

# Sodium Chloride (NaCl)-Induced Physiological Alteration and Oxidative Stress Generation in *Pisum sativum* (L.): A Toxicity Assessment

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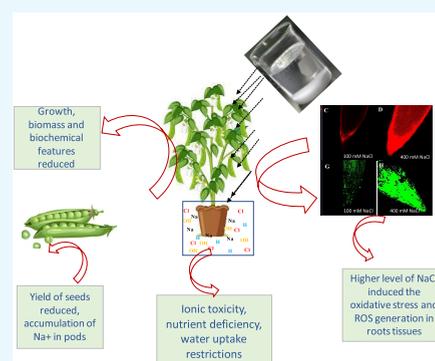


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**ABSTRACT:** Salinity stress has a deleterious impact on plant development, morphology, physiology, and biochemical characteristics. Considering the NaCl-induced phytotoxicity, current investigation was done to better understand the salt-tolerant mechanisms using *Pisum sativum* L. (pea) as a model crop. Generally, NaCl resulted in a progressive decrease in germinative attributes and physiological and biochemical parameters of *P. sativum* (L.). The 400 mM NaCl level had a higher detrimental effect and reduced the germination rate, plumule, radicle length, and seedling vigor index (SVI) by 78, 89, 84, and 77%, respectively, under *in vitro*. Furthermore, after 400 mM NaCl exposure, physiological and enzymatic profiles like root dry biomass (71%), chl-a (66%), chl-b (54%), total chlorophyll (45%), and nitrate reductase activity (NRA) (59%) of peas were decreased. In addition, a NaCl dose-related increase in soluble protein (SP) and sugar (SS), Na<sup>+</sup> and K<sup>+</sup> ions, and stressor metabolites was recorded. For instance, at 400 mM NaCl, SP, SS, Na<sup>+</sup> ion, K<sup>+</sup> ion, root proline, and malondialdehyde (MDA) contents were significantly and maximally elevated by 65, 33, 84, 79, 85, and 89%, respectively, compared to the control (0 mM NaCl). Data analysis indicated that greater doses of pesticides dramatically increased reactive oxygen species (ROS) levels and induced membrane damage through production of thiobarbituric acid reactive substances (TBARS), as well as increased cell injury. To deal with NaCl-induced oxidative stress, plants subjected to higher salinity stress showed a considerable build-up in antioxidant levels. As an example, ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) were maximally and significantly ( $p \leq 0.05$ ) increased by 68, 80, 74, and 58%, respectively, after 400 mM NaCl exposure. The propidium iodide (PI)-stained and NaCl-treated plant roots corroborated the damaging effect of salinity-induced stress on the cell membrane, which was observed under a confocal laser microscope (CLSM). The cells exposed to 400 mM NaCl had maximum fluorescence intensity, indicating that higher level of salts can cause pronounced cell damage and reactive oxygen species (ROS) generation. The increases in superoxide ion (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content in NaCl-treated plant tissues indicated the elevation of ROS with increasing salt levels. This finding revealed that salt stress can cause toxicity in plants by causing alteration in metabolic activity, oxidative injury, and damage to cell membrane integrity.



## INTRODUCTION

Salinity can arise as a result of improper irrigation, drainage, or fertilizer application, and it is most prevalent in protected farming.<sup>1</sup> Plants cultivated in saline areas have a number of disadvantages. The very first and foremost is increased osmotic stress caused by excessive salt concentrations in the soil solution, which reduces the soil's water potential.<sup>2</sup> The other drawback is increased concentrations of sodium (Na<sup>+</sup>) and chloride (Cl<sup>-</sup>) ions, which causes ionic imbalance by accumulating Na and Cl in tissues and inhibiting the mineral nutrient uptake.<sup>3</sup> Although plant species differ in their mechanisms of salinity tolerance,<sup>4</sup> salinity stress eventually reduces the plant development.<sup>5</sup> Excessive levels of soil salt can impede seed germination and seedling growth due to the combined effects of high osmotic pressure and specific ion toxicity.<sup>6</sup> The production of many plant species declines when exposed to excessive salinity, which is generally linked to a

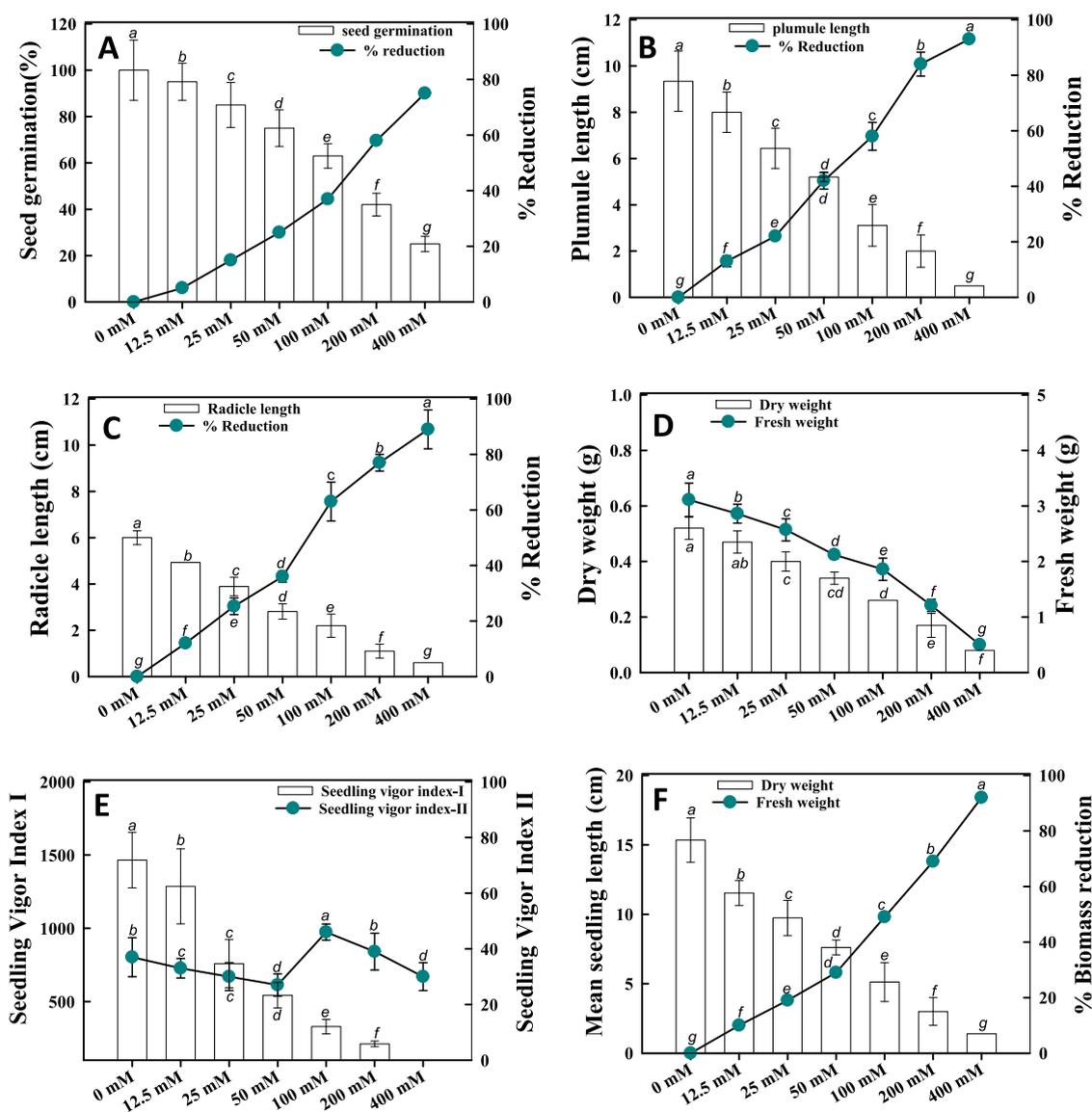
drop in photosynthetic capability. A decrease in chlorophyll formation can also cause a decrease in photosynthesis when the environment is salinized.<sup>7</sup> In salt-sensitive plants, salinity decreases/damages chlorophyll formation,<sup>8,9</sup> decreases the photosynthetic pigments,<sup>10</sup> reduces the photosynthetic<sup>11</sup> and transpiration rates,<sup>12</sup> and reduces stomatal conductance,<sup>13</sup> while it shows an increasing effect on salt-tolerant plants. Salinity inhibits plant development by altering the turgor, photosynthesis, and enzyme activity and it may cause leaf mortality in older leaves.<sup>14,15</sup>

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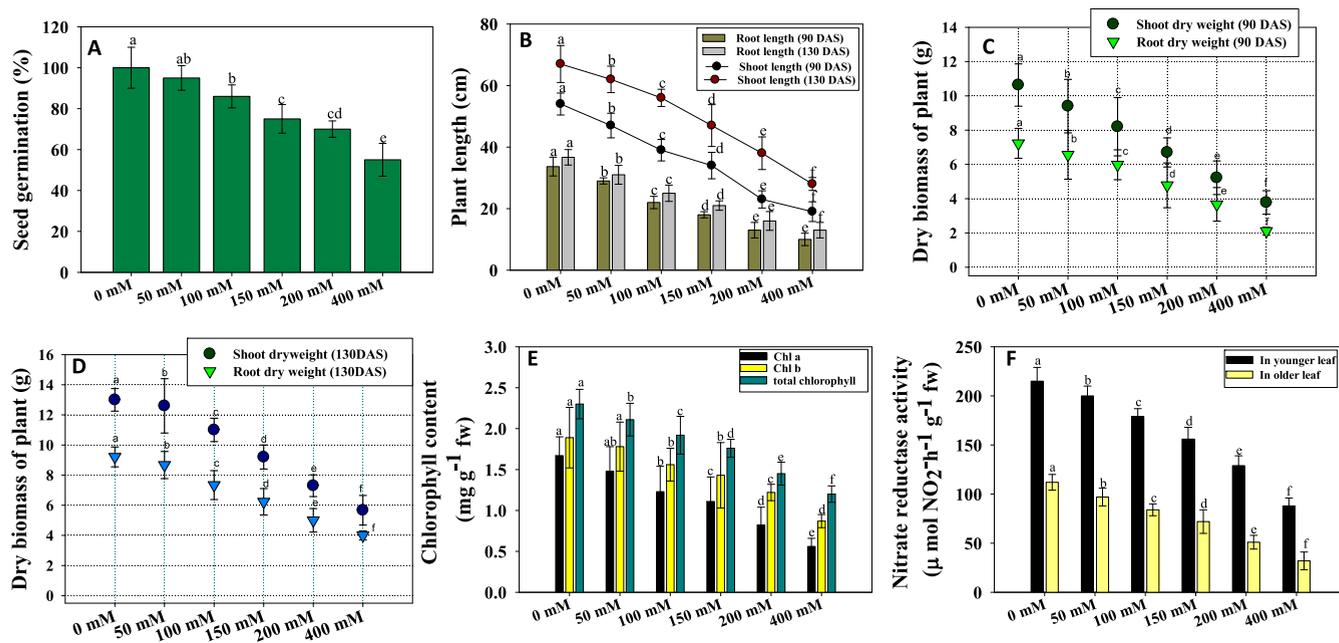
**Figure 1.** Effect of increasing NaCl concentrations on germination attributes and seedling parameters of peas germinated on soft agar plates treated with 0, 2, 50, 100, 150, 200, and 400 mM NaCl; percentage seed germination (A), plumule length (B), radicle length (C), dry weight (D), seedling vigor index (E), and mean seedling length (F). Each value is a mean of three replicates. Mean values followed by different letters are significantly different at  $p \leq 0.05$  according to Duncan's multiple range (DMRT) test. Vertical bars represent means  $\pm$  SD ( $n = 3$ ), and error bars represent standard deviation (SD).

After salt stress, apoptosis (like degradation of DNA) has been reported, resulting in successive nuclear degradation, cell death, and retardation in the root development.<sup>16</sup> Salinity stress more often causes a build-up of reactive oxygen species (ROS) in the roots and leaf tissues of plants at the cellular level.<sup>17</sup> Plants have evolved a variety of complex physiological and metabolic processes to cope with severe environments, including a high number of stress-responsive genes and the production of varied functional proteins via a complex signal transduction network.<sup>18</sup> Plants can respond to oxidative stress by increasing their antioxidant defenses. In aerobic organisms, harmful byproducts of metabolic processes include superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $OH^\bullet$ ), and other reactive oxygen species (ROS).<sup>19</sup> The coordinated functioning of ROS-scavenging pathways from different cellular compartments may play a vital role in plant

salt tolerance by controlling the amount of ROS in cells, minimizing cellular damage, and managing the ROS.<sup>20,21</sup>

Plants use a variety of antioxidant mechanisms to keep away these toxic chemicals. Plants can regulate the activity of various antioxidant enzymes and metabolites to keep ROS at a safe level when they are stressed.<sup>22</sup> Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and peroxidase (POX) activity levels are critical for a plant's response to salinity stress.<sup>23</sup> These enzymes and metabolites not only protect the plants from cellular damage but also regulate ROS levels to ensure that their metabolic activities are optimum.<sup>24</sup> Furthermore, when plants are stressed by salt, they produce more secondary metabolites such as soluble solids, sugars, organic acids, proteins, and amino acids.

Pea (*Pisum sativum* L.) is a widely farmed vegetable and pulse crop. It is cultivated in the vicinity of 1.1 million hectares worldwide, with a total production of 9.2 million tonnes and a



**Figure 2.** Effect of increasing NaCl concentrations on biological parameters, photosynthetic pigments, and enzymatic activity of pea plants raised in pot soils treated with 0, 2, 50, 100, 150, 200, and 400 mM NaCl; seed germination (A), plant length (B), dry biomass at 90 DAS (C), dry biomass at 130 DAS (D), chlorophyll content (E), and nitrate reductase activity (F). Each value is a mean of three replicates where each replicate constituted three plants/pots. Mean values followed by different letters are significantly different at  $p \leq 0.05$  according to the DMRT test. Vertical bars represent means  $\pm$  SD ( $n = 3$ ), and error bars represent SD.

yield of 8.35 tonnes per hectare.<sup>25</sup> Since pea has high levels of proteins, carbohydrates, vitamins, and minerals such as iron (Fe), calcium (Ca), potassium (K), and phosphorous (P) in their nutritional composition, this therefore makes it a valuable human dietary component.<sup>26</sup> It is also used to reduce cardiovascular problems due to its low fat, salt, and cholesterol content.<sup>27</sup>

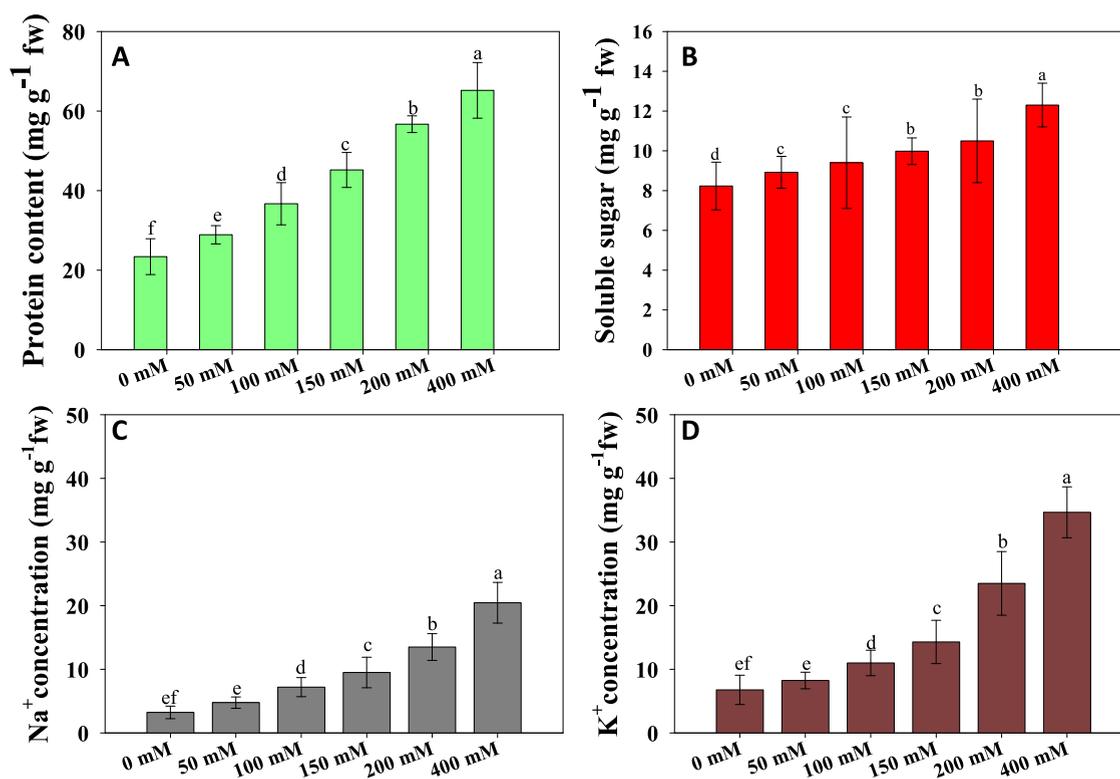
The current study, which used *P. sativum* L. (garden pea) as a test/model crop, was designed to address the serious concerns connected with salinity in legume cultivation. The present work aimed at (i) evaluating the effect of increasing level of NaCl on germination attributes of pea seeds cultivated both *in vitro* and *in vivo*, (ii) assessing the salt-induced stress on biological features (growth, length, and dry biomass) of peas, (iii) estimating the photosynthetic attributes (chlorophyll and carotenoid) and nitrate reductase (NR) activity in NaCl-treated pea foliage, (iv) determining the soluble protein, soluble sugar, relative leaf water content (RLWC) and  $\text{Na}^+/\text{K}^+$  ions in NaCl-induced peas, (v) evaluating the responses of increasing NaCl levels on plant stress markers (proline and malondialdehyde content) and antioxidant enzyme activity, and (vi) assessing the NaCl-induced oxidative stress, cellular damage, and ROS generation ( $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  content) in pea organs.

## RESULTS AND DISCUSSION

**NaCl Negatively Affected the Germination Attributes and Vigor Indices of Pea Seedling.** Germination is a complex biological process that requires several elements to operate simultaneously for a seedling to emerge. Water intake is essential to activate the hydrolytic enzymes that metabolize the seeds' stored nutrients into simple molecules, which are needed for cell growth and differentiation. The presence of different abiotic stresses including salinity has an inhibitory

effect on seed germination. Salinity affects germination via changing the osmotic component, which affects the ionic component, i.e., build-up of Na and Cl.

For the endurance and upholding of plant species, the capacity of their seeds to sprout under a higher level of NaCl in the soil is critical. Seed germination occurs in saline ecosystems after heavy precipitation, i.e., when the soil salinity is low.<sup>28</sup> Here, *P. sativum* (L.) seeds germinated in the presence of 400 mM NaCl showed an 80% reduction when compared to the control (Figure 1A). Increasing levels of salts can result in osmotic and/or particular toxicity, which can lower the percentage of seeds germination. Seeds treated with increasing levels of salt had shorter plumule and radicle lengths than untreated control seeds. For instance, a maximum and considerable decrease in length of plumule (87%), radicle (74%), fresh weight (56%), and dry biomass (64%) was recorded at 400 mM NaCl (Figure 1B–D). Like our observation, Baruah and Das<sup>29</sup> observed delayed germination in the presence of elevated metal and salt concentrations. Similarly, different levels of salt stress affected the germination efficiency and seedling development in sorghum.<sup>30</sup> The degree of activity and performance of seeds during germination and seedling emergence is determined by the seedling vigor index. As a result, the most important physiognomies of the seeds to be employed for cultivation are seed germination and seedling vigor indices. In this study, with the increasing level of salt concentrations (0–400 mM NaCl), seedling vigor and stress tolerance indices dropped steadily (Figure 1E,F). Similarly, differing levels of NaCl have been shown to have a negative/reducing influence on seedling germination, vigor indices, and growth parameters of a leguminous plant *Vigna radiata*.<sup>31</sup> Furthermore, *Cucurbita pepo* (L.) seeds treated with NaCl had reduced germination, vigor indices, biological features, and dry biomass.<sup>32</sup> During the process of seed germination, ROS are



**Figure 3.** Effect of increasing concentrations (0–400 mM) of NaCl on soluble protein content (A), soluble sugar (B), Na<sup>+</sup> ion concentration (C), and K<sup>+</sup> ion concentration (D) of pea plants grown under pot-house conditions. Each value is a mean of three replicates where each replicate constituted three plants/pots. Mean values followed by different letters are significantly different at  $p \leq 0.05$  according to the DMRT test. Vertical bars represent means  $\pm$  SD ( $n = 3$ ), and error bars represent SD.

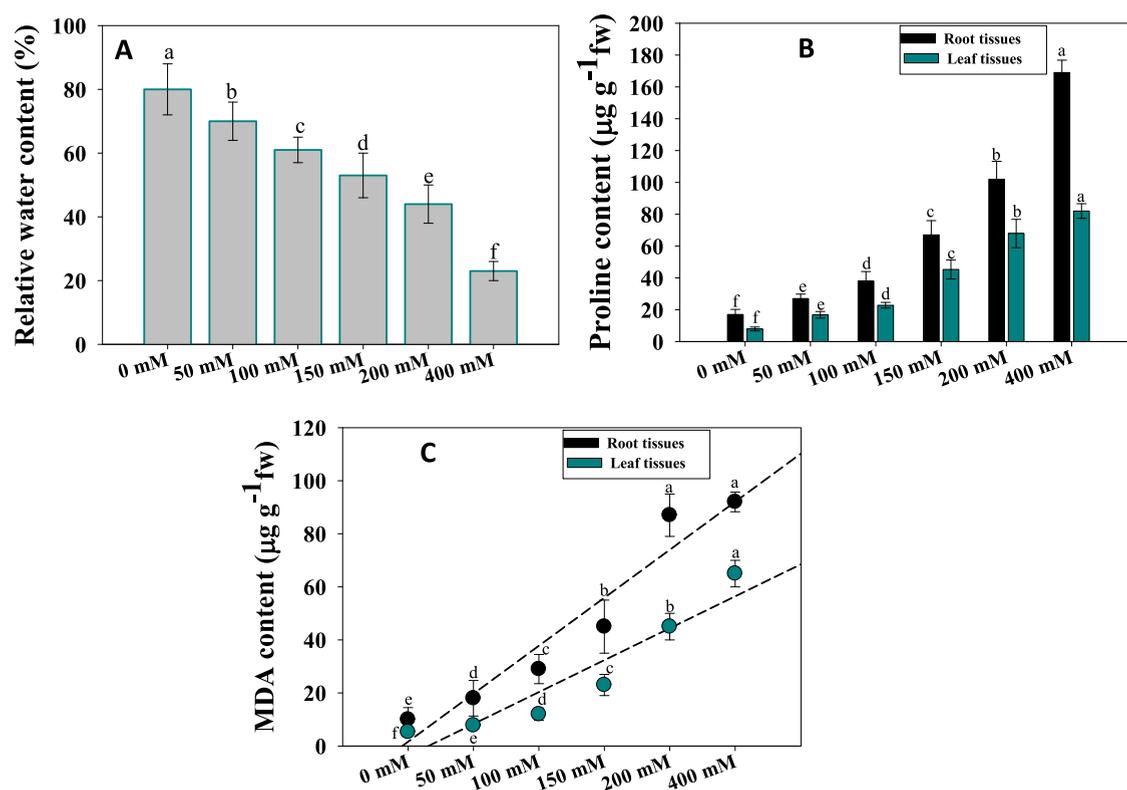
produced by plasma membrane-bound peroxisomes, glyoxisomes, and NADPH oxidases.<sup>33</sup> The increased ROS levels impair the cellular lipids, proteins, and nucleic acids. As a result, seed germination may be possible only if ROS production is properly regulated.<sup>34</sup> The first step in the germination process is to hydrate the stored ingredients. Following hydration, the process of water intake activates metabolic activities, resulting in the leakage of solutes.<sup>35</sup> The osmotic components of salinity have a considerable detrimental impact on the hydration of the embryo, cotyledon, and endosperm.<sup>36</sup>

The seed reserves are engaged in the turnover and de novo synthesis of macromolecules, as well as embryonic development and elongation.<sup>37</sup> Many studies have shown that increasing salt stress causes seed germination to be delayed and the percentage of germination to be reduced. The delayed seed germination and a considerable decrease in percent germination due to the exposure to salinity-induced stress are reported.<sup>38</sup> Furthermore, at quantities above the species' tolerance threshold, salt can completely impede seed germination.<sup>39</sup>

**NaCl Reduced the Morphological Features (Growth and Dry Biomass) of Peas.** The process of seed germination involves physiological, metabolic, and molecular mechanisms that are required for the expansion of the embryonic axis. The fundamental components of a plant's life cycle are seedling germination and establishment. Plant density, homogeneity, and management options in crop production are all influenced by seedling establishment.<sup>35</sup> Germination can often be influenced by a variety of abiotic stress elements including salinity, drought, heavy metals, etc.<sup>38</sup>

All of the growth characteristics investigated were influenced by the presence of NaCl in the rooting medium, and the high NaCl level had maximum detrimental effect. For instance, at 200 mM NaCl, germination rate (in pot soils), root length (RL), and dry weight (DW) of roots were significantly reduced by 50, 70, and 65%, respectively, compared to the untreated control (Figure 2A–D). Salt has a stifling and reducing impact on plant growth might possibly be due to (i) decreasing the soil solution's osmotic potential surrounding the roots, (ii) increasing the concentrations of certain ions in tissues that are damaging, and (iii) modifying the nutrient status of the needed ions for overall physiological processes of plants. Several researchers have stated that salinity has a negative impact on crop plants. In this regard, Khator et al.,<sup>40</sup> for example, found that NaCl caused oxidative stress and biochemical, physiological, and morphological alterations in two legumes. Similarly, in two varieties of green gram cultivated in various amounts of NaCl under pot-house conditions, salts had a negative effect on water relations, ion build-up, and plant nutrients.<sup>41</sup> The reduction/suppression in biological features of plants under increasing salinity stress may be possibly due to the lower water potential, ion toxicity, and imbalance excreted by NaCl.

**Photosynthetic Attributes and Nitrate Reductase (NR) Activity under NaCl Stress. Photosynthetic Pigments.** As the concentration of NaCl increased from 0 to 400 mM, the chlorophyll content in pea leaf was decreased in a comparable manner and a higher salinity level had the maximum depressive effect. For instance, 400 mM NaCl caused a maximum and significant ( $p \leq 0.05$ ) reduction of 66, 54, and 48% in chl-a, chl-b, and total chlorophyll content, respectively, compared to



**Figure 4.** Relative leaf water content (A), proline (B), and MDA content (C) accumulated in plant tissues detached from pea raised in pot soils treated with 0, 2, 50, 100, 150, 200, and 400 mM NaCl. Each value is a mean of three replicates where each replicate constituted three plants/pots. Mean values followed by different letters are significantly different at  $p \leq 0.05$  according to the DMRT test. Vertical bars represent means  $\pm$  SD ( $n = 3$ ), and error bars represent SD.

the control (Figure 2E). It has also been noted that a decrease in chlorophyll content is accompanied by an increase in salt concentration. The accumulation of Na and Cl ions in the leaves could be linked to the reduction of chlorophyll concentration. The loss of chlorophyll as a result of salt stress is a common occurrence that results in the disordering of chlorophyll synthesis and the appearance of chlorosis in plants.<sup>42</sup> Due to the loss of enzymes important for the production and synthesis of leaf pigments, salt stress has a negative impact on the photosynthetic apparatus, resulting in decreased synthesis of carotenoid and chlorophyll.<sup>43</sup> Furthermore, by boosting the activity of chlorophyll degrading enzyme chlorophyllase, NaCl stress decreases the chlorophyll molecules of plants, causing a breakdown of the chloroplast structure and instability of pigment–protein complexes.<sup>44</sup> Furthermore, the high NaCl level negatively influences the pigment composition in plant foliage by (i) suppressing the electron transport (ii) inactivating the reaction sites in photosystem II (PS-II),<sup>45</sup> (iii) obstructing the oxygen-evolving complex (OEC) and (iv) destroying the electron transfer capacity on the donor side of PS-II.<sup>46</sup> In addition, increasing concentrations of sodium ( $\text{Na}^+$ ) and chloride ( $\text{Cl}^-$ ) ions in nonstomatal leaf tissues can also cause a harmful impact on photosynthesis-limiting metabolic activities.<sup>47</sup>

**Nitrate Reductase (NR) Activity.** In higher plants, nitrate reductase (EC 1.6.6.1) is the first and most important enzyme involved in the nitrate assimilation pathway. In tissue, its catalytic activity is complexly regulated in response to many environmental factors. The post-translational changes of the nitrate reductase protein cause fast modulation of NR activity by a variety of external stimuli. Because enzyme nitrate

reductase (NR) is sensitive to Na and Cl ions, it is a good indicator of NaCl-induced toxicity. While measuring the NR activity in peas, it was observed that with the increasing NaCl concentration in the rooting media, the activity of NR was steadily dropped in both young and old pea leaves (Figure 2F). The detrimental effect of NaCl on NRA was more evident for salt-sensitive bean plants than for salt-tolerant cotton plants, according to Gouia et al.<sup>48</sup> The toxicity of Na and Cl, as well as low nitrate availability, could explain the inhibition of NRA. Changes in nitrate reductase activity in response to salinity were associated with an increase in the enzyme's activation status in roots and a decrease in the phosphorylated enzyme pool in the cytosol. Many factors influence how salt affects NR activity including plant species, nitrogen supply availability, salt content, and time duration of stress exposure to plants. Like present observation, exposure to increasing levels of NaCl significantly decreased the activity of NR in roots and leaf tissues of various agriculturally important vegetable crops including *Solanum lycopersicum* L. (tomato), *Cucumis sativus* L. (cucumber),<sup>49</sup> *Zea mays* L. (maize),<sup>50</sup> *Beta vulgaris* L. (sugar beet),<sup>51</sup> *V. radiata* L. (green gram),<sup>52</sup> *Cicer arietinum* L. (chickpea),<sup>53</sup> etc.

**Soluble Protein (SP) and Sugar (SS) under NaCl Stress.** The soluble protein concentration in pea plants was increased considerably ( $p < 0.05$ ) when exposed to NaCl. In the absence of salt (under control), the amount of soluble protein extracted from pea tissues was  $23.4 \mu\text{g mL}^{-1}$ , which, however, increased by  $65.2 \mu\text{g mL}^{-1}$  (64%) at 400 mM NaCl (Figure 3A). The following factor may account for the elevated soluble protein level generated by NaCl. Salt promotes the production of various primitive proteins while also increasing

the expression of multiple genes. The findings of this study revealed that plants exposed to salt stress had higher soluble protein levels than plants exposed to nonsaline environments. Proteins accumulated in plants cultivated in saline settings may work as a nitrogen storage form that is reutilized once the stress has passed, as well as play an osmotic regulatory role. Osmotic stress is a key method for plants to cope with salt stress, and it is induced by high salinity levels. It is a critical process for sustaining the water content of cells by increasing net solute concentrations or lowering the cell water potential through osmotic adjustment.<sup>54</sup> The two possible physiological responses to osmolyte build-up under stress are (i) lowering the cell's osmotic potential and (ii) stabilizing the membranes and macromolecular structure.<sup>55</sup>

Under NaCl stress, soluble proteins are important for osmotic correction and can provide a storage form of nitrogen. Sugars that are soluble in water serve as key osmolytes in maintaining cell homeostasis.<sup>56</sup> Soluble sugars appear to play a protective role in the membrane as well as osmotic adjustment in the root systems. Here, with increasing salt concentrations (0–400 mM NaCl), the quantity of soluble sugar was also increased in plant tissues (Figure 3B). Under NaCl stress, an increase in soluble sugar concentration could be due to the increased production of certain stress-related proteins.<sup>57</sup> Under NaCl stress, modifications in soluble sugars are accompanied by changes in CO<sub>2</sub> absorption, enzyme activity, and gene expression.<sup>58</sup> In *Nitraria tangutorum*, Liu et al.<sup>59</sup> found that saline stress caused an increase in total soluble sugars and total soluble proteins. When K<sup>+</sup> concentration is low, carbohydrates lead to an increase in osmotic pressure in stomata, permitting stomatal opening.<sup>60</sup> Likewise, a remarkable increase in the soluble sugar content in *Tagetes minuta* (L.) plants was observed when exposed to 200 mM NaCl concentration.<sup>61</sup>

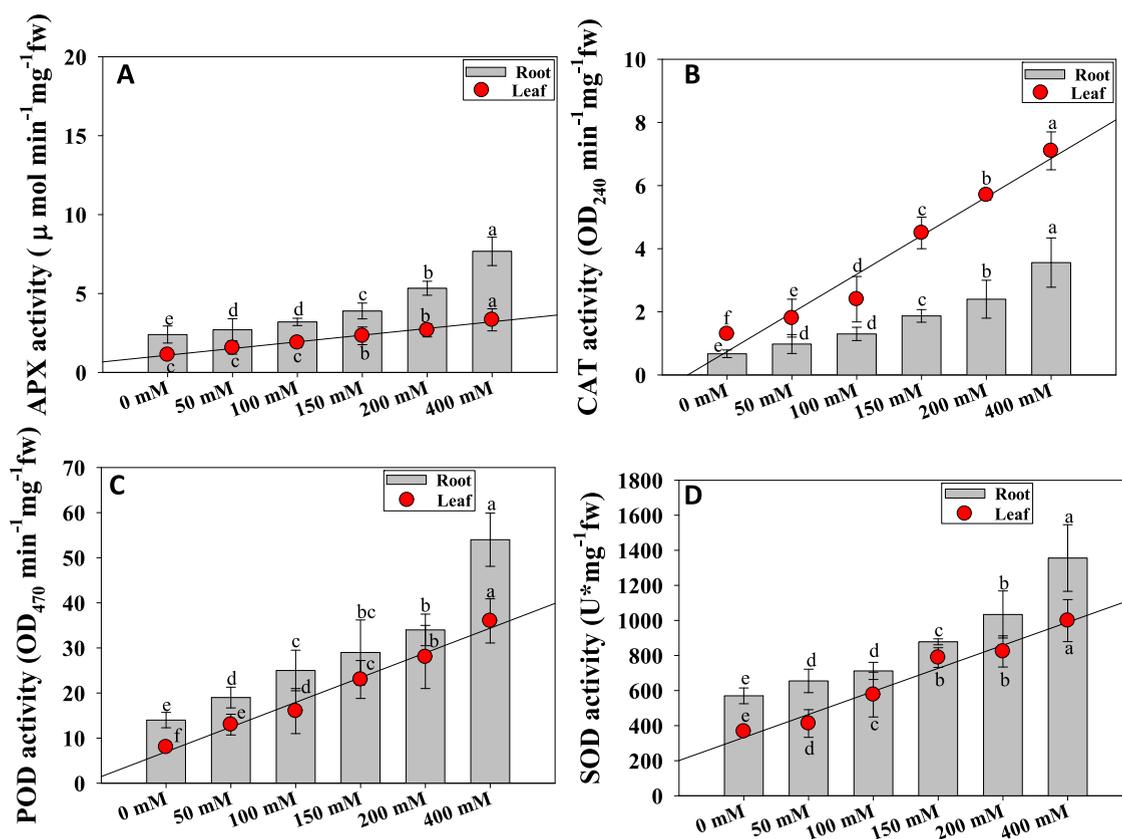
**Effect of NaCl on Na<sup>+</sup> and K<sup>+</sup> Ion Concentration and Relative Leaf Water Content (RLWC) in Peas.** When plants are exposed to salt, sodium ions (Na<sup>+</sup>) contend with potassium ions (K<sup>+</sup>), resulting in nutritional and metabolic disturbance that ultimately caused the death of plant cells. To address these difficulties, the amounts of Na<sup>+</sup> and K<sup>+</sup> in the leaf tissues of salt-treated pea plants were examined. With increasing salt concentrations, the build-up of Na<sup>+</sup>/K<sup>+</sup> increased continuously. At the 400 mM NaCl level, for example, a maximum build-up of 20.4 and 34.6 mg g<sup>-1</sup> fw in Na<sup>+</sup> and K<sup>+</sup> concentrations was observed (Figure 3C,D). A high K<sup>+</sup>/Na<sup>+</sup> ratio is one of the markers of good salt stress defense mechanisms.<sup>62</sup> Also, it is well known that the ability of plants to withstand salt stress is highly dependent on the condition of their K<sup>+</sup> nutrition.<sup>63</sup> Increased K<sup>+</sup> supply in the root environment may help to alleviate the loss of plant biomass caused by salt.

At high salinity levels (400 mM), peas showed the maximum and considerable reduction (71%) in RLWC (Figure 4A). The accumulation of poisonous ions such as Na<sup>+</sup> and Cl<sup>-</sup>, which reduces the leaf expansion and stomata closure, resulting in a decrease in intracellular CO<sub>2</sub> partial pressure, which could easily explain these findings.<sup>64</sup> Salinity reduces the capacity of plants to absorb water, resulting in a quick fall in the growth rate and a plethora of biochemical changes comparable to those seen during water shortage.<sup>65</sup>

**NaCl Changed the Proline and Malondialdehyde (MDA) Content in Peas.** To maintain the ionic balance, cytoplasm accumulates the low molecular mass compounds in the vacuoles because suitable solutes do not interfere with

normal physiological reactions and instead replace water in biological reactions. The increasing NaCl treatment generated an increase in proline content in the leaves of the investigated pea plant. The lower level of salt caused the minimum uptake of proline in plant tissues which, however, increased at greater NaCl concentrations. As an example, at 400 mM NaCl, 169 μg proline g<sup>-1</sup> fw was accumulated in pea roots, which is 90% higher than that in the control (Figure 4C). The ability of the cell to sustain its turgor pressure at low water potential improves when the osmotic potential is lowered by osmolyte build-up in response to stress. This appears to be required for physiological tasks such as photosynthesis, enzyme activity, and cell expansion. In salt-stressed plants, proline, which is abundant in higher plants, accumulates in greater quantities than other amino acids.<sup>66</sup> Under stressful conditions, induction or activation of proline biosynthesis enzymes, as well as decreased proline oxidation to glutamate, decreased the proline consumption in protein synthesis and increased protein turnover, which could all contribute to proline accumulation.<sup>67</sup> Osmotic and salinity stresses regulate the expression of genes encoding important enzymes for proline synthesis (P5C synthase; EC 2.7.2.11, P5C reductase; EC 1.5.1.2) and proline oxidation (proline dehydrogenase; EC 1.4.3), which leads to an increase or a decrease in proline concentration in plant tissue.<sup>68</sup> During early poststress metabolism, proline appears to be the predominant source of energy and nitrogen, and proline accumulation appears to supply energy for growth and survival, resulting in salinity or alkalinity tolerance. Proline synthesis and accumulation generated by salt may have worked as a compatible solute, allowing plant tissues to survive stress, according to the research. Like the current finding, various researchers have reported the salinity-induced increase in the concentration of proline. For example, the increasing NaCl treatment significantly increased the proline content in *Brassica juncea* (L.) plants.<sup>69</sup>

The production of malondialdehyde (MDA) is often utilized as a broad measure of the degree of lipid peroxidation caused by oxidative stress. Salt stress can cause oxidative stress, which can lead to the formation of lipid peroxidation products such as MDA. Like other stressor molecules (proline), the content of MDA in the roots and leaves tissues of pea plants under NaCl stress increased with increasing concentrations of salt, indicating cumulative damage (Figure 4D). The findings presented are in line with previous studies.<sup>70</sup> The increased quantity in malondialdehyde, as well as O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> levels, suggested that NaCl promoted oxidative stress in plant organs of pea. It is believed that the contents of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> increased first and subsequently declined in the root, while they always increased in the leaves, as revealed by studying the tendency of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>. Because roots are the earliest and most vulnerable plant organ to be exposed to contaminants and pressures. As a result, physiological and metabolic abnormalities, as well as toxic symptoms, first manifested themselves in the roots. Similar to this, increased salinity stress adversely affected the *Portulaca oleracea* (L.) plants where the amounts of proline and MDA significantly increased with increasing NaCl concentrations.<sup>71</sup> The increase in the quantity of proline and MDA in pea plants under saline conditions suggested that increasing salinity increased organic matter's contribution to osmotic adjustment. Plants respond to soil salinity by adjusting their osmotic balance, which is a critical component of their physiological system.<sup>72</sup>

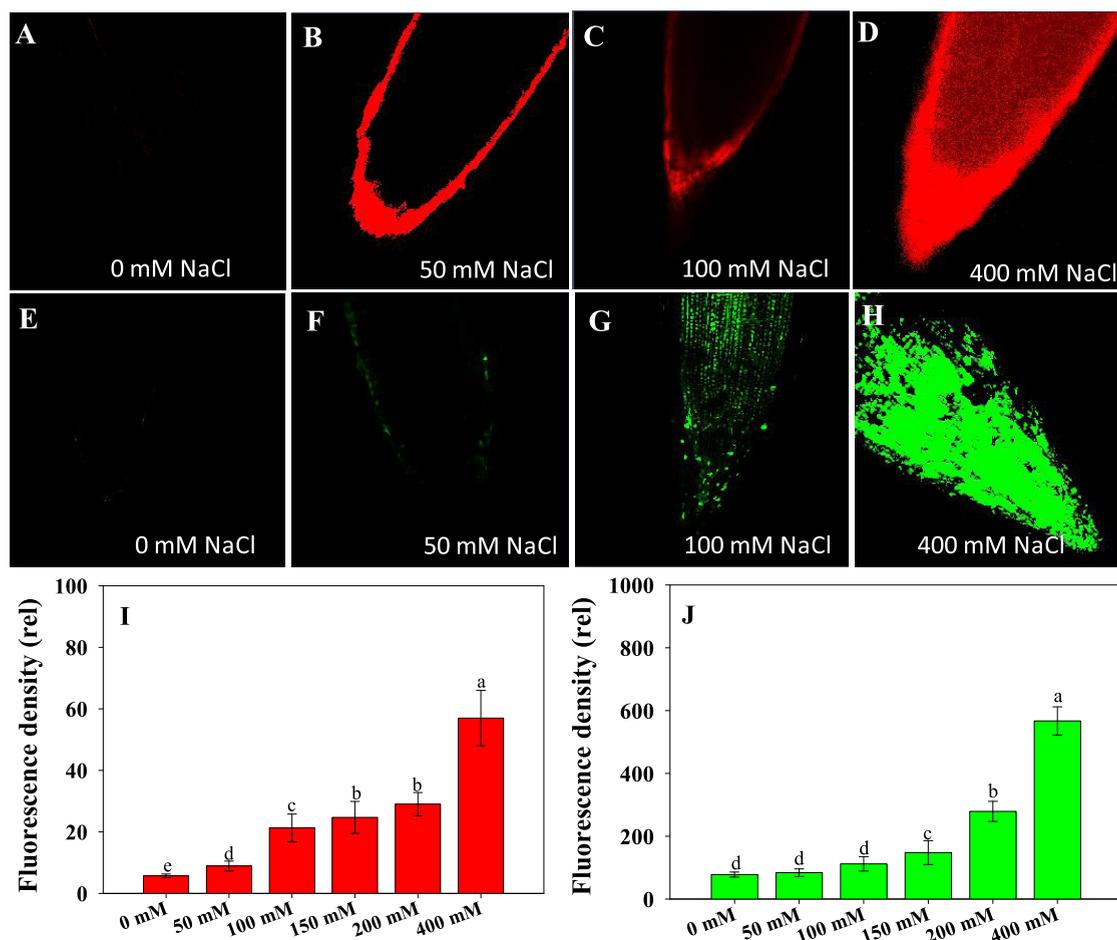


**Figure 5.** Antioxidative defense enzymes; ascorbate peroxidase (APX) (A), catalase (CAT) (B), peroxidase (POD) (C) and superoxide dismutase (SOD) (D) extracted from root and leaf tissues of pea plants detached from pot soils treated with 0, 2, 50, 100, 150, 200, and 400 mM NaCl. Each value is a mean of three replicates where each replicate constituted three plants/pots. Mean values followed by different letters are significantly different at  $p \leq 0.05$  according to the DMRT test. Vertical bars represent means  $\pm$  SD ( $n = 3$ ), and error bars represent SD.

**NaCl Modulated Antioxidant Enzymatic Activity in Peas.** In response to injury, plants have efficient scavenging systems.<sup>20</sup> By stabilizing the ROS levels in plants, the antioxidant enzymatic system protects the plant cells.<sup>73</sup> Superoxide dismutase (SOD) is the cell's first line of defense against ROS since the superoxide radical is a precursor to a variety of other highly reactive species, so maintaining a stable state of superoxide concentration via SOD is a crucial defensive mechanism.<sup>74</sup> Under stressful situations, peroxidase (POD) activity reflects the changes in cell wall mechanical characteristics and cell membrane integrity in plant leaves.<sup>75</sup> Catalase (CAT) is the most widely distributed oxidoreductase, converting  $\text{H}_2\text{O}_2$  to  $\text{O}_2$  and  $\text{H}_2\text{O}$ .<sup>76</sup> An adapted ROS-scavenging system including CAT, POD, SOD, ascorbate peroxidase (APX), and glutathione reductase (GR) might provide some protection from oxidative damage under salt-stressed circumstances.<sup>77</sup> With increasing NaCl concentrations, the antioxidant enzymatic activities in root and leaf tissues of pea plants were progressively increased. As an example, APX, CAT, POD and SOD activities in root tissues were maximally increased by 68, 80, 74, and 58%, respectively, at 400 mM NaCl with respect to untreated control plants (Figure 5A–D). Further, it has been noted that under salinity stress, root tissues of peas accumulated more antioxidants compared to leaf tissues. In this case, taking an example of CAT activity, at 400 mM NaCl, root tissues had  $7.1\text{ mg g}^{-1}\text{ fw}$ , while,  $3.56\text{ mg g}^{-1}\text{ fw}$  was recorded for leaf tissues. The higher antioxidant levels indicate that they are actively involved in scavenging ROS generated by NaCl toxicity, implying that the plants have a

high ability to withstand salt stress due to the well-functioning antioxidant defensive mechanism. Furthermore, NaCl had a more pronounced effect on the root system as the quantities of CAT and POD were larger in roots than in leaves, which can be explained by the fact that NaCl comes into direct contact with the roots and is largely absorbed via the root system. Only a limited percentage of high-concentration NaCl stored in roots makes it to the leaves. As a result, roots are subjected to more oxidative stress than leaves. Salinity-induced increases in antioxidant enzyme activity may protect the biological molecules of studied pea plants from  $\text{O}_2$ -induced damage.<sup>78</sup> Also, this improvement would have aided in the removal of ROS from pea seedlings. The build-up of antioxidant enzymes in some species under stress conditions is attributable to their tolerance ability, which is not the same in all plant species.<sup>79</sup> Salinity stress boosted the antioxidant enzymes such as polyphenol oxidase (PPO), catalase, and superoxide dismutase (SOD) in *Sesuvium portulacastrum* L.<sup>80</sup>

**NaCl-Induced Cell Damage and ROS Generation in Pea Roots.** To study the salinity-induced oxidative damage, roots of pea plants were exposed to 0–400 mM NaCl. Further, cell damage and ROS production in salinity stressed plant samples, roots were stained with propidium iodide (PI) and 3,3'-diaminobenzidine (DAB) and dichloro-dihydro-fluorescein diacetate (DCFH-DA), respectively. Increasing red fluorescence in roots is a sign of cellular damage. It is very complicated to assess the fluorescence in control roots (since these dyes are only taken up by dead tissues/cells) (Figure 6A–D). The confocal microscopy images revealed that varying



**Figure 6.** (A–D) CLSM images showing the cellular damage in root tissues of peas exposed to different concentrations of NaCl as revealed by increased PI fluorescence (left to right: roots treated with 0, 50, 100, and 400 mM NaCl, respectively). (E–H) Reactive oxygen species (ROS) generation in roots of pea plants treated with increasing salt levels as revealed by staining with 3,3'-diaminobenzidine (DAB) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) treatment (left to right: roots treated with 0, 50, 100 and 400 mM NaCl, respectively). (I, J) Quantification of dose-dependant increase in cell damage and ROS generation. Each value is a mean of three replicates where each replicate constituted three plants/pots. Mean values followed by different letters are significantly different at  $p \leq 0.05$  according to the DMRT test. Vertical bars represent means  $\pm$  SD ( $n = 3$ ), and error bars represent SD.

NaCl levels showed variable patterns of fluorescence intensity. The lower level (50 mM) of fluorescence intensity showed that minimum concentrations of salts can also induce cellular damage. With an increase in salt concentrations, the intensity of red fluorescence increased considerably. Being a fluorescent chemical/intercalating agent, PI is very often applied to stain DNA molecules and can be utilized as an alternate agent to evaluate cell membrane damage.<sup>81</sup> Damage to the cell membrane integrity caused morphological alteration in cells. PI staining results corroborated the damaging effect of salinity-induced stress on the cell membrane in pea root tips. The cells exposed to 100 mM NaCl had lower fluorescence intensity, indicating that lower levels of salts can also cause cell damage. With higher NaCl concentrations and longer treatment times, the harmful impact became more pronounced. When salt stress is temporary or adjustable at the seedling stage, nucleotides could be produced by DNA breakdown and reallocated for shoots and new root formations. Here, salinity stress resulted in cell death. Also, the growth of roots may be hampered as a result of this cell loss. Similar to this observation, a salinity-induced increase in ROS production in root tissues of rice cultivars has been reported.<sup>82</sup> Likewise, Li et al.<sup>83</sup> have also observed that higher concentrations of NaCl induced the

induction of antioxidant enzymes and increased ROS production in meristematic root tips of *Oryza sativa* (L.).

Plants go through a number of stress acclimation processes, including gene regulation in oxidative stress responses, which results in stimulation of antioxidant enzymes, to protect cells from excessive accumulation of reactive oxygen species caused by numerous environmental stresses. When a cell is under stress, distinct forms of ROS are produced in different compartments. The activation of antioxidant enzyme genes in response to oxidative stress is an important indicator for further research into plant antioxidant defense systems. The effect of increasing salt levels on ROS production in pea roots was qualitatively examined using *in vivo* histochemical labeling with fluorescent dyes. When root tissues were subjected to increasing NaCl concentrations, staining with 3,3'-diaminobenzidine (DAB) and treatment with dichloro-dihydrofluorescein diacetate (DCFH-DA) demonstrated an increase in ROS formation (in the form of an increase in green color), with increased ROS generation in the root tip region, compared to untreated controls (Figure 6E–H). Salinity-induced oxidative stress in different plant species has been reported. For example, NaCl caused oxidative stress by increasing the accumulation of hydrogen peroxide in *Triticum*

*aestivum* (wheat) seedlings, which was observed after staining the root tissues with fluorescent probe DCFH-DA.<sup>84</sup> In a study, Hernandez et al.<sup>85</sup> examined the accumulation of hydrogen peroxide in the roots tissues of *Brassica oleracea* (L.) exposed to long- and short-term NaCl stress. The increase in fluorescence of DCFH-DA-stained roots indicated that light appears to reside in the cytoplasm and apoplast of root tip cells. Also, H<sub>2</sub>O<sub>2</sub> appears to be mostly found in the mitochondrial cristae and external membrane.

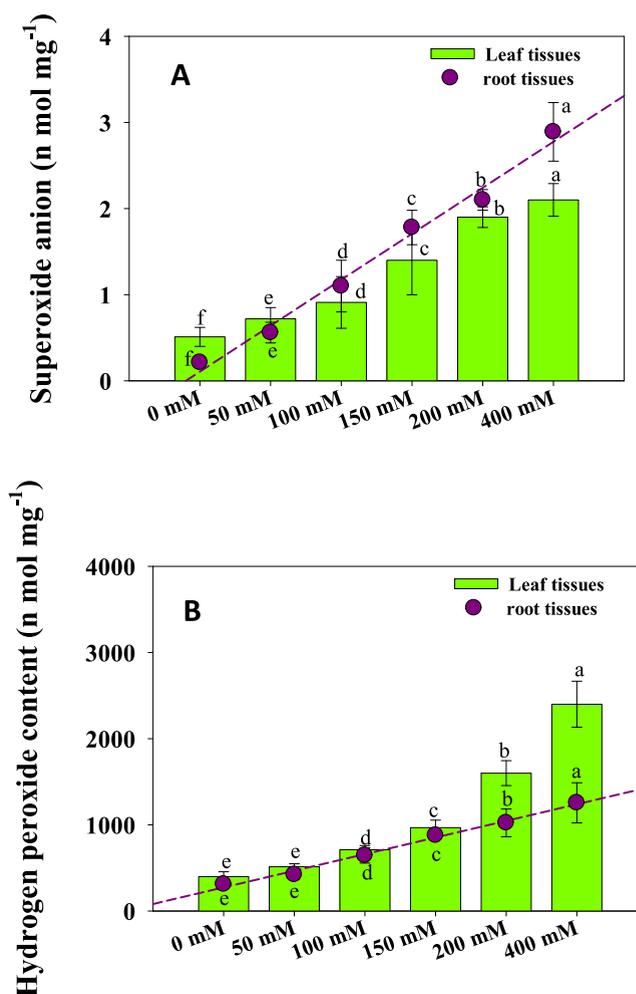
#### NaCl-Induced ROS Generation, Superoxide Ion (O<sub>2</sub><sup>-</sup>), and Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) Content in Pea Organs.

In the presence of NaCl stress, reactive oxygen species (ROS) including -OH (hydroxyl radicals), O<sub>2</sub><sup>-</sup>, and H<sub>2</sub>O<sub>2</sub> are increased.<sup>17</sup> ROS interact with other biological components, causing oxidative damage such as lipid peroxidation, protein degradation, and DNA damage.<sup>86</sup> The current study found that salt treatment increased the O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> contents of peas, which was related to the decreased integrity of the plasma membrane in plants. Plant plasma membranes are thought to be the first biological structures to be impacted by the toxicity of environmental stressors including salts.<sup>87</sup> ROS can affect the biomolecules and cause cell membrane lipid peroxidation. As a result of the damage to cell membranes, the selectivity of cell membranes has diminished.

Considering these, the impact of increasing salts on superoxide anion (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) concentrations in leaf and root tissues of pea plants was observed. The amounts of superoxide anions in both the plant organs were upsurged significantly ( $p \leq 0.05$ ) with increasing NaCl treatments. The O<sub>2</sub><sup>-</sup> content reached the maximum level at 400 mM NaCl. Similarly, salt-induced production of H<sub>2</sub>O<sub>2</sub> in root and leaf tissues of peas varied with different NaCl concentrations. A similar trend was recorded for hydrogen peroxide, i.e., as the level of NaCl increased, the production of H<sub>2</sub>O<sub>2</sub> was also increased and high NaCl levels caused the maximum production. At 400 mM NaCl, the H<sub>2</sub>O<sub>2</sub> contents in root and leaf tissues of peas were maximally increased by 75 and 83%, respectively, when compared to the untreated control (Figure 7A,B). Similar to our observation, different levels of salts significantly induced the production of oxidative stress (H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>) on rapeseed (*Brassica napus* L.) cultivars.<sup>88</sup> Additionally, Hernández et al.<sup>89</sup> found an exceptionally high increase in apoplastic hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content and O<sub>2</sub><sup>-</sup> production in NaCl-treated pea leaves that caused necrotic lesions.

## CONCLUSIONS

Plants have evolved various strategies to reduce the damage caused by nonessential high NaCl exposure. When the concentration of NaCl inside the cells becomes too high, a defense mechanism kicks in to protect the cells from oxidative stress, which can cause cell death as well as stress-induced adaptation and survival. In the current findings, as the level of salinity increased, the number of damaged and dead cells in plant tissues also increased. The growth of pea root severely slowed down, which could be attributed to the death of large root cells as a result of the high NaCl concentration and prolonged NaCl exposure. In addition, the ROS levels significantly increased with an increase in NaCl concentration. Hence, when the level of ROS excessively increased, the plant cell membrane system was injured, resulting in changes in MDA and osmotic regulatory chemicals. Significant changes in antioxidant enzymatic activities, lipid peroxidation, and cell



**Figure 7.** Effect of increasing NaCl concentrations on ROS content in pea plants raised in pot soils treated with 0, 2, 50, 100, 150, 200, and 400 mM NaCl; O<sub>2</sub><sup>-</sup> content (A) and H<sub>2</sub>O<sub>2</sub> content (B). Each value is a mean of three replicates where each replicate constituted three plants/pots. Mean values followed by different letters are significantly different at  $p \leq 0.05$  according to the DMRT test. Vertical bars represent means  $\pm$  SD ( $n = 3$ ), and error bars represent SD.

damage have been observed in peas, and they can be used as biomarkers to determine the extent of damage in plants caused by NaCl. Conclusively, the information gathered here could be useful in deciphering the tolerance mechanism in agriculturally important edible crops under NaCl-stressed conditions.

## EXPERIMENTAL SECTION

### Assessment of Seedling Germination Attributes of Peas in the Presence of NaCl Stress.

*P. sativum* seeds were sterilized for 1 min in a 0.5% sodium hypochlorite (NaOCl) solution. After that, distilled water was used to wash them twice. In Petri dishes (9 cm), 10 mL of the test solution was added to one disc of Whatman No. 1 filter paper. Parafilm was used to prevent evaporation on the dishes. The germination experiment was carried out in incubators at 25/15 °C for 20 days in four repetitions with 25 seeds in each treatment under a 12 h light/12 h dark photoperiod. The seedlings grew in 50, 100, 200, 300, and 500 mM NaCl, as well as pure water. Seeds were counted every day, and when the radicle appeared, they were judged to have germinated and removed from the Petri dishes.

**Salt Treatment and Culturing of Pea.** Seeds of *P. sativum* (L.) variety (var. Arkil) were purchased from the local seed market. Seeds were washed, rinsed, and desiccated at room temperature after being disinfected/sterilized using NaOCl (2%). The pot experiment was conducted in a pot-house condition. The solution of sodium chloride (NaCl) was produced in double-distilled water (DDW) at concentrations of 0, 50, 100, 200, and 400 mM and applied as a presowing application to moisten the soil (7.6 pH value, EC = 0.863 mv cm<sup>-2</sup>, % (organic carbon) OC = 5.17 g kg<sup>-1</sup>, total nitrogen (N) = 0.76 g kg<sup>-1</sup>, total P = 12.3 mg kg<sup>-1</sup>, K = 14.08 mg kg<sup>-1</sup>, Mg = 13.01 mg kg<sup>-1</sup>, water holding capacity (mL g<sup>-1</sup>) = 0.512, calcium (mg kg<sup>-1</sup>) = 10.15, sodium (mg kg<sup>-1</sup>) = 7.61, carbonate (mg kg<sup>-1</sup>) = 22.9, bicarbonate (mg kg<sup>-1</sup>) = 10.7, cation exchange capacity (cmol kg<sup>-1</sup>) = 14.2, anion exchange capacity (cmol kg<sup>-1</sup>) = 5.7) at least 1 day prior to sowing (20 cm in length and 24 cm in diameter) containing 5 kg of unsterilized soil. Each test concentration was repeated three times, and the pots were placed in a fully random block configuration. Seedlings were thinned after germination, and 15 days following emergence, two uniform healthy pea seedlings were kept in each pot. Pots were irrigated on a regular basis and kept in open field conditions. The crop was harvested at two different stages: 90 and 130 days after sowing (DAS). The whole study was performed for 2 successive years, and each individual experiment with identical/similar treatment was repeated for 2 consecutive years to validate the reproducibility and accuracy of data.

**Effect of NaCl on Seed Germination and Biological Features (Length and Dry Biomass) of Peas.** The NaCl-treated pea plants were harvested at 90 and 130 DAS, and morphological parameters like the length of plant organs (root and shoot), fresh weight, and dry biomass were measured. Plant samples were dried in an oven at 80 °C for 2 days and then weighed to determine dry biomass.

**Estimation of Chlorophyll Content and Nitrate Reductase Activity.** The accumulation of leaf photosynthetic molecules (chlorophyll and carotenoid) in NaCl-treated and untreated pea plants was estimated following the methods previously described by Arnon<sup>90</sup> and Kirk and Allen.<sup>91</sup>

**Nitrate Reductase (NR) Activity.** The activity of nitrate reductase (NR; EC 1.6.6.1) was determined using the intact tissue technique as previously described by Siddiqui et al.<sup>92</sup> The freshly detached NaCl-treated leaf samples were incubated in a solution comprising 2.5 mL of phosphate buffer (pH 7.5), 0.2 M potassium nitrate, and 5% iso-propanol. The reaction was calorimetrically determined by adding 1% sulfanilamide and 0.2% N-1-naphthylethylene-diamine di-hydrochloride. A calibration curve was used to compare absorbance measurements taken at 540 nm. Nanomoles NO<sub>2</sub>/g per FW (fresh weight) per hour were used to measure NR activity.

**Determination of Soluble Protein (SP) and Soluble Sugar (SS) Contents.** In this study, the soluble protein concentration was determined by means of Bradford's<sup>93</sup> technique using a reference solution of bovine serum albumin (BSA). At the end of each time interval (7 days) of the NaCl treatment, the fresh roots and leaves from each treatment (six seedlings) were rinsed in distilled water, dried, and placed in a mortar with 5 mL 0.05 M PBS (pH 7.8). The homogenate was centrifuged at 10 000g (for 20 min), and the supernatant was utilized to determine the soluble protein level. The amount of soluble protein per g of fresh weight was calculated.

**Determination of Leaf Relative Water Content (LRWC) and Na<sup>+</sup> and K<sup>+</sup> Concentrations.** The salt-treated leaf samples were cut, weighed, and stored in DDW for 3 h to get the turgid weight for the measurement of the relative leaf water content (RLWC). After that, the samples were oven-dried for 24 h at 80 °C until they reached a constant weight.<sup>94</sup> The RLWC was calculated using the following formula

$$\text{RLWC (\%)} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100$$

Leaf powder was homogenized and transferred to Erlenmeyer flasks in six different treatments. A 6.0 mL solution of nitric acid (HNO<sub>3</sub>) + perchloric acid (HClO<sub>4</sub>) was added to this mixture. After that, the samples were digested in a water bath (at 40 °C) until the volume of the sample was decreased to 1.0 mL. The residual volume was brought up to 100 mL with DDW after digestion. The concentrations of Na<sup>+</sup> and K<sup>+</sup> were calculated in the sample.<sup>95</sup>

**Proline and MDA Content Estimation.** For determination of stress, biomarker, i.e., proline content accumulated in peas plants raised in soil amended with elevated level of salts was used.<sup>96</sup> Further, for estimation of the malonaldehyde (lipid peroxidation) content, the freshly detached roots and leaves (500 mg) were homogenized using a prechilled mortar and pestle with 10 mL of 5% (w/v) trichloroacetic acid (TCA; C<sub>2</sub>HCl<sub>3</sub>O<sub>2</sub>) (SRL Pvt. Ltd. India) and centrifuged at 12 000g for 20 min at 4 °C. The supernatant (2.0 mL) was added to a tube containing 2.0 mL of 0.67% (w/v) thiobarbituric acid (TBA; C<sub>4</sub>H<sub>4</sub>N<sub>2</sub>O<sub>2</sub>S) (Hi-media, Pvt. Ltd. India). Then, the tubes were heated in a water bath at 100 °C for 30 min and rapidly cooled to 4 °C in an ice bath to terminate the reaction, and afterward, the reaction mixture was centrifuged at 10 000g for 10 min at 4 °C. The absorbance of the supernatant was measured at 532, 600, and 450 nm. The MDA content was calculated using the extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup>.<sup>97</sup>

MDA level (μmol/L)

$$= 6.45 \times (\lambda_{532} - \lambda_{600}) - 0.56 \times \lambda_{450}$$

**Antioxidant Enzyme Estimation in NaCl-Treated Peas.** For the estimation of the antioxidant enzymatic activity in pea plants, 0.5 g of freshly detached roots and leaves of NaCl-treated plants was homogenized in 50 mM phosphate buffer having a pH value of 7.8 under cold conditions after being pulverized with a mortar and pestle. The homogenized mixture was centrifuged at 12 000g for 10 min at 4 °C after being filtered through four layers of muslin cloth. Then, the prepared samples were used for the analysis of ascorbate peroxidase (APX; 1.11.1.11), catalase (CAT; 1.11.1.6), and guaiacol peroxidase (GPX, EC 1.11.1.7) activities (refer to the [Supporting Information](#) for detailed descriptions).

**Assessment of NaCl-Induced Cell Damage and ROS Generation.** *Root Staining with Propidium Iodide (PI).* The undamaged root tips of peas strained by NaCl were subjected to various doses of NaCl to investigate the cellular damage in root tip cells of peas affected by NaCl (0–400 mM). For the assessment, root samples were stained in the dark at room temperature with propidium iodide (PI) and then rinsed three times with sodium phosphate buffer (PBS) for 3 min each time (pH 7.8).<sup>98</sup> Using the analysis and measure feature of ImageJ software, the fluorescence density of ten undamaged root tips of peas under NaCl stress was evaluated to study the distribution (NIH, Bethesda, MD). A confocal laser scanning

microscope with an excitation maximum at 535 nm and a fluorescence emission maximum at 617 nm was used to analyze the immunofluorescent specimens.

**Determination of  $O_2^-$  and  $H_2O_2$  Concentrations.** To determine the concentration of ROS generation in NaCl-treated pea tissues, the previously used method of Velikova et al.<sup>99</sup> was applied. To determine the hydrogen peroxide ( $H_2O_2$ ) content in NaCl-exposed peas, 500 mg of plant samples (roots and leaves) was homogenized (experiment conducted in an ice bath) in 5.0 mL of acetone solution. Thereafter, the mixture solution was centrifuged (at 10 000g for 20 min at 4 °C). From this extract, 1.0 mL was taken and mixed with 0.20 mL of titanium sulfate ( $TiO(SO_4)$ ; 5%) and 0.2 mL of ammonia. The mixture solution was centrifuged at 10 000g for 20 min at 4 °C after it had precipitated. After rinsing three times with acetone, 5.0 mL of 2.0 M  $H_2SO_4$  (sulfuric acid) was added. The absorbance of the solution was read at 415 nm until the sediments dissolved, and the  $H_2O_2$  content was determined using a reference curve.

According to Sun et al.,<sup>100</sup> the rate of  $O_2^-$  generation was evaluated by measuring the generation of nitrite from hydroxylamine in the presence of  $O_2^-$ . For the assay, 0.2 g of frozen leaves (in an ice bath) was extracted with 2.0 mL of 50 mM PBS (pH 7.8). The centrifugation of this mixture was done at 12 000g (for 20 min at 4 °C), thereafter, 0.5 mL supernatant was mixed with 0.5 mL of 50 mM PBS (pH 7.8) and 1.0 mL of 1 mM hydroxylamine hydrochloride ( $HONH_2 \cdot HCl$ ). After 20 min in a water bath at 25 °C, 1 mL of sulfanilic acid ( $C_6H_7NO_3S$ ; 17 mM) and 1 mL of 1-aminonaphthalene ( $C_{10}H_9N$ ; 7.0 mM) were added to the mixture. After that, the solution was centrifuged for 3 min at 12 000g at 25 °C after being incubated for 20 min in a water bath at 25 °C. The absorbance of the supernatant was measured at 530 nm, and the amount of  $O_2^-$  in the sample was determined using the standard curve.

**Statistical Analysis.** To analyze the data statistically, each plant treatment was repeated three times. The data was analyzed using SPSS and Sigma Plot 10.0 software. To evaluate the significance at  $p \leq 0.05$ , one-way analysis of variance (ANOVA) and Student's *t*-test were utilized in the statistical study.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c01427>.

Details of physiochemical properties of experimental soil and detailed methodology for the antioxidant enzymatic activity (PDF)

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## Notes

The authors declare no competing financial interest.

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## ■ REFERENCES

- (1) Phogat, V.; Mallants, D.; Cox, J. W.; Šimůnek, J.; Oliver, D. P.; Awad, J. Management of soil salinity associated with irrigation of protected crops. *Agric. Water Manage.* **2020**, *227*, No. 105845.
- (2) Maggio, A.; De Pascale, S.; Angelino, G.; Ruggiero, C.; Barbieri, G. Physiological response of tomato to saline irrigation in long-term salinized soils. *Eur. J. Agron.* **2004**, *21*, 149–159.
- (3) Ebrahimi, R.; Bhatla, S. C. Effect of sodium chloride levels on growth, water status, uptake, transport, and accumulation pattern of sodium and chloride ions in young sunflower plants. *Commun. Soil Sci. Plant Anal.* **2011**, *42*, 815–831.
- (4) Islam, F.; Xie, Y.; Farooq, M. A.; Wang, J.; Yang, C.; Gill, R. A.; Zhu, J.; Zhou, W. Salinity reduces 2, 4-D efficacy in *Echinochloa crusgalli* by affecting redox balance, nutrient acquisition, and hormonal regulation. *Protoplasma* **2018**, *255*, 785–802.
- (5) Gupta, B.; Huang, B. Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. *Int. J. Genomics* **2014**, *2014*, No. 701596.
- (6) Akbarimoghaddam, H.; Galavi, M.; Ghanbari, A.; Panjehkeh, N. Salinity effects on seed germination and seedling growth of bread wheat cultivars. *Trakia J. Sci.* **2011**, *9*, 43–50.
- (7) Ali, Y.; Aslam, Z.; Ashraf, M. Y.; Tahir, G. R. Effect of salinity on chlorophyll concentration, leaf area, yield and yield components of rice genotypes grown under saline environment. *Int. J. Environ. Sci. Technol.* **2004**, *1*, 221–225.
- (8) Siddiqui, M. H.; Alamri, S. A.; Al-Khaishany, M. Y.; Al-Qutami, M. A.; Ali, H. M.; AL-Rabiah, H.; Kalaji, H. M. Exogenous application of nitric oxide and spermidine reduces the negative effects of salt stress on tomato. *Hortic., Environ., Biotechnol.* **2017**, *58*, 537–547.
- (9) Heidari, M. Effects of salinity stress on growth, chlorophyll content and osmotic components of two basil (*Ocimum basilicum* L.) genotypes. *Afr. J. Biotechnol.* **2012**, *11*, 379–384.
- (10) Long, M.; Shou, J.; Wang, J.; Hu, W.; Hannan, F.; Mwamba, T. M.; Farooq, M. A.; Zhou, W.; Islam, F. Ursolic acid limits salt-induced oxidative damage by interfering with nitric oxide production and oxidative defense machinery in rice. *Front. Plant Sci.* **2020**, *11*, 697.
- (11) Redondo-Gómez, S.; Wharmby, C.; Castillo, J. M.; Mateos-Naranjo, E.; Luque, C. J.; De Cires, A.; Luque, T.; Davy, A. J.; Enrique Figueroa, M. Growth and photosynthetic responses to salinity in an extreme halophyte, *Sarcocornia frutescens*. *Physiol. Plant.* **2006**, *128*, 116–124.
- (12) Tian, F.; Hou, M.; Qiu, Y.; Zhang, T.; Yuan, Y. Salinity stress effects on transpiration and plant growth under different salinity soil levels based on thermal infrared remote (TIR) technique. *Geoderma* **2020**, *357*, No. 113961.
- (13) Lotfi, R.; Ghassemi-Golezani, K.; Pessaraki, M. Salicylic acid regulates photosynthetic electron transfer and stomatal conductance of mung bean (*Vigna radiata* L.) under salinity stress. *Biocatal. Agric. Biotechnol.* **2020**, *26*, No. 101635.
- (14) Hussain, S.; Shaikat, M.; Ashraf, M.; Zhu, C.; Jin, Q.; Zhang, J. Salinity Stress in Arid and Semi-Arid Climates: Effects and

Management in Field Crops. In *Climate Change and Agriculture*, IntechOpen, 2019; Vol. 13.

(15) Kataria, S.; Verma, S. K. Salinity Stress Responses and Adaptive Mechanisms in Major Glycophytic Crops: The Story So Far. In *Salinity Responses and Tolerance in Plants*, Springer: Cham, 2018; Vol. 1, pp 1–39.

(16) Ling, T.; Zhang, B.; Cui, W.; Wu, M.; Lin, J.; Zhou, W.; Huang, J.; Shen, W. Carbon monoxide mitigates salt-induced inhibition of root growth and suppresses programmed cell death in wheat primary roots by inhibiting superoxide anion overproduction. *Plant Sci.* **2009**, *177*, 331–340.

(17) Banerjee, A.; Roychoudhury, A. Abiotic Stress, Generation of Reactive Oxygen Species, and Their Consequences: An Overview. In *Reactive Oxygen Species in Plants: Boon or Bane? Revisiting the Role of ROS*, Wiley, 2018; pp 23–50.

(18) Fraire-Velázquez, S.; Rodríguez-Guerra, R.; Sánchez-Calderón, L. Abiotic and Biotic Stress Response Crosstalk in Plants. In *Abiotic Stress Response in Plants—Physiological, Biochemical and Genetic Perspectives*, IntechOpen, 2011; pp 3–26.

(19) Madkour, L. H. Function of reactive oxygen species (ROS) inside the living organisms and sources of oxidants. *Pharm. Sci. Anal. Res. J.* **2019**, *2*, No. 180023.

(20) Czarnocka, W.; Karpinski, S. Friend or foe? Reactive oxygen species production, scavenging and signaling in plant response to environmental stresses. *Free Radical Biol. Medic.* **2018**, *122*, 4–20.

(21) Miller, G.; Suzuki, N.; Ciftci-Yilmaz, S.; Mittler, R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant, Cell Environ.* **2010**, *33*, 453–467.

(22) Siddiqui, M. H.; Mohammad, F.; Khan, M. N.; Al-Whaibi, M. H.; Bahkali, A. H. Nitrogen in relation to photosynthetic capacity and accumulation of osmoprotectant and nutrients in *Brassica* genotypes grown under salt stress. *Agric. Sci. China* **2010**, *9*, 671–680.

(23) García-Caparrós, P.; De Filippis, L.; Gul, A.; Hasanuzzaman, M.; Ozturk, M.; Altay, V.; Lao, M. T. Oxidative stress and antioxidant metabolism under adverse environmental conditions: a review. *Bot. Rev.* **2021**, *87*, 421–466.

(24) Shao, H. B.; Chu, L. Y.; Shao, M. A.; Jaleel, C. A.; Hong-mei, M. Higher plant antioxidants and redox signaling under environmental stresses. *C. R. Biol.* **2008**, *331*, 433–441.

(25) FAOSTAT. *Peas Green Area and Production*, 2004. <http://faostat.org/>.

(26) Dahl, W. J.; Foster, L. M.; Tyler, R. T. Review of the health benefits of peas (*Pisum sativum* L.). *Br. J. Nutr.* **2012**, *108*, S3–S10.

(27) Verma, A. K.; Banerjee, R.; Sharma, B. D. Quality characteristics of low fat chicken nuggets: effect of salt substitute blend and pea hull flour. *J. Food Sci. Technol.* **2015**, *52*, 2288–2295.

(28) Yadav, S.; Irfan, M.; Ahmad, A.; Hayat, S. Causes of salinity and plant manifestations to salt stress: a review. *J. Environ. Biol.* **2011**, *32*, 667.

(29) Baruah, B. K.; Das, M. Effect of paper mill effluent on plankton population of wetland. *Environ. Ecol.* **1997**, *15*, 770–777.

(30) Rajabi Dehnavi, A.; Zahedi, M.; Ludwiczak, A.; Cardenas Perez, S.; Piernik, A. Effect of salinity on seed germination and seedling development of sorghum (*Sorghum bicolor* (L.) Moench) genotypes. *Agronomy* **2020**, *10*, 859.

(31) Dutta, P.; Bera, A. K. 2014. Effect of NaCl salinity on seed germination and seedling growth of mungbean cultivars. *Legume Res.* **2014**, *37*, 161–164.

(32) Siddiqui, M. H.; Al-Whaibi, M. H.; Faisal, M.; Al Sahli, A. A. Nano-silicon dioxide mitigates the adverse effects of salt stress on *Cucurbita pepo* L. *Environ. Toxicol. Chem.* **2014**, *33*, 2429–2437.

(33) Janků, M.; Luhová, L.; Petřivalský, M. On the origin and fate of reactive oxygen species in plant cell compartments. *Antioxidants* **2019**, *8*, 105.

(34) El-Maarouf-Bouteau, H.; Sajjad, Y.; Bazin, J.; Langlade, N.; Cristescu, S. M.; Balzergue, S.; Baudouin, E.; Bailly, C. Reactive oxygen species, abscisic acid and ethylene interact to regulate sunflower seed germination. *Plant, Cell Environ.* **2015**, *38*, 364–374.

(35) Bewley, J. D.; Bradford, K. J.; Hilhorst, H. W.; Nonogaki, H. Germination. In *Seeds*, Springer: New York, 2013; pp 133–181.

(36) Almansouri, M.; Kinet, J. M.; Lutts, S. Effect of salt and osmotic stresses on germination in durum wheat (*Triticum durum* Desf.). *Plant Soil* **2001**, *231*, 243–254.

(37) Ali, A. S.; Elozeiri, A. A. Metabolic Processes During Seed Germination. In *Advances in Seed Biology*, IntechOpen, 2017; pp 141–166.

(38) Fernández, I. C. D.; Luque, E. G.; Mercado, F. G.; Pedrosa, W. Influence of temperature and salinity on the germination of *Limonium tabernense* Erben from Tabernas Desert (Almería, SE Spain). *Flora* **2016**, *218*, 68–74.

(39) Nasri, N.; Kaddour, R.; Rabhi, M.; Plassard, C.; Lachaal, M. Effect of salinity on germination, phytase activity and phytate content in lettuce seedling. *Acta Physiol. Plant.* **2011**, *33*, 935–942.

(40) Khaton, K.; Mahawar, L.; Shekhawat, G. S. NaCl induced oxidative stress in legume crops of Indian Thar Desert: an insight in the cytoprotective role of HO1, NO and antioxidants. *Physiol. Mol. Biol. Plants* **2020**, *26*, 51–62.

(41) Sultana, M. S.; Halim, M. A.; Hossain, F.; Karim, M. A.; Hossain, M. T. 2019. Effects of sodium chloride salinity on water relations and ion accumulation in two mungbean varieties differing in salinity tolerance. *J. Asiatic Soc. Bangladesh* **2019**, *45*, 45–54.

(42) Sharma, A.; Kumar, V.; Shahzad, B.; Ramakrishnan, M.; Singh Sidhu, G. P.; Bali, A. S.; Handa, N.; Kapoor, D.; Yadav, P.; Khanna, K.; Bakshi, P.; et al. Photosynthetic response of plants under different abiotic stresses: a review. *J. Plant Growth Regulation* **2020**, *39*, 509–531.

(43) Turan, S.; Tripathy, B. C. 2015. Salt-stress induced modulation of chlorophyll biosynthesis during de-etiolation of rice seedlings. *Physiol. Plant.* **2015**, *153*, 477–491.

(44) Punia, H.; Tokas, J.; Malik, A.; Singh, S.; Phogat, D. S.; Bhuker, A.; Mor, V. S.; Rani, A.; Sheokand, R. N. Discerning morpho-physiological and quality traits contributing to salinity tolerance acquisition in sorghum [*Sorghum bicolor* (L.) Moench]. *South Afr. J. Bot.* **2021**, *140*, 409–418.

(45) Mehta, P.; Jajoo, A.; Mathur, S.; Bharti, S. Chlorophyll a fluorescence study revealing effects of high salt stress on Photosystem II in wheat leaves. *Plant Physiol. Biochem.* **2010**, *48*, 16–20.

(46) Kalaji, H. M.; Rastogi, A.; Živčák, M.; Brestic, M.; Daszkowska-Golec, A.; Sitko, K.; Alsharafa, K. Y.; Lotfi, R.; Stypiński, P.; Samborska, I. A.; Cetner, M. D. Prompt chlorophyll fluorescence as a tool for crop phenotyping: an example of barley landraces exposed to various abiotic stress factors. *Photosynthetica* **2018**, *56*, 953–961.

(47) Biswal, B.; Joshi, P. N.; Raval, M. K.; Biswal, U. C. Photosynthesis, a global sensor of environmental stress in green plants: stress signalling and adaptation. *Curr. Sci.* **2011**, *101*, 47–56.

(48) Gouia, H.; Ghorbal, M. H.; Touraine, B. Effects of NaCl on flows of N and mineral ions and on NO<sub>3</sub>-reduction rate within whole plants of salt-sensitive bean and salt-tolerant cotton. *Plant Physiol.* **1994**, *105*, 1409–1418.

(49) Martínez, V.; Cerda, A. Nitrate reductase activity in tomato and cucumber leaves as influenced by NaCl and N source. *J. Plant Nutr.* **1989**, *12*, 1335–1350.

(50) Sacala, E.; Biegun, A.; Demczuk, A.; Grzyz, E. Effect of NaCl and supplemental calcium on growth parameters and nitrate reductase activity in maize. *Acta Soc. Bot. Pol.* **2005**, *74*, 119–123.

(51) Dias, M. A.; Costa, M. M. Effect of low salt concentrations on nitrate reductase and peroxidase of sugar beet leaves. *J. Exp. Bot.* **1983**, *34*, 537–543.

(52) Shahi, S.; Srivastava, M. Influence of foliar application of manganese on growth, pigment content, and nitrate reductase activity of *Vigna radiata* (L.) R. Wilczek under salinity. *J. Plant Nutr.* **2018**, *41*, 1397–1404.

(53) Garg, N.; Singla, R. Nitrate reductase activity in roots and leaves of chickpea cultivars under salt stress. *Spanish J. Agric. Res.* **2005**, *248*–252.

(54) Hessini, K.; Martínez, J. P.; Gandour, M.; Albouchi, A.; Soltani, A.; Abdelly, C. Effect of water stress on growth, osmotic adjustment,

cell wall elasticity and water-use efficiency in *Spartina alterniflora*. *Environ. Exp. Bot.* **2009**, *67*, 312–319.

(55) Slama, I.; Abdelly, C.; Bouchereau, A.; Flowers, T.; Savouré, A. Diversity, distribution and roles of osmoprotective compounds accumulated in halophytes under abiotic stress. *Ann. Bot.* **2015**, *115*, 433–447.

(56) Saddhe, A. A.; Manuka, R.; Penna, S. Plant sugars: Homeostasis and transport under abiotic stress in plants. *Physiol. Plant* **2021**, *171*, 739–755.

(57) Rosa, M.; Prado, C.; Podazza, G.; Interdonato, R.; González, J. A.; Hilal, M.; Prado, F. E. Soluble sugars: Metabolism, sensing and abiotic stress: A complex network in the life of plants. *Plant Signal. Behav.* **2009**, *4*, 388–393.

(58) Antunes, W. C.; Provart, N. J.; Williams, T. C.; Loureiro, M. E. Changes in stomatal function and water use efficiency in potato plants with altered sucrolytic activity. *Plant, Cell Environ.* **2012**, *35*, 747–759.

(59) Liu, W.; Zhang, Y.; Yuan, X.; Xuan, Y.; Gao, Y.; Yan, Y. Exogenous salicylic acid improves salinity tolerance of *Nitraria tangutorum*. *Russ. J. Plant Physiol.* **2016**, *63*, 132–142.

(60) Morant-Manceau, A.; Pradier, E.; Tremblin, G. Osmotic adjustment, gas exchanges and chlorophyll fluorescence of a hexaploid triticale and its parental species under salt stress. *J. Plant Physiol.* **2004**, *161*, 25–33.

(61) Moghaddam, M.; Farhadi, N.; Panjtandoust, M.; Ghanati, F. Seed germination, antioxidant enzymes activity and proline content in medicinal plant *Tagetes minuta* under salinity stress. *Plant Biosyst.* **2020**, *154*, 835–842.

(62) Hauser, F.; Horie, T. A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K<sup>+</sup>/Na<sup>+</sup> ratio in leaves during salinity stress. *Plant, Cell Environ.* **2010**, *33*, 552–565.

(63) Parida, A. K.; Das, A. B. Salt tolerance and salinity effects on plants: a review. *Ecotoxicol. Environ. Saf.* **2005**, *60*, 324–349.

(64) James, R. A.; Rivelli, A. R.; Munns, R.; Von Caemmerer, S. Factors affecting CO<sub>2</sub> assimilation, leaf injury and growth in salt-stressed durum wheat. *Funct. Plant Biol.* **2002**, *29*, 1393–1403.

(65) Munns, R. Comparative physiology of salt and water stress. *Plant, Cell Environ.* **2002**, *25*, 239–250.

(66) de Freitas, P. A. F.; de Carvalho, H. H.; Costa, J. H.; de Souza Miranda, R.; da Cruz Saraiva, K. D.; de Oliveira, F. D. B.; Coelho, D. G.; Prisco, J. T.; Gomes-Filho, E. Salt acclimation in sorghum plants by exogenous proline: physiological and biochemical changes and regulation of proline metabolism. *Plant Cell Rep.* **2019**, *38*, 403–416.

(67) Fichman, Y.; Gerdes, S. Y.; Kovács, H.; Szabados, L.; Zilberstein, A.; Csonka, L. N. Evolution of proline biosynthesis: enzymology, bioinformatics, genetics, and transcriptional regulation. *Biol. Rev.* **2015**, *90*, 1065–1099.

(68) Claussen, W. Proline as a measure of stress in tomato plants. *Plant Sci.* **2005**, *168*, 241–248.

(69) Siddiqui, M. H.; Mohammad, F.; Khan, M. N. Morphological and physio-biochemical characterization of *Brassica juncea* L. Czern. & Coss. genotypes under salt stress. *J. Plant Interact.* **2009**, *4*, 67–80.

(70) de Azevedo Neto, A. D.; Prisco, J. T.; Enéas-Filho, J.; de Abreu, C. E. B.; Gomes-Filho, E. Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environ. Exp. Bot.* **2006**, *56*, 87–94.

(71) Hnilickova, H.; Kraus, K.; Vachova, P.; Hnilicka, F. Salinity stress affects photosynthesis, malondialdehyde formation, and proline content in *Portulaca oleracea* L. *Plants* **2021**, *10*, 845.

(72) Azooz, M. M.; Shaddad, M. A.; Abdel-Latef, A. A. Leaf growth and K<sup>+</sup>/Na<sup>+</sup> ratio as an indication of the salt tolerance of three sorghum cultivars grown under salinity stress and IAA treatment. *Acta Agron. Hung.* **2004**, *52*, 287–296.

(73) Ejaz, S.; Fahad, S.; Anjum, M. A.; Nawaz, A.; Naz, S.; Hussain, S.; Ahmad, S. Role of osmolytes in the mechanisms of antioxidant defense of plants. *Sustainable Agric. Rev.* **2020**, *39*, 95–117.

(74) Wang, Y.; Branicky, R.; Noë, A.; Hekimi, S. Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. *J. Cell Biol.* **2018**, *217*, 1915–1928.

(75) Miteva, E.; Hristova, D.; Nenova, V.; Maneva, S. Arsenic as a factor affecting virus infection in tomato plants: changes in plant growth, peroxidase activity and chloroplast pigments. *Sci. Hortic.* **2005**, *105*, 343–358.

(76) Olson, K. R.; Gao, Y.; DeLeon, E. R.; Arif, M.; Arif, F.; Arora, N.; Straub, K. D. Catalase as a sulfide-sulfur oxido-reductase: an ancient (and modern?) regulator of reactive sulfur species (RSS). *Redox Biol.* **2017**, *12*, 325–339.

(77) Hasanuzzaman, M.; Bhuyan, M. H. M.; Anee, T. I.; Parvin, K.; Nahar, K.; Mahmud, J. A.; Fujita, M. Regulation of ascorbate-glutathione pathway in mitigating oxidative damage in plants under abiotic stress. *Antioxidants* **2019**, *8*, 384.

(78) Chen, Q.; Zhang, M.; Shen, S. Effect of salt on malondialdehyde and antioxidant enzymes in seedling roots of Jerusalem artichoke (*Helianthus tuberosus* L.). *Acta Physiol. Plant.* **2011**, *33*, 273–278.

(79) Alamri, S.; Hu, Y.; Mukherjee, S.; Aftab, T.; Fahad, S.; Raza, A.; Ahmad, M.; Siddiqui, M. H. Silicon-induced postponement of leaf senescence is accompanied by modulation of antioxidative defense and ion homeostasis in mustard (*Brassica juncea*) seedlings exposed to salinity and drought stress. *Plant Physiol. Biochem.* **2020**, *157*, 47–59.

(80) Kannan, P. R.; Deepa, S.; Kanth, S. V.; Rengasamy, R. Growth, osmolyte concentration and antioxidant enzymes in the leaves of *Sesuvium portulacastrum* L. under salinity stress. *Appl. Biochem. Biotechnol.* **2013**, *171*, 1925–1932.

(81) Liao, T. T.; Jia, R. W.; Shi, Y. L.; Jia, J. W.; Wang, L.; Chua, H. Propidium iodide staining method for testing the cytotoxicity of 2, 4, 6-trichlorophenol and perfluorooctane sulfonate at low concentrations with Vero cells. *J. Environ. Sci. Health, Part A* **2011**, *46*, 1769–1775.

(82) Islam, F.; Farooq, M. A.; Gill, R. A.; Wang, J.; Yang, C.; Ali, B.; Wang, G. X.; Zhou, W. 2, 4-D attenuates salinity-induced toxicity by mediating anatomical changes, antioxidant capacity and cation transporters in the roots of rice cultivars. *Sci. Rep.* **2017**, *7*, No. 10443.

(83) Li, J. Y.; Jiang, A. L.; Zhang, W. Salt stress-induced programmed cell death in rice root tip cells. *J. Integr. Plant Biol.* **2007**, *49*, 481–486.

(84) Liu, L.; Huang, L.; Lin, X.; Sun, C. Hydrogen peroxide alleviates salinity-induced damage through enhancing proline accumulation in wheat seedlings. *Plant Cell Rep.* **2020**, *39*, 567–575.

(85) Hernandez, M.; Fernandez-Garcia, N.; Diaz-Vivancos, P.; Olmos, E. A different role for hydrogen peroxide and the antioxidative system under short and long salt stress in *Brassica oleracea* roots. *J. Exp. Bot.* **2010**, *61*, 521–535.

(86) Su, L. J.; Zhang, J. H.; Gomez, H.; Murugan, R.; Hong, X.; Xu, D.; Jiang, F.; Peng, Z. Y. Reactive oxygen species-induced lipid peroxidation in apoptosis, autophagy, and ferroptosis. *Oxid. Med. Cell. Longevity* **2019**, No. 5080843.

(87) Ahmad, R.; Hussain, S.; Anjum, M. A.; Khalid, M. F.; Saqib, M.; Zakir, I.; Hassan, A.; Fahad, S.; Ahmad, S. Oxidative Stress and Antioxidant Defense Mechanisms in Plants Under Salt Stress. In *Plant Abiotic Stress Tolerance*, Springer: Cham, 2019; pp 191–205.

(88) Huang, Q.; Farooq, M. A.; Hannan, F.; Chen, W.; Ayyaz, A.; Zhang, K.; Zhou, W.; Islam, F. Endogenous nitric oxide contributes to chloride and sulphate salinity tolerance by modulation of ion transporter expression and reestablishment of redox balance in *Brassica napus* cultivars. *Environ. Exp. Bot.* **2022**, *194*, No. 104734.

(89) Hernández, J. A.; Ferrer, M. A.; Jiménez, A.; Barceló, A. R.; Sevilla, F. Antioxidant systems and O<sub>2</sub>·<sup>-</sup>/H<sub>2</sub>O<sub>2</sub> production in the apoplast of pea leaves. Its relation with salt-induced necrotic lesions in minor veins. *Plant Physiol.* **2001**, *127*, 817–831.

(90) Arnon, D. I. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* **1949**, *24*, 1.

(91) Kirk, J. T. O.; Allen, R. L. Dependence of chloroplast pigment synthesis on protein synthesis: effect of actidione. *Biochem. Biophys. Res. Commun.* **1965**, *21*, 523–530.

(92) Siddiqui, M. H.; Mohammad, F.; Khan, M.; Al-Wahaibi, M. H. Cumulative effect of nitrogen and sulphur on *Brassica juncea* L. genotypes under NaCl stress. *Protoplasma* **2012**, *249*, 139–153.

- (93) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
- (94) Barrs, H. D.; Weatherley, P. E. A re-examination of the relative turgidity technique for estimating water deficits in leaves. *Aust. J. Biol. Sci.* **1962**, *15*, 413–428.
- (95) Holiday, E. R.; Preedy, J. R. K. The precision of a direct-reading flame photometer for the determination of sodium and potassium in biological fluids. *Biochem. J.* **1953**, *55*, 214.
- (96) Bates, L. S.; Waldren, R. P.; Teare, I. D. Rapid determination of free proline for water-stress studies. *Plant Soil* **1973**, *39*, 205–207.
- (97) Heath, R. L.; Packer, L. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* **1968**, *125*, 189–198.
- (98) Koyama, H.; Toda, T.; Hara, T. Brief exposure to low-pH stress causes irreversible damage to the growing root in *Arabidopsis thaliana*: pectin–Ca interaction may play an important role in proton rhizotoxicity. *J. Exp. Bot.* **2001**, *52*, 361–368.
- (99) Velikova, V.; Edreva, A.; Loreto, F. Endogenous isoprene protects *Phragmites australis* leaves against singlet oxygen. *Physiol. Plant.* **2004**, *122*, 219–225.
- (100) Sun, X.; Luo, X.; Zhang, X.; Xie, J.; Jin, S.; Wang, H.; Zheng, X.; Wu, X.; Xie, Y. Enhanced superoxide generation on defective surfaces for selective photooxidation. *J. Am. Chem. Soc.* **2019**, *141*, 3797–3801.