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Gamma Irradiation-Induced Hormesis Enhances Salinity Tolerance During Germination and Early Seedling Growth of Canola (*Brassica napus* L.)

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Abstract

Salinity stress severely constrains canola (*Brassica napus* L.) germination and early seedling establishment. This study investigated whether low dose gamma irradiation could alleviate salinity induced damage during germination. Canola seeds were pretreated with 0, 25, 50, 75 and 100 Gy gamma radiation and germinated under 0, 50, 100, or 150 mM NaCl. Salinity significantly reduced germination percentage, seedling vigor, root and shoot growth, and photosynthetic pigment content while increasing oxidative stress markers including hydrogen peroxide (H₂O₂), malondialdehyde (MDA) and electrolyte leakage (EL). However, pretreatment with 25–50 Gy, markedly alleviated these effects. The 50 Gy treatment improved germination, enhanced seedling growth, increased chlorophyll and carotenoid contents and stimulated antioxidant enzyme activities (catalase, peroxidase, superoxide dismutase, ascorbate peroxidase). It also promoted accumulation of osmoprotective compounds (proline, soluble sugars, soluble proteins) while reducing lipid peroxidation and hydrogen peroxide accumulation. Higher doses (75–100 Gy) provided limited protection or intensified stress damage. Multivariate analyses confirmed the superiority of 50 Gy in maintaining physiological and biochemical homeostasis under salinity. These findings demonstrate that low dose gamma irradiation appears to act as an effective seed priming strategy, activating antioxidant defenses and osmotic adjustment mechanisms to enhance salinity tolerance. This is a potentially sustainable approach with considerable potential for improving canola establishment under saline conditions.

Keywords: Hormesis, Antioxidant enzymes, Osmoprotectants, Oxidative stress, Seed priming

Introduction

Canola (*Brassica napus* L.), a member of the Brassicaceae family, is one of the most important edible oilseed crops worldwide and ranks among the leading sources of vegetable oil production. In addition to its value as an oilseed crop, canola is widely used in biofuel production, animal feed, and human nutrition. Its inclusion in crop rotation systems contributes to improved soil fertility and supports sustainable agricultural practices. Global canola production increased by approximately 61% between 2004 and 2019, reflecting its growing importance in modern agriculture. More recently, the versatility of canola has expanded further, with applications as a leafy vegetable, forage crop, and ornamental plant [1; 2].

Despite its agricultural importance, canola production is increasingly challenged by environmental stresses, particularly in arid and semiarid regions where soil salinity is widespread. The accumulation of soluble salts in the soil severely restricts plant growth and productivity. Although canola is generally considered moderately tolerant to salinity [3], its early developmental stages are especially sensitive to salt stress. Salinity can impair seed germination, hinder seedling establishment, disrupt photosynthesis, and reduce nutrient uptake, ultimately leading to substantial yield losses [4; 1].

Salinity is among the most serious abiotic stresses limiting agricultural productivity worldwide. This phenomenon results from the excessive accumulation of soluble salts in the soil or root zone and affects both irrigated and rainfed agricultural systems [5]. Currently, approximately 836 million hectares of cultivated land are affected by salinity, and projections suggest that more than half of the world's arable land could be impacted by salinization by 2050 [6; 7]. Salinity adversely influences plant growth throughout the entire cycle, but the germination stage is particularly vulnerable. Since successful germination forms the foundation of crop establishment and productivity, improving salt tolerance during this stage is essential for sustainable crop production in saline environments [8].

The reduction in seed germination under saline conditions is primarily attributed to two closely interconnected factors, namely osmotic stress and ion toxicity. High salt concentrations decrease the osmotic potential of the germination medium, thereby reducing water uptake by seeds, delaying imbibition, and consequently inhibiting or postponing germination. In addition, excessive accumulation of sodium (Na^+) and chloride (Cl^-) ions disrupts ion homeostasis, impairs cellular metabolism and enzyme activities, and interferes with essential physiological and biochemical processes required for successful germination. Collectively, these adverse effects significantly reduce germination percentage, delay germination, and impair subsequent seedling vigor [9; 10].

To overcome the negative impacts of salinity, several seed enhancement technologies have been developed. Among these methods, seed priming is recognized as one of the most effective and economical methods for improving germination performance under stressful environmental conditions [11; 12]. Seed priming involves controlled hydration that initiates the metabolic processes required for germination without allowing radicle emergence [13]. This treatment enhances germination uniformity, accelerates seedling establishment, and improves seed vigor through the activation of metabolic pathways and optimization of water uptake dynamics [14].

Various priming techniques have been successfully employed, including hydropriming, halopriming, and osmopriming, each designed to improve seed performance under abiotic stress conditions [15; 16].

In recent years, gamma irradiation has emerged as a promising seed-priming approach for improving stress tolerance. When applied at low doses, gamma radiation can induce beneficial physiological and molecular responses that increase plant resilience. These responses include the activation of antioxidant defense systems, improved osmotic regulation, and the upregulation of genes associated with stress adaptation [17]. Previous studies have shown that gamma-irradiated seeds often exhibit higher germination rates and greater seedling vigor under saline conditions. These improvements are largely attributed to increased activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), which help limit oxidative damage caused by reactive oxygen species (ROS). Furthermore, increased accumulation of osmoprotectants, including proline and soluble sugars, contributes to membrane stabilization, maintenance of cellular homeostasis, and improved tolerance to environmental stress [18].

Therefore, the objective of this study was to evaluate the dose-dependent effects of gamma irradiation on salinity tolerance during canola germination. Morphological, physiological, biochemical, and antioxidant responses were examined to identify the most effective irradiation dose and to clarify the mechanisms underlying radiation-induced stress tolerance.

Results

Germination indices

Salinity stress strongly inhibited the germination of canola seeds, and the magnitude of this reduction progressively increased with increasing NaCl concentration. The germination percentage decreased significantly from the nonsaline control to the highest salinity level (150 mM NaCl). Nevertheless, gamma irradiation pretreatment moderated this inhibitory response in a dose-dependent manner. Seeds exposed to low irradiation doses (25 and particularly 50 Gy) consistently presented higher germination percentages than nonirradiated seeds across all salinity levels did, whereas irradiation doses of 75 and 100 Gy failed to provide similar protection and frequently resulted in reduced germination performance (Fig. 1). These findings demonstrate a clear hormetic response, whereby low-dose irradiation enhances seed germination, whereas higher doses become progressively detrimental. The beneficial influence of low-dose gamma irradiation became increasingly evident as the salinity increased. Under moderate salinity (50 and 100 mM NaCl), irradiation at 50 Gy resulted in relatively high germination percentages compared with those of the untreated controls, indicating that this dose effectively alleviated the adverse effects of salt stress on germination capacity. Even under severe salinity (150 mM NaCl), seeds receiving 50 Gy retained greater germination ability than did those in the corresponding nonirradiated treatment. In contrast, pretreatment with 100 Gy consistently resulted in the lowest percentage of germination under saline conditions (Fig. 1).

Seedling growth exhibited a response pattern similar to that observed for germination. Increasing salinity significantly reduced the RL, PL, SL and biomass accumulation, confirming that early seedling development is highly sensitive to salt stress (Table 1). These seedlings also accumulated the highest dry biomass, with radicle, plumule, and seedling dry weights of 1.18, 2.05, and 3.23 mg, respectively. The reduction was particularly evident at 100 Gy, where the seedling length decreased to 6.06 cm. Similarly, the SDW decreased from 3.23 mg in the control to 1.90 mg following 100 Gy irradiation, confirming the inhibitory effect of excessive radiation on biomass production. Salinity further intensified these reductions in all growth-related characteristics. In untreated seedlings exposed to 50 mM NaCl, the RL decreased to 4.84 cm, whereas the PL and SL decreased to 4.59 and 9.44 cm, respectively. Increasing the salinity to 100 mM NaCl produced additional reductions, with the SL reaching 8.21 cm, whereas severe salinity (150 mM NaCl) reduced the SL to only 2.85 cm. A comparable decline was observed for dry matter accumulation, where the seedling dry weight decreased from 3.23 mg under nonsaline conditions to 0.96 mg at 150 mM NaCl. Although the absolute values of root and shoot growth still decreased with increasing salinity, the rate of reduction was considerably lower in the seedlings pretreated with 25 or 50 Gy than in the untreated plants. Under 150 mM NaCl combined with 100 Gy irradiation, the RL decreased to only 0.91 cm, the PL decreased to 0.78 cm, and the SL decreased to 1.69 cm, representing the lowest values recorded among all the treatment combinations. Similar reductions occurred in biomass production, where the radicle, plumule, and seedling dry weights decreased to 0.18, 0.39, and 0.57 mg, respectively (Table 1).

The remaining germination indices also responded significantly to both salinity and gamma irradiation. The mean germination time (MGT) increased progressively as the salinity increased, indicating delayed germination under saline conditions. In contrast, the germination rate (GR) and seedling vigor index (SVI) decreased continuously with increasing NaCl concentration, reflecting slower and less vigorous seedling establishment. Under control conditions, untreated seeds presented the shortest mean germination time (2.49 days) and the highest germination rate (0.63 seeds/day) and vigor index (5.87). Increasing irradiation doses under nonsaline conditions gradually prolonged MGT while simultaneously reducing the GR and SVI, again suggesting that excessive irradiation negatively affected the physiological quality of seeds. The most pronounced inhibitory response occurred following 100-Gy irradiation, where MGT increased to 7.40 days, whereas GR and SVI decreased to 0.26 seed days⁻¹ and 2.65, respectively (Table 2).

Compared with high-dose irradiation, exposure to 25 and 50 Gy substantially moderated these responses by shortening the mean germination time and maintaining comparatively higher GR and VI across all salinity levels. Among all irradiation treatments, 50 Gy consistently produced the most favorable germination performance under saline conditions, confirming that this irradiation dose effectively enhanced seed vigor during the critical establishment phase. The observed improvement in seedling vigor following low-dose irradiation was accompanied by better biomass accumulation and greater seedling elongation, suggesting that enhanced germination translated directly into superior early growth performance. Because seedling vigor integrates both germination capacity and seedling development, its consistent improvement under 25–50 Gy indicates that gamma irradiation enhanced not only the initiation of germination but also the subsequent establishment of healthy seedlings.

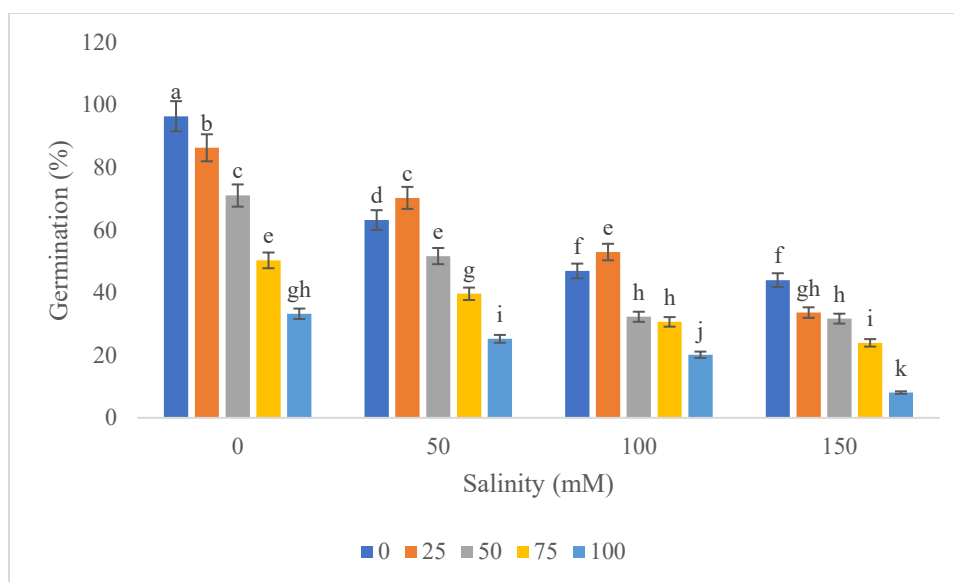


Table 1. Effect of Gamma pretreatment on the length and dry weight of canola radicle, plumule and seedling under salt stress conditions

Salinity (mM)	Gamma (Gy)	RL (cm)	PL (cm)	SL (cm)	RDW (mg)	PDW (mg)	SDW (mg)
0	0	5.88±0.35a	5.52±0.12a	11.40±0.12a	1.18±0.020a	2.05±0.042a	3.23±0.23a
	25	5.35±0.32ab	4.8±0.11b	10.15±0.10b	1.00±0.017b	1.88±0.039b	2.88±0.21b
	50	4.59±0.83c	4.44±0.10c	9.03±0.10c	0.90±0.015c	1.71±0.035c	2.61±0.20c
	75	3.65±0.22f	4.08±0.09d	7.74±0.09ef	0.83±0.014d	1.53±0.031d	2.36±0.18d
	100	2.81±0.17hi	3.25±0.07f	6.06±0.07gh	0.68±0.011f	1.22±0.025e	1.900±0.15e
50	0	3.77±0.23f	4.00±0.09d	7.77±0.10e	0.56±0.009h	1.04±0.021g	1.60±0.15e
	25	4.41±0.27d	4.59±0.10bc	9.00±0.08c	0.73±0.012e	1.25±0.026e	1.98±0.18d
	50	4.84±0.29b	3.70±0.08e	8.54±0.08d	0.62±0.010 g	1.14±0.023f	1.76±0.16d
	75	3.01±0.18 h	3.40±0.08f	6.41±0.07 g	0.52±0.009i	0.93±0.019 h	1.45±0.13ef
	100	2.31±0.14j	2.71±0.06 h	5.02±0.06i	0.42±0.007j	0.74±0.015ij	1.16±0.11gh
100	0	4.44±0.22d	3.27±0.07f	7.71±0.08ef	0.35±0.005l	0.72±0.015j	1.10±0.12 fg
	25	4.05±0.24e	3.77±0.08e	7.82±0.07d	0.49±0.009i	0.87±0.018 h	1.36±0.15e
	50	3.47±0.21 fg	3.03±0.06 g	6.50±0.07f	0.42±0.007j	0.79±0.016i	1.21±0.14ef
	75	2.76±0.17i	2.79±0.06 h	5.55±0.06 g	0.38±0.006k	0.65±0.013k	1.00±0.11gh
	100	2.12±0.13k	2.22±0.05i	4.34±0.05 h	0.29±0.004mn	0.52±0.010 m	0.81±0.09ij
150	0	1.71±0.10j-l	1.14±0.03j	2.85±0.03i	0.31±0.005 m	0.65±0.013k	0.96±0.11gh
	25	1.56±0.09k-m	0.99±0.02jk	2.55±0.02j	0.26±0.004no	0.59±0.012kl	0.85±0.10hi
	50	1.33±0.08lm	0.92±0.02k	2.25±0.02k	0.24±0.004op	0.54±0.011lm	0.78±0.09ij
	75	1.06±0.06 m	0.84±0.02kl	1.91±0.02 l	0.22±0.004p	0.48±0.010 m	0.70±0.08jk
	100	0.91±0.05n	0.78±0.02 l	1.69±0.01m	0.18±0.003q	0.39±0.008n	0.57±0.07k

The means with same Duncan's multiple range test letters in each column are not statistically significant ($P \leq 0.01$). Data are average of four replications $\pm$ SE.

Table 2. Effect of Gamma pretreatment on germination indices of canola seedlings under salt stress conditions

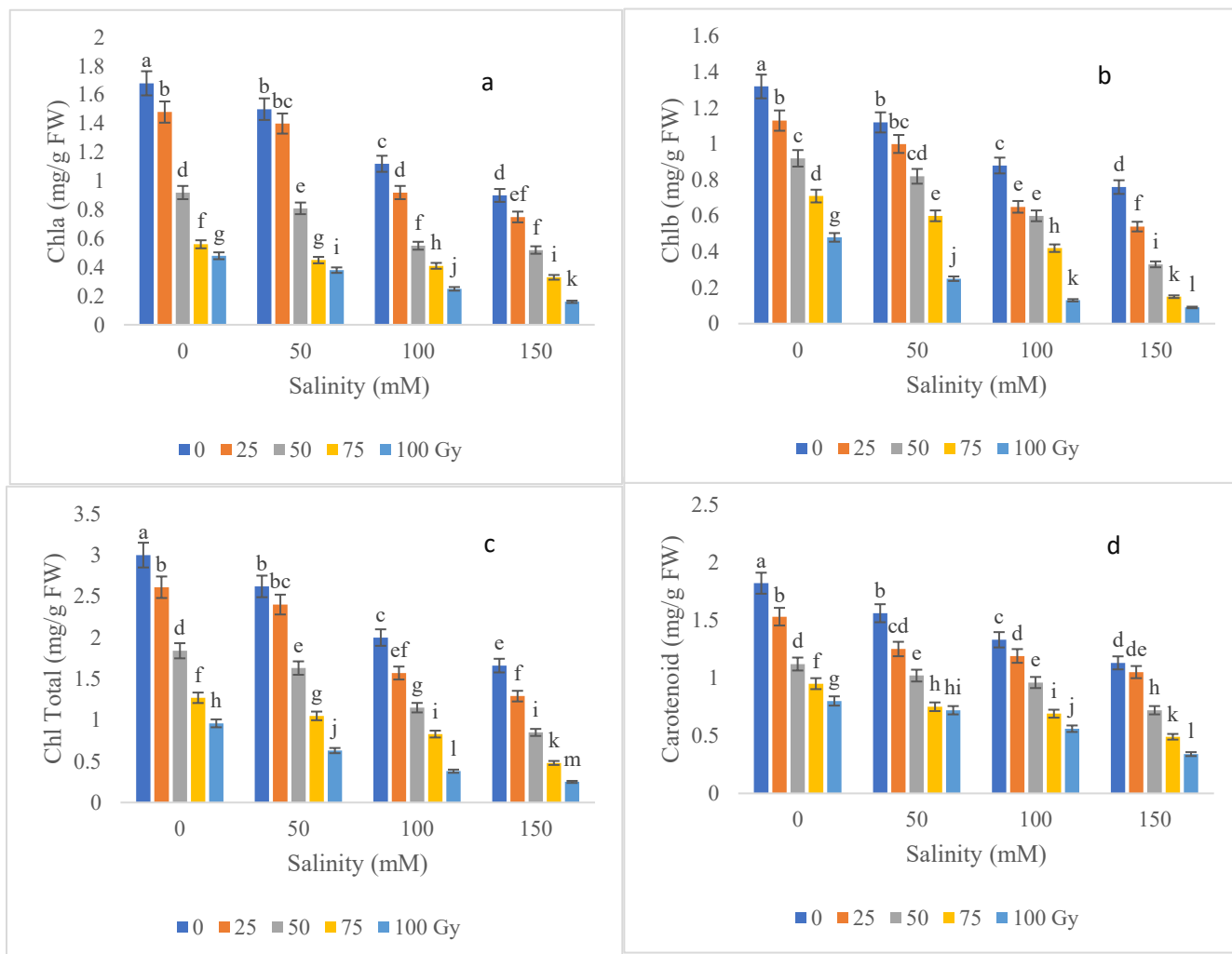
Salinity (mM)	Gamma (Gy)	MGT (days)	GR (seeds/day)	VI
0	0	2.49±0.07 l	0.63±0.016a	1098.7±0.17a
	25	3.01±0.08kl	0.59±0.014b	875.9±0.15b
	50	4.05±0.10i	0.46±0.013bc	641.5±0.13c
	75	5.08±0.12 g	0.32±0.012de	389.6±0.10e
	100	7.40±0.16e	0.26±0.009 g-i	201.6±0.09
50	0	3.91±0.09 h	0.52±0.011e	491.1±0.14
	25	3.52±0.08i	0.55±0.013cd	632.6±0.13c
	50	5.09±0.12 g	0.29±0.011e-g	441.5±0.10d
	75	6.39±0.16e	0.26±0.010 g-i	254.2±0.08f
	100	8.06±0.19c	0.21±0.008jk	126.7±0.08
100	0	4.53±0.11 h	0.35±0.010f-h	361.9±0.08e
	25	4.07±0.10i	0.42±0.011ef	414.3±0.07d
	50	5.90±0.14f	0.20±0.009 g-i	210.0±0.06f
	75	7.40±0.18d	0.17±0.008ij	170.2±0.05 g
	100	9.33±0.23b	0.11±0.007kl	87.5±0.04 h
150	0	4.58±0.11 h	0.38±0.010f-h	125.4±0.06 g
	25	5.10±0.13 g	0.25±0.009hi	85.8±0.05 h
	50	6.64±0.16e	0.13±0.009ij	71.3±0.04i
	75	8.33±0.20c	0.12±0.008jk	45.8±0.03j
	100	11.50±0.26a	0.08±0.006 l	13.7±0.03k

The means with same Duncan's multiple range test letters in each column are not statistically significant ($P \leq 0.01$). Data are average of four replications $\pm$ SE.

Photosynthetic Pigments

Photosynthetic pigment concentrations were also significantly influenced by both salinity stress and gamma irradiation. The chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid contents progressively decreased as salinity increased, indicating substantial impairment of the photosynthetic apparatus during early seedling growth. The reduction was evident across all the pigment classes and became particularly severe at 150 mM NaCl, where the pigment concentrations reached their minimum values (Fig. 2).

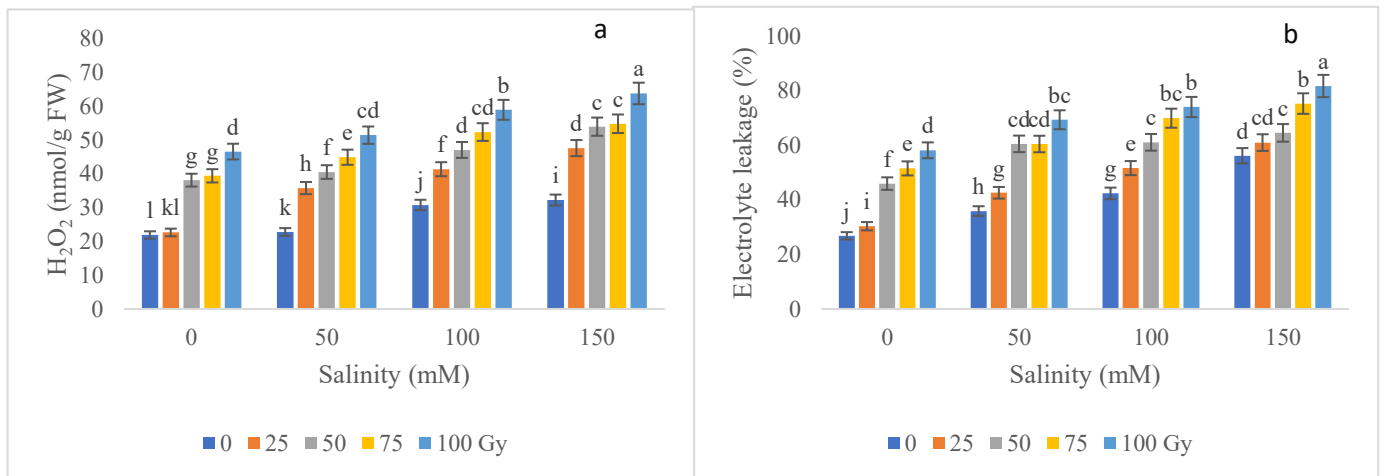
Gamma irradiation substantially modified pigment responses depending on the irradiation dose. Pretreatment with 25 and especially 50 Gy effectively preserved the chlorophyll and carotenoid contents under saline conditions, resulting in consistently higher pigment concentrations than those observed in untreated seedlings. The seedlings receiving 50 Gy generally presented the greatest chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid contents across all the salinity treatments, indicating superior maintenance of their photosynthetic capacity. In contrast, irradiation doses of 75 and 100 Gy failed to preserve pigment concentrations (Fig 2).



Both electrolyte leakage (EL) and hydrogen peroxide (H_2O_2) accumulation were significantly influenced by salinity level, gamma irradiation dose, and their interaction. As the NaCl concentration increased from 0 to 150 mM, both oxidative stress indicators increased progressively. Conversely, gamma irradiation clearly influenced oxidative stress in a dose-dependent manner. Pretreatment with low irradiation doses, particularly 25 and 50 Gy, significantly alleviated oxidative injury under saline conditions, whereas higher doses (75 and 100 Gy) failed to provide comparable protection and, in several cases, intensified oxidative damage (Fig 3).

Hydrogen peroxide accumulation exhibited a strong positive relationship with salinity severity. Under nonsaline conditions, the seedlings maintained relatively low H_2O_2 concentrations regardless of irradiation treatment, reflecting a balanced intracellular redox state. However, increasing salinity progressively increased H_2O_2 production, reaching its maximum level at 150

mM NaCl. Gamma pretreatment effectively moderated this response within the optimal irradiation range. Seedlings originating from seeds irradiated with 25 and particularly 50 Gy consistently accumulated less hydrogen peroxide than did untreated seedlings exposed to the same salinity level. This reduction became increasingly evident under moderate and severe salinity, where the difference between low-dose irradiated and untreated plants was greatest. Consistently lower H_2O_2 concentrations were observed after 50 Gy pretreatment (Fig 3a). The protective effect of 50 Gy was particularly evident under moderate and severe salinity, where membrane injury was substantially lower than that in untreated controls. In contrast, seedlings treated with 75 or 100 Gy exhibited electrolyte leakage values approaching those of severely stressed nonirradiated seedlings (Fig 3b).

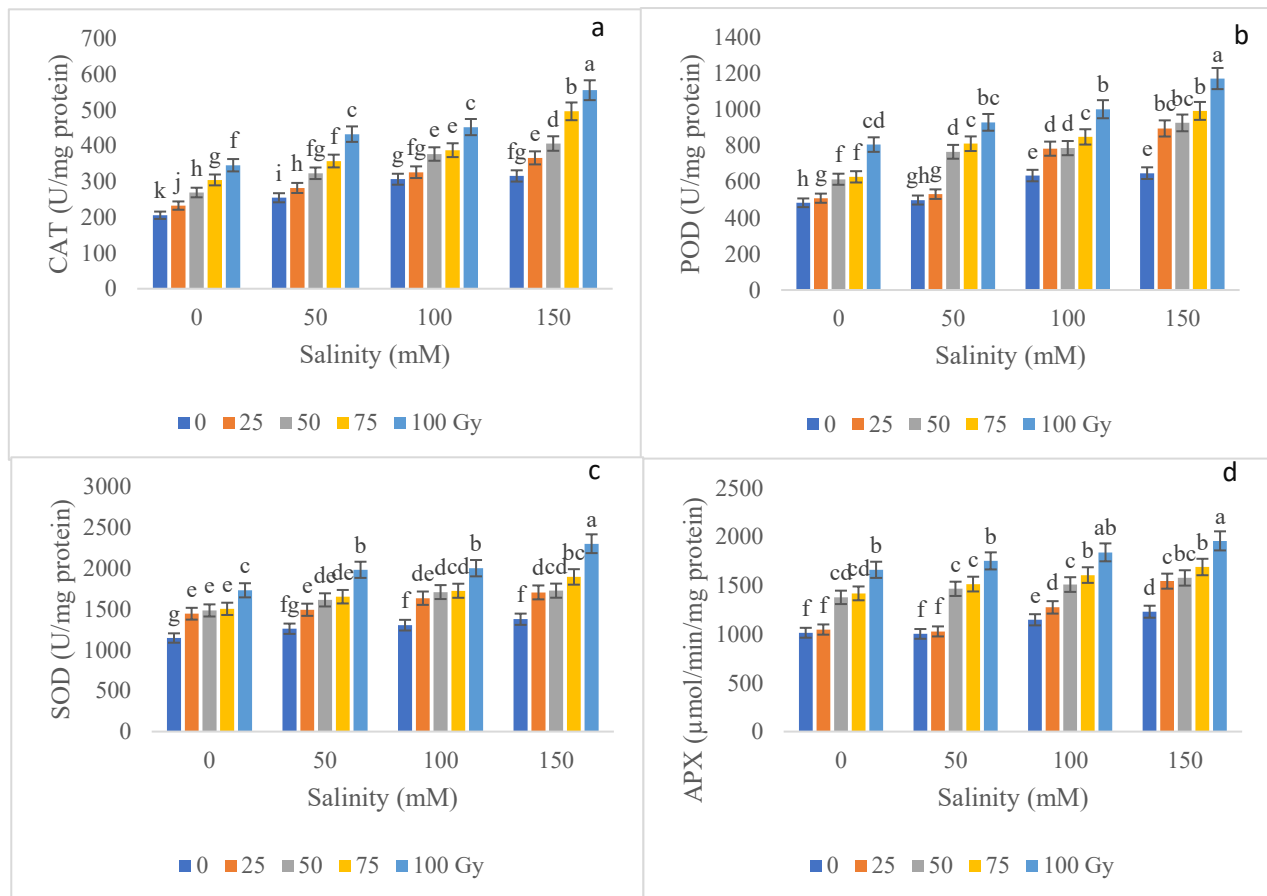


Antioxidant enzyme activity

The enhanced protection against oxidative damage was accompanied by significant changes in antioxidant enzyme activity. The activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and ascorbate peroxidase (APX) were significantly affected by salinity stress and gamma irradiation. Salinity alone stimulated the activities of all four enzymes, indicating the activation of the enzymatic antioxidant defense system in response to elevated ROS production. Enzyme activities generally increased progressively with increasing NaCl concentration and reached their highest levels under severe salinity (Fig. 4).

The catalase activity increased markedly with increasing salinity and was further enhanced by Gamma pretreatment. Although untreated seedlings responded to salinity by increasing CAT

activity, seedlings pretreated with 25 and 50 Gy consistently presented significantly greater enzyme activity at each salinity level. Among all the treatments, 50 Gy resulted in the highest CAT activity, particularly under 100 and 150 mM NaCl, indicating that this irradiation dose effectively increased hydrogen peroxide detoxification (Fig. 4a). The peroxidase activity exhibited a similar trend. The POD activity increased gradually with increasing salinity and reached its maximum under severe salt stress. Gamma pretreatment further amplified this response, particularly following exposure to 25 and 50 Gy. Seedlings receiving 50 Gy consistently presented the greatest POD activity across the salinity gradient, whereas high-dose irradiation produced considerably smaller increases (Fig. 4b). SOD also responded strongly to the combined effects of salinity and irradiation. Salinity alone stimulated SOD activity, reflecting increased production of superoxide radicals during oxidative stress. However, pretreatment with low Gamma doses substantially amplified this response. The seedlings irradiated with 50 Gy presented the highest SOD activity throughout the experiment (Fig. 4c). A comparable pattern was observed for ascorbate peroxidase activity. APX activity increased significantly with increasing salinity, and gamma irradiation further stimulated enzyme activity within the optimal irradiation range. Once again, 50 Gy consistently produced the greatest APX activity under saline conditions, whereas treatments receiving 75 or 100 Gy resulted in considerably lower enzyme activities (Fig. 4d).

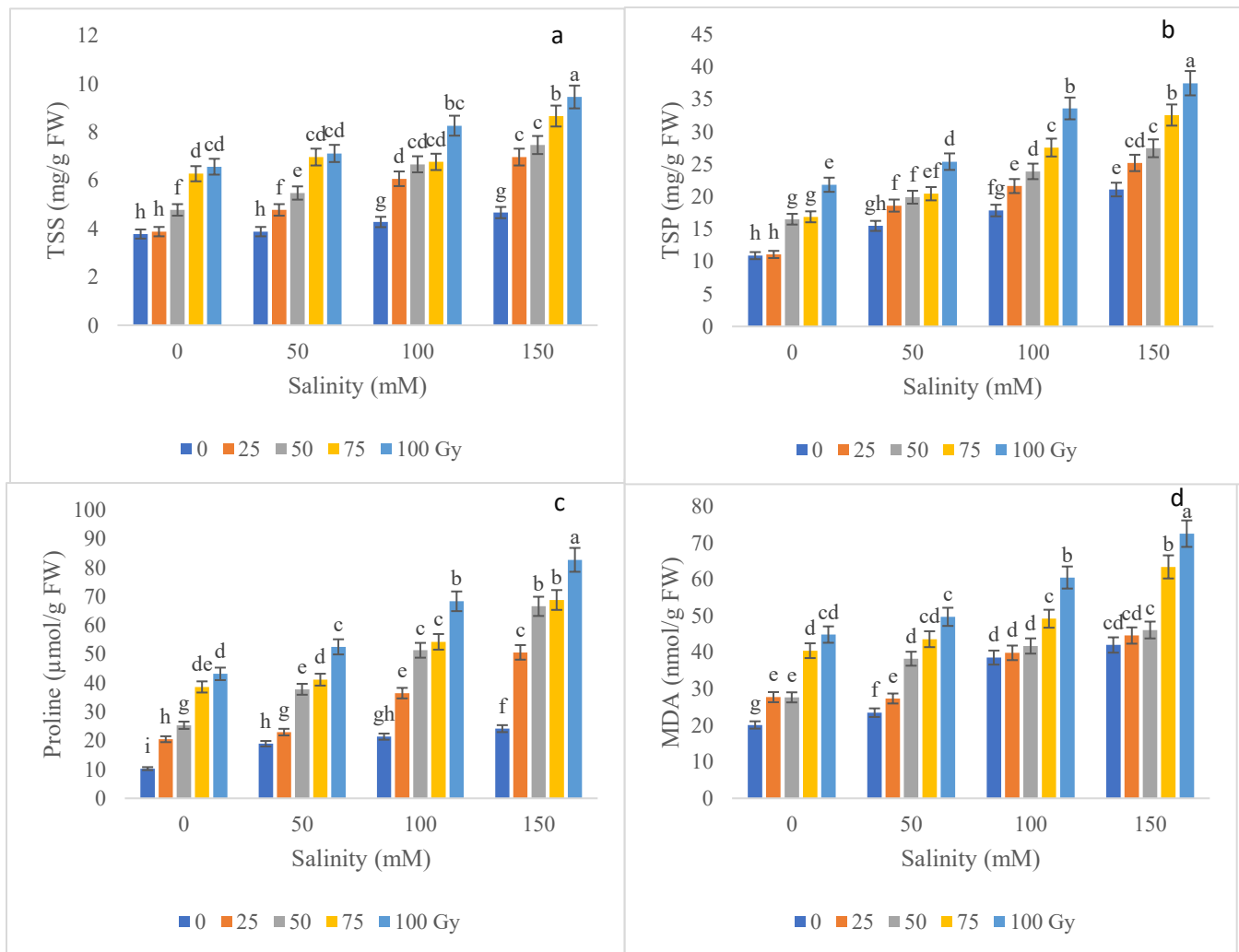


Biochemical assay

In addition to antioxidant defense, salinity induced substantial changes in several biochemical constituents associated with osmotic adjustment and stress tolerance. The total soluble sugars (TSSs), total soluble proteins (TSPs), proline, and malondialdehyde (MDA) all responded significantly to salinity and gamma irradiation, although the direction of their responses differed according to their physiological functions (Fig. 5).

The accumulation of compatible osmolytes increased progressively with increasing salinity. The TSSs gradually increased from nonsaline conditions to 150 mM NaCl. Gamma pretreatment significantly increased sugar accumulation within the optimal irradiation range. Seedlings treated with 25 and particularly 50 Gy consistently accumulated greater quantities of soluble sugars than did untreated seedlings under identical salinity conditions. This increase was most pronounced under severe salinity, where 50 Gy resulted in the highest TSSs concentration among all the treatments (Fig. 5a). The TSPs content exhibited a similar pattern. Protein accumulation increased steadily as salinity increased and was further stimulated by low-dose gamma irradiation. The greatest TSPs concentrations were consistently recorded in seedlings receiving 50 Gy, whereas irradiation doses of 75 and 100 Gy resulted in comparatively smaller increases (Fig. 5b).

Proline accumulation presented one of the strongest responses to salinity. Its concentration increased dramatically with increasing NaCl concentration, reflecting the activation of osmotic adjustment mechanisms under stress. Gamma irradiation further increased proline accumulation, particularly at 25 and 50 Gy. The greatest proline concentrations were consistently observed under severe salinity following 50 Gy pretreatment, whereas higher irradiation doses produced comparatively weaker responses (Fig. 5c). Unlike osmoprotective metabolites, malondialdehyde also increased progressively with increasing salinity but responded inversely to optimal gamma irradiation. Nonirradiated seedlings accumulated progressively greater quantities of MDA as the salt concentration increased, confirming enhanced membrane lipid oxidation during oxidative stress. Pretreatment with 25 and especially 50 Gy significantly reduced MDA accumulation across all salinity levels (Fig. 5d).



Multivariate analysis

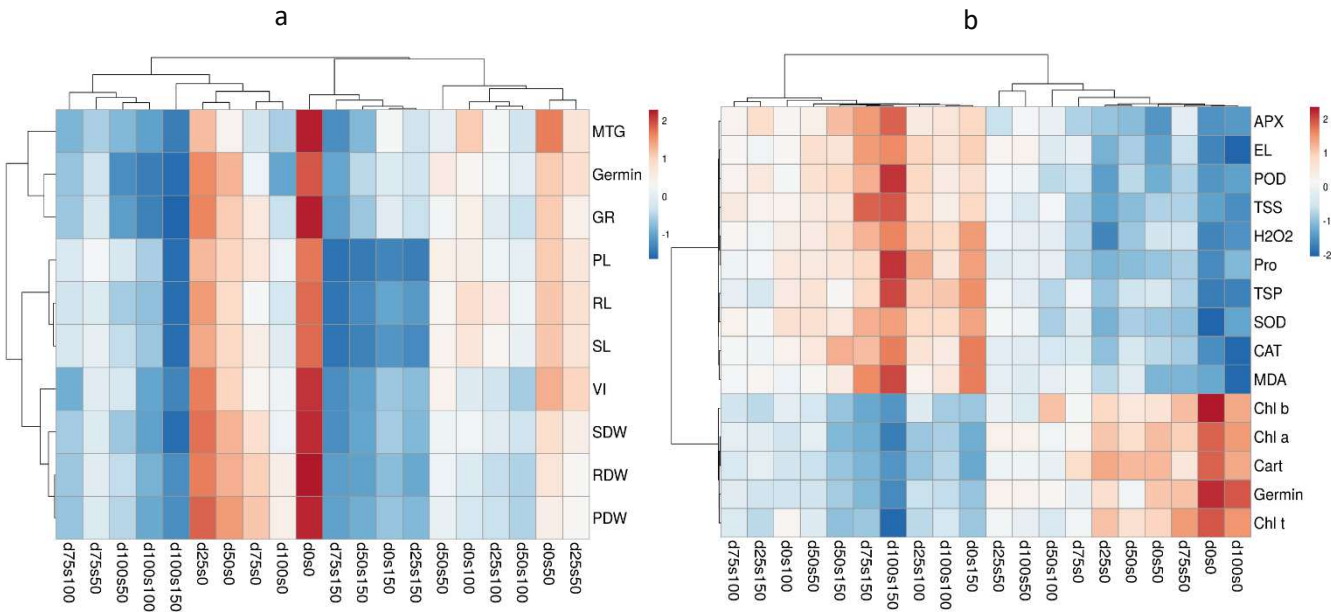
Hierarchical clustering on the basis of germination and morphophysiological traits revealed three well-defined treatment groups, indicating substantial differences in seedling performance among the experimental treatments. The first cluster consisted primarily of nonsaline control treatments together with seedlings originating from seeds exposed to low Gamma irradiation doses (25 and 50 Gy) under nonsaline, mild, and moderate salinity conditions. This cluster was characterized by superior germination performance, rapid seedling establishment, greater root and shoot elongation, greater seedling vigor, and increased biomass accumulation. The close associations among these treatments indicate that low-dose Gamma irradiation successfully maintained normal growth even under moderate salinity, producing response patterns comparable to those observed under favorable environmental conditions. In contrast, the second cluster included seedlings exposed to severe salinity (150 mM NaCl) without Gamma pretreatment. These treatments were characterized by poor germination performance, prolonged mean germination time, reduced seedling growth,

and markedly lower biomass accumulation. Furthermore, they presented elevated proline accumulation, reflecting a pronounced stress response. The separation of these treatments from the favorable growth cluster illustrates the severe detrimental impact of high salinity on early seedling establishment (Fig. 6a).

The third cluster consisted primarily of seedlings receiving high gamma irradiation doses (75 and 100 Gy) under saline conditions. These treatments clustered closely with severely stressed nonirradiated plants rather than with low-dose irradiated plants, indicating that excessive gamma exposure failed to mitigate the detrimental effects of salinity. Instead, high irradiation doses produced physiological responses characteristic of stressed seedlings, suggesting that irradiation beyond the optimal range imposed additional metabolic disturbances that limited seedling performance. The clustering pattern therefore provides clear evidence that only low irradiation doses were capable of improving early seedling establishment, whereas excessive doses diminished the beneficial effects of irradiation. The relationships among individual germination and growth variables further supported these treatment groupings. The germination percentage, germination rate, vigor index, root length, shoot length, seedling length, and biomass-related traits clustered closely together, indicating strong positive associations among these variables. Their close proximity suggests that improvements in one growth parameter were generally accompanied by simultaneous improvements in the remaining growth-related characteristics. In contrast, the mean germination time and proline accumulation were positioned separately from these growth traits, reflecting their negative associations with seedling performance. In particular, proline was inversely related to most growth parameters, indicating that increased proline accumulation was associated with greater physiological stress rather than increased growth potential (Fig. 6a).

Hierarchical clustering of physiological and biochemical variables produced a comparable classification pattern. Three major response groups were clearly distinguished according to their biochemical characteristics and oxidative status. The first cluster included nonsaline controls together with seedlings treated with 25 and 50 Gy under low to moderate salinity. These treatments were characterized by relatively high concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids, combined with lower hydrogen peroxide accumulation, reduced electrolyte leakage, and minimal lipid peroxidation. Consequently, these treatments resulted in superior physiological performance and greater tolerance to salinity stress. The second cluster consisted predominantly of severely saline treatments without Gamma pretreatment. These seedlings presented elevated levels of oxidative stress markers, including hydrogen peroxide, electrolyte leakage, and malondialdehyde, together with increased antioxidant enzyme activities and increased accumulation of osmoprotective compounds. Moreover, the photosynthetic pigment concentrations markedly decreased. This clustering pattern reflects the substantial metabolic adjustments required for seedlings to survive under severe salinity, where the activation of antioxidant defense mechanisms occurs concurrently with increased oxidative damage. The third cluster included seedlings exposed to higher irradiation doses under saline conditions. These treatments closely resembled severely stressed controls, which presented persistent oxidative stress, reduced pigment concentrations, and relatively high levels of oxidative damage. The proximity of these plants to severe saline controls further confirmed that excessive irradiation did not effectively protect the photosynthetic apparatus or cellular membranes against salt-induced

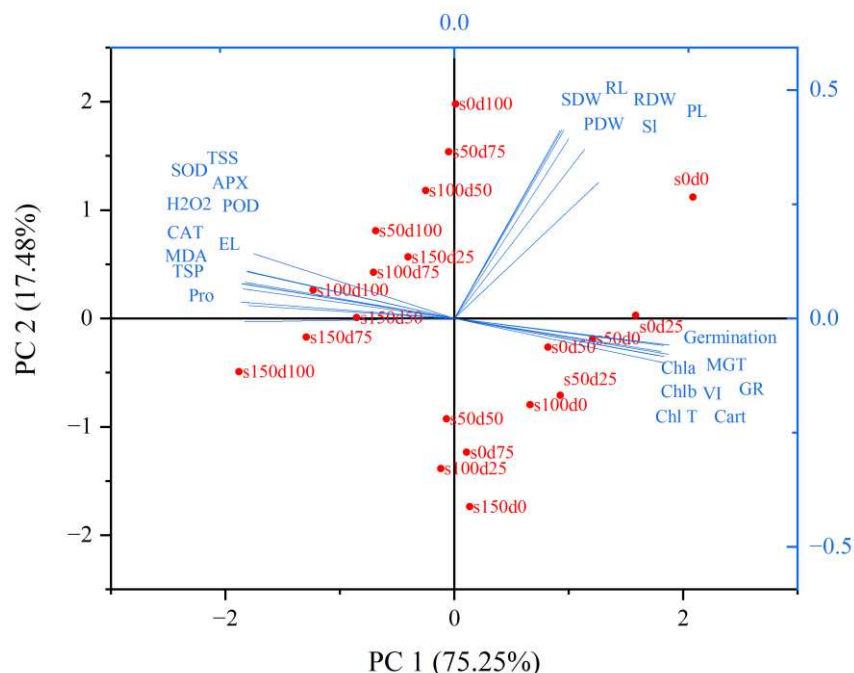
injury. Moreover, antioxidant enzymes and osmoprotective metabolites clustered closely with oxidative stress indicators, whereas photosynthetic pigments formed a distinct group that was negatively associated with oxidative damage. This distribution clearly demonstrated the antagonistic relationship between photosynthetic efficiency and oxidative stress during salinity exposure (Fig. 6b).



Principal component analysis further clarified the relationships among the treatments and measured variables. The first two principal components explained 92.73% of the total experimental variation, indicating that these components adequately represented the overall response of canola seedlings to salinity stress and gamma irradiation. Principal component one (PC1) accounted for 75.25% of the total variance, whereas principal component two (PC2) explained an additional 17.48%, together providing excellent discrimination among treatment combinations (Fig. 7).

PC1 primarily separated growth- and photosynthesis-related traits from oxidative stress- and defense-associated variables. The germination percentage, germination rate, vigor index, root length, shoot length, seedling biomass, chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid contents presented strong positive loadings on PC1, indicating that these characteristics contributed substantially to superior seedling performance. In contrast, hydrogen peroxide, electrolyte leakage, malondialdehyde, proline, total soluble sugars, total soluble proteins, and antioxidant enzyme activities (CAT, POD, SOD, and APX) were negatively associated with this component. The opposite orientation of these vectors demonstrates a strong inverse relationship between plant growth and physiological stress responses. Principal component two primarily reflected secondary differences among treatments related to the magnitude of biochemical

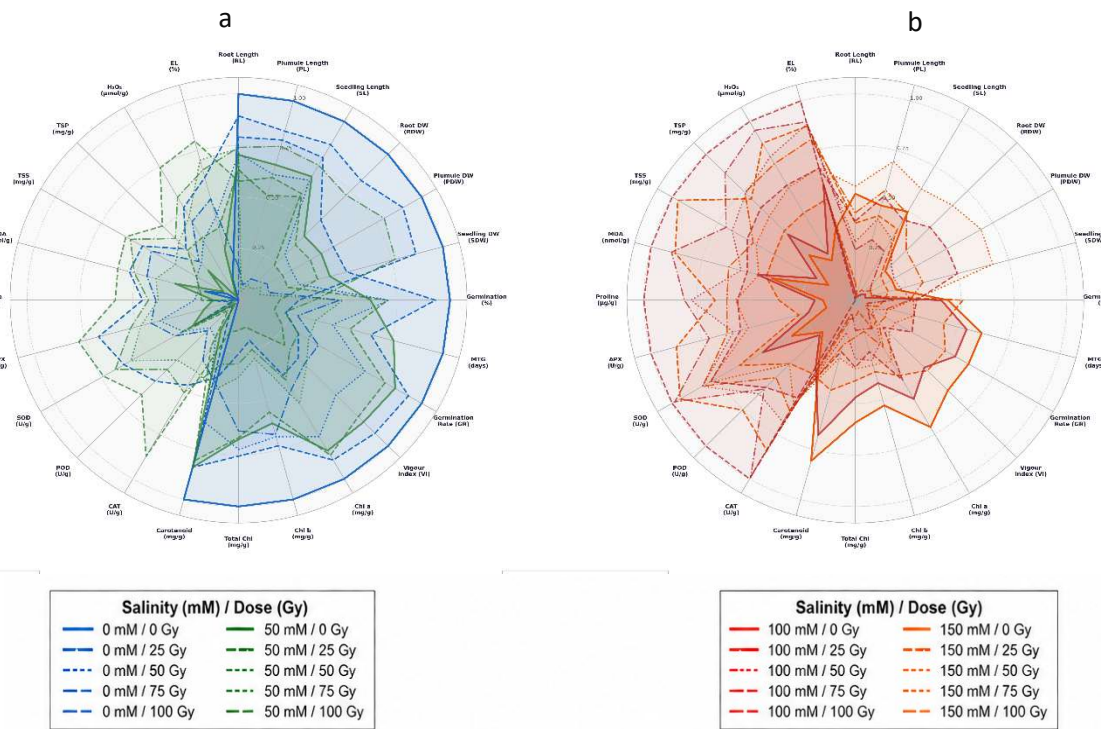
adjustments rather than overall stress severity. Although PC2 contributed less to the total variation than PC1 did, it further separated intermediate treatments according to their antioxidant capacity and osmotic adjustment responses, providing additional discrimination among irradiation doses under similar salinity conditions (Fig. 7).



Radar plot analysis provided a comprehensive visualization of the integrated responses of all the measured variables simultaneously. Under nonsaline conditions and mild salinity (0–50 mM NaCl), seedlings pretreated with 25 and particularly 50 Gy exhibited broad and symmetrical radar profiles characterized by high germination capacity, vigorous seedling growth, greater biomass accumulation, elevated chlorophyll concentrations, and relatively low oxidative stress indicators. These expanded profiles indicate balanced physiological performance across multiple traits and illustrate the overall improvement induced by optimal Gamma pretreatment. As the salinity increased to 100 and 150 mM NaCl, the radar profiles decreased markedly, particularly for growth-related and photosynthetic traits, whereas the levels of oxidative stress indicators, antioxidant enzyme activities, and osmoprotective metabolites increased considerably. This pattern reflects the shift from normal physiological growth to stress-defense mechanisms as environmental stress intensifies. Nevertheless, seedlings treated with 50 Gy consistently maintained broader radar profiles than did untreated or highly irradiated seedlings under equivalent salinity conditions. The larger profile area indicates that optimal gamma irradiation simultaneously preserved several physiological processes rather than improving only individual traits. In contrast, seedlings exposed to 75 and 100 Gy irradiation presented irregular and contracted radar profiles under saline conditions. Although some antioxidant responses remained elevated, these treatments failed to maintain balanced physiological performance because reductions in growth, biomass, and photosynthetic pigments outweighed the activation of defense mechanisms. Consequently, their

radar profiles closely resembled those of severely stressed nonirradiated seedlings, further confirming the limited protective value of excessive irradiation (Fig 8).

Overall, the multivariate analyses consistently demonstrated that treatment effects were highly coordinated across all measured characteristics. Regardless of the statistical approach employed, seedlings receiving 25–50 Gy, particularly 50 Gy, clustered with favorable physiological and morphological traits, whereas severe salinity and excessive irradiation were consistently associated with oxidative damage, impaired photosynthetic performance, and reduced growth.



Discussion

Salinity is one of the major abiotic stresses limiting seed germination and early seedling establishment in canola (*Brassica napus* L.). The present findings indicate that low-dose gamma irradiation, particularly 25 and 50 Gy, alleviated the detrimental effects of salinity during early growth, suggesting that gamma irradiation enhances salt tolerance through coordinated physiological and biochemical adjustments.

The inhibition of germination and seedling growth under salinity agrees with previous reports in canola and other Brassicaceae species [19; 20; 1]. Reduced osmotic potential together with excessive Na⁺ and Cl⁻ accumulation restricts water uptake, disturbs ion homeostasis, and suppresses metabolic processes required for germination and seedling development [21]. Similar responses have been reported in Brassica species, highlighting the high sensitivity of the germination stage to salinity despite the moderate salt tolerance of canola [22].

The beneficial effects of gamma irradiation were strongly dose dependent. While 25 and 50 Gy improved seedling performance under both saline and non-saline conditions, higher doses (≥ 100 Gy) were ineffective or inhibitory, reflecting the hormetic nature of low-dose irradiation [23]. Mild radiation-induced ROS production can act as a signaling stimulus that activates defense pathways without causing irreversible damage, thereby enhancing stress preparedness [17].

Salinity-induced reductions in chlorophyll and carotenoid contents are consistent with reports linking salt stress to chloroplast disruption and oxidative degradation of photosynthetic pigments [20]. The recovery of pigment levels following low-dose irradiation suggests improved protection of the photosynthetic apparatus, likely through enhanced antioxidant capacity and preservation of chloroplast integrity [24; 25].

Oxidative stress represents a major consequence of salinity. Increased H₂O₂, MDA, and electrolyte leakage reflected ROS-induced membrane damage, whereas gamma pretreatment markedly reduced these indicators, especially at 50 Gy. This protection was accompanied by enhanced activities of CAT, SOD, POD, and APX, which collectively maintain cellular redox homeostasis through efficient ROS detoxification. Similar stimulation of antioxidant metabolism by low-dose gamma irradiation has been reported in wheat, maize, and other crops supporting the hypothesis that irradiation primes antioxidant defense before stress exposure [26; 17].

Besides antioxidant protection, improved osmotic adjustment also contributed to salinity tolerance. The enhanced accumulation of proline, soluble sugars, and soluble proteins following gamma irradiation is consistent with their established roles in osmotic regulation, membrane stabilization, and stress adaptation [11; 27]. Similar responses have been observed in previous studies of radiation-induced stress tolerance, indicating that gamma priming reinforces multiple protective pathways simultaneously [26; 28].

Cluster analysis further supported these physiological responses by grouping seedlings treated with 25–50 Gy under mild to moderate salinity separately from severely stressed treatments. The negative association between proline and growth traits suggests that excessive proline accumulation primarily reflects stress severity rather than improved performance, whereas the

close association among germination and growth traits indicates coordinated protection following optimal gamma pretreatment.

Overall, the results support a model in which low-dose gamma irradiation functions as a priming stimulus that activates antioxidant defense, osmolyte accumulation, and stress-responsive metabolic pathways through transient ROS signaling [29; 30]. These responses preserve membrane integrity and photosynthetic capacity while improving osmotic regulation, ultimately enhancing germination and early seedling establishment under saline conditions. This interpretation is consistent with increasing evidence supporting radiation-induced hormesis as a practical strategy for improving crop stress tolerance [31], including reports in wheat, potato, and *Chlorella sorokiniana* [26; 32; 23].

Collectively, these findings demonstrate that low-dose gamma irradiation functions as an effective seed-priming strategy capable of increasing salinity tolerance through integrated physiological and biochemical mechanisms. Therefore, gamma irradiation at 25–50 Gy, especially 50 Gy, represents a promising, environmentally friendly, and nonchemical approach for improving canola establishment under saline conditions. Future studies should validate these findings in greenhouse and field environments and further investigate the molecular and genetic mechanisms underlying gamma irradiation-induced stress tolerance to facilitate its practical application in sustainable crop production.

Material and Methods

Plant material and experimental conditions

Seeds of the canola cultivar Okapi (*Brassica napus* L. cv. Okapi) were obtained from the Seed and Plant Improvement Institute, Karaj, Iran. Thirty seeds were placed in each Petri dish lined with sterile filter paper, and the appropriate NaCl solutions were added according to the experimental treatments. The Petri dishes were incubated in a germination chamber at $25 \pm 1^\circ\text{C}$ under a 16-h light/8-h dark photoperiod to provide uniform conditions for seed germination and seedling growth. For the determination of morphological traits, photosynthetic pigments, oxidative stress indicators, antioxidant enzyme activities, and other biochemical parameters, the seedlings were sampled 14 days after the initiation of germination. At the sampling stage, seedlings from each experimental unit were harvested and immediately used for the respective biochemical and physiological analyses.

Experimental design and treatment

In this experiment, 4 levels of salinity stress (0, 50, 100, and 150 mM) with saline solution and 5 levels of gamma ray pretreatment (0, 25, 50, 75, and 100 Gy) were used. The experiment was conducted as a factorial in a completely randomized design (CRD) with four replications. Salinity stress treatment was applied by irrigating the seeds with water containing different concentrations of salt solution, and irrigation was carried out in the same manner until the end of the experiment. To prevent possible salt accumulation, the filter papers were replaced every three days. Seeds were surface-sterilized by immersion in 70% (v/v) ethanol for 1 min, followed by 1% (w/v) sodium hypochlorite solution for 10 min, thoroughly rinsed three to five times with sterile distilled water and then used for the experiment.

Gamma irradiation

Uniform seeds were randomly divided into two experimental groups: (1) nonirradiated control seeds and (2) seeds subjected to gamma irradiation treatment. Seeds were irradiated using a Cobalt-60 (^{60}Co) gamma irradiator at the Nuclear Agriculture Research School, Nuclear Science and Technology Research Institute (NSTRI), Atomic Energy Organization of Iran. Irradiation was performed at a dose rate of approximately $77.35 \text{ mGy min}^{-1}$, with the samples positioned at the calibrated irradiation distance determined by the facility to deliver the required absorbed dose uniformly. Following irradiation, the seeds were stored under ambient laboratory conditions for 24 h and subsequently used for germination or planting experiments. In this study four different radiation doses, 25, 50, 75, and 100 Gy, were applied.

Germination indices

At the end of the experiment, the germination percentage (GP), mean germination time (MGT), germination rate (GR) and seedling vigor index (SVI) were calculated. Germination tests were completed in 14 days by applying ISTA rules [33].

$$\text{GP (\%)} = \frac{\text{number of germinated seeds}}{\text{total number of seeds}} \times 100 \text{ [34].}$$

$$\text{GR} = \frac{\sum n_i}{t_i} \text{ [}\sum n_i \text{ = Total number of germinated seeds, and } t_i \text{ = Number of days after germination] [34].}$$

$$\text{MGT: } \frac{\sum D_n}{\sum n} \text{ D = days counted from the beginning of the test, n = number of seeds germinated on day D [35].}$$

$$\text{VI} = [\text{Seedling length (cm)} \times \text{GP (\%)}] \text{ [36].}$$

Photosynthetic Pigments

Photosynthetic pigments were determined following Lichtenthaler and Buschmann (2001). Fresh leaf tissue (0.1 g) was homogenized in 5 mL of 80% acetone and centrifuged at $10,000 \times g$ for 10 min at 4°C . The absorbance of the supernatant was read at 663, 645, and 470 nm. The chlorophyll

a, chlorophyll b, total chlorophyll, and carotenoid contents were calculated via standard formulas and expressed as mg/g fresh weight [37].

Oxidative stress markers

Electrolyte leakage (EL): Electrolyte leakage was measured following Lutts et al. (1996) [38]. Fresh leaf tissue (0.2 g) was washed and incubated in 15 mL of deionized water at room temperature for 24 h. The initial conductivity (EC_1) was recorded, the samples were autoclaved (121°C, 20 min), and the final conductivity (EC_2) was measured. EL (%) was calculated as $(EC_1/EC_2) \times 100$. All measurements were performed in triplicate.

Hydrogen peroxide (H_2O_2): The hydrogen peroxide (H_2O_2) content was quantified via the potassium iodide assay. Fresh plant samples (0.5 g) were homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) under chilled conditions, followed by centrifugation at $10,000 \times g$ for 10 minutes at 4°C. The resulting supernatant was then neutralized to pH 7 via 10 mM potassium phosphate buffer. Subsequently, 1 mL of the neutralized extract was combined with 1 mL of 1 M potassium iodide (KI) and 0.1 mL of 10 mM potassium phosphate buffer (pH 7.0), vortexed thoroughly, and incubated in darkness at ambient temperature for 20 minutes. Absorbance readings were taken at 350 nm via spectrophotometry. The H_2O_2 concentration was determined by reference to a standard calibration curve generated from known H_2O_2 concentrations ranging from 0 to 50 μM and expressed as micromoles per gram of fresh weight. All measurements were performed in triplicate, in accordance with the methodology described by Junglee et al. (2014) [39].

Antioxidant enzyme activity

Fresh plant tissues (0.5 g) were homogenized in cold extraction buffer according to the method of Bradford (1976), and the resulting homogenate was centrifuged at $12,000 \times g$ for 20 min at 4°C. The supernatant was collected and used for the determination of antioxidant enzymes activity [40].

Catalase (CAT): Catalase activity was determined following the protocol established by Aebi (1984) [41]. Fresh plant tissue (0.5 g) was homogenized in 5 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, and the homogenate was centrifuged at $10,000 \times g$ for 10 minutes at 4°C to obtain the enzyme extract. The reaction mixture, with a total volume of 3 mL, contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM hydrogen peroxide (H_2O_2), and 100 μL of the enzyme extract. The decomposition of H_2O_2 was monitored by measuring the decrease in absorbance at 240 nm over a 1-minute interval. Catalase activity was calculated using an extinction coefficient of $39.4 \text{ ppm}^{-1} \text{ cm}^{-1}$. All the assays were conducted in triplicate to ensure reproducibility.

Peroxidase (POD): Peroxidase activity was determined following the methodology described by Chance and Maehly (1955) [42]. Briefly, 0.5 g of plant tissue was homogenized in 5 mL of 50 mM potassium phosphate buffer (pH 7.0) supplemented with 1 mM EDTA and 1% polyvinylpyrrolidone (PVP). The homogenate was then centrifuged at $12,000 \times g$ for 15 minutes at 4°C, and the resulting supernatant was collected for analysis. The enzymatic reaction mixture (3 mL total volume) consisted of 50 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol,

10 mM hydrogen peroxide (H_2O_2), and 50 μL of the enzyme extract. The increase in absorbance at 470 nm, corresponding to the formation of tetraguaiacol, was monitored over a 1-minute interval. POD activity was quantified via an extinction coefficient of $26.6 \text{ ppm}^{-1} \text{ cm}^{-1}$. All the assays were conducted in triplicate to ensure reproducibility.

Superoxide Dismutase (SOD): Superoxide dismutase activity was determined following the method described by Giannopolitis and Ries (1977) [43]. The plant tissue samples (0.5 g) were homogenized in 5 mL of 50 mM potassium phosphate buffer (pH 7.8) supplemented with 0.1 mM EDTA and 1% polyvinylpyrrolidone (PVP). The homogenate was then centrifuged at $15,000 \times g$ for 20 minutes at 4°C , and the resulting supernatant was collected for analysis. The reaction mixture, with a total volume of 3 mL, contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 0.1 mM EDTA, 2 μM riboflavin, and 50–100 μL of the enzyme extract. The samples were exposed to fluorescent light at an intensity of 4000 lux for 15 minutes, after which the absorbance was measured at 560 nm. One unit of SOD activity was defined as the quantity of enzyme required to inhibit 50% of NBT photoreduction. All the assays were performed in triplicate to ensure reproducibility.

Ascorbate peroxidase (APX): Ascorbate peroxidase activity was assessed according to the method described by Nakano and Asada (1981) [44]. Fresh plant tissue samples (0.5–1 g) were homogenized in 5 mL of 50 mM potassium phosphate buffer (pH 7.0) supplemented with 1 mM EDTA, 1% polyvinylpyrrolidone (PVP), and 1 mM ascorbic acid. The homogenate was then centrifuged at $12,000 \times g$ for 15 minutes at 4°C to obtain the enzyme extract. The enzymatic reaction mixture (3 mL) comprised 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM hydrogen peroxide (H_2O_2), and 100 μL of the enzyme extract. The oxidation of ascorbate was monitored by measuring the decrease in absorbance at 290 nm over a 1-minute interval via a spectrophotometer. APX activity was calculated utilizing an extinction coefficient of $2.8 \text{ ppm}^{-1} \text{ cm}^{-1}$. All the assays were conducted in triplicate to ensure reproducibility.

Biochemical assay

Total soluble sugars (TSSs): The soluble sugar content was quantified via the phenol–sulfuric acid assay as described by Chow and Landhäusser (2004) [45]. Fresh plant tissue samples (0.5 g) were homogenized in 5 mL of 80% ethanol, followed by incubation at 80°C for 30 minutes. The homogenates were subsequently centrifuged at $10,000 \times g$ for 10 minutes at 4°C , after which the supernatants were collected. The residual pellets were subjected to two additional extractions under identical conditions. The combined supernatants were then evaporated to dryness under vacuum at 40°C and reconstituted in 5 mL of distilled water. For the assay, aliquots of 100 μL were combined with 1 mL of 2% phenol solution and 5 mL of concentrated sulfuric acid, vortexed thoroughly, and incubated at ambient temperature for 30 minutes. Absorbance readings were taken at 490 nm using a spectrophotometer, with glucose serving as the calibration standard. The soluble sugar concentrations were determined from the standard curve and expressed as milligrams of glucose equivalents per gram of fresh weight. All the measurements were conducted in triplicate to ensure reproducibility.

Total soluble protein (TSPs): The soluble protein content in plant tissues was quantified via the Bradford assay as described by Jones et al. (1989) [46]. Briefly, 100–200 mg of tissue was homogenized in 0.1 N NaOH (1:10 w/v), incubated for 30 min, and centrifuged at $10,000 \times g$ for 10 min at 4°C. The supernatant (100 µL) was mixed with Bradford reagent (diluted 1:4 with water containing 3 mg/mL PVP), incubated for 5 min, and the absorbance was measured at 595 nm. The protein concentration was determined from a bovine serum albumin standard curve (0–20 µg/mL) and expressed as mg/g fresh weight.

Proline: Proline concentration was determined following the method described by Bates et al. (1973) [47]. Fresh plant samples (0.5 g) were homogenized in 10 mL of 3% (w/v) sulfosalicylic acid and then centrifuged at $10,000 \times g$ for 10 minutes at 4°C. The resulting supernatant was filtered through Whatman No. 2 filter paper. Two mL of the filtrate was subsequently combined with 2 mL of ninhydrin reagent (prepared by dissolving 1.25 g of ninhydrin in 30 mL of glacial acetic acid and 20 mL of phosphoric acid) and 2 mL of glacial acetic acid. The mixture was thoroughly mixed and incubated in a water bath at 100°C for 1 hour. The reaction was halted by cooling the tubes on ice for 5 minutes, followed by extraction with 4 mL of toluene. A 1 mL aliquot of the toluene layer containing the chromophore was transferred to a new tube, and the absorbance was measured at 520 nm via a spectrophotometer. The proline content was quantified with reference to a standard curve generated with L-proline and expressed as micromoles per gram of fresh weight. All measurements were performed in triplicate.

Malondialdehyde (MDA): MDA content, which serves as a marker for lipid peroxidation, was quantified via the thiobarbituric acid-reactive substances (TBARS) assay as described by Hodges et al. (1999). Fresh plant tissue weighing 0.5 g was homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) while being kept on ice. The homogenate was then centrifuged at $10,000 \times g$ for 10 minutes at 4°C, after which 1 mL of the supernatant was combined with 4 mL of 0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA. This mixture was subjected to heating at 95°C for 30 minutes, subsequently cooled on ice for 5 minutes, and then centrifuged again at $10,000 \times g$ for 10 minutes at 4°C to achieve clarification. The absorbance was measured at 532 nm and adjusted for nonspecific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was determined via an extinction coefficient of $155 \text{ ppm}^{-1} \text{ cm}^{-1}$ and reported as nmol g^{-1} of fresh weight, with the assays conducted in triplicate [48].

Data analysis

The effects of salinity, gamma irradiation, and their interaction were evaluated using two-way ANOVA. Mean comparisons were performed using Duncan's multiple range test at $P < 0.01$. Data normality and homogeneity of variance were checked before analysis. For statistical analysis, SAS 9.4 software was used. Microsoft Excel was used to create the graphs. Radar analysis and PCA were performed by using R studio version R-4.3.0.

Data Availability

Some or all data that support the findings of this study are available from the corresponding author upon reasonable request.

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890 **Authors' contributions**

891 S.M, H.K.S and A.B: Designed and performed the experiments and wrote a draft version of the
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900 The authors declare that all studies comply with the guidelines and laws related to plant ethics in
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Figure legends

Fig. 1. Effects of salinity stress and different doses of gamma radiation on canola germination percentage. The means with the same Duncan's multiple range test letters in each column are not significantly different ($P \leq 0.01$). The data are the average of four replications $\pm$ SE.

Fig. 2. Effects of salinity stress and different doses of gamma radiation on the contents of chlorophyll a (**a**), chlorophyll b (**b**), total chlorophyll (**c**) and carotenoids (**d**) in canola seedlings. The means with the same Duncan's multiple range test letters in each column are not significantly different ($P \leq 0.01$). The data are the average of four replications $\pm$ SE.

Fig. 3. Effects of salinity stress and different doses of gamma radiation on the hydrogen peroxide content (**a**) and electrolyte leakage (**b**) in canola seedlings. The means with the same Duncan's multiple range test letters in each column are not significantly different ($P \leq 0.01$). The data are the average of four replications $\pm$ SE.

Fig. 4. Effects of salinity stress and different doses of gamma radiation on the activities of the enzymes CAT (**a**), POD (**b**), SOD (**c**) and APX (**d**) in canola seedlings. The means with the same Duncan's multiple range test letters in each column are not significantly different ($P \leq 0.01$). The data are the average of four replications $\pm$ SE.

Fig. 5. Effects of salinity stress and different doses of gamma radiation on TSSs (**a**), TSPs (**b**), Proline (**c**) and MDA (**d**) contents in canola seedlings. The means with the same Duncan's multiple range test letters in each column are not significantly different ($P \leq 0.01$). The data are the average of four replications $\pm$ SE.

Fig. 6. Heatmap and hierarchical clustering of morphophysiological (**a**) and physiological (**b**) parameters in canola (*Brassica napus* L.) seedlings under salinity stress and gamma irradiation pretreatment. (d: 0–100 Gy) and salinity stress levels (S: 0–150 mM).

Fig. 7. Multivariate principal component analysis of growth, photosynthetic, osmotic, and oxidative stress-related traits in canola seedlings exposed to salinity stress and gamma irradiation.

Fig. 8. Comparative radar plot of normalized growth, physiological, osmotic, and oxidative stress-related traits in canola seedlings subjected to salinity and gamma irradiation treatments. Changes in traits related to the control and 50 mM salinity treatments (**a**) and changes in traits related to the 100 and 150 mM salinity treatments (**b**).