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# Research Article
# Interaction Between Salt Stress and Drought Stress on Some Physiological Parameters in Two Pea Cultivars

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## Abstract

**Background and Objective:** Salinity is a key factor limiting agricultural production around the world and often occurs at the same time as drought stress. Conventional selection in saline conditions has generally failed, in part due to the large variability of natural saline soils resulting from different salinity and drought. The purpose of the work was to assess the combined effect of drought and salinity in 2 pea (*Pisum sativum* L.) cultivars cv *Lincoln* and cv *Douce de provence*. **Materials and Methods:** Seedlings were cultured on liquid medium without or with NaCl, 75 mM in controlled conditions. Fourteen days old, salt treated plants were subjected to drought (PEG 6000). After 7 days of treatment, growth parameters, mineral content ($Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$) and chlorophyll content were measured. Peroxidases and catalase activities was assayed on gels. **Results:** The combined action of drought and salt restricted leaf biomass more pronounced in cv *D. provence* than in cv *Lincoln*. This growth decrease was accompanied by a restriction in leaf hydration and $K^+$ uptake, concomitant with low $Na^+$ accumulation in cv *D. provence* leaves. Considering the response of antioxidant enzymes to combined stress, leaves guaiacol peroxidase activity showed an increase as compared to control in much more pronounced in cv *Lincoln* than in cv *D. provence*. **Conclusion:** Both pea cultivars exhibited a stimulation of catalase activity with higher in cv *D. Provence* than in cv *Lincoln*. Thus, cv *D. lincoln* had a higher osmotic drought stress than cv *D. provence*.

Key words: Antioxidant defense, combined stress, drought, growing, mineral nutrients, *Pisum sativum* L. salinity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Field pea (*Pisum sativum* L.) is widely cultivated as an important pulse crop on a global basis for human nutrition and stock-feed consumption. This species is also used for forage production[1], in rotations with cereals for provision of soil nitrogen[2], and to provide disease breaks.

Salt and water stresses due to drought and soil salinity are the extremely important abiotic stresses in limiting the yield of food crops in the world[3]. Soil salinity is a grave danger to global crop that limits agricultural production globally[4], and is present in 6% of the world's soil area and 20% of irrigated soil[5]. In addition to a natural increase in saline soils especially in arid and semi-arid areas due to partial precipitation, high temperature and evapotranspiration, insufficient organization of freshwater and clearing of soil for dry land cultivation contributes to augmented salt stress in vegetation. Because salinity is mainly important for arid and semi-arid parts and because of the increasing frequency of droughts in many other parts of the world, salinity usually occurs at the same time as drought stress[6]. Both abiotic stresses decrease the potential water in the soil and the ability of plants to absorb water, reducing the rate of cell expansion in growing tissues, the stomatal conductance and thus photosynthetic rate[3]. While dehydration also reduces nutrient availability, nutrient uptake by roots and transport from roots to shoots, saline soils further diminish plant development most often through the vegetative phase due to definite ion toxicities and ionic imbalances[7] which was projected as the 2 time representation of saline stress[8].

Since salinity is mainly significant for arid and semi-arid areas, plants have developed tolerance strategies for both salt and water stress[9]. Salinity often induces osmotic adjustment, which is considered an important mechanism for maintaining water absorption and cell turgor under stress conditions[10].

The improvement of the antioxidant defense system is considered effective in the development of resistance and adaptive characteristics in plants against drought stress. Many research findings confirm that enhanced activities of antioxidant enzyme components decrease oxidative damage, and develop and improve drought tolerance and plant resistance[11,12].

In this study we investigated the effect of osmotic stress induced by a combination of salt and PEG 6000 on the growth and antioxidant enzymes of two cultivars of pea: *Lincoln* and D. provence.

## MATERIALS AND METHODS

**Plant materials, growth conditions and salt and drought treatments:** The seeds of two pea (*Pisum sativum* L.) of commercial cultivars widely used in Tunisia come from the Seed Legume Laboratory of the Tunisian National Institute for Agriculture Research. The experiment was conducted from October, 2017 to October, 2018. *Douce de Provence* (*D. Provence* thereafter) has a shorter breeding cycle (45 days) than *Lincoln* (60 days). The seeds were surface sterilized with 96% (v/v) ethanol for 3 min and 2% (w/v) sodium hypochlorite for 5 min. After rinsing they were allowed to germinate on a filter paper in 9 cm diameter Petri dishes, moistened with distilled water or NaCl, 75 mM. The Petri dishes were stored for 4 days at room temperature (24±2°C) under dark conditions. Vigorous seedlings were selected and transferred to plastic pots and were grown in hydroponic culture containing 1/5 strength nutrient solution of Long Ashton[13]. The NaCl, 75 mM was added to the nutritive solution for half of plants. The conditions of the growth chamber were set at 25/18°C day/night, 80% relative humidity and 150 mmol $m^{-2}$ $sec^{-1}$ light intensity with a photoperiod of 14 h.

Fourteen days later, plants of salt medium were separated into two groups: The first was maintained on the same sline medium (S) and the second was supplemented with polyethylene glycol (PEG 6000) having a hydric potential -0.5 MPa. This solution is named (S+D) medium. Eight plants of each treatment were harvested 7 days later.

**Biomass measurements and inorganic ions assays:** At the harvest, individual plants were divided into leaf, stem and root fractions. Fresh weights (FW) were immediately determined. The samples were then dried in a forced draft oven at 70°C for 48 h and dry weights (DW) were determined. Leaf surface area was measured using a scanner and Optimas® software. Water content was calculated utilizing the following equation with FW, fresh weight and DW, dry weight:

$$WC = \frac{FW\text{-}DW}{DW}$$

Ions were extracted with 0.5% $HNO_3$. Sodium and potassium were assayed by flame photometry (Eppendorf) with butane-air flame, calcium by acetylene-air flame, magnesium by atomic absorption spectrophotometry (VARIAN 220 FS) and $Cl^-$ by coulometry (Butcher Cotlove chloridometer), according to manufacturers' instructions.

**Analysis of total chlorophyll content:** Total chlorophylls were extracted from fresh leaves of 8 different individuals in 80% acetone in the dark and assayed photometrically according to Arnon[14].

**Enzyme extraction and assays:** Leaf sample (200 mg) was frozen in liquid nitrogen and finely ground by pestle in a chilled motor, the frozen powder was added to 0.6 mL of phosphate buffer (pH 7.0). The homogenate was centrifuged at 13000×g for 30 min at 4°C and supernatant was used for enzymatic activity measurements. The protein concentration was determined according to Bradford[15] using bovine serum albumin as a standard.

Enzyme activities were analyzed after native gel electrophoresis of the supernatant. Guaiacol peroxidase (GPX, EC 1.11.1.7), stacking gel was 5% acrylamide in pH 6.8, 0.5 M Tris-HCl buffer, and resolving gel was 5% acrylamide in pH 8.8, 1.5 M Tris-HCl buffer. Catalase (EC 1.11.1.6) was identified on 6% acrylamide gels. The same Tris-glycine analysis buffer 23 mM, pH 8.3 was used for the three enzymatic systems. The migration was carried at 4°C at 120 V, with a pH 4.0, 8 mM glycine buffer as analysis buffer, using the Mini-protean Bio-Rad system. To show the activity of peroxidase, the gels were incubated for 30 min in the dark in 50 mL of pH 4, 100 mM acetate buffer containing 1% (w/v) guaiacol. They were then transferred to 50 mL acetate buffer (100 mM, pH 5) containing 25 mg of 3-amino-9-ethylcarbazole, 25 mL of N,N-dimethyl formamide (99%, v/v), 1 mL 0.1 M $CaCl_2$ and 0.5 mL $H_2O_2$ (30%, v/v). Gel staining for catalase activity was performed according to Chandlee and Scandalios[16] after pretreatment in 0.01% (v/v) $H_2O_2$ for 10 min. The staining mixture contained 1% (w/v) $FeCl_3$ and 1% (w/v) $K_3Fe(CN)_6$ in distilled water.

**Statistical analysis:** Statistical analysis were performed with StatisticaTM Software, using ANOVA and the Newman-Keuls test for post hoc mean comparison.

## RESULTS

**Biomass production:** After 21 d of culture in salt, the leave dry weight exhibited marked reductions only in *D. provence* (70% of control) (Fig. 1). cv *Lincoln* appeared more tolerant to salinity in the nutrient solution than cv *D. provence*. Under the combined action of drought and salt, *D. provence* leaves were more affected than those from Lincoln and the decrease in dry weight reached 48 and 18% of control, respectively (Fig. 1).

The water content on a dry matter basis of both pea cultivars decreased with salinity and when both stresses were combined, with much more marked impact for the second constraint and for cv *D. provence* (48% of control) (Fig. 1). Moreover, we noticed early leaf wilting of the latter cultivar on PEG, announcing osmotic effects due to this organic osmoticum.

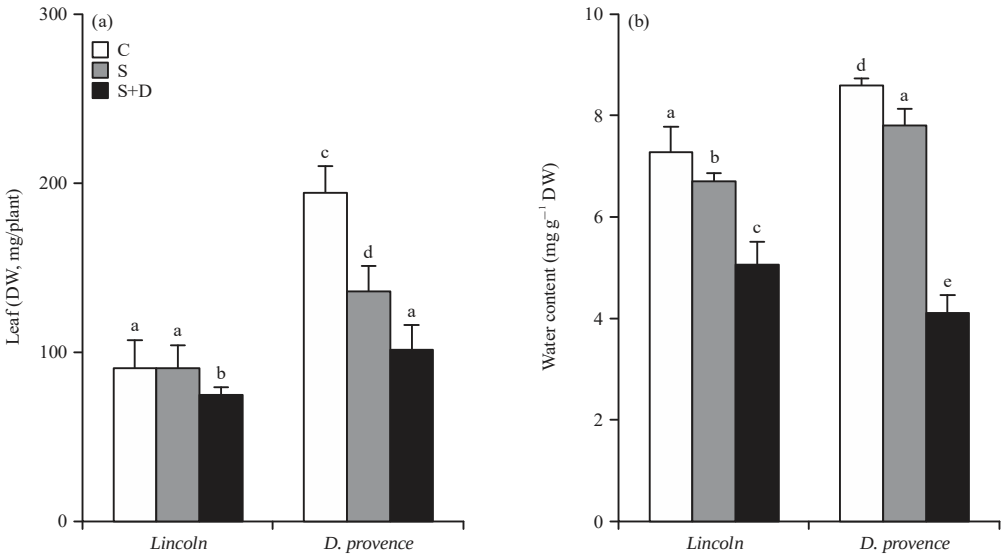


Fig. 1(a-b): Combined effect of NaCl and PEG 6000 on (a) Leaf dry weight (DW, mg/plant) and (b) Water content (mL g$^{-1}$ DW) in 2 pea cultivars, *Lincoln* and *D. provence*

Fourteen days old salt-treated plants were grown for 7 days in the absence (S) or in the presence (S+G) of PEG 6000 (C) plants grown in the absence of both NaCl (75 mM) and PEG 6000, mean of 8 plants and confidence intervals for p = 0.05, mean sharing a same letter are not significantly different at p = 0.01 (ANOVA and mean comparison with Newman-Keuls test)

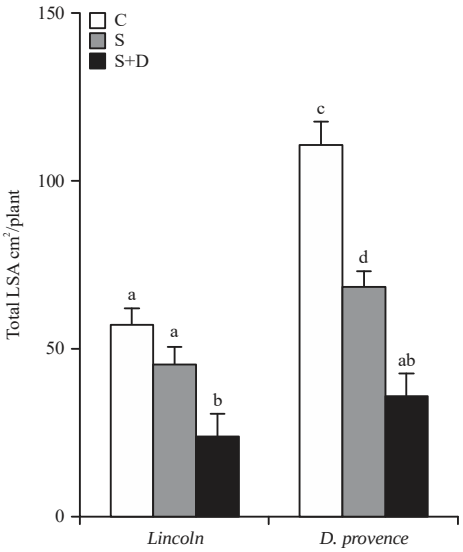


Fig. 2: Combined effect of NaCl and PEG 6000 on total leaf surface area ($cm^2$/plant) in 2 pea cultivars, *Lincoln* and *D. provence*

Fourteen days old salt-treated plants were grown for 7 days in the absence (S) or in the presence (S+G) of PEG 6000. (C) plants grown in the absence of both NaCl (75 mM) and PEG 6000, mean of eight plants and confidence intervals for p = 0.05, mean sharing a same letter are not significantly different at p = 0.01 (ANOVA and mean comparison with Newman-Keuls test)

Total area of leaves/plant was limited in the presence of NaCl to about 81 and 61% of control, in *Lincoln* and *D. provence*, respectively (Fig. 2). However, this effect was not statistically significant (p = 0.05) in *Lincoln*. Salt presence in the culture medium of plants subjected to drought strongly restricted leaf expansion, more active in cv *D. provence* (32% of control) than in cv *Lincoln* (42% of control) (Fig. 2).

**Salt accumulation:** Great contents of $Na^+$ and $Cl^-$ were accumulated in treated plants, more than 1.5 mmol $g^{-1}$ DW in leaves of both cultivars (Fig. 3). Combined effect of drought and salinity (S+D treatment) greatly reduced sodium and chloride accumulation in cv *Lincoln* leaves, as compared to cv *D. provence* leaves.

**Mineral nutrition:** In the leaves of cv *Lincoln* and cv *D. provence*, accumulation of $K^+$, $Ca^{2+}$ and $Mg^{2+}$ was reduced by salt treatment compared to control in both pea cultivars, *Lincoln* and *D. provence* (Fig. 4). Combined salinity+drought stress partially mitigated the limitations caused by NaCl to potassium content only in *Lincoln*. In leaves of *D. provence*, it gave rise to an accumulation of $K^+$ almost equal to that of the salt treatment. In combined salt and drought treatment,

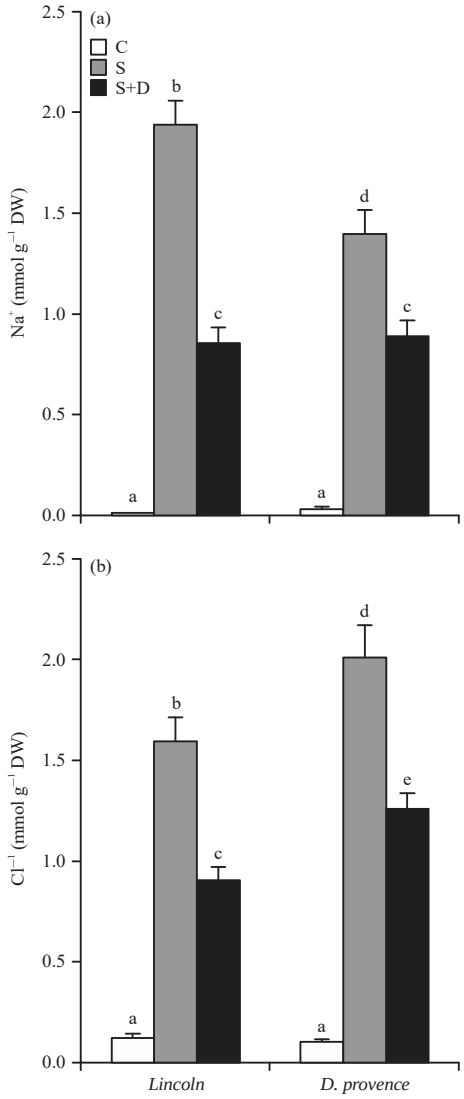


Fig. 3(a-b): Combined effect of NaCl and PEG 6000 on salt accumulation in two pea cultivars, *Lincoln* and *D. provence*

Fourteen days old salt-treated plants were grown for 7 days in the absence (S) or in the presence (S+G) of PEG 6000 (C) plants grown in the absence of both NaCl (75 mM) and PEG 6000, mean of eight plants and confidence intervals for p = 0.05, mean sharing a same letter are not significantly different at p = 0.01 (ANOVA and mean comparison with Newman-Keuls test)

$Ca^{2+}$ and $Mg^{2+}$ accumulation was similar to that of salt-treated plants for both cultivars with a small decrease for $Ca^{2+}$ accumulation in *D. provence* leaves (Fig. 4).

**Total chlorophylls:** Treatment with NaCl did not change total chlorophylls in *Lincoln* leaves, but stimulated an increase in cv *D. provence* (Fig. 5). The effects of the two constraints when combined increased the chlorophyll content in both cultivars.

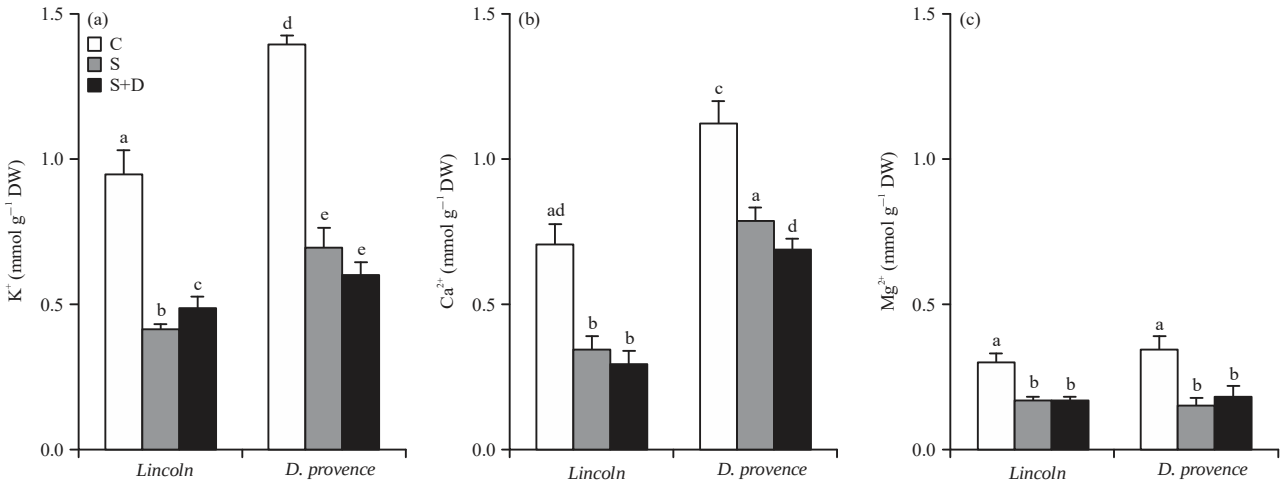


Fig. 4(a-c): Combined effect of NaCl and PEG 6000 on mineral nutrition (a) $K^+$, (b) $Ca^{+2}$ and (c) $Mg^{2+}$ in two pea cultivars, *Lincoln* and *D. provence*

Fourteen days old salt-treated plants were grown for 7 days in the absence (S) or in the presence (S+G) of PEG 6000 (C) plants grown in the absence of both NaCl (75 mM) and PEG 6000, mean of 8 plants and confidence intervals for p = 0.05, mean sharing a same letter are not significantly different at p = 0.01 (ANOVA and mean comparison with Newman-Keuls test)

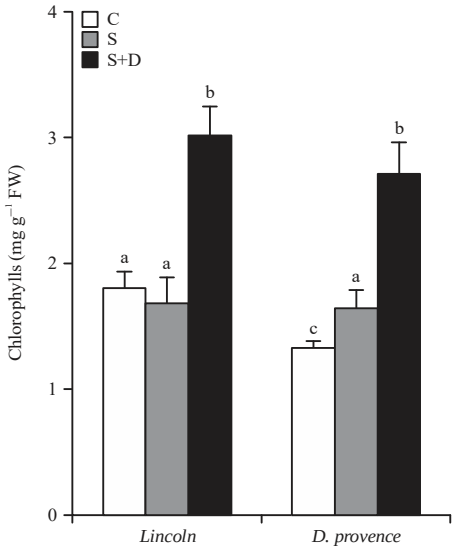


Fig. 5: Combined effect of NaCl and PEG 6000 on total chlorophylls in 2 pea cultivars, *Lincoln* and *D. provence*

Fourteen days old salt-treated plants were grown for 7 days in the absence (S) or in the presence (S+G) of PEG 6000 (C) plants grown in the absence of both NaCl (75 mM) and PEG 6000, mean of eight plants and confidence intervals for p = 0.05. Mean sharing a same letter are not significantly different at p = 0.01 (ANOVA and mean comparison with Newman-Keuls test)

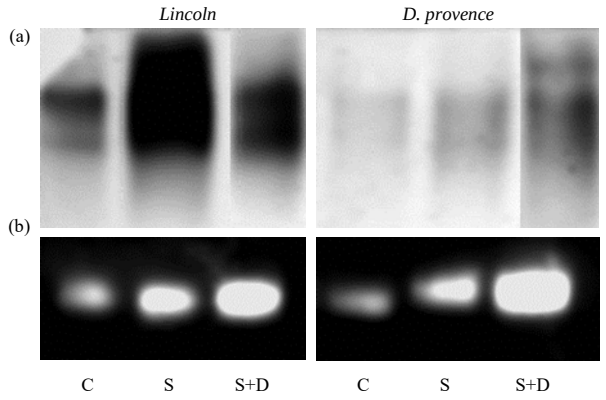


Fig. 6(a-b): Combined effect of NaCl and PEG 6000 on leaf antioxidant enzyme activities in 2 pea cultivars, *Lincoln* and *D. provence*. Electropherograms of antioxidant enzymes in extracts from pea leaves, (a) Guaiacol peroxidase and (b) Catalase

Fourteen days old salt-treated plants were grown for 7 days in the absence (S) or in the presence (S+G) of PEG 6000 (C) plants grown in the absence of both NaCl (75 mM) and PEG 6000

**Leaf antioxidant enzyme activities:** The electrophoretic examination illustrated that independently of the treatment, GPX activity was higher in cv *Lincoln* than in cv *D. provence*. In addition, this activity considerably stimulated in cv *Lincoln* under NaCl; however, in cv *D. provence*, it appears to remain unaffected or slightly stimulated (Fig. 6a). Under both water deficit and salinity stress, GPX activity decreased in cv *Lincoln* compared to salt treatment, but increased in cv *D. provence* (Fig. 6a).

Catalase (CAT) is implicated in scavenging hydrogen peroxide created by dismutation of superoxide anions catalyzed by superoxide dismutase enzyme. The

electrophoretic examination discovered higher CAT activity in response to salt stress and much more elevated under the interaction stress of salinity and drought both in cultivars (Fig. 6b).

## DISCUSSION

Salinity and drought are 2 environmental stresses that often occur concurrently in arid areas. The capacity to overcome various and concurrent stresses is of big importance for the plant development and endurance in environmental constraints[17]. Despite the presence of different methods for application of water stress, polyethylene glycol 6000 was used in this study for the reason that a cytorrhytic instead of plasmolytic reduce water potential effect can be imposed with solutions including a high molecular weight solute such as polyethylene glycol with molecular mass 6000 or above[18]. Polyethylene glycol 6000 or above cannot penetrate the pores of plant cells[19]. In addition, PEG is a good choice to impose a lower water potential than mannitol, an often used solute, as it has been shown that mannitol is absorbed by the plant cells and may have adverse effects on development[20].

Destructive conventional samplings do not allow dissecting the three mechanisms of tolerance to salinity: Excluding $Na^+$, tissue tolerance against $Na^+$ and osmotic tolerance[21]. In a previous work[22], seedlings of 2 pea cultivars *Lincoln* and *D. provence* grow for 14 days on ordinary medium were disputed for 21 days with NaCl using a split-root system. Processing complete salt (S/S) resulted growth inhibition more marked in cv *Lincoln* than in cv *D. provence*[22]. The opposite was observed in this work, explaining the effect of seeds irrigation with saline solution. Combined effect of drought and salt stresses causes a decrease in dry weight, especially in cv *D. provence* which shows a particular sensitivity to this organic osmoticum. In general, it is accepted that the key destructive factors at the cellular stage in water-deficit and salt are the toxic and osmotic effects of salt[23].

The interaction between salinity and drought stresses diminished water potential in barley[24]. These results showed that salinity considerably diminished water content in leaves, with a remarkable impact when plants subjected to NaCl and PEG 6000 in the culture medium, more pronounced in cv *D. provence* than in cv *Lincoln* (Fig. 1). The main differentiation among low water potential situation due to salinity against water deficit is the whole quantity of water accessible. Plants have a ability to regulate their osmotic potential, which avert defeat of turgor and produce a minor water potential, which allows the plants to access water in soil solution for development[25].

As for ion effects, concentrations of $Na^+$ and $Cl^-$ accumulation in plants subjected to combined stress were greatly lower than those treated only with NaCl. This may propose that salinity cannot lead to effect of ions due to salinity under combined stress, signifying that the exclusion of Na and $Cl^-$ cannot always be a tolerance of plants to salt stress, which is maintained by Genc *et al.*[26].

The increased salt treatments cause an increase in $Na^+$ and $Cl^-$ and a decrease in $Ca^{2+}$, $K^+$ and $Mg^{2+}$ leaves in several plants[27]. Plant physiology studies have shown that throughout salt stress, early symptoms on plant development are due to water stress, while effects of salt-specific only occur much later (from a few days to a few days)[28,29].

Chlorophyll content in leaves is a key parameter for characterization of physiological yield of plants[30]. Decrease in photosynthesis is often related to the decrease of pigment content caused by inhibition of their synthesis or stimulated damage as well as chloroplasts destruction[31]. In the present study, salt stress did not change total chlorophylls in leaves of cv *Lincoln*, but stimulated an increase in cv *D. provence* ones (Fig. 5). The concentrations of total chlorophylls in plant tissue when the two constraints where combined were higher in the two pea cultivars which can be attributed to a capacity and carbohydrate formation under water deficit and salinity stresses. Augmentation of chlorophyll content as a result of salt constraint was observed in a previous study[32].

Environmental constraints restrictive photosynthesis may augment oxygen-induced cellular damage as a result of augmented ROS generation[33,34]. The level of damage by ROS is related to the equilibrium between ROS product and its elimination by these antioxidant systems[35,36]. It has been found that a relationship between the antioxidant capacity and NaCl tolerance was established by assessment of tolerant cultivars with sensitive cultivars in pea plants[22]. In the present study, the peroxidases and catalase activity was increased in leaves under salt stress and combination of 2 stresses stress, but pea cultivars response pattern was different. The most induction of enzyme activity was observed in salt-treated cv *Lincoln* and in cv *D. provence* under drought stress in combination with salinity. The results suggest that different regulatory mechanisms may exist in the regulation of antioxidant enzyme activity under different situations. Several studies have related oxidative defense systems with salt tolerance. On the other hand, the regulation of a number of oxidative defenses can be a secondary response to salt stress[37]. It has been shown that the production of organic osmolytes through plant stresses such as NaCl or water deficit develops osmoregulation and protects the enzymatic function in the cytosol[38], as validated in recent transgenic studies[39]

including tobacco[40] and potato[41]. Little study has been conducted to date to classify QTLs controlling creation of different osmoprotectants, except proline accumulation studies[42]. The efficacy of various osmolytes in improving salinity tolerance may also vary from one species to another[43] which may provide another way to identify genes that are useful for pea tolerance to salt stress.

## CONCLUSION

In conclusion, an important finding of our study is that a higher sensitivity to osmotic drought stress, induced by a combination of NaCl and PEG 6000, of cv *D. provence* compared to cv *Lincoln* was evident from the higher reduction in growth, water content, mineral nutrition mainly $K^+$ and $Ca^{2+}$ accumulation, which was concomitant with low $Na^+$ accumulation in cv *D. provence* leaves.

## SIGNIFICANCE STATEMENT

This study discovers the Interaction between salt stress and drought stress in 2 pea cultivars *Lincoln* and *D. provence*. These 2 cultivars showed, in a previous study, different responses of plants subjected to salt stress. This study will help the researcher to uncover the most tolerant cultivar for salt responses as well as for water stress and the combined effect on a species growing as a significant crop on a universal basis for human nutrition. Thus, a cultivar shows a degree of tolerance to combined stress, may be retained.

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