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Effect of foliar application of amino acids on the salinity tolerance of tomato plants cultivated under hydroponic system

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ABSTRACT

Salinity is one of the most critical problems faced by agriculture in all the arid and semi-arid climates in the world. Many biostimulant-producing companies utilize different raw materials to palliate the negative effects of salinity on crops. Some of these active materials are amino acids (AAs). In this study, the effect of the foliar application of free amino acids or as a mixture of them was studied in tomato plants *cv* "Optima", grown in a controlled environment growth chamber in a hydroponic system with Hoagland's solution with added 50 mM NaCl. The following treatments were applied: i) Control (-salt), ii) salt (+salt), and the salt treatments with amino acids (AAs + salt): iv) L-Arg, v) L-Pro, vi) Glu, vii) L-Trp, viii) L-Met + L-Arg, ix) L-Met + L-Trp, x) Glu + L-Pro. At the end of the assay, vegetative growth parameters, relative water content, mineral nutrient content, carbohydrates and organic solutes and chlorophylls were measured. The results showed that salinity decreased the growth of the plants, but those treated with the **L-Met, Pro** + **Glu and Met** + **Trp** reversed the negative effect of salinity. Also, this result was not due to differences in the concentration of Cl- or Na^+ in the leaves, or to changes in the water status of the plants, but to a greater accumulation of total soluble sugars induced by the application of AAs, which could have de-activated the reactive oxygen species created by the toxicity of these ions. The results of this experiment also highlight the antagonistic or synergistic effects between the AAs, which should be taken into account by fertilizer producers.

1. Introduction

Faced with the increase in the world's population and the ensuing demand for food, together with the negative effects of climate change on the productivity of crops, the main objective of agricultural research has been refocused into acquiring new scientific knowledge related with the mechanisms involved in the tolerance of abiotic stress in plants in order to design agronomic strategies that help crops live and thrive with these stresses (Safdar et al., 2019). About 40% of the world's surface is found in arid regions, which forces agricultural production to depend on irrigation. Under these conditions, and in order to palliate the lack of good water resources, water from aquifers is utilized, which contain a high concentration of dissolved salts. These salts, along with a high evapotranspiration rate, a low leaching capacity of some soils and the use of high quantities of fertilizers, and/or sea water intrusion into aquifers and agricultural land near the coast and marshes, have increased the salinization of agricultural soils (Plaut et al., 2013; Safdar et al., 2019).

Soil salinity is one of the environmental factors that greatly affects agricultural productivity and the quality of the harvest (Zhang et al., 2019). It is estimated that about 20% of the area in the world dedicated to irrigation is affected by salinity (Kader and Lindberg, 2010), especially in the most productive areas such as California, the southern part of Asia, and a great part of the Mediterranean area (FAO, 2019). Thus, soil and water salinity have become a true threat for agriculture, as most of the cultivated plants, among them tomato (*Solanum lycopersicum* L.), are affected, to a greater or lesser degree, by this environmental factor. The negative effect of salinity on crop growth is due to the combination of various factors (Hasegawa et al., 2000; Zhu, 2001; Syvertsen and Garcia-Sanchez, 2014), including: i) the water deficit due to osmotic effects, as a high concentration of salts in the soil results in the establishment of a zone with low water potential, which

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makes difficult the absorption of water through the plant's roots; ii) the creation of a specific ionic toxicity that is normally associated to an excessive absorption of Na^+ and Cl- ions; and iii) the excess of Na^+ and Cl- in the soil results in a decreased absorption of mineral nutrients or disequilibrium with K^+, Ca^{2+}, nitrate or phosphate (Negrao et al., 2017). At the same time, all of these factors lead to the negative alteration of different physiological processes such as water relations, net assimilation rate of CO_2, light-gathering processes, antioxidant capacity, among others, which result in a decrease in both production and commercial quality of the crops.

In order to adapt to conditions of salinity plants utilize a series of mechanisms, among them the synthesis of amino acids such as Proline and/or glycine betaine (an ammonium quaternary compound). Proline is involved in processes of osmotic adjustment, protection of cellular membranes and deactivation of reactive oxygen species (ROS), at the same time that it acts as a nitrogen reserve compound (Dar et al., 2016). Of the metabolic routes that are involved in the synthesis of proline (glutamate or ornithine pathway), it has been demonstrated that those that begin with the synthesis of the amino acid glutamate are the main ones in situations of osmotic stress (Rhodes et al., 1986). The sulfur-containing amino acid methionine is a nutritionally important essential amino acid and the precursor of several metabolites that regulate plant growth and responses to the environment (Amir et al., 2002). This amino acid, through the S-adenosyl methionine (SAM) route, regulates the synthesis of secondary metabolites, polyamines and ethylene, and together with an amine group, help plants withstand abiotic stresses (Capaldi et al., 2015). From the 21 proteinogenic AAs, arginine has the greatest amount of nitrogen, making this amino acid an important reserve and manner of transport of this element. Also, this amino acid is the precursor of polyamines and nitric oxide; therefore, it also plays crucial roles in regulating developmental processes, as well as the responses to biotic and abiotic stress (Winter et al., 2015). L-Tryptophan is known to be a physiological precursor of auxins and melatonin in higher plants, and research has shown that L-Tryptophan has a more positive effect on plant growth and yield as compared to auxins themselves (Mustafa et al., 2018). L-Tryptophan may act as an osmolyte or ion transport regulator, modulating stomatal opening and diminishing the harmful effects of heavy metals (Hildebrandt et al., 2015).

The application of biostimulants is one of the most popular strategies utilized today for mitigating the negative effects of salinity on crops (Rouphael et al., 2017; Yakhin et al., 2017). This is due to the product's ability to decrease the absorption and transport of Cl- to the aerial part of the plants, to protect the photosynthetic machinery, to maintain a good nutritional equilibrium, to reduce the reactive oxygen species (ROS) and to favor the osmotic adjustment processes, among other factors (Calvo et al., 2014; Du Jardin, 2015; Colla et al., 2015; Rouphael and Colla, 2020). The biostimulants can be formulated from a wide range of materials, but one of the most utilized to combat salinity are protein hydrolysates. Thus, beneficial effects have been observed with the application of animal protein hydrolysates in persimmon (*Diospyros kaki* L.f.) (Visconti et al., 2017), alfalfa plant hydrolysates in maize (*Zea mays* L.) (Ertani et al., 2013), or plant hydrolysates in tomato (*Solanum lycopersicum* L.) (Cerdán et al., 2009) or lettuce (*Lactuca sativa* L.) cultivation (Lucini et al., 2015). However, the products formulated with materials coming from the hydrolysis of animal or plant proteins have some inconveniences (Colla et al., 2015), such as: i) the destruction of some beneficial AAs, such as tryptophan, cysteine and threonine, ii) the conversion of AAs to their acidic forms such as asparagine and glutamine, iii) appearance of non-desired AAs. Numerous researchers from research centers and biostimulant companies have observed that the foliar application of individual amino acids can promote the mechanisms of adaptation of plants to salinity. The amino acid that has been most-commonly used in foliar applications to alleviate the effects of salinity is proline. The applications of proline have been beneficial in mustard crops (*Brassica juncea* L.) (Ahmad et al., 2015), wheat (*Triticum* L.) (Amir et al., 2002; Talat et al., 2013; Ami et al., 2020) and rice (*Oryza sativa* L.) (Siddique et al., 2015). To a lesser degree, other amino acids have been applied such as glycine betaine (Estaji et al., 2019), arginine (Badi et al., 2018), and cysteine (Genisel et al., 2014), with promising results. In other abiotic stresses, beneficial AAs applications have also been reported: drought, application of proline in oats (*Avena sativa* L.) (Ghafoor et al., 2019), L-Ornithine in sugar beet (Beta vulgaris var. saccharifera L.) (Hussein et al., 2019), and salicylic acid and L-Tryptophan in maize (Rao et al., 2012), absorption of nutrients, L-Glycine and L-Glutamine, individual and combined in lettuce (Lucini et al., 2015), Cd toxicity, application of aspartic acid in rice plants (Rizwan et al., 2017), low temperatures, application of glutamic acid in Kimchi cabbage (*Brassica campestris* spp. *Pekinensis*) (Lee et al., 2017). However, there is a lack of studies which have compared the application of AAs individually or in a mixture. To manufacture biostimulants efficiently, it would be wise to understand the function of each of the AAs found in nature and to know if an interaction between them exists. The objective of this study was to evaluate the effect of the foliar application of AAs (Arginine, Methionine, Glutamine, Proline and Tryptophan), either individually or as a mixture, on tomato plants grown with saline water, in order to: i) elucidate which of these free amino acids can increase the tolerance to salinity of these plants, ii) estimate possible tolerance mechanisms induced by these AAs, and iii) determine synergistic or antagonistic effects between AAs in mixtures.

2. Materials and methods

2.1. Growing Conditions and Plant Material

The experiment was performed with tomato plants (*Solanum lycopersicum* L.) cultivar "Óptima". For this, plants from a commercial nursery (Baby Plant, S.L. Murcia, Spain) were utilized, and these were grown in hydroponics in a grown chamber located at the CEBAS-CSIC (Espinardo, Murcia, Spain) under controlled conditions. The photoperiod was set for 16 hours of light with an intensity of 500 $\mu mol\ m^{-2}\ s^{-1}$, the temperature was set at 25 °C day/night, with a relative humidity of 60%. The seedlings were grown in 9 L containers with a Hoagland nutrient solution, composed of: 0.54 $g\ L^{-1}$ of KNO_3, 0.84 $g\ L^{-1}$ of $Ca(NO_3)_2$, 0.14 $g\ L^{-1}$ of KH_2PO_4, 0.25 $g\ L^{-1}$ of $MgSO_4$, 0.2 $g\ L^{-1}$ of Fe, and 0.2 $g\ L^{-1}$ of a micronutrient mix (Hidromix S, Valagro). This nutrient solution had an EC = 2 $dS\ m^{-1}$, and the pH was adjusted weekly to pH 5.5-6.5 with 1 M NaOH. These containers had a constant flow of air to avoid conditions of hypoxia during the experimental period. The experiment lasted 42 days; firstly plants were germinated until four true leaves were fully developed (25 days), then they were transferred to hydroponic cultivation system for 10 days for acclimatization. The experiment started after the acclimatization period (7 days), and the plants were 10 days under salt condition.

2.2. Salinity treatment

The experiment was comprised by a set of plants grown with a Hoagland nutrient solution without salinity (control; EC = 2 $dS\ m^{-1}$) and another set of plants grown with this solution but to which NaCl was added at a concentration of 50 mM (EC = 8 $dS\ m^{-1}$). The EC was monitored daily with a conductivity meter (LAQUAtwin EC-22; Kioto, Japón).

2.3. Preparation, and application of AAs and mixtures during the salinity stress period

Of the 21 proteinogenic AAs, the following were selected: Glutamic acid (Glu), L-Proline (L-Pro), L-Arginine (L-Arg), L-Methionine (L-Met) and L-Tryptophan (L-Trp), which were applied foliarly, individually or in a mixture, to the salt-treated plants, with the following independent treatments: i) Control (-salt), ii) saline (+salt), iii) L-Met + salt, iv) L-

Arg + salt, v) L-Pro + salt, vi) Glu + salt, vii) L-Trp + salt, viii) L-Met + L-Arg + salt, ix) L-Met + L-Trp + salt, x) Glu + L-Pro + salt. For the application of the selected AAs, the individual stock solutions were first prepared for each one of them (Glu, Pro, Arg, Met and Trp) with a concentration of 5% (v/v). In second place, for the foliar application of each amino acid: i) for the individual treatments, a 10 mL L^{-1} dose of the stock solution was utilized, and ii) for the AAs mixtures, a solution was prepared starting with the two stock solutions, having in mind that the final dose of each was also 10 mL L^{-1}. Before the application, all the products were adjusted to a pH of 5.5-5.6, and Tween-20, whose composition is a saturated fatty acid ester and polyoxyethylene sorbitan, was added for a final concentration of 0.1% (v/v); this surfactant improves the adherence of the sprayed solution. The application of these products was conducted an hour before the salt treatment. For this, the products were foliarly sprayed onto the plants in a manner that the leaves were completely covered with the solution. For each treatment, 4 containers with 3 plants each were utilized. In total, 120 plants were utilized, and each container with its three plants was considered to be an experimental unit. The containers were randomly distributed in the growth chamber.

2.4. Growth parameters and relative water content

At the end of the experiment (6 days after the foliar application), the plants were harvested, and the leaves, stems and roots were weighed separately (g, fresh weight; fw). Afterwards, they were thoroughly washed with deionized water and dried in an oven at 60 °C for at least 48 hours. Then, the samples were weighed again (g, dry weight; dw), and they were milled until obtaining a fine powder for their posterior analysis in the lab. For the quantification of the relative water content (RWC), 1 cm leaf disks were taken from each plant, weighed (g fw) and placed in a rack with distilled water for 16 hours in the dark and at room temperature for them to become turgid. Posteriorly, they were weighed again (g, turgid weight; tw), and dried in an oven at 60 °C for 16 hours, after which they were weighed again (g dw). With this data, the water content of the shoots was calculated with the equation: [(fw – dw)/(tw – dw)] x 100, expressed as a %.

2.5. Leaf chlorophyll concentration

To determine the concentration of total chlorophylls in the leaf, the spectrophotometry method proposed by Hansmann (1973) was utilized with some modifications. Discs of 1 cm in diameter were taken from the aerial part of the plant and suspended in a volume of 5 mL acetone-water at 90% (v/v) as the pigment extraction solvent. They were agitated and left in the dark at 4 °C for 24 hours. Afterwards, the optical density of the supernatant was measured at 665, 645 and 630 nm, verifying that there was no turbidity or particles present. The solvent solution was utilized as the blank. For quantification, the equation proposed by Parsons and Strickland (1963) was utilized:

$$C_a \text{ (mg } L^{-1}) = 11.6 \text{ O.D.}665 - 1.31 \text{ O.D.}645 - 0.14 \text{ O.D.}630$$

$$C_b \text{ (mg } L^{-1}) = 20.7 \text{ O.D.}645 - 4.34 \text{ O.D.}665 - 4.42 \text{ O.D.}630$$

$$C_c \text{ (mg } L^{-1}) = 55.0 \text{ O.D.}630 - 4.64 \text{ O.D.}665 - 16.3 \text{ O.D.}645$$

Where C_a, C_b and C_c are the concentrations of chlorophylls a, b and c, respectively, and O.D. is the average optical density. The concentration of chlorophyll was expressed in mg g^{-1} leaf fw.

2.6. Biochemical parameters

At the end of the experiment, the concentrations of proline, carbohydrates (total soluble sugars, reducing sugars and starch) and the concentration of quaternary ammonium compounds (QAC) were quantified in the leaves. Proline was extracted from the dry leaf tissue with sulfosalicylic acid (3%) and was quantified according to the protocol described by Bates et al. (1973). On their part, the total soluble and reducing sugars were also extracted from the dry leaf tissue with ethanol (80%) and quantified in accordance by protocols described by Hodge and Hofreiter (1962), Nelson (1944) and Somogy (1952). Also, the concentration of starch from the dry tissue was determined with the method described by Haissig and Dickson (1979). The QAC were extracted and quantified from the dry material following the protocol by Grieve and Grattan (1983). The results were expressed in mg g^{-1} dw.

2.7. Mineral analysis

A nutritional analysis of the dry and milled leaf samples was conducted. The concentration of Cl- and NO_3- was measured using a silver ion titration chloridometer (Corning 926 Chloridometer; Sherwood, UK) and a Dionex DX-600 HPLC (California, EE.UU.), respectively, after extraction with distilled water. On the other hand, the concentration of Na^+ and Ca^{2+} was analyzed through inductively coupled plasma (ICP) spectrophotometry (Iris Intrepid II, Thermo Electron corporation, Franklin, USA), after digestion with HNO_3:H_2O_2 (5:3 by volume) in a microwave (CERM Mars Xpress, North Carolina, USA).

2.8. Statistical analysis

The experiment utilized a single-factor design (treatment with AAs), and therefore, the statistical analysis of the data was performed with an analysis of variance (ANOVA) with the statistical program SPSS version 24 (Chicago, IL, USA). When the variables were found to be significant ($p < 0.05$), the treatment means were separated utilizing Tukey's multiple range test. The values presented for each treatment are the means of 4 experimental repetitions (every experimental unit are three replicate plants). Principal component analysis (PCA) and cluster analysis (CA) were also performed. Cluster analysis was applied to the standardized data for hierarchical associations employing Ward's method for agglomeration and the squared Euclidean distance as the dissimilarity measure.

3. Results

3.1. Plant growth parameters

As expected, the salt treatment (+salt) significantly reduced vegetative growth as compared to the control plants (-salt). The total dry biomass of the plants was reduced by 31% as compared to the control plants (-salt), and this reduction was due to the biomass reduction of the shoots (32%) as well as the root (30%). The total dry biomass of the plants treated with the AAs Glu, Trp, Pro, Arg and Arg + Met was similar to the plants in the + salt treatment, with a reduction of 53-61%, relative to the control treatment (Fig. 1). However, the application of the treatments **Met, Met + Trp, and Pro + Glu** mitigated the reduction in vegetative growth due to salinity. In this last treatment, there were no significant differences with the control plants.

For the growth results for each plant tissue, it can be observed that the shoot was significantly reduced in all plants from the salt treatments except for the Pro + Glu treatment, relative to the non-salinized plants. Also, the plants from the Met + Trp and Met treatments had a vegetative growth that was greater than those from the salt treatment without AAs, with the growth similar to the rest of treatments, except for the L-Pro plants, with a significantly less growth. In the case of the roots, the L-Met, Met + Trp and Pro + Glu treatments did not show a decreased growth, while the L-Pro, Met + Arg and L-Arg had a 15% decrease in growth, although their growth was greater than in the salt treatments without AAs or the salt treatments with Glu and L-Trp. The changes occurred in the growth of shoot and roots, resulted in alterations in the root/shoot ratio. In general, a progressively decreasing trend was observed in the following order:

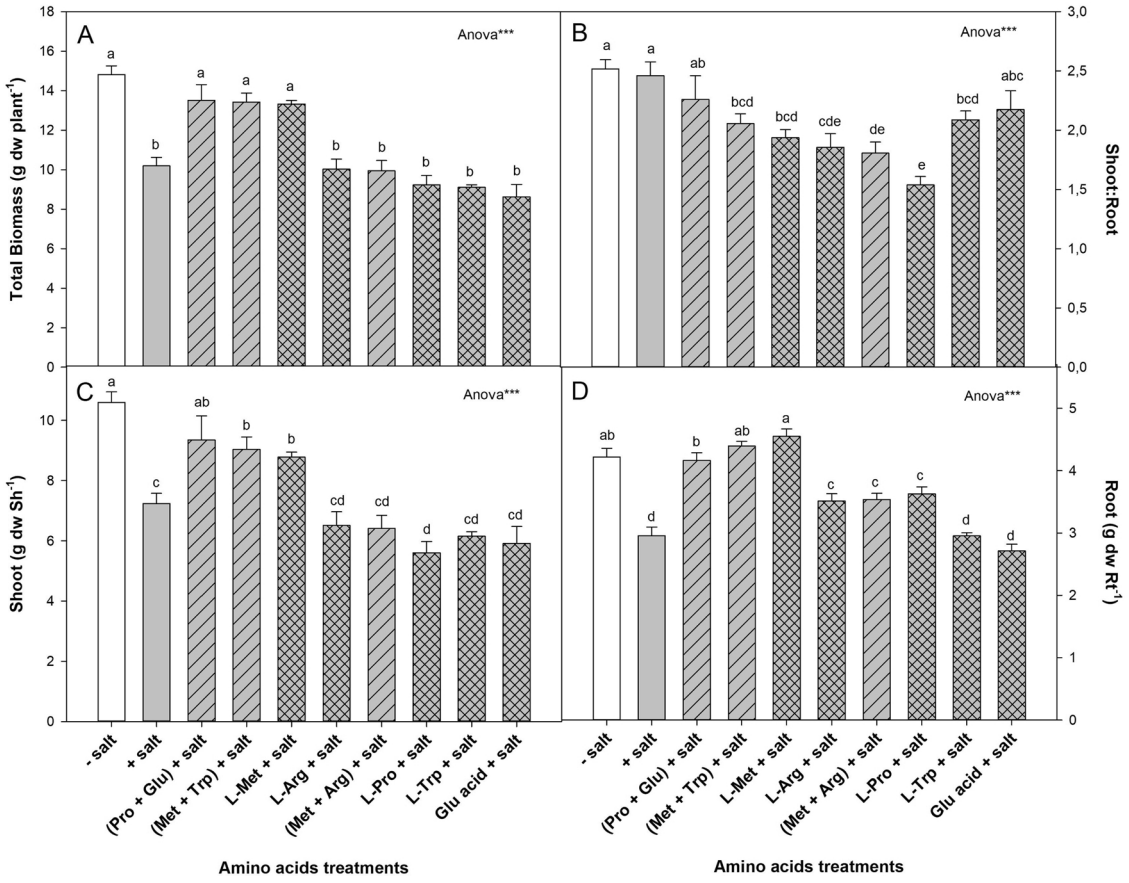


Fig. 1. Growth parameters: **(A)** Total Biomass, **(B)** Shoot:Root ratio, **(C)** Shoot, and **(D)** Root of tomato plants *var.* Óptima watered with saline water (50 mM) and foliarly treated with different amino acids (glutamic acid (Glu), L-Tryptophan (L-Trp), L-Proline (L-Pro), L-Arginine (L-Arg) and L-Methionine (L-Met); and their mixtures: Met + Arg, Met + Trp and Pro + Glu). In the ANOVA: *** indicates significant differences at $p < 0.001$. The different *lower case letters* indicate significant differences between the treatments at $p < 0.05$ established by Tukey's multiple range test. The vertical bar indicates the standard error of the mean ($n = 4$).

-salt > +salt > Pro + Glu > Glu > L-Trp > Met + Trp > L-Met > L-Arg > Met + Arg > L-Pro.

3.2. Relative water content (RWC)

The RWC data had a range between 69% in the control treatment and 46% of the salt treatment without AAs, with significant differences between these two treatments (Fig. 2). The RWC of the salt treatments with amino acids were found between the previously-mentioned values. The Pro + Glu, Met + Trp, L-Met, Met + Arg and Glu treatments had a tendency to decrease the RWC, but without significant differences with the non-salinized control treatment. The L-Arg and L-Pro treatments had a RWC that was significantly lower than the control treatment, but these values were higher than the + salt treatment. Non-significant differences were observed between L-Trp and + salt treatments.

3.3. Concentration of mineral nutrients in leaves

The salt treatments significantly increased the concentration of Cl- and Na^+ in the leaves with respect to the control treatment plants, independently of the treatments with or without AAs (Fig. 3). In the case of chloride, the salt-treated plants with AAs had a range of concentration between 1.3-2.1 (%, mg 100 mg^{-1} dw). All AAs treatments, except for L-Trp, reported non-significant differences between them or between the + salt treatment. The plants treated with the amino acid tryptophan significantly increased their concentration with respect to the rest of the treatments, reaching a concentration of 2.2%.

In the case of Na^+, a similar response as that of Cl- was observed. The salinity applied increased its concentration in the leaves in all the

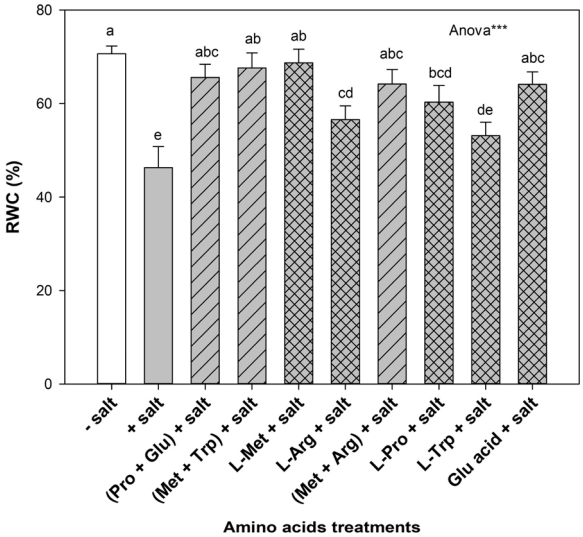


Fig. 2. RWC of tomato plants *var.* Óptima watered with saline water (50 mM) and foliarly treated with different amino acids (glutamic acid (Glu), L-Tryptophan (L-Trp), L-Proline (L-Pro), L-Arginine (L-Arg) and L-Methionine (L-Met); and their mixtures: Met + Arg, Met + Trp and Pro + Glu). In the ANOVA: *** indicates significant differences at $p < 0.001$. The different *lower case letters* indicate significant differences between the treatments at $p < 0.05$ established by Tukey's multiple range test. The vertical bar indicates the standard error of the mean ($n = 4$).

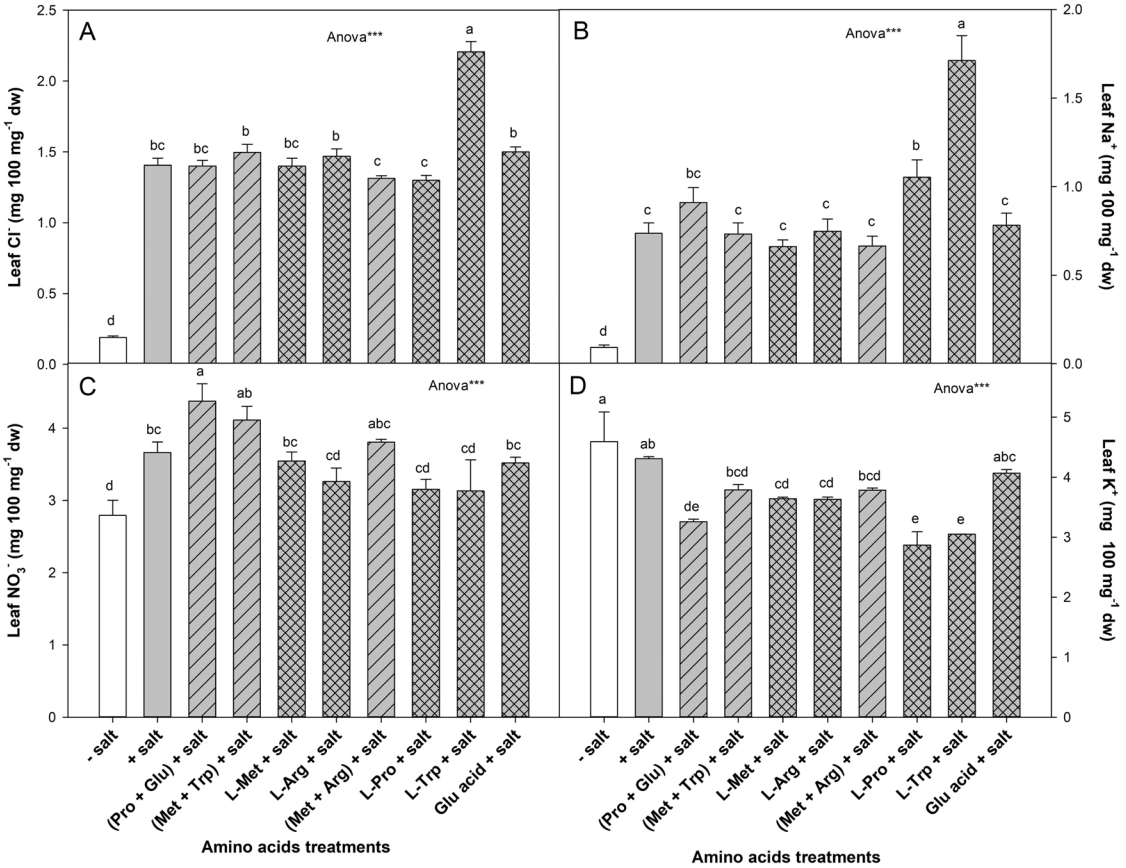


Fig. 3. Mineral nutrition. Concentration of **(A)** Cl-, **(B)** Na^+, **(C)** NO_3-, and **(D)** K^+ of tomato plants *var.* Óptima watered with saline water (50 mM) and foliarly treated with different amino acids (glutamic acid (Glu), L-Tryptophan (L-Trp), L-Proline (L-Pro), L-Arginine (L-Arg) and L-Methionine (L-Met); and their mixtures: Met + Arg, Met + Trp and Pro + Glu). In the ANOVA: *** indicates significant differences at $p < 0.001$. The different *lower case letters* indicate significant differences between the treatments at $p < 0.05$ established by Tukey's multiple range test. The vertical bar indicates the standard error of the mean ($n = 4$).

treatments, independently of the application or not of the amino acids. No significant differences were observed between the plants to which no AAs were applied (+salt treatment), and the treatments with Glu, L-Arg, L-Met, Met + Arg, Met + Trp and Pro + Glu (Fig. 3). However, the L-Pro and L-Trp treatments had a significantly higher leaf Na^+ concentration than plants from the + salt treatments, with the L-Trp plants reaching the highest value (1.7%).

The range in foliar concentration of nitrate was found to be between 2.8-4.4%. The trend found was that the salt treatment, independently of the application of AAs, tended to increase the concentration of nitrate as compared to the control treatment (-salt), with the highest value observed in Pro + Glu plants. However, in this trend, there were no significant differences between the -salt and L-Arg, L-Pro, and L-Trp treatments.

The concentration of K^+ significantly decreased with the application of all the AAs treatments with respect to the control treatment, except for Glu. However, in the plants from the salt treatment without AAs, K^+ was similar to the control plants. Among the treatments with amino acids, where a decrease of K^+ was observed (L-Pro, L-Trp, Pro + Glu, L-Arg, L-Met, Met + Arg and Met + Trp), the lowest value was observed for the treatments with L-Pro and L-Trp, although without significant differences with respect to Pro + Glu.

3.4. Organic solutes, carbohydrates and chlorophylls

The concentration of proline tended to increase in the salt treatments as compared to the control treatment (-salt), with this increase being significant for all the treatments except for Pro + Glu and L-Trp. In the treatments with AAs, L-Arg, Met + Arg and L-Pro had a similar but higher concentration than the rest of the treatments (Fig. 4). The concentration of quaternary ammonium compounds (QAC) ranged between 0.7-1.4 mg g^{-1} dw. The salt treatment without AAs did not show significant differences with the control (-salt), although + salt plants had significantly higher values than L-Pro plants.

The total chlorophyll concentration, as compared to the control treatment, significantly decreased with the salt treatment without AAs and with the Met + Trp, Met + Arg, L-Pro, L-Trp and Glu treatments (Table 1). In these treatments, the plants treated with Pro + Glu, Met + Arg, and L-Met, had a greater concentration of chlorophylls that those to which no AAs were applied.

The concentration of total soluble sugars increased significantly in the Pro + Glu, Met + Trp and L-Pro treatments with respect to the rest of the treatments, where no differences were observed between them. The concentration of reducing sugars was significantly higher in the plants from the L-Pro, Glu and Pro + Glu treatments, although this last did not show significant differences with Met + Trp, relative to -salt and + salt treatments. Leaf concentration of starch, Pro + Glu treatment had higher concentration than those from L-Met, L-Arg and Glu, which concentration was significantly lower than for control treatments (-salt and + salt).

3.5. Principal components analysis (PCA) and cluster analysis

For a better and simpler visual interpretation of all the data, a principal component analysis and a cluster analysis were conducted (Fig. 5). The first five components explained 93% of the variability, and the first three, represented by PC1, PC2 and PC3, explained 76%. The PC1 component explained 41% of the variability observed, thus

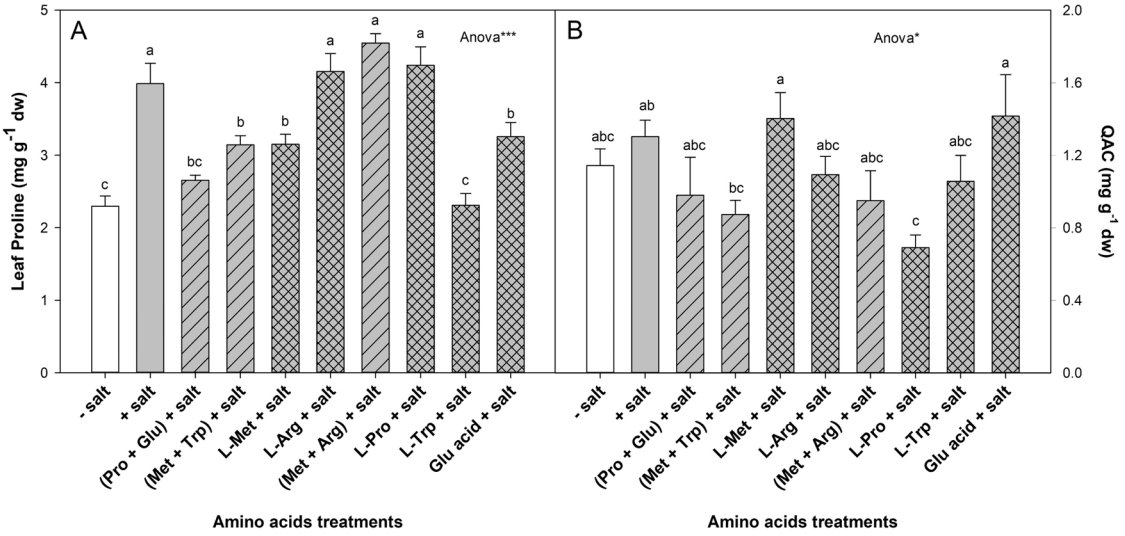


Fig. 4. (A) Proline and **(B)** QAC concentration quantified in leaves of tomato *var.* Óptima plants watered with saline water (50 mM) and foliarly treated with different amino acids (glutamic acid (Glu), L-Tryptophan (L-Trp), L-Proline (L-Pro), L-Arginine (L-Arg) and L-Methionine (L-Met); and their mixtures: Met + Arg, Met + Trp and Pro + Glu). In the ANOVA: *** indicates significant differences at $p < 0.001$. The different *lower case letters* indicate significant differences between the treatments at $p < 0.05$ established by Tukey's multiple range test. The vertical bar indicates the standard error of the mean ($n = 4$).

Table 1
Concentration of chlorophylls, carbohydrates and other osmolytes in leaves of tomato plants *var.* Óptima watered with saline water (50 mM) and foliarly treated with different amino acids and their mixtures.

Treatment	Soluble sugars (mg g^{-1} dw)	Reducing sugars (mg g^{-1} dw)	Starch (mg g^{-1} dw)	Total Chl (mg g^{-1} fw)
- Salt	38.84 c	10.65 c	22.68 bc	1.64 a
+ Salt	49.97 bc	13.79 c	29.30 b	0.93 de
Pro + Glu + Salt	66.20 a	25.17 b	39.49 a	1.41 ab
Met + Trp + Salt	70.27 a	15.79 bc	15.77 cd	1.32 bc
L-Met + Salt	65.67 a	11.83 c	8.22 e	1.54 ab
L-Arg + Salt	39.80 bc	13.71 c	10.97 de	0.96 de
Met + Arg + Salt	46.57 bc	9.98 c	17.27 cd	1.12 cd
L-Pro + Salt	47.3 bc	78.89 a	17.28 cd	1.00 d
L-Trp + Salt	52.03 b	11.27 c	17.24 cd	0.69 e
Ac. Glu + Salt	46.27 bc	25.31 b	14.78 de	1.05 cd
ANOVA	***	***	***	***

In the ANOVA: *** indicates significant differences at $p < 0.001$. The different *lower case letters* indicate significant differences between the treatments at $p < 0.05$ established by Tukey's multiple range test. The vertical bar indicates the standard error of the mean ($n = 4$).

showing that the variability was fundamentally due to the total biomass, growth of the shoots and the root, relative water content, total chlorophylls and foliar Na concentration. The PC2 component explained 20% of variability, with the parameters associated to it shown in Fig. 5.

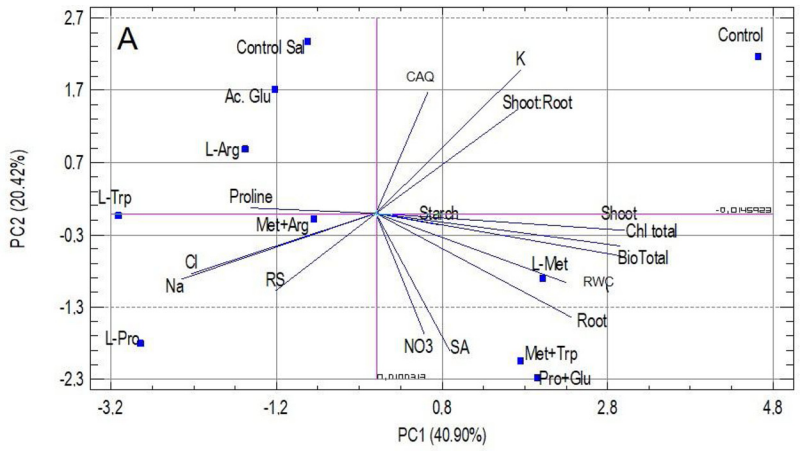
In light of these results, it can be confirmed that the PCA demonstrates that there were differences in the results of the different treatments tested (AAs and their combinations). The cluster analysis shows results that are similar to those from the PCA. The treatments of L-Met, L-Met + Trp and the Pro + Glu showed results that were the most similar to the control treatment (-salt), and therefore, their tolerance was greater than the rest of the treatments.

4. Discussion

Under salinity conditions, the growth of the plants is affected as result of the alterations in their physiological and chemical processes, and this leads to decreases in the production and harvest quality of the crops (Chrysargyris et al., 2018). Many fertilizer companies aim to formulate biostimulant products, which could help to mitigate the negative effects of abiotic stresses such as salinity. To manufacture these products, AA-rich active materials are utilized, but as these materials are composed by a great variety of organic molecules, the role that these AA-based biostimulants have in plants is unknown. In this study, the individual application of 4 AAs and 3 double mixtures was analyzed in tomato plants under salinity conditions in order to better understand the effect of the AAs in the tolerance to salinity of tomato plants.

In our experiments, as expected, salinity decreased the overall growth of the tomato plants. But the individual application of L-Met and the Pro + Glu and Met + Trp mixtures reversed this effect, so that the salinity in the nutrient solution scarcely affected the total dry biomass of the plants. In fact, this parameter did not show significant differences with respect to the control plants. The beneficial effect of the application of L-Met, Pro + Glu and Met + Trp was not observed with the rest of AAs treatments, although no negative effects were found either. Even the application of L-Arg, Met + Arg and L-Pro induced a greater growth of the root with respect to the salt-treated plants without the application of AAs.

The high concentration of NaCl in the irrigation water can result in i) the loss of turgor of the leaves as a result of the osmotic effect, ii) Cl- and Na^+ toxicity in the different plant tissues, iii) and a possible nutritional unbalance (Khan et al., 2000; Acosta-Motos et al., 2017; Hussain et al., 2018). The degree of tolerance to salinity of the plants depends on their ability to mitigate some of these factors (Ghalati et al., 2020). In our assay, as previously mentioned, the plants treated with the AAs L-Met, Pro + Glu and Met + Trp had a greater tolerance to salinity with respect to the rest of the salt treatments. According to the data on the relative water concentration (Fig. 2), the foliar concentration of Cl- and Na^+ (Fig. 3) and the concentration of K^+ and NO_3-, this greater tolerance to salinity was not related to the accumulation of Cl- in the leaves, the water status or the nutritional status of the plants, as a relationship between these parameters and the growth of the plants was not observed. Nevertheless, in the plants that received the treatments with L-Met, Pro + Glu and Met + Trp, the concentration of total soluble sugars increased, which could have reduced the oxidative stress produced by the toxicity of Cl- and Na^+ accumulated in the leaves. In fact, in these plants, the concentration of total Chl was barely reduced with salinity, which is an indication that the Cl- and/or Na^+ toxicity in the leaves was less than for the rest of the treatments, despite a similar concentration of these two ions accumulating in all salinized plants.



Principal components	1	2
Characters	Eigenvectors	
BioTotal	0.383	-0.128
Shoot	0.389	-0.051
Root	0.305	-0.310
Shoot:Root	0.223	0.311
RWC	0.297	-0.206
Chl total	0.381	-0.097
Cl^-	-0.288	-0.181
Na^+	-0.304	-0.197
K^+	0.226	0.426
NO_3^-	0.075	-0.361
Proline	-0.195	0.016
CAQ	0.082	0.359
SA	0.115	-0.411
RS	-0.156	-0.230
Starch	0.100	-0.035

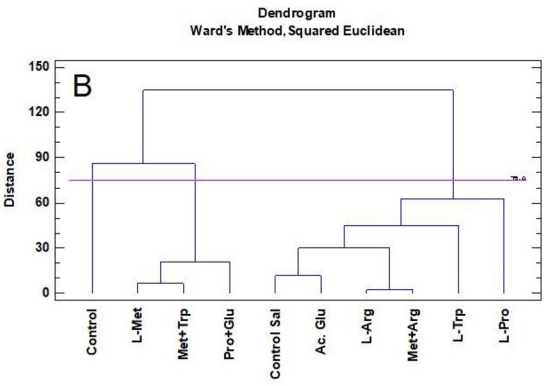


Fig. 5. (A) Principal component analysis (PC1 and PC2) and **(B)** cluster analysis (CA) of tomato plants *var.* Óptima watered with saline water (50 mM) and foliarly treated with different amino acids (glutamic acid (Glu), L-Tryptophan (L-Trp), L-Proline (L-Pro), L-Arginine (L-Arg) and L-Methionine (L-Met); and their mixtures: Met + Arg, Met + Trp and Pro + Glu) in a growth chamber.

The synthesis and accumulation of organic solutes are common responses of the adaptation of plants to water deficit as well as salt stress (osmoregulation). These compounds include AAs such as proline, quaternary ammonium compounds, poly-alcohols and sugars such as sucrose. These compounds, aside from acting as osmoregulators, have an antioxidant capacity that helps plants de-activate ROS (Caverzan et al., 2019). According to our data, the following hypothesis can be rised: The increase on salt tolerance in plants treated with L-Met and Met + Trp could have been due to relation of these two AAs with polyamines metabolism. It is known that these compounds (polyamines) are involved in the tolerance of the plants to salinity (Ke et al., 2018), and that L-Met is a precursor in the biochemical synthesis route of these compounds (Duarte-Sierra et al., 2019). In the case of the Pro + Glu treatment, the presence of Glu could help plants tolerate salinity thanks to this amino acid modulating the equilibrium of ions and boosting the antioxidant systems under salt stress, as confirmed by the results obtained by Guo et al. (2017), in an experiment where poly-γ-glutamic acid (γ-PGA) was applied exogenously to wheat plants grown with 150 mM NaCl.

Another important conclusion that could be extracted from these experiments is related with i) the osmoregulatory capacity of the AAs, ii) the accumulation of proline in the plants, and iii) the effect of tryptophan on the accumulation of Cl- and Na^+ in the leaves. Starting with the first, one of the negative effects of salinity in plants is the dehydration of the tissues. In order to impede this, the plants increase the synthesis of osmoregulatory substances, such as proline and glycine-betaine (Núñez-Vázquez et al., 2017). Thus, this experiment also revealed that other amino acids such as L-Met, L-Arg and Glu can also induce osmoregulation, since RWC of the salinized L-Met, L-Arg and Glu plants was greater than those from the salt treated plants without AAs. L-Trp, however, did not have this osmoregulating capacity as observed in Fig. 2.

It is known that tomato plants synthesize proline when subjected to salt stress (Kishor et al., 2015; Siddiqui et al., 2020), but its accumulation is not enough for it to have a beneficial effect (Gagneul et al., 2007; Ghars et al., 2008; Bendaly et al., 2016; Mansour and Ali, 2017), and also, this accumulation increases as the plants are more affected by the stress, so that the concentration of proline can be used as an negative indicator of tolerance, where the higher levels indicate less tolerance (*data not published*). In our experiments, this can be confirmed, as the more tolerant plants had the least concentration of proline. In fact, those plants did not have a decreased growth due to salinity, (L-Met, Pro + Glu and Met + Trp), and the concentration of proline was significantly lower than the other salt treatments.

L-Trp, L-Phe and L-Tyr are aromatic AAs derived from the shikimic acid pathway. This metabolic route plays a vital role against pathogenic microorganisms and herbivores, as it is involved in the mechanisms of defense that could also act against abiotic stresses (Marco, 2006; Ávalos-García and Pérez-Urria, 2011; Bilal et al., 2018). These types of amino acids are utilized in the manufacture of new biostimulant products that stimulate and activate the plant's defense systems against pathogens. In our assay, it was observed that the application of these AAs had a detrimental effect on the plants, such as the accumulation of the toxic ions Cl- and Na^+ (Fig. 3). However, this effect did not have a critical impact on our experiments, as the total dry biomass was similar in plants under salt stress without amino acids and L-Trp-treated plants. It would be interesting to study if this could have negative repercussions on the development of the crop in the long term, and it would be important to study which mechanisms are responsible for L-Trp increasing the concentration of these ions in the plants.

In this assay, it was also verified that when an amino acids mixture was applied to the tomato plants grown under salt stress conditions, the interaction between the AAs should be taken into account. Antagonistic and synergistic effects could be observed from our experimental data, thus, further research should be conducted on the physiological mechanisms involved. The antagonistic effect could be observed in the

case of L-Met and L-Arg. When L-Met was applied to the plants either as a single amino acid or in a mixture with L-Trp, the result was very different than when applied in a mixture with L-Arg. This amino acid reversed the positive effect of L-Met on the growth of the plants. It is also important to observe that the effect of L-Trp on the concentration of Cl- and Na^+ was reversed with the Met + Trp mixture, as Met + Trp plants had a concentration of Cl- and Na^+ lower that L-Trp plants under salt stress conditions. This synergistic effect was also observed in the application of the Pro + Glu mixture. The effects of its application were completely different to the effects observed after the individual application of these two amino acids. The mixture of these two AAs stimulated the vegetative growth as compared to their individual application.

5. Conclusions

In the response of the tomato cultivar "Optima" plants to salinity, it was observed that when AAs were applied foliarly, the type of amino acid and type of amino acid mixture played a fundamental role, demonstrating that different types of interactions are possible between them. Of all the treatments applied, the L-Met, Pro + Glu and Met + Trp treatments reversed the negative effects of salinity, and this was possibly due to the increase in the concentration of total soluble sugars, whose involvement in mitigating oxidative stress caused by the toxicity of Cl- and Na^+ are well-proven (Guan-fu et al., 2011). Also, negative interactions were observed between L-Met with L-Arg and L-Trp, while L-Pro and Glu had synergistic effects. In the future, it would be interesting to evaluate the agronomic behavior of the tomato crop when these treatments are applied in real-world field conditions, and overall, to identify the physiological mechanisms involved in the antagonistic or synergistic interaction of the AAs.

Declaration of Interest Statement

This statement is to confirm that the co-authors of the manuscript: "Effect of foliar application of amino acids on the salinity tolerance of tomato plants cultivated under hydroponic system" Marina Alfosea-Simón, Ernesto A. Zavala-Gonzalez, Jose M. Camara-Zapata, Juan J. Martínez-Nicolás, Inmaculada Simón, Silvia Simón-Grao, Francisco García-Sánchez have no interests to declare.

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