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ARTÍCULO CIENTÍFICO

Extract of *Heterotheca inuloides* Cass. as a biostimulant of the growth and quality of *Rubus idaeus* L. under salt stress

Extracto de *Heterotheca inuloides* Cass. como bioestimulante del crecimiento y calidad de *Rubus idaeus* L. bajo de estrés salino

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ABSTRACT

Raspberry (*Rubus idaeus* L.) is susceptible to various environmental stressors, which reduces fruit yield and quality. The aim of this study was to evaluate the effect of arnica (*Heterotheca inuloides* Cass.) extract on raspberry plants under salt stress conditions. Plants were subjected to four sodium chloride (NaCl) concentrations (0 mM, 30 mM, 50 mM, and 80 mM), with or without the application of arnica extract (1,000 mg L^{-1}). A control group without NaCl or extract application was also included. In plants exposed to salt stress, the application of *H. inuloides* extract led to increased concentration of anthocyanins, phenols, flavonoids, total sugars, and antioxidant activity (ABTS), as well as enhanced enzymatic activity of superoxide dismutase (SOD) and peroxidase (POD) in fruits of the Adelita raspberry variety. These findings suggest that *H. inuloides* extract can strengthen both enzymatic and non-enzymatic antioxidant systems in raspberries, offering a potential eco-friendly strategy to mitigate the adverse effects of salinity on crop productivity and fruit quality. raspberry plants under salt stress strengthens the enzymatic and non-enzymatic antioxidant system in var raspberry fruits var. Adelita.

KEYWORDS

Agriculture organic; bioproducts; enzymatic compounds; non-enzymatic compounds.

RESUMEN

La frambuesa (*Rubus idaeus* L.) se ve afectada por diferentes tipos de estrés, entre ellos se encuentra el estrés salino; esto provoca una disminución en la producción y calidad del fruto. El objetivo de este estudio fue evaluar el efecto del extracto de árnica en plantas de frambuesa bajo condiciones de estrés salino (cuatro concentraciones de cloruro de sodio, 0 mM, 30 mM, 50 mM y 80 mM) y con aplicación de extracto de árnica (1,000 mg L^{-1}) y plantas sin aplicación de cloruro de sodio y sin aplicación de extracto. En plantas expuestas a estrés salino con la aplicación de extracto de árnica aumenta la concentración de antocianinas, fenoles, flavonoides, actividad antioxidante por ABTS, azúcares totales, enzimas antioxidantes como superóxido dismutasa y peroxidasa en frutos de frambuesa var. Adelita. Estos resultados sugieren que la aplicación de extracto de *H. inuloides* en plantas de frambuesa bajo estrés salino fortalece el sistema antioxidante enzimático y no enzimático en frutos de frambuesa var. Adelita.

PALABRAS CLAVE

Agricultura orgánica; bioproductos; compuestos enzimáticos; compuestos no enzimáticos.

Introduction

Raspberry (*Rubus idaeus* L.) is a berry rich in nutritional and bioactive compounds (Kotula et al., 2022). Its popularity is mainly attributed to its content of fructose, glucose, proteins, minerals (K, Ca, Mg, Fe, Zn and Mn), ascorbic acid, carotenoids and phenolic compounds such as anthocyanins, flavonoids and phenolic acids. Due to these compounds, there is a high demand for this fruit (Schulz and Chim, 2019). Its health-promoting properties include anti-inflammatory, anticancer, anti-vascular, and neurodegenerative effects (Sigala-Aguilar et al., 2023).

However, one of the main limitations to raspberry growth and production is soil salinity (Neocleous and Vasilakakis, 2007). Salinity restricts water uptake by plants, leading to cellular dehydration, ionic toxicity, nutritional imbalance, and increase production of reactive oxygen species (ROS), which affect lipids, proteins and DNA causing cell damage (González-García et al., 2021).

As an alternative to address salinity stress, the use of biostimulants has gained interest. Plant-derived secondary metabolites, particularly phenolic compounds (e.g., gallic acid, ascorbic acid, ferulic acid, vanillic acid), and phytohormones (e.g., indolacetic acid, brasinosteroids, methyl-jasmonate) have shown biostimulant activity in mitigating salinity stress (Ben et al., 2021). Several studies have explored the biostimulant potential of plant extracts. For example, Hassanein et al. (2019), used *Moringa oleifera* Lam. and *M. peregrena* (Forssk.) Fiori LC. extracts as biostimulants in sweet basil under saline stress, observing increased growth, fresh weight and dry weight of sprouts. Ertani et al. (2013) examined a *Medicago sativa* L. extract on salt-stressed maize and reported enhanced biomass. Grajkowski & Ochmian (2007) tested three biostimulants —Atonik SL, Biochikol 020 PC and Tytanit—on raspberry cultivars (Pokusa, Polka and Poranna Rosa) resulting in increased firmness, size, total soluble solids, and vitamin C. Sigala et al. (2023) applied humic and fulvic acids, as well as algal extracts, to raspberry plants and found increased levels of flavonoids, anthocyanins, palmitic acid and °Brix. Garza-Alonso et al. (2022) used *M. oleifera* extracts on strawberry plants, achieving greater firmness, total soluble solids, vitamin C, anthocyanin content and yield.

In this context, the biostimulant effect of botanical extracts is believed to be associated with the availability of bioactive molecules such as phenolic compounds, osmoprotectans, antioxidants, vitamins, and phytohormones. These compounds have been reported in arnica (*Heterotheca inuloides* Cass.) (Rodríguez-Chávez et al., 2017), a plant abundantly cultivated in Mexico and traditionally used for its anti-inflammatory, analgesic and ulcerogenic properties (Gené et al.,1998).

Therefore, the objective of this study was to evaluate the effect of arnica extract on raspberry plants under salt stress, focusing on agronomic performance, physicochemical characteristics, enzymatic activity, and secondary metabolites with antioxidant potential.

Materials and methods

Plant material

The aerial parts of arnica, specifically leaves and flowers, were collected from the municipality of San Diego Texmelucan, Puebla, Mexico (19°17′3.52″ N, 98°26′19.86″ W, 2580 masl). The region has a temperate sub-humid climate with summer rainfall (1,991 mm in 2023). The plant material was transported to the post-harvest laboratory of the Institute of Agricultural Sciences (ICAp) of the Universidad Autónoma del Estado de Hidalgo (UAEH), where it was frozen at -75°C for 48 h (Model ULT2540-9-A41, Ultra-freezer, Thermo Scientific 303, Massachusetts, United States of America). Subsequently, the samples were freeze-dried (Model 79480, Labconco Freeze-Dryer, Kansas City, United States of America) and ground into a fine powder using a blade mill (Model RTSCH GM-200, Haan, Germany) at 9,000 rpm for 50 s.

Preparation of arnica extract

Dried and powdered arnica leaves and flowers were mixed with 1,000 mL of 80 % ethanol and macerated for 7 days. The mixture was then filtered through Whatman No. 2 filter paper. The solvent was removed under reduced pressure using a rotary evaporator (Model BUCHI R-215, Buchegg, Switzerland) connected to a vacuum pump (Model BUCHI Vacuum Pump V-700, Buchegg, Switzerland), operating at 45 °C and 75 rpm. The resulting extract was stored at 5 °C. Extraction yield was calculated using the following formula, as described by García et al. (2010):

$$\%Y = \frac{IW - FW}{IW} * 100 \quad (1)$$

Where:
%Y= Percentage of yield
IW= initial weight
FW = final weight

Characterization of arnica extract

Total phenols

Total phenols content was determined using the Folin-Ciocalteu method, as described by Singleton et al. (1999), based on an oxide-reduction reaction. For sample preparation, 80% ethanol was used as the solvent. Subsequently, 0.5 mL of the sample was mixed with Folin Ciocalteu reagent diluted to 50% in water. After standing for 7 min, then 7.5% sodium carbonate solution was added, and the reaction mixture was incubated in the dark for 60 min. Absorbance was then measured at 725 nm using a spectrophotometer (Model 6715 UV/visible, Jenway, Techne Inc, San Diego, California, United States of America). Results were presented in gallic acid equivalents per mg of extract (mg GAE g^{-1} of extract).

Total flavonoids

Flavonoid content was determined according to the method of Rosales et al. (2011), using the same ethanol solvent for sample preparation. A 2 mL aliquot of the extract was mixed with sodium nitrite and water, and the mixture was allowed to stand in the dark for 5 min. Then, aluminium trichloride and 1 mL of sodium hydroxide were added, and the mixture was left to react for another 20 min. Absorbance was measured at 415 nm using the same spectrophotometer. Results were expressed as mg quercetin equivalents of dry weight (mg QE/100 g of dry weight).

Antioxidant activity (DPPH and ABTS)

Antioxidant activity was evaluated using two methods: DPPH (2,2-Diphenyl-1-picrylhydrazyl) for lipophilic compounds, and ABTS•+ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) for both lipophilic and hydrophilic compounds.

The $ABTS^{\bullet+}$ assay followed the protocol of Re et al. (1999). The $ABTS^{\bullet+}$ radical was generated by mixing 7 mM ABTS with 2.45 mM potassium persulphate and allowing the solution to react in the dark with constant stirring for 16 h. Then, 0.1 mL of the extract was mixed with $ABTS^{\bullet+}$ solution and allowed to react for 6 min. Absorbance was measured at 734 nm. Results were expressed in equivalent micromoles of Trolox per gram of dry weight (μM TE g^{-1} DW).

For the DPPH method, the protocol of Brand-Williams et al. (1995) was used. A 6 x 10^{-5} M ethanolic solution of DPPH was prepared and stirred in complete darkness for 2 h. Then, 0.5 mL of the extract was added to the DPPH solution and incubated for 1h at 4°C. Absorbance was measured at 517 nm. Results were expressed in equivalent micromoles of Trolox per gram of dry weight (μM TE g^{-1} DW).

Crop growth and experimental conditions

The experiment was conducted from March to October 2022 in the greenhouse of ICAp (UAEH). Greenhouse conditions were maintained at 22 ± 2°C with a relative humidity of 70%. Raspberry seedlings of the 'Adelita' variety from Michoacan, Mexico, were transplanted into 4 L black polyethylene bags. The substrate consisted of a 1:1 (v/v) mixture of peat moss and perlite (agrolite). Plants were irrigated daily with 1,000 mL of Steiner nutrient solution (Steiner, 1961), applied progressively: 25% during the vegetative stage, 50% during flowering, 75% during fruit set, and 100% during fruit filling and harvesting, according to the methodology of Hernández-Soto et al. (2024).

Preparation of arnica extract solution

One g of ethanolic arnica extract was diluted with 2 mL of ethanol, followed by the addition of 1,000 mL of distilled water and 1 mL L^{-1} of Bionex (adherent). The solution was stirred at 350 rpm at 15°C for 25 min using a magnetic shaker (Velp Scientifica, Multistirrer Series, Texas, United States of America).

Design of treatments

Three sodium chloride concentrations, 30, 50 and 80 mM of sodium chloride were used and five treatments were performed: T1: Control (0 mM NaCl, no arnica extract); T2: 30 mM NaCl + arnica extract (1,000 mg L^{-1}); T3: 50 mM NaCl + arnica extract (1,000 mg L^{-1}); T4: 80 mM NaCl + arnica extract (1,000 mg L^{-1}); T5: 0 mM NaCl + arnica extract (1,000 mg L).

Crop management

Foliar applications of arnica extract were carried out at 30, 45, 60, 75 and 90 days after transplanting. Each application involving spraying 75 mL of the extract solution evenly on the upper and lower surfaces of the leaves. Fruits were harvested 17 weeks after transplanting, at commercial maturity (fully red fruit color).

Study variables

Agronomic variables

Plant heigth was measured using measuring tape. The number of flowers and number of fruits per plant was counting manually. Fruit weight was recorded using a digital scale (Model Scout Pro OHAUS, New Jersey, United States of America). Stem diameter, equatorial diameter, and polar diameter of fruits were measured with a digital vernier (Model CALDI-6MP, Truper, Ciudad de Mexico, Mexico).

Physicochemical variables

Total soluble solids (TSS) were measured with a digital refractometer (PR ATAGO Co. Ltd., Minato, Tokyo, Japan), and expressed in ºBrix. pH was determined using a digital potentiometer (Model 550 Oakton, Virginia, United States). Fruit color parameters (L*, a* and b*) were measured using a HunterLab colorimeter (Model CM508d, Minolta Camera Co. Ltd., Osaka, Japan), following Monroy-Gutiérrez et al. (2017). Titratable acidity was determined using the method of Association of Official Agricultural Chemists (1931). 10 g of fruit were blended (Model BLSTBC4129-'13, Osterizer blender Classic, Wisconsin, United States of America), with 50 mL of distilled water. The total volume was measured, and 10 mL of the homogenate were titrated with 0.1 N NaOH using three drops of 1% phenolphphthalein as an indicator. Acidity percentage was calculated using the following formula:

$$\%citricacid = (mLNaOH * NNaOH * meq * VT100)/(A * g)$$

Where: *mL NaOH*= milliliters of sodium hydroxide spent on titration.
N= is the normal of sodium hydroxide.
meq= milliequivalent of citric acid (0.064).
TV= corresponds to the volume of the prepared sample.
A= Aliquot taken for measurement.
g= weight of the sample

Processing of fruit samples for biochemical analysis

Fruits were stored in an ultra-freezer at -75 °C (Model ULT2540-9-A41, Thermo Scientific 303, San Diego, California, United States of America), freeze-dried (Model 79480, Labconco, Kansas City, United States of America) for 72 h, ground using a blade mill (Model RTSCH GM-200, Grindomix, Haan, Germany) and stored at 5 °C until further analysis.

Fruit variables analyzed

The methodologies for determining total phenols (Singleton et al., 1999), total flavonoids (Rosales et al., 2011) and antioxidant activity via ABTS (Re et al., 1999) and DPPH (Brand-Williams et al., 1995), were the same as those described in the characterization of arnica extract section.

Anthocyanins

Total anthocyanin content was determined using the method of Giusti and Wrolstad (2001). A lyophilized sample was mixed with 2 mL of 80% ethanol and centrifuged at 4,000 x *g* for 10 min. Then, 400 µL of supernatant was mixed 1,600 µL of buffer solution (sodium acetate and potassium chloride). After vortexing, absorbance was measured 510 and 700 nm using a microplate reader (Model AMR-100, ALLSHENG Mark, Shanghai, China). The total anthocyanin content was expressed as mg 100 g^{-1} DW.

Vitamin C

Vitamin C content was determined according to Dürüst et al. (1997). The sample was mixed with 10 mL of 3% metaphosphoric acid solution (v/v), sonicated for 15 min in an ultrasonic bath (Model 32V118A, Branson, Freeport, United States of America), and centrifuged at 10,000 x *g* for 10 min. A 2 mL aliquot of the supernatant was added to a pH 4 buffer solution (1:1 mixture of glacial acetic acid and 5% sodium acetate), followed by the addition of 3 mL dichloroindophenol and 15 mL xylene. Absorbance was measured at 520 nm (Model 6715 UV/Vis, Jenway, Techne Inc, Staffordshire, United Kingdom). Results were expressed as mg of ascorbic acid equivalents per gram of dry weight (mg AA g^{-1} DW).

Total, reducing, and non-reducing sugars

Total sugar content was determined following Montaño-Herrera et al. (2022). Lyophilized sample was mixed with 10 mL of distilled water and centrifuged at 10,000 rpm for 10 min at 4 °C (Model ST 16R, Thermo Scientific, San Diego, California, United States of America). The supernatant was combined with 5 mL of anthrone, boiled for 10 min, cooled to room temperature and absorbance measured at 625 nm (Model 6715 UV/Visible, Jenway, Techne Inc, San Diego, California, United States of America). Results were expressed as mg of glucose equivalent per gram of dry weight (mg GE g^{-1} DW).

Reducing sugars was quantified using the 3,5 dinitrosalicylic acid (DNS) (Ávila et al., 2012). A known amount of lyophilized sample was mixed with 0.5mL of DNS reagent, boiled for 5 min, cooled in ice, diluited with 5 mL of distilled water, and absorbance measured at 540 nm (Model 6715 UV/Visible, Jenway, Techne Inc, San Diego, California, United States of America). Non-reducing sugars were calculated by subtracting reducing sugars from total sugars. Results were expressed in mg of glucose equivalent per gram dry weight (mg GE g^{-1} DW).

Enzymatic antioxidant activity

The activities of superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6) were determined following Balois-Morales et al. (2008). A known amount of the sample was mixed with 5 mL of a 0.05 M phosphate buffer (pH 7.8), and centrifuged at 10,000 x *g* for 15 minutes at 4 °C. SOD activity was assessed by inhibition of the nitro blue tetrazolium (NBT) photochemical reaction. The results were expressed in international units per gram of protein (U mg^{-1} protein), where 1 U is the amount of enzyme needed to cause 50% inhibition of NBT reduction. Catalase activity was determined by the change in absorbance at 240 nm for 3 min. Ascorbate peroxidase (APx; EC 1.11.1.11) activity was measured using the method of Proietti et al. (2013), and calculated using the extinction coefficient of ascorbate (2.8 mM^{-1} cm^{-1}).

Guaiacol peroxidase (POD; EC 1.11.1.7) activity was measured by monitoring the oxidation of guaiacol to tetraguaiacol, according to Vanegas et al. (2022). Tetraguaiacol formation was recorded every 10 s for 3 min at a wavelength of 470 nm. The activity of polyphenol oxidases (PPOs; E.C. 1.14.18.1 and E.C. 1.10.3.1) was followed by the oxidation of catechol to o-diquinone at a wavelength of 470 nm for 3 min, according to the method proposed by Vanegas et al. (2022). The results of these enzymes were expressed in international units per gram of protein (U mg^{-1} protein). Protein content was determined using the method of Pedrol and Ramos (1976), with bovine serum albumin (BSA) as the standard.

Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide content was determined according to Junglee et al. (2014), using a H_2O_2 calibration curve. Results were expressed as nanomoles of H_2O_2 per gram of dry weight (nmol g^{-1} DW).

Malondialdehyde concentration (MDA)

Lipid peroxidation was measured by quantifying Malondialdehyde using the thiobarbituric acid (TBA) method (Pasquariello et al., 2013). Absorbance of the MDA-TBA complex was measured at 532 nm, and the concentration calculated using the molar extinction coefficient of 1.56 mM^{-1} cm^{-1}. Results were expressed as millimoles of malondialdehyde per gram of dry weight (mM MDA g^{-1} DW).

Proline

Proline content was determined following the modified protocol of Bates et al. (1973). Fifty milligrams of lyophilized tissue was homogenized in 1,000 μL of 3% sulfosalicylic acid, centrifuged at 15,000 x *g* at room temperature for 5 min, and filtered on Whatman No. 2 paper. Then, 200 μL of the filtrate was mixed with 200 μL of acid ninhydrin and 200 μL of glacial acetic acid, and incubated at 100 °C for 1 h. The reaction was stopped in an ice bath, and the mixture was extracted with 1000 μL toluene and vortexed for 20s. The toluene phase was recovered, brought to room temperature, and absorbance was read at 250 nm. A standard curve of 1-proline was used for quantification. Results were expressed as mg g^{-1} DW.

Statistical analysis

Nine plants per treatment were evaluated using a completely randomized block design. All analyses were performed in triplicate. A normality test was conducted to verify that data were met the assumptions

Table 1. Yield, total phenols, total flavonoids and antioxidant activity of the 80% ethanolic extract of *Heterotheca inuloides*.

Extract	Solvent	Yield (%)	Phenols (mg GAE g^{-1})	Flavonoids (mg QE g^{-1})	ABTS (μM TE g^{-1} DW)	DPPH (μM TE g^{-1} DW)
H. inuloides	80% Ethanol	12.82±0.50	69.71± 2.02	228.62±0.63	345.48±0.37	154.97±0.40

GAE: Gallic acid equivalents; QE: Quercetin equivalent; TE Trolox equivalent. Data are expressed as mean ± standard error (n= 9).

for ANOVA. Subsequently, an analysis of variance (ANOVA) and Fisher's LSD test ($\alpha = 0.05$) were applied to agronomic, physicochemical, bioactive compounds and antioxidant enzyme variables using Infostat *software* (v2020).

RESULTS AND DISCUSSION

Arnica extract

These yield (Table 1) was higher than those obtained by García-Pérez et al. (2016), which may be attributed to differences in the solubility of secondary metabolites depending on the solvents system, extraction technique, and plant material used (Dirar et al., 2019). Variations in phenolic and flavonoid content (Table 1), as well as antioxidant activity, are largely influenced by the type of solvent, polarity, extraction conditions, and plant part used for extraction (Wakeel et al., 2019).

Morphology

As shown in Figure 1, raspberry plants grown under no salinity but treated with arnica extract exhibited vigorous growth and healthy leaves, plants subjected to 30mM NaCl+EXHi showed chlorosis on apical leaves; those under 50mM NaCl+EXHi exhibited chlorosis on basal leaves; and plants under 80mM NaCl+EXHi displayed necrotic lesions and severe damage, including plant death.

In general, there were effects on the biomass and morphological traits of raspberry plants exposed to NaCl and EXHi. Salt stress reduced the plant's ability to absorb water, negatively impacting leaf transpira-

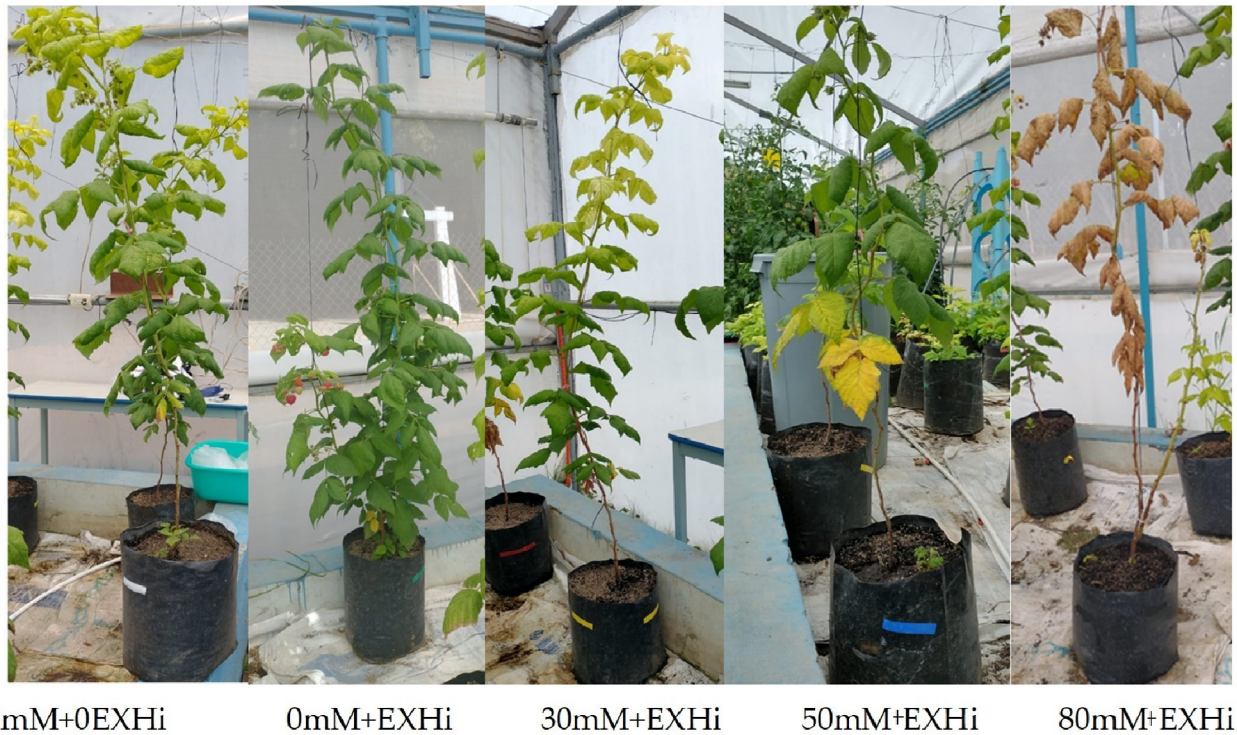

Figure 1. Morphological appearance of raspberry (*Rubus idaeus*) treated with arnica extract (*Heterotheca inuloides*) and subjected to salt stress (NaCl). Treatments: Treatments: 0mM+0EXHi: Plants without NaCl or arnica extract (control); 30mM+EXHi: Plants treated with 30 mM NaCl and arnica extract (1,000 mg L^{-1}); 50mM+EXHi: plants treated with 50 mM NaCl and arnica extract; 80mM+EXHi: Plants treated with 80mM NaCl and arnica extract; 0mM+EXHi: Plants treated with arnica extract but without NaCl.

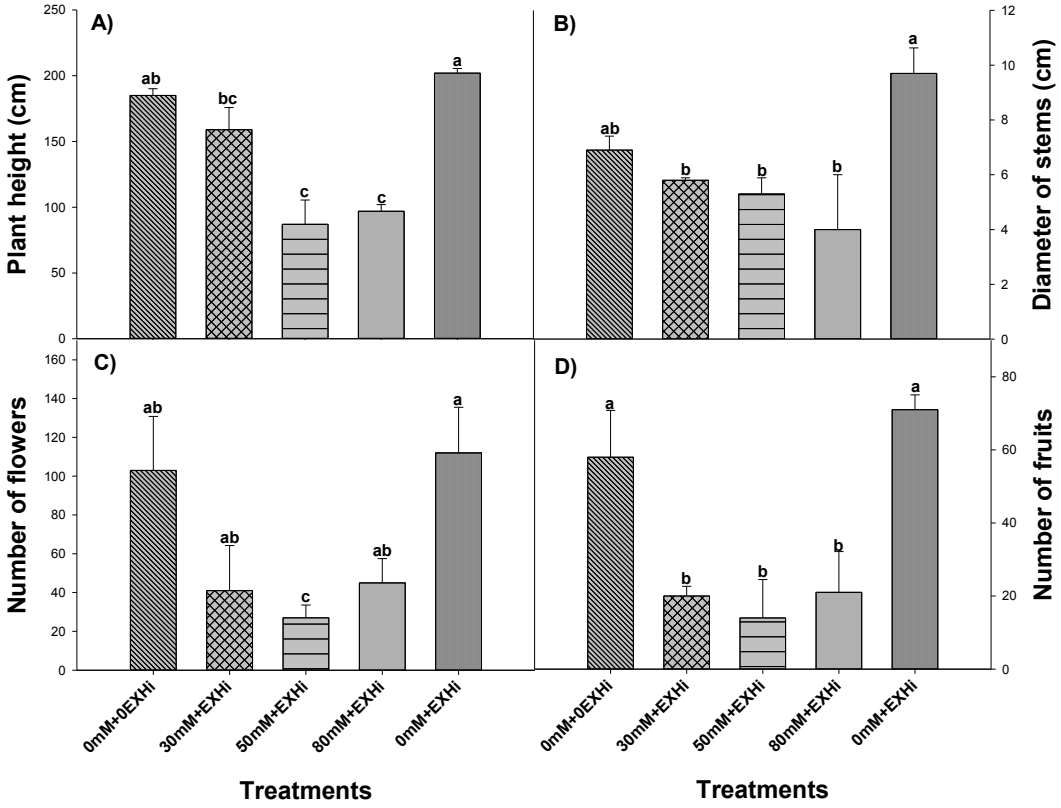

Figure 2. Agronomic variables of raspberry (*Rubus idaeus*) plants treated with arnica (*Heterotheca inuloides)* extract and subjected to salt stress: (A) plant height, (B) stem diameter, (C) number of flowers, (D) number of fruits. Treatments: 0mM+0EXHi: Plants without NaCl or arnica extract (control); 30mM+EXHi: Plants treated with 30 mM NaCl and arnica extract (1,000 mg L^{-1}); 50mM+EXHi: plants treated with 50 mM NaCl and arnica extract; 80mM+EXHi: Plants treated with 80mM NaCl and arnica extract; 0mM+EXHi: Plants treated with arnica extract but without NaCl. Values with different letters in each column indicate statistically significant differences among treatments and control according to Fisher's LSD ($\alpha = 0.05$). Data are expressed as mean ± standard error (n= 9).

tion and photosynthesis (Hand et al., 2017). Moreover, it can stimulate ethylene synthesis, leading to leaf senescence and a reduced leaf area (Acosta-Motos et al., 2017). On the other hand, application of EXHi enhanced the morphological development of raspberry plants. This improvement can be attributed to the antioxidant compounds in the extract, such as kaempferol and catechins, which promote leaf expansion, parenchyma thickening, cell turgor, and structural stability (Ahmad et al., 2022).

Agronomic variables

Plants biostimulated with arnica extract (0mM+EXHi) exhibited increases of 9.20% and 40.60% in plant height and stem diameter respectively, compared to the control (0mM+0EXHi). In contrast, salinity stress reduced plant height by 14.00% (30mM+EXHi); 53.00% (50mM+EXHi) and 48.00% (80mM+EXHi). Stem diameter also decreased by 14.50% (30mM+EXHi), 23.20% (50mM+EXHi) and 42.00% (80mM+EXHi), respectively (Figure 2 A, B). These reductions are typical of osmotic stress caused by salinity, which limits water uptake (Bistgani et al., 2019), induces cell dehydration, ionic toxicity, and leads to ROS accumulation, causing oxidative damage to lipids, proteins, and nucleic acids (González-García et al., 2021).

However, foliar application of arnica extract mitigated these effects and improved growth traits, likely due its content of macronutrients (K, P, Ca) and phytohormones like indole acetic acid and gibberellins, which influence plant development when applied exogenously (Arif et al., 2023). In line with this, Neocleous and Vasilakakis (2007) reported a decrease in photosynthetic capacity and growth in raspberry plants under saline conditions. Meanwhile, Hassanein et al. (2019), demonstrated that the application of *M. oleifera* and *M. peregrena* extracts increased branching in sweet basil under similar stress.

Physicochemical variables of the fruits

Raspberry fruits from plants treated with arnica extract and without salt stress (0mM+EXHi) exhib-

ited the greatest fruit size and weight, with increases of 15.1% in length and 12.8% in weight, compared to fruits from control plants (0mM+EXHi) (Figure 3A, 3F). In contrast, fruits from plants exposed to high salinity (80mM NaCl) and treated with arnica extract (80mM+EXHi) displayed smaller dimensions and lower weight, showing reductions of 3.9% in length, 14.6% in diameter and 33.7% in weight (Figure 3 A, 3C) compared to the control. These negative effects can be attributed to excessive accumulation of sodium and chloride ions in plant tissues, which decreases cell turgor pressure, impair water relations, and promote the generation of reactive oxygen species. These ROS, in turn, damage essential biomolecules such as proteins, lipids and DNA (Pinedo-Guerrero et al., 2020). However, the application of arnica extract mitigated the negative effects of salt stress and improved fruit size and weight. This enhancement is likely due to the cytokinins and phytohormones present in the extract, which are known to stimulate cell division and expansion, thereby promoting fruit development. These results are consistent with previous findings in other crops: Nasir et al. (2016) observed similar effects in mandarina, and Ahmed et al. (2019) reported improved

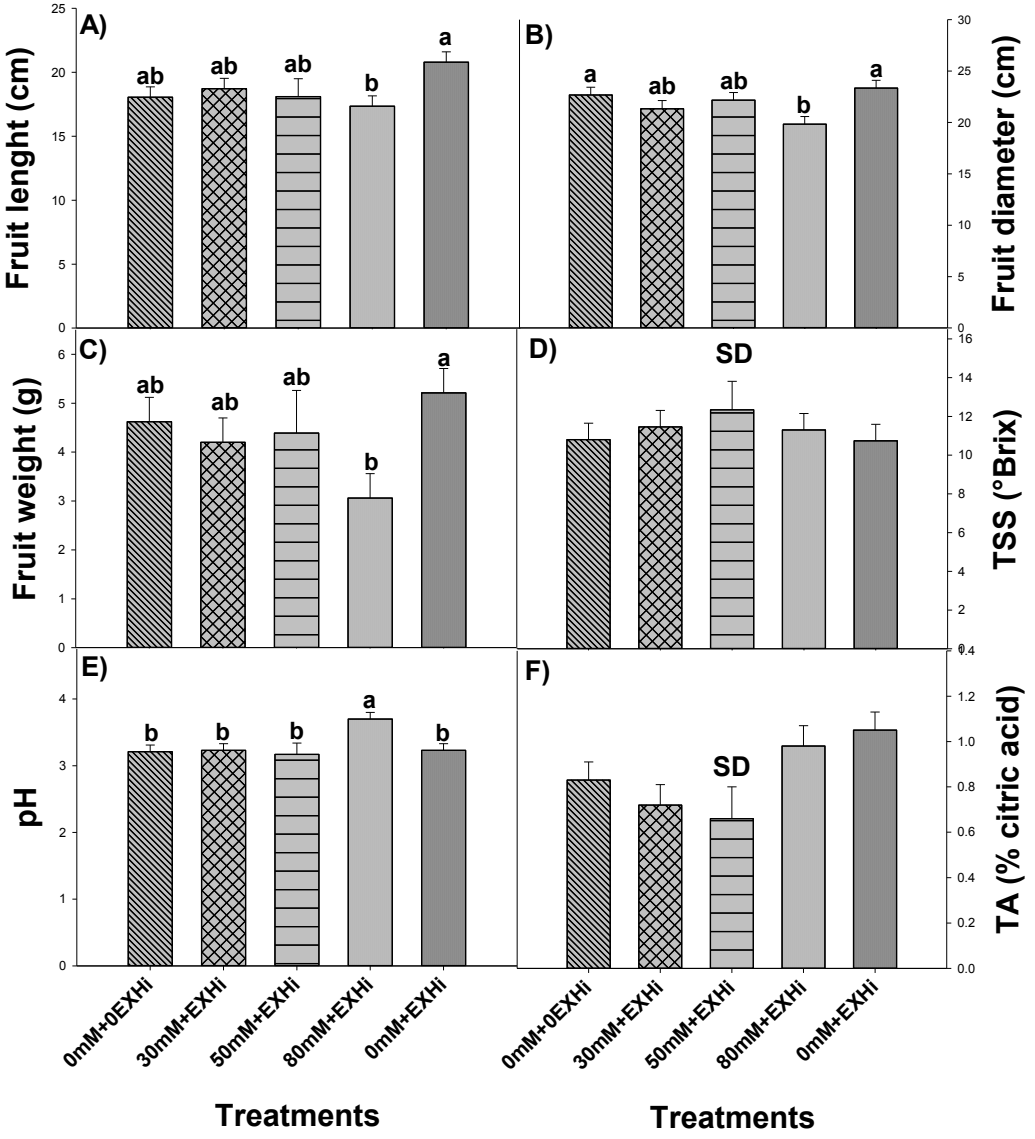

Figure 3. Physico-chemical characteristics of raspberry (*Rubus idaeus*) fruits treated with arnica (*Heterotheca inuloides*) extract and subjected to salt stress (NaCl): (A) fruit length, (B) fruit diameter, (C) fruit weight, (D) total soluble solids (TSS), (E) hydrogen potential (pH), and (F) titratable acidity (TA). Treatments: 0mM+0EXHi: Plants without NaCl or arnica extract (control); 30mM+EXHi: Plants treated with 30 mM NaCl and arnica extract (1,000 mg L^{-1}); 50mM+EXHi: plants treated with 50 mM NaCl and arnica extract; 80mM+EXHi: Plants treated with 80mM NaCl and arnica extract; 0mM+EXHi: Plants treated with arnica extract but without NaCl. Values with different letters in each column indicate statistically significant differences between the differences among treatments and control according to Fisher's LSD (α = 0.05). Data are expressed as mean ± standard error (n= 9).

Table 2. Color parameters (L*, °Hue, Croma) of raspberry (*Rubus idaeus*) 'Adelita' variety fruits under salt stress and arnica (Heterotheca inuloides) extract application.

Treatments	Color		
	L*	°Hue	Croma
0mM+0EXHi	39.00±0.68[a]	13.92±0.70[a]	19.14±1.04[a]
30mM+EXHi	40.09±0.73[a]	13.90±0.76[a]	19.04±1.13[a]
50mM+EXHi	40.29±1.44[a]	12.65±1.50[ab]	18.40±2.21[ab]
80mM+EXHi	38.88±1.73[a]	9.98±1.80[b]	12.50±2.65[b]
0mM+EXHi	40.83±0.73[a]	11.95±0.76[ab]	16.57±1.12[ab]

Treatments: 0mM+0EXHi: Plants without NaCl or arnica extract (control); 30mM+EXHi: Plants treated with 30 mM NaCl and arnica extract (1,000 mg L^{-1}); 50mM+EXHi: plants treated with 50 mM NaCl and arnica extract; 80mM+EXHi: Plants treated with 80mM NaCl and arnica extract; 0mM+EXHi: Plants treated with arnica extract but without NaCl. Values with different letters within each column indicate statistically significant differences among treatments and control according to Fisher's LSD ($\alpha = 0.05$). Data are expressed as mean ± standard error (n= 9).

cucumber growth and yield following the application of *M. oleifera* xtracts in saline conditions.

In relation to the total soluble solids content (TSS), although no significant differences were found between treatments (Figure 3D), fruits from plants treated with an intermediate dose of salt and extract application (50mM+EXHi) showed a higher °Brix or TSS content. This increase may be due to salt-induced biosynthesis of soluble sugars during maturation (Schulz and Chim, 2019). The values found in raspberry fruits were higher than those previously reported.

Concerning the hydrogen potential (pH), the highest value (3.70) was recorded in the fruits from plants treated with the highest salt concentration and extract application (80mM+EXHi). No significant differences were observed between plants exposed to a low and medium salt concentration with extract application (30mM+EXHi and 50mM+EXHi), and plants treated with arnica without salt application (Figure 3E). These changes are attributed to the transformation of organic acids into simple sugars (Pinedo-Guerrero et al. 2020), as well as the imbalance between K^+/Na^+ ions that maintain pH stability in fruits (González-García et al., 2021). Additionally, soil, climate, salinity conditions, and the fruit maturity stage (Denaxa et al., 2022) influence pH. In this regard, Turmanidze et al. (2017) reported lower pH values for raspberry fruits.

In titratable acidity (TA), although no significant differences were observed, the lower acidity content was recorded in fruits from plants treated with the lowest salt concentration and extract application (Figure 3F). Titratable acidity decreases in parallel with the fruit maturation since organic acids are used as substrates in the respiration process (González-García et al., 2021). It is worth mentioning that acidity increase is associated with salinity concentrations, crop management, crop type, and environmental conditions (Abdelgawad et al., 2019).

Color

Regarding color, the highest values of chroma (purity) and hue (tone) were observed in raspberry fruits without extract and salt application (0mM+0EXHi), and in fruits treated with the lowest salt concentration ans extract application (30mM+EXHi). However, no significant differences were observed in the L* values (fruit brightness). A higher L* value indicates a brighter fruit, which is preferred by consumers (Alves et al., 2023) (Table 2).

A decrease of 28.31% in hue and 35.64% in chroma was observed in fruits subjected to high salt concentration and arnica extract stimulation (80mM+EXHi), compared to the control plants. Zushi et al. (2009) indicated that abiotic stress disrupts normal metabolic processes such as fruit senescence. According to Stavang et al. (2015), color is a key parameter for consumer acceptability, and changes in color reflect degradative reactions during processing and storage.

Bioactive compounds of fruits

With respect to the content of total phenols and flavonoids, no statistically significant differences were found among fruits from raspberry plants exposed to different NaCl concentrations with arnica extract application (30mM+EXHi, 50mM+EXHi and 80mM+EXHi) (Figure 4A, 4B). However, a slight increase was observed in phenolic content: 19.6% (30mM+EXHi), 20.4% (50mM+EXHi), and 21.9% (80mM+EXHi), and in fla-

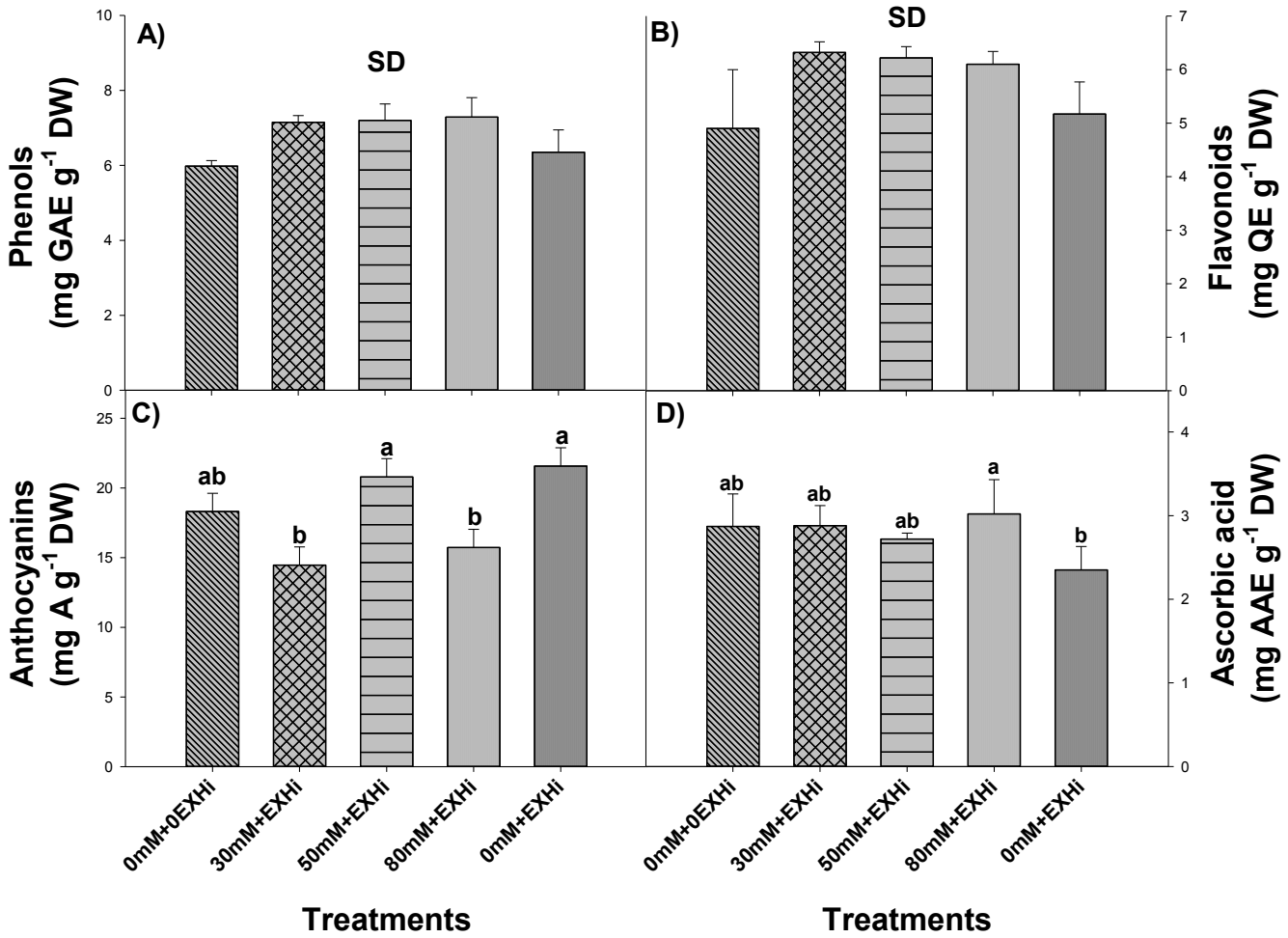

Figure 4. Total phenols, total flavonoids, anthocyanins and ascorbic acid of raspberry (*Rubus idaeus*) fruits treated with arnica (*Heterotheca inuloides*) extract and subjected to salt stress (NaCl): (A) phenols, (B) flavonoids, (C) anthocyanins and (D) ascorbic acid. Treatments: 0mM+0EXHi: Plants without NaCl or arnica extract (control); 30mM+EXHi: Plants treated with 30 mM NaCl and arnica extract (1,000 mg L^{-1}); 50mM+EXHi: plants treated with 50 mM NaCl and arnica extract; 80mM+EXHi: Plants treated with 80mM NaCl and arnica extract; 0mM+EXHi: Plants treated with arnica extract but without NaCl. Values with different letters in each column indicate statistically significant differences among treatments and control according to Fisher's LSD ($\alpha = 0.05$). Data are expressed as mean ± standard error (n= 9).

vonoids: 28.9% (30mM+EXHi), 26.9% (50mM+EXHi) and 24.4% (80mM+EXHi), compared to the control (0mM+0EXHi). This may be explained by the fact that plants synthesize phenolic acids and flavonoids under salt stress, which helps mitigate environmental stress.

Sharma et al. (2019), reported that abiotic stress activates the phenylpropanoid pathway, leading to the accumulation of phenolic compounds capable of scavenging reactive oxygen species. Therefore, increased phenols and flavonoids can help reduce oxidative stress in plants. The application of plant-based biostimulants has been shown to enhance the production of antioxidant compounds. For instance, applying exogenous EXHi to raspberry plants may contribute phenolic compounds such as gallic acid and quercetin, which inhibit DNA oxidation, and kaempferol, which traps ROS (Ferrari, 2004). Drobek et al. (2019) reported that *M. oleifera* extracts alleviated salt stress by increasing phenol and flavonoid levels, thereby reducing ROS accumulation.

Regarding anthocyanin content, raspberry fruits from plants under salinity conditions showed a decrease of 21.03% and 14.15% for 30mM+EXHi and 80mM+EXHi, respectively, compared to the control (Figure 4C). This reduction may be attributed to the presence of salt, which may affect the expression of genes for enzymes involved in anthocyanin biosynthesis. On the other hand, anthocyanin levels may be influenced by climatic conditions and the maturity stage of the fruits (Palonen and Weber, 2019).

In contrast, the application of arnica extract increased anthocyanin content in raspberry fruits by 17.80%, compared to the control. This effect may be due to the antioxidant propierties of the extract, which help

mitigate stress through the upregulation of related genes and increased activity of key enzymes (Ben et al., 2021), such as phenylalanine ammonia lyase (PAL), chalcone isomerase (CHI), and glucose (UDP), all involved in anthocyanin biosynthesis (Deng et al., 2019).

With respect to ascorbic acid content (Figure 4D), a 5.22% increase was observed in fruits from plants treated with a low NaCl concentration and arnica extract (80mM+EXHi). It has been reported that salt application in nutrient solutions can enhance ascorbic acid levels (Zhang et al., 2016). This suggests that ascorbic acid plays a key role in plant adaptation to saline conditions by either promoting its biosynthesis or prevening its degradation (Leon-Calvario et al., 2020).

Antioxidant capacity

Antioxidant capacity, as measured by the ABTS assay (Figure 5A), was 21.44% in fruits from plants treated with a low NaCl concentration and arnica extract (30mM+EXHi), compared to untreated plants under salt stress. According to Ben et al. (2021), plant extracts are efficient sources of ROS eliminators, which are essential for mitigating oxidative damage and maintaining cellular homeostasis. Specifically, EXHi contains antioxidants of biostimulating interest such a kaemptferol, quercetin, and α-tocopherol (Rodríguez-Chávez et al., 2017).

Similarly, Nasir et al. (2016) reported increased antioxidant levels in mandarin fruits with Moringa extract. Likewise, Ahmed et al. (2019), observed a rise in antioxidant content in pumpkins treated with *M. oleifera* under salt stress. These effects are attributed to the broad range of antioxidant compounds found in plant leaves, which, when applied exogenously to crops, stimulate both primary and secondary metabolism, resulting in greater antioxidant accumulation in the fruit.

With regard to the antioxidant activity by DPPH, no significant statistical differences were found between treatments (Figure 5B). These results are consistent with those reported by Martínez et al. (2020), who also found no impact of salt stress on antioxidant activity in both wild and domesticated tomato cultivars. This lack of impact could be attributed to the environmental conditions in which the crop is grown, as these can influence the composition of phenolic compounds in the plant, thereby affecting its antioxidant capacity (Ordóñez-Díaz et al., 2021).

Total sugars, reducing sugars, and non-reducing sugars

In terms of total sugar content, an increase was observed in the fruits of plants under salt stress conditions with the application of extract (Figure 6A), showing values

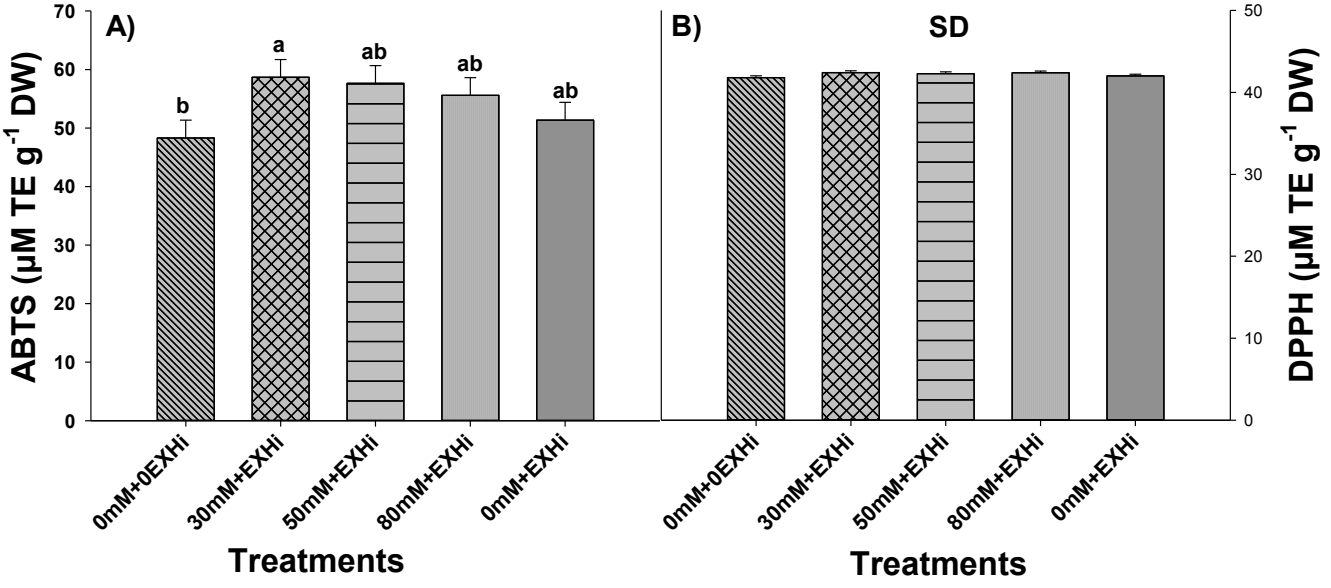

Figure 5. Antioxidant activity by ABTS (lipophilic-hydrophilic) and DPPH (hydrophilic) of raspberry (*Rubus idaeus*) fruits treated with arnica (*Heterotheca inuloides*) extract and subjected to salt stress (NaCl): (A) ABTS, (B) DPPH. Treatments: 0mM+0EXHi: Plants without NaCl or arnica extract (control); 30mM+EXHi: Plants treated with 30 mM NaCl and arnica extract (1,000 mg L^{-1}); 50mM+EXHi: plants treated with 50 mM NaCl and arnica extract; 80mM+EXHi: Plants treated with 80mM NaCl and arnica extract; 0mM+EXHi: Plants treated with arnica extract but without NaCl. Values with different letters in each column indicate statistically significant differences among treatments and control according to Fisher's LSD ($\alpha = 0.05$). Data are expressed as mean ± standard error (n= 9).

of 6.7% (30mM+EXHi), 6.7% (50mM+EXHi), and 8.9% (0mM+EXHi) compared to the control. This may be due to the high sugar and starch content of some plant extracts, which, when applied to crops, facilite the translocation of sugars and carbohydrates into fruits by promoting the activation of enzymes involved in biochemical reactions, thus increasing total sugar content (Arif et al., 2023). These results are consistent with those of Mona (2013), who found that applying *M. oleifera* extract to rocket (*Eruca vesicaria subsp. sativa*) increased total sugar content. Regarding reducing and non-reducing sugars (Figure 6B, 6C) no significant statistical differences were observed. This could be due to the influence of factors such as solar radiation, temperature, water availability, soil mineral content, crop irrigation and fertilization (Fu et al., 2015).

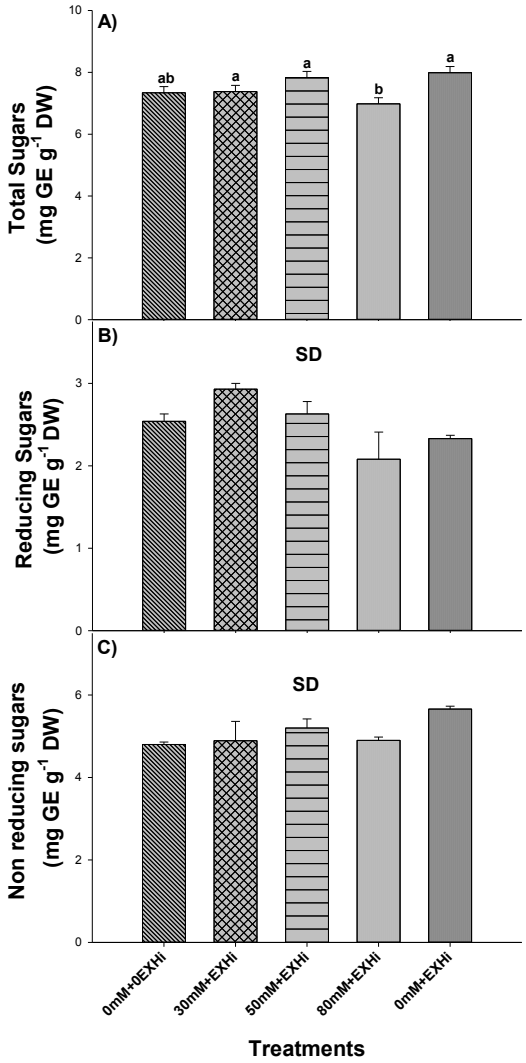

Figure 6. Total sugars, reducing sugars and non-reducing sugars content of raspberry (*Rubus idaeus*) fruits treated with arnica (*Heterotheca inuloides*) extract and subjected to salt stres (NaCl): (A) total sugars, (B) reducing sugars, (C) non-reducing sugars. Treatments: 0mM+0EXHi: Plants without NaCl or arnica extract (control); 30mM+EXHi: Plants treated with 30 mM NaCl and arnica extract (1,000 mg L^{-1}); 50mM+EXHi: plants treated with 50 mM NaCl and arnica extract; 80mM+EXHi: Plants treated with 80mM NaCl and arnica extract; 0mM+EXHi: Plants treated with arnica extract but without NaCl. Values with different letters in each column indicate statistically significant differences among treatments and control according to Fisher's LSD ($\alpha = 0.05$). Data are expressed as mean ± standard error (n= 9).

Enzymatic antioxidant compounds

To maintain ROS homeostasis and reduce oxidative damage caused by salt stress, plants have developed an enzymatic antioxidant system that includes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APx). Enzyme activity is mainly observed modified for SOD and POD, this may be due stress generated by the enlargement of NaCl and compounds present in EXHi (Figure 7A, 7B). For SOD, the increase was 62.50% (30mM+EXHi), 175.00% (50mM+EXHi), 200.00% (80mM+EXHi) and 250.00% (0mM+EXHi). POD activity increased by 73.13%, 51.67%, 30.97% and 15.16% in the treatments 30mM+EXHi, 50mM+EXHi, 0mM+EXHi and 80mM+EXHI, respectively. It is known that salt stress generates a high production of ROS, and these molecules can be eliminated by overexpression of antioxidant enzymes genes such as SOD and POD. These genes can serve as indicators to predict and confirm the stress in plants (Hao et al., 2021). In this sense, the exogenous application of plant extracts improves salt tolerance in crops through signaling and gene expression pathways such as *GIPC, MOCA1, ANNEXIN4, ANN4, HKT1, NTHK1, SOS2, PKB5, SOS3* and *SCaBP8* (Ahmad et al., 2022). The signal transduction of these genes regulates ionic homeostasis in membranes, thereby improving resistance to salt (Hao et al., 2021). These results are consistent with those of ElSayed et al. (2022), where an increase in the enzymatic activity of pumpkin was observed following the application of extracts from *Cupressus macrocarpa* Hartw. ex Gord.

The antioxidant enzymes CAT and APx did not show significant statistical differences between treatments (Figure 7B, 7D). However, there was a decrease of 3.04% (30mM+EXHi) and 17.58% (80mM+EXHi) for CAT. As for Apx, the recorded decreases were 10.41% (80mM+EXHi), 15.4% (30mM+EXHi), 18.67% (0mM+EXHi), and 36.71% (50mM+EXHi) relative to the control. This may be due to the presence of quercetin and catechins in arnica extract (Rodríguez-Chávez et al., 2017), compounds that trap superoxide

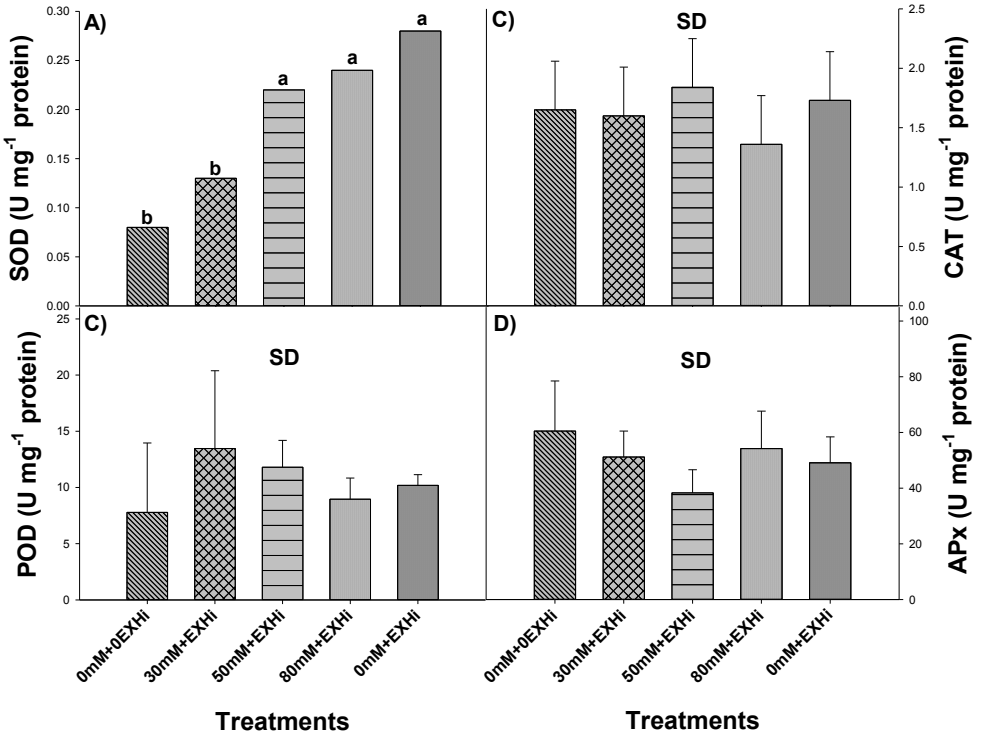

Figure 7. Enzymatic antioxidant activity by SOD (superoxide dismutase), CAT (catalase), POD (peroxidase) and APx (ascorbate peroxidase) of fruits of raspberry (*Rubus idaeus*) fruits treated with arnica (*Heterotheca inuloides*) extract and subjected to salt stress (NaCl): (A) SOD, (B) CAT, (C) POD, (D) APx. Treatments: 0mM+0EXHi: Plants without NaCl or arnica extract (control); 30mM+EXHi: Plants treated with 30 mM NaCl and arnica extract (1,000 mg L^{-1}); 50mM+EXHi: plants treated with 50 mM NaCl and arnica extract; 80mM+EXHi: Plants treated with 80mM NaCl and arnica extract; 0mM+EXHi: Plants treated with arnica extract but without NaCl. Values with different letters in each column indicate statistically significant differences among treatments and control according to Fisher's LSD ($\alpha = 0.05$). Data are expressed as mean ± standard error (n= 9).

radicals (Ferrari, 2004). As a result, the activity of detoxifying enzymes such as ascorbate peroxidase and catalase may decrease. Additionally, the long-term exposure of plants to salt stress leads to various genomic adjustments, and such genetic variation can result in negative regulation, or even no expression at all (Ben et al., 2021). Furthermore, gene expression is regulated according to the growth stage and environmental conditions (González-Gordo et al., 2022).

Stress markers

The activity of the stress markers, including polyphenol oxidase (PPO), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA), was found to be modified in raspberry fruits subjected to different salinity concentrations and the application of arnica extract (Figure 7A, 7C). Salinity stress inhibited the enzymatic activity of PPO by 28.60% for 30mM+EXHi and 50mM+EXHi, as well as 57.20% for 80mM+EXHi and 0mM+EXHi compared to the control. In this context, Ben et al. (2021) note that plant extracts contain antioxidant compounds capable of trapping superoxide radicals, thus preventing an increase in stress markers. Among these compounds are quercetin and catechins, which are present in arnica extract (Rodríguez-Chávez et al., 2017). Additionally, it has been reported that PPO activity may decrease or increase in response to salinity, with these changes depending on the type of crop and the specific mechanisms of plant adaptation to salt stress (Barbieri et al., 2012).

Regarding H_2O_2, a decrease in the activity of this stress marker was observed by 33.80% (30mM+EXHi), 36.60% (50mM+EXHi), 52.10% (80mM+EXHi) and 50.70% (0mM+EXHi). These results suggest that protection against oxidative stress induced by salt differed among treatments, possibly because the fruits were better protected from oxidative damage caused by salt. Moreover, the antioxidant enzymatic properties of raspberries can be influenced by various external factors such as light exposure, storage temperatures, and the natural volatile compounds present in the fruits (Martínez et al., 2021).

The MDA content increased by an average of 10.29% (30mM+EXHi), 15.37% (80mM+EXHi)

and 17.40% (0mM+EXHi) compared to the control (0mM+0EXHi). These results indicate that a high concentration of MDA reflects a loss of integrity and damage to cell membranes under salt stress due to excess ROS (González-García et al., 2022). In particular, the highest MDA concentration in raspberry fruits was observed with the application of EXHi, which may be due to a lower tolerance of raspberries to this micronutrient (Hernández-Fuentes et al., 2023).

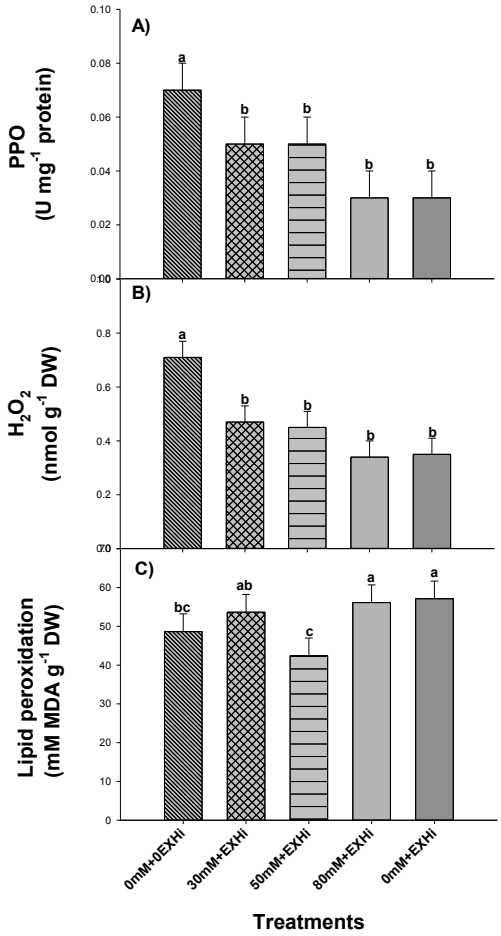

Figure 8. Stress markers for PPO (polyphenol oxidase), hydrogen peroxide (H_2O_2) and lipid peroxidation (MDA) of raspberry (*Rubus idaeus*) fruits treated with arnica (*Heterotheca inuloides*) extract and subjected to salt stress (NaCl): (A) PPO, (B) H_2O_2, (C) MDA. Treatments: 0mM+0EXHi: Plants without NaCl or arnica extract (control); 30mM+EXHi: Plants treated with 30 mM NaCl and arnica extract (1,000 mg L^{-1}); 50mM+EXHi: plants treated with 50 mM NaCl and arnica extract; 80mM+EXHi: Plants treated with 80mM NaCl and arnica extract; 0mM+EXHi: Plants treated with arnica extract but without NaCl. Values with different letters in each column indicate statistically significant differences among treatments and control according to Fisher's LSD (α = 0.05). Data are expressed as mean ± standard error (n= 9).

Proline

Proline content decreased in raspberry plant fruits following the application of arnica extract and salt treatments (Figure 8), whit reductions of 64.05% (30mM+EXHi), 41.49% (50mM+EXHi), 74.29% (80mM+EXHi) and 9.95% (0mM+EXHi). However, proline concentration was maintained in treatments with EXHi compared to fruits from plants without arnica extract or salt application. This decrease may be attributed to the fact that proline does not play a central role in protection mechanisms against salinity-induced ROS (Marček et al., 2015). Additionally, it has been reported that salinity-sensitive cultivars tend to accumulate lower amounts of proline (Pirlak and Eşitken, 2004).

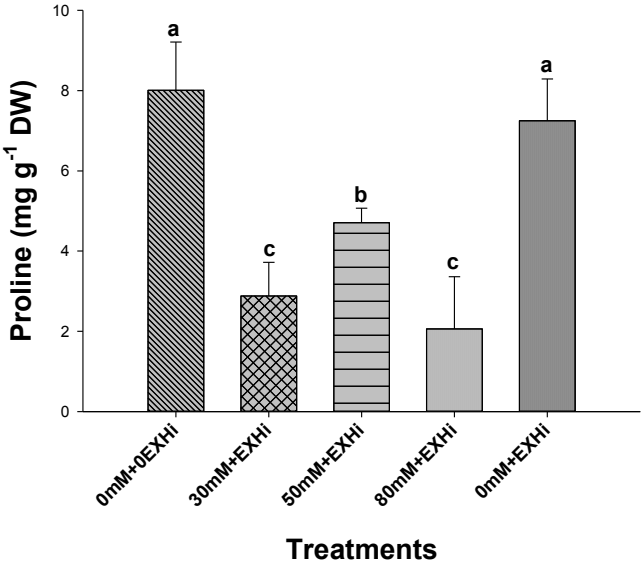

Figure 9. Proline of raspberry (*Rubus idaeus*) fruits treated with arnica (*Heterotheca inuloides*) extract and subjected to salt stress s (NaCl). Treatments: 0mM+0EXHi: Plants without NaCl or arnica extract (control); 30mM+EXHi: Plants treated with 30 mM NaCl and arnica extract (1,000 mg L^{-1}); 50mM+EXHi: plants treated with 50 mM NaCl and arnica extract; 80mM+EXHi: Plants treated with 80mM NaCl and arnica extract; 0mM+EXHi: Plants treated with arnica extract but without NaCl. Values with different letters in each column indicate statistically significant differences among treatments and control according to Fisher's LSD (α = 0.05). Data are expressed as mean ± standard error (n= 9).

CONCLUSIONS

This research demonstrated that the application of a hydroalcoholic extract from *Heterotheca inuloides* leaves had positive effects on raspberry plants not exposed to salt stress, promoting increases in plant height, stem diameter, fruit size, fruit weight and overall yield. In plants subjected to salt stress, the application of *Heterotheca inuloides* extract enhanced the content of total phenols, flavonoids, antioxidant activity (ABTS), and total sugars, as well as the enzymatic activity of superoxide dismutase (SOD) and peroxidase (POD) in raspberry fruits.

These findings suggest that the hydroalcoholic extract of *Heterotheca inuloides* represents a viable and

environmentally friendly alternative to mitigate the adverse effects of salinity in raspberry cultivation. Its application strengthens and activates the enzymatic and non-enzymatic defense systems in raspberry fruits, particularly in the variety Adelita. Furthermore, future research should explore the extract´s effects on other plant species under salt stress and consider refining the statistical approach for a more accurate identification of the observed interactions.

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