

## Role of active transport of potassium to leaves in the mechanisms of tolerance to salinity in common bean (*Phaseolus vulgaris* L.)

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### Abstract

Biochemical markers are of great utility in screening for salt tolerance of crops. In common beans (*Phaseolus vulgaris*), lower levels of proline under stress have been associated with a better stress resistance of cultivars. In the present study, the responses to salinity have been analysed in six cultivars of common beans: four local landraces from Spain and two experimental lines from Cuba. Proline was used for ranking the relative tolerance of the cultivars, confirming a previous study which reported as more stress-tolerant two of the Spanish landraces. Total soluble sugars concentrations varied with treatments and between genotypes, but it was difficult to assess their role in stress tolerance of the analysed plants. Sodium concentration in leaves was the lowest in one of the two salt-resistant cultivars, and potassium did not vary or even increased under salt stress in all of them, except for the most susceptible one, where a drop of this cation was registered under 150 mM NaCl. Changes in malondialdehyde (MDA) contents did not indicate salt-induced membrane peroxidation resulting from secondary oxidative stress; consequently, accumulation of total phenolic compounds and flavonoids, as an antioxidant defence mechanism, was not detected. These results highlight the reliability of using proline as a biochemical marker of salt stress in common beans and the importance of the mechanism related to potassium transport to leaves in conferring stress tolerance to some common bean cultivars.

**Keywords:** bean cultivars; potassium transport; proline; salt stress; sodium exclusion

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### Introduction

The formidable challenge for agriculture represented by global warming and other harmful effects of climate change makes studies on stress tolerance in crops particularly relevant. An ever-growing human population, which will reach almost 10 x 10<sup>9</sup> people by the middle of the century, will need around 70% more food and plant products provided by agriculture (Dwivedi *et al.*, 2016). Agriculture, in areas with an arid and semi-arid climate, and soon also in other parts of the world that will be affected by climate change, relies on the extensive use of irrigation, which triggers in time the so-called secondary salinisation. This is produced by

the accumulation of toxic ions in the soil from the irrigation water and is causing an enormous loss of arable land every year (Daliakopoulos *et al.*, 2016). Highly productive elite germplasm developed to provide high yields under optimal cultivation conditions (irrigation, high agrochemical inputs) may not cope with environmental restrictions imposed by climate change (Fita *et al.*, 2015). Climate-smart agriculture can rely on improving cultural technologies, such as adjusting planting and harvesting time, genetic engineering approaches or the use of cultivars or new crops better adapted to environments prone to abiotic stress (Raza *et al.*, 2019). Landraces developed as a result of the human-mediated and natural selection of traits adapted to local conditions, often suboptimal or even highly stressful. They shelter a high phenotypic variability, and some are tolerant to abiotic or biotic stresses (Mercer and Perales, 2010). Therefore, landraces represent a valuable source of allelic richness that may have an important role in the breeding of new varieties and also have an additional market value as they are increasingly demanded by consumers as healthier and tastier (Hurtado *et al.*, 2014). Screening for stress tolerance in local landraces is achieving more and more relevance (Arteaga *et al.*, 2019, 2020). However, the mere knowledge of which varieties are more tolerant to stress is not sufficient; the key is to analyse the mechanisms supporting this tolerance. Plants respond to stress factors by the activation of a series of conserved responses at the cellular, tissue, organ and whole plant levels (Larcher, 2003). Plant growth, especially the reduction of their fresh weight and water content, are optimal parameters indicating the level of stress affecting the plants since these processes depend on the metabolic and physiological capacity for adaptation and acclimatisation to environmental conditions. Under salt stress, the levels of ions, especially potassium and sodium, both in roots and leaves, are also good indicators of stress tolerance in plants. Compatible solutes, also called osmolytes, are diverse chemical compounds, which contribute to the osmotic adjustment and play osmoprotective roles, acting as low-molecular-weight chaperons in the stabilisation of proteins, membranes and other macromolecular structures under conditions of cell dehydration, as well as in reactive oxygen species (ROS) detoxification (Hussain *et al.*, 2008; Szabados and Savouré, 2010). Excessive ROS accumulation that occurs under different types of stresses in plants causes oxidative stress, which activates antioxidant systems, both enzymatic and non-enzymatic, for preventing or reducing oxidative damage to proteins, membranes and DNA (Apel and Hirt, 2004; Türkan and Demiral, 2009). Amongst the major non-enzymatic antioxidants are phenolic compounds, especially flavonoids, which have a marked antioxidant activity since they undergo oxidation before other compounds and therefore protect against oxidant attacks.

Common bean (*Phaseolus vulgaris* L.) is an essential legume in human nutrition (Broughton *et al.*, 2003), with Mesoamerican and Andean origin (Gepts and Debouck, 1991; Rendón-Anaya *et al.*, 2017), but cultivated since centuries in Europe, where a secondary centre of diversification emerged in the Iberian Peninsula (Pinheiro *et al.*, 2007). Common bean is a glycophyte, sensitive to salt stress, as the vast majority of crops. However, not all cultivars respond equally to salt stress, some showing a better adjustment than others to saline soils (Gama *et al.*, 2007; Kaymakanova and Stoeva, 2008; Al Hassan *et al.*, 2016).

This study aims to analyse the mechanisms of response to salt stress in a selection of cultivars (both tolerant and susceptible to salinity) regarding (i) accumulation of osmolytes, (ii) ionic homeostasis, (iii) levels of malondialdehyde (MDA) as an indicator of oxidative stress, and (iv) accumulation of total phenolic compounds and flavonoids as representative non-enzymatic antioxidants.

## Materials and Methods

### *Plant material*

The study included four local landraces of *Phaseolus vulgaris* from Spain ('BGV001191', 'BGV001581', 'BGV004161' and 'BGV015856') and two experimental lines from Cuba ('E-125' and 'Milagro VIII') from INIFAT (Alexander Humboldt Institute for Basic Research in Tropical Agriculture)

and 'IIHDL' (Liliana Dimitrova Horticultural Research Institute, La Habana, Cuba). The seeds were provided by the Germplasm bank of the Institute for the Conservation and Improvement of Valencian Agrodiversity (COMAV) of the Polytechnic University of Valencia, Spain, and the Bioplants Center, University of Ciego de Ávila, Cuba, respectively. A previous study (Arteaga *et al.*, 2020) reported that cultivars 'BGV004161', 'BGV015856' were relatively more salt and drought tolerant, whereas the others, in particular, 'E125' were more susceptible to these two types of stress.

**Table 1.** Origin of the common bean cultivars analysed

Genebank's code	Country	Origin	Name of the cultivar
BGV001191 <sup>a</sup>	Spain	Almería	Judía
BGV001581 <sup>a</sup>	Spain	Palma de Mallorca	Judía de careta
BGV004161 <sup>a</sup>	Spain	Plasencia, Cáceres	
BGV015856 <sup>a</sup>	Spain	Alicante	Habichuela del barco
E125 <sup>b</sup>	Cuba	IIHLD	
Milagro VIII <sup>b</sup>	Cuba	INIFAT	Milagro Villareñoa

<sup>a</sup> Local landrace; <sup>b</sup> experimental lines

#### *Plant growth and stress treatments*

Seeds were germinated in trays with peat, perlite and vermiculite (2:1:1). Once the first true leaves were formed, seedlings were transplanted to individual 1.6 L pots with the same substrate and irrigated with Hoagland's nutrient solution (Hoagland and Arnon, 1950). Salt treatments were started when the plants reached a height of ca. 20 cm by watering twice a week with aqueous solutions of 50, 100 and 150 mM NaCl, or with deionised water for the control treatments (Figure 1). Treatments were extended for 18 days in the greenhouse under the following conditions: long-day photoperiod (16 h of light and 8 h of darkness), temperature of 23 °C during the day and 17 °C at night, and relative humidity ranging between 50% and 80%.



**Figure 1.** Plants exposed to saline stress treatments

#### *Osmolyte quantification*

Proline (Pro) determination was performed following the classical method described by Bates *et al.* (1973) with small laboratory modifications. Fresh leaf material was extracted in a 3% (w/v) sulfosalicylic acid solution, then mixed with acid ninhydrin, incubated for one hour at 95 °C, cooled on ice and extracted with two volumes of toluene. After collecting the upper organic phase, its absorbance was read at 520 nm, with toluene used as a blank. Total soluble sugars (TSS) were extracted from dry leaf material with 80% (v/v) methanol, mixed on a rocker shaker for 24 h and then quantified spectrophotometrically at 490 nm,

following the phenol/sulphuric acid method (Dubois *et al.*, 1956). The concentrations of TSS were expressed as “mg equivalent of glucose” per g DW.

#### *Monovalent ions measurements*

Samples were extracted by incubating 0.15 g of ground dry leaf material in 25 mL of water for one hour at 95 °C in a water bath (Weimberg, 1987), followed by cooling the sample on ice and filtration through a 0.45 µm filter (Gelman Laboratory, PALL Corporation). Sodium and potassium were measured in a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA).

#### *MDA and non-enzymatic antioxidants quantification*

Malondialdehyde (MDA), total flavonoids (TF) and total phenolic compounds (TPC) were determined in 80% (v/v) methanol extracts of 100 mg of fresh plant material. MDA was determined following the protocol of Hodges *et al.* (1999). Extracts were mixed with 0.5% thiobarbituric acid (TBA), prepared in 20% TCA (or with 20% TCA without TBA for the controls), and were then incubated at 95 °C for 20 min. After stopping the reaction on ice, the supernatant's absorbance was measured at 532 nm. The non-specific absorbance at 600 and 440 nm was subtracted, and the MDA concentration was calculated with the equations described in Hodges *et al.* (1999).

Total phenolic compounds (TPC) were quantified as described in Blainski *et al.* (2013) by reaction with the Folin-Ciocalteu reagent. The extracts were mixed with the reagent and sodium carbonate and left in the dark for 90 min. Absorbance was recorded at 765 nm, and the results were expressed in equivalents of gallic acid, used as a standard (mg eq GA g<sup>-1</sup> DW).

Total flavonoids (TF) were measured by reaction of the methanol extracts with NaNO<sub>2</sub> followed by AlCl<sub>3</sub> at a basic pH (Zhishen *et al.*, 1999). Absorbance was measured at 510 nm, and the TF contents were expressed in equivalents of catechin used as a standard (mg eq C g<sup>-1</sup> DW).

#### *Statistical analysis*

Data were analysed using the software Statgraphics Centurion v.16 (Statpoint Technologies, Warrenton, VA, USA). A two-way analysis of variance (ANOVA) was performed for all traits analysed to check the interaction between the genotype and the treatments in all cultivars. The post-hoc Tukey test (p<0.05) was used to check the effect of treatment in each genotype separately.

## **Results**

#### *Osmolytes*

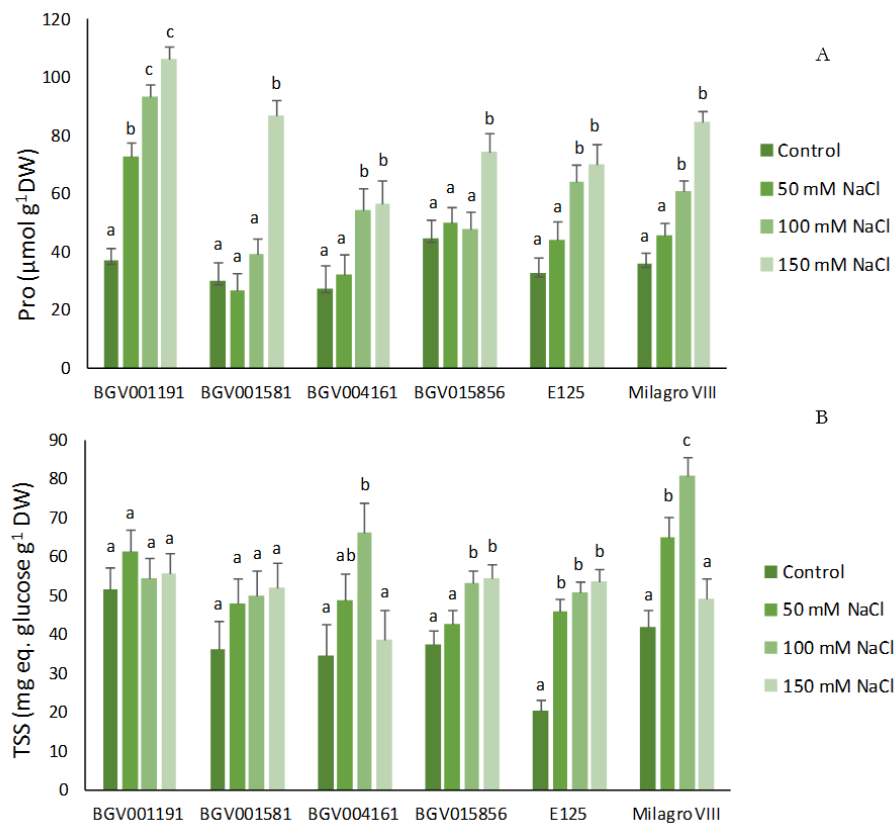
Two main types of osmolytes have been analysed in the six accessions of common bean. Proline (Pro) increased in response to salt stress in all cultivars, and differences with respect to other treatments were significant in the presence of 150 mM NaCl, for all cultivars. However, a considerable difference in Pro concentration was noticed between cultivars, with maximal values ranging from 56 µmol g<sup>-1</sup> DW in ‘BGV004161’ to 106 µmol g<sup>-1</sup> DW in ‘BGV001191’. Also, there was a clear difference in the relative increase of Pro contents, in relation with its background values in the non-stressed controls, which were more pronounced in ‘BGV001191’ and ‘BGV001581’, whereas the smallest increase was registered in ‘BGV004161’ and ‘BGV015856’ (Figure 2A).

Total soluble sugars (TSS) did not vary significantly in the cultivars ‘BGV001191’ and ‘BGV001581’. In ‘BGV004161’ and ‘Milagro VIII’, TSS showed an increase under 50 and 100 mM NaCl, followed by a drop at 150 mM NaCl. In cultivars ‘BGV015856’ and ‘E125’, TSS increased in parallel to the concentration of NaCl applied, but not all differences were significant (Figure 2B).

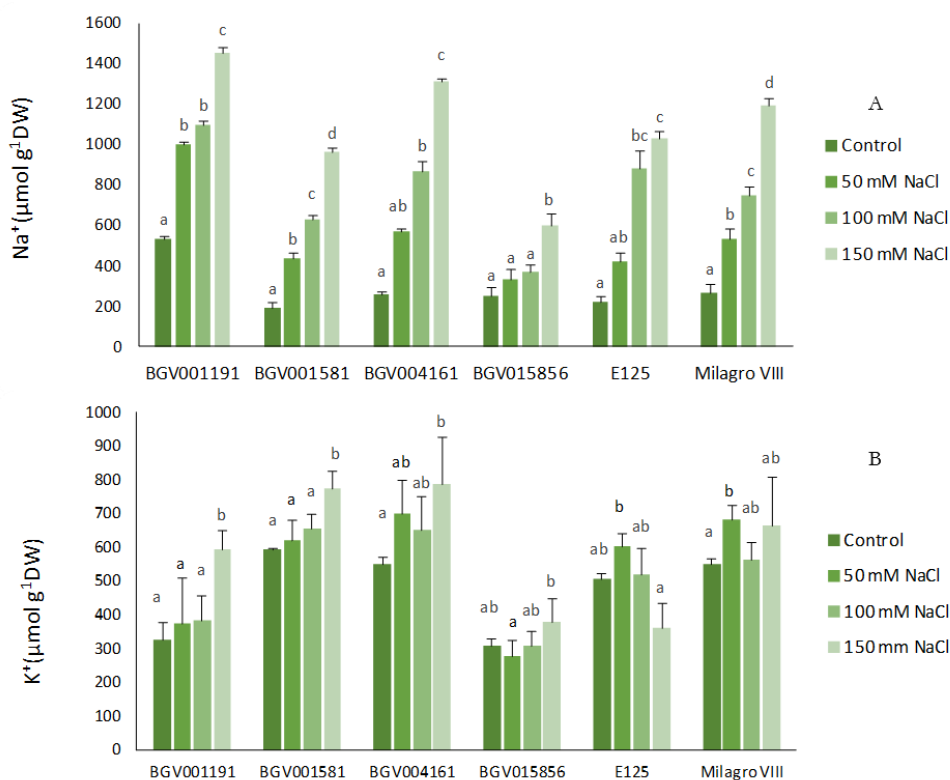
*Monovalent ions*

Na<sup>+</sup> increased in all plants subjected to salt stress in parallel to the concentration of NaCl applied. The lowest levels of foliar Na<sup>+</sup> under salt stress were found in the cultivar ‘BGV015856’ and the highest in ‘BGV001191’, but in the latter, the relative increase was small, as Na<sup>+</sup> concentration was also high in the control plants. The variation of Na<sup>+</sup> levels in plants treated with 150 mM NaCl, in comparison to non-stressed plants, ranged from 2.3-fold in ‘BGV015856’ and 2.72-fold in ‘BGV001191’ to over 4-fold in the remaining cultivars (Figure 3A).

K<sup>+</sup> levels in leaves did not vary significantly at low or moderate external salinities and generally increased in the plants treated with the highest NaCl concentrations, except for cultivar ‘E125’, in which a reduction of leaf K<sup>+</sup> contents was observed in the presence of 150 mM NaCl. The highest relative increase in K<sup>+</sup> (1.8-fold), was registered in cultivar ‘BGV001191’, characterised by lower values of this cation in control plants (Figure 3B).



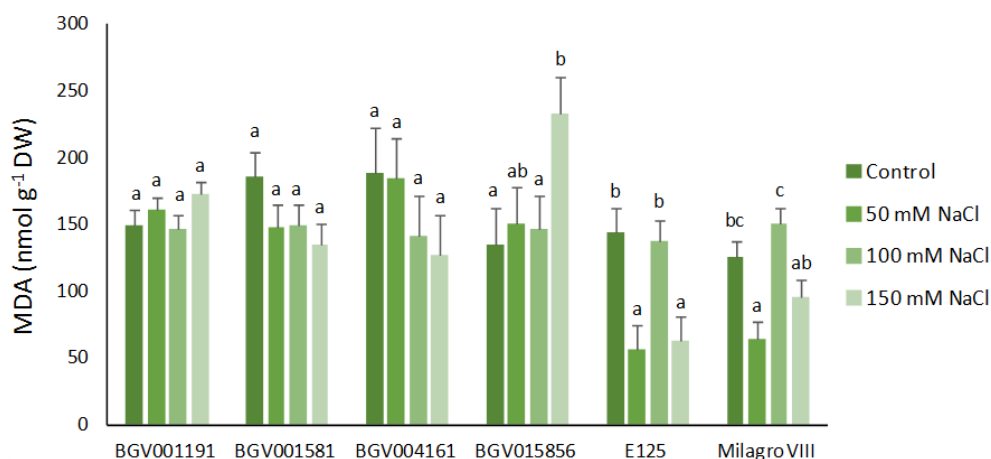
**Figure 2.** Variation of osmolytes contents in response to salt stress in the six cultivars of common beans analysed; (A) Proline; (B) Total soluble sugars. Mean ± SE values are shown (n = 5). Same lowercase letters within each column indicate homogeneous groups between treatments according to the Tukey test (p < 0.05)



**Figure 3.** Monovalent cation content variation in response to salt stress in the six cultivars of common beans analysed: (A) Sodium; (B) Potassium  
 Mean ± SE values are shown (n = 5). Same lowercase letters within each column indicate homogeneous groups between treatments according to the Tukey test (p < 0.05)

*MDA and non-enzymatic antioxidants quantification*

Malondialdehyde (MDA) leaf contents showed no statistically significant variation in cultivars ‘BGV001191’, ‘BGV 001581’, or ‘BGV004161’, and displayed fluctuations not related to the concentration of salt applied in the remaining cultivars. The only significant increase was registered in plants of the cultivar ‘BGV015856’ subjected to 150 mM NaCl (Figure 4).

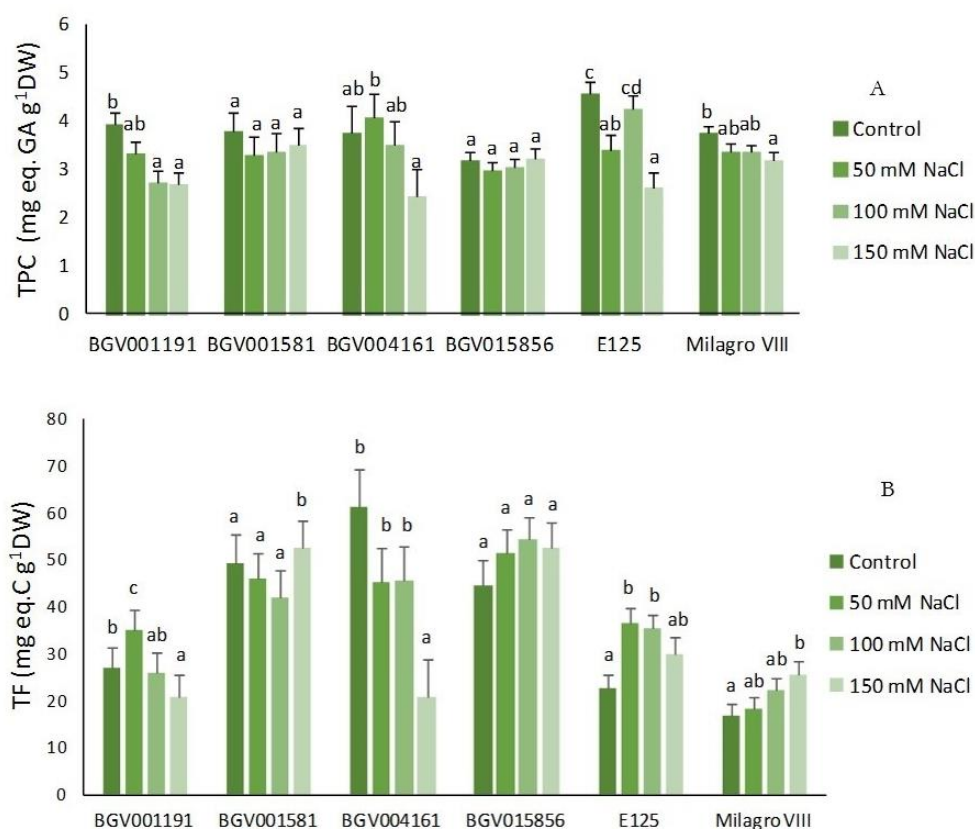


**Figure 4.** Malondialdehyde content variation in response to salt stress in the six cultivars of common beans analysed  
 Mean ± SE values are shown (n = 5). Same lowercase letters within each column indicate homogeneous groups between treatments according to the Tukey test (p < 0.05)

The antioxidant compounds analysed in the present work were the total phenolic compounds (TPC) and total flavonoids (TF). The first presented a variation that could not be correlated with the levels of salinity applied to the plants. In general, the highest levels of TPC were found in control plants, except for cultivar 'BGV004161', where values were slightly higher in plants from the 50 mM NaCl treatment (Figure 5A). In the case of TF, compared to the non-stressed controls, a decrease under salt stress was observed in cultivars 'BGV001191' and 'BGV004161', no variation was found in 'BGV015856', and an increase was detected in 'BGV001581', 'E125' and 'Milagro VIII'. However, only in the latter cultivar, the increase was gradual, in parallel to the rise in the concentration of NaCl (Figure 5B).

#### Factorial ANOVA

A two-way ANOVA was performed considering the effect of cultivar, treatment and their interaction. The effect of cultivar was significant for all parameters analysed, except for total phenolic compounds (TPC), and that of treatment for all traits evaluated except malondialdehyde (MDA) and total flavonoids (TF). Regarding the interaction of the two factors, it was significant for all parameters, except for Na<sup>+</sup>, which showed a similar pattern of variation, increasing in all cultivars under salt stress, although its concentrations were different between treatments. The statistical analysis confirmed the previous observation, revealing that osmolytes and monovalent cations are reliable stress parameters and at the same time allow a proper differentiation of cultivars. Of these categories, proline and potassium are optimal according to their lower residual values.



**Figure 4.** Variation in antioxidant compounds contents in response to salt stress in the six cultivars of common beans analysed; (A) Total phenolic compounds; (B) Total flavonoids. Mean  $\pm$  SE values are shown (n = 5). Same lowercase letters within each column indicate homogeneous groups between treatments, according to the Tukey test (p < 0.05)



**Table 2.** Percentages of the sum of squares (SS) from the two-way analysis of variance (ANOVA) of cultivar, treatment, and their interactions, for the indicated parameters

Parameter	Cultivar (C)	Treatment (T)	Interaction (C x T)	Residual
Pro	21.63***	44.57***	11.57***	22.24
TSS	13.43***	24.73***	17.08**	44.74
Na <sup>+</sup>	21.17***	42.93***	4.82	31.08
K <sup>+</sup>	57.88***	6.19***	10.83***	25.10
MDA	24.43***	3.33	26.86***	45.38
TPC	6.55	14.53***	18.32*	60.58
TF	48.26***	1.25	15.95***	34.52

\*\*\*, \*\*, and \* indicate significant at  $p < 0.001$ ,  $p < 0.01$ , and  $p < 0.05$ , respectively

## Discussion

The six cultivars investigated here were the subject of a previous study regarding salt and drought tolerance in a large number of cultivars (Arteaga *et al.*, 2020). Out of the 47 cultivars analysed in the previous work, 'BGV004161' and 'BGV015856' appeared as more stress-tolerant and were characterized by reduced levels of proline under stress, whereas the cultivar 'E125' was one of the most susceptible to stress and showed a significant increase of proline under both water and salt stress. The results reported here confirmed that the more tolerant genotypes identified in our previous study were those that experimented the smallest increase in the concentration of proline in leaves in response to increasing salinity. Proline is one of the commonest compatible solutes accumulated by plants under different types of stress (Szabados and Savouré, 2010; Grigore *et al.*, 2012). Besides its essential role in osmotic adjustment as an osmolyte, Pro also has a function in osmoprotection, by directly stabilising sub-cellular structures such as membranes and proteins, in free-radical scavenging or as a signalling molecule in stress responses (Hayat *et al.*, 2012; Kavi Kishor *et al.*, 2014; Rana *et al.*, 2017). Proline accumulation has been reported in beans under different stressful conditions, from the presence of herbicides (Mackay *et al.*, 1990) or heavy metals (Zengin and Munzuroglu, 2005) to drought (Ashraf and Iram, 2005; Rosales *et al.*, 2012; Morosan *et al.*, 2017) and salinity (Jiménez-Bremont *et al.*, 2006; Nagesh and Devaraj, 2008; Al Hassan *et al.*, 2016). Higher Pro levels were reported in the more stress-tolerant cultivars in several studies comparing two or a few cultivars (Misra and Gupta, 2005; Cárdenas-Avila *et al.*, 2006; Kaymakonova and Stoeva, 2008; Herrera Flores *et al.*, 2012; Ghanbari *et al.*, 2013), whereas others, based on a larger number of cultivars, revealed higher Pro levels in the more susceptible ones (Domínguez *et al.*, 2014; Arteaga *et al.*, 2020). In the present study, the levels of Pro increased with increasing salinity in all cultivars, but its absolute values were lower in the more salt-resistant 'BGV004161' and 'BGV015856' cultivars. On the contrary, Pro contents were higher, and the increase more pronounced, in the remaining four cultivars, which in the previous analysis were ranked as more susceptible to stress.

Total soluble sugars (TSS) play an essential role in osmotic adjustment under stress in many plant species and act as signalling molecules regulating the expression of different genes involved in photosynthesis, sucrose metabolism and osmolyte biosynthesis (Rosa *et al.*, 2009). However, as TSS are direct products of photosynthesis and are involved in many metabolic processes, their specific role in stress tolerance is sometimes difficult to be assessed (Gil *et al.*, 2011). Salinity was reported to increase levels of TSS in leaves (Abdallah *et al.*, 2016) and in fruits after ripening (Yin *et al.*, 2010) in different species. Although there is no clear correlation between TSS and the salt treatments applied (Al Hassan *et al.*, 2016), leaf concentrations of some specific sugars considerably increased under stress in different bean cultivars, such as glucose and inositol (Bahena-Betancour *et al.*, 2006) or *myo*-inositol (Al Hassan *et al.*, 2016). Accumulation of sucrose and starch in the pericarp has also been observed (Tazuke *et al.*, 2009). In this study, a gradual increase of TSS



in parallel to the concentration of NaCl has been detected only in the stress susceptible cultivar 'E125'. For a better understanding of the TSS variation in this cultivar, separation, identification and quantification of individual soluble carbohydrates, for example by HPLC analysis, would be required to provide a more in-depth insight.

An essential mechanism related to salt stress responses is ion absorption and transport. The primary response in glycophytes in conditions of high soil salinity is generally blocking the transport of toxic Na<sup>+</sup> ions from roots to aboveground organs, contrary to dicotyledonous halophytes, which usually actively transport the ions to the leaves and accumulate them in their vacuoles as cheap osmotica (Flowers *et al.*, 1986; Flowers and Colmer, 2008). It is known that plants of the genus *Phaseolus* can exclude sodium from the shoots, even in the presence of relatively high NaCl concentrations in the soil (Seemann and Critchley, 1985; Bayuelo-Jiménez *et al.*, 2012), and that mechanisms that restrict the transport of Na<sup>+</sup> to the aerial part of the plants are more efficient in the relatively more tolerant cultivars (Al Hassan *et al.*, 2016). This seems to be also the case of the more stress-resistant cultivar 'BGV015856' analysed here, which showed the lowest levels of Na<sup>+</sup> under salt stress. Sodium accumulation is generally associated with a drop in K<sup>+</sup> levels, mostly due to the competition of the two cations for the same membrane transport systems (Tester and Davenport, 2003). Potassium is regarded as the 'physiological' cation, whose deficiency has adverse effects on photosynthesis, protein biosynthesis and turgor driven movements (Gierth and Mäser, 2007). However, in all cultivars included in the present study, except for the most salt-sensitive 'E125', K<sup>+</sup> leaf contents did not decrease with increasing external salinity, even rose significantly over control levels at high salt concentrations. Maintaining relatively high cellular K<sup>+</sup> concentrations under salt stress conditions is a well-known fundamental mechanism of tolerance (Shabala and Cuin, 2007). Increased K<sup>+</sup> in foliar tissue is the result of the activation of K<sup>+</sup> transport to the leaves at high salinity, which may be regarded as an important mechanism of tolerance in this species as in many others (Volkov *et al.*, 2003; Schiop *et al.*, 2015; Kozminska *et al.*, 2018).

Salinity, like other types of stress, is usually associated with secondary oxidative stress. MDA, a final product of membrane lipid peroxidation is considered as a reliable marker of oxidative stress (Del Rio *et al.*, 2005), as one of the symptoms of oxidative damage is cell membrane degradation. However, no significant increase in MDA was detected in salt-treated plants, with the exception of plants from the 150 mM NaCl treatment of cultivar 'BGV015856', indicating that generally they were not affected by salt-induced oxidative stress. These results suggest that, under the specific conditions of our experiments, possible mechanisms of tolerance based on the activation of antioxidant systems are not relevant in the analysed cultivars. This would explain the lack of correlation of TPF and TF contents with the relative salt tolerance of the tested cultivars and the concentrations of salt applied. In other bean cultivars and under different conditions, however, there is evidence that these secondary metabolites may increase in response to abiotic stress treatments (Taibi *et al.*, 2016; Rahma *et al.*, 2019).

## Conclusions

Proline is a reliable marker of salt stress in common beans, its leaf contents increasing to higher levels in the more stress-susceptible cultivars. One of the main mechanisms conferring a relative salt resistance to common beans appears to be the active transport of potassium to leaves, maintaining its levels stable or even increasing in response to salt stress, thus partly compensating the deleterious effects of toxic Na<sup>+</sup> accumulation. This mechanism contributed to the relatively higher tolerance of two local landraces from Spain as compared to the other cultivars analysed in the present work. However, under the specific salt stress treatments applied to the plants, tolerance mechanisms based on the activation of antioxidant systems are not relevant since no secondary oxidative stress was generated.

### Authors' Contributions

Conceptualisation: MB and OV; Data curation: SA; Formal analysis: JP; Funding acquisition: OV; Investigation: SA; Methodology: SA, OV; Project administration: OV; Resources: OV and MB; Software: JP; Supervision: MB and OV; Validation: JP; Visualisation: SA; Writing - original draft: SA and MB; Writing - review and editing: JP and OV. All authors read and approved the final manuscript.

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### Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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