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Pretreatment with High-Dose Gamma Irradiation on Seeds Enhances the Tolerance of Sweet Osmanthus Seedlings to Salinity Stress

Xingmin Geng *, Yuemiao Zhang, Lianggui Wang and Xiulian Yang

College of Landscape Architecture, Nanjing Forestry University, Nanjing 210037, China; lgwang2017@163.com (Y.Z.); wlg@njfu.com.com (L.W.); yangxl339@sina.com (X.Y.)

* Correspondence: xmgeng@njfu.edu.cn; Tel.: +86-15951902586

Received: 16 April 2019; Accepted: 7 May 2019; Published: 10 May 2019

Abstract: The landscape application of sweet osmanthus (*Osmanthus fragrans* (Thunb.) Lour.) with flower fragrance and high ornamental value is severely limited by salinity stress. Gamma irradiation applied to seeds enhanced their tolerance to salinity stress as reported in other plants. In this study, *O. fragrans* 'Huangchuang Jingui' seeds were pretreated with different doses of gamma irradiation, and tolerance of the seedlings germinated from the irradiated seeds to salinity stress and the changes of reactive oxygen species (ROS) production and ROS scavenging systems induced by gamma irradiation were observed. The results showed that seed pretreatment with different doses of gamma irradiation enhanced the tolerance of sweet osmanthus seedlings to salinity stress, and the positive effect induced by gamma irradiation was more remarkable with the increase of radiation dose (50–150 Gy). The pretreatment with high-dose irradiation decreased O_2^- production under salinity stress and mitigated the oxidative damage marked by a lower malondialdehyde (MDA) level, which could be related to the significant increase of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities in the seedlings germinated from the irradiated seeds compared to the corresponding control seedlings. In addition, the accumulation of proline in the irradiated seedlings may contribute to enhancing their tolerance to salt stress by the osmotic adjustment. The study demonstrated the importance of regulating plant ROS balance under salt stress and provided a potential approach to improve the tolerance of sweet osmanthus to salt stress.

Keywords: plant tolerance; reactive oxygen species; antioxidant activity; proline

1. Introduction

Salinity has been threatening more and more land in the world [1,2]. Soil salinity greatly affects plant growth, development and productivity, thus posing a serious threat to agricultural and landscape plants in many regions of the world. Salt stress increases the concentration of toxic ions in plant cells, causes ion homeostasis disruption and then results in oxidative damage with excess generation of reactive oxygen species (ROS) [3].

ROS, including free radicals like O_2^- and OH·, and non-radicals like H_2O_2 and $^1O_2$, can be generated in the process of aerobic metabolism. Under favorable conditions, ROS production is controlled at basal levels and is beneficial to plants by supporting cellular proliferation, physiological function and viability [4]. However, the accumulation of ROS was accelerated by various environmental stresses such as salinity, drought, heat and high light [5–8], which caused damage to protein, DNA and lipid and thereby affecting normal cellular function [9,10]. Plants possess specific mechanisms to detoxify ROS which include enzymatic antioxidants such as multiple superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate

reductase (DHAR) and non-enzymatic antioxidants such as ascorbic acid (AA) and glutathione (GSH). SOD enzymatically disproportionate O_2^- (the primary product of oxygen reduction) into H_2O_2 and O_2 [11], and the SOD-catalyzed reaction provides the initial defense against ROS in plant cells. POD and CAT catalyze the conversion of H_2O_2 to water and O_2. APX, GR, DHAR, AA and GSH regulate mainly ROS by the ascorbate-glutathione (ASC-GSH) cycle. Under stresses, keeping higher activities of enzymatic antioxidants or the level of non-enzymatic antioxidants contributes to plant tolerance to environmental stresses [4,6].

Gamma rays belong to ionizing radiation and interact to atoms or molecules to produce free radicals in cells, which affects plant cellular structure and metabolism, e.g., the dilation of thylakoid membranes, alteration in photosynthesis, modulation of the antioxidative system, and accumulation of phenolic compounds [12–14]. It is possible that ^{60}Co-γ radiation could keep more complete cellular structures of the irradiation-induced mutants under chilling treatment than wild-type plants [14]. It has been reported that the activities of scavenging enzymes, such as SOD, POD and CAT, are generally increased in various plant species by the treatment of ionizing radiation [15–17]. ROS scavenging systems are important inducible factors for improving stress resistance through gamma-irradiation [18–20]. For example, gamma irradiation could help plants acclimate to lethal salinity by increasing antioxidant enzymes in irradiation-pretreated plants and alleviating oxidative damage [19,20].

Sweet osmanthus is a representative species of the genus *Osmanthus*, evergreen ornamental plants with excellent fragrance, color and shape. Sweet osmanthus has been cultivated at least for 2500 years in China. It was introduced into Japan, then to England in 18th century, and is now cultivated in Southeast Asia and many European countries [21]. However, soil salinity adversely affects the landscape application and geographic distribution of sweet osmanthus. The objective of this study was to explore a new approach to improve the salt tolerance of sweet osmanthus and determine how seed pretreatment with gamma irradiation improves the tolerance of sweet osmanthus through influencing antioxidant activity and ROS balance.

2. Materials and Methods

2.1. Seed Collection, Gamma Irradiation and Storage

Seeds of *O. fragrans* 'Huangchuan Jingui' (a cultivated variety) were collected on the campus of Nanjing Forestry University (Nanjing, Jiangsu Province) in May 2013. The seeds were randomly divided into four groups: Group 1 as non-irradiated controls and Group 2, 3 and 4 exposed to gamma irradiation with three irradiation doses. Gamma irradiation was conducted using a ^{60}Co-γ gamma source at a dose rate of 1.3 Gy/min, and the doses of exposure in this study were 50, 100 and 150 Gy. After irradiation, the non-irradiated and irradiated seeds were pretreated with 0.1% gibberellin (GA_3) for 24 h in order to break seed dormancy quickly and stored in wet sand at 4 °C before germination. The germinated seeds were sown in soil (1:1, v/v, mixture of garden soil and peat moss) in October 2013 and grown in greenhouse. Two years later, these plants were used in the following experiments of salt stress and tolerance assays.

2.2. Salt Stress and Tolerance Assays

Salinity stress was carried out using the method of water culture. Ten seedlings from each treatment (0, 50, 100, 150 Gy irradiation) with uniform size were chosen and grown in glass bottles (10 cm diameter and 8 cm high, one seedling in one bottle) filled with Hoagland solution with sodium chloride at different concentration, 20, 40, 60, 80, 100 and 120 mmol/L, sequentially. All seedlings were exposed to each concentration for three days before being moved to the next level.

Morphological changes of the seedlings due to salt stress were observed in the process of salt stress treatment, and the injury index was recorded at each concentration of salt stress. The extent of salinity injury was divided into five grades as shown in Table 1. The salinity injury index was calculated according to the following formula:

Table 1. Degree of leaf damage under salinity stress.

Grade	Salinity Damage Level	Description
0	No damage	No morphological damage symptoms of the whole plant
1	Mild	Less than 20% of the leaves have scorched margin and dehydration symptoms
2	Moderate	Nearly 50% of the leaves scorch, yellow with rust or wither
3	Severe	More than 50% of the leaves scorch, yellow with rust or wither
4	Very severe	More than 90% of the leaves scorch and wither or even the whole plant dies

Injury index = (Σ injury grades × corresponding number of seedlings)/(the highest grade × total number of seedlings); Injury rate = (Number of seedlings with morphological symptoms of salinity injury/total number of seedlings) × 100%. The plants were continuously monitored and observed for 1 week after salt stress. This same experiment was conducted twice again to confirm the results.

2.3. Sampling for Physiological Index

As described above, ten non-irradiated and ten irradiated seedlings were sequentially exposed to salt stress with different concentrations of NaCl solution for three days. Leaves from the ten individual non-irradiated and ten irradiated seedlings were collected after every concentration of salinity treatment and were stored in a −80 °C freezer for the measurement of O_2^- content, antioxidant enzyme activity, proline, malondialdehyde (MDA) and soluble proteins. This experiment was conducted three times to confirm the results.

2.3.1. Measurement of O_2^- Content and MDA Level

The content of O_2^- in leaves was determined by the hydroxylamine hydrochloride method according to Ke et al. [22] with minor modifications. Frozen leaves (0.3 g of fresh weight) were ground in 6 mL of potassium phosphate buffer (65 mM, pH 7.8), and centrifuged for 15 min at 10,000 rpm. The supernatant (1 mL) was mixed with 0.2 mL of 10 mmol/L hydroxylamine hydrochloride, then incubated for 20 min at 25 °C. After 1 mL of 17 mmol/L sulfanilic amide and 1 mL of 7 mmol/L α-naphthylamine were added, the mixture was further incubated for 20 min at 25 °C. The reaction mixtures were extracted with the same volume of chloroform (3.2 mL), and the absorbance was determined at 530 nm. Sodium nitrite was used to make a standard solution for calculating the content of O_2^-. MDA level was determined by the thiobarbituric acid (TBA) method as previously described [23].

2.3.2. Determination of Antioxidant Enzyme Activity and Proline Level

Leaves (0.3 g of fresh weight) were homogenized in a mortar and pestle with 2 mL of ice-cold phosphate buffer (50 mM, pH 7.8). The homogenate was centrifuged at 9000 rpm and 4 °C for 20 min. The supernatant was used for measuring the activities of SOD, POD and CAT. The procedure was conducted at 4 °C.

SOD activity was assayed by monitoring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) using the method of Dhindsa et al. [24]. The reaction mixture (3 mL) contained 1.5 mL of phosphate buffer (50 mM, pH 7.8), 0.5 mL of 0.1 mM EDTA, 0.5 mL of 130 mM methionine, 0.5 mL of 0.5 mM NBT, 0.5 mL of 0.02 mM riboflavin, and 0.05 mL of enzyme extract. Riboflavin was added last, and the reaction mixtures were illuminated under 4000 Lm/m^2 for 30 min. The reaction was stopped by switching off the light and the tubes were covered with a black cloth. Non-illuminated and illuminated reactions without the supernatant served as calibration standards. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

POD activity was measured following the method of Zhang and Kirkham [25] with some modifications. The enzyme extract (0.02 mL) was added to the reaction mixture containing 0.3% guaiacol solution and 3% hydrogen peroxide solution. The reaction was started by adding the enzyme extract and the absorbance increase at 470 nm in 5 min was recorded.

CAT activity was assayed in a reaction mixture containing phosphate buffer (pH 7.8), 0.1 mol/L H_2O_2 and enzyme extract. The reaction was initiated by adding the enzyme extract. The decrease of H_2O_2 was monitored at 240 nm in at least 4 min.

Proline was determined using colorimetric methods [26] as previously described [27].

2.4. Data Analyses

Statistical analyses were carried out using SPSS17.0 software (SPSS Company, Chicago, IL, USA) to calculate the mean and standard deviation. The Duncan's multiple range test (DMRT) was applied to test the significance in differences among treatments ($P < 0.05$).

3. Results

3.1. Tolerance in Irradiated Seedlings to Salinity Stress

To determine the level of enhanced tolerance to salt stress induced by gamma irradiation, we investigated the morphological changes of two-year-old sweet osmanthus seedlings developed from the irradiated seeds. The injury degree induced by salinity stress was represented by the injury index and injury rate. As shown in Table 2, the injury degree of sweet osmanthus seedlings was gradually aggravated when exposed to successively increasing NaCl concentration of 20, 40, 60, 80, 100 and 120 mmol/L, and the injury degree in non-irradiated seedlings was significantly different from the irradiated ones. The non-irradiated and irradiated seedlings at lower dose appeared to show slight injury with yellowing of basic leaves under 20 mmol/L salt stress. The similar injury symptom in 150-Gy gamma irradiated seedlings was not observed until exposed to 40 mmol/L NaCl. The non-irradiated seedlings suffered from moderate injury when NaCl concentration was increased to 40 mmol/L: nearly 50% of the leaves scorched, yellowed with rust or withered, and the salt injury rate reached 100%. Similar injury symptom in 50-Gy and 100-Gy gamma irradiated seedlings was observed when exposed to 60 mmol/L NaCl. However, 150-Gy gamma irradiated seedlings suffered from moderate injury under 80 mmol/L NaCl. When salt concentration was increased to 100 mmol/L, all seedlings appeared to show very severe injury symptom: 100% of the leaves scorched, withered, or the whole plant died.

Table 2. Effects of different dose of gamma irradiation on the salt injury index and injury rate of *O. fragrans* 'Huangchuan Jingui' under salt stress.

NaCl Concentration (mmol/L)	Salt Injury Index				Salt Injury Rate (%)			
	0 Gy	50 Gy	100 Gy	150 Gy	0 Gy	50 Gy	100 Gy	150 Gy
0	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
20	0.08 ± 0.01 a	0.07 ± 0.01 a	0.07 ± 0.01 a	0.00 ± 0.00 b	63.33 a	30.00 b	26.67 c	0.00 d
40	0.55 ± 0.05 a	0.37 ± 0.03 b	0.36 ± 0.01 b	0.28 ± 0.04 c	100.0 a	90.00 b	70.00 c	33.33 d
60	0.70 ± 0.05 a	0.56 ± 0.06 b	0.52 ± 0.06 b	0.40 ± 0.04 c	100.0 a	100.0 a	96.67 b	80.00 c
80	0.90 ± 0.02 a	0.74 ± 0.05 b	0.67 ± 0.05 c	0.54 ± 0.04 c	100.0 a	100.0 a	100.0 a	100.0 a
100	0.90 ± 0.01 a	0.89 ± 0.01 a	0.78 ± 0.06 c	0.85 ± 0.03 b	100.0 a	100.0 a	100.0 a	100.0 a
120	0.98 ± 0.01 a	0.98 ± 0.01 a	0.95 ± 0.01 b	0.97 ± 0.01 a	100.0 a	100.0 a	100.0 a	100.0 a

Note: Different normal letters within the same row indicate significant difference among different doses of gamma irradiation at 0.05 level ($P < 0.05$).

3.2. *Effects of High-Doses Gamma Irradiation on ROS Level and Lipid Peroxidation in Response to Salt Stress*

ROS production can be accelerated by various environmental stresses. As shown in Figure 1A, O_2^- production was accelerated as the increase of salt concentrations, and the O_2^- level in gamma-irradiated seedlings was lower than non-irradiated seedlings, except in response to 20 mmol/L salt stress (Figure 1A).

As shown in Figure 1B, MDA level in the gamma-irradiated seedlings was higher than the level observed in the non-irradiated seedlings under control condition, which indicated that gamma-ray irradiation applied on the seeds resulted in oxidative damage of cell membrane in sweet osmanthus seedlings to some extent. The MDA level in the gamma-irradiated seedlings under 20~60 mmlo/L and 120 mmol/L salt stress was lower than in the non-irradiated seedlings, and no significant difference in MDA level between the gamma-irradiated seedlings and the control seedlings was observed under 80~100 mmlo/L salt stress.

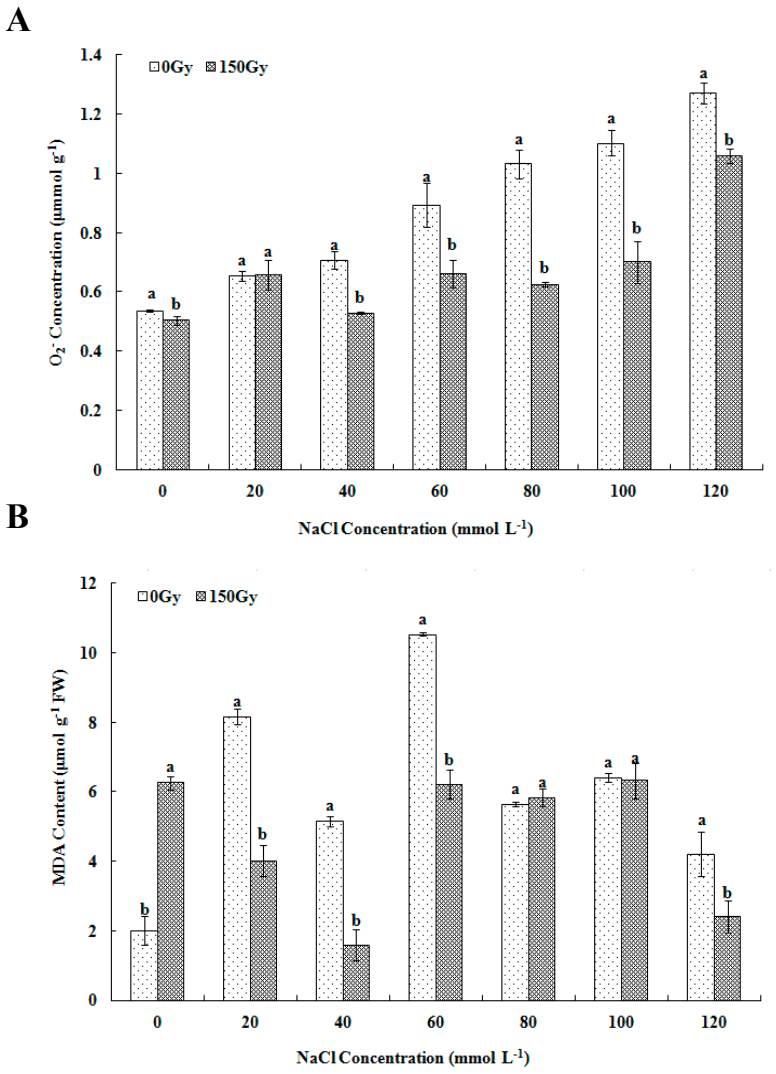

Figure 1. Effects of high-dose gamma irradiation on O_2^- production (**A**) and MDA level (**B**) in *Osmanthus fragrans* (Thunb.) Lour. seedlings in response to salt stress. Note: 0 Gy was used as the control; 150 Gy was the seedlings germinated from 150-Gy gamma irradiated seeds. Means with different letters above bars were significantly different at $P < 0.05$ between the control and the irradiation treatment.

3.3. *Effects of High-Doses Gamma Irradiation on ROS Scavenging Systems in Response to Salt Stress*

SOD activity in the control and the irradiated seedlings under salt stress was gradually enhanced along with the increasing of salt concentrations and reached the peak in the control seedlings under 60 mmol/L NaCl or in the irradiated seedlings under 80 mmol/L NaCl (Figure 2A). The pretreatment with 150-Gy gamma irradiation increased the SOD activities in seedlings under salt stress compared with the levels observed in the corresponding control seedlings, and the increase was statistically significant ($P < 0.05$) under stress with 40 or 80~120 mmlo/L NaCl.

POD activity increased initially and thereafter declined along with the increasing of salt concentrations. Maximum value in the control seedlings was observed under 40 mmol/L NaCl, but the peak in the irradiated seedlings appeared under 60 mmol/L NaCl. POD activity in the irradiated seedlings was significantly enhanced under higher concentration of NaCl (≥60 mmol/L) (Figure 2B). Exposed to successively increasing salt stress, the activity of CAT in the irradiated seedlings was increased compared with the non-irradiated seedlings, except in response to 80 mmlo/L NaCl (Figure 2C).

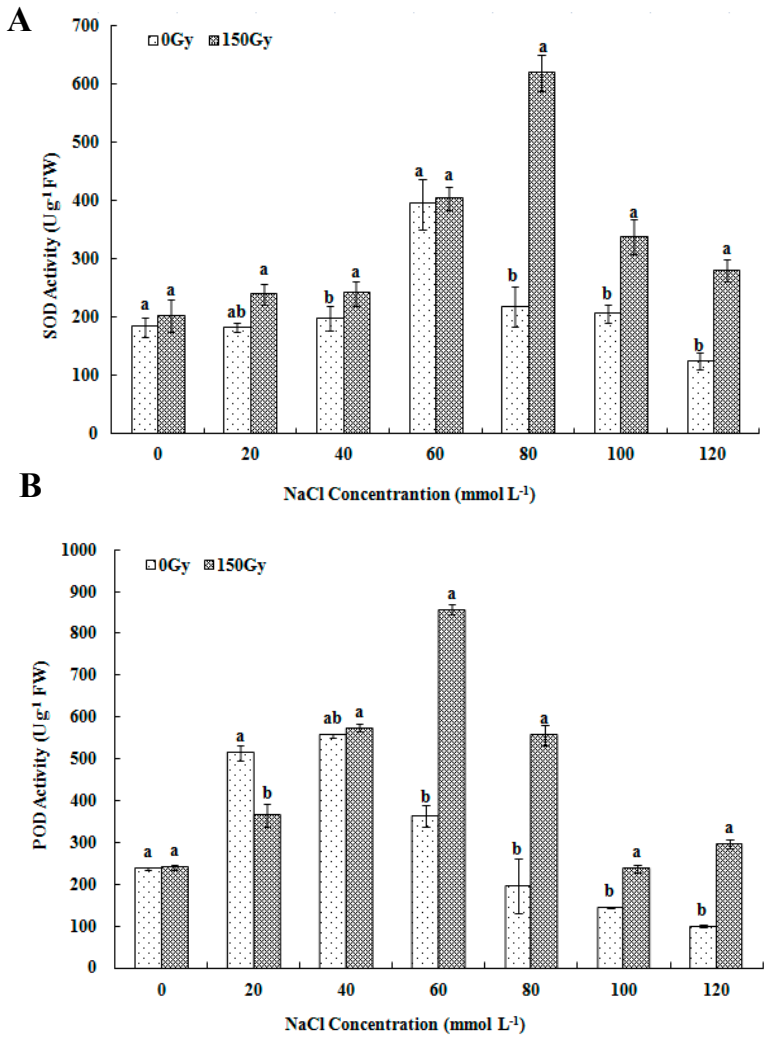

Figure 2. *Cont.*

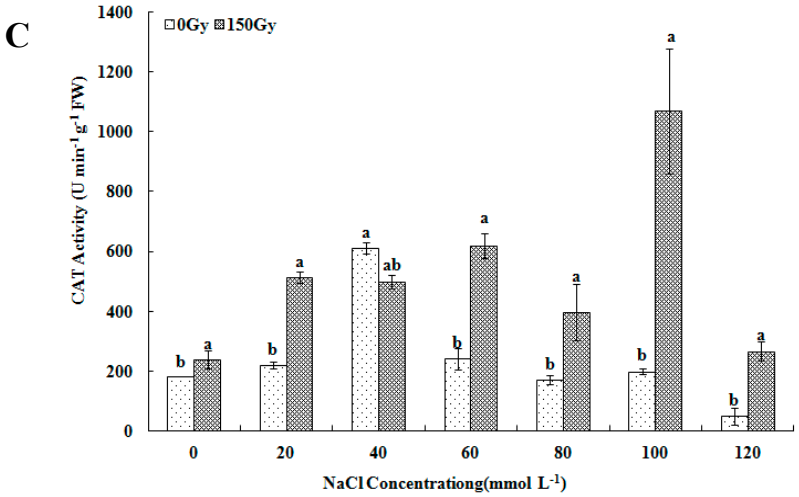

Figure 2. Effects of high-dose gamma irradiation on the activity of SOD (**A**), POD (**B**) and CAT (**C**) in *Osmanthus fragrans* seedlings in response to salt stress. Note: 0 Gy was used as the control; 150 Gy was the seedlings germinated from 150-Gy gamma irradiated seeds. Means with different letters above bars were significantly different at $P < 0.05$ between the control and the irradiation treatment.

3.4. Effects of High-Doses Gamma Irradiation on Proline Level in Response to Salt Stress

The proline level in the irradiated seedlings was lower than that in the non-irradiated seedlings in control and 20 mmol/L NaCl condition (Figure 3). More accumulation of proline in the irradiated seedlings was observed when exposed to higher concentration of salt stress. And the proline content in irradiated seedlings increased with the increase of salt stress.

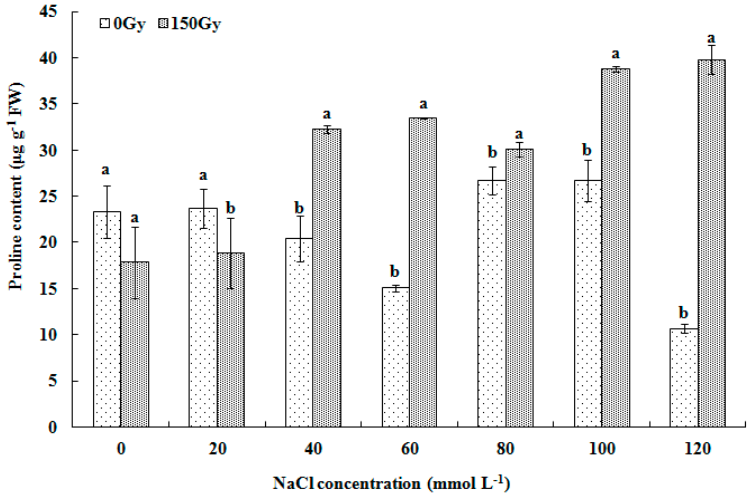

Figure 3. Effects of high-dose gamma irradiation on proline level in *Osmanthus fragrans* seedlings in response to salt stress. Note: 0 Gy was used as the control; 150 Gy was the seedlings germinated from 150-Gy gamma irradiated seeds. Means with different letters above bars were significantly different at $P < 0.05$ between the control and the irradiation treatment.

4. Discussion and Conclusions

The effects of gamma irradiation on the morphological changes and biological responses of plants are dependent on radiation doses [13]. Low-dose gamma irradiation improves seed germination and seedling growth, but adverse effects are induced by high-dose [28]. We previously reported that 50-Gy gamma irradiation displayed the maximal positive effects on seed germination and seedling growth of sweet osmanthus [29], and a high dose resulted in the decrease of seed germination and the poor

growth of seedlings, even leading seedlings to death. In the present study, the seed pretreatment with different doses of gamma irradiation enhanced the tolerance of sweet osmanthus seedlings to salinity stress, and the survival of seedlings developed from the seeds irradiated with higher doses of gamma-ray showed stronger tolerance to salinity stress (Table 2). The result is different from the previous reports in rice [30], sweet potato [31] and *arabidopsis* seedlings [20], in these studies gamma irradiation at relative low dose improved the seedling growth under salinity stress. The differences of the response to irradiation dose were hypothesized to be related to the sensitivity to radiation. It has been well documented that there are great differences of sensitivity to irradiation between the taxa from the level of varieties of the same species to main plant divisions [32]. The resistance to irradiation of sweet osmanthus may be stronger than other plants mentioned in front. In addition, in the present study salt tolerance experiments were carried out after two years of seed radiation and normal cultivation. As reported by Zaka et al. [33], the effects induced by irradiation were often not reproducible or only transitory. The higher radiation dose may maintain the radiation effect for a longer time. According to the current research results, it is uncertain whether higher doses of radiation cause genotype variation and alter plant resistance genetically and stabilize radiation effects, which requires further research in the later stage.

Salinity stress resulted in the accumulation of excess O_2^- (Figure 1). If excessive O_2^- cannot be removed in time, it will produce ·OH and $^1O_2$ with strong activity and toxicity, and result in membrane lipid peroxidation and oxidative damage to the cell membrane [34]. The pretreatment with high-dose gamma irradiation suppressed ROS production in salinity-stressed conditions and mitigated lipid peroxidation and the damage of cell membrane as manifested by the lower O_2^- and MDA level in the irradiated seedlings compared with the controls (Figure 1). The enhanced defense against ROS damage under high-dose gamma irradiation was probably due to the increased activities of antioxidant enzymes. POD, SOD and CAT activities were all significantly increased to different extents in response to salinity stress (Figure 2). The responses of antioxidant enzymes to salinity stress in the irradiated seedlings are consistent with other reported studies [20,35]. We also found another *O. fragrans* "Zi Yin gui", whose tolerance to salinity stress was enhanced similarly to "Huangchuan Jingui" and the defense response against ROS damage was induced by pretreatment with different doses of gamma irradiation; although the up-regulated extent of every single antioxidant enzyme was not exactly the same (data not shown). As reported by Ashraf [6], the extent to which the activities of enzymatic antioxidants and the level of non-enzymatic antioxidants were up-regulated under salinity stress is highly variable among plant species and even between two cultivars of the same species. The response and activation of antioxidant systems induced by irradiation exposure are dependent upon the γ-ray dose rate, the doses of gamma rays and the developmental stages [36,37]. The ASC-GSH cycle system were the main ROS-scavenging systems in the chloroplasts [11], the activities of APX and GR were found to be higher in the salt-tolerant cultivars of potato than those in the salt-sensitive cultivars [38]. ASC-GSH cycle also plays an important role in plant salt tolerance, hence further research on this system is necessary in the future in order to fully understand the effect of gamma irradiation on salinity tolerance of sweet osmanthus.

The proline level was significantly increased in the irradiated sweet osmanthus seedlings when exposed to higher concentration of salt stress (Figure 3). Proline is one of the major osmolytes regulating osmotic adjustment in plants exposed to osmotic stresses, and active accumulation of proline is associated with salinity tolerance in various plant species [5]. Proline contributes to membrane stability and mitigates the effect of NaCl on cell membrane disruption [39]. The proline content was also increased by irradiation in sweet potato [31], *arabidopsis* [20] and sugarcane [40]. Proline, other than being an osmoprotectant, can act as a singlet oxygen quencher and scavenger of hydroxyl radicals [41], and may be important in preventing oxidative damage caused by ROS when it accumulates during the exposure of the plants to adverse environmental conditions. Hossain and Fujita [42] provide evidence for the role of proline, which can protect against salt-induced oxidative

damage by reducing H_2O_2 and lipid peroxidation levels, and by enhancing antioxidant defense and methylglyoxal detoxification systems.

In summary, our study provides a new approach to improve the tolerance of sweet osmanthus to salt stress by the application of irradiation, and proves that enhanced tolerance to salinity stress is closely related to the ROS-scavenging system.

Author Contributions: X.G. directed the project, drafted the first version of the manuscript, finalized figures and manuscript revision; Y.Z. conducted salinity stress experiments, collected morphological and physiological data on salinity tolerance, prepared draft figures; L.W. initiated the project, contributed to manuscript writing and finalizing; X.Y. supervised the experiments on physiological analyses and contributed to the manuscript writing.

Funding: This work was funded by the Agriculture-Forestry Plant Germplasm Resources Exploration, Innovation and Utilization Project of the National Twelve-Five Science and Technology Support Program (2013BA001B).

Acknowledgments: The authors thank the NJFU Greenhouse staff for supporting the cultivation of sweet osmanthus seedlings and graduate students Na Li for assistance in radiation treatment methods and concentrations.

Conflicts of Interest: The authors declare no conflict of interest.

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