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MORINGA OLEIFERA ALUMINUM TOLERANCE PRODUCED BY GAMMA IRRADIATION THROUGH IN VITRO CULTURE

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SUMMARY

Moringa oleifera Linn. is a plant with significant potential as a functional food. For mutation, the gamma rays irradiation has been proven effective in producing prominent characteristics such as tolerance to aluminum (Al) stress. Therefore, the present study aimed to assess the genetic variability of *M. oleifera* plant by exposing to gamma rays irradiation at various doses. Selection of Al tolerant genotypes was carried out in *in vitro* under Al stress conditions by adding $AlCl_3$ to the media at the rate of 0, 50, 100, 250, and 500 mg/L. The selection was made for prominent accessions of *M. oleifera* capable of producing high yields under abiotic stress conditions. In addition to agronomic parameters, the organic acids content were observed to select the tolerant accessions with metabolic profile of Al stress tolerant accessions. Gamma irradiation applied to *M. oleifera* shoot culture produced new traits, as shown by the molecular dendrogram of ISSR markers, where the 10 and 20 Gy treatments had varied genetic diversity compared to the wild type. Additionally, gamma irradiation at 10 and 20 Gy increased the tolerance of *M. oleifera* culture to 100 and 250 mg/L of $AlCl_3$.

Keywords: *Moringa oleifera* L., gamma radiation, *in vitro* culture, organic acid, Al tolerance

Key findings: Plant cultures showed $AlCl_3$ tolerance in the *M. oleifera* clones based on growth traits and the organic acids produced. This advancement could potentially allow the use of suboptimal land with acidic soil for *M. oleifera* propagation in the future.

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INTRODUCTION

Moringa oleifera L. is a plant native to South Asia and belongs to the family Moringaceae (Oyeyinka and Oyeyinka, 2018). It is a small, fast-growing, evergreen tree growing in tropical regions and prevalent in the Himalayan areas of Pakistan, India, Bangladesh, and Afghanistan. This plant has also achieved extensive distribution across several countries, thriving in diverse regions globally, including tropical and subtropical zones. In addition, it has significant potential in various sectors due to its utilization as a functional food, animal feed, and medicinal plant (Toyosi *et al*., 2021). Presently, Moringa plants cultivation thrives in numerous tropical and subtropical areas globally. Indonesia additionally supports this plant through its sustainable production and domestication (Toyosi *et al*., 2021; Rudiyanto *et al*., 2024). *M. oleifera* leaves and seeds contain various chemical components, including fatty acids, tocopherol, carotene, and phenolics (Ziani *et al*., 2019).

M. oleifera propagation has used traditional methods, such as, seedlings and cuttings. However, Debnath and Juran (2020) reported several complexities with traditional propagation, such as, limited explants, lethargic growth, and susceptibility to various pests and diseases. According to Sarkar (2010), in vitro culture has numerous advantages, including the ability to generate uniform, pest-free seeds independent of season, and potential for rapid development.

Arable land reduction due to industrialization and urbanization posed a significant obstacle to propagating *M. oleifera*. The use of suboptimal land becomes an alternative option to address this challenge. According to Fanindi *et al*. (2020), Indonesia has approximately 108.8 million ha of suboptimal land areas as acid dry land, with 62.6 million ha still a potential for agricultural use. However, several abiotic issues such as low soil pH and high aluminum (Al) saturation, inhibit the efficient use of thatland. These characteristics inhibit the photosynthetic process and plant growth and development, resulting in reduced crop yields (Xu *et al*., 2018; Fanindi *et al*., 2020). Plants' absorption of aluminum hinders numerous metabolic and physiological processes, resulting in inhibited plant development and decreased agricultural output (Liu *et al*., 2023).

Suboptimal land can be effectively functional by planting prominent *M. oleifera* clones tolerant to abiotic stress conditions. Moreover, the selection of clones from different genotypes with Al stress tolerance is achievable through manipulating somatic cells via in vitro culture using gamma irradiation. The ISSR markers enable the detection of any genetic alterations induced by radiation in the culture of *M. oleifera*. According to Abdulhafiz *et al*. (2018), molecular markers serve to identify the genetic variants in targeted mutants, enabling accurate and efficient discrimination between irradiated and non-irradiated plants at the DNA level (Due *et al*., 2019).

Gamma irradiation has benefitted to induce mutation in several crop plants, resulting in the development of traits with tolerance to different abiotic stress conditions. According to Moussa (2011), soybean plants subjected to irradiation showed enhanced plant resilience to drought and reduced the crop yield losses caused by abiotic stress factors. Ramabulana *et al*., (2017) reported gamma irradiation of *M. oleifera* increased the concentration of glucomoringin compounds and the derivative metabolites.

Based on previous research studies, the presented work aimed to assess the genetic variability of *M. oleifera* plant through exposure to gamma ray irradiation at various doses. Gamma ray irradiation for mutation has shown effective in producing prominent characteristics, such as, tolerance to Al stress. The confirmation of the mutation was successful through ISSR markers, with the selection of Al-tolerant mutants performed via in vitro culture by adding $AlCl_3$. The selection further transpired to identify the prominent clones with high yields under abiotic stressful environmental circumstances. Plant culture observed with tolerance depended on growth parameters and the levels of organic acids produced. Chang and Liao (2016) and Ramabulana *et al*. (2017) discussed the most prevalent organic acid molecules in numerous

plant species, including citric, oxalic, and acetic acids. The results will provide valuable information in the production of prominent *M. oleifera* clones tolerant to the stress caused by $AlCl_3$. This advancement could potentially allow the use of suboptimal land with acidic soil for propagating *M. oleifera* in the future.

MATERIALS AND METHODS

Materials and experimental design

M. oleifera shoots were sample explants radiated with gamma rays at 0, 5, 10, and 20 Gy. Ensuring stability had the shoots subcultured six times. The basal medium used was Driver and Kuniyuki Walnut (DKW) media supplemented with 0, 50, 100, 250, and 500 mg/L of $AlCl_3$, according to the treatments tested (Driver and Kuniyuki, 1984; Rudiyanto *et al*., 2021). The tools used were the plant tissue culture equipment, with the experiment carried out using a completely randomized factorial design. The number of replications was 12, with a total of 240 experimental units. Data recorded on various Variables at the 0–6 weeks after culture (WAC) included shoot height, number of shoots, petioles, and roots. Meanwhile, raw and dry weights, including citric, oxalic, and acetic acids reached measuring at the 6 WAC.

Study procedure

The surviving *M. oleifera* culture exposure to gamma radiation at 0, 5, 10, and 20 Gy wascollected. The analysis process included DNA extraction, primer identification, DNA amplification, electrophoresis, and molecular data analysis (Kumar *et al*., 2009). Subsequently, DNA isolation used a modified CTAB (cetyltrimethylammonium bromide) technique (Healey *et al*., 2014). A 100-mg leaf sample incurred mixing with 700 µL CTAB extraction buffer solution and 10 mg of PVP (polyvinylpyrrolidone), followed by crushing using a mortar. After leaves disintegrated and thoroughly pulverized, the resulting mixture transfer into a 2 mL tube followed and vigorously mixed until homogenized. The solution underwent a water bath set at 60 °C for 30 min. For DNA purification, adding 700 µL purification buffer/CIA buffer (chloroform: isoamyl alcohol = 24:1 v/v) ensued, followed by vortexing for 15 s. The fraction's separation utilized a centrifuge at 10,000 rpm for five minutes.

Polymerase chain reaction (PCR) contained 2.5 µL sample DNA, 5 µL PCR mix, and 2.5 µL primer. Applying the Esco Thermal Cycler 2012 PCR equipment ran for DNA amplification, with initial denaturation (pre-PCR) performed at 94 °C for 5 min in a single cycle. Subsequently, conducting 40 cycles consisted of denaturation at 94 °C for 1 min, annealing (primer attachment) at 50 °C for 1 min, and extension at 72 °C for 2 min. After 40 cycles, a final extension at 72 °C for 10 min concluded the process, followed by cooling to 4 °C (Kumar *et al*., 2009). The reaction results received electrophoresis on an agarose gel with a 1.5% concentration. The electrophoresis results visualization employed the UV transilluminator to observe the band pattern.

The organic acid content analysis, specifically citric, oxalic, and acetic acids, engaged the HPLC. The sample's mixing with a solution contained 70% alcohol, 30% acetonitrile, and type-I distilled water in a ratio of 50%:30%:20% distilled water. Subsequently, the sample bore shaking for two hours using a shaker and centrifuged for 10 min at 10,000 rpm. The liquid fraction passing through a filter utilized the Whatman filter paper No. 4, with the filtrate separated using a Sep-Pak® C18 type cartridge. The outcomes of the extraction process were the samples used to examine organic acids, with an injection volume of 10 µL. Using the Refractive Index detector continued on the column type, which was Coregel 87H3. The mobile phase consisted of a solution containing 5 mM H_2SO_4. The experimental process progressed at a consistent flow rate of 0.6 mL/min while maintaining a temperature of 80 °C (Das *et al*., 2014).

Data analysis

The quantitative data obtained from various observations underwent the analysis of

variance (ANOVA) F-Test at a 95% significant level. When significant differences exist, further analysis continued using the Duncan's Multiple Range Test (DMRT). ISSR markers' polymorphism analysis scoring consisted of each band represented a character with an assigned value based on its presence or absence. Subsequently, transforming the scores into binary data continued to cluster analysis on matrix data to generate a dendrogram using the UPGMA (unweighted pair group method arithmetic) algorithm (Cruz *et al*., 2014). The hierarchical cluster heatmap analysis employed the Metaboanalyst 5.0 (https://www.metaboanalyst.ca/).

RESULTS

Various irradiation doses applied to *M. Oleifera* cultures assessed the culture responses to gamma irradiation. The DNA band patterns obtained from electrophoresis using ISSR markers on *M. oleifera* samples irradiated with gamma rays at the doses of 0, 5, 10, and 20 Gy appear in Figure 1A. Among 15 primers used, the DNA bands with a sequence length ranging from 500 to 1500 bp succeeded amplification. The gel images also revealed the molecular profiles of irradiated plant cultures, wherein L represents the ladder indicating the molecular size of DNA fragments (Figure 1A). The control group (1) showed the baseline genetic makeup without irradiation. Samples exposed to 5 Gy (2) and 10 Gy (3) irradiation exhibited apparent variations in the band pattern compared with the control, suggesting dose-responsive genetic variability. The 20 Gy treatment (4) further strengthened these variations, highlighting the efficiency of gamma radiation in inducing mutagenesis. The negative control (5), included to authenticate the experiment's accuracy, showed no unintended DNA amplification, confirming the reliability of the results. The distinct banding patterns across different irradiation doses reflected the genetic diversity induced by gamma radiation, with potential implications for selecting *M. oleifera* variants with desirable traits.

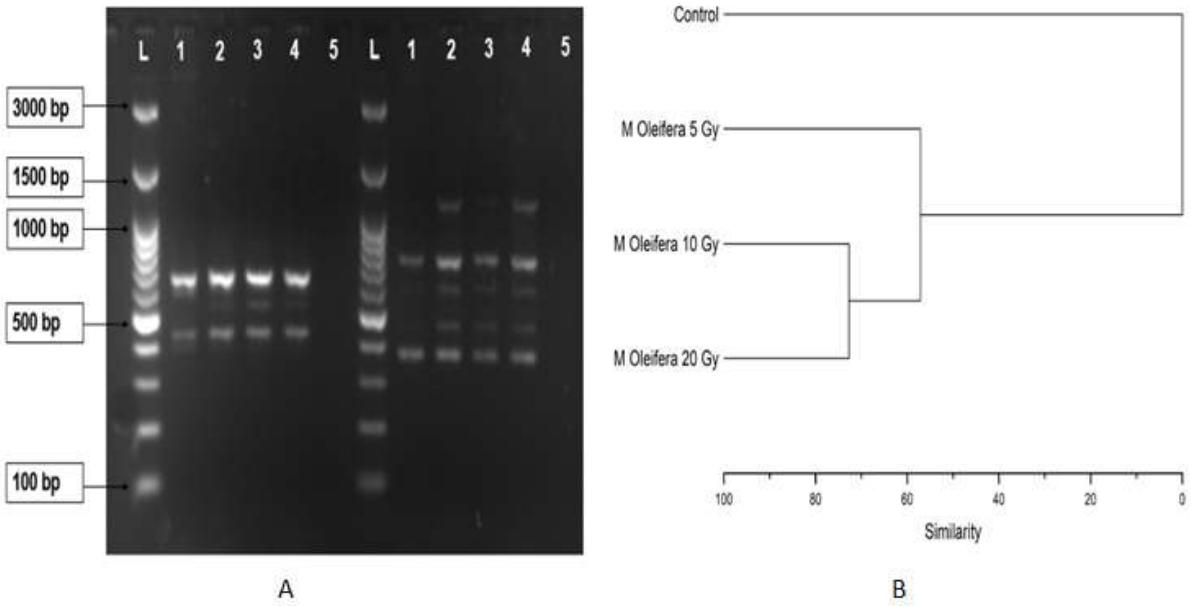

Figure 1. DNA band pattern resulting from electrophoresis with ISSR markers for *M. oleifera* irradiated with gamma ray of 0, 5, 10, and 20 Gy (A). The dendrogram of the similarity coefficient of *M. oleifera* shoots exposed to gamma radiation at 0, 5, 10, and 20 Gy based on ISSR markers and a hierarchical cluster analysis using UPGMA (unweighted pair group method arithmetic) algorithm (B).

Table 1. DNA band amplifications in *M. oleifera* shoot culture exposed to different doses of gamma rays (0, 5, 10, and 20 Gy) using ISSR markers.

No.	Primers	Temp. (°C)	Polymorphic bands	Monomorphic bands	Number of bands
A	5′-CACACACACACAGG-3′	37	1	2	3
B	5′-CACACACACACAAC-3′	35	3	2	5
C	5′-CTCTCTCTCTCTCTCTTG-3′	47.8	1	3	4
D	5′-CTCTCTCTCTCTCTCTGC-3′	50.9	4	1	5
E	5′-GTGGTGGTGGC-3′	31	2	3	5
F	5′-CTCGTCGTCGC-3′	31	1	1	2
G	5′-CTCTCTCTCTCTCTCTAC-3′	43.4	2	1	3
H	5′-CACACACACACAGG-3′	37	2	1	3
I	5′-CTCCTCCTCGC-3′	31	4	1	5
J	5′-CACACACACACAGT-3′	35	1	2	3
K	5′-GAGAGAGAGAGACC-3′	37	0	4	4
L	5′-GTGTGTGTGTGTCC-3′	37	4	0	4
M	5′-GTGTGTGTGTGTGG-3′	37	2	1	3
N	5′-CACACACACACAAG-3′	35	3	0	3
O	5′-GAGGAGGAGGC-3′	31	2	2	4
Number of bands			32 (57.14%)	24 (42.86 %)	56 (100%)

The dendrogram generated from the similarity coefficients of *M. oleifera* shoots subjected to gamma irradiation at the doses of 0, 5, 10, and 20 Gy occurs in Figure 1B. The clustering analysis used ISSR markers, as executed with the UPGMA method (Cruz *et al*., 2014). The dendrogram represents the genetic distances between the treated and control groups, providing visual evidence of the induced genetic variability. Notably, the *M. oleifer*a cultures exposed to 10 and 20 Gy treatments formed a distinct cluster, indicative of a higher degree of genetic variation than the control and the 5 Gy irradiation treatments. The genetic variability reached further substantiation by the polymorphism levels indicated in Table 1. Among the 56 analyzed bands, 32 bands (57.14%) were polymorphic, while 24 bands (42.86%) were monomorphic. The genetic diversity demonstrated 75% similarity between the 10 and 20 Gy irradiation groups, separated them from the control group with no exposure to gamma rays. The cultures treated with 5 Gy exhibited a 55% genetic similarity with the control group, established a baseline for mutagenesis sensitivity, and suggested an optimal dose for enhancing genetic diversity without overly compromising plant vitality.

The mean values for shoot height and the number of shoots, petioles, and roots of *M. oleifera* radiated to gamma rays at 0, 5, 10, and 20 Gy, in combination with 0, 50, 100, 250, and 500 mg/L of $AlCl_3$ are available in Table 2. At 10 Gy, radiation without $AlCl_3$ had the highest number of petioles, with an average value of 12.75 ± 0.66. This value was also significantly different from other treatments, except at 10 Gy radiation and 100 mg/L of $AlCl_3$. The 5 and 10 Gy treatments, including 10 Gy with 10 mg/L $AlCl_3$, produced the highest number of roots, while the treatment with 500 mg/L $AlCl_3$ generated the least number of roots in *M. oleifera* (Table 2).

On examining the effects of gamma irradiation combined with varying concentrations of $AlCl_3$ on the organic acid production in *M. oleifera* (Table 3), results reveal a dose and concentration-dependent response at 6 WAC. Notably, at lower irradiation doses (0 and 5 Gy), a slight increase in citric, oxalic, and acetic acid contents occurred, suggesting an initial rise in metabolic activity in response to mild stress conditions. However, this response escalated at 10 Gy with 100 mg/L $AlCl_3$, where the citric acid content peaked significantly, indicating a possible role in conferring aluminum stress

Table 2. Average shoot height, number of shoots, number of petioles, and number of roots in *M. oleifera* irradiated with gamma ray of 0, 5, 10, and 20 Gy in combination with 0, 50, 100, 250, and 500 mg/L of $AlCl_3$ at 6 WAC.

Radiation (Gy)	$AlCl_3$ (mg/L)	Shoot height	Number of shoots	Number of petioles	Number of roots
0	0	7.91±0.45a-d	2.50±0.23b	8.83±0.73cd	2.08±0.34bcd
	50	6.33±0.42de	1.92±0.26bcd	10.58±1.25bc	1.67±0.26c-f
	100	7.46±0.43bcd	2.33±0.26bc	8.67±0.75cde	1.00±0.25e-i
	250	4.63±0.56fg	1.17±0.17e	6.67±0.38e-i	0.67±0.22f-i
	500	3.24±0.49g	1.08±0.08e	5.58±0.53g-j	0.08±0.08i
5	0	8.23±0.67abc	2.00±0.25bcd	9.08±0.53cd	2.83±0.30ab
	50	6.30±0.60de	1.33±0.19de	7.00±0.67d-h	1.67±0.40c-g
	100	6.74±0.35cde	1.75±0.35cde	7.50±0.61d-g	1.33±0.33de-h
	250	3.42±0.44g	1.17±0.11e	5.33±0.31hij	0.67±0.22fghi
	500	2.84±0.35g	1.08±0.08e	4.83±0.49ij	0.67±0.22fghi
10	0	8.75±0.33ab	3.50±0.19a	12.75±0.66a	3.58±0.36a
	50	8.17±0.51ab	2.50±0.31b	9.00±0.94cd	3.08±0.34a
	100	8.65±0.65de	3.25±0.22a	11.25±0.79ab	2.67±0.31abc
	250	5.51±0.70fg	1.17±0.11e	5.83±0.61g-j	2.67±0.31abc
	500	3.16±0.22g	1.08±0.08e	4.50±0.40j	1.17±0.24d-h
20	0	8.43±0.47abc	1.67±0.22cde	8.83±0.47cd	2.08±0.34bcd
	50	9.28±1.01a	1.50±0.23de	8.67±0.51c-f	1.67±0.47c-f
	100	9.18±0.70ab	1.67±0.28cde	8.67±0.51c-f	1.67±0.47c-f
	250	6.35±0.49de	1.17±0.17e	5.83±0.61ghij	1.75±0.25cde
	500	4.40±0.48fg	1.08±0.08e	4.25±0.33j	0.58±0.23hi

Note: Numbers followed by the same letter in the same column are not significantly different based on Duncan's multiple range test at α = 5%. Presented values are means ± standard error (SE) of 12 replicates.

Table 3. The average content of citric acid, oxalic acid, and acetic acid in *M. oleifera* irradiated with gamma ray of 0, 5, 10, and 20 Gy in combination with 0, 50, 100, 250, and 500 mg/L of $AlCl_3$ at 6 WAC.

Radiation (Gy)	$AlCl_3$ (mg/L)	Citric acid (mg/mL)	Oxalic acid (mg/mL)	Acetic acid (mg/mL)
0	0	0.0040±0.0017e	0.0000±0.0000c	0.8150±0.7249d
	50	0.0183±0.0159cde	0.0343±0.0343b	1.1773±1.1098bcd
	100	0.0282±0.0081cde	0.0213±0.0064b	0.8110±0.7965d
	250	0.0220±0.0029cde	0.0750±0.0293b	1.2050±1.0706bcd
	500	0.0113±0.0012e	0.0503±0.0270b	1.1253±1.0691bcd
5	0	0.0243±0.0070cde	0.0007±0.0007c	0.9653±0.8979cd
	50	0.0288±0.0046cde	0.0007±0.0007c	1.0257±0.8672bcd
	100	0.0543±0.0058bcd	0.2233±0.1502a	1.1813±1.0229bcd
	250	0.0553±0.0109bcd	0.0428±0.0110b	2.1880±0.1507abc
	500	0.0590±0.0168bc	0.0717±0.0130b	1.1217±0.7838bcd
10	0	0.0163±0.0015de	0.0137±0.0137b	1.1337±0.8213bcd
	50	0.0470±0.0066b-e	0.0313±0.0313b	1.2453±1.1699bcd
	100	0.1213±0.0227a	0.0727±0.0260b	2.6660±0.2114a
	250	0.1577±0.0097a	0.1267±0.0636ab	2.2503±0.1767ab
	500	0.0727±0.0063b	0.0457±0.0203b	1.4430±0.6456bcd
20	0	0.0187±0.0090cde	0.0400±0.0400b	1.0773±0.9497bcd
	50	0.0840±0.0318b	0.0800±0.0800b	0.6830±0.6076d
	100	0.1483±0.0143a	0.0197±0.0099b	2.6033±0.1522a
	250	0.1220±0.0051a	0.0570±0.0154b	1.0467±0.8882bcd
	500	0.0570±0.0154bcd	0.0428±0.0110b	0.9977±0.8392bcd

Note: Numbers followed by the same letter in the same column are not significantly different based on Duncan's multiple range test at α = 5%. Presented values are means ± standard error (SE) of 12 replicates.

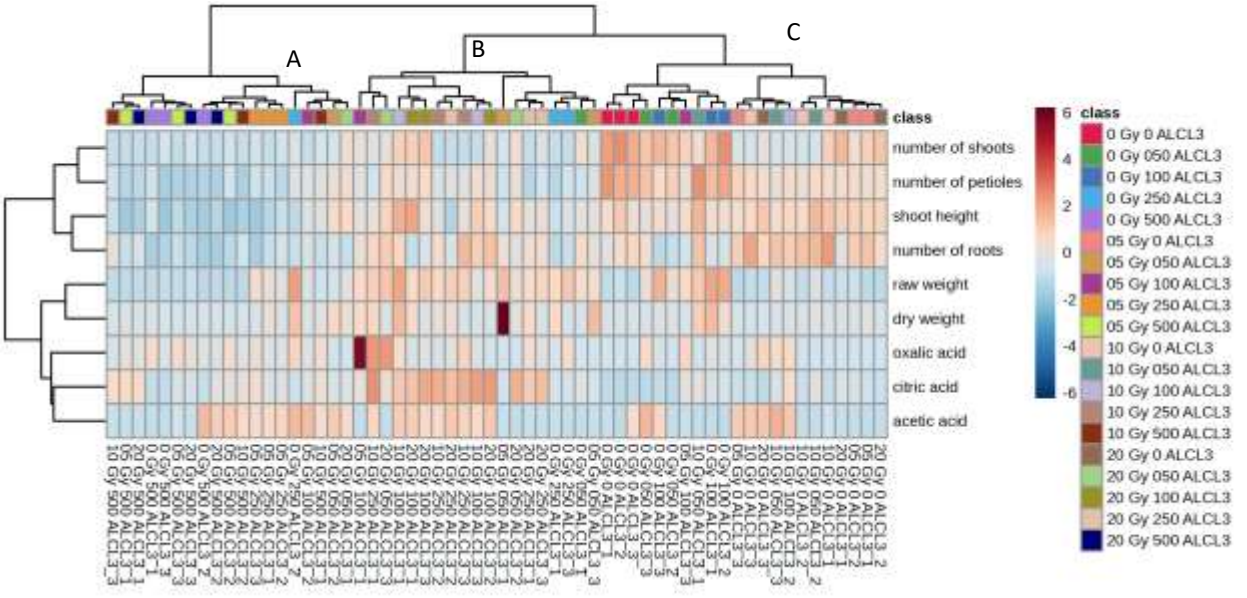

Figure 2. Clusters between observation parameters: shoot height, number of shoots, number of petioles, number of roots, raw weight, dry weight, citric acid, oxalic acid, and acetic acid in *M. oleifera* irradiated with gamma ray of 0, 5, 10, and 20 Gy in combination with 0, 50, 100, 250, and 500 mg/L of $AlCl_3$ at 6 WAC.

tolerance. At a higher irradiation dose of 20 Gy, despite the presence of 250 mg/L $AlCl_3$, a less-pronounced increase in citric acid content existed, implying a threshold beyond which additional stress may not lead to further upregulation of metabolic defense mechanisms. The acetic acid content followed a similar trend, while the oxalic acid concentration remained relatively unaffected by higher doses of $AlCl_3$. These results denote a critical balance between irradiation dose and $AlCl_3$ concentration in modulating the organic acids mediated response to abiotic stress conditions in *M. oleifera*.

A comprehensive cluster analysis also proceeded to elucidate the impact of gamma irradiation combined with aluminum stress on *M. oleifera* (Figure 2). This analysis revealed the clustering of *M. oleifera*'s phenotypic and biochemical parameters following exposure to gamma rays at dosages of 0, 5, 10, and 20 Gy with $AlCl_3$ concentrations. The hierarchical clustering illustrated three distinct groupings — A, B, and C — signifying intrinsic similarities among the various treatments. These were also in relation to critical growth determinants, such as, shoot height, the number of shoots, petioles, and roots, raw weight, dry weight, and ratios of pivotal organic acids. Notably, within Group B, a convergence of shoot responses manifested to mid-level irradiation doses and intermediate $AlCl_3$ concentration, indicating a potential spot for inducing stress tolerance. Moreover, the emergence of Group C, marked by lower doses of both irradiation and $AlCl_3$, highlighted conditions that could foster the optimal growth and stress resilience, which was crucial for advancing the cultivation of *M. oleifera* in challenging environmental settings.

DISCUSSION

Gamma irradiation is an effective inducer of genetic variation, as demonstrated in the presented study on *M. oleifera*, and ISSR markers unveiled the distinct polymorphisms post irradiation. The ISSR ability to detect DNA polymorphisms, as highlighted by Reddy *et al*. (2012), provided a robust tool to evaluate the extent of genetic diversity induced by gamma

rays. Consistent with the observations by Wang *et al*. (2017), the latest results corroborate the efficacy of ISSR markers in detecting DNA polymorphism among the mutants induced by gamma irradiation. Gamma irradiation has proven effective in developing polymorphism, with ISSR analysis revealing significant genetic variation in the presented study. The degree of polymorphism detected aligns with previous reports by Taheri *et al*. (2014), further supporting the notion of ISSR markers as instrumental in discerning the genetic diversity arising through mutagenesis.

As evidenced by the study results and substantiated by Wang *et al*. (2017), varied doses of gamma irradiation generated a wide range of genetic diversity in *M. oleifera*. The dendrogram presented in Figure 1B, also replicate these diversities, illustrating distinct genetic distances between irradiated and control plants of *M. oleifera*. The said induced diversity, highlighted by the separation of irradiated plants into discrete clusters, revealed the gamma irradiation potential in enhancing genetic variation, crucial in breeding programs.

The presented results further indicate *M. oleifera*'s response to gamma radiation and $AlCl_3$ treatments manifests a complex interplay of the growth metrics (Table 2). The exposure to increasing doses of gamma radiation, particularly at 10 and 20 Gy, combined with varying concentrations of $AlCl_3$, has shown a remarkable impact on shoot length and the number of shoots, petioles, and roots. Notably, the treatment with 10 Gy of radiation and 100 mg/L of $AlCl_3$ resulted in a promising increase in shoot length and number of roots, suggesting a dose-specific resilience to aluminum stress.

The induced tolerance was evident in the number of shoots and roots, which gain support from past findings. The photosynthetic apparatus of two sorghum cultivars demonstrated adaptability to aluminum stress, exhibiting considerable variations in physiological responses between Al-tolerant and Al-sensitive genotypes (Peixoto *et al*., 2002). In the latest study, the plant's capability to sustain shoot production and maintain root development under combined abiotic stress conditions signal at inherent mechanisms that confer tolerance, similar to the progressive and sustainable adjustment in the photosynthetic apparatus in sorghum (Peixoto *et al*., 2002).

Furthermore, the apparent response at a 20 Gy dose with 250 mg/L $AlCl_3$ indicated a threshold where radiation may no longer strengthen the resilience, as the shoot height does not show significant variation. Olivares *et al*. (2009) reported Al poisoning causes an imbalance in nutrients absorption, such as, Ca, Mg, P, and K. This phenomenon also inhibited cell division (Muhammad *et al*., 2018), photosynthesis, respiration, and metabolism in plant (Peixoto *et al*., 2002), leading to reduced plant growth and development (Lu *et al*., 2020; Fanindi *et al*., 2020). Comparatively, Olivares *et al*. (2009) also identified *Pterolepis glomerat*a as an aluminum accumulator, and their findings on Al-tolerance in the plant may also support the presented observations on the *M. oleifera*, with varied tolerance at different radiation doses and $AlCl_3$ concentrations.

In *M. oleifera,* the investigation of organic acid profile provides insight into the mechanism underlying plant responses to abiotic stress conditions (Table 3). The results indicated gamma irradiation, combined with $AlCl_3$ treatment, transforms the concentration of key organic acids, such as, citric, oxalic, and acetic acids. A well-documentation of the presence of these organic acids, particularly citric acid, disclosed an association with aluminum tolerance due to their ability to chelate aluminum ions, thus, reducing toxicity. The latest results revealed a dose-dependent increase in organic acid concentration, notably citric acid, with a peak at the irradiation dose of 10 Gy with 100 mg/L $AlCl_3$, aligning with trends observed in other stress-resilient plants (Riaz *et al*., 2018). These results also substantiate the findings of Vega *et al*. (2022), who outlined the chelating efficacy of carboxylic acids in mitigating heavy metal stress. These chelators, particularly citric acid, play a crucial role by forming stable complexes with Al^{3+} ions, a physiological response noted for mitigating the detrimental effects of Al in crop plants.

Comparing the organic acid profile with growth parameters, the positive correlation existed between the increased organic acid levels and improved growth parameters under certain stress conditions. This indicated an enhanced tolerance capability, as organic acid production is a known defense response to aluminum stress (Riaz *et al*., 2018). The recent results were also parallel to those reported by Igamberdiev and Eprintsev (2016), where increased organic acid production, specifically citric acid, linked to the amelioration of aluminum toxicity through internal detoxification mechanism.

The cluster analysis further revealed the association between the organic acid profile and the observed clusters of tolerance (Figure 2). The grouping within the clusters suggested a specific adaptation to Al stress, which supports the defense strategy and the plants, i.e., *Lotus corniculatus,* extrude more oxalic acid to counter Al toxicity (Santos *et al*., 2022). This root exudation mechanism proved positively correlated with reduced Al accumulation in root tissues, signifying an apparent relationship between the organic acid response and tolerance mechanism.

Overall, the present results signify gamma irradiation, apart from inducing genetic variation, may also organize the plants for Al stress resilience, enhancing their capacity to produce and secrete organic acids necessary for Al chelation and tolerance. These observations offer a promising outlook for the implementation of irradiation in breeding programs to develop Al-tolerant genotypes, potentially echoing the positive correlation between organic acid exudation rates and reduced Al accumulation in other plant species (Rahman *et al*., 2024). Therefore, the presented results proposed gamma irradiation, combined with specific $AlCl_3$ treatments, induce genetic variability in *M. oleifera* and tend to prepare the plants for improved tolerance to aluminum toxicity through alterations in organic acid metabolism (Al-Karboli and Al-Janabi, 2024; Moneim and Al-Anbari, 2024).

CONCLUSIONS

The promising study elucidated the versatile responses of *M. oleifera* to gamma irradiation and aluminum chloride stress through a comprehensive analysis using ISSR markers, cluster analysis, and metabolic profiling. The results provided more understanding of how the *M. oleifera* manages abiotic stress conditions. Enhanced production of specific organic acids, particularly citric acid at optimal gamma irradiation doses, has indicated a potential mechanism of tolerance against aluminum stress. This aligns with the current scientific narratives that highlight the importance of organic compounds in mediating plant stress responses.

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