AL-KAUNIYAH: Jurnal Biologi, 19(2), 2026, 446-459
Website: http://journal.uinjkt.ac.id/index.php/kauniyah
P-ISSN: 1978-3736, E-ISSN: 2502-6720

CROSS-TOLERANCE RESPONSES OF GAMMA Co^{60} IRRADIATION-DERIVED SUGARCANE MUTANTS TO SALINITY AND OSMOTIC STRESS UNDER *IN VITRO* CONDITIONS

RESPONS TOLERANSI SILANG MUTAN TEBU HASIL IRADIASI GAMMA Co^{60} TERHADAP CEKAMAN SALINITAS DAN OSMOTIK PADA KONDISI *IN VITRO*

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Submitted: 14 May 2026; Revised: 26 May 2026; Accepted: 3 June 2026

Abstract

Sugarcane (*Saccharum officinarum* L.) cultivation is increasingly expanding into marginal lands affected by salinity and drought stress, requiring the development of stress-tolerant cultivars. This study aimed to evaluate the cross-tolerance responses of gamma irradiation-derived sugarcane mutants to salinity and osmotic stress under *in vitro* conditions. The experiment was arranged in a split-plot design with three replications. The main plot consisted of two putative mutant clones (11.201 and 11.401) and one wild-type clone (11.000) of sugarcane cultivar NXI 1–3. Salinity stress was induced using NaCl concentrations of 0, 50, 100, 150, 200, and 250 mM, while osmotic stress was induced using polyethylene glycol (PEG) concentrations of 0, 5, 10, 15, 20, and 25%. Observed parameters included explant survival percentage and the numbers of shoots, leaves, and roots. Both NaCl and PEG treatments significantly reduced microshoot growth. However, clone 11.201 consistently showed better survival and growth performance under both stress conditions compared with the wild type and clone 11.401. Clone 11.201 maintained its growth under 200 mM NaCl, yielding an average of 33.67 ± 13.58 shoots/explant, 143.00 ± 61.51 leaves/explant, and 2.33 ± 2.08 roots/explant. It also exhibited limited leaf production at 15% PEG (3.33 ± 5.77 leaves/explant. The results indicate the potential occurrence of cross-tolerance associated with gamma irradiation-induced variation.

Keywords: Abiotic stress; Cross-tolerance; Gamma irradiation; PEG, Salinity; Sugarcane

Abstrak

Budi daya tebu (Saccharum officinarum L.) saat ini semakin diarahkan ke lahan marginal yang mengalami cekaman salinitas dan kekeringan, sehingga diperlukan kultivar yang toleran terhadap cekaman abiotik. Penelitian ini bertujuan untuk mengevaluasi respons toleransi silang mutan tebu hasil iradiasi gamma terhadap cekaman salinitas dan osmotik pada kondisi in vitro. Penelitian ini dilaksanakan dengan rancangan petak terpisah dengan tiga ulangan. Petak utama terdiri atas dua klon mutan putatif (11.201 dan 11.401) serta satu klon nonmutan (wild type/klon 11.000) dari tebu kultivar NXI 1–3. Cekaman salinitas diinduksi menggunakan NaCl dengan konsentrasi 0, 50, 100, 150, 200, dan 250 mM, sedangkan cekaman osmotik diinduksi menggunakan polyethylene glycol (PEG) dengan konsentrasi 0, 5, 10, 15, 20, dan 25%. Parameter yang diamati meliputi persentase eksplan hidup, jumlah tunas, daun, dan akar. Perlakuan NaCl dan PEG secara nyata menurunkan pertumbuhan tunas mikro tebu. Namun, klon 11.201 secara konsisten menunjukkan persentase hidup dan pertumbuhan yang lebih baik dibandingkan dengan tipe nonmutan dan putatif mutan 11.401 pada kedua kondisi cekaman. Klon 11.201 mampu tumbuh pada cekaman salinitas 200 mM NaCl, menghasilkan rata-rata 33,67 ± 13,58 tunas/eksplan, 143,00 ± 61,51 daun/eksplan, dan 2,33 ± 2,08 akar/eksplan. Klon ini juga masih mampu membentuk daun secara terbatas pada cekaman 15% PEG (3,33 ± 5,77 daun/eksplan). Hasil penelitian menunjukkan adanya potensi toleransi silang yang berkaitan dengan variasi hasil iradiasi gamma.

***Kata Kunci**: Cekaman abiotik; Iradiasi gamma; PEG; Salinitas; Tebu; Toleransi silang*

Permalink/DOI: http://dx.doi.org/10.15408/kauniyah.v19i2.51294

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is one of the most important plantation crops in tropical countries, serving as a primary source of sugar and bioenergy (Roslinda et al., 2022). However, national sugar production in Indonesia remains insufficient to meet increasing domestic demand. In 2023, sugar production declined to 2.27 million tonnes, representing a 5.60% decrease compared with 2022 (Badan Pusat Statistik, 2024). Meanwhile, national sugar consumption has reached approximately 7.6 million tonnes annually, resulting in continued dependence on sugar imports (Respati & Darmawan, 2023). Increasing sugar production, therefore, becomes essential to support national food and energy security.

One strategic approach to increasing sugar production is the utilisation of marginal lands, including drought-prone and saline areas. Sugarcane cultivation in Indonesia is increasingly shifting toward rain-fed drylands that frequently experience water deficit stress (Hartati & Yuniyati, 2022). In addition, coastal saline lands also possess considerable agricultural potential despite their high salt content (Bariyyah, 2015; Ermiana et al., 2021; Parnidi et al., 2016). However, the successful utilisation of these sub-optimal lands largely depends on the availability of sugarcane cultivars tolerant to multiple abiotic stresses, particularly salinity and drought (Lisdyayanti et al., 2019).

The development of stress-tolerant cultivars can be accelerated through mutation breeding combined with *in vitro* selection techniques. Somaclonal variation induced through tissue culture and mutagenesis has been widely recognised as an effective approach for generating novel genetic variability associated with environmental stress tolerance (Duta-Cornescu et al., 2023; Lailani & Kuswandi, 2023). Among various mutagenic agents, Co^{60} gamma irradiation is widely used because it induces random genetic changes that may produce superior adaptive traits (Riviello-Flores et al., 2022). Previous studies have shown that gamma irradiation can generate plant mutants with enhanced tolerance to abiotic stresses, including salinity stress (Lisdyayanti et al., 2019). Nevertheless, effective screening methods are required to identify promising mutants from irradiated populations.

In vitro selection offers a rapid and controlled approach for evaluating plant responses to abiotic stress. Salinity stress can be simulated through the addition of NaCl to the culture medium, while drought stress can be mimicked using polyethylene glycol (PEG), a stable and non-toxic osmotic agent that reduces water potential and limits water availability to plant tissues (Gul et al., 2022; Riviello-Flores et al., 2022; Samanhudi et al., 2021; Suhesti et al., 2022). The integration of gamma irradiation with *in vitro* selection, therefore, provides a promising strategy for the early identification of sugarcane genotypes adapted to sub-optimal environments.

The sugarcane cultivar NXI 1–3 is known for its high productivity, adaptability, and tolerance to several environmental constraints, including dryland conditions with limited water availability (Alfian et al., 2015). This cultivar originated from the improvement of the Victoria Milling cultivar VMC 86-550 through polycross breeding (Hartatie & Safira, 2022). In the present study, putative mutant clones of NXI 1–3 derived from Co^{60} gamma irradiation at doses of 20 Gy (11.201) and 40 Gy (11.401) were evaluated under salinity and osmotic stress conditions.

Although several studies have reported the use of mutagenesis or *in vitro* selection for abiotic stress tolerance in sugarcane, information regarding the cross-tolerance responses of gamma irradiation-derived mutants to both salinity and osmotic stress remains limited, particularly in cultivar NXI 1–3. Therefore, this study aimed to evaluate the responses of putative mutant clones and their wild-type counterparts of sugarcane NXI 1–3 to NaCl- and PEG-induced stresses under *in vitro* conditions, and to identify potential clones with superior tolerance to multiple abiotic stresses. The findings of this study are expected to contribute to the development of sugarcane cultivars suitable for cultivation on marginal lands, thereby supporting sustainable sugar production in Indonesia.

MATERIALS AND METHODS

Experimental Design

This study aimed to identify sugarcane clones tolerant to *in vitro* salinity and drought stresses. Two experiments were conducted using a completely randomised design (CRD) arranged in a split-plot design with three replications. The main plots consisted of two putative mutant clones (11.201

and 11.401) and a wild-type clone (11.000) of sugarcane cultivar NXI 1–3. For the salinity stress experiment, the subplots consisted of NaCl concentrations of 0, 50, 100, 150, 200, and 250 mM. Meanwhile, for the osmotic stress experiment, the subplots consisted of polyethylene glycol (PEG) concentrations of 0, 5, 10, 15, 20, and 25%.

Explant Preparation

The plant material consisted of sugarcane microshoots obtained from the culture collection of the Plant *In Vitro* Culture Laboratory, Faculty of Biology, Universitas Jenderal Soedirman. The microshoots were regenerated from calli irradiated with gamma rays from Cobalt-60 at doses of 20 and 40 Gy. Microshoots were maintained on MS medium (Phytotech M519) supplemented with 20 g L^{-1} sucrose and 15 µM BAP (Sigma-Aldrich B3408) and solidified with 0.25% Phytagel (Sigma-Aldrich P8169). Cultures were incubated at 24 °C under continuous light. Explants used in this study were 2-cm-long microshoots bearing five leaves and no roots.

Microshoot explants were aseptically cultured on hormone-free MS medium (MS0) supplemented with 2% sucrose and solidified with 0.8% agar. Cultures were incubated at 24 °C under continuous light for 12 days to obtain a similar growth phase.

Plant *In Vitro* Culture Procedures

The basal medium consisted of MS medium supplemented with 2% sucrose. For salinity stress induction, NaCl was added to the medium at concentrations of 0, 50, 100, 150, 200, and 250 mM. The media were solidified with 0.25% Phytagel (Sigma-Aldrich P8169). For osmotic stress induction, PEG was added to the basal medium at concentrations of 0, 5, 10, 15, 20, and 25%. Liquid media were used, and explants were supported with viscose sponges. Each treatment consisted of 50 mL of medium per bottle with three replicates. The pH of all media was adjusted to 5.8 before sterilisation in an autoclave at 121 °C and 0.15 MPa for 20 min. Explants of two putative mutant clones (11.201 and 11.401) and one wild-type clone (11.000) were transferred aseptically into the treatment media, one microshoot per bottle, and sealed tightly. Cultures were maintained at 24 °C under continuous fluorescent light for 16 weeks and monitored throughout the experiment.

Data Collection and Analysis

In both experiments, the number of shoots, leaves, and roots was recorded after 16 weeks of culture. Morphological responses were documented photographically using a Samsung S20 FE camera. Data were analysed using analysis of variance (ANOVA) with DSAASTAT version 1.514 at 95% and 99% confidence levels. Mean comparisons were performed using Duncan's multiple range test (DMRT) at the 95% confidence level.

RESULTS

Sugarcane Clone Responses to Salinity Stress

The analysis of variance showed that variation between individual sugarcane clones, the NaCl concentration, and the interaction between these two had significant effects on the shoot, leaf, and root numbers of sugarcane NXI 1–3 at 16 weeks after planting (Table 1). These findings show the value of *in vitro* selection in assessing putative mutant lines of sugarcane for salt tolerance.

Table 1. The ANOVA results on the effects of sugarcane clones, NaCl concentration, and their interaction on the number of shoots, leaves, and roots of NXI 1–3 sugarcane clones at16 weeks after planting

Source of variants	Number of shoots	Number of leaves	Number of roots
Sugarcane clones	0.000**	0.000**	0.003**
NaCl concentrations	0.000**	0.000**	0.000**
Interaction between sugarcane Clones and NaCl concentration	0.000**	0.004**	0.000**

Note: Numbers followed by: ** shows highly significance difference (p-value <0.01); * shows significance difference (p-value <0.05)

The DMRT results (Table 2) showed that clone 11.201 exhibited the highest salinity tolerance, indicated by superior shoot, leaf, and root production, which were significantly different from those of other clones. Figure 1 depicts the morphological attributes of the three sugarcane clones cultivated under 50 mM NaCl at 16 weeks after planting, in which clone 11.201 demonstrated superior growth relative to the wild type and clone 11.401.

Table 2. The DMRT results on the average number of shoots, leaves, and roots of NXI 1–3 sugarcane clones showed by different clones at 16 weeks after planting (n= 18)

Sugarcane clones	Number of shoots	Number of leaves	Number of roots
11.000	15.94 ± 19.73^{b}	66.28 ± 81.93^{b}	2.06 ± 2.95^{a}
11.201	64.89 ± 42.28^{a}	275.00 ± 173.76^{a}	4.22 ± 7.31^{a}
11.401	26.28 ± 41.01^{b}	90.67 ± 140.67^{b}	0.00 ± 0.00^{b}

Note: The average numbers in the same column followed by different letters indicate significant differences at DMRT 5%

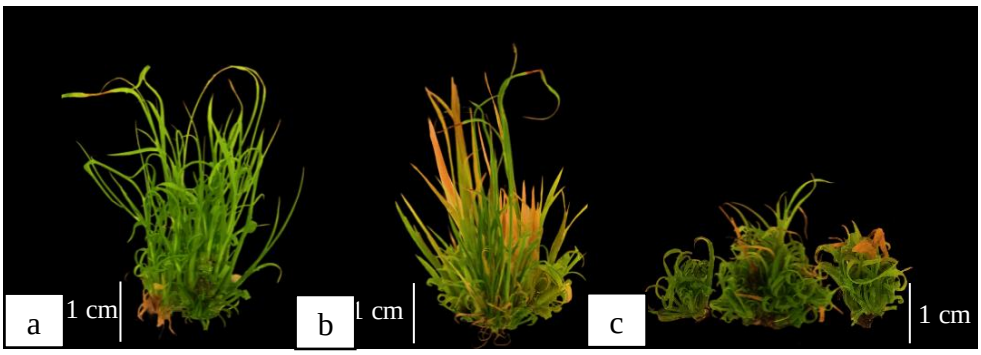

Figure 1. The appearances of three sugarcane clones on a NaCl concentration of 50 mM, at 16 weeks after planting: wild type 11.000 (a); putative mutant 11.201 (b); and putative mutant 11.401 (c)

The DMRT results in Table 3 showed that the highest salt concentration able to be tolerated was 200 mM. Elevated NaCl concentration significantly reduced shoot, leaf, and root numbers across clones. At 250 mM NaCl, no explants survived, indicating this concentration exceeded the tested clones' tolerance threshold.

Table 3. The DMRT results on the average number of shoots, leaves, and roots of NXI 1–3 sugarcane clones as a response to salt concentration at 16 weeks after planting (n= 9)

NaCl concentrations (mM)	Number of shoots	Number of leaves	Number of roots
0	84.78 ± 37.87^{a}	306.56 ± 148.74^{a}	7.56 ± 10.08^{a}
50	60.56 ± 24.40^{a}	246.33 ± 90.58^{a}	3.11 ± 3.79^{b}
100	32.56 ± 42.52^{b}	154.89 ± 212.13^{b}	0.44 ± 0.77^{c}
150	25.11 ± 43.49^{bc}	108.44 ± 187.83^{b}	0.67 ± 1.15^{c}
200	11.22 ± 19.44^{cd}	47.67 ± 82.56^{bc}	0.78 ± 1.35^{c}
250	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}

Note: The average numbers in the same column followed by different letters indicate significant differences at DMRT 5%

The DMRT results (Table 4) demonstrated that clone 11.201 maintained its growth at 200 mM NaCl, yielding an average of 33.67 ± 13.58 shoots explant^{-1}, 143.00 ± 61.51 leaves explant^{-1}, and 2.33 ± 2.08 roots explant^{-1}. In contrast, clones 11.000 and 11.401 did not survive at this concentration. The appearances of sugarcane clones under different NaCl concentrations are shown in Figure 2. These observations highlight that the enhanced salt tolerance of clone 11.201 is distinctly evident at elevated NaCl levels. These findings collectively highlight the pronounced effect of salinity on sugarcane growth.

Table 1. The DMRT results on the average number of shoots, leaves, and roots of NXI 1–3 sugarcane clones as a response to the interaction between clones and NaCl concentration at 16 weeks after planting (n= 3)

Sugarcane clones × NaCl concentrations	Number of shoots	Number of leaves	Number of roots
11.000 × 0 mM	46.00 ± 38.51^{bc}	186.67 ± 150.11^{b}	3.67 ± 1.53^{bc}
11.000 × 50 mM	32.67 ± 15.01^{cd}	143.00 ± 59.35^{bc}	7.33 ± 5.03^{b}
11.000 × 100 mM	17.00 ± 29.44^{de}	68.00 ± 117.78^{cd}	1.33 ± 2.31^{cd}
11.000 × 150 mM	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
11.000 × 200 mM	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
11.000 × 250 mM	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
11.201 × 0 mM	121.67 ± 44.30^{a}	473.00 ± 195.70^{a}	19.00 ± 7.21^{a}
11.201 × 50 mM	78.00 ± 32.08^{abc}	312.00 ± 128.31^{ab}	2.00 ± 3.46^{cd}
11.201 × 100 mM	80.67 ± 48.27^{abc}	396.67 ± 252.90^{ab}	0.00 ± 0.00^{d}
11.201 × 150 mM	75.33 ± 39.55^{abc}	325.33 ± 242.29^{ab}	2.00 ± 3.46^{cd}
11.201 × 200 mM	33.67 ± 13.58^{cd}	143.00 ± 61.51^{bc}	2.33 ± 2.08^{cd}
11.201 × 250 mM	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
11.401 × 0 mM	86.67 ± 6.11^{ab}	260.00 ± 18.33^{ab}	0.00 ± 0.00^{d}
11.401 × 50 mM	71.00 ± 8.54^{abc}	284.00 ± 34.18^{ab}	0.00 ± 0.00^{d}
11.401 × 100 mM	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
11.401 × 150 mM	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
11.401 × 200 mM	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
11.401 × 250 mM	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}

Note: The average numbers in the same column followed by different letters indicate significant differences at DMRT 5%

Sugarcane Clone Responses to PEG-Induced Osmotic Stress

In this experiment, shoots and leaves are formed, but no roots were observed. The ANOVA results on the effects of variation between sugarcane clones, PEG concentration, and their interaction on the number of shoots, leaves, and roots of NXI 1–3 sugarcane clones at 16 weeks after planting (Table 5) showed that PEG concentration significantly affected shoot and leaf numbers, whereas clone identity did not. The interaction between clones and PEG concentrations significantly controlled the number of leaves formed.

Table 5. The ANOVA results on the effects of sugarcane clones, PEG concentration, and their interaction on the number of shoots, leaves, and roots of NXI 1–3 sugarcane clones at 16 weeks after planting

Source of variants	Number of shoots	Number of leaves
Sugarcane clones	0.581	0.502
PEG concentrations	0.000**	0.000**
Interaction between sugarcane clones and PEG concentration	0.335	0.045*

Note: Numbers followed by: ** shows highly significance difference (p-value <0.01); * shows significance difference (p-value <0.05)

Table 6. The DMRT results on the average number of shoots and leaves of NXI 1–3 sugarcane clones as a response to PEG concentration at 16 weeks after planting (n= 9)

PEG concentrations (%)	Number of shoots	Number of leaves
0	20.89 ± 2.67^{a}	65.11 ± 24.13^{a}
5	8.44 ± 5.23^{b}	51.56 ± 47.93^{a}
10	3.78 ± 2.99^{c}	16.33 ± 11.85^{b}
15	0.33 ± 0.58^{d}	1.11 ± 1.92^{c}
20	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
25	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}

Note: The average numbers in the same column followed by different letters indicate significant differences at DMRT 5%

The DMRT results in Table 6 showed that increasing PEG concentration was associated with significant reductions in shoot and leaf formation. At 15% PEG, a minority of explants generated

shoots (0.33 ± 0.58 shoots explant^{-1}) and leaves (1.11 ± 1.92 leaves explant^{-1}), indicating partial osmotic stress tolerance. No growth occurred at PEG concentrations of 20% and 25%.

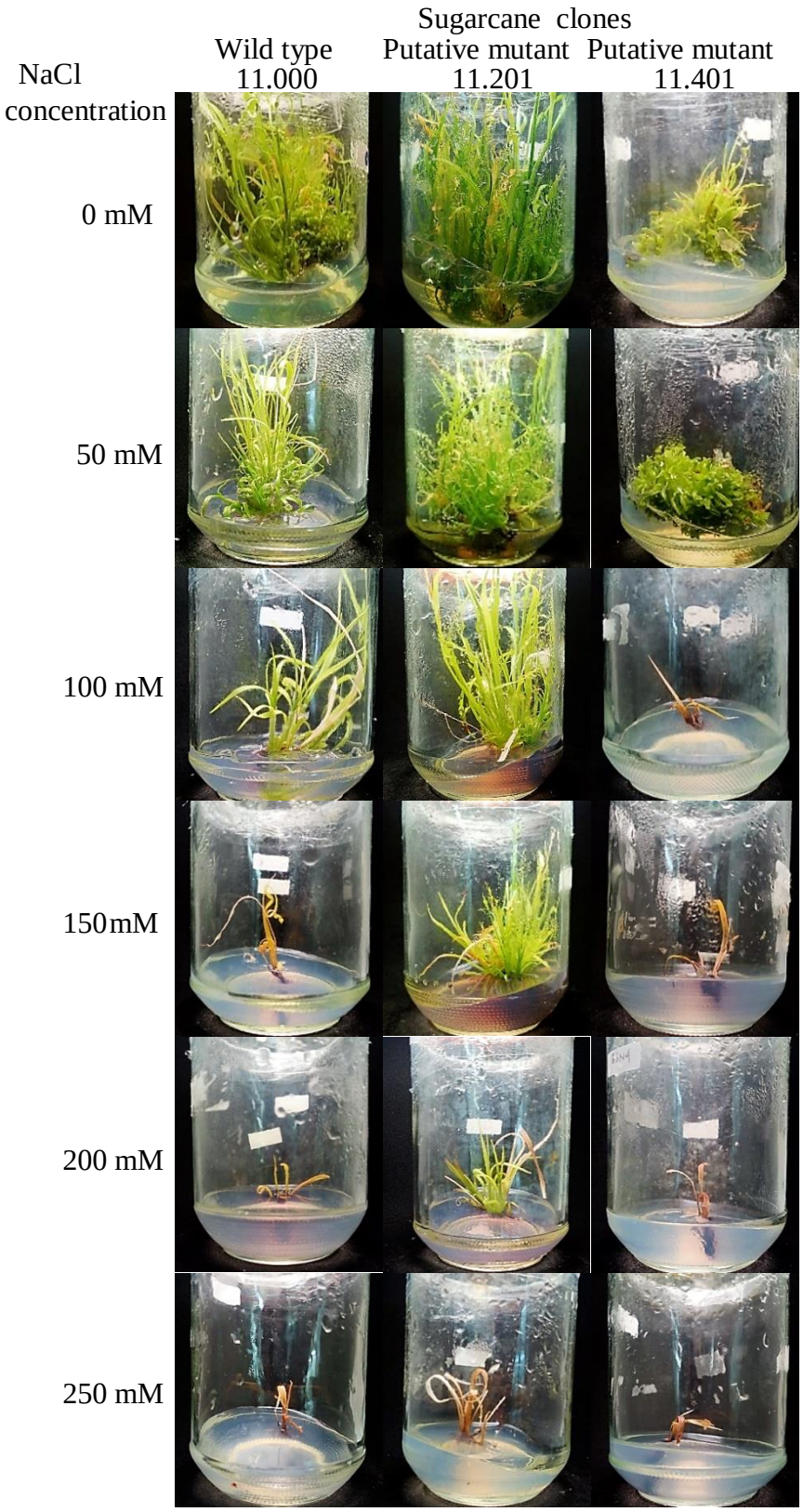

Figure 2. Growth of 3 sugarcane clones at increasing NaCl concentration, 16 weeks after plan

The DMRT results in Table 7 demonstrated that clone 11.201 exhibited limited leaf production at 15% PEG (3.33 ± 5.77 leaves explant^{-1}), whereas the remaining clones showed no growth under this condition. These findings indicate that clone 11.201 exhibits greater tolerance to PEG-induced

osmotic stress, particularly at elevated PEG concentrations. The appearances of sugarcane clones under different PEG concentrations are shown in Figure 3.

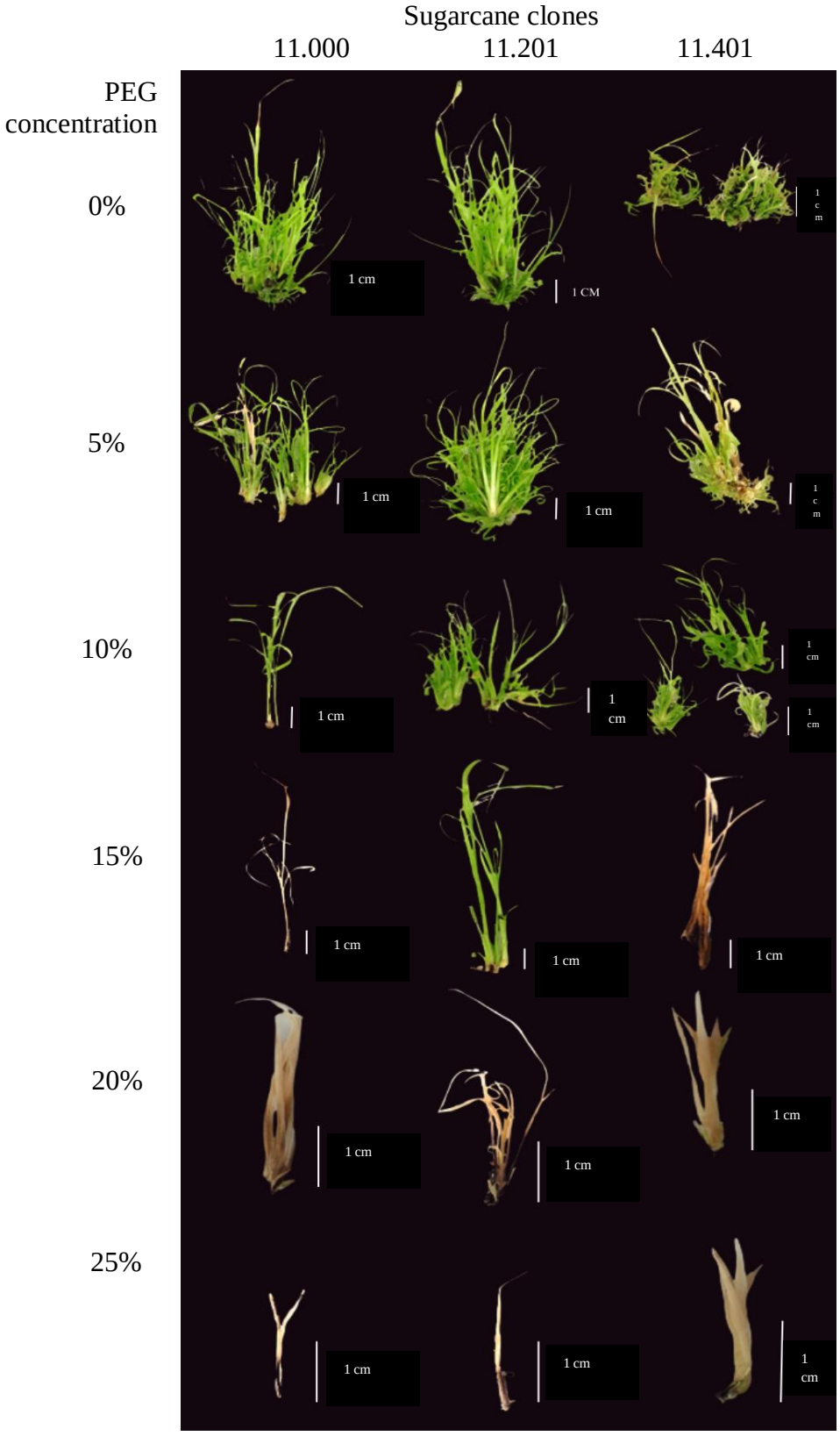

Figure 3. Shoot and leaf growth of three sugarcane clones at various PEG concentration levels, 16 weeks after planting

Table 7. The DMRT results on the average number of shoots and leaves of NXI 1–3 sugarcane clones as a response to the interaction between clones and PEG concentration at 16 weeks after planting (n= 3)

Sugarcane clones × PEG concentrations	Number of Leaves
11.000 × 0%	39.67 ± 25.32^{abc}
11.000 × 5%	106.33 ± 102.18^{a}
11.000 × 10%	3.00 ± 5.20^{d}
11.000 × 15%	0.00 ± 0.00^{d}
11.000 × 20%	0.00 ± 0.00^{d}
11.000 × 25%	0.00 ± 0.00^{d}
11.201 × 0%	87.67 ± 61.04^{ab}
11.201 × 5%	31.00 ± 11.79^{bc}
11.201 × 10%	20.33 ± 14.64^{cd}
11.201 × 15%	3.33 ± 5.77^{d}
11.201 × 20%	0.00 ± 0.00^{d}
11.201 × 25%	0.00 ± 0.00^{d}
11.401 × 0%	68.00 ± 16.00^{ab}
11.401 × 5%	17.33 ± 30.02^{cd}
11.401 × 10%	25.67 ± 27.68^{cd}
11.401 × 15%	0.00 ± 0.00^{d}
11.401 × 20%	0.00 ± 0.00^{d}
11.401 × 25%	0.00 ± 0.00^{d}

Note: Numbers in the same column followed by different letters indicate significant differences at the 95% confidence level

DISCUSSION

Effects of Gamma Irradiation on Somaclonal Variation in Sugarcane

Clone 11.201 was identified as a putative mutant of the NXI 1–3 sugarcane cultivar following exposure of callus tissue to 20 Gy Co^{60} gamma irradiation. The superior performance of this clone under both salinity and osmotic stress conditions suggests that gamma irradiation successfully generated genetic variation associated with abiotic stress tolerance. Similar findings have been reported in other sugarcane cultivars, where gamma irradiation at approximately 20 Gy effectively induced phenotypic and genetic variation, including in Kidang Kencana and Co J6 4 (Kaur et al., 2016; Suhesti et al., 2021). However, the optimal irradiation dose varies among genotypes. Yasmeen et al. (2017), for example, reported that 10 Gy was more effective for inducing variation in the NIA-98 cultivar, indicating that radiosensitivity is strongly genotype-dependent. Higher irradiation doses may increase mutation frequency and genetic diversity, but excessive exposure can also reduce regeneration capacity and cellular viability (Díaz-Juárez et al., 2022). Therefore, the effectiveness of gamma irradiation depends on achieving a balance between mutation induction and tissue survival.

Gamma irradiation induces mutations through the interaction of high-energy radiation with cellular components, particularly DNA (Astuti et al., 2020). This interaction may cause single- and double-strand DNA breaks, disrupt replication and transcription processes, and interfere with normal cell division and growth (Ma et al., 2021). In addition, prolonged *in vitro* culture combined with irradiation exposure can elevate reactive oxygen species (ROS) accumulation, resulting in oxidative stress that further contributes to genetic instability and somaclonal variation (Mullins et al., 2021). Such conditions may trigger chromosomal rearrangements, nucleotide substitutions, deletions, and epigenetic modifications, including DNA hypermethylation and hypomethylation (Krishna et al., 2016). These molecular and epigenetic alterations are likely responsible for the emergence of novel phenotypes with altered physiological responses to environmental stress.

The morphological variation observed in irradiated clones, particularly differences in leaf morphology (Figure 1), further supports the occurrence of irradiation-induced genetic changes. Alterations in chloroplast DNA (cpDNA) and other genomic regions associated with photosynthesis, metabolism, and plant development may contribute to these phenotypic modifications (Fadli et al., 2018). Importantly, the improved tolerance of clone 11.201 under both NaCl- and PEG-induced stress suggests that the induced mutations may affect common physiological pathways involved in osmotic

adjustment and stress adaptation. This finding indicates the potential occurrence of cross-tolerance, in which a single genotype exhibits adaptive responses to multiple abiotic stresses. Therefore, the combination of gamma irradiation and *in vitro* selection appears to be a promising strategy for generating sugarcane genotypes with broader environmental adaptability for cultivation on marginal lands.

Sugarcane Clone Responses to Salinity Stress

Under control conditions (0 mM NaCl), explants exhibited the highest growth performance, producing an average of 84.78 ± 37.87 shoots, 306.56 ± 148.74 leaves, and 7.56 ± 10.08 roots per explant. These values were not significantly different from those observed at 50 mM NaCl, suggesting that low salinity levels did not substantially inhibit explant growth. Similar observations were reported by Junandi et al. (2019), who demonstrated that moderate NaCl concentrations (34.22–68.43 mM) could still support normal plant growth. Although Na^+ and Cl^- are generally classified as non-essential elements, low concentrations may contribute to osmotic regulation and cellular homeostasis under certain conditions (Sukerta et al., 2024). These findings show the value of *in vitro* selection in assessing putative mutant lines of sugarcane for salt tolerance, which is consistent with previous observations by Wati et al. (2022).

Increasing NaCl concentrations progressively suppressed explant regeneration and organ development. Nevertheless, explants exposed to 200 mM NaCl remained viable and were still capable of producing shoots, leaves, and roots, indicating the presence of adaptive tolerance mechanisms in certain sugarcane clones. Similar findings were reported by Anitha et al. (2015), who observed that sugarcane clones CoC671 and CoC24 maintained osmotic balance under 200 mM NaCl through increased proline accumulation. In contrast, Yunita et al. (2020) reported that BL sugarcane failed to survive at salinity levels of 150–200 mM, highlighting substantial genotype-dependent variation in salt tolerance.

Salinity stress limits plant growth through both osmotic and ionic effects. Excessive Na^+ accumulation disrupts K^+ uptake, impairs enzyme activity, and disturbs cellular ion homeostasis, ultimately inhibiting plant growth and development (Breś et al., 2022). To maintain ionic balance, plants activate stress-responsive signalling pathways, particularly Ca^{2+}-mediated signalling and the Salt Overly Sensitive (SOS) pathway, which regulate Na^+ exclusion and intracellular ion equilibrium. The ability of several irradiated clones to survive under high salinity conditions suggests that gamma irradiation may have induced genetic modifications associated with improved ionic regulation and osmotic adjustment.

The present findings are consistent with Sigit (2016), who reported that BL sugarcane irradiated with 20 Gy gamma rays tolerated salinity up to 205 mM NaCl and continued producing new leaves. Likewise, Nikam et al. (2014) found that Co740 sugarcane irradiated with 40 Gy gamma rays survived under 200 mM NaCl stress. Collectively, these studies indicate that gamma irradiation can generate beneficial genetic variation associated with salinity tolerance, although the effectiveness of mutation induction remains dependent on genotype and irradiation dose (Lisdyayanti et al., 2019).

At the molecular level, salinity tolerance is controlled by complex regulatory networks involving stress-responsive genes and hormonal signalling pathways. Salt stress stimulates membrane-associated receptors and secondary messengers such as Ca^{2+} and reactive oxygen species (ROS), which subsequently regulate protein kinases and phosphatases involved in stress signalling. Increased abscisic acid (ABA) accumulation activates transcription factors, including AP/ERF, bHLH, bZIP, DREB, MYB, NAC, WRKY, and zinc finger proteins, which coordinate adaptive responses such as osmotic adjustment, ROS detoxification, and stomatal regulation (Hussain et al., 2012; Rai et al., 2024). Therefore, the improved performance of clone 11.201 under salinity stress may be associated with enhanced regulation of these physiological and molecular defence mechanisms.

Sugarcane Clone Responses to PEG-Induced Osmotic Stress

Analysis of variance demonstrated that PEG concentration significantly affected shoot and leaf formation in NXI 1–3 sugarcane at 16 weeks after planting. In general, increasing PEG concentration

reduced explant growth and regeneration capacity, confirming that osmotic stress severely restricts plant development under *in vitro* conditions. Under control conditions (0% PEG), explants produced the highest numbers of shoots and leaves, with averages of 20.89 ± 2.67 shoots and 65.11 ± 24.13 leaves per explant. Leaf production at 5% PEG did not differ significantly from the control treatment, indicating that mild osmotic stress had limited effects on leaf development. However, PEG concentrations above 15% completely inhibited shoot and leaf formation. Similar responses have been reported previously, where PEG-induced water deficit reduced nutrient translocation, cell expansion, and organ development (Manalu et al., 2024; Oguz et al., 2022; Rahayu et al., 2005).

Comparable findings were reported by Hapsari et al. (2017), who observed delayed leaf development at 10% PEG, while Sabatini et al. (2022) demonstrated that PEG concentrations exceeding 10% markedly reduced leaf formation despite continued explant survival. Dewani and Rahayu (2023) also reported that PEG concentrations of 5–15% reduced leaf number by more than 50% compared with the control treatment. In the present study, root formation was absent even under non-stress conditions. This response was likely associated with prolonged exposure to benzylaminopurine (BAP) during culture. Continuous BAP application may elevate endogenous cytokinin levels and suppress root initiation by altering the hormonal balance. Previous studies have shown that BAP is rapidly metabolised into stable glucoside derivatives, such as 3G-BAP and 9G-BAP, which inhibit root induction and maintain shoot proliferation (Feng et al., 2017; Kieber & Schaller, 2018; Reinert & Yeoman, 1982; Schaller et al., 2014).

Unlike the salinity experiment, PEG-induced osmotic stress did not produce statistically significant differences between mutant and wild-type clones. This finding suggests that tolerance mechanisms against osmotic stress may be more complex and not solely determined by irradiation-induced variation. Hartati et al. (2021) reported enhanced drought tolerance in gamma-irradiated sugarcane under PEG treatment, whereas Purwito et al. (2016) demonstrated that irradiation could also reduce growth performance depending on the nature of the induced mutation. Such contrasting responses indicate that gamma irradiation may generate both beneficial and deleterious mutations affecting stress-responsive pathways differently across genotypes (Pan et al., 2017).

Despite the overall reduction in growth, clone 11.201 remained viable at 15% PEG and continued producing leaves, indicating a certain degree of osmotic stress tolerance. Similar observations were reported by Le Roux (2019), who found that irradiated sugarcane clones survived under PEG concentrations up to 15%. The survival capacity of clone 11.201 under both NaCl and PEG stress suggests the possible occurrence of cross-tolerance mechanisms, whereby common physiological pathways contribute to adaptation against multiple abiotic stresses.

Physiologically, osmotic stress triggers complex signalling networks involving Ca^{2+}, ABA, ROS, MAPK, and CDPK pathways. Reduced cellular water potential activates Ca^{2+} influx through osmosensitive channels, stimulating ABA accumulation and the expression of stress-responsive genes. ABA signalling promotes osmotic adjustment through proline accumulation, stomatal regulation, and activation of antioxidant enzymes such as SOD, CAT, and APX, which detoxify excessive ROS (Pramudya & Pamungkas, 2022). In addition, ABA regulates genes encoding protective proteins, including LEA proteins, aquaporins, and dehydrins that maintain cellular water balance under drought conditions (Yang et al., 2021).

Gamma irradiation may further influence drought adaptation by modifying transcriptional regulation associated with stress tolerance. Transcription factors such as WRKY, NAC, bZIP, and MYB play important roles in ABA signalling, antioxidant defence, and developmental regulation during stress adaptation (Li et al., 2024; Tolosa & Zhang, 2020). Therefore, the improved performance of clone 11.201 under both salinity and osmotic stress conditions may result from irradiation-induced genetic variation that enhances stress-responsive regulatory pathways. These findings support the potential application of gamma irradiation combined with *in vitro* selection for the development of sugarcane genotypes adapted to marginal environments affected by salinity and drought stress.

CONCLUSION

NaCl-induced salinity and PEG-induced osmotic stresses significantly inhibited the growth of sugarcane cultivar NXI 1–3 under *in vitro* conditions. However, the putative mutant clone 11.201 generated gamma Co^{60} irradiation consistently showed better survival and growth performance under both NaCl- and PEG-induced stress compared to the wild type and clone 11.401. Clone 11.201 maintained its growth under 200 mM NaCl, yielding an average of 33.67 ± 13.58 shoots/explant, 143.00 ± 61.51 leaves/explant, and 2.33 ± 2.08 roots/explant. It also exhibited limited leaf production at 15% PEG (3.33 ± 5.77 leaves/explant) indicating the potential occurrence of cross-tolerance associated with gamma Co^{60} irradiation-induced variation. These findings demonstrate that gamma Co^{60} irradiation combined with *in vitro* selection is a promising approach for the early screening and development of sugarcane genotypes tolerant to multiple abiotic stresses for cultivation on marginal lands.

ACKNOWLEDGMENTS

The authors wish to thank the Rector and the Head of the Research and Public Service Institute of Jenderal Soedriman University for partially funding this research through the Unsoed Applied Research Scheme, Contract Number: 26.338/UN23.35.5/PT.01/II/2024.

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