moderate yellow-green	Greyish olive green
0	100	-	100	-	100	-
40	50	50	60	40	-	100
50	100	-	100	-	100	-
60	100	-	100	-	100	-
70	100	-	100	-	100	-

The interaction between M1 and M2 sorghum mutants was depicted in Fig. 3. The M1 mutant showed a negative linear curve with a low gradient on the character of plant height, while the M2 mutant showed a cubic curve in Fig. 3a. In the character of stem diameter, the curve formed by M1 mutant generation was positive linear with a low slope, while the M2 generation produced a cubic curve in Fig. 3b. Both M1 and M2 mutant generations showed a quadratic curve response in the variable of sugar brix, with an opposite direction orientation in Fig. 3c. Based on the panicle fresh weight character, the M1 generation displayed a relatively stagnant curve, while the M2 generation showed a cubic curve with a high slope and standard deviation in Fig. 3d.

In the second generation (M2) of sorghum mutants, there was three notable genetic diversity revealed, viz., seed form, panicle form and leaf colour. The result shown in Table 5 implied that only 40 Gy gamma rays irradiated sorghum mutants that showed different on seed form (50 Moderate circulars and 50 Big circulars), panicle form (60 Moderate open, 40 Moderate compacts) and leaf colour (100 greyish olive green). Meanwhile, on the 50, 60 and 70 dosages, the mutant has the same phenotype as the wild type. All mutant on these dosages has 100 moderate circular, 100 Moderate open and 100 Moderate yellow-green (Table 5).

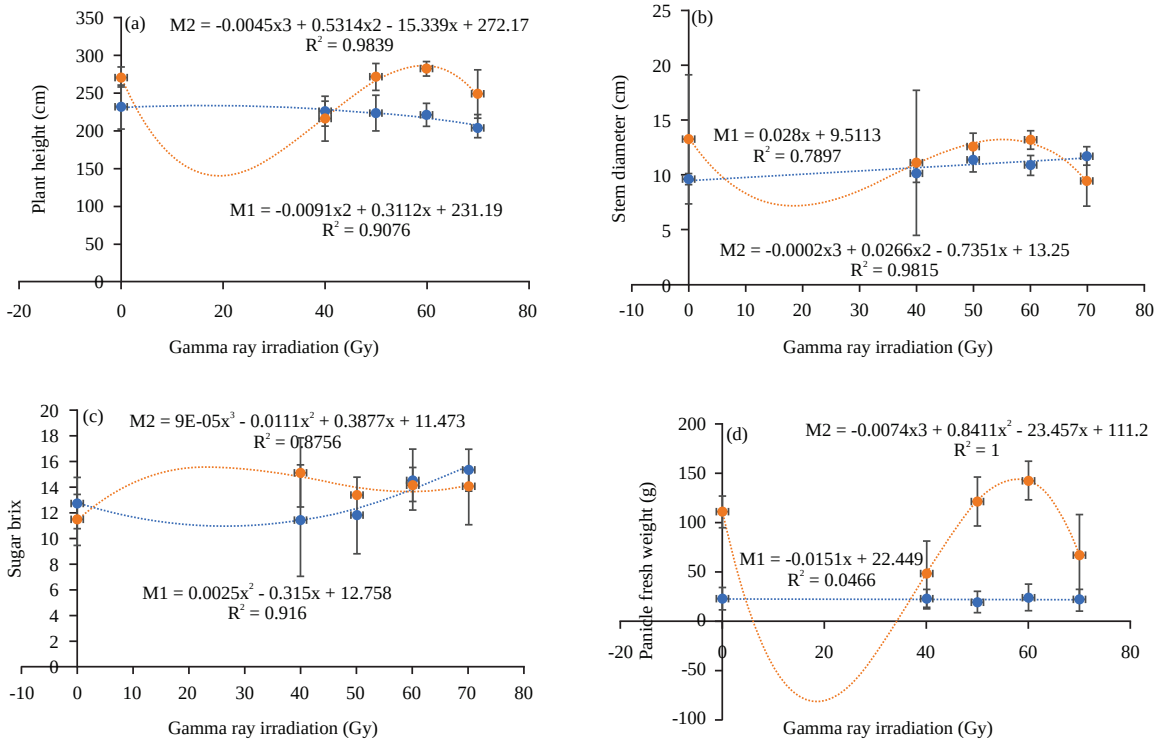

Fig. 3(a-d): Response curve of interaction between M1 and M2 sorghum mutants on measured variables
(a) Plant height, (b) Stem diameter, (c) Sugar brix and (d) Panicle fresh weight

The M2 generation was also subjected to nutritional profile analysis. The nutritional profile of M2 sorghum seemed to form a dynamic pattern in response to gamma-ray irradiation in Fig. 4. All M2 mutants irradiated with gamma-ray showed a higher fat content rather than control, except the 50 Gy mutants in Fig. 4a. The highest fat content was found in the 60 and 70 Gy mutants, i.e., 2.5%, compared to the control that was only 2.0%. The ash and protein content also increased in all M2 mutants rather than controls in Fig. 4b-c. The highest ash content was measured in the 60 Gy mutants (1.71%), then followed by 50 Gy mutants (1.59%), 70 Gy mutants (1.58%) and 40 Gy mutants (1.51%). In terms of protein, the highest result was noted in mutants irradiated with 70 Gy (9.49%) and then followed by 40 Gy (9.40%), 50 Gy mutants (9.11%) and 60 Gy mutants (8.12%). In the opposite pattern to ash and protein content, the carbohydrate and amylose content of all M2 mutants was reduced as the effect of gamma-ray irradiation. The carbohydrate content of the M2 mutant varied from 72.67-74.39% and it was lower than control, i.e., 75.5% in Fig. 4d. The amylose content of control was 33.98% and this value was higher rather than all M2 mutants that varied from 11.20-31.05% in Fig. 4e. The highest reduction of both carbohydrate and amylose content was measured in 60 Gy mutants with only 72.67% carbohydrate and 11.20% amylose content. The content of amylopectin and dietary fibre displayed an increase in all M2 mutants than controls. The normal Suri 3 Agritan (control) composed of 66.01% amylopectin, whereas 60 Gy irradiation could increase that nutritional content up to 88.79% in Fig. 4f. Similarly, the control plant only composed of 1.86% dietary fibre, while the 70 Gy irradiated plant had a 3.28% dietary fibre in Fig. 4g. The result of the ratio of amylose to amylopectin was similar to the pattern of amylose content, i.e., reduced as the effect of gamma-ray irradiation, with the lowest result for about 0.12 was found in 60 Gy mutant (Fig. 4g).

DISCUSSION

Biotechnology based sorghum improvement program became the latest technology that popularly applied to this cereal crop[28]. As part of biotechnology, *in vitro* culture combined with mutation induction has been previously reported to accelerate plant breeding programmes, because *in vitro* tissue culture could increase the effectiveness of induced mutation[29] and the efficiency of plant breeding[28]. *In vitro* culture applied in the present study was significantly affected the growth performance of sorghum mutant plantlets. The modification of MS to MSBA significantly

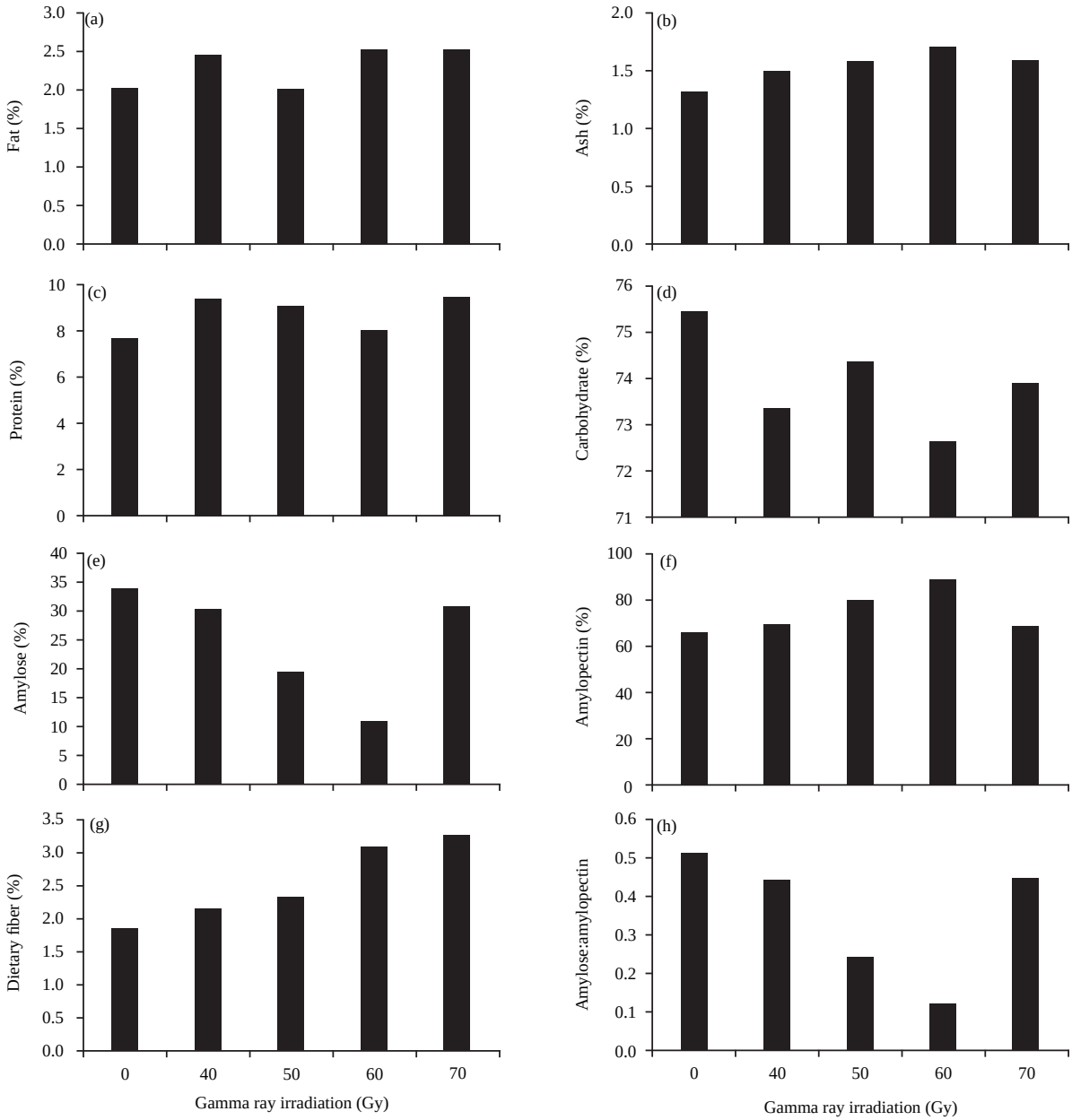

Fig. 4(a-h): Nutritional compound in the 2nd generation (M2) sorghum mutants irradiated with different gamma-ray doses

(a) Fat content, (b) Ash content, (c) Protein content, (d) Carbohydrate content, (e) Amylose content, (f) Amylopectin content, (g) Dietary fibre content and (h) Amylose: amylopectin

declined the shoot growth performance. In agreement, earlier studies[30] also reported the reduction of rice seedlings treated with Benzyl Adenine (BA). In contrast, the previous studies[31-33] showed the significance of BA treatment on plant growth. The effect of BA on the plantlet root part relatively increased root growth performance, which implied that BA might eliminate the negative effect of gamma-ray irradiation. The gamma-ray induced mutation could produce undesired cell damage in M1 generation[34], thus the addition of BA to growing media was hoped to eliminate cell damage and senescence incidence[35].

Field evaluation during two generations of gamma-ray irradiated mutants showed that the panicle length was the only character with no significant variance of interaction, which implied that different irradiation doses in the two generations produced a relatively similar response pattern.

In contrast, other agronomical characters such as plant height, stem diameter, sugar brix and panicle fresh weight significantly differed as to the effect of interaction between irradiation dose and mutant generation. This finding was in agreement with an earlier study[36] on sunflowers. Therefore, further analysis of the interaction through PCA and response curve was still required.

The PCA became an important tool to have a better understanding of the breeding program since this program produced big data. PCA was frequently used to observe the diversity in big data[37-39]. The big data was compressed into a simpler dimension, without changing most of the diversity that existed in the initial data[40,41]. In other words, PCA is a statistical technique to reveal the underlying correlations within a large number of variables[42]. Correlation used to understand the relationship between two variables or more, e.g., the correlation between leaf morphological characters[43], phytochemical content[44] and yield and growing location[45].

For better understanding, the result of PCA was expressed in a biplot. The PCA biplot in Fig. 2 displayed a clear variance of the result in between mutant generations. The M2 generation visually showed a broader diversity compared to the M1 that seemed to be highly concentrated in the certain part, implicitly reporting that M1 had more stable characters. Previous studies used PCA to understand genetic diversity[46] and evaluate the edible quality[47].

The result of PCA was in agreement with the response curve of Fig. 3 that showed a cubic pattern in the M2 generation, while M1 had a linear curve. In general, the higher degree of curve pattern associated with the complexity of the variation in between observed levels. This finding was similar to a previous study[48] that reported M2 generation underwent more fluctuating changes compared to M1. M1 tended to be more of a dominant mutation with fewer guarantees that it could be passed to a subsequent generation[49]. In contrast, this happened in M2 where the mutation segregation pattern could be seen and the seed was free from epigenetic mutations. Therefore, the selection of mutant genotypes was more favourable to conduct in the M2 generation.

In the M2 generation, the best treatment with a high genetic diversity was found both at 40 and 70 Gy mutants (Fig. 2c). However, the additional qualitative approaches related to the form of seed and panicle and leave colour emphasized that only 40 Gy treatments showed a clear variation. The present study also revealed the variation of nutritional profile in M2 mutant generation. The M2 mutant displayed a dynamic nutrition profile in response to different irradiation levels, where the increase of irradiation levels generally associated with the increase of fat, protein, ash, amylopectin and dietary fibre content; and the decrease of carbohydrate and amylose levels. One of the nutritional variables required to design grain sorghum is protein content[50]. Thus, the increase of protein content in all obtained mutants was a promising result, since the elder; namely, Suri 3 Agritan was classified as grain sorghum rather than sweet sorghum. Amylose and amylopectin were two important variables determining sorghum texture, either fluffy or waxy. The gamma-ray mutations in the present study seemed to produce waxier sorghum rather than the elder, as indicated by the lower ratio of amylose to amylopectin, which implied the dominant amylopectin than amylose. Lower amylose content is desired for designing sorghum as a staple food and industrial need since lower amylose improves carbohydrate digestibility and ethanol fermentation[51]. Amylose content was reported to be higher in grain sorghum rather than sweet varieties[52]. The variation of nutritional content revealed in the present findings was following previous studies in sorghum as the effect of different genotypes[53]. The success story of gamma-ray induced mutations in present findings produced a broader genetic diversity leading to the variance of agronomic character and nutritional content. Genetic variability is an important input for further development of new high yielding sorghum varieties[54,55], especially with an improvement in terms of nutritional content[53].

CONCLUSION

In short, *in vitro*, growing media and gamma-ray irradiation doses significantly affected the plantlet mutant growth performance, where the increase of irradiation dose impeded the mutant growth and the addition of BA was significantly reduced shoot growth but favoured for the root part. Field evaluation revealed the presence of significant variance on agronomic characters as the effect of different mutant generations and irradiation dose. A broad diversity was found in the M2 generation after 40 and 70 Gy irradiation doses. The 40 Gy mutants displayed a qualitative variation in different parts of the plant. Nutritional profiling analysis in M2 concluded that the increase of fat, protein, ash, amylopectin and dietary fibre content associated with the increase of irradiation dose and the decrease of carbohydrate and amylose levels.

SIGNIFICANCE STATEMENT

This study discovered that BA potentially used to decrease the damage of irradiation mutation in sorghum mutant and both 40 and 70 Gy irradiation dose produced broader genetic diversity of mutant lines, revealing the potential to have desired mutant with an increase of fat, protein, ash, amylopectin and dietary fibre content. This effort became an important milestone and be beneficial for the sorghum development program. This study will help the researchers to uncover the critical areas of gamma-ray induced mutation in Indonesian sorghum that many researchers were not able to explore. Thus a new theory on the optimum gamma-ray irradiation dose for obtaining broader genetic diversity in sorghum may be arrived at.

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