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PHENOLIC COMPOUNDS AND AOX ACTIVITIES IN CHARD (*BETA VULGARIS* L. VAR. CICLA) ELICITATED WITH HYDROGEN PEROXIDE IN OPEN FIELD

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ABSTRACT

Objective: The main objective of this study was to enhance the functional food properties of Swiss chard (*Beta vulgaris* var. cicla L.) through controlled elicitation using hydrogen peroxide (H_2O_2).

Theoretical Framework: Chard is a highly demanded leafy vegetable due to its low cost, ease of production, and high nutritional value, containing minerals, soluble sugars, fatty acids, vitamins, and phenolic compounds. Plants respond to these stressors leading to the production of antioxidant (AOX) compounds that mitigate stress-induced damage. Hydrogen peroxide (H_2O_2) exogenous application can stimulate specialized metabolite production and enhance antioxidant capacity in plant tissues.

Method: Swiss chard plants were subjected to seven H_2O_2 concentrations. It was evaluated $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, Catalase (CAT), Superoxide dismutase (SOD), Phenylalanine ammonia-lyase (PAL) enzymatic activity, total phenolic content and concentration, and Nitrate concentration.

Results and Discussion: The 75 mM H_2O_2 treatment acted as a bio-stimulant, significantly increasing antioxidant activity and the synthesis of phenolic compounds without negatively affecting nitrate concentration. Enzymatic activity of PAL was enhanced, indicating an upregulation of secondary metabolism, leading to higher levels of phenolic compounds and antioxidants in the plant.

Research Implications: This study provides an alternative approach to improving leafy vegetable quality using a non-toxic, cost-effective elicitor (H_2O_2). The results suggest that controlled H_2O_2 application could be implemented in agriculture to enhance plant metabolic responses, leading to higher antioxidant content and better functional properties in crops.

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Originality/Value: This study demonstrates a novel bio-stimulation approach that does not compromise nitrate concentrations, addressing concerns related to excess nitrate accumulation in leafy greens.

Keywords: Elicitation, Eustress Factor, Enzymatic Activity, Hydrogen Peroxide, Nitrates.

COMPOSTOS FENÓLICOS E ATIVIDADES ANTIOXIDANTES (AOX) EM ACELGA (*BETA VULGARIS* L. VAR. CICLA) ELICITADOS COM PERÓXIDO DE HIDROGÊNIO EM CAMPO ABERTO

RESUMO

Objetivo: O principal objetivo deste estudo foi aprimorar as propriedades funcionais da acelga suíça (*Beta vulgaris* var. cicla L.) por meio da elicitação controlada com peróxido de hidrogênio (H_2O_2).

Referencial Teórico: A acelga é uma hortaliça folhosa altamente demandada devido ao seu baixo custo, facilidade de produção e alto valor nutricional, contendo minerais, açúcares solúveis, ácidos graxos, vitaminas e compostos fenólicos. As plantas respondem a esses estressores produzindo compostos antioxidantes (AOX) que ajudam a mitigar os danos causados pelo estresse. A aplicação exógena de peróxido de hidrogênio (H_2O_2) pode estimular a produção de metabólitos especializados e aumentar a capacidade antioxidante nos tecidos vegetais.

Método: As plantas de acelga suíça foram submetidas a sete concentrações de H_2O_2. Foram avaliadas a atividade das DPPH• e ABTS•+, catalase (CAT), superóxido dismutase (SOD), atividade enzimática da fenilalanina amônia-liase (PAL), teor e concentração total de compostos fenólicos, e concentração de nitratos.

Resultados e Discussão: Os resultados obtidos revelaram [sintetize os principais resultados da pesquisa]. Na seção de discussão, esses resultados são contextualizados à luz do referencial teórico, destacando-se as implicações e relações identificadas. Possíveis discrepâncias e limitações do estudo também são consideradas nesta seção.

Implicações da Pesquisa: Este estudo oferece uma abordagem alternativa para melhorar a qualidade de hortaliças folhosas utilizando um elicitor não tóxico e de baixo custo (H_2O_2). Os resultados sugerem que a aplicação controlada de H_2O_2 pode ser implementada na agricultura para estimular as respostas metabólicas das plantas, resultando em um maior teor de antioxidantes e melhores propriedades funcionais nas culturas.

Originalidade/Valor: Este estudo demonstra uma nova abordagem de bioestimulação que não compromete as concentrações de nitratos, abordando preocupações relacionadas ao acúmulo excessivo de nitratos em hortaliças folhosas.

Palavras-chave: Atividade Enzimática, Elicitação, Fator de eustresse, Nitratos, Peróxido de Hidrogênio.

COMPUESTOS FENÓLICOS Y ACTIVIDADES DE AOX EN ACELGA (*BETA VULGARIS* L. VAR. CICLA) ELICIDADAS CON PERÓXIDO DE HIDRÓGENO EN CAMPO ABIERTO

RESUMEN

Objetivo: El objetivo principal de este estudio fue mejorar las propiedades funcionales de la acelga suiza (*Beta vulgaris* var. cicla L.) mediante una elicitación controlada utilizando peróxido de hidrógeno (H_2O_2).

Marco Teórico: La acelga es un vegetal de hojas altamente demandado debido a su bajo costo, facilidad de producción y alto valor nutricional, que contiene minerales, azúcares solubles, ácidos grasos, vitaminas y compuestos fenólicos. Las plantas responden a estos factores de estrés produciendo compuestos antioxidantes (AOX) que mitigan los daños inducidos por el estrés. La aplicación exógena de peróxido de hidrógeno (H_2O_2) puede estimular la producción de metabolitos especializados y mejorar la capacidad antioxidante en los tejidos vegetales.

Método: Las plantas de acelga fueron sometidas a siete concentraciones de H_2O_2. Se evaluaron DPPH• y $ABTS^{\bullet+}$, catalasa (CAT), la superóxido dismutasa (SOD), fenilalanina amoníaco-liasa (PAL), el contenido y la concentración total de fenoles, y la concentración de nitratos.

Resultados y Discusión: El tratamiento con 75 mM de H_2O_2 actuó como un bioestimulante, aumentando significativamente la actividad antioxidante y la síntesis de compuestos fenólicos sin afectar negativamente la concentración de nitratos. La actividad enzimática de la PAL se vio aumentada, lo que indica una regulación positiva del metabolismo secundario, lo que llevó a niveles más altos de compuestos fenólicos y antioxidantes en la planta.

Implicaciones de la Investigación: Este estudio ofrece un enfoque alternativo para mejorar la calidad de los vegetales de hoja utilizando un elicitor no tóxico y rentable (H_2O_2). Los resultados sugieren que la aplicación controlada de H_2O_2 podría implementarse en la agricultura para mejorar las respuestas metabólicas de las plantas, lo que llevaría a un mayor contenido de antioxidantes y mejores propiedades funcionales en los cultivos.

Originalidad/Valor: Este estudio demuestra un enfoque novedoso de bioestimulación que no compromete las concentraciones de nitratos, abordando las preocupaciones relacionadas con la acumulación excesiva de nitratos en los vegetales de hoja.

Palabras clave: Elicitación, Factores de Eustrés, Actividad Enzimática, Peróxido de Hidrogeno, Nitratos.

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1 INTRODUCTION

Chard is a glycophytic plant found worldwide and is used as a leafy green vegetable in the human diet due to its health benefits and mineral composition. Its leaves are rich in volatile compounds, and its nutritional composition includes soluble sugars, dietary fiber, fatty acids, and minerals (Mzoughi et al., 2019). Additionally, its pigment composition consists of β-carotene, lycopene, and chlorophyll. Chard exhibits hypoglycemic effects on α-glucosidase and α-amylase activities, as well as antimicrobial activity. It is rich in Ca, Fe, K, and Mg. Furthermore, chard, a member of the Amaranthaceae family, provides provitamin A, vitamins such as K1, B2, B3, B5, B6, C, and E, and folates (Bozokalfa et al., 2011). Chard is widely consumed in traditional dishes and is also used as a medicinal plant for treating diabetes, cancer, heart, kidney, and liver diseases, as well as for stimulating the hematopoietic and immune systems (Bahremand 2018). The phenolic composition of Swiss chard includes phenolic acids, flavonoids, flavonols, tannins, and orthodiphenols. Among the phenolic acids, p-coumaric acid, myricitrin acid, and rosmarinic acid are also present (Mzoughi et al., 2019). Additionally, chard shoots contain high amounts of carotenoids (Barickman & Kopsell, 2016). Chard is highly demanded for food products, including infant formulations (Calderón et al., 2020), and is recognized for its sweetness, quality, and flavor attributes (Caracciolo et al., 2020).

On the other hand, elicitors are known to enhance the production of secondary and specialized metabolites in plants. These elicitors can be of biological origin, such as cells, molecules, and specific compounds. Hydrogen peroxide (H_2O_2) is part of cellular metabolism

and plays a role in cell defense systems and biosynthetic oxidative reactions (Vázquez-Hernández et al., 2019a). The aim of the present study was to evaluate the effect of exogenous applications of H_2O_2 on enzymatic defense-related parameters, such as total phenols, flavonoids, and antioxidant (AOX) capacity in chard.

2 THEORETICAL FRAMEWORK

H_2O_2 participates in signal transduction, activating gene expression, enzymatic activities, and apoptosis (Sun et al., 2016). Exogenous treatments with H_2O_2 can mitigate biotic stress factors such as salinity, drought, and heavy metal toxicity. Plant tissues can tolerate high concentrations of H_2O_2, up to 10×10^5 µM and show resistance to excess light and cooling stress (Sathiyaraj et al., 2014). 'Hormesis' in plants is a dose-response phenomenon where low doses have a positive effect, while high doses can be detrimental (Vargas-Hernandez et al., 2017). Other non-monotonic dose-response relationships, such as biphasic and multiphasic responses, differ from hormesis in that they lack a stimulatory effect and show a decrease in the damaging stress effect as the toxic dose increases. These are considered non-hormetic responses, or paradoxical effects (Erofeeva, 2020). Stress factors applied to chard have been shown to increase the production of specialized metabolites. For example, the evaluation of 100 kg N/ha of nitrogen fertilization resulted in superior fresh weight (FW), taller and leafier plants, and increased nitrate and ascorbic acid content and yield (Miceli and Miceli 2014). Exposure to yellow light (234 µmol $m^{-2}s^{-1}$) increased dry mass (Casierra-Posada et al., 2014). Moreover, chard has shown resistance to salt stress. The response to 50 and 100 mM of an alkaline salt mixture ($NaHCO_3$ and Na_2CO_3 in a 9:1 molar ratio) was determined in chard, where proline and glycinebetaine (GB) content increased. Treatments with GB enhanced glycinebetaine content to 224 µmol g^{-1} DW at 100 mM (Liu et al., 2013). Therefore, chard was selected as a model crop due to its nutritional value, being suitable for both human consumption and animal fodder. It is worth mentioning that overpopulation has led to increased production and a rise in the inherent nutrients of each species. This is essential to meet dietary requirements for tissue maintenance and restoration (Kant, 2018).

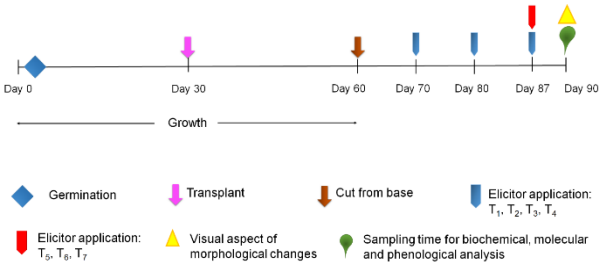
3 METHODOLOGY

3.1 PLANT MATERIAL AND GROWTH CONDITIONS

The substratum for seedlings was compound of earthworm humus, dry compost and vermiculite 2:1:1 and watered every day. Thirty-day-old seedlings were transplanted (Figure 1). The crop was cultivated in an open field. Grooves of 14 x 50 m were used with drip irrigation by gravity. The plants were irrigated each third day for one hour. During the experiment, a 21 °C average temperature, 5.80 mm of evaporation and it no was precipitation. After 60 d of transplanting, plant growth variables measured were leaf width and stem height in cm (measured with a graduated ruler), and plant weight in g (weighed with a balance Etekcity).

Figure 1

Experimental design. Plants were divided in eight blocks. Treatments were the following: T1- Control (distilled water), T2- 75 mM H_2O_2, T3- 150 mM H_2O_2, T4- 200 mM H_2O_2, T5- 225 mM H_2O_2, T6- 450 mM H_2O_2, T7- 600 mM H_2O_2.



3.2 STRESS TREATMENTS WITH EUSTRESS FACTOR APPLICATION

After 30 d of transplanting the chard plants were cut and sprayed with six doses of H2O2 and water distilled as control (Mejía-Teniente et al., 2019). Leaves samples were taken three-d post-elicitation and immediately frozen in liquid nitrogen and storage at −80°C until further biochemical and molecular determinations (Vázquez-Hernández et al., 2019b).

3.3 TOTAL POLYPHENOL CONTENT (TPC)

Chard leaves from each treatment were randomly selected, separated and ground in liquid nitrogen. Next, 1 g of chard leaves ground samples were macerated with methanol:water

in a 1:10 ratio (w/v). The suspensions were put in a sonicator (Bransonic M2800-CPX-HE, Emerson, Ferguson, Missouri, USA) for 30 min and then was centrifuged at 10,000 rpm for 15 min at 4 °C. The resultant supernatants were frizzed at 4 °C in dark vials until use. Total phenols were determined by the Folin-Ciocalteu method according to Vergara-Castañeda et al. (2010) employing gallic acid as standard. Shortly, 140 µL of the extract was mixed with 460 µL of distilled water and 250 µL of the Folin-Ciocalteu (1 N) reagent. After 5 min, 1250 µL of 20 % (w/v) sodium carbonate was added. The mixture was shaken in a vortex and incubated for 2 h in darkness. The absorbance was measured at 760 nm. The results were expressed as mg equivalent of gallic acid per gram of fresh weight (mg EGA/g).

3.4 ANTIOXIDANT ACTIVITY (AOX)

The antioxidant activity obtained from each treatment was evaluated through the 1,1-diphenyl-2-picrylhydrazyl (DPPH•) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) assays.

3.4.1 $DPPH^{\bullet}$ radical scavenging activity (DRSA) assay

Radical scavenging activity was verified according to the method by Chen et al. (2012), with a few modifications. 0.5 mL sample solution aliquot (600 µg mL^{-1}) was added to 0.5 mL of $DPPH^{\bullet}$ radical (0.1 mmol L^{-1}) in methanol solution. The mixture was shaken vigorously and incubated for 30 min in the dark at room temperature; absorbance was measured at 515 nm. Methanol, rather of $DPPH^{\bullet}$, was used for the blank, while deionized water, instead of sample, was used for the control. DRSA was calculated by the following equation (1):

$$DRSA\ (\%) = \left[1 - \left(A_{sample} - {}^{A_{blank}}/_{A_{control}}\right)\right] x100 \qquad (1)$$

3.4.2 $ABTS^{\bullet+}$ radical scavenging activity (ARSA) assay

ARSA was measured according to the method by Re et al. (1999), with modifications. The $ABTS^{\bullet+}$ radical cation was generated by mixing ABTS stock solution (7 mmol L^{-1}) with potassium persulfate (2.45 mmol L^{-1}) and the resulting mixture rested in the dark at room temperature for 12 h before use. The $ABTS^{\bullet+}$ radical solution was diluted in 0.15 mol L^{-1}

phosphate buffered saline (PBS) pH 7.4 (~ 1:35) to read an absorbance of 0.70 ± 0.02 at 734 nm. About 3 mL of this diluted solution was mixed with 150 µL of a sample (600-µg mL^{-1}). Absorbance was read under conditions of darkness. PBS, instead of $ABTS^{\bullet+}$ was used for the blank; while deionized water was used for the control. ARSA was calculated by the following equation (2):

$$ARSA\ (\%) = \left[1 - \left(A_{sample} - {}^{A_{blank}}/_{A_{control}}\right)\right] x100 \qquad (2)$$

3.5 ENZYME ACTIVITY ASSAYS

3.5.1 Samples preparation of enzymatic assays

Chard leaves dried samples (0.5 g) were homogenized with 1 mL of cold extraction buffer and the slurry was centrifuged at 13,000 rpm for 20 min at 4°C. The supernatants were used to measure the enzyme activities. Protein concentration in enzymatic extracts was determined according to the method reported by Bradford (1976), using bovine serum albumin as standard for protein concentration (Sigma-Aldrich, St. Louis, Missouri, USA).

3.5.2 Catalase activity assay

Catalase activity (CAT; EC 1.11.1.6) was assessed spectrophotometrically by measuring the decrease rate of H_2O_2 at 240 nm according to the method described by Afiyanti & Chen (2014). The reaction was carried out by adding 0.95 mL 50 mM potassium phosphate buffer (pH 8.0), 0.05 mL enzymatic extract and 0.1 mL 100 mM H_2O_2. The change in absorbance was measured at 240 nm, for 1 min, and estimated to determine the rate of decomposition of H_2O_2 by CAT. One unit of CAT decomposes 1 µmol of H_2O_2 per min at pH 8.0 and 25°C. The data were expressed as U/mg protein.

3.5.3 Superoxide dismutase activity assay

Superoxide dismutase activity (SOD; EC 1.15.1.1) was assessed by the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) according to the method described by Hayat et al. (2018). The reaction was realized by adding 1.5 mL 50 mM potassium phosphate

buffer (pH 7.8), 0.3 mL 0.1 mM EDTA, 0.3 mL 0.13 M methionine, 0.3 mL 0.02 mM riboflavin, 0.05 mL enzymatic extract and 0.25 mL distilled water. The mixture was exposed to fluorescent light (86.86 µmol $m^{-2} \cdot s^{-1}$) for 20 min. Subsequently, the solution absorbance was measured at 560 nm. One unit of SOD inhibits reduction of NBT by 50% at pH 7.8 and 25 °C. The data were expressed as U/mg protein.

3.5.4 Phenylalanine ammonia-lyase (PAL) activity

Phenylalanine ammonia-lyase activity (PAL; EC 4.3.1.5) was assessed spectrophotometrically according to the method described by Toscano et al. (2018). L-Phenylalanine was used as the substrate, and the released cinnamic acid was quantified by absorbance at 290 nm. The reaction was carried out by adding 2.3 mL of 0.1 M borate buffer (pH 8.8 containing 10 mM L-phenylalanine) and 0.2 mL enzymatic extract. The mixture was incubated at 40°C for 1 h and the reaction was stopped by the addition of 0.5 mL of 5 N HCl. Finally, the solution absorbance was measured at 290 nm. One unit of PAL releases 1 µmol of cinnamic acid per min at pH 8.8 and 40°C. The data were expressed as (U/mg protein).

3.6 SALICYLIC ACID METHOD FOR NITRATE DETERMINATION

Determination of nitrate content by the salicylic acid method was done as follow. Aliquots 0.2 ml of the extracts were pipetted into 50 mL Erlenmeyer flasks, and mixed thoroughly with 0.8 mL of 5% (w/v) salicylic acid in concentrated H_2S0_4 (SA-H_2SO_4). After 20 min at room temperature, 19 mL of 2N NaOH were added slowly to raise the pH above 12. Samples were cooled to room temperature and absorbance at 410 nm was determined in a spectrophotometer. Standards containing 1 to 60 µg NO^{-3}-N in a 0.2 mL aliquot were analysed. For extracts from lyophilized plant tissue, a blank of 0.2 ml H_2O and the normal reagents was normally sufficient. For fresh-tissue extracts, a separate blank was required for each sample because of pigmentation in the extracts. The blank consisted of the extract, 0.8 mL of concentrated H_2SO_4 (without salicylic acid), and 19 mL of 2N NaOH. Nitrate-N concentrations were expressed as µg NO^{-3}-N per g dry or fresh weight (ppm). The SA-H_2SO_4-reagent was made fresh at least once each week and stored in a brown bottle. Nitrate standards were stored at 4°C (Cataldo et al., 1975).

3.7 ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC) ANALYSIS

The phenolic composition was done according to Figueroa et al. (2018) with modifications. Shortly, 1 g of chard leaves ground samples were macerated with methanol:water in a 1:10 ratio (w/v). The aliquots were put in a sonicator (Bransonic M2800-CPX-HE, Emerson, Ferguson, Missouri, USA) for 30 min and then was centrifuged at 14,000 rpm for 15 min at 4 °C. The resultant supernatants were frizzed at 4 °C in dark vials until use. The extracts were reconstituted in ethanol-water (1:1, v/v, HPLC grade) at a concentration of 10,000 mg L^{-1} and filtered using an Acrodisc of 0.45 μm pore mesh and 25 mm diameter (Agilent Technologies). Chard leaves were analysed in triplicate and assessed in an Agilent 1260 series Rapid Resolution Liquid Chromatographer coupled to a diode-array detector (DAD) were used for the chromatographic determination. The chromatographic separation was performed in a Zorbax Eclipse Plus C18 column (4.6 × 150 mm, 1.8 μm) at a flow rate of 0.5 mL/min using an injection volume of 10 μL. The mobile phases were acidified water (0.1 % formic acid, v/v) and acetonitrile as solvent A and B, respectively. The following multi-step linear gradient was used in order to achieve efficient separation: 0.0 min [A:B 95/5], 25.0 min [A:B 50/50], 33.0 min [A:B 0/100], and 36.0 min [A:B 95/5]. Finally, initial conditions were kept for 4 min at the end of each analysis to equilibrate the system before the subsequent injection. The column temperature and auto-sampler compartment were set at 25 °C and 4 °C, respectively.

Detection was performed in negative ionization mode over a mass range from 100–1700 m/z, and the detection window was set to 100 ppm. Ultrahigh pure nitrogen was used as drying and nebulizing gas. The operating parameters were as follows: drying gas temperature, 325 °C; drying gas flow rate, 10 L min^{-1}; nebulizer pressure, 172 kPa; nebulizer gas temperature, 400°C; nebulizer gas flow, 12 L min^{-1}; capillary, 4000 V; fragmentor, 130 V; nozzle voltage, 500 V; skimmer, 45 V and octopole radiofrequency voltages, 750 V. In order to maintain mass accuracy during the runtime, all spectra were corrected by means of continuous infusion of Agilent TOF mixture containing two references masses: m/z 112.985587 (trifluoroacetate anion) and 1033.988109 (adduct of hexakis (1H,1H, 3H-tetrafluoropropoxy) phosphazine or HP-921). Both reference ions provided accurate mass measurements typically better than 2 ppm. All operations were processed through MassHunter Qualitative Analysis B.06.00 (Agilent Technologies, Palo Alto, CA, USA).

3.8 STATISTICAL ANALYSIS

A completely blocks randomized experimental design was used to evaluate the effect of foliar application of elicitors on morphological changes, AOX, phenolic compounds and enzymatic activity in chard leaves. The arrangement of the experiment was seven treatments with eight blocks and five experimental units per treatment. Data were subjected to analysis of variance (ANOVA) and Fisher´s least significant difference (LSD) tests ($P < 0.05$) using the Statgraphics® Centurion XVI statistical software (StatPoint Technologies Inc., Bedford, MA, USA, 2010).

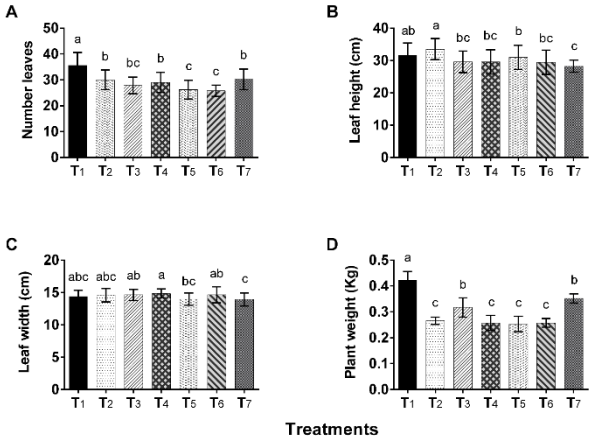
4 RESULTS AND DISCUSSIONS

4.1 INDUCTION OF PHENOLOGICAL CHANGES

H_2O_2 plays an important role as a disinfectant when is exposed to soil and organic matter, it releases oxygen and enhances the photosynthesis process. Additionally, it is a secondary product of photosynthesis in chloroplasts (Gollan & Aro, 2020). The results obtained show that exogenous application of H_2O_2 has a statistically significant effect on leaf height at the 75 mM treatment compared to others, but not compared to the control. In other phenotypic variables, such as the number of leaves and plant weight, the control treatment showed statistically better values than the others (Figure 2).The results and discussions of an article must be presented in a clear and organized manner, based on the data collected and the analyzes carried out during the study. Initially, the results must be presented in an objective and concise way, using tables, graphs and statistics, if applicable, to highlight the main findings. Then, in the discussion section, the results are interpreted in light of existing literature, highlighting similarities, differences and implications for theory and practice. Furthermore, limitations of the study and possible directions for future research are discussed. It is essential that both the results and the discussion are based on solid evidence and that they contribute significantly to the advancement of knowledge on the topic addressed.

Figure 2

Effect of H_2O_2 on morphological variables in chard cultivated after 60 days after transplanting in open field: A) number leaves, B) Leaf height, C) Leaf width and D) plant weight. T1- Control (distilled water), T2- 75 mM H_2O_2, T3- 150 mM H_2O_2, T4- 200 mM H_2O_2, T5- 225 mM H_2O_2, T6- 450 mM H_2O_2, T7- 600 mM H_2O_2. Different letters indicate the significance differences between means using Tukey test (α =0.05).



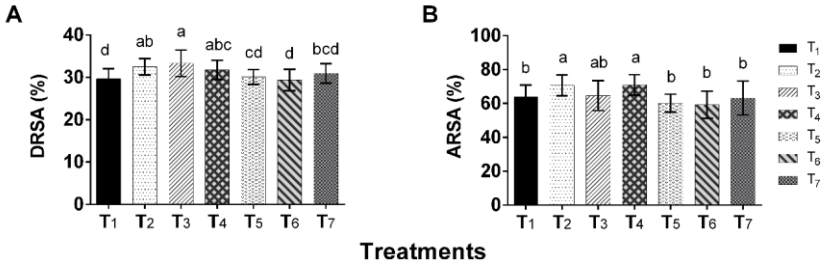
There is no research on the application of H_2O_2 in Swiss chard, but other stress factors have been studied with similar results. Liu et al. (2013) reported a decrease in Swiss chard growth under NaCl 50 mM (1.7%) and Na_2SO_4 50 mM (8.7%) concentrations. Bahremand (2018) determined the minimum seed germination temperature at 1.92°C, the optimal at 25°C, and the maximum at 45.84°C, with a pH of seven. In hydroponic crops, Barickman & Kopsell (2016) applied 25% NH_4-N: 75% NO_3-N (105 mg L^{-1} N), achieving higher fresh weight and dry matter accumulation compared to 50% NH_4-N: 50% NO_3-N, due to the preferential absorption of NO_3-N and its role in photosynthesis and nitrogen metabolism (Mzoughi et al., 2019). Additionally, Mzoughi et al. (2019) measured 0.21 mg 100 g^{-1} FW of chlorophyll a and 0.06 mg 100^{-1} g FW of chlorophyll b in wild Swiss chard leaves, essential for reducing ROS and protecting DNA from oxidative damage caused by H_2O_2. Ekinci et al. (2020) mitigated drought stress in chard with NO 100 and 150 µM, achieving the highest chlorophyll content with NO 150 µM (33% watered) and improved plant development. Schuller et al. (2018) evaluated soils with CsCl 100 kBq ^{134}Cs m^{-2}, finding a lower transfer factor in chard stalks than in leaves in K-fertilized umbric andosol soils. However, Swiss chard is considered a hyperaccumulator plant in shoots.

4.2 ANTIOXIDANT ACTIVITY (AOX)

The results of methanolic extracts of chad leaves shown AOX over increased of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals (Figure 3). The T2, T3 and T4 are the best treatments, as on DPPH as on ABTS being superior to the control less in ABTS were control and T3 have not significate difference.

Figure 3

A) $DPPH^{\bullet}$ radical scavenging activity (DRSA, %) and B) $ABTS^{\bullet+}$ radical scavenging activity (ARSA, %) of methanolic extracts from chard leaves. T1- Control (distilled water), T2- 75 mM H_2O_2, T3- 150 mM H_2O_2, T4- 200 mM H_2O_2, T5- 225 mM H_2O_2, T6- 450 mM H_2O_2, T7- 600 mM H_2O_2. Different letters indicate the significance differences between means using Tukey test (α =0.05). GAE (Gallic acid equivalents) where the experiments were conducted.



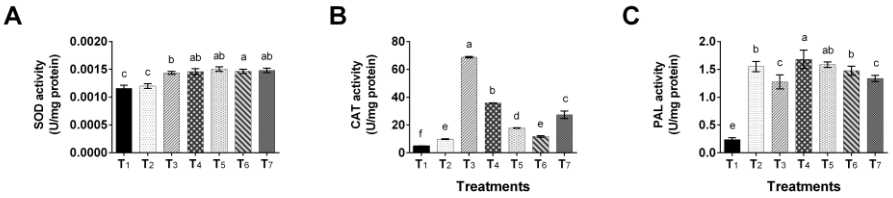
Mzoughi et al. (2019) reported in ethanolic extract leaf chard a $DPPH^{\bullet}$ (EC50= 0.75 mg mL^{-1}) and $ABTS^{+\bullet}$ (EC50= 1.22 mg mL^{-1}) generated AOX, to preserve themselves from oxidative pressure. AOX activity of phenols is obtained from the permanence of a hydroxyl group, consequently of the donation of protons to the free radicals and neutralizes them. The significant difference treatments between DRSA and ARSA suggest different origins of the AOX. As a stress, answer due to the hydrogen peroxide concentrations over Swiss chard leaves is called polynomial or non-monotomic. After the AOX determinations, it was checked if this activity was due to the enzymatic activities relates to stress that could act directly over them due to the hydrogen peroxide production or for the contrary, if it was an active answer which was concern of synthesis di novo of the plants on the secondary metabolites (Agathokleous, 2018).

4.3 ENZYMES ESSAYS

Climate conditions influence crop morphology, yield, and biochemical attributes, H_2O_2 mitigates stress by enhancing antioxidant enzyme activities (SOD, CAT, POD) and stimulating the phenylpropanoid pathway (PAL, CHI) (Vijayalakshmi et al., 2016). Exogenous H_2O_2 application on chard leaves produced significant differences between doses (Figure 4). SOD activity was highest at 225 mM H_2O_2, while CAT activity peaked at 150 mM H_2O_2. Similarly, PAL activity was significantly enhanced at 150 mM H_2O_2. All H_2O_2 treatments induced a significant SOD activity increase, except at 75 mM H_2O_2 (Figure 4A). CAT activity was higher in all treatments compared to the control (Figure 4B). PAL activity also showed a highly significant response in all treatments (Figure 4C). These results suggest an induced stress response through increased antioxidant activity and metabolite synthesis due to H_2O_2 exposure. This was consistent with the Ekinci et al. (2020) results, drought stress reduced SOD, CAT, and POD activity, with NO 150 µM through seed and foliar treatment in swiss chard was the most effective but negatively affecting plant weight.

Figure 4

Enzymatic activity in chard leaves after 60 days after transplanting post-elicitation with H_2O_2. A) SOD activity, B) CAT activity, C) PAL activity. T1- Control (distilled water), T2- 75 mM H_2O_2, T3- 150 mM H_2O_2, T4- 200 mM H_2O_2, T5- 225 mM H_2O_2, T6- 450 mM H_2O_2, T7- 600 mM H_2O_2. Different letters indicate the significance differences between means using Tukey test (α =0.05).



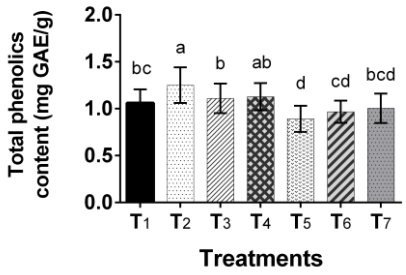
4.4 TOTAL PHENOLIC COMPOUNDS

In this study, the 75 mM H_2O_2 treatment resulted in the highest phenolic content in chard leaves (Figure 5). This is noteworthy since, at the same concentration, antioxidant enzyme activity was lower in the 75 mM treatment compared to others, suggesting a distinct stress response mechanism induced by exogenous H_2O_2 application. The results suggest that plants

maintain a precise balance, where low H_2O_2 dosages promote antioxidant capacity, but a gradual increase triggers de novo synthesis of secondary metabolites (Ekinci et al., 2020). Mzoughi et al. (2019) found 96.58 mg/g GAE of total phenolics in wild Tunisian Swiss chard, higher than in this study; also identified myricitrin acid (4.08 mg/g extract) and p-coumaric acid (3.53 mg/g extract) as major compounds.

Figure 5

Total polyphenols content (TPC, mg GAE/g) of methanolic extracts from chard leaves. Results are plotted as mean ± standard deviation. T1- Control (distilled water), T2- 75 mM H_2O_2, T3- 150 mM H_2O_2, T4- 200 mM H_2O_2, T5- 225 mM H_2O_2, T6- 450 mM H_2O_2, T7- 600 mM H_2O_2. Different letters indicate the significance differences between means using Tukey test (α =0.05). GAE (Gallic acid equivalents).



4.4.1 Individual phenolic compounds identification

The analysis of phenolic compounds by UPLC was done only in treatments T1 (distiller water), T2 (75 mM H_2O_2) and T4 (200 mM H_2O_2). Due to the treatments, two and four were the ones with the highest PAL activity and the highest amount of phenols obtained in the previous analyses. In this study, ellagic acid and catechin were identified through a comparison with the retention time of standards analysed as shown in Table 1 (peak 1: 2.4 min and peak 2: 10.8 min) which agrees with other reports (Ninfali & Angelino, 2013).

Table 1

Phenolic profile of ethanolic extracts from Swiss chard leaves elicitated (μg/g).

No.	Compound	Retention time (min)	T1 - Control	T2 - 75 mM	T4 - 200 mM
1	Ellagic acid	2.42	$12.39±1.44^b$	$23.03±2.16^a$	$11.35±1.66^b$
2	Gallic acid	7.49	$1.5±0.25^b$	$0.61±0.11^c$	$1.84±0.07^a$
3	4-hydrophenylacetic acid	9.81	$0.23±0.07^c$	$0.75±0.09^a$	$0.57±0.07^b$
4	Catechin	10.84	$2.88±0.6^c$	$15.35±1.21^a$	$6.13±0.51^b$
5	Epicatechin gallate	11.79	$1.97±0.2^c$	$14.77±1.02^a$	$6.2±0.89^b$

Different letters indicate the significance differences between means using Tukey test (α =0.05).

Ellagic acid was the most abundant phenolic acid in T2 (23.03 µg/g extract), followed by gallic acid (1.84 µg/g extract), with values similar to those reported by Mzoughi et al. (2019) for Swiss chard. Additionally, nitrate content was analyzed in T1, T2, and T4 to determine whether H_2O_2 application influenced nitrate levels in Swiss chard (Table 2).

Table 2

Level of nitrates presents in Swiss chard leaves elicitated.

Treatments	T1	T2	T4
Nitrates (g/100 g)	13.74 ± 1.22^{b}	11.82 ± 1.12^{c}	16.27 ± 0.51^{a}

Different letters indicate the significance differences between means using Tukey test (α =0.05).

NO_3^- is a primary nitrogen source for plants, essential for photosynthesis, biomass production, and gene regulation related to transport and assimilation. Additionally, H_2O_2 activates defense mechanisms via redox-signaling, while NO_3^-, formed from NO_2^-, can trigger protective responses under stress conditions. Nitrate accumulation in plants depends on soil nutrients, fertilizer application timing, and light intensity, with low light favoring accumulation due to reduced nitrate metabolism (Bonasia et al., 2017). The analyzed variables showed non-monotonic dose-response relationships, where higher H_2O_2 doses did not always yield the lowest results. This aligns with previous studies, like Erofeeva (2020) found non-monotonic responses in *B. pendula*, *T. cordata*, and *T. officinale* under various pollutant doses. (Agathokleous, 2018) observed U-shaped dose-response curves in *P. vulgaris*, *F. crenata*, *H. vulgare*, and *H. annuus* under different abiotic stresses exhibited hormesis responses.

5 CONCLUSION

Under the experimental conditions, the results suggest that peroxide hydroxide application confer highest functional potential to the chard. This highest potential is due to that is induced highest antioxidant capacity for ABTS and DPPH, highest phenols quantity without affecting weight neither number of leaves that are important variables due the chard in yield production. From the all treatments, the one with 75 mM confer the results previously pointed on chard and can be increased the antioxidant capacity. This treatment did not affect nitrates concentration, also in this treatment were shown the highest quantification of phenols.

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