Effect of acetylsalicylic acid and ammonium sulphate on productive and physiological parameters in *Stipa caudata* under water shortage conditions

Julio MOLINA[1,2*], Sara GONZÁLEZ-ORENGA[1,3], Oscar VICENTE[3],
Monica BOSCAIU[1*], Josep V. LLINARES[1], Francisco ZAMBRANO[4],
Claudia SANTIBÁÑEZ[5]

[1]*Universitat Politècnica de València, Instituto Agroforestal Mediterráneo (IAM), Camino de Vera 14, 46022 Valencia, España; sagonor@doctor.upv.es; mobosnea@eaf.upv.es (coresponding autor); jollipa@qim.upv.es*
[2]*Universidad Mayor, Escuela de Agronomía, Facultad de Ciencias, Santiago 8580745, Chile; julio.molina@umayor.cl (*corresponding author)*
[3]*Universitat Politècnica de València, Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV), Camino de Vera 14, 46022 Valencia, España, ovicente@upvent.upv.es*
[4]*Hémera Centro de Observación de la Tierra, Escuela de Agronomía, Facultad de Ciencias, Santiago 8580745, Chile; francisco.zambrano@umayor.cl*
[5]*Universidad Mayor, Escuela de Ingeniería en Medio Ambiente y Sustentabilidad, Facultad de Ciencias, Santiago 8580745, Chile; claudia.santibanez@umayor.cl*

Abstract

Stipa caudata is a grass native to low rainfall areas in Argentina and Chile, considered an excellent potential candidate for biofuel production or soil restoration programmes. This study aimed at analysing the effects of ammonium sulphate (AMS) and acetylsalicylic acid (ASA) on the productivity and biochemical traits of plants of this species under water scarcity conditions. The experimental work was carried out on plants grown outdoors using a randomised block plot design. Several yield and biochemical parameters related to resistance to water scarcity were analysed in plants treated with AMS or ASA. Plants in the treatments with ASA and AMS had higher total chlorophyll content than the others. Concerning ion content, water-restricted plants treated with AMS had similar values to irrigated plants. Regarding the osmoprotectants and antioxidants, treated plants had increased concentrations of proline and total flavonoids. Under water stress, plants had higher APX activity and there was an A x B interaction for CAT and SOD activity. The results obtained show that the use of ASA and AMS in some crops or in environmental restoration programmes could be a useful tool to cope with future climate scenarios of water scarcity.

Keywords: oxidative stress; *Stipa caudata*; water shortage condition

Introduction

Chile's latest climate change projections predict that there will be an increase in temperatures and a decrease in rainfall in much of the north and centre of the country, leading to desertification and reduced water

availability for plants (IPCC, 2000; Masson-Delmotte, 2018). Water stress is one of the leading environmental problems affecting crops. It is associated with several physiological and biochemical cellular effects, including oxidative damage by increased reactive oxygen species (ROS) production, reducing plant growth and productivity (Osakabe *et al.*, 2014; Sun *et al.*, 2020).

Stipa caudata Trin. is a grass native to Chile and Argentina, adapted to areas of low annual rainfall (100 - 300 mm/year) and agriculturally infertile and eroded soils. The species is a good candidate for soil restoration and biofuel production programmes. Plants tolerant to water stress can respond through a variety of physiological changes, such as an increase of osmoprotectant levels or the activation of antioxidant systems. Under stress conditions, plants increase the synthesis of osmolytes, such as proline, an amino acid involved in responses to various stresses (Magdy *et al.*, 2017; Vargas-Ortiz *et al.*, 2021). Antioxidant mechanisms are based on the synthesis of metabolites with strong antioxidant activity, such as phenolic compounds and especially the subgroup of flavonoids, and the activation of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) or glutathione reductase (GR), which help to maintain the plant's metabolic functions, minimising inhibition of growth (Apel and Hirt, 2004).

To mitigate environmental constraints in dry areas, the use of various additives has shown promising results. For example, nitrogen and sulphur are essential mineral elements for plants, partly because they participate in the synthesis of glutathione, a tripeptide involved in the detoxification of ROS (Neuberg *et al.*, 2010). Moreover, since nitrogen assimilation is crucial for ion uptake and amino acids and proteins synthesis, ammonium sulphate is one of the most important nitrogen fertilisers. On the other hand, acetylsalicylic acid (ASA) is a precursor of salicylic acid, a phytohormone that plays an essential role in plant signalling in response to various stresses, including water stress (Nazar *et al.*, 2015).

However, there is very little information on the physiological responses for increased resistance and biomass production under water deficit conditions in *Stipa caudata*. Even less is known on the possible effects of sustainable strategies such as ammonium sulphate (AMS) or acetylsalicylic acid (ASA) application to water-stressed plants. This study represents a first assessment of the resilience of *Stipa caudata* to water stress and on the alleviation of this stress by sustainable and low-cost strategies.

Therefore, this work aimed at evaluating the effects of the application of AMS and ASA on biomass production and activation of specific stress tolerance mechanisms of *Stipa caudata* plants under water deficit conditions by (i) analysis of growth parameters in control and stressed plants; (ii) quantification of leaf contents of total chlorophyll, ions and osmolytes; (iii) determination of total phenolic compounds and flavonoids concentrations in shoots; (iv) measurement of the specific activities of several antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase).

Materials and Methods

Study site and experimental conditions

The experiment was conducted under field conditions in September 2018, at the Bosque Santiago facilities, Ministry of Housing and Urbanism, Government of Chile, located at Camino La Pirámide 6.000, Huechuraba, Metropolitan Region, Chile (33° 22′ 38.6″ S 70° 36′ 3.36.8″ W). The temperature range was between 28 to 31.6 °C, with an average of 22 °C and a mean relative humidity of 67.2%, without precipitation (Agromet, 2018).

Analyses were carried out in the Faculty of Sciences of the Universidad Mayor, Santiago, Chile and COMAV, Polytechnic University of Valencia, Spain.

Experimental design and treatments

Stipa caudata plants were extracted from the ground in La Pintana, Metropolitan Region, Santiago de Chile (33° 26′ 40.42″ S; 70° 39′ 3.43″ W), where they grow spontaneously. Subsequently, they were

transferred to 10 L pots filled with soil sampled from the piedmont of the Rinconada de Los Andes commune, V Region of Valparaíso, Chile (32° 52′ 00.42′ S 70° 43′ 37.29′ W; Suppl. Table 1).

Before starting the experiment, the plants were conditioned for six weeks in the open, watering them daily up to field capacity (FC) with irrigation water from the Bosque Santiago facility. Basal fertilisation was performed 23 days after transplanting with a nutrient solution based on 0.1% total N and 3% K at a dose of 1.5 L/ha. The same dose was repeated on day 37 after transplanting. The plants were pruned twice and cut at the height of 10 cm from the ground; the first pruning was performed on the same day as the first basal fertilisation and the second on the day starting the treatments.

The experimental design used was a randomised block plot design (RBLP) with four replicates per treatment. The main plot corresponded to the water status (with or without irrigation), and the sub-plot corresponded to the ammonium sulphate and acetylsalicylic acid treatments.

Plants were treated at the beginning of the experiment in 10 L plastic pots containing 19% clay, 26% lime and 55% sand andisol soil (pH = 7,2, EC = 0,47 dS/m, C_{ox} = 1,5 %) with 0.5 mM ASA (0.09 g/plant) diluted in 30 mL of distilled water and 2 g per pot AMS in irrigated and non-irrigated groups. In the treatment with irrigation, plants were watered every two days until reaching the pot weight corresponding to FC. In the water stress treatment, the stress period consisted of withholding irrigation for 14 days, when ca. 90% of the non-irrigated plants showed symptoms of severe general chlorosis. Each plant of *Stipa caudata* in a pot represented one experimental unit (n = 32). Table 1 indicates the experimental design consisting of four randomised blocks, each with two variants: with and without irrigation.

Table 1. The randomised blocks used for the growth of *Stipa caudata* in plots andisol soil under irrigation (Irrigated) and water stress (WS)

Main Plot 1		Main Plot 2		Main Plot 3		Main Plot 4	
Irrigated	WS	Irrigated	WS	Irrigated	WS	Irrigated	WS
T1	T3	T4	T2	T3	T3	T1	T2
T4	T4	T1	T1	T2	T1	T4	T4
T2	T2	T2	T4	T1	T2	T2	T1
T3	T1	T3	T3	T4	T4	T3	T3

T1 = ASA + AMS; T2 = ASA; T3 = AMS; T4 = Control.

Evaluation of plant morphological and biochemical traits

The length of five vegetative shoots per plant was determined using a ruler. The number of spikes was also registered every week. At the end of the experiment, plants were harvested, part of the aerial biomass was frozen, and the rest was dried in an oven at 65 °C to a constant weight.

Total chlorophyll content was measured according to a previously described method (Walinga *et al.*, 1989). Stem material (0.1 g fresh weight) was crushed in a mortar in the presence of liquid N_2. For extraction, 1 ml of 80% cold acetone was used. Samples were shaken at 4 °C overnight and then centrifuged at 12,000 rpm for 10 min. The supernatant was diluted 10-fold in 80% acetone. A spectrophotometer (VWR UV-1600PC) was used for the measurements.

Ion (Na^+, Cl^-, K^+ and Ca^{2+}) contents were determined according to Weimberg (1987), in leaf water extracts obtained by heating ground, dry plant material (0.15 g in 10 mL of water) in a water bath for 1 h at 95 °C. Cations were analysed with a flame photometer (Corning 410 C), and Cl^- was measured with a chloride analyser (Corning 926).

Proline (Pro) content was determined at the end of the experimental period in 0.2 g fresh plant material by the ninhydrin-acetic acid method described by Bates (1973). Briefly, Pro was extracted in 3% aqueous sulphosalicylic acid, and the extract was mixed with acid ninhydrin solution, incubated for 1 h at 95 °C, cooled on ice and then extracted with two volumes of toluene. The absorbance of the organic phase was measured at 520 nm, using toluene as a blank. Pro concentration was expressed as mmol g^{-1} DW.

The MDA content was analysed in 0.1 g of fresh leaf material, ground to a fine powder in a mortar and extracted with 1.5 ml of 80% methanol. The samples were shaken gently overnight at 7 °C. The supernatant was collected by centrifugation at 13,300 *g* for 10 min at 4 °C and stored at -20 °C until used in the assays. MDA in the extracts was quantified by the trichloroacetic/thiobarbituric acid method as described previously (Hodges *et al.*, 1999).

Total phenolic compounds (TPC) and total flavonoids (TF) were measured in the same methanol extracts used for MDA determination. TPCs were quantified by measuring absorbance at 765 nm after reaction with the Folin-Ciocalteu reagent (Blainski *et al.*, 2013), and expressed as gallic acid equivalents (mg eq GA / g DW), used to obtain the standard curve. TF were measured following the procedure described by Zhisen *et al.* (1999), based on the nitration of aromatic rings bearing a catechol group and their reaction with $AlCl_3$ under alkaline conditions. After the reaction, the absorbance of the sample was measured at 510 nm, and flavonoids contents were expressed as catechin equivalents (mg eq. Catec. g^{-1} DW).

Activity of the antioxidant enzymes

Crude protein extracts were prepared from the leaf material, stored frozen at -75 °C, as described by Gil *et al.* (2014). The protein concentration in the extracts was determined according to Bradford (1976) using the Bio-Rad reagent and bovine serum albumin (BSA) as the standard.

Superoxide dismutase (SOD) activity was determined according to a published protocol (Beyer and Fridovich, 1987) by spectrophotometric monitoring of nitroblue tetrazolium (NBT) photoreduction inhibition. The reaction mixtures (1 ml) contained 50 mM potassium phosphate buffer, pH 7.8, 9.9 mM L-methionine, 58 mM NBT, 0.025% (v/v) Triton X-100, 2.4 mM riboflavin (as a source of superoxide radicals) and the protein extract. After adding riboflavin, the reaction mixtures were irradiated (300 mmol m^{-2} s^{-1}, provided by three compact fluorescent lamps (Osram DULUX PRO 23 W) for 10 min at 25 °C, and the absorbance was measured at 560 nm using a non-irradiated solution mixture as blank. One SOD unit was defined as the amount of enzyme causing 50% inhibition of NBT photoreduction under the assay conditions.

Catalase (CAT) activity was determined following the decrease in absorbance at 240 nm that accompanied H_2O_2 consumption after adding protein extracts to a solution of 10 mM H_2O_2 in 50 mM Tris-HCl (pH 7.0). A CAT unit was defined as the amount of enzyme that decompose one mmol of H_2O_2 per minute at 25 °C (Aebi, 1984).

Ascorbate peroxidase (APX) activity was determined by measuring the decrease in absorbance at 290 nm as ascorbate is oxidised in the reaction (Nakano and Asada, 1981). One APX unit was defined as the amount of enzyme required to consume one mmol of ascorbate per minute at 25 °C.

Glutathione reductase (GR) activity was determined according to Connell and Mullet (1986) following the oxidation of NADPH, the cofactor in the GR-catalysed reduction of oxidised glutathione (GSSG). In a final volume of 1 ml, the reaction mixtures contained 100 mM Hepes, pH 7.5, 1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM GSSG and the protein extracts. The reactions were initiated by adding NADPH to a final concentration of 0.2 mM. Samples were incubated at 25 °C, and the decrease in absorbance at 340 nm was measured after 25 min. Control reactions without protein extract were incubated in parallel to correct for non-enzymatic oxidation of NADPH. One GR unit was defined as the amount of enzyme that will oxidise one mmol of NADPH per minute at 25°C.

Statistical analysis

The experiments results were analysed using linear and mixed models to assess whether there were significant differences between treatments, and a Fisher's test for comparison of means, with 95% confidence, to determine differences between treatments. The temporal correlation for the variables shoots growth, the number of spikes, pH and EC were considered in the model when running the analyses. The statistical analysis was conducted using InfoStat, version 2019 (University of Cordoba, Argentina).

Results

Vegetative variables

Lengths of stems were checked every week during the stress treatment. Figure 1 shows the interaction between Treatments AMS/ASA (A) x Water condition (B) for the stem length increment at the end of the experiment. Plants treated with the irrigated AMS and ASA mixture showed the greatest increase in length. In turn, plants with AMS, ASA or the mixture of both, presented the highest levels of stem length regardless of the hydric condition in which they were found. Finally, control plants without treatments and without irrigation showed the lowest values for stem length increase.
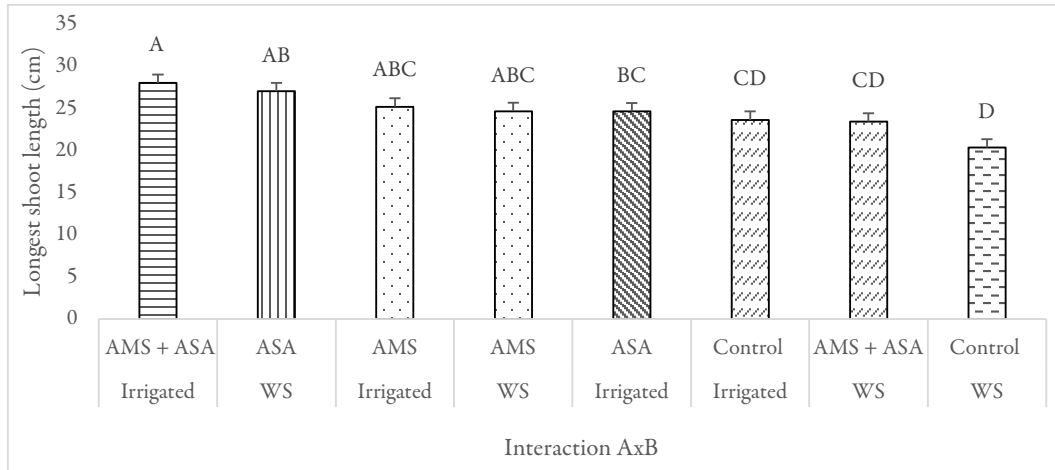

Figure 1. Interaction between Treatments (A) x Water condition (B) for the mean increase in length of *Stipa caudata*
Different letters indicate significant differences according to Fisher's test (p< 0.05). Vertical bars indicate standard error.

Figure 2 shows the number of spikelets measured at the end of the experiment. Control irrigated plants treated with AMS + ASA showed an increase in the number of spikelets per plant compared to other plants. At the same time, under water restriction conditions, plants treated with AMS showed the highest number of spikelets per plant. However, when statistical analyses were run taking into account all irrigated and water-stressed plants, only the effect of treatment was significant, but not that of the water regime. Of all treatments, the smallest number of inflorescences was registered in plants treated with ASA, significantly different from that in control plants (Figure 2).

Chlorophyll content

Total chlorophyll content measured in shoots of *S. caudata* in the four treatments is indicated in Figure 3. As for the previous trait analysed, the absence of irrigation did not significantly decrease the content of total chlorophyll, but the effect of the treatment was significant. Plants grown on soils fertilised with the mixture of AMS and ASA had significantly higher values of total chlorophyll concentrations than control plants or those from the treatments with AMS and ASA separately.
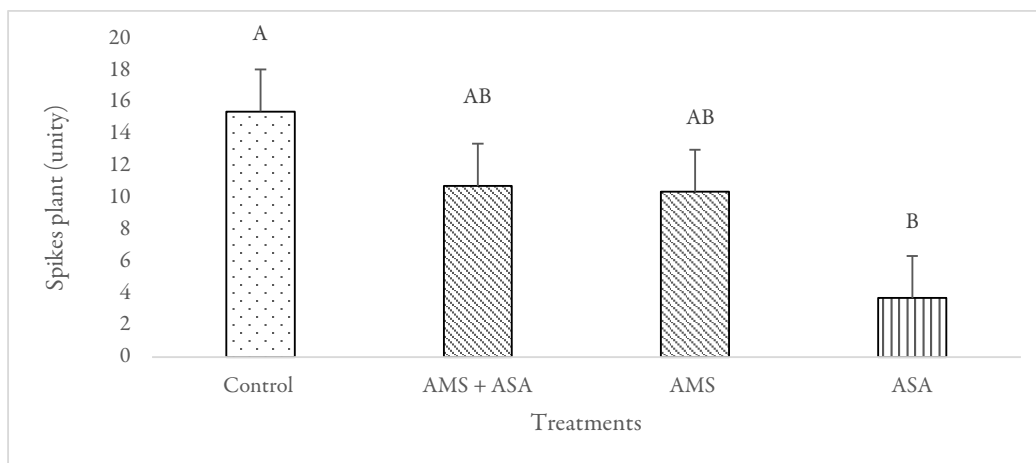
Figure 2. Mean spikelet number per plant in *Stipa caudata* at the end of the experiment
Different letters indicate significant differences according to Fisher's test (p<0.05). Vertical bars indicate standard error.
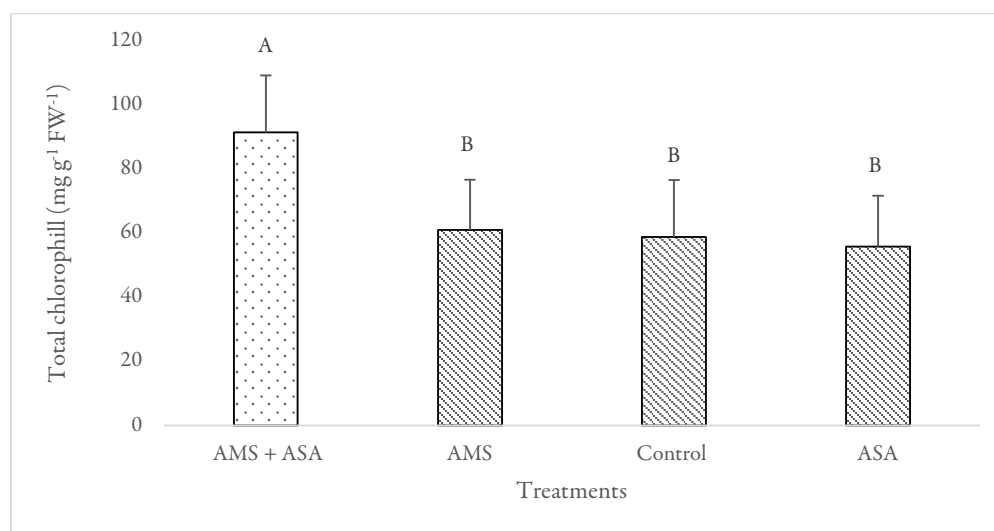

Figure 3. Total chlorophyll content in shoots of *Stipa caudata* at the end of the experiment
Different letters indicate significant differences according to Fisher's test (p<0.05). Vertical bars indicate standard error.

Ion contents of shoots

Monovalent and divalent cations (Na^+, K^+, and Ca^{2+}) and the monovalent anion Cl^- were quantified in the shoots of plants at the end of the experiment. Generally, the effect of treatments and water conditions were not significant.

The only significant interaction between the type of treatment (A) and the water condition (B) was found for the shoots Ca^{2+} content, as shown in Figure 4.

Table 2. Concentrations of mono- and divalent ions in shoots of *Stipa caudata* at the end of the experiment

Water condition (A)	Treatments (B)	Na$^+$ (μmol g^{-1} DW)	Cl$^-$ (μmol g^{-1} DW)	K$^+$ (μmol g^{-1} DW)	Ca$^+$ (μmol g^{-1} DW)
Irrigates	AMS + ASA	197.19±38,61	85.12±25,75	183.59±49,94	4.44±2,75 b
Irrigates	AMS + Control	121.02±38,61	87.00±25,75	187.43±49,94	3.90±2,75 b
Irrigates	ASA	130.12±21,72	115.47±25,75	171.07±49,94	14.30±2,75 a
Irrigates	Control	199.29±38,61	155.69±25,75	282.49±67,04	7.09±2,75 ab
WS	AMS + ASA	115.46±38,61	84.81±25,75	225.43±49,94	3.73±2,75 b
WS	AMS	175.26±38,61	122.16±25,75	221.58±49,94	9.62±2,75 ab
WS	ASA	98.63±21,72	84.81±25,75	150.30±49,94	3.46±2,75 b
WS	Control	129.70±38,61	104.78±25,75	223.92±67,04	3.79±2,75 b
Statistics					
Treatments A		N.S	N.S	N.S	N.S
Water Condition B		N.S	N.S	N.S	N.S
AxB		N.S	N.S	N.S	*

Statistical significance of the effect of treatment (A) and water condition (B) and their interaction (A x B) is indicated.

* Indicates significant differences according to Fisher's test ($p < 0.05$). N.S indicated no significative differences.
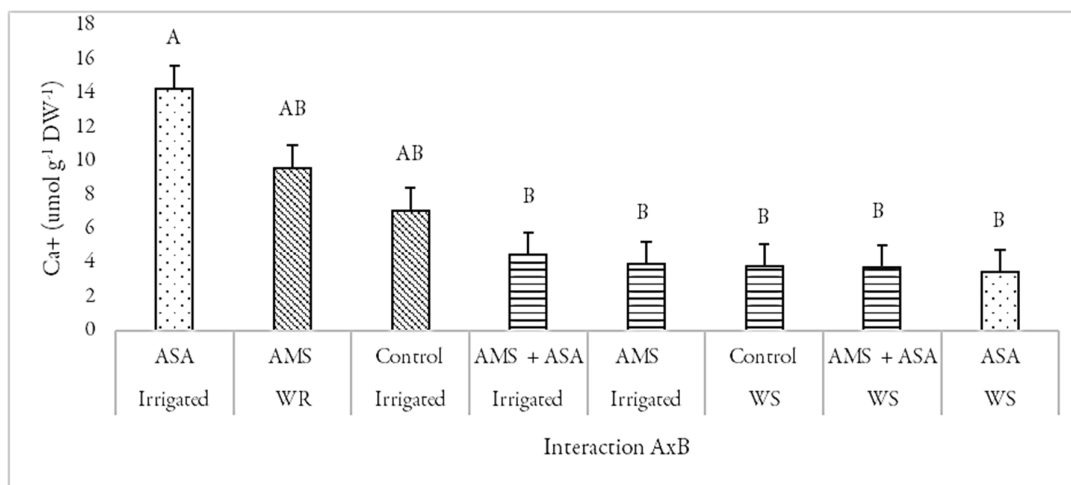

Figure 4. Interaction between Treatment (A) x Water condition (B) for Ca^{2+} content in shoots of *Stipa caudata* at the end of the experiment

Different letters indicate significant differences according to Fisher's test ($p = 0.05$). Vertical bars indicate standard error.

Biochemical variables

Several biochemical characteristics, which generally are considered as suitable markers of stress, were measured in shoots of all plants at the end of the treatments and their concentrations are indicated in Table 3.

Variation of the concentration of proline was dependent only on the water regime, but not on the treatments applied. Water stress induced a significant increase in proline concentrations in plants under all treatments (Figure 5a), but interaction between Treatment (A) x Water Stress (B) had no statistical significance. Malondialdehyde (MDA) increased in all watered-stressed plants (Table 3 and Figure 5b) but as for proline neither treatment or interactions between treatments and water conditions were significant.

7

Table 3. Concentrations of proline (Pro), malondialdehyde (MDA), total phenolic compounds (TFC) and total flavonoids (TF) in shoots of *Stipa caudata* at the end of the experiment.

Water condition (A)	Treatments (B)	TFC (mg eq. GA g^{-1} FW)	TF (mg eq.catequin g^{-1} FW)	Pro (mg g^{-1} FW)	MDA (nmol g^{-1} FW)
Irrigated	AMS + ASA	11.87±3,24 c	4.73±1,03	1.66±0,23	28.13±2,92
Irrigated	AMS	13.86±3,24 bc	5.10±1,03	0.58±0,25	13.89±2,54
Irrigated	ASA	26.05±3,70 a	8.45±1,19	0.10±0,05	48.02±2,48
Irrigated	Control	17.65±3,24 abc	5.80±1,03	0.21±0,03	47.05±45,54
WS	AMS + ASA	21.20±3,24 ab	7.67±1,03	5.70±0,02	109.05±56,48
WS	AMS	25.01±3,24 a	7.46±1,03	2.65±0,02	95.42±45,54
WS	ASA	19.73±3,24 abc	8.27±1,03	1.79±0,53	120.53±48,92
WS	Control	21.23±3,24 ab	7.62±1,03	5.03±0,20	64.99±45,54
Statistics					
Treatments A		N.S	N.S	N.S	N.S
Water Condition B		*	*	*	*
AxB		*	N.S	N.S	N.S

Statistical significance of the effect of treatment (A) and water condition (B) and their interaction (A x B) is indicated.
* Indicates significant differences according to Fisher's test ($p < 0.05$). N.S indicated no significative differences.
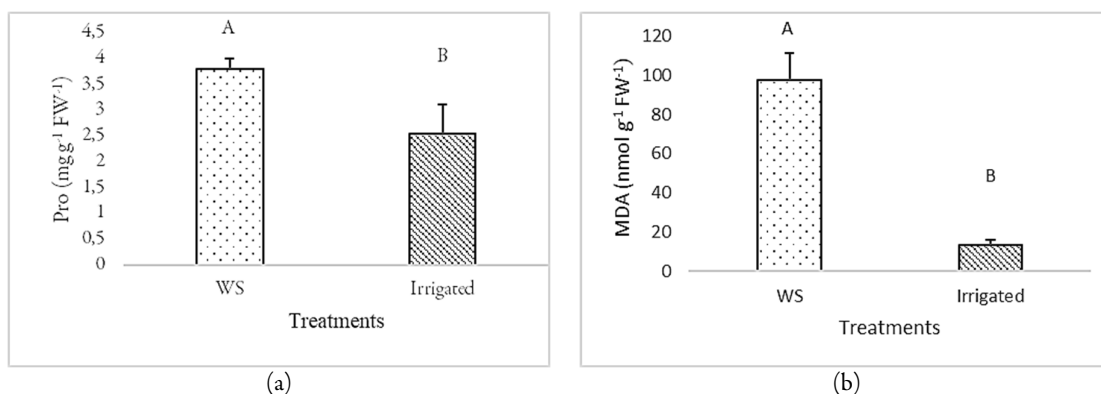

(a)   (b)

Figure 5. (a) and (b) show the effect of hydric condition on proline concentration (Pro) and malondialdehyde (MDA) in shoots of *Stipa caudata* at the end of the experiment
Different letters indicate significant differences according to Fisher's test ($p = 0.05$). Vertical bars indicate standard error.

Regarding the non-enzymatic antioxidants, the pattern of variation according to the water regime was similar for total phenolics (TFC) and total flavonoids (TF), with an increase in water-stressed plants from all treatments. In the case of TFC, there were detected also significant differences according to the different treatments and the interaction between treatments and water regime. Figure 6 shows the interaction of Treatments (A) x Water condition (B) for TFC content in shoots measured at the end of the experiment. The total of plants treated with ASA, AMS or the mixture of both in the water restricted condition, together with plants treated with ASA with irrigation, presented higher levels of TFC than plants treated with the ASA-AMS mixture with irrigation.
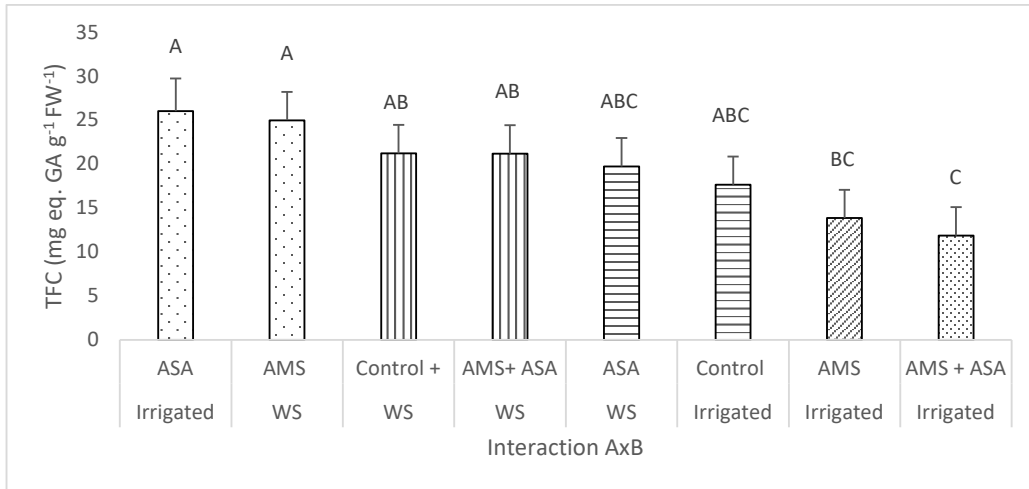
Figure 6. Interaction of treatments (A) x water condition (B) for total phenolic compounds (TFC) content in shoots of *Stipa caudata* at the end of the experiment
Different letters indicate significant differences according to Fisher's test (p= 0.05). Vertical bars indicate standard error.

Figure 7 shows Total Flavonoids (TF) in *Stipa caudata* shoots analysed at the end of the experiment. There were significant differences with respect to the water condition (B), where plants under water stress condition (WS) presented higher levels of TF than irrigated plants.
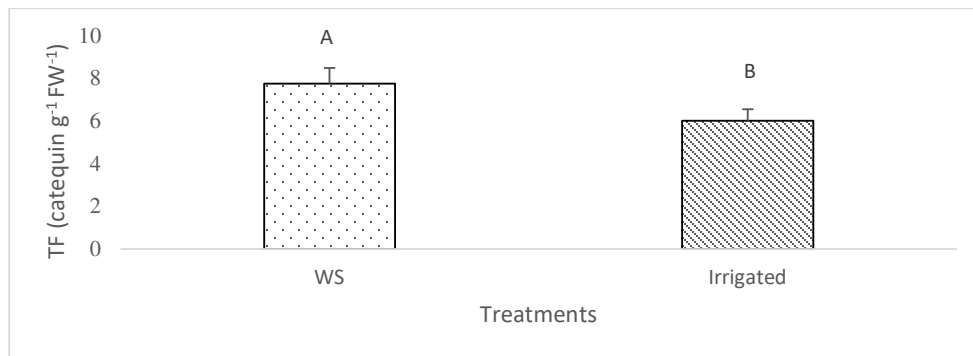

Figure 7. Interaction of treatments (A) x water condition (B) for total flavonoids (TFC) in shoots of *Stipa caudata* at the end of the experiment
Different letters indicate significant differences according to Fisher's test (p= 0.05). Vertical bars indicate standard error.

Antioxidant enzyme activity
Table 4 summarises the activity of Catalase (CAT), Ascorbate Peroxidase (APX), Superoxide Dismutase (SOD) and Glutathione Reductase (GR) in shoots of *Stipa caudata* measured at the end of the experiment. There was an interaction between treatments (A) x water condition (B) for CAT and SOD activity, together with significant differences for water condition (B) with respect to APX activity.

Table 4. The activity of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) in shoots of *Stipa caudata* at the end of the experiment

Water Condition (A)	Treatments (B)	CAT (U mg^{-1} protein)	APX (U mg^{-1} protein)	SOD (U mg^{-1} protein)	GR (U mg^{-1} protein)	MDA (nmol g^{-1} FW^{-1})
Irrigated	AMS + ASA	17628.67±1641,17 ab	186.04±99,09	251.90±221,99 ab	4246.75±2042,51	28.13±48,92
Irrigated	AMS	13847.5±824,35 ab	130.03±81,55	785.35±85,52 a	3370.65±1403,38	13.89±45,54
Irrigated	ASA	10633.0±1165,8 c	365.81±98,17	32.50±120,95 b	9670.38±1939,16	48.02±56,48
Irrigated	Control	20029.5±2010,01 a	45.77±15,18	881.27±221,99 a	5555.92±1829,99	47.05±45,54
WS	AMS+ ASA	15505.0±824,35 bc	35.00±15,55	654.75±271,88 ab	5684.81±2864,33	109.05±56,48
WS	AMS	16622.25±582,90 ab	428.62±99,09	728.33±69,83 a	5165.0±1027	95.42±45,54
WS	ASA	16946.00±824,35 ab	558.96±81,55	767.30±120,95 a	5665.19±1404	120.53±48,92
WS	Control	16193.0±1641,17 ab	777.63,63±75,18	848.70±221,99 a	6018.36±1826,35	64.99±45,54
Statistics						
Treatments A		N.S	N.S	*	N.S	N.S
Water Condition B		*	*	N.S	N.S	*
AxB		*	N.S	*	N.S	N.S

Statistical significance of the effect of treatment (A) and water condition (B) and their interaction (A x B) is indicated.

As can be seen in Figure 8, there is an interaction between the hormone/mineral x water condition treatments. There were no significant differences in plants with the hormone/mineral mixture irrigated and not irrigated with respect to the rest of the treatments. There were also no significant differences between control treatments and the rest, regardless of the water condition of the plants. Irrigated plants from the treatments with ASA showed the lowest SOD activity levels, significantly different of all other values calculated, whereas the highest were determined in irrigated and water stressed plants from control treatments.
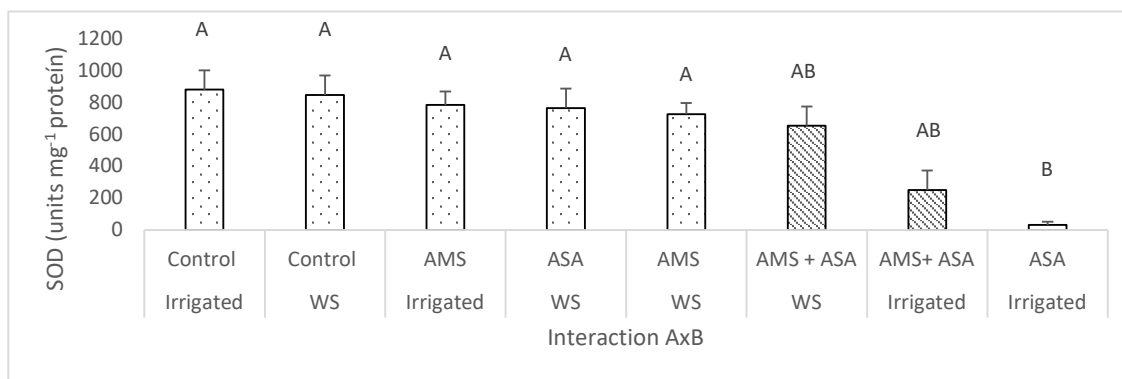

Figure 8. Interaction of treatments (A) x water condition (B) for the activity of superoxide dismutase (SOD) activity in shoots measured at the end of the experiment (TFC)
Different letters indicate significant differences according to Fisher's test (p= 0.05). Vertical bars indicate standard error.

ASA irrigated plants showed also the lowest levels of CAT activity compared to the other treatments. On the other hand, irrigated plants from control had the highest CAT activity (Figure 9).

Figure 10 shows the APX activity measured in *Stipa caudata* shoots at the end of the experiment. Significant differences were found with respect to the water condition (B), where water-restricted plants (WS) showed higher levels of APX than irrigated plants but differences between treatments or interactions water regime x treatments were both not significant.
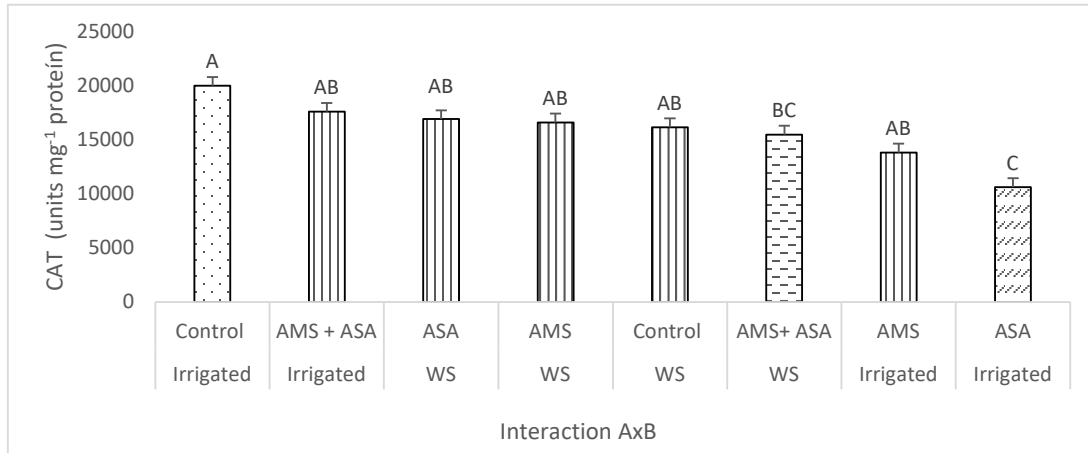

Figure 9. Interaction of treatments (A) x water condition (B) for the activity of Catalase (CAT) activity in shoots measured at the end of the experiment (TFC)
Different letters indicate significant differences according to Fisher's test (p= 0.05). Vertical bars indicate standard error.
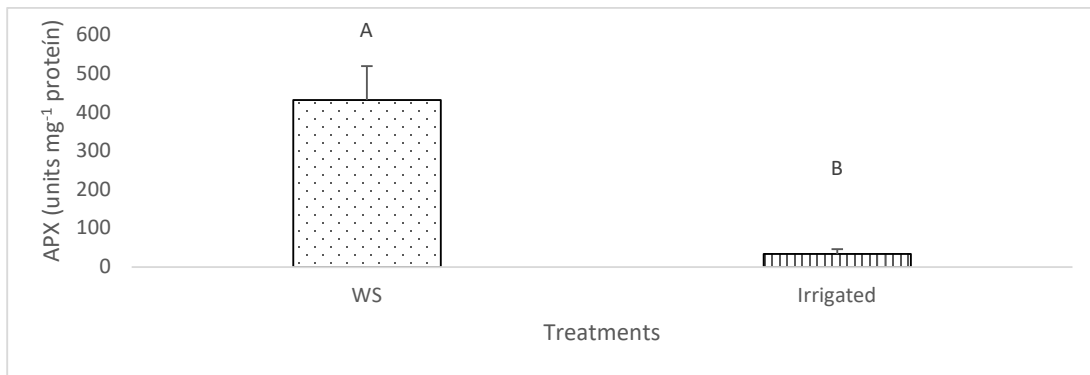

Figure 10. Interaction of treatments (A) x water condition (B) for the activity of ascorbate peroxidase (APX) activity in shoots measured at the end of the experiment (TFC)
Different letters indicate significant differences according to Fisher's test (p= 0.05). Vertical bars indicate standard

Discussion

Knowledge of the mechanisms involved in the response of plants to water restrictions is essential to optimise water use in scenarios of climatic uncertainty. The use of phytohormones and mineral elements to enhance plant resistance to water stress could increase or at least reduce biomass loss under limiting conditions. This research presents an unprecedented set of results in *Stipa caudata* treated with acetylsalicylic acid and ammonium sulphate under different water conditions.

11

Productive and physiological variables

The above results show interaction AxB in the studied factors. It was observed that plants from AMS and ASA under water restriction regime produced similar stem height as the irrigated ones. In turn, AMS plants showed the highest chlorophyll contents.

Positive results on height and biomass of water stressed plants treated with ASA or AMS have been reported in tomato (Rodriguez et al., 2020), bean (Seranatna *et al.*, 2000), wheat (Kareem *et al.*, 2017), chickpea (Hussain *et al.*, 2020) and *Eragrostis plana* (Bastiani *et al.*, 2021). However, several studies (Kabiri and Naghizadeh, 2015) found no effect in height of barley. On the other hand, water-restricted plants had higher MDA contents than irrigated plants. In turn, plants with AMS and ASA showed higher total chlorophyll content than the rest of the treatments. Similar observations have been reported with AMS or ASA treatments in maize (Gunes *et al.*, 2007), *Allium sativum* (Zeinali and Moradi, 2015), mustard (Giansoldati *et al.*, 2012) and chickpea (Hussain *et al.*, 2020).

The above results suggest that applications of ASA or AMS could increase some growth parameters in water-stressed plants, as they may have an effect on stomatal closure and maintenance of water flow related to growth (Vargas-Ortiz *et al.*, 2021). It is likely that low-dose applications of these supplements at the onset of a water deficit could also have a synergistic effect on stress defence systems. In this sense, Jopia *et al.* (2020) showed that water deficit could decrease growth by reducing the water potential. Stomatal closure and reduced water flow lead to physiological and biochemical changes also through the production of reactive oxygen species (ROS). Elevated ROS concentrations increase lipid peroxidation, programmed cell death and decrease photosynthetic functions due to chlorophyll degradation (Okuma *et al.*, 2011). Similar findings have been described in nutritionally stressed plants (Molina and Covarrubias, 2019). However, the results indicated that AMS and ASA alleviated the effects of water reduction on vegetative growth and chlorophyll production. Recent reports have shown synergistic relationships between AMS and nitrogen-stimulated nitrate reductase activity, which is involved in the synthesis of nitric oxide precursor of ammonium sulphate, thus alleviating water stress symptoms by enhancing defence systems and water potential (Kaya, 2021).

Ion accumulation

Of all ions analysed, statistical analysis revealed a significant AxB interaction only for Ca^{2+} content. However, it was observed that when AMS was present water-restricted and irrigate plants showed similar Ca^{2+} accumulation in shoots.

The results suggest that ammonium sulphate applications could increase Ca^{2+} accumulation under water deficit. The uptake and transport of dissolved Ca^{2+} in the soil solution occurs by transpiration following differences in water potentials with respect to the atmosphere. Ca^{2+} is an essential mineral element that acts as a cellular second messenger signaling physiological and transcriptional responses to stress conditions (Kudlak *et al.*, 2010). It also acts by enhancing stomatal regulation and activating a number of enzymes. Recent studies have shown that nitrogen is an element that promotes stress resistance by acting synergistically with Ca^{2+} accumulation (Zhang *et al.*, 2018).

Osmoprotectants and non-enzymatic antioxidants

Although generally treated plants had higher concentration of Pro and TF, only the effect of water regime was statistically significant. At the same time, a significant between the water regime and treatment was detected for TCF. However, it was observed that plants with treatments and water restriction presented similar values in terms of TCF increases with respect to irrigated plants.

Positive results on osmoprotectant accumulation have been widely described in stressed plants of the genus *Stipa* (Yang *et al.*, 2021). In the same direction, other results showed that ASA or ammonium sulphate increase proline concentration, flavonoids and phenolic compounds in Poaceae, such as *Festuca arundinacea* (Neuberg *et al.* 2010) or in dicots such as *Simarouba glauca* (Awate *et al.*, 2014) and chickpea (Hussain *et al.*, 2020).

The above results suggest that applications of ASA or ammonium sulphate to plants under water deficit could stimulate the synthesis of osmolytes and antioxidant compounds in their cells, such as proline, flavonoids and phenolic compounds. These compounds have a high capacity to degrade reactive oxygen species (ROS) produced under stress (Farooq *et al.*, 2009; Akinci and Lösel, 2012). Proline is a protein precursor with antioxidant activity that acts in detoxification and protection through cellular homeostasis by providing a proper redox balance. Proline also induces a number of genes responsible for the activation of antioxidant enzymes, improving the water potential in plants (Neuberg *et al.*, 2010; Nazar *et al.*, 2015). On the other hand, phenols and flavonoids can chelate transition metal ions, directly scavenge active molecular oxygen species and quench lipid peroxidation by trapping the alkoxide radical. In addition, flavonoids and phenylpropanoids are oxidised by peroxidase and act in the H_2O_2 uptake system, phenolic / AsA / POD (Kaya, 2021). Other results on different species and different experimental conditions indicate that ASA or ammonium sulphate enhance the synthesis of proline, flavonoids and phenolic compounds against water stress (Nazar *et al.*, 2015; Kaya, 2021).

Antioxidant enzyme activity

Increases in ROS such as O_2, O_3 or H_2O_2 occur in plants under stressful conditions. These radicals produce lipid peroxidation in the cell and at high concentration may cause cell death. As a defence, organisms respond by increasing the activity of antioxidant enzymes such as SOD, CAT and APX, with SOD acting in a first line of action by catalysing the dismutation of O_2^- to H_2O_2 through its neutralisation by the addition of two hydrogen ions reducing the formation of OH^- via the Haber-Weiss reaction. On the other hand, CAT and APX catalyse the conversion of H_2O_2 to H_2O and O_2 alleviating the damage (Apel and Hirt, 2004; Das and Roychoudhury, 2014; Noctor *et al.*, 2018; Farooq *et al.*, 2020).

Of the four enzymes analysed, only higher APX activity was detected under water restriction. On the other hand, there was an AxB interaction for CAT and SOD activity. The pattern of variation in the antioxidant enzymes varies according to the species studied or the type of stress applied. In a species of the same genus, *S. lagascae*, increases in APX and GR but decreases in SOD activity were reported under saline conditions (Abdellaoui *et al.*, 2017), whereas the activity of the antioxidant enzymes varied in Juncus not only in dependence of the species, but also within species according to the applied water or salt stress treatments (Al Hassan *et al.*, 2017).

Regarding the effect of acetylsalicylic acid and AMS on antioxidant enzymes activity, there are no reports in the genus Stipa and only a few published data in monocotyledonous plants. Wheat showed higher activity of SOD, CAT and APX when treated with ASA (Hassanein *et al.*, 2015). However, no clear trends in enzymatic activity under abiotic stresses treated with ASA have been reported. In this respect, whereas a decrease in SOD, CAT was found in *Oryza sativa* under water stress (Guo *et al.*, 2007) in *Spartina alterniflora* were reported increases in the activity of CAT, SOD and APX with ammonium treatments (Hessini et al., 2013) and no clear trends in antioxidant enzyme activity in maize (Zhang *et al.*, 2007).

Our findings suggest that applications of ASA or AMS could enhance SOD and CAT activity in some species under stressful conditions. SOD and CAT are enzymes in charge of systematically degrading ROS and reducing their toxic level to lower and lower levels.

Conclusions

Our findings indicate that water stress had a stronger effect on the growth of plants grown in the absence of AMS and acetylsalicylic acid or their combination. Plants treated with these compounds, or their combination had increased concentrations of proline and total flavonoids, but treated plants under water restriction showed similar values in terms of total phenolics. The activity of APX was enhanced under water restriction conditions, and an AxB interaction for CAT and SOD activity was detected. However, plants

treated with acetylsalicylic acid and ammonium sulphate under water restriction showed similar reductions in the activity of both enzymes compared to irrigated/ASA.

The results obtained show that these additives can improve responses to water deficit in *Stipa caudata*, a species with great potential in environmental restoration or biofuel production programmes. However, several questions remain open about the relative role of ASA and AMS in other physiological and biochemical mechanisms such as the regulation of xylem water potential, photosynthetic activity and aerial dry matter production.

Authors' Contributions

Conceptualisation, OV and CS; methodology, JM and SGP, software, JM..; validation, JM, SGO and FZ; formal analysis, JM; investigation, JM, SGO; JL resources, CS; data curation, JM; writing—original draft preparation, JM; writing—review and editing, MB and OV; visualisation, JM; supervision, MB, JL and CS; project administration, CS; funding acquisition, JM and CS.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

Conflict of Interests

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

References

Aebi H (1984). Catalase *in vitro*. Methods in Enzymology 105:121-126. *https://doi.org/10.1016/S0076-6879(84)05016-3*

Akıncı Ş, and Lösel DM (2012). Plant water-stress response mechanisms. In: Rahman M, Hasegawa H (Eds). Water Stress InTech Press, Rijeka, Croatia, pp 15-42.

Agromet (2018). Chilean climate variables. Retrieved December 30 2021 from *https://www.agromet.cl*

Apel K, Hirt H (2004). Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annual Reviews in Plant Biology 55:373-399. *https://doi.org/10.1146/annurev.arplant.55.031903.141701*

Abdellaoui R, Boughalleb F, Chebil Z, Mahmoudi M, Belgacem AO (2017). Physiological, anatomical and antioxidant responses to salinity in the Mediterranean pastoral grass plant *Stipa lagascae*. Crop Pasture Science 68(9):872-884. *https://doi.org/10.1071/CP16365*

Al Hassan M, Estrelles E, Soriano P, López-Gresa MP, Bellés JM, Boscaiu M, Vicente O (2017). Unraveling salt tolerance mechanisms in halophytes: A comparative study on four Mediterranean *Limonium* species with different geographic distribution patterns. Frontiers in Plant Science 8. *https://doi.org/10.3389/fpls.2017.01438*

Awate PD, Gaikwad DK (2014). Influence of growth regulators on secondary metabolites of medicinally important oil yielding plant Simarouba glauca DC. Journal of Physiology and Biochemistry 10(1):222-229.

Bates LS, Waldren RP, Teare ID (1973). Rapid determination of free proline for water-stress studies. Plant and Soil 39(1):205-207. *https://doi.org/10.1007/BF00018060*

Bastiani MO, Roma-Burgos N, Langaro AC, Salas-Perez RA, Rouse CE, Fipke MV, Lamego FP (2021). Ammonium sulfate improves the efficacy of glyphosate on South African lovegrass (*Eragrostis plana*) under water stress. Weed Science 69:167-176. *https://doi.org/10.1017/wsc.2020.97*

Beyer WF, Fridovich I (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Annals of Biochemisry 161:559-566. *https://doi.org/10.1016/0003-2697(87)90489-1*

Blainski A, Lopes GC, De Mello JCP (2013). Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from *Limonium brasiliense* L. Molecules 18(6):6852-6865. *https://doi.org/10.3390/molecules18066852*

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Annal of Biochemistry 72(1-2):248-254. *https://doi.org/10.1016/0003-2697(76)90527-3*

Connell JP, Mullet JE (1986). Pea chloroplast glutathione reductase: purification and characterisation. Plant Physiology 82(2):351-356. *https://doi.org/10.1104/pp.82.2.351*

Das K, Roychoudhury A (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Frontiers in Plant Science 2:1-13. *https://doi.org/10.3389/fenvs.2014.00053*

Farooq M, Wahid A, Kobayashi NSMA, Fujita DB, Basra SMA (2009). Plant drought stress: effects, mechanisms and management. Journal of Sustainable Agricu;ture 153-188. *https://doi.org/10.1007/978-90-481-2666-8_12*

Farooq A, Bukhari SA, Akram NA, Ashraf M, Wijaya L, Alyemeni MN, Ahmad P (2020). Exogenously applied ascorbic acid-mediated changes in osmoprotection and oxidative defense system enhanced water stress tolerance in different cultivars of safflower (*Carthamus tinctorious* L.). Plants 9:104. *https://doi.org/10.3390/plants9010104*

Gil R, Bautista I, Boscaiu M, Lidón A, Wankhade S, Sánchez H, ... Vicente O (2014). Responses of five Mediterranean halophytes to seasonal changes in environmental conditions. AoB Plants 6.

Giansoldati V, Tassi E, Morelli E, Gabellieri E, Pedron F, Barbafieri M (2012). Chemosphere nitrogen fertiliser improves boron phytoextraction by *Brassica juncea* grown in contaminated sediments and alleviates plant stress. Chemosphere 87:1119-1125. *https://doi.org/10.1016/j.chemosphere.2012.02.005*

Gunes A, Inal A, Alpaslan M, Eraslan F, Bagci EG, Cicek N (2007). Salicylic acid induced changes on some physiological parameters symptomatic for oxidative stress and mineral nutrition in maize (*Zea mays* L.) grown under salinity. Journal of Plant Physiology 164:728-736. *https://doi.org/10.1016/j.jplph.2005.12.009*

Guo B, Liang YC, Zhu Y, G, Zhao FJ (2007). Role of salicylic acid in alleviating oxidative damage in rice roots (*Oryza sativa*) subjected to cadmium stress. Environmental Pollution 147(3):743-749. *https://doi.org/10.1016/j.envpol.2006.09.007*

Hassanein RA, Amin AAE, Rashad ESM, Ali H (2015). Effect of thiourea and salicylic acid on antioxidant defense of wheat plants under drought stress. International Journal of ChemTech Research 7(01):346-354.

Hessini K, Hamed KB, Gandour M, Mejri M, Abdelly C, Cruz C (2013). Ammonium nutrition in the halophyte Spartina alterniflora under salt stress: evidence for a priming effect of ammonium?. Plant Soil 370(1):163-173. *https://doi.org/10.1007/s11104-013-1616-1*

Hodges DM, Delong JM, Forney CF, Prange RK (1999). Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207:604-611. *https://doi.org/10.1007/s004250050524*

Hussain I, Rasheed R, Ashraf MA, Mohsin M, Shah SMA, Rashid DA, Akram M, Nisar J, Riaz M (2020). Foliar applied acetylsalicylic acid induced growth and key biochemical changes in chickpea (*Cicer arietinum* L.) under drought stress. Dose-Response 18:1-13. *https://doi.org/10.1177/1559325820956801*

IPCC (2018). Global warming of 1.5 C. An IPCC Special Report on the impacts of global warming of 1.5 C°. 1, 5. Retrieved 2021 December 12 from *https://www.ipcc.ch/site/assets/uploads/sites/2/2019/05/SR15_Citation.pdf*

Ismail Mofizur and Hiroshi Hasegawa (2012). Water Stress. InTech, Rijeka Croatia.

Jopia A, Zambrano F, Pérez-Martínez W, Vidal-Páez P, Molina J, Mardones F. de la H (2020). Time-series of vegetation indices (VNIR/SWIR) derived from sentinel-2 (A/B) to assess turgor pressure in kiwifruit. ISPRS International Journal of Geo-Information 9:1-18. *https://doi.org/10.3390/ijgi9110641*

Kaya C (2021). Nitrate reductase is required for salicylic acid-induced water stress tolerance of pepper by upraising the AsA-GSH pathway and glyoxalase system. Physiologia Plantarum 172:351-370. *https://doi.org/10.1111/ppl.13153*

Kareem F, Rihan H, Fuller M (2017). The effect of exogenous applications of salicylic acid and molybdenum on the tolerance of drought in wheat. 2017. Agricultural Research & Technology: Open Access Journal 9. *https://10.19080/ARTOAJ.2017.09.555768*

Kabiri R, Naghizadeh M (2015). Exogenous acetylsalicylic acid stimulates physiological changes to improve growth, yield and yield components of barley under water stress condition. Journal of Plant Physiology and Breeding 5(1):35-45.

Kudlak J, Batistic O, Hashimoto K (2010). Calcium signals: the lead currency of plant information processing. Plant Cell 22:541-563. *https://doi.org/10.1105/tpc.109.072686*

Masson-Delmotte V, Zhai, P, Pörtner H, Roberts D, Skea, J, Shukla PR, Waterfield T (2018). global warming of 1.5 C. An IPCC Special Report on the impacts of global warming of 1.5 C°. 1, 5. Retrieved 2021 December from *https://www.ipcc.ch/site/assets/uploads/sites/2/2019/09/IPCC-Special-Report-1.5-SPM_es.pdf*

Magdy M, Mansour F, Farouk E (2017) Evaluation of proline functions in saline conditions. Phytochemistry Review 140. *https://doi.org/10.1016/j.phytochem.2017.04.016*

Molina J, Covarrubias JI (2019). Influence of nitrogen on physiological responses to bicarbonate in a grapevine rootstock. Journal of Soil Science and Plant Nutrition 19:305-312. *https://doi.org/10.1007/s42729-019-00030-1*

Nazar R, Umar S, Khan NA, Sareer OS (2015). Salicylic acid supplementation improves photosynthesis and growth in mustard through changes in proline accumulation and ethylene formation under drought stress. South African Journal of Botany 98:84-94. *https://doi.org/10.1016/j.sajb.2015.02.005*

Nakano Y, Asada K (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiology 22(5):867-880. *https://doi.org/10.1093/oxfordjournals.pcp.a076232*

Neuberg M, Pavlíková D, Pavlík M, Balík J (2010). The effect of different nitrogen nutrition on proline and asparagine content in plant. Plant Soil Environment 56:305-311. *https://doi.org/10.17221/47/2010-PSE*

Noctor G, Reichheld J, Foyer CH (2018). ROS-related redox regulation and signaling in plants. In: Seminars in Cell & Developmental Biology 80:3-12. Academic Press. *https://doi.org/10.1016/j.semcdb.2017.07.013*

Okuma E, Hossain MA, Munemasa S, Uraji M, Nakamura Y, Mori IC, Murata Y (2011). Involvement of extracellular oxidative burst in salicylic acid-induced stomatal closure in *Arabidopsis*. Plant, Cell & Environment 434-443. *https://doi.org/10.1111/j.1365-3040.2010.02253.x*

Osakabe, Y, Osakabe K, Shinozaki K, Tran LS (2014). Response of plants to water stress. Frontiers in Plant Science 5:86. *https://doi.org/10.3389/fpls.2014.00086*

Rodrigues L, Emilaine A, Prado R, Oliveira R, De Ferreira, E (2020). Mechanisms of cadmium-stress avoidance by selenium in tomato plants. Ecotoxicology 594-606. *https://doi.org/10.1007/s10646-020-02208-1*

Senaratna T, Touchell D, Bunn E, Dixon K (2000). Acetyl salicylic acid (aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. Plant Growth Regulation 30:157-161. *https://doi.org/10.1023/A:1006386800974*

Sun Y, Wang C, Chen HYH, Ruan H (2020). Response of plants to water stress: A meta-analysis. Frontiers in Plant Science 11:1-8. *https://doi.org/10.3389/fpls.2020.00978*

Vargas-Ortiz E, Ramírez-Tobias HM, González-Escobar JL, Gutiérrez-García AK, Bojórquez-Velázquez E, Espitia-Rangel E, Barba de la Rosa AP (2021). Biomass, chlorophyll fluorescence, and osmoregulation traits let differentiation of wild and cultivated Amaranthus under water stress. Journal of Photochemistry and Photobiology B: Biology 220. *https://doi.org/10.1016/j.jphotobiol.2021.112210*

Walinga I, Van Vark W, Houba VJG, Van der Lee JJ (1989). Soil and plant analysis. Plant. Part 7.

Weimberg R (1987). Solute adjustments in leaves of two species of wheat at two different stages of growth in response to salinity. Physiologia Plantarum 70:381-388. *https://doi.org/10.1111/j.1399-3054.1987.tb02832.x*

Yang D, Ni R, Yang S, Pu Y, Qian M, Yang, Y (2021). Functional characterisation of the Stipa purpurea P5CS gene under drought stress conditions. International Journal of Molecular Sciences 22(17):9599. *https://doi.org/10.3390/ijms22179599*

Zeinali A, Moradi P (2015). The effects of humic acid and ammonium sulfate foliar spraying and their interaction effects on the qualitative and quantitative yield of native garlic (*Allium sativum* L). Journal of Applied Environmental and Biological Sciences 4:205-211.

Zhang L, Li S, Zhang H, Liang Z (2007). Nitrogen rates and water stress effects on production, lipid peroxidation and antioxidative enzyme activities in two maize (*Zea mays* L.) genotypes. Journal of Agronomy and Crop Science 193(6):387-397. *https://doi.org/10.1111/j.1439-037X.2007.00276.x*

Zhang T, Yang J, Sun Y, Kang Y, Yang J, Qi Z (2018). Calcium deprivation enhances non-selective fluid-phase endocytosis and modifies membrane lipid profiles in Arabidopsis roots. Journal of Plant Physiology 226:22-30. *https://doi.org/10.1016/j.jplph.2018.04.002*

Zhishen J, Mengcheng T, Jianming W (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chemistry 64:555-559.