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Physiological and biochemical changes in leaves of interspecific Prunus rootstock (GF677) exposed to different external K, Ca, and Na supply

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Keywords: Calcium, Potassium, GF677 rootstock, anthocyanin, Polyamine.

Abbreviations: A, net photosynthetic rate; Car, carotenoids; Chl (a+b), total chlorophyll; gs, stomatal conductance; TAA, total antioxidant activity.

ABSTRACT

Physiological and biochemical changes of interspecific hybrid almond rootstock GF677 exposed to different external K+, Ca2+ and Na+ were investigated in this study. Plants were grown either with a complete nutrient solution containing 10 mM K+ and Ca2+ without or with 75 mM NaCl and 75 mM NaCl alone. Growth, gas exchange, leaf pigments, free polyamine, proline, total sugar contents, total polyphenols as well as total antioxidant activities were determined. A significant reduction of biomass was detected in GF677 in response to additional KCl and CaCl2 in the culture medium. Nevertheless, the best results in terms of photosynthetic rate, photosynthetic pigment concentrations and mineral nutrition (limitation of Na+ accumulation and maintenance of Ca2+ and K+ leaf content) were obtained in response to the combined Ca2+ + NaCl or K+ + NaCl treatment. However, the most important was the fact that the addition of K+ under salinity increased the chlorophyll content, the Chla and Cha/Chlb ratio. Such increment was more pronounced compared to that induced by Ca2+ addition in saline medium. Furthermore, our results showed that the exogenous supply of K+ under saline conditions increase the petunidin-3-glucoside leaf content. The high accumulation of this component is considered as a more important source of antioxidants than others components. Interestingly, the results showed a significant accumulation of Putriscine, proline and sugar under K+ + NaCl treatment in GF677. The addition of Ca2+ or K+ alone or with NaCl enhanced the polyphenols accumulations in leaves of GF677 compared to unstressed plants.

INTRODUCTION

Potassium and calcium are essential macronutrients and normally the most abundant cations in plants. Potassium is essential for many metabolic processes since it is a major contributor to cell turgor enzymes activation, cell expansion, and neutralization of non-diffusible negatively charged ions and membrane polarization regulation of osmotic pressure, stoma movement and tropisms

[1]. Calcium plays a crucial role in controlling cell membrane permeability and selectivity. It is well known that calcium functions as a regulator of plant cell metabolism [2]. These cationic elements are acquired by plant roots from the soil solution. Deficiency or toxicity in soils of any of them reduces plant growth, crop yield, and the quality of plant food stuff as well as selected mineral concentration [3]. Roots of all plant species can take up K and Ca present in their cationic forms in the rhizosphere, although soil properties and the intensity of crop harvesting determine the phytoavailability of these elements [4]. In deficient soils, essential mineral elements can be supplied to crops as fertilizers due to the importance of the yield in agriculture. However, for both economic and environmental reasons, it is clear that fertilizers should be used with caution. Conversely, high concentrations of mineral elements in the soil solution can occur on certain soil types with concomitant negative effects on crop production [5]. Plants must balance uptake, utilization and storage of mineral elements in order to maintain proper ion homeostasis, and this can be affected by adverse conditions, such as nutritional imbalances in soils caused by salinity. Under some conditions, electrons and excitation energy not used in photosynthesis can be channeled to molecular O2 and there is an overproduction of reactive oxygen species (ROS) in cell compartments. These ROS can react with lipids, proteins, and nucleic acids, resulting in oxidative damage [6,7]. Maintenance of redox status requires a strict balance between ROS production and detoxification, and to protect against the toxic effect of ROS, plants have developed a complex antioxidant system that can be non-enzymatic, such as anthocyanin. Previous studies have presented evidence for the ability of anthocyanins to provide photoprotection under stressful conditions. There is evidence that anthocyanin protect the photosynthetic apparatus from photoinhibition by absorbing green light and thereby reducing excess excitation energy [8]. Since anthocyanins are not photosynthetic pigments, their presence should act antagonistically to Chl for photon absorption, thus reducing photosynthesis. Although this is disadvantageous for a healthy leaf under optimal conditions, it may become important under stress, when a reduction in excitation pressure of Chl is needed. Therefore, anthocyanin may play a photoprotective role by directly eliminating ROS during photooxidative stress [9]. In addition, in such stress conditions, the production of polyphenols is related to the leaf carbon economy. Their accumulation is enhanced when carbon production overtakes the metabolic demand for growth [10]. The antioxidant activity of polyphenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [9] .

The compatible solutes, such as proline, sugars and polyamines (PAs) were recognized to play important roles in plant adaptation to various kinds of environmental stresses. In recent years, considerable attention has been focused on the study of changes in PAs levels in plants subjected to environmental stresses [11]. In higher plants, the major forms of PAs are diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm), presenting in the free, soluble conjugated and insoluble bound forms. Soluble conjugated PAs are those which are covalently conjugated to small molecules such as phenolic acids, and insoluble bound PAs are those which are covalently bound to various macromolecules such as nucleic acids and proteins. Researches have shown that the changes in PAs levels were closely associated with tolerance of plants to stresses [11]. Zrig et al. reported that PAs were salt tolerance traits in almond tree under salt stress [12]. Moreover, it has been suggested that PAs participated in cellular defence against oxidative damage through the inhibition of lipid peroxidation [13]. The aim of this study was to investigate the effect of external K+ and Ca2+ supply on the accumulation and distribution of these elements in leaves of GF677 as well as the accumulation, proline, sugars, polyphenols and the possible roles of antioxidant activity of anthocyanin, carotenoids and polyamines in defense reaction of GF677 against stress by excess of Ca2+ or K+.

MATERIALS AND METHODS

Plant Material And Salt Treatments

The present study was performed on eight months-old rooted cuttings of an hybrid Prunus rootstocks almond rootstock GF677 (Prunus amygdalus x Prunus persica), and did between april and june in Gabes zone location. The plants were cultivated in perforated-4L-plastic-pots containing desert dune-sand under controlled conditions (temperature: 25±2°C; light intensity (PAR): 500-700 µMm-2s-1), while relative air humidity ranged between 49% and 60%. During the first 3 weeks of cultivation, the plants received a complete nutrient solution prepared with deionized water and the addition of salts to have an initial total ion concentration of 4.5 mM and electrical conductivity (EC) of 2.77 dS m-1 (consisting of N, 1.8 mM, P, 0.35 mM, K, 0.64 mM, Ca, 1.0 mM, Mg, 0.35 mM, S, 0.35 mM, Fe, 0.03 mM, Zn, 0.4 mM, Mn, 5 mM, Cu, 0.1 mM and B, 0.023 mM). Plants were subjected to one of the following six treatments: (i) control (nutrient solution), (ii) control + 10 mM KCl, (iii) control + 8 mM CaCl2, (iv) salt stress (nutrient solution + 75 mM NaCl), (v) salt stress + 10 mM KCl, and (vi) salt stress + 10 mM CaCl2, as described in **(Table 1).** The experimental design was a completely randomized block with four replicates per treatment (each pot contained one plant being a replicate). These plants were harvested after four weeks of initiating the treatments. Fully expanded leaves from each plant were harvested in the morning (between 9 to 11 a.m. local time), weighted and divided into two batches; one was frozen in liquid nitrogen and then stored at -80 ° C for biochemical analyses; the other was washed in de-ionized water, dried at 80°C in a forced-air oven for 48 hours and ground into a fine powder to pass through a 30-mesh screen for ion analyses.

Growth And Ions Selectivity

Before the treatments were imposed, the tip of main the shoot of each plant was marked to be able later to assess shoot extension during the period of the experiment. After four weeks of treatment, the plants were harvested and divided into stems, leaves and roots. The latter were washed free of soil particles. The number of leaves was recorded for each plant. The materiel

was then dried for 48h at 80 C° and total dry weights were determined. For ion analyses, 1g of dry ground leaves from each plant was extracted with 20 mL of 0.1M HNO3. After filtration, Na^+, Ca^{2+}, K^+ and Mg^{2+} contents were determined with an atomic absorption spectrometer (Avanta, GBC, Australia).

Table 1. Irrigation solution composition.

Treatment N°	Irrigation solution composition
Control (C)	Basal nutriment solution : control (0)
C+$CaCl_2$	C + 10 mM $CaCl_2$
C+KCl	C + 10 mM KCl
NaCl (S)	C + 75 mM NaCl
S+$CaCl_2$	C + 75 mM NaCl + 10 mM $CaCl_2$
S+KCl	C + 75 mM NaCl + 10 mM KCl

Gas Exchange Measurements

Gas exchange measurements were carried out after 30 days of salt treatment. Net photosynthetic rate (*A*) and stomatal conductance (*Gs*) of upper mature leaves were measured with a portable photosynthesis system (Lcp *pro*+, ADC Systems Ltd, UK) under ambient conditions (PAR was 500-700 µmol $m^{-2}s^{-1}$ and air temperature was 25±2°C).

Determination Of Chlorophylls And Carotenoids

Leaf chlorophyll and carotenoids contents were determined by using the method of [14]. In short, 0.5 g of fresh leaves were grounded in liquid nitrogen to a fine powder in a pre-cooled mortar and homogenized for 30 s in 5 mL of 95.5% acetone. The pigments' concentrations were estimated from absorbance at 647 nm and 664 nm. A solution of 95.5% acetone was used as a blank. Pigment concentrations were calculated as follows: Chl *a* (mg/g FW) = [12.7× (A664) _ 2.69× (A647)] × (0.5×5), Chl *b* (mg/g FW) = [22.9× (A647) _ 4.69× (A664)] × (0.5×5).

Total carotenoids were extracted in duplicates according to Mínguez-Mosquera and Hornero-Méndez [15]. One gram of frozen leaf tissue was briefly extracted with acetone and shaken with diethyl ether and 10% NaCl. Two phases were obtained; the lipophilic phase was washed with Na_2SO4 (2%), saponified with 10% KOH in MeOH, and the pigments were subsequently extracted with diethyl ether, evaporated and then made up to 25 mL with acetone. Total carotenoids were estimated by reading the absorbance at 450 nm in a UNICAM Helios- spectrophotometer (Cambridge, UK), and expressed as mg of β-carotene equivalent per kg fresh weight, taking into account the molar absorption coefficient ($\varepsilon^{1\%}$ cm) of 2560. The results are presented as means± SE.

Anthocyanin Content

Leaf anthocyanins were determined according to Serrano et al. [16]. Total anthocyanin was calculated using as a standard cyanidin 3-glucoside (molar absorption coefficient of 23.900 L cm^{-1} mol^{-1} and molecular weight of 449.2 g mol^{-1}) and the results, expressed as mg of anthocaynin per kg fresh weight, were the mean ± SE of duplicate determinations made on each one of four samples.

Leaf individual's anthocyanin as cyanidin-3.5-glucoside and petunidin-3-glucoside assayed by high performance liquid chromatography coupled with a diode array detector (HPLC-DAD) [16]. One millilitre from the extracts obtained for total anthocyanin quantification was filtered through 0.45 µm Millipore filter and then injected into a Hewlett-Packard HPLC series 1100 equipped with a C18 Supelco column (Supelcogel C-610H, 30 cm x 7.8 mm, Supelco Park, Bellefonte, USA) and detected by absorbance at 510 nm. The peaks were eluted by a gradient using the following mobile phases: 95% water + 5% MeOH (A); 88% water + 12% MeOH (B); 20% water + 80% MeOH (C); and MeOH (D) at a rate of 1 mL min^{-1}. Peaks were identified using authentic standards by comparison of the retention times and peak spectral analysis. The anthocyanin standards were provided by García-Viguera et al. [17].

Antioxidant Activity

The total antioxidant activity (TAA) was quantified also in duplicate for each subsample according to Serrano et al. [18]. which enables the determination of TAA due to hydrophilic compounds (HTAA) in the same extraction. Briefly, 1 g of fresh leaves were homogenized in 5 mL of 50 mM phosphate buffer pH 7.8 and 3 mL of ethylacetate, and then centrifuged at 15.000 rpm for 15 min at 4°C. two fractions were obtained, the lower fraction was used for H-TAA quantification. TAA was determined using the enzymatic system composed of the chromophore 2.2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), the horse radish peroxidase enzyme (HRP) and its oxidant substrate (hydrogen peroxide), in which $ABTS^{\bullet+}$ radicals are generated and monitored at 730 nm. The decrease in absorbance after adding the extracts was proportional to TAA of the sample. A calibration curve (0 – 20 nM) was performed with Trolox (R)-(+)-6-hydroxy-2.5.7.8-tetramethylcroman-2-carboxylic acid (Sigma, Madrid, Spain) in aqueous media for H-TAA. The results are expressed as the mean± SE in mg of Trolox equivalent kg^{-1} fresh weight.

Proline Content

0.2 g of frozen leaves were ground to a fine powder in precooled mortar with liquid nitrogen homogenized with 5 mL of 3% aqueous sulfosalicylic acid and centrifuged at 8000×g for 15 min. Two ml of acid-ninhydrin and 2 mL of glacial acetic acid

were added to 2 mL of the homogenate in a test tube. The mixture was then incubated at 100°C for 1h, after which the reaction was stopped by placing the test tube in an ice bath. Four ml of toluene were added to each test tube and vortexed for 20 s. The absorbance at 520 nm of the organic toluene phase containing the chromophore was used to quantify the amount of proline as described Bates et al. [19]

Soluble Sugars Concentration

Total soluble sugars in leaves were determined in each replicate according to the method of Robyt and White [20]. Plant material (0.2 g) was extracted in 80% methanol solution. The extract was incubated for 30 mn at 70 °C. This extract was used for the estimation of total soluble sugars. Plant extract was taken and 5 mL of sulphuric acid were added mixed vigorously. DO was read at 645 nm on a spectrophotometer

Phenolic Compounds Content

Phenolic compounds were extracted according to Tomás-Barberán et al. .[21] using water: MeOH (2:8) containing 2 mM NaF and quantified using the Folin–Ciocalteu reagent [22]. The results were expressed as mg gallic acid equivalent per kg fresh weight of duplicate determinations made on each subsample.

Free Polyamines Content

For each sample, 1 g fresh leaves was extracted with 10 mL of 5% cold perchloric acid with 1.6 hexanediamine (100 nmol g^{-1} of tissue) added as an internal standard. The homogenate was then centrifuged for 30 min at 20000×g. A 2 mL aliquot of the supernatant was used to determine free polyamines by benzoylation, and derivatives analysed by HPLC according to Serrano et al. [23]. The elution system consisted of MeOH/H20 (64:36) solvent, running isocratically with a flow rate of 0.8 mL min^{-1}. The benzoylpolyamines were eluted through a reversed-phase column (LiChroCart 250-4.5 µm) and detected by absorbance at 254 nm. A relative calibration procedure was used to determine the polyamines in the samples. using 1.6 hexanediamine as the internal standard and standard curves covered the range 1–320 nM. The calibration curves were y = 10.66x + 170.00, r^2 = 0.94 for Put, y = 10.19x−39.96 r^2 = 0.96 for Spd, and y = 11.52x−4.32, r^2 = 0.90 for Spm.

Statistical Analyses

Variance of data was analyzed with GLM procedure of SAS software (SAS Institute. 1996) for a Randomized Complete Block design with four replicates. Where applicable means were separated by Duncan's Multiple Range Test($P \leq 0.05$).

RESULTS

Growth, Ca^{2+}, K+, Mg^{2+} And Na^{+} Leaf Content

The shoot extension and total dry weight displayed significant reductions under increased levels of NaCl alone and with 10 mM of $CaCl_2$ or KCl treatments versus to control **(Table 2)**. However, the combination of $CaCl_2$ with NaCl alleviated the symptoms of the individual salt stresses only in the shoot extension. In fact, the shoot extension showed 30% reductions in 75 mM of NaCl in comparison with the control. Such reductions decreased to 23% with the treatment of 10 mM $CaCl_2$ + 75 mM NaCl. Inclusion of 10 mM KCl in the medium in the presence of 75 mM NaCl significantly increased the shoot extension compared to 75 mM NaCl. However, both supplementary calcium and potassium chloride decreased the leaf number compared to unstressed plants. Sodium concentration Na^{+} in plant tissues increased in the high NaCl treatment, but it was reduced by supplementary calcium or potassium chloride **(Table 3)**. Concentration Mg^{2+} in the leaves was lower in plants grown at high NaCl than those in unstressed treatment. Application of both supplementary calcium corrected Mg^{2+} deficiency in GF677. Concentrations of Ca^{2+} and K^{+} in the leaves were lower in plants grown at high NaCl than those in unstressed ones **(Table 3)**. Application of both supplementary calcium corrected Ca^{2+}, K^{+} and Mg^{2+} deficiency **(Table 3)**.

Table 2. Leaves, shoot and total dray weight, shoot extension and leaf number of GF677 almond rootstock grown in a medium without NaCl (control), containing 75 mM NaCl (NaCl (S)), or containing KCl or $CaCl_2$ alone or together with 75 mM NaCl: control + 10 mM KCl (C+KCl), NaCl+10 mM KCl (S+KCl), control+ 10 mM $CaCl_2$ (C+$CaCl_2$) or NaCl+10 mM $CaCl_2$ (S+$CaCl_2$). Each point represents the mean (± SE) of four replicates; values marked by different small letters are significantly different at P < 5%

NaCl (mM)	Leaves DW(g)	Shoot DW(g)	Total DW(g)	Shoot extension	Leaf number
0	4.77 ± 0.0a	9.56 ± 0.4a	14.37±0.7a	40,66±3,9a	136±7,0a
CaCl2(10mM)	1,84±0,00b	3,96±0,11b	6,31±0,01b	35±3ab	64±11,8c
KCl(10mM)	2,01±0,25b	3,83±0,16b	5,79±0,1b	39±1a	63,75±5,7c
75	1.64 ± 0.2b	3.69 ± 0.5b	5.65 ±0.5b	28 ± 1,3c	74,33±2,5b
Ca (10 mM) + 75mM NaCl	0.77± 0.3c	2.77 ± 0.2c	4.87±0.2bc	30±0b	48,33±6,7bc
KCl(10 mM) + 75mM NaCl	0.83 ± 0.2c	2.22 ± 0.3c	3.45 ± 0.5c	31,66±2,25b	50±3,5bc

Table 3. Ion concentrations in the leaves of GF677 grown in a medium without NaCl (control), containing 75 mM NaCl (NaCl (S)), or containing KCl or CaCl2 alone or together with 75 mM NaCl: control + 10 mM KCl (C+KCl), NaCl+10 mM KCl (S+KCl), control+ 10 mM CaCl2 (C+CaCl2) or NaCl+10 mM CaCl2 (S+CaCl2). Different letters indicate significant differences at P < 5% between treatments.

	Traitement	Leaves
Ca^{2+} ($\mu eq.g^{-1}DW$)	0	610.81a
	CaCl2 (10mM)	692,79a
	KCl (10mM)	588,595b
	75 mM	413.40b
	CaCl(10mM)+NaCl(75mM)	521.34ab
	KCl (10mM)+NaCl(75mM)	494.88ab
K^{+} ($\mu eq.g^{-1}DW$)	0	869.95a
	CaCl2(10mM)	677,32 b
	KCl(10mM)	919,56a
	75 mM	572.38b
	CaCl(10mM)+NaCl(75mM)	663.43b
	KCl (10mM)+NaCl(75mM)	822.57a
Na^{+} ($\mu eq.g^{-1}DW$)	0	800.87b
	CaCl2(10mM)	505,09bc
	KCl(10mM)	370,16b
	75 mM	1260.55a
	CaCl(10mM)+NaCl(75mM)	639.01b
	KCl (10mM)+NaCl(75mM)	801.46b
Mg^{2+} ($\mu eq.g^{-1}DW$)	0	274.18ab
	CaCl2(10mM)	317a
	KCl(10mM)	273ab
	75 mM	221.81c
	CaCl(10mM)+NaCl(75mM)	274.00ab
	KCl (10mM)+NaCl(75mM)	226.00ab

Gas Exchange Measurements

The CO_2 assimilation rate decreased generally at the highest level of salinity **(Figure 1).** Elevated K^+ prompted photosynthesis at 75 mM NaCl dramatically from 12.9 to 16 mol CO_2 m^{-2} s^{-1}, whilst additional Ca^{2+} resulted in increased from 12.9 to 15 mol CO_2 m^{-2} s^{-1}. Stomatal conductance (gs) and transpiration rate decreased significantly in 75 mM NaCl treatment compared to the control, however, the exogenous supply of Ca^{2+} or K^+ increased the both parameters. Further, the addition of the same treatments under salt condition decreased the stomatal conductance to low level, but, did not affect significantly the rate of transpiration **(Figure 1).**

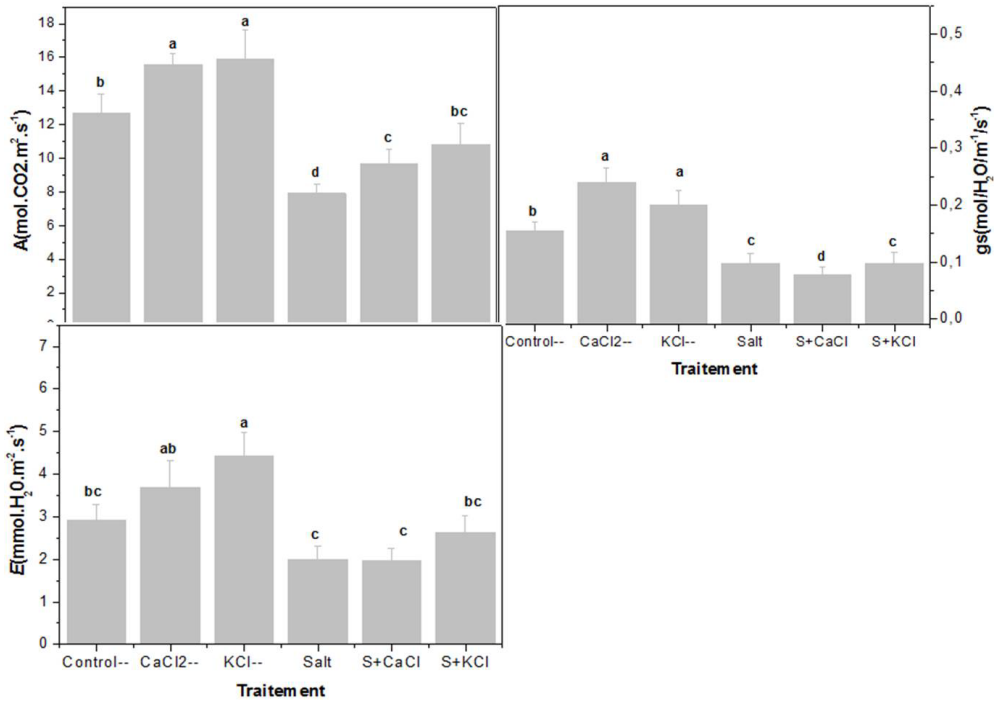

Figure 1. Net photosynthesis (*A*), stomatal conductance (gs) and transpiration rate (*E*) in leaves of GF677 grown in a medium without NaCl (control), containing 75 mM NaCl (NaCl (S)), or containing KCl or $CaCl_2$ alone or together with 75 mM NaCl: control + 10 mM KCl (C+KCl), NaCl+10 mM KCl (S+KCl), control+ 10 mM $CaCl_2$ (C+$CaCl_2$) or NaCl+10 mM $CaCl_2$ (S+$CaCl_2$). Each point represents the mean (± SE) of three replicates, values marked by different small letters are significantly different at P < 5%.

Chlorophylls And Carotenoids

According to the measurements reported in **(Figure 2)**, salt stress reduced significantly the pigment contents of GF677 ($p<0.05$). The addition of $CaCl_2$ to NaCl-treated plants increased total chlorophyll and Chl*a*. However, the ratio of Chl *a/b* was not significantly different with that recorded in NaCl treatment ($P > 0.05$) (Fig. 2). The addition of K^+ resulted in an increasing in total chlorophyll, Chl*a* and Chl*a*/Chl*b* ratio in leaves compared to that recorded in stress plants by 75 mM NaCl. Proper elevation of Ca^{2+} or K^+ concentration with 75 mM NaCl could not alleviate the carotenoids accumulation and the Chl/Car ratio in leaves **(Figure 2)**.

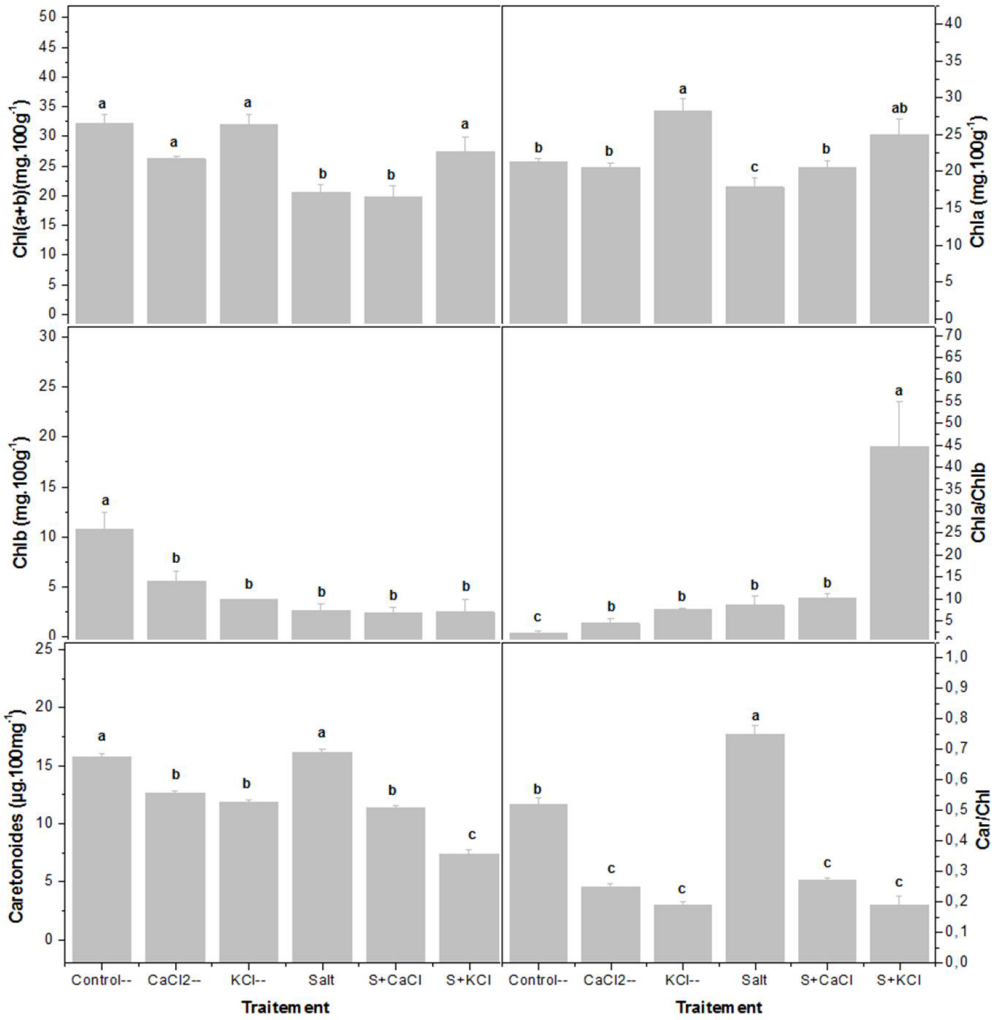

Figure 2. Total chlorophyll (Chl*a*+*b*), chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*), total carotenoids contents, as well as Chl*a*/*b* and carotenoids/Chl*a*+*b* ratios, in leaves of GF677 grown in a medium without NaCl (control), containing 75 mM NaCl (NaCl (S)), or containing KCl or $CaCl_2$ alone or together with 75 mM NaCl: control + 10 mM KCl (C+KCl), NaCl + 10 mM KCl (S + KCl), control + 10 mM $CaCl_2$ (C+$CaCl_2$) or NaCl+10 mM $CaCl_2$ (S+$CaCl_2$). Each point represents the mean (± SE) of three replicates, values marked by different small letters are significantly different at P < 5%.

Anthocyanin Content

Anthocyanin concentration reduced by the addition of KCl or $CaCl_2$, while the content was not affected by saline treatment **(Figure 3)**. The leaf cyanidin-3.5-glucoside was not altered by all treatments. Petunidin-3-glucoside leaf content increased to reach control level under salinity when Ca^{2+} and K^+ were added. Under the same conditions, the Petunidin/Chl ratio decreased significantly, while the Cyanidin/Chl ratio still unchangeable for all treatments.

Free Polyamines

The free polyamines detected in this study were spermidine and putrescine, but spermine was not detected. The putrescine content in leaves of GF677 increased greatly with supplemental of K^+ with Na^+, while, the spermidine content still unchangeable in stressed plants **(Figure 4)**.

Proline Content

Proline content increased significantly with saline treatment ($P<0.05$) in leaves of GF677. Interestingly, the proline content increased to a great extent with the addition of either Ca^{2+} or K^+ to the NaCl solution by 60% and 80% respectively compared to control **(Figure 4)**. However, the exogenous supply of $CaCl_2$ or KCl alone did not affect the proline content.

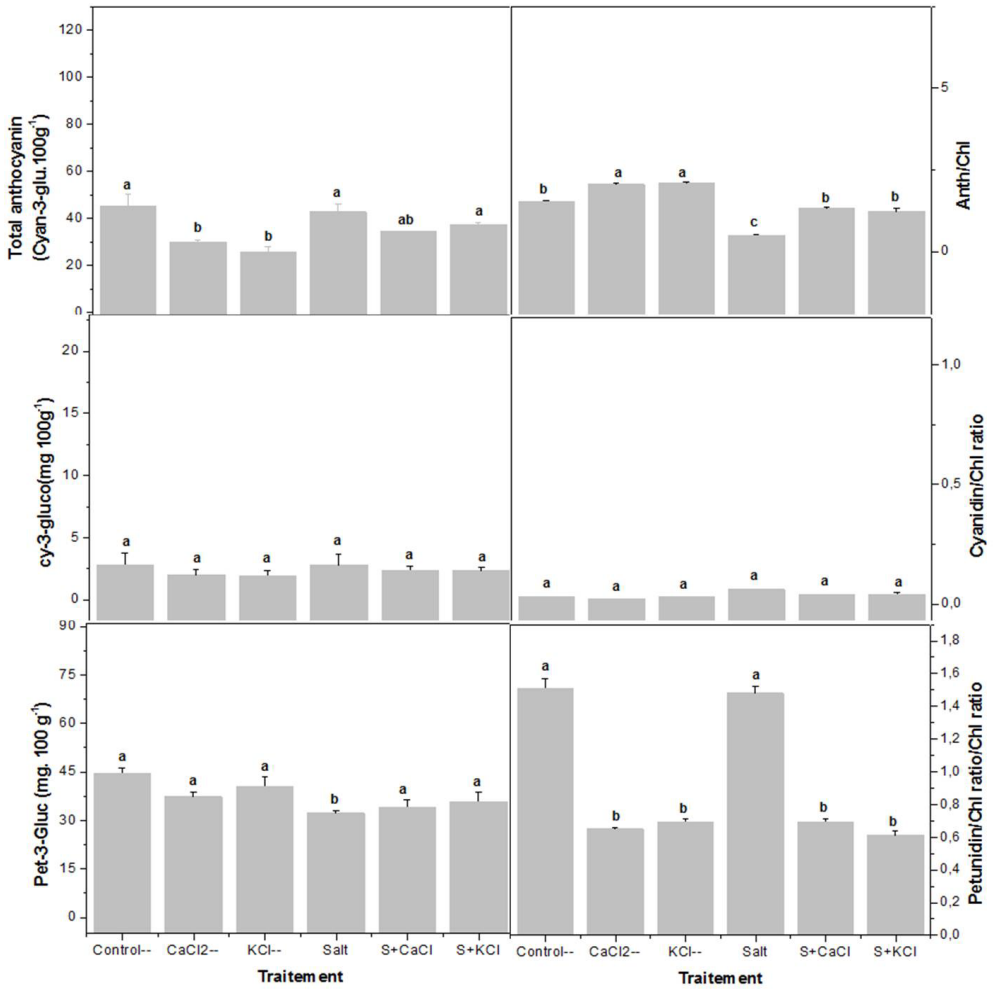

Figure 3. Total anthocyanin, and individual anthocyanin (cyanidin 3.5-glucoside and petunidin 3-glucoside), as well as anthocyanin/Cha+*b*, cyanidin /Cha+*b* and petunidin /Cha+*b* ratios in leaves of GF677 grown in a medium without NaCl (control), containing 75 mM NaCl (NaCl (S)), or containing KCl or $CaCl_2$ alone or together with 75 mM NaCl: control + 10 mM KCl (C+KCl), NaCl+10 mM KCl (S+KCl), control + 10 mM $CaCl_2$ (C+$CaCl_2$) or NaCl + 10 mM $CaCl_2$ (S+$CaCl_2$). Each point represents the mean (± SE) of three replicates, values marked by different small letters are significantly different at P < 5%.

Total Soluble Sugars

One of the most important mechanisms exerted by higher plants under salt-stress conditions is the accumulation of compatible solutes such as soluble sugars. In the present study, the amount of soluble sugars decreased with the addition of KCl or $CaCl_2$ in unstressed plants **(Figure 4)**. The soluble sugars accumulation was higher in KCl + NaCl treatment when compared to NaCl-treated plants. However, the exogenous $CaCl_2$ in salinity did not affect the total soluble sugars leaf content as compared to control.

Polyphenols

In the present study, an increase in polyphenols accumulation in GF677 ($p<0.05$) under salinity was observed **(Figure 4)**. Polyphenol content was diminished after the addition of $CaCl_2$ and KCl, anyhow, $CaCl_2$ or KCl with NaCl increased polyphenols content when compared to control.

Total Antioxidant Activity

The total antioxidant activity has been increased in $CaCl_2$ or KCl treated plants compared to control. Moreover, the addition of $CaCl_2$ or KCl to NaCl-plant treated increased the total antioxidant activity **(Figure 4)**.

DISCUSSION

Plants require nutrients for a normal physiological functioning and nutritional imbalances have an effect on crop yield and the nutritional quality of vegetable products. Likewise, nutrient stresses negatively affect plant growth and development with plant biomass data widely used as an agricultural index to define stress tolerance .In the present study, significant reduction of biomass was detected in GF677 following the addition of KCl and $CaCl_2$ in the culture medium. For instance, the adding of Ca^{2+} and K^+ seemed to affect the growth of GF677 rootstock as reported in several Mediterranean plant species adapted to low nutrient availability [24]. Exogenous use of various chemicals to alleviate the toxic effects of saline stress may have implications

both from theoretical and practical perspectives [25]. In the present study, the salt exposed to plants treated with both K^+ and Ca^{2+} affected the plant growth. This lower growth might be due to the Cl^- toxicity since all salts used here were in chloride form [24]. Consequently, addition of Ca^{2+} or K^+ to salinised soil to the critical level resulted in recovery of nutrition imbalances. In Fact, they hugely decreased the leaf Na^+ and Cl^- contents of the NaCl-treated GF677, and somewhat increased the leaf Ca^{2+}, K^+ and Mg^{2+} contents. Despite such nutrition's enhancement and the alleviation of the NaCl toxicity, the growth of GF677 did not positively respond to fertilizers inputs in the culture medium. Our results showed that in GF677 fed with 10 mM KCl or $CaCl_2$ had significantly lower Na^+ and Cl^- concentrations in their leaves. Similar results were found in tomato plants [26] and rice [27]. In fact, it seems that the GF677 limited the Na^+ and Cl^- transport to their shoots when they are treated with the exogenous calcium and potassium under salt stress. In fact higher Ca^{2+} concentrations in the substrate improves the K^+/Na^+ selectivity by shifting the uptake ratio in favour of K^+ in the expense of Na^+ [24].

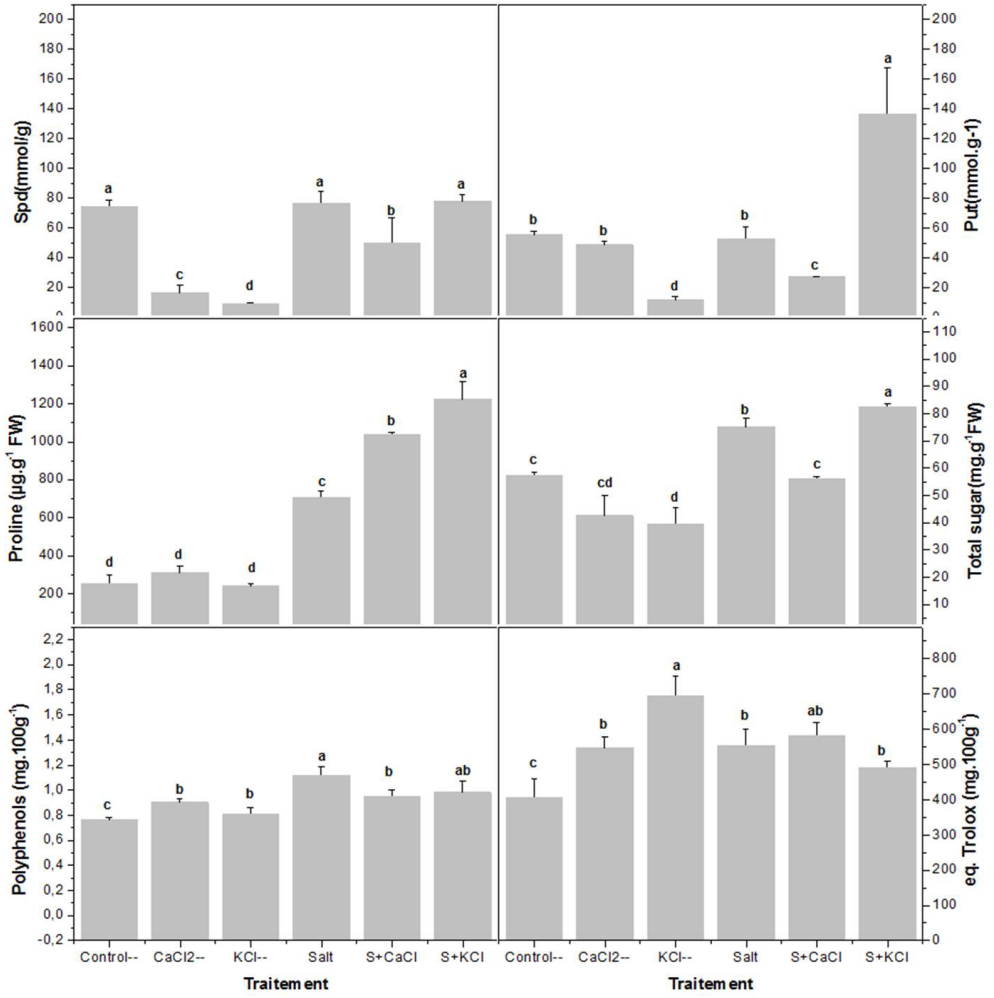

Figure 4. Spermidine, putricine, proline, total sugar, , total polyphenols contents as well as the total antioxidant activity (TAA) in leaves of GF677 grown in a medium without NaCl (control), containing 75 mM NaCl (NaCl (S)), or containing KCl or $CaCl_2$ alone or together with 75 mM NaCl: control + 10 mM KCl (C+KCl), NaCl+10 mM KCl (S+KCl), control+ 10 mM $CaCl_2$ (C+$CaCl_2$) or NaCl+ 10 mM $CaCl_2$ (S+$CaCl_2$). Each point represents the mean (± SE) of three replicates, values marked by different small letters are significantly different at P < 5%.

In the present study, we showed that potassium and calcium supply increased several parameters (CO_2 net assimilation rate, stomatal conductance and transpiration rate) of GF677 photosynthetic activity. Regardless of the growth reduction under high supply of calcium and potassium in growth medium, it appeared that exogenous Ca^{2+} and K^+ (10 mM) were beneficial for higher photosynthesis efficiency. Because of salt stress impairs K^+ or Ca^{2+} uptake of plants, it has been suggested that K^+ and Ca^{2+} deficiency might be a contributor factor to salt-induced oxidative stress and related cell damage. Enhanced photosynthetic rate in the NaCl-treated plants under Ca-enriched condition might be attributed to the protection against chlorophyll loss, caused by NaCl. However, the addition of Ca^{2+} or K^+ to salinity has significantly ameliorated the detrimental effect of salinity in GF677, showing that the photosynthetic apparatus was capable of adapting to the higher salinity in the presence of external Ca^{2+} or K^+. The ability of the plant to photosynthesize in such condition is affected by leaf chlorophyll content in GF677. Under nonsaline conditions supplemental Ca^{2+} adversely affected to some extent the Chlb but did not affect significantly Chla and total chlorophyll. Studies have described a range of plant species with varying responses to supplemental Ca^{2+} from positive to negative effects [28]. Nevertheless, under salinity supplemental Ca^{2+} significantly increase Chla contents of leaves and the total chlorophyll. However, the most important was the fact that the addition of K^+ under salinity increased the chlorophyll content and the Chla, which could be related to the enhanced of the assimilation rate. The great increasing in the Cha/Chl*b* ratio with the supplemental

K^+ under salinity in leaves of GF677 rootstock indicates also less emphasis on light harvesting in relation to the rates of PSII photochemistry under stress. Increased chlorophyll contents due to supplying soil with K^+ have been reported in cucumber and pepper plants grown at high salinity [29]. This effect is attributable to different roles carried out by K in photosynthetic metabolism [30].In this present investigation, it appeared that the KCl supplement was better than $CaCl_2$ for protecting the chlorophyll content under salt stress. Besides, it was reported that the leaf pigments as carotenoids and anthocyanin can play an important photo protector role in some rootstocks and thus protecting plants against salt stress [11]. Thus, it appears that in this almond rootstock, the external supplemental of Ca^{2+} or K^+ in saline solution decrease the importance role of carotenoids in the photo protection. We suggested that this antioxidant started degrading, which suggests oxidative stress in chloroplasts [31]. It seemed that the role protective of carotenoids was replaced by different antioxidant. The higher Anth/Car ratio in GF677 when stressed plants fed with KCl indicated that the anthocyanin was implicated in the mechanism of photo protection in this hybrid. Under unstressed conditions, the supplemental calcium decreased the total anthocyanin, but did not affect significantly the cyanidin and petunidin accumulation. In other studies, it was reported that the addition of $CaCl_2$ partly relieved the inhibition of cyaniding and petunidin accumulation [32]. Whilst, the external Ca^{2+} supply to salinity enhance the petunidin-3-glucoside leaf content compared to stressed plants. Furthermore, our results suggested that GF677 with high accumulation of petunidin-3-glucoside content are a more important source of antioxidant phytochemicals than others components. The cell protection of petunidin was the most effective than the others phenolic component such as cyanidin. In addition, under same condition, the petunidin/Chl ratio decreased too suggesting that this individual anthocyanins protected chlorophylls against reactive oxygen species and this should confer some photo protection for their chloroplasts. We suggested that the accumulation of these components improved by the external supplement of Ca^{2+} plays a protective role and became one of the most important photo protection mechanisms that help this hybrid to prevent photo oxidative stress. Similarly, in the present investigation, the addition of KCl in stressed condition alleviates the harmful effect of NaCl on photosynthetic activity by enhancing the antioxidant activity of Petudinin. Thus, it appeared that also the external supplement of K^+ in stressed conditions plays a protective role and became one of the most important photo protection mechanisms that help this hybrid to prevent photo oxidative stress caused by high salinity.

Furthermore, other soluble N containing compounds such as other amino acids, polyamines and soluble proteins could protect plant tissues against osmotic stress and has an important role in the regulation of plant growth [33].The marked decrease of Spd contents observed in GF677 with increasing concentrations of $CaCl_2$ and KCl in the salt medium might rely on either a decreased rate of their biosynthesis or an enhanced degradation leading to the formation of D1-pyrroline and g-aminobutyric acid (GABA). It has been shown that Ca^{2+} and K^+ inhibited arginine decarboxylase (ADC) activity from various plant sources and this inhibitory effect was more evident with Ca^{2+} than with K^+ [34]. Interestingly, the results showed a significant accumulation of putrescine with K^+ and salinity treatment in GF677. Our results suggest that an excess of K^+ under high salinity may be responsible for an ionic imbalance which may induce polyamine accumulation but it is not possible to assess that this effect was due to K^+ by itself, or to chloride accumulation which was shown to be higher under KCl with NaCl than under NaCl stress in the former rootstock.

Survival under stressful conditions depends on the plant's ability to perceive the stimulus, generate and transmit signals, and instigate biochemical changes that adjust the metabolism accordingly. Accumulation of organic solutes/osmolytes in plants contributes to osmotic adjustment that is important for physiological machinery by which plants tolerate biotic and abiotic stresses [35]. Among the studied organic osmolytes, proline showed the highest accumulation leaf subjected to (K + NaCl) and (Ca^{2+}+ NaCl) treatments, when compared to NaCl treatment alone. Our results showed that almond tree accumulated more such osmotica under under KCl + NaCl treatments seems to be induced by the interaction between K^+ and arginine, as K^+ may enhances arginase activity and the ensuing ornithine is used throughout the proline pathway [36].

The high parallelism in the endogenous changes of PAs and Pro, which were observed in GF677 after the addition of the supplemental concentration of calcium and potassium with the salt lethal concentration (75 mM NaCl), emphasises the role played by the metabolic relationship between Pro and PAs. In other works, these results suggest that the metabolic pathways of PAs and Pro, are strongly co-ordinated, and the metabolic fluxes through these pathways becomes competitive only when the stress level becomes lethal for cells [37]. The synchronism in Pro and PAs changes also supports the recent suggestion that PAs metabolism might exert a direct or indirect action on Pro biosynthesis [37].Similar variation in accumulation of soluble sugars was also seen under same treatment (K^+ + NaCl). In fact, sugars are also considered to play a major role in osmoregulation under abiotic stress conditions. Total soluble carbohydrates are important solutes that accumulate in cytosol under salt stress and may thus contribute to plant survival [38]. In our study, it appeared that the hybrid GF677 seemed to take advantage of the excess of K^+ in salt solution to increase the total sugars especially in their leaves as reported in winter wheat [12].

The addition of Ca^{2+} or K^+ alone or with NaCl enhanced the polyphenols accumulations in leaves of GF677 compared to unstressed plants. The role of Ca^{2+} in phenolic metabolism has been demonstrated in several works [39]. In this study, we suggested that the application of Ca^{2+} and NaCl together increased the phenylalanine ammonia-lyase activity and therefore the accumulation of phenols promoting resistance to salt stress. This increase in total polyphenols under an excess on K^+ may be due to the increase in the total sugars. This phenomenon led to the idea that polyphenols can lower the Ψ_s and allow leaves to maintain a low Ψ_w helping the plant to withstand sub-optimal water levels [39]. The antioxidant activity of phenolic compounds is mostly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers

[40]. Whilst, the total activity antioxidant in leaves of GF677 was maintaining stable, which appeared that, this hybrids benefit to the external calcium and potassium in the saline solution, which can inhibit the photodamage to PSII by inhibiting the repair of the damage to PSII.

The results from the present study illustrate that application of K or Ca improved the salt tolerance of almond rootstock GF677 through protection of photosynthetic pigments and organic solute/osmolyte accumulation, regardless the negatively response of growth attributes to fertilizers inputs in the culture medium. The findings also indicated that combination of K and Na^+ could play a synergetic role in regulating several properties conditioning plant salt resistance by enhancing the antioxidant activity of Petudinin, polyphenols and Putrescine. Besides, it appeared that the supply of K^+ under salinity was more effective than Ca^{2+} supply to improve the osmoregulation by accumulation of proline and soluble sugars.

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