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Research Article

In vitro selection of osmotic stress-tolerant Gerbera jamesonii shoots following induced mutagenesis with N-nitroso-N-methylurea and sodium azide

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ABSTRACT

Osmotic stress is a major abiotic constraint that limit ornamental plant growth and quality. Conventional breeding for osmotic tolerance is restricted by lengthy breeding cycles, highlighting the need for alternative strategies such as *in vitro* mutagenesis combined with osmotic screening. The ornamental crop gerbera(*Gerbera jamesonii*) is highly sensitive to water deficit. In this study, four-week-old *in vitro* shoot clusters of *G. jamesonii* "Jent" were treated with N-nitroso-N-methylurea (NMU) or sodium azide (NaN_3) at concentrations of 0.05–0.25% for 5–20 min and then cultured on MS medium supplemented with 40 g/L mannitol to simulate osmotic stress. Regeneration frequency, growth traits, photosynthetic pigments, gas exchange, and biochemical markers were evaluated six weeks after treatment. Regeneration declined with increasing mutagen concentrationand exposure time, with the sharpest reduction observed at 0.20–0.25% for 20 min. Four osmotic-tolerant lines were identified, three derived from NaN_3 treatment and one from NMU treatment. Under osmotic stress conditions, Lines 1 and 2 (NaN_3, 0.15% for 15 min) exhibited superior performance, maintaining leaf area and fresh weight at levels comparableto those of non-mutagen-treated shoots under non-stressed conditions. These lines maintained higher chlorophyll levels and displayedonly approximately15% reduction in photosynthesis, without asignificant increase in respiration. oluble sugar and proline contents, particularly in Lines 3 (NaN_3, 0.15% for 20 min) and 4 (NMU, 0.10% for 20 min), increased under osmotic stress, whereasstarch and protein contents decreased, most notably in Line 4. Our findings indicate that NaN_3 treatment, particularly at 0.15% for 15 min,is an effective condition for generating osmotic-tolerant gerbera lines, providing promising germplasm for future breeding and functional studies.

Key words: Osmotic-Tolerant, Gerbera, Mutagenesis, N-Nitroso-N-Methylurea, Sodium Azide

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INTRODUCTION

Osmotic stress is one of the most critical abiotic factors limiting plant growth and productivity worldwide, particularly under the increasing pressure of global climate change[1]. In gerbera (*Gerbera jamesonii*), an ornamental crop highly sensitive to water deficit, osmotic stress markedly reduces vegetative growth and flower yield. Previous studies have reported reductions of 20–35% in leaf area and stem length, along with decreases of 15–25% in plant biomass under water-deficit conditions. More critically, osmotic stress can lead to a 20–50% reduction in flower number and a 15–30% decrease in flower diameter, significantly diminishing marketable yield and flower quality[2,3]. Therefore, improving osmotic tolerance in gerbera is of considerable practical importance. However, conventional breeding approaches are often constrained by the species' narrow genetic base, long breeding cycles, and the complex, quantitative nature of osmotic tolerance traits[4,5].

In this context, i*n vitro* culture techniques combined with induced mutagenesis provide a valuable alternative for enhancing genetic variability and enabling early-stage screening of stress-tolerant variants. Chemical mutagenesis has been widely employed as an effective strategy to generate novel traits related to stress adaptation. Among chemical mutagens, N-nitroso-N-methylurea (NMU) and sodium azide (NaN_3) are frequently used because of their high mutagenic efficiency and their ability to induce point mutations that may result in beneficial stress-tolerance phenotypes[6]. For preliminary screening under controlled conditions, osmotic-related stress is commonly simulated *in vitro* using mannitol-induced osmotic stress, a well-established approach that mimic key components of osmotic stress at the cellular and physiological levels[7,8]. Numerous studies have demonstrated a strong correlation between osmotic stress tolerance observed *in vitro* and tolerance expressed at later developmental stages, support-

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ing the relevance of this method for early-stage selection [9–11].

In mutation breeding programs, early identification of stress-tolerant variants typically relies on physiological and biochemical markers associated with osmotic adaptation. Traits such as chlorophyll retention and photosynthetic capacity reflect the ability to maintain carbon assimilation and limit photooxidative damage under water deficit, while respiration stability indicates metabolic adjustment and energy balance during stress [12]. In addition, the accumulation of proline and soluble sugars serves as a key indicator of osmotic adjustment and cellular protection, while starch content reflects carbon reserve dynamics that support stress survival and recovery [13,14]. These markers are widely used because they are sensitive, quantifiable, and suitable for rapid screening under *in vitro* conditions. Although chemical mutagenesis has been applied in gerbera primarily to induce ornamental and morphological variation, studies focusing on mutagenesis-based screening for osmotic-related stress tolerance in this species remain limited [15,16]. To our knowledge, the effects of NaN_3 and NMU on *in vitro* regeneration and stress-related physiological responses of gerbera under osmotic-associated conditions have not been systematically reported. Consequently, information is lacking on how mutagen-treated gerbera tissues respond to osmotic-related stress during early developmental stages, particularly with respect to regeneration capacity and physiological performance.

In the present study, we aim to evaluate the responses of NaN_3- and NMU-treated gerbera shoots to mannitol-induced osmotic stress *in vitro*, with emphasis on shoot regeneration, growth performance, and selected physiological and biochemical indicators. By providing experimental data under controlled stress conditions, this work contributes to addressing this knowledge gap and supports the use of *in vitro* osmotic-related stress as a preliminary screening approach for identifying osmotic-responsive gerbera variants.

MATERIALS AND METHODS

Plant material

Four-week-old shoot clusters of *Gerbera jamesonii* L. cultivar "Jent"cultured *in vitro* on MS medium, were used in this study.

Investigation of the effects of NMU and NaN_3 on shoot regeneration under osmotic stress

Shoots approximately 1.0 cm in height were excised from four-week-old *in vitro* shoot clusters and treated with sterilized NMU or NaN_3 at concentrations of 0.05%, 0.10%, 0.15%, 0.20%, and 0.25% for 5, 10, 15, or 20 min. Following treatment, the explants were rinsed five times with sterile distilled water and transferred to Erlenmeyer flasks containing 50 mL of MS medium supplemented with 40 g L^{-1} mannitol, 0.5 mg L^{-1} BA, and 0.1 mg L^{-1} IAA to impose osmotic stress. For comparison, non-mutagen-treated shoots were cultured on MS medium without mannitol (wild type (WT), non-stressed) or on MS medium supplemented with mannitol (WT, osmotic-stressed). Cultures were maintained at 25 ± 2 °C and 75 ± 5% relative humidity, under a 12-h photoperiod with a light intensity of 3000 ± 200 lux. After six weeks of culture, shoot regeneration frequency was recorded.hoot clusters exhibiting superior growth performance, characterized by uniform shoot development and increased biomass accumulation, with a biomass ratio (mutant shoot biomass / non-mutant control biomass under stress) greater than 1, were selected for subsequent physiological and biochemical analyses.

Physiological and biochemical assessment

Gas exchange determination

A closed-chamber system (0.75 L) equipped with a CO_2 analyzer (EA80, Extech, USA) was usedto evaluate gas exchange [17]. The initial CO_2 concentration in the chamber was set to approximately 420 ppm and adjusted by gradually introducing CO_2 generated from the reaction between HCl and $NaHCO_3$. Measurements were conducted over a 15-min period. Net photosynthetic activity was measuredunder 3000 lux illumination (LED lamp, Philips; positioned 10 cm from the samples), whereas respiratory activity was measured under dark conditions. Allmeasurements were performedunder controlled environmental conditions(30 ± 1 °C and 75 ± 2% relative humidity).

Photosynthetic pigment determination

Ethanolic extracts of finely ground leaf samples were used for pigment analysis [18]. Following homogenization and centrifugation, absorbance was measured at 664, 648, and 470 nm.These values were used to estimate chlorophyll a, chlorophyll b, and carotenoid contents.

Sugar and starch content determination

oluble sugarwereextractedfrom ethanol homogenates of leaves. amples were reacted with phenol and concentrated H_2SO_4, and absorbance was measured at 490 nm [19]. The insoluble residue was processed separately for starch determination. After conversion of starch into soluble sugars, quantification was performed using the DNS method, with absorbance measured at 540 nm [20] . oth soluble sugar and starch concentrationswere calculated usingcalibration curves.

Protein and proline content determination

Proteins were extracted from leaf tissues in phosphate buffer (pH 7.5), and the extractwerecentrifuged. Protein content in the supernatant was determined using the Bradford method, with absorbance measured at 595 nm and bovine serum albumin used as the standard [21]. Proline was quantified from leaf material extracted in 70% ethanol. After centrifugation at 6000 rpm for 15 min, the supernatant was treated with ninhydrin solution, incubated in a boiling water bath for 20 min, cooled, and measured at 520 nm [22]. Proline standards were used to construct the calibration curve.

Statistical analysis

All experimental data were analyzed using analysis of variance (ANOVA) in SPSS software (SPSS Inc., USA). Each treatment consisted of 5 independent biological replicates, with 50 explants per replicate. Mean comparisons were conducted at the 5% significance level using Duncan's Multiple Range Test (DMRT). Data are expressed as mean ± standard deviation (SD), and SD values are shown as error bars in the figures.

RESULTS

Effects of NMU and NaN_3 on shoot regeneration under osmotic stress

The effects of NMU and NaN_3 concentration and exposure duration on shoot regeneration of gerbera under osmotic stress are presented in Figure 1. Overall, shoot regeneration declined progressively with increasing mutagen concentration and exposure time. At a given concentration, prolonged exposure (15–20 min) consistently resulted in lower regeneration percentages than shorter treatments (5–10 min). Notably, at the longest exposure duration (20 min), shoot regeneration was severely suppressed, and no regenerated shoots were observed (Figure 1).

Under NMU treatments, shoot regeneration at 0.05% remained relatively high after 5 min (approximately 85%) and 10 min (approximately 75%) but declined sharply to approximately 50% and 40% following 15- and 20-min exposures, respectively. A pronounced reduction was observed at 0.10–0.15% NMU, where regeneration percentages ranged from 40% to 60% under short exposure times (5–10 min) but dropped markedly to below 25% when exposure increased to 15–20 min. At 0.20% NMU, shoot regeneration remained consistently low—below 40% regardless of exposure duration—indicating a strong inhibitory effect at elevated doses. A similar dose- and time-dependent reduction in shoot regeneration was observed following NaN_3 treatment; however, its inhibitory effects were generally less severe than those induced by NMU. At 0.05% and 0.10% NaN_3, regeneration rates remained relatively high, exceeding 50% across all exposure times. From 0.15% NaN_3 onward, a clear distinction emerged between short (5–10 min) and long (15–20 min) exposure groups, with regeneration rates of approximately 75% in the former and declining to approximately 25% in the latter. This trend was further accentuated at 0.20% NaN_3, where shoot regeneration reached approximately 35% after 5–10 min of exposure but dropped sharply to approximately 10% following 15–20 min treatments.

A total of 6,120 regenerated shoots were screened, of which only four lines (0.07%) met the selection criteria and were identified as putative osmotic-tolerant variants based on their superior morphological performance under mannitol-induced stress (Figure 2). Of these, three lines originated from NaN_3 treatments and were designated as Line 1, Line 2, and Line 3, corresponding to treatment with 0.15% NaN_3 for 15 min (Lines 1 and 2) and 20 min (Line 3). The remaining line was derived from treatment with 0.10% NMU for 20 min and was designated as the NMU line (Line 4). Under osmotic stress conditions, these selected lines exhibited distinct morphological differences compared with non-mutagen-treated shoots (WT, osmotic-stressed).

Although the overall growth of the mutant lines was inferior to that of non-mutagen-treated shoots cultured under non-stressed conditions (WT, non-stressed), their morphological performance was markedly superior to that of non-mutagen-treated shoots subjected to osmotic stress. Specifically, non-mutagen-treated shoots exposed to osmotic stress exhibited pronounced growth inhibition, characterized by reduced shoot size and fewer and narrower leaves compared with those grown under non-stressed conditions. Bycontrast, NaN_3-derived lines (Lines 1–3) producedvigorous shoot clusters with greener, broader leaves and a more compact

growth habit under the same stress conditions. Among these, Lines 1 and 2 showed the most pronounced tolerance, displaying robust growth and healthy shoot clusters that closely resembled the morphology of non-mutagen-treated shoots cultured under non-stressed conditions. Line 3 also exhibited improved leaf development and shoot growth under osmotic stress, although its overall vigor was slightly lower than that observed in Lines 1 and 2. Bycontrast, the NMU-derived line exhibited distinct growth abnormalities, including curled and malformed leaves, reduced shoot elongation, and diminished overall vigor. These traits indicate that, although this line survived under osmotic stress conditions, its growth and developmental stability were substantially compromised.

To further evaluate the growth performance of regenerated lines under osmotic stress conditions, shoot number, leaf area, and fresh weight were determined. The findings are summarized in Table 1. Under normal condition , non-mutant plants exhibited the highest values across all traits, with average of 10.43 shoots, a leaf area of 11.82 cm^2, and a fresh weight of 3.10 g. Osmotic stress caused a significant reduction in the growth of non-mutant plants, with shoot number, leaf area, and fresh weight decreasing to 4.26, 5.25 cm^2, and 1.69 g, respectively.

Among the mutant lines, Lines 1 and 2 (0.15% NaN_3 for 15 min) showed the best performance under osmotic stress conditions, producing 8.14 and 8.26 shoots, respectively. These valueswere significantly higher than those produced bythe wildtype under osmotic stress. These linesalso maintained large leaf areas (11.66 and 9.93 cm^2) and fresh weights (2.95 and 2.98 g), comparableto those of the wildtype under non-stressed conditions (11.82 cm^2 and 3.10 g). Under osmotic stress, Line 3 (0.15% NaN_3 for 20 min) exhibited moderate performance, producing on average 6.31 shoots, a leaf area of 9.88 cm^2, and a fresh weight of 2.71 g. These values were significantly higher than those ofnon-mutant plants but lower than those of Lines 1 and 2. Bycontrast, the NMU-derived line (0.10% NMU for 20 min) exhibited poor growth under stress, producing on average only 1.04 shoots and a fresh weight of 1.72 g; these values were statistically similar to those of stressed non-mutant plants. Notably, its leaf area (7.77 cm^2) remained higher than that of non-mutant plants under osmotic stress, suggesting a partial but limited tolerance response.

Within each column, means labeled with different letters differ significantly at $p < 0.05$.

Screening regenerated shoots for osmotic stress tolerance

Osmotic stress markedly altered pigment composition and gas exchange parameters in all mutant lines compared with the wildtype (Figure 3). Chlorophyll a content declined substantially under osmotic stress conditions, reaching approximately 1.05 mg g^{-1} FW in Line 4, whereas Lines 1, 2, and 3 maintained relatively higher levels, ranging from approximately1.45 to 1.72 mg g^{-1} FW. Chlorophyll b and carotenoid contents followed a similar trend, with higher levels observed in Lines 1 and 2 than in Lines 3 and 4. Notably, Line 4 exhibited the lowest chlorophyll and carotenoid contents among all lines. Consistent with the reduction in pigment content, the photosynthetic rate was inhibited under osmotic stress. Compared with the wildtype under non-stressed conditions (approximately 10.12 mg CO_2 g^{-1} FW h^{-1}), photosynthetic rates decreased to approximately 9.10 mg CO_2 g^{-1} FW h^{-1} in Lines 1 and 2, approximately 7.21 mg CO_2 g^{-1} FW h^{-1} in Line 3, and approximately 5.32 mg CO_2 g^{-1} FW h^{-1} in Line 4 under osmotic stress. Bycontrast, osmotic stress markedly increased respiration in the wildtype, from approximately 2.51 to 4.83 mg CO_2 g^{-1} FW h^{-1}. Notably, respiration rates in Lines 1 and 2 did not differ significantly from those of the non-stressed wildtype, whereas Lines 3 and 4 exhibited elevated respiration rates.

Osmotic stress markedly increased total soluble sugar content, reaching approximately 33 mg g^{-1} FW under stress conditions compared with 18 mg g^{-1} FW in the wildtype under non-stressed conditions (Figure 4). Among the mutant lines, Lines 3 and 4 exhibited the highest accumulation, ranging from 20 to 25 mg g^{-1} FW, whereas Lines 1 and 2 maintained total soluble sugar levels comparable to the wildtype under non-stressed conditions. tarch content in Lines 1 and 2 wascomparable to that in the non-stressed WT and significantly higher than that in the stressed WT, as well as higher than in Lines 3 and 4. Bycontrast, Line 4 exhibited the lowest starch content (approximately 20 mg g^{-1} FW), similar to that of the stressed WT. Line 4 showed the highest level of proline among all lines, exceeding thoseof Lines 1–3 and the non-stressed WT, and was comparable to the stressed WT. Conversely, Lines 1 and 2 exhibited the lowest proline content, similar to that of the non-stressed WT.

DISCUSSION

The present study demonstrates that chemical mutagenesis combined with *in vitro* osmotic stress screening is an effective approach for generating novel

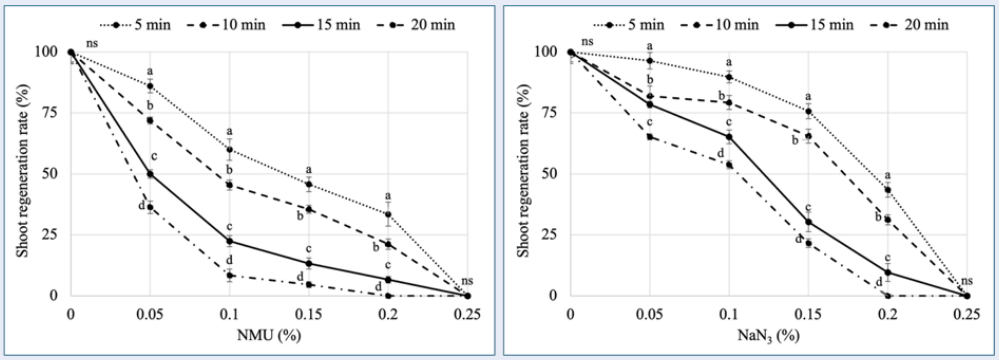

Figure 1: Effects of treatment duration and concentrations of NMU and NaN_3 on the shoot regeneration rate of gerbera under osmotic stress after six weeks of culture. Means within the same treatment concentration followed by different letters differ significantly at $p < 0.05$.

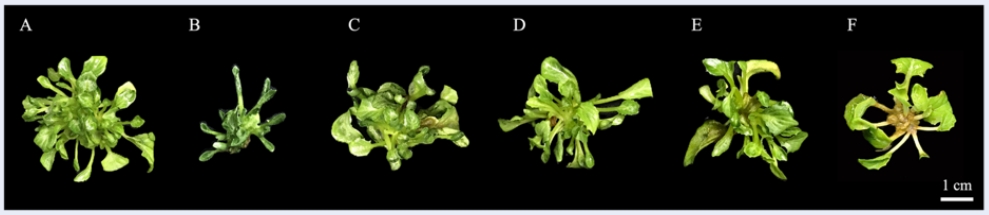

Figure 2: Morphology of regenerated shoot clusters after six weeks of culture. (A) Non-mutagen-treated shoots cultured under non-stressed conditions (WT, non-stressed); (B) Non-mutagen-treated shoots cultured under osmotic stress conditions (WT, osmotic-stressed); (C–E) NaN_3-derived mutant lines (Lines 1, 2, and 3) cultured under osmotic stress conditions; (F) NMU-derived mutant line (Line 4) cultured under osmotic stress conditions.

Table 1: Effects of treatment duration and concentrations of NMU and NaN_3 on the leaf area of gerbera under osmotic stress after six weeks of culture.

Shoot cluster origin	Shoot number	Leaf area (cm^2)	Fresh weight (g)
WT, non-stressed	10.43 ± 1.21^a	11.82 ± 0.32^a	3.10 ± 0.04^a
WT, osmotic-stressed	4.26 ± 0.36^d	5.25 ± 0.08^d	1.69 ± 0.03^c
Line 1 (0.15% NaN_3; 15 min), osmotic-stressed	8.14 ± 0.55^b	11.66 ± 0.28^a	2.95 ± 0.05^a
Line 2 (0.15% NaN_3; 15 min), osmotic-stressed	8.26 ± 0.67^b	9.93 ± 0.11^b	2.98 ± 0.04^a
Line 3 (0.15% NaN_3; 20 min), osmotic-stressed	6.31 ± 0.21^c	9.88 ± 0.19^b	2.71 ± 0.02^{ab}
Line 4 (0.10% NMU 20 min), osmotic-stressed	1.04 ± 0.06^e	7.77 ± 0.10^c	1.72 ± 0.03^c

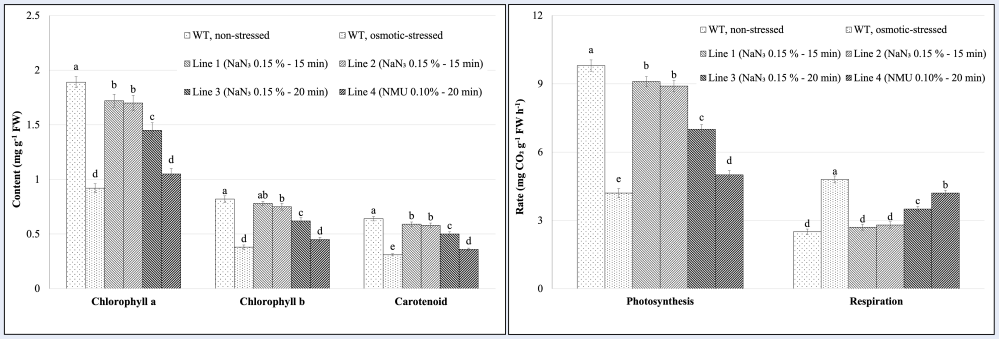

Figure 3: Photosynthetic pigments (left), photosynthetic rate (right), and respiration rate (right) in non-mutagen-treated plants under non-stressed conditions, and in non-mutagen-treated plants and NaN_3- and NMU-derived mutant lines under osmotic stress. Different lowercase letters above the bars indicate significant differences at $p < 0.05$.

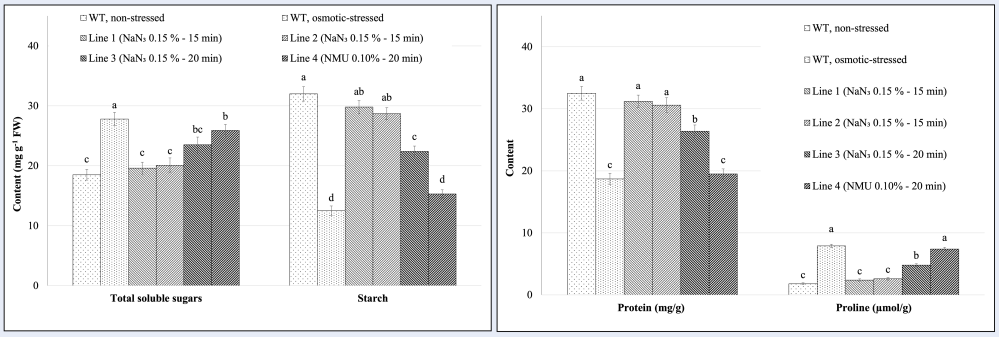

Figure 4: Total soluble sugar and starch content (left), and protein and proline content (right) in non-mutagen-treated plants under non-stressed conditions, and in non-mutagen-treated plants and NaN_3- and NMU-derived mutant lines under osmotic stress. Different lowercase letters above the bars indicate significant differences at $p < 0.05$.

gerbera variants with improved osmotic tolerance. Both NMU and NaN_3 treatments significantly affected shoot regeneration in a dose- and time-dependent manner, with regeneration frequency declining sharply at higher concentrations and longer exposure times (Figure 1). This finding is consistent with previous reports in wheat and sugar beet, where increasing the mutagen dose beyond the optimum level reduced cell survival and organogenesis due to cytotoxic effects [23,24]. Notably, NaN_3-treated cultures producedthree viable osmotic stress-tolerant lines, whereas NMU treatment produced only one line. This difference reflects the distinct mutagenic mechanisms of the two chemicals. NMU, a strong alkylating agent, introduces O^6-methylguanine and other alkyl adducts that cause extensive point mutations and even frameshifts, often leading to deleterious phenotypes [25]. The NMU-derived line in this study displayed curled leaves, reduced elongation, and a 55% reductionin photosynthetic activity, suggesting substantial functional disruption at the genomic level (Figure 2 and Figure 3). Bycontrast, NaN_3 is metabolized in plant cells to an azido metabolite that induces predominantly GC→AT transitions, resulting inmore targeted point mutations with lower background damage [26]. This mechanism likely explains the higher regeneration frequencies and more stable phenotypes observed in the NaN_3-derived lines. Similar resulthave been reported in quinoa and okra, where NaN_3 efficiently generated useful mutations with minimal growth penalty [27,28].

Physiologically, the NaN_3-derived lines, particularly Lines 1 and 2, exhibited a strong capacity to sustain shoot proliferation, leaf expansion, and fresh biomass under mannitol-induced stress, with values comparable to those observed under non-stressed conditions. This performance was associated with the maintenance of higher chlorophyll a and carotenoid con-

tents, resulting in only an approximately 15% reduction in photosynthetic rate and no significant increase in respiration (Figure 3). The preservation of photosynthetic pigments likely contribute to the stability of the photosynthetic electron transport chain, thereby limiting excessive reactive oxygen species (ROS) production and protecting cellular structures under water-deficit conditions [1]. In particular, higher carotenoid levels play a crucial photoprotective role by scavenging singlet oxygen and dissipating excess excitation energy through non-photochemical quenching. This mechanism prevents chlorophyll from entering an overexcited state that would otherwise promote ROS formation and cause photodamage to photosystem II [11,12]. By safeguarding chlorophyll molecules and maintaining the functional integrity of the photosynthetic apparatus, carotenoids indirectly support sustained photosynthetic activity and carbon assimilation under osmotic stress [29]. Such coordinated regulation of photosynthetic pigment retention and photosynthetic activity is widely recognized as a key characteristic of osmotic-tolerant genotypes. Similar responses have been reported in NaN_3-induced mutant lines of pineapple "MD2", where a marked increase in chlorophyll a content was accompanied by a proportional enhancement of photosynthetic rate, resulting in improved growth performance under osmotic stress [30]. By contrast, the NMU-derived Line 4 exhibited severe pigment degradation, elevated respiration rates, and pronounced reductions in starch and protein contents, indicating a metabolic imbalance characterized by carbon starvation and enhanced proteolysis, which are responses typically associated with osmotic-sensitive phenotypes [12].

At the biochemical level, distinct metabolic patterns were observed among the lines. Lines 3 and 4 showed stress-associated responses, whereas Lines 1 and 2 maintained profiles similar to the non-stressed WT (Figure 3). Proline accumulation is widely recognized as an adaptive response to abiotic stress, primarily contributing to osmotic adjustment and the maintenance of cellular turgor. It has also been suggested to play roles in membrane stabilization and stress recovery, potentially through interactions with cellular components such as phospholipids and proteins. In addition, proline has been implicated in cellular redox balance; however, its function as a direct antioxidant remains debated, with some studies indicating a limited role in scavenging ROS [13,31]. Nevertheless, excessive proline accumulation accompanied by marked starch depletion, as observed in Line 4, may indicate heightened stress perception rather than an efficient adaptive strategy. Adequate starch reserves serve as essential carbon and energy source that support maintenance metabolism and post-stress recovery, including the reactivation of growth and repair processes once favorable conditions are restored. Lines that retain higher starch levels may therefore sustain metabolic homeostasis more effectively under osmotic stress [32]. By contrast, Lines 1 and 2 maintained moderate levels of soluble sugars together with relatively higher starch reserves, suggesting more balanced carbohydrate partitioning that supports both osmotic regulation and sustained carbon supply for growth and post-stress recovery. Comparable physiological and biochemical patterns have been documented in several osmotic-tolerant crop species, including *Arachis hypogaea*, *Brassica parachinensis*, and *Brassica integrifolia*, further supporting the relevance of these traits as reliable indicators of osmotic-related stress tolerance [33,34].

Overall, the superior performance of NaN_3-derived Lines 1 and 2 underscores the efficiency of NaN_3 mutagenesis at 0.15% for 15 min in producing variants with stable growth, photosynthetic resilience, and effective osmotic regulation. Similar success with NaN_3 mutagenesis has been reported in maize, cowpea, and sugarcane, where mutant lines exhibited improved osmotic tolerance while maintaining high yield potential [35,36]. These results indicate that combining chemical mutagenesis with *in vitro* stress screening is a promising strategy for expanding genetic variability and developing valuable germplasm resources for breeding osmotic-resilient gerbera cultivars. The use of mannitol-induced osmotic stress *in vitro*, although widely employed for preliminary screening, may not fully capture the complexity of osmotic conditions encountered under natural or field environments. In addition, the absence of mutagen-only controls limits the ability to distinguish between the direct effects of mutagenesis and stress-induced responses. Moreover, the selected lines have not yet been validated under *ex vitro* or greenhouse conditions. Therefore, future studies should incorporate molecular confirmation of mutation events, include appropriate controls, and evaluate the performance of selected lines under more realistic water-deficit conditions to ensure their stability and practical applicability.

CONCLUSIONS

This study demonstrates that chemical mutagenesis using NaN_3 and NMU can generate gerbera variants with improved osmotic-related stress tolerance, although regeneration efficiency declined at higher mutagen concentrations and longer exposure times. Among the four mutant lines obtained, NaN_3-derived

Lines 1 and 2 exhibitedthe best performance, maintaining growth, photosynthetic activity, and biomass comparable to the wild type, together with relatively balanced metabolic responses under osmotic stress. In particular, treatment with 0.15% NaN_3 for 15 min appears promising for developing osmotic-responsive gerbera germplasm. However, molecular validation of mutation induction should be conducted prior to subsequent evaluation of these lines under greenhouse water-deficit conditions to ensure their reliability and practical applicability.

ABBREVIATIONS

FW: Fresh weight
MS: Murashige & Skoog
NaN_3: Sodium azide
NMU: N-Nitroso-N-Methylurea
ROS: Reactive oxygen species
SPSS: Statistical package for the social sciences

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Conceptualization: Thang Thanh Tran; Methodology: Thang Thanh Tran; Formal analysis and investigation: Linh Minh Hong Tran; Writing - review and editing: Hien Thi Thanh Tran. All authors read and approved the final manuscript.

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