

Article

# Changes in the Content of Free Phenolic Acids in Maize and Sunflower Leaves Treated with Sodium Cholate

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**Abstract:** Maize (*Zea mays* L.) and sunflower (*Helianthus annuus* L.) seedlings were treated with sodium cholate (NaC, concentrations 20, 40, 60, and 80 mg L<sup>-1</sup>), and the content of free phenolic acids in leaf samples was determined. The hypothesis of this work was that NaC causes oxidative stress in these seedlings and thus activates the plant's defense response in the form of increased synthesis of secondary biomolecules, phenolic acids, which participate in the removal of free radicals and the mitigation of oxidative stress. The idea was that bile salt, with elicitor activity, could influence the preparation of the plant's defense system for a subsequent stress. The determination of nine phenolic acids (gallic acid—GA, protocatechuic acid—PCA, chlorogenic acid—CHA, *p*-hydroxybenzoic acid—PHBA, caffeic acid—CA, vanillic acid—VA, syringic acid—SA, *p*-coumaric acid—*p*-CA, ferulic acid—FA) was performed by the HPLC method. The results showed increased concentrations of GA, PCA, VA, SA, *p*-CA, and FA in maize leaves treated with 60 and 80 mg L<sup>-1</sup> NaC compared to the control, after the 5th and 7th days. In sunflower leaves, this pattern only appears in PCA and PHBA. It was also shown that CHA was the most abundant free phenolic acid in maize treated with NaC, and CHA, PHBA, and SA in sunflower. Concentrations of 40 and 60 mg L<sup>-1</sup> NaC induced the highest amount of free phenolic acids in maize, and 20 and 40 mg L<sup>-1</sup> NaC in sunflower.

**Keywords:** bile acids; elicitor; oxidative stress

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## Introduction

Phenolic acids are secondary metabolites of plants with various functions and are the most widespread group of phenolic compounds. Their role in a plant's nutrition, fertility, growth, and

protection has been well known for centuries ([Pratyusha, 2022](#)). These compounds are very important for plants because they participate in defense mechanisms in various ways. They exhibit antioxidant activity as reducing agents, free radical scavengers, and quenchers of singlet oxygen formation, as well as other activities, such as anticarcinogenic activity ([Ghasemzadeh and Ghasemzadeh, 2011](#)). The antioxidant activity of phenols is reflected in their ability to donate hydrogen or an electron that delocalizes within the aromatic structure ([Fernandez-Panchon et al, 2008](#)).

Various abiotic stresses that affect plants in the field and disrupt their growth and development, and that affect crop yield (e.g., extreme temperatures, salt stress, drought, and heavy metals), lead to an imbalance in the metabolism of reactive oxygen (ROS) and nitrogen (RNS) species. This leads to oxidative stress, damage to macromolecules, but ultimately to cell death. The plant's defense response is then activated, initiating the synthesis of metabolites involved in defense—secondary biomolecules ([Chaki et al, 2020](#)).

It has been found that bile acids trigger plant defense responses by acting as elicitors ([Koga et al, 2006](#); [Zarattini et al, 2017](#)). Although they do not originate from plants, but only from human or animal metabolism, bile acids could be very useful for protecting plants from pests, especially because this biochemical approach is more environmentally friendly than the pesticides used today. The use of bile acids to protect plants from disease is based on their availability, as they are derived from natural sources (e.g. manure), which could significantly support sustainable agriculture. So far, the effects of cholic and deoxycholic acid have been examined. It has been found that the salt of cholic acid, sodium cholate (NaC), triggers oxidative stress in some plants ([Malenčić et al, 2012](#)), and it has been hypothesized that by inducing mild oxidative stress in maize through the treatment with NaC, subsequent (e.g., biotic) stress can be reduced and a faster defense response can be achieved. However, this particular study addresses salt stress induced in maize and sunflower seedlings by treatment with NaC.

Maize (*Zea mays* L.) and sunflower (*Helianthus annuus* L.) were selected for this study due to their widespread use worldwide and high nutritional value. Maize is rich in carbohydrates, making it a good source of energy, and it is also beneficial for human nutrition because it contains various proteins, fats, fibers, vitamins, etc. ([Saeed and Saeed, 2020](#)). Sunflower is the main oilseed crop. Its cultivation is important for the production of sunflower oil, which is highly represented in the human diet ([Belikina, 2021](#)). Various pests negatively affect the yield of these crops, and it is desirable to find a healthier approach to addressing this problem rather than relying on harmful pesticides to increase yields. So, the idea was to induce mild oxidative stress in these crops at early stages (while they are still seedlings) by treating them with NaC, which would prepare the plants for possible pathogen infection by inducing the production of secondary defense biomolecules. This treatment with NaC could be applied preventively to seedlings to prevent later infections and increase maize and sunflower yields.

In this study, changes in the content of free phenolic acids in maize and sunflower leaves treated with different concentrations of NaC were examined to determine the effect of NaC on plant secondary metabolism and on free phenolic acid content as one of the parameters of oxidative stress. After this experiment, the next one is planned, in which plants will be treated with NaC and then infected with pathogenic microorganisms to test whether NaC enhances the plant's faster defense against pathogens.

## Materials and Methods

### *Experiment setup and treatment*

Laboratory-grown seedlings were used for this study. Maize and sunflower seeds were sterilized with 1.8% NaClO for 5 min ([Sun et al, 2007](#)) and sown in sand preheated for 2 h at 180 °C. According to the method of [Malenčić et al.](#) (2012), after seven days, the seedlings were transferred to pots with Hoagland's nutrient solution (HS), which consisted of several components ([Table 1](#)) dissolved in water.

Maize seedlings were grown in HS for an additional week before treatment with NaC, and sunflower seedlings for an additional two weeks.

**Table 1:** The components of HS used for growing plants.

No.	Component	Concentration (g L <sup>-1</sup> )
1.	MgSO <sub>4</sub> ·7H <sub>2</sub> O	24.65
2.	Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	70.85
3.	KH <sub>2</sub> PO <sub>4</sub>	13.6
4.	KNO <sub>3</sub>	10.11
5.	<u>FeEDTA:</u>	
	• FeSO <sub>4</sub> ·7H <sub>2</sub> O	2.78
	• Na <sub>2</sub> EDTA·2H <sub>2</sub> O	3.73
6.	Microelements:	
	• H <sub>3</sub> BO <sub>3</sub>	0.2845
	• MnSO <sub>4</sub> ·4H <sub>2</sub> O	0.152
	• ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.23
	• CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.0075
	• (NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	0.099

Concentrations of 20, 40, 60, and 80 mg L<sup>-1</sup> NaC in HS were prepared. Sodium cholate was used instead of cholic acid due to its higher water solubility. Two-week-old maize seedlings and three-week-old sunflower seedlings were transferred to prepared NaC solutions, where the seedlings were grown for 1, 3, 5, or 7 days when leaf sampling was performed. The control seedlings were not treated with NaC and grew only in HS. Therefore, at each sampling time, there was one control and four treatments, so there were a total of 20 samples, each with three replicates.

### ***Sample preparation***

The collected leaves were frozen at -70 °C, and lyophilized the next day at -56 °C and 1 bar for 24 h. Then, 0.02 g of lyophilized leaf samples were weighed in triplicate. One mL of 50% methanol was added, and the samples were left in the refrigerator overnight. After that, another 1.5 mL of 50% methanol was added, placed on a vortex, an ultrasonic bath for 30 min, and then centrifuged at 4000 rpm for 5 min. The supernatant was separated, and an additional 1 mL of 50% methanol was added to the remaining precipitate, mixed on a vortex, centrifuged again, and the supernatant was separated again. The procedure was repeated once more, three times in total. The collected fractions of the water-methanol mixture were evaporated to dryness using a rotary vacuum evaporator (RVC 2-25 CDplus, Martin Christ, Osterode am Harz, Germany). The dry residue was dissolved in a mixture of methanol: 0.3% acetic acid (50:50, v/v), maize in 1.5 mL of the mixture, and sunflower in 1 mL, so the final weight/volume ratio for the maize was 1:75 (g mL<sup>-1</sup>) and for the sunflower 1:50 (g mL<sup>-1</sup>). The samples were filtered through a syringe filter (Chromafil Xtra RC-45/25, 0.45 µm, Ø25, Macherey-Nagel, Düren, Germany) ([Kurilich and Jurik, 1999](#)).

### ***HPLC method***

Phenolic acids were separated and quantitatively determined by the HPLC method. The HPLC instrument SpectraSystem (Thermo Fisher Scientific, Inc., Waltham, MA, USA) consisted of a quaternary gradient pump (P4000), a degasser (SCM1000), an autosampler (AS3000) with a column heater, a UV/Vis detector (UV2000), and an FL detector (FL3000). ChromQuest 5.0 Software was used for data

processing. Gradient elution mode was used with a mixture of 0.3% acetic acid and methanol as the mobile phase (Table 2). A Hypersil BDS C18 column (250 × 4.6, 5 μm) was used as the stationary phase. Phenolic acids were monitored at 280 and 320 nm using a UV/Vis detector and with excitation at 266 nm and emission at 350 nm using the FL detector.

**Table 2.** Percentage of mobile phase components (gradient elution)

<b>t (min)</b>	<b>0.3% Acetic acid (%)</b>	<b>Methanol (%)</b>
0	93	7
7	80	20
12	65	35
18	65	35
20	40	60
25	20	80
27	0	100
30	0	100
35	93	7
42	93	7

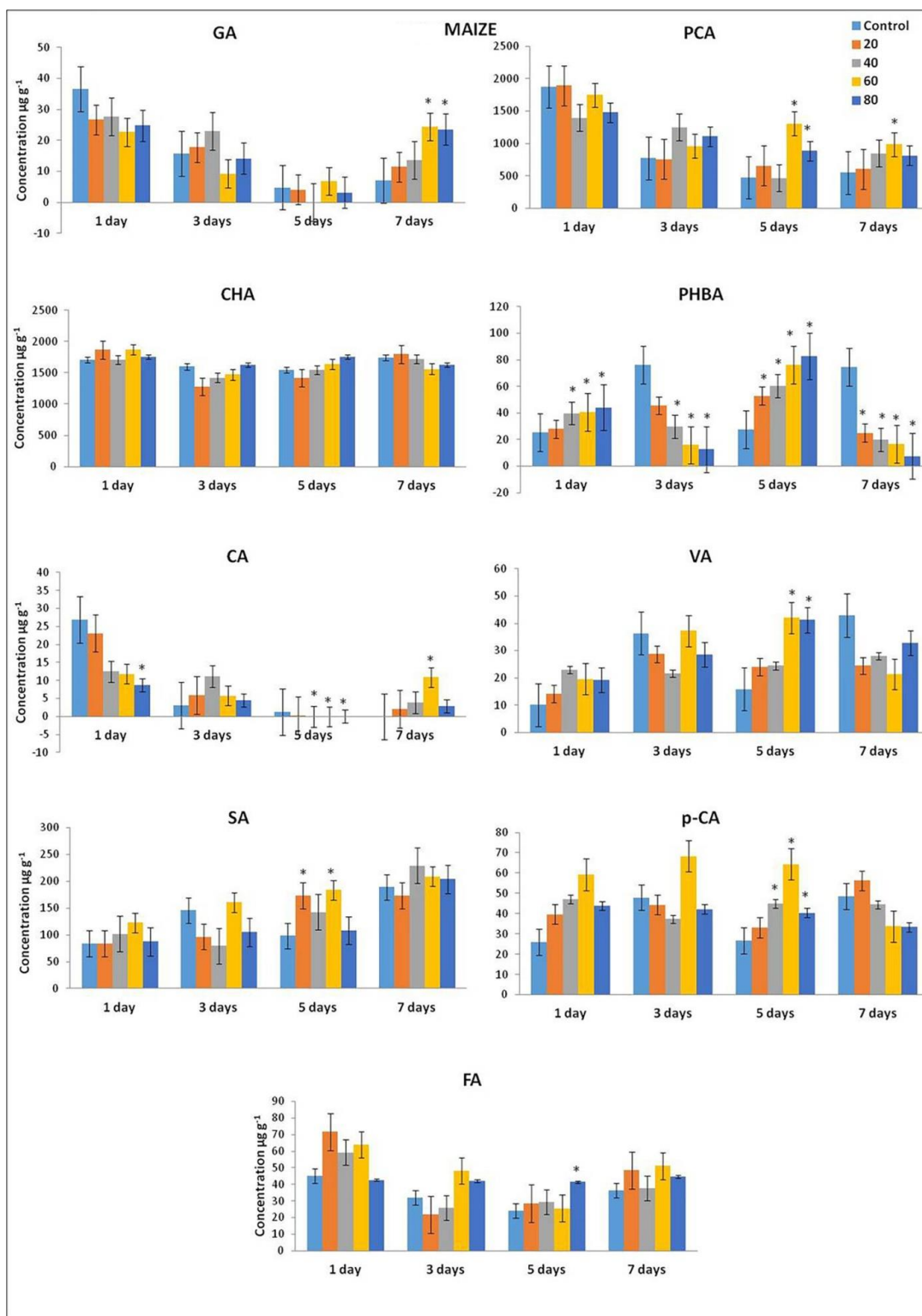
The injection volume was 20 μL, the tray temperature was 20 °C, and the column temperature was 30 °C. The mobile-phase flow rate was 0.6 mL min<sup>-1</sup>, and the analysis was completed after 42 min. Nine standard solutions in the concentration range of 0.1–16 μg/mL were used for chromatographic identification and for constructing calibration curves, which were used for the quantification of the analyzed phenolic acids (Table S1 and Figure S1 in Supplementary data): gallic acid (GA), protocatechuic acid (PCA), chlorogenic acid (CHA), caffeic acid (CA), *p*-coumaric acid (*p*-CA), and ferulic acid (FA) monitored at UV/Vis detector, vanillic acid (VA) at FL detector, and *p*-hydroxybenzoic acid (PHBA), and syringic acid (SA) at both detectors.

### **Statistical analysis**

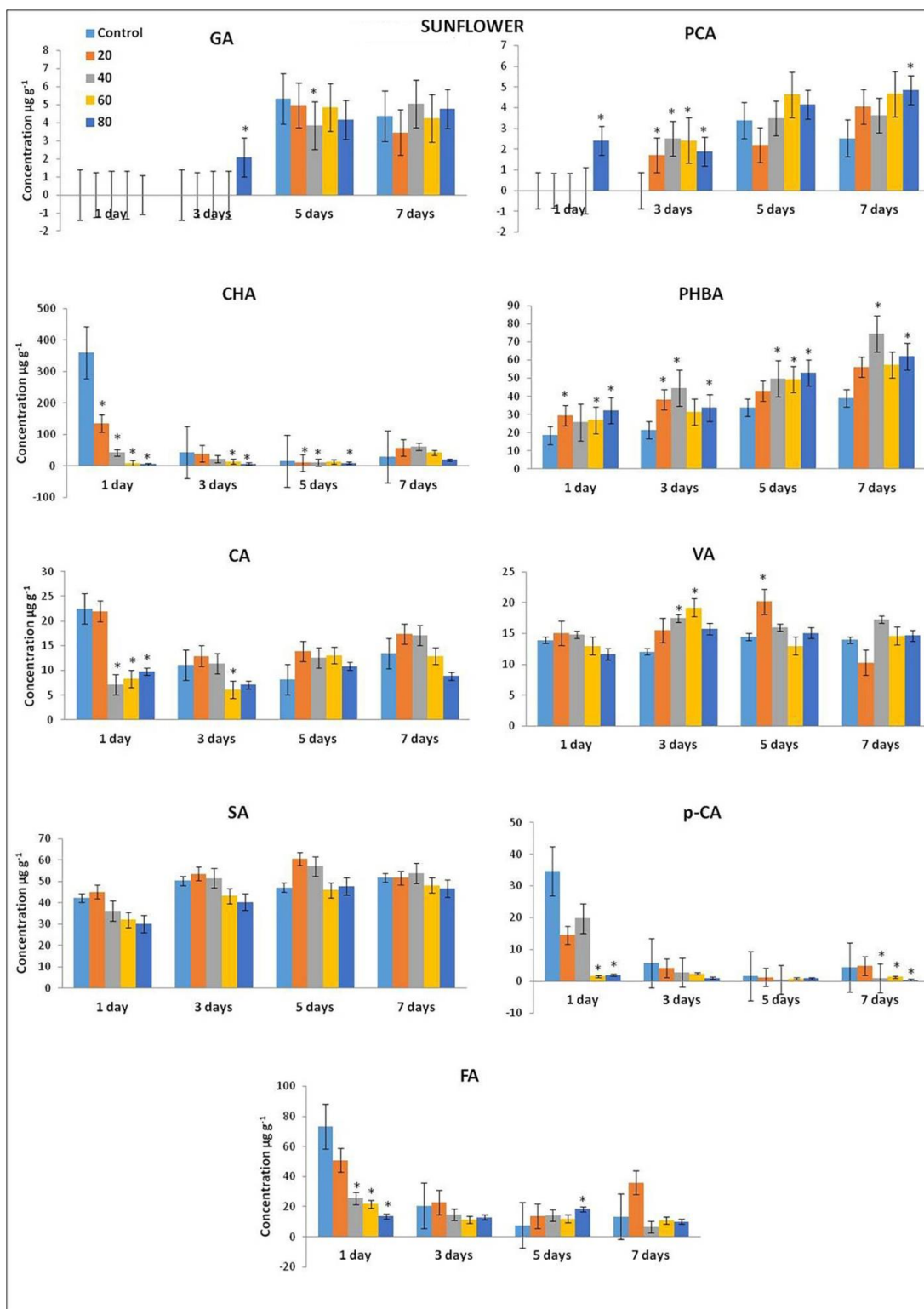
One sample consisted of eight seedlings per pot, and each pot represented one of three replicates. All results are presented as mean ± standard error, *n* = 3. One-way ANOVA analysis and Fisher LSD test were used to determine a statistically significant difference between treatments and controls. The software Statistica – 14.0.0.15 (TIBCO Software Inc., Palo Alto, CA, USA) was used, and *p*-values < 0.05 were considered significant.

### **Results**

The numerical values of the peak areas and concentrations of all nine measured phenolic acids are given in Table S2 and Table S3 in the Supplementary data. Figures 1 and 2 show the concentration profiles over seven days of maize and sunflower treatment with 20, 40, 60, and 80 mg L<sup>-1</sup> NaCl.



**Figure 1.** Concentration ( $\mu\text{g g}^{-1}$ ) of phenolic acids in maize leaves treated with 20, 40, 60, and 80  $\text{mg L}^{-1}$  sodium cholate (NaC), during seven days. The results marked with an asterisk are statistically significantly different from the control ( $p < 0.05$ ). The vertical lines on each bar of the histogram represent the standard error. Abbreviations: GA—gallic acid, PCA—protocatechuic acid, CHA—chlorogenic acid, PHBA—*p*-hydroxybenzoic acid, CA—caffeic acid, VA—vanillic acid, SA—syringic acid, *p*-CA—*p*-coumaric acid, FA—ferulic acid.



**Figure 2.** Concentration ( $\mu\text{g g}^{-1}$ ) of phenolic acids in sunflower leaves treated with 20, 40, 60, and 80  $\text{mg L}^{-1}$  sodium cholate (NaC), during seven days. The results marked with an asterisk are statistically significantly different from the control ( $p < 0.05$ ). The vertical lines on each bar of the histogram represent the standard error. Abbreviations: GA—gallic acid, PCA—protocatechuic acid, CHA—chlorogenic acid, PHBA—*p*-hydroxybenzoic acid, CA—caffeic acid, VA—vanillic acid, SA—syringic acid, *p*-CA—*p*-coumaric acid, FA—ferulic acid.

In maize leaves ([Figure 1](#)), the concentration of GA was significantly higher compared to the control only after seven days of treatment with 60 and 80 mg L<sup>-1</sup> NaC. PCA concentration was also significantly increased only after five and seven days compared to the control and after treatment with higher NaC concentrations (after 5 days with 60 and 80 mg L<sup>-1</sup> and after 7 days with 60 mg L<sup>-1</sup>). A similar pattern is observed for VA, SA, *p*-CA, and FA. The concentrations of these four phenolic acids were also significantly increased compared to the control after the 5th day of treatment, mainly with higher concentrations of NaC (60 and/or 80 mg L<sup>-1</sup>), and in the case of SA and *p*-CA, also after the treatment with one of the lower concentrations (20 or 40 mg L<sup>-1</sup>). The remaining phenolic acids, CHA, PHBA, and CA, are exceptions to that pattern. Namely, none of the NaC treatments caused a significant change in CHA concentration compared to the control during the entire treatment period. Then, the concentration of PHBA was higher compared to the control after the 1st and 5th days of the treatment, whereas after the 3rd and 7th days it was lower, with the difference being statistically significant across almost all NaC concentrations. Finally, the concentration of CA was significantly lower compared to the control after the 1st day of treatment with 80 mg L<sup>-1</sup> NaC. It was not detected after the 5th day of treatment with 40, 60, and 80 mg L<sup>-1</sup> NaC, but increased after the 7th day after treatment with 60 mg L<sup>-1</sup> NaC. Catechin (CTH) was also tested but was not detected in any maize or sunflower samples; therefore, it was excluded from the results of this study.

Unlike maize, in sunflower leaves ([Figure 2](#)), there was no recurring pattern in the concentration of phenolic acids. However, one similarity observed was that no significant difference in SA concentration was found between control and treated sunflower samples, as was the case for CHA in maize. The concentrations of PCA, PHBA, and VA were increased compared to the control, but these increases were not strictly at the same sampling times or due to treatment with the same concentrations of NaC. In this respect, the results of sunflower differ from maize, since the increase in the concentration of most phenolic acids in maize mainly occurred after 5 and/or 7 days and only at higher NaC concentrations. Another difference was that, for four out of nine phenolic acids, a decrease was measured in sunflower compared to the control. More precisely, there were increased concentrations of CHA, CA, *p*-CA, and FA (but only after the first day). GA was not detected in sunflower after the 1st and the 3rd day, except for the treatment with 80 mg L<sup>-1</sup> after the 3rd day, when the GA concentration was increased compared to the control, and the same was noted for PCA concentration after the 1st day of treatment with the same concentration of NaC.

## Discussion

As expected, given their different genetic backgrounds, the results from maize and sunflower samples obtained in the present study are different. Six out of nine measured phenolic acids (GA, PCA, VA, SA, *p*-CA, and FA) in maize have one common pattern, which is significantly increased concentrations in the second half of the experiment (after five and seven days), only after treatment with higher concentrations of NaC. This pattern cannot be applied to sunflower samples, except for PCA and PHBA. However, unlike maize, sunflower had several undetectable phenolic acids, e.g., GA after one and three days of the treatment and PCA after one day. This confirms that the same source of stress activates different metabolic responses in different plants.

Plants have evolved metabolic pathways to combat various stresses, particularly through the biosynthesis of secondary metabolites, which are normally present in plants in certain amounts, regardless of whether the plants are under stress ([Mattila and Kumpulainen, 2002](#)). Other authors have also determined the phenolic profiles of maize and sunflower across different genotypes or following exposure to specific abiotic stresses. [Mesarović et al. \(2017\)](#) determined the free phenolic acid profile in maize leaves with different grain colors. They separated and detected five free phenolic acids, GA, PCA, CA,

*p*-CA, and FA, with the last three being the most abundant in maize leaves. According to the literature, the examination of the phenolic profile in sunflower leaves is not as extensive as in florets (Ye et al, 2015), kernels, and shells (Weisz et al, 2009). Furthermore, Pajak et al. (2014) found that free phenolic acids CA, CHA, and GA occur in the highest amounts in sunflower seeds and sprouts. In the present study, free phenolic acids CHA, PCA, and SA were most abundant in untreated maize seedlings (control, after seven days), and SA, PHBA, and CHA in untreated sunflower seedlings.

When a plant is exposed to abiotic stress, it activates a range of biochemical responses that enhance the synthesis and accumulation of phenolic compounds as part of its defense mechanisms. An increase in phenolic acids and other compounds due to drought stress was found in the leaves of the *Amaranthus tricolor* (Sarker and Oba, 2018). Additionally, drought stress led to the accumulation of PHBA and VA in rice leaves compared to the control (Quan et al, 2016), which is consistent with the results of the present study for both maize and sunflower after treatment with NaC. Salt stress is known to induce the production of ROS in plants, leading to oxidative stress (Chowdhary et al, 2022). An increased concentration of phenolic acids may be associated with increased free radical production and oxidative stress, as their function is to combat this condition. It has also been found that salt stress causes the accumulation of phenolic acids, as reported by Minh et al. (2016) who obtained an increase in VA, PCA, FA and *p*-CA in rice, and by Mohammadkhani (2018) who obtained an increase in GA, PHBA, VA, SA, CA, *p*-CA, FA and others in grapevine leaves and roots after treatment with 50 mM NaCl. In the present study, in maize leaf samples treated with all four concentrations of NaC (20, 40, 60, and 80 mg L<sup>-1</sup>) and at all four sampling times (1, 3, 5, and 7 days), CHA had the highest concentration, and in sunflower leaf samples, CHA, PHBA, and SA had the highest concentration.

In Table 3, the average concentrations of all free phenolic acids measured in maize and sunflower leaves, after seven days of treatment with 20, 40, 60, and 80 mg L<sup>-1</sup>, are shown. By comparing how certain concentrations of NaC affected phenolic acids, it was observed that the highest concentrations of free phenolic acids were induced by 40 and 60 mg L<sup>-1</sup> in maize, and 20 and 40 mg L<sup>-1</sup> in sunflower. Thus, low to moderate concentrations of NaC induced the highest levels of free phenolic acids in these crops.

**Table 3.** Average values of all phenolic acid concentrations (µg g<sup>-1</sup>) induced by different concentrations of NaC in maize and sunflower, seven days after treatment.

	20 mg L <sup>-1</sup> NaC	40 mg L <sup>-1</sup> NaC	60 mg L <sup>-1</sup> NaC	80 mg L <sup>-1</sup> NaC
Maize	304.5	327.5	323.6	309.9
Sunflower	26.8	26.7	21.9	19.1

## Conclusions and Recommendations

The results of this study showed that maize and sunflower respond differently to NaC. In maize, the defense response appears to be activated only after the 5th day of treatment and only at higher concentrations of NaC, which is not the case for sunflower. The study also confirmed that NaC increases the content of certain phenolic acids in maize and sunflower leaf samples compared to the control, indicating oxidative stress in the tested plants. Moreover, it was shown that CHA was the most abundant free phenolic acid in maize and (with PHBA and SA) in sunflower, after treatment with NaC. Other than that, 40 and 60 mg L<sup>-1</sup> NaC induce the highest amount of free phenolic acids in maize leaves, and 20 and 40 mg L<sup>-1</sup> in sunflower leaves. The results of this study indicate that these NaC concentrations have potential as a sustainable protective treatment to maize and sunflower when applied in the field at early growth stages, as they induce mild oxidative stress that enhances plant defense against subsequent pest attacks.

**Supplementary materials:** Table S1: Data for the calibration curve of standard phenolic compounds. Their initial concentration was 1 mg mL<sup>-1</sup>; Table S2: Peak areas and concentrations of phenolic

compounds in maize leaves from seedlings treated with sodium cholate (20, 40, 60, and 80 mg mL<sup>-1</sup>); Table S3: Peak areas and concentrations of phenolic compounds in sunflower leaves from seedlings treated with sodium cholate (20, 40, 60 and 80 mg mL<sup>-1</sup>); Figure S1: Calibration curves of standard solutions for determining phenolic profiles.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Author Contributions:** JŠE developed the original idea and conceptualized the manuscript, MC carried out the field measurements, MC and KK performed laboratory analysis, MC and KK processed the data and performed the statistical analysis, JŠE and KK performed review and editing, JŠE supervised the research and helped to draft the manuscript, MC wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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