Biol. Stud. 2024; 18(2): 125–137 • doi:https://doi.org/10.30970/sbi.1802.778
www.http://publications.lnu.edu.ua/journals/index.php/biology

UDC: 57.04: 581.1

# THE EFFECT OF SALICYLIC ACID ON THE CONTENT OF ASCORBIC ACID AND PHENOLIC COMPOUNDS IN WHEAT PLANTS

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Kobyletska, M., & Kavulych, Y. (2024). The effect of salicylic acid on the content of ascorbic acid and phenolic compounds in wheat plants. *Studia Biologica*, 18(2), 125–137. doi:10.30970/sbi.1802.778

**Background.** Salicylic acid is an important phytohormone in plants, influencing various functions such as senescence, respiration, and stress resistance. Despite extensive studies, the role of salicylic acid in stress, its effects under normal conditions are poorly understood. This study explores the influence of salicylic acid on the biosynthesis of important biochemical compounds such as ascorbic acid, rutin, and other phenolic compounds in wheat (*Triticum aestivum* L.), aiming to elucidate potential applications in agriculture.

**Materials and Methods.** Wheat variety “Podolyanka” was treated with 0.05 mM salicylic acid and grown under controlled conditions. Biochemical analyses were conducted on the 7th, 10th and 20th days of growth using the spectrophotometric method for the determination of ascorbic acid, rutin, total phenolic compounds, anthocyanins, flavonoids, and xanthones. Methods included chromatography on the plate with silicagel for rutin.

**Results and Discussion.** Salicylic acid treatment significantly increased the ascorbic acid content in wheat shoots at all studied stages. There was also a notable increase in rutin content in the early growth phase. However, the content of other phenolic compounds, such as xanthones, generally decreased under salicylic acid treatment. Interestingly, anthocyanin content was increased, suggesting a complex interaction within the biosynthetic pathways influenced by salicylic acid. The study also revealed correlations among different phenolic compounds, indicating intertwined metabolic pathways.

**Conclusion.** Salicylic acid enhances the biosynthesis of specific phenolic compounds such as ascorbic acid and rutin in wheat, which can have implications for

agricultural practices aiming at improving plant resilience and nutritional quality. The differential impact of SA on various phenolic compounds underscores the complexity of plant biochemical pathways and emphasizes the need for further research to fully understand these interactions and their practical applications.

***Keywords:*** *Triticum aestivum* L., salicylic acid, phenolic compounds, ascorbic acid, rutin, anthocyanins, xanthones, flavonoids

## INTRODUCTION

Salicylic acid (SA) is an important phytohormone with multifunctional stress-protective properties, the effect of which has been actively studied in recent years (Koo *et al.*, 2020; Yang *et al.*, 2023). Being an endogenous signal molecule of phenolic nature, it participates in various physiological processes of plants, such as senescence, closing and opening of stomata, respiration, photosynthesis, seed germination and formation of resistance to biotic and abiotic stressors (Kaya *et al.*, 2023; Torun *et al.*, 2022). Most researchers investigate the processes that occur in plants during SA treatment under the influence of stress factors that change the functioning of the plant organism (Khan *et al.*, 2015; Song *et al.*, 2023; Yang *et al.*, 2023; Sangwan *et al.*, 2022). However, the effects of SA on plant metabolism under normal conditions remain poorly understood. A deeper insight into these mechanisms will expand the understanding of the effects of SA on plants and allow for its wider application in agricultural practice. Considering this, the aim of our work was to investigate the influence of SA on the content of such biologically active compounds as ascorbic acid, rutin, flavonoids, anthocyanins, xanthones and the total content of phenolic compounds in wheat plants.

## MATERIALS AND METHODS

**Plant materials and growth conditions.** Wheat plants (*Triticum aestivum* L.) var. Podolyanka (Institute of Plant Physiology and Genetics of the National Academy of Science of Ukraine, V. M. Remeslo Myronivka Institute of Wheat of NAAS, https://sops.gov.ua/reestr-sortiv-roslin) was used for laboratory vegetative experiments. The seeds were sterilized in 1 % potassium permanganate solution for 20 min, after which their surface was rinsed with water at least twice. Then, the seeds were soaked for 5 h in 0.05 mM salicylic acid (SA) (Sphera Sim, Lviv, Ukraine) solution or distilled water (control). The optimal concentration of SA was determined experimentally, based on the previous data (Malyk, 2019; Kavulych, 2019) and literature reports (Yuan & Lin, 2008; Guo, 2019).

**Seed germination and plant growing.** The seeds of the selected variety were germinated on filter paper in Petri dishes at 23 °C for 3 days in the dark using a thermostat. Uniform seedlings were transferred into pots filled with sterile sand supplemented with Hoagland's nutrient solution (Hoagland & Arnon, 1950) and grown under controlled conditions (16/8-h photoperiod) for 3 weeks. Control plants were grown without SA treatments; experimental plants were grown from seeds, treated with SA. The content of phenolic compounds, flavonols, and ascorbic acid in plant shoots was determined on the 7th, 10th, and 20th days of plant growth.

**Determination of the ascorbic acid content.** The plant shoots (15.00 ± 0.01 g) were milled vigorously with 2.0 % metaphosphoric acid after the volume was adjusted

to 25.0 mL with the same acid. After that, the extract was centrifuged for 15 min at 3000 rpm. Then, the sample of 5.0 mL, each with 0.5 mL of 0.025 % dichlorophenolindophenol solution, was measured with a stopwatch on a spectrophotometer at a wavelength of 530 nm against 2.0 % metaphosphoric acid. At the same time, 0.5 mL of 2. % metaphosphoric acid with 0.5 mL of dye (control) was added. Changes in the color intensity of the test solution are proportional to the amount of ascorbic acid. The amount of ascorbic acid content was calculated in mg/g of the plant material (Musienko, 2001).

**Estimation of rutin content.** Leaves were fixed at 100 °C for 15 min and put into the drying oven at 38 °C to obtain dry matter. 50–100 mg of each sample were homogenized and transferred to a test tube with 2 mL of methanol. The mixture was kept for 1 hour for extraction. After 1 hour, the mixture was centrifuged at 3000 g for 5 minutes. The supernatant was used for the next steps of rutin analysis. A series of standard solutions of rutin (concentrations 0.5, 1, 2, 4 mg/mL) and 0.5 µL extract were dropped on the plate with silicagel (Sorbfil). The solvent system for the separation of flavonoid compounds was ethyl acetate – acetonitrile – 35 % formic acid (13:5:2, v/v/v). After drying the plates with a hot air stream, visualization was performed with 0.1 % $TiOSO_4$; chromatograms were read at 450 nm using ULAB 101 Spectrophotometer (Sytar, 2014; Smirnow, 2012).

**Estimation of flavonoid content.** Plant material was fixed at 105 °C for 15 min and dried at 40 °C to dry matter in an oven (UOSLab-100, Ukraine). Sample 25 mg of the dried plant material was extracted with 1 mL of absolute methanol (Sphera Sim, Lviv, Ukraine) for 24 h. Total flavonoid content was determined using 0.2 % zirconyl (IV) nitrate hydrate ($ZrO(NO_3)_2 \cdot 2H_2O$) (Sphera Sim, Lviv, Ukraine), and rutin as a standard, with aluminum chloride by the differential spectrophotometry method (ULAB 101, China). The reference cuvette contained the plant extract (50 µL) and 3.5 mL of deionized water. The sample cuvette was prepared when the plant extract was added to 3 mL of deionized water and 0.5 mL of zirconyl nitrate hydrate. The absorbance was measured at 397.6 nm after 15 min incubation at 25 °C (Petry, 2011; Smirnow, 2012).

**Total phenolic content analyses.** The content of phenolic compounds in the plant shoots extracts were determined by the Folin-Ciocalteu method (Perez *et al.*, 2023). Briefly, the reaction mixture by mixing 0.5 mL of ethanol solution, 0.5 mL of 10 % Folin-Ciocalteu reagent (dissolved in water) and 1 mL of 7.5 % $NaHCO_3$ solution was prepared. A blank solution was also prepared. The samples were then stored at room temperature for 60 min. Gallic acid (GA) standard solutions were prepared using the same procedure. Absorbance readings were performed at 725–730 nm with ULAB 101 Spectrophotometer (Bobo-García, 2015).

**Determination of anthocyanin content.** Anthocyanin was extracted and estimated by the method of Beggs and Wellmann (Beggs, 1994) with some minor modifications. A portion of plant material (500 mg) was crushed and homogenized with the addition of 10 mL of hydrochloric acid and methanol in a ratio of 100:1. The homogenate was left for a day in the dark at a temperature of 5 °C. Absorbance was measured at 530 nm with ULAB 101 Spectrophotometer. The quantity of anthocyanin was calculated using cyanidin-3-glucoside coefficients – the major anthocyanin in buckwheat (molar extinction coefficient of 26 900 $L \cdot cm^{-1} \cdot mol^{-1}$ and molecular weight of 449.2 $g \cdot mol^{-1}$) (Jaleel, 2009).

**Colorimetric determination of xanthone content**. The quantitative content of xanthones was determined using the spectrophotometric method. For quantification of the xanthone content a calibration curve was prepared with mangiferin. A systematic approach was followed, starting with the weighing of 0.5 g of ground plant material and the addition of 50 mL of 60 % ethanol. The extraction was carried out in a water bath for 1 hour, and the process was repeated twice for 30 min each. The combined extract was then evaporated almost to dryness, and the residue was dissolved in a small amount of 70 % ethanol. A systematic amount of 0.05 mL of the alcohol extract was applied to chromatographic paper and dispersed in an ascending method in 40 % acetic acid. The chromatogram was dried, and the appearance of orange bands was systematically observed. Stained areas were cut out and filled with 60 % ethanol, extracting for 6 hours. It was then filtered into graduated tubes, and the volume of eluates was measured. For mangiferin, optical density was measured at a wavelength of 319 nm. (Krivut, 1976; Joubert, 2008, 2012).

**Statistical Analysis.** Each experiment was performed in five replications. The mean and standard deviation values were calculated by JMP Pro (https://www.jmp.com/en_us/home.html) and Microsoft Office Excel (https://www.microsoft.com). Statistical significance of the difference was evaluated with Student's *t*-test (P <0.05) (https://www.jmp.com/en_us/home.html). To determine the dependency of content of ascorbic acid, rutin, phenolic content, flavonoids, xanthone, anthocyanin correlation analysis was used. We also used regression analysis to determine the relationship among ascorbic acid and rutin content.

## RESULTS AND DISCUSSION

Confirming our assumption, we observed that salicylic acid increases ascorbic acid content, likely due to the common pathways of synthesis of flavonoids and salicylic acid. This increase in ascorbic acid content was observed on the 7th, 10th, and 20th days of growth. Linear growth was also recorded in the control, but the ascorbic acid content was still higher with SA treatment by 7, 10, and 20 times after ten days of growth (**Fig. 1**).

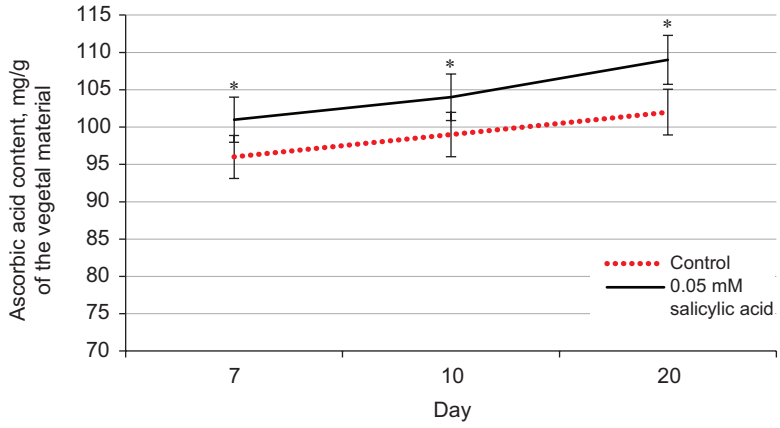


**Fig.1.** Effect of seed pretreatment with 0.05 mM salicylic acid (SA) on ascorbic acid content in wheat shoots, laboratory experiment. Mean values ± SD of three independent experiments are presented. * – statistically significant differences non-pretreatment vs. pretreatment groups, p < 0.05

Importantly, the biosynthesis of phenolic compounds increases under biotic and abiotic stress. This finding aligns with our previous studies (Kavulych, 2019; Kobyletska, 2022). However, treatment with SA in biotic stress conditions usually reduces the content of other phenolic compounds. Furthermore, the biosynthesis of flavonoids is often stimulated to a greater extent in stress-sensitive species than in stress-tolerant ones, suggesting potential applications in plant stress management. We can assume that exogenous SA in a particular concentration without stress conditions increases the content of phenolic compounds and other antioxidants, such as ascorbic acid (**Fig. 1**).

Both SA and flavonoids are phenylpropanoids, which have antioxidant activity and are synthesized from phenylalanine via cinnamic acid, an intermediate in the shikimic acid pathway. Flavonols represent a subgroup of flavonoids and are primarily synthesized from dihydroflavonols by flavonol synthase (EC 1.14.11.23). The synthesis of anthocyanins can precede the synthesis of rutin, which occurs through 6,8-dihydroxykaempferol (**Fig. 2**). The results indicate that pretreatment with SA promotes an increased rutin content at the initial stages of plant growth (**Fig. 3**).

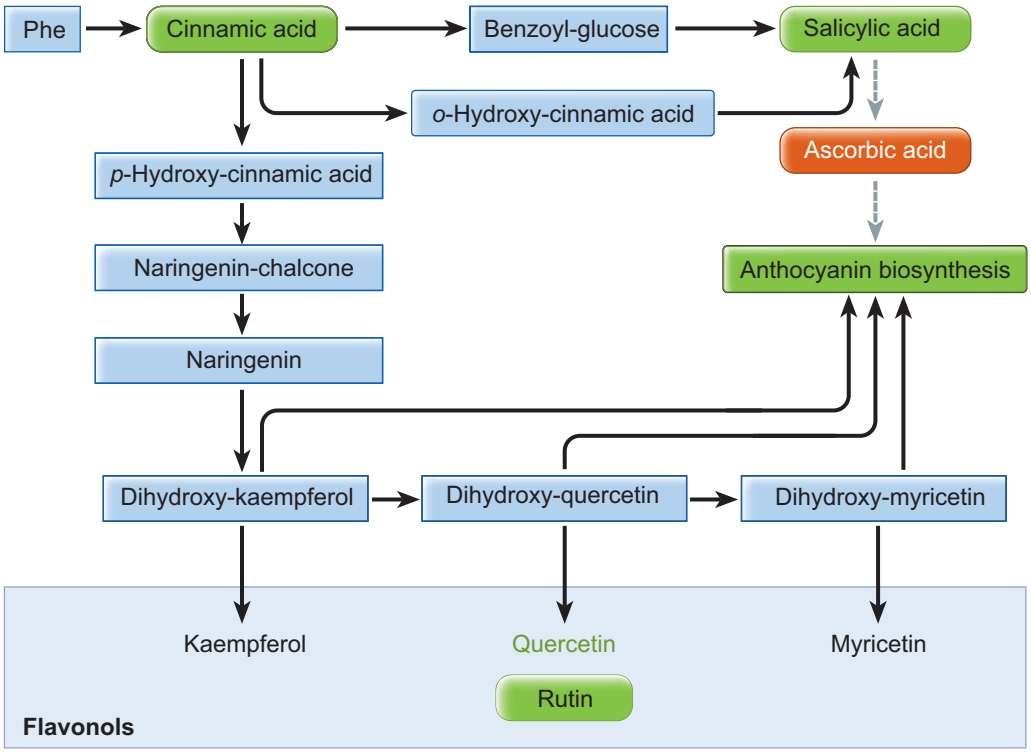


**Fig. 2.** Schematic presentation of salicylic acid and flavonoid biosynthesis and the possible role of ascorbic acid. Explanation in the text. Created in BioRender.com and modified from Gondor, 2016

As mentioned above, SA can regulate the content of phenolic compounds under the influence of stress factors. In **Figure 4**, except for anthocyanins, phenolic compounds' total content and xanthones, and flavonoids' content decreased under pretreatment

conditions with SA. In most experiments, SA pretreatment increases the anthocyanin content by increasing the activity of chalcone synthase (CHS) and induces anthocyanin synthesis. SA positively regulates anthocyanin biosynthesis, but the concentration of SA plays an important role in anthocyanin response. SA decreases anthocyanin content potentially due to its high accumulation in the vacuole compartment.

Surprisingly, SA increased the rutin content (**Fig. 3**), but did not positively affect the flavonoid content (**Fig. 4**).

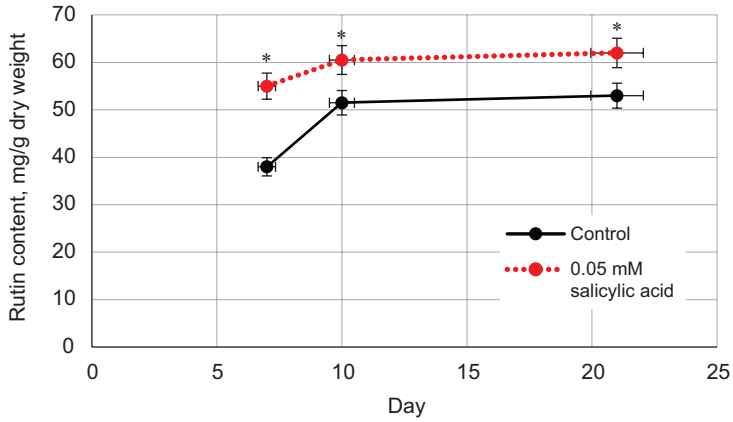


**Fig. 3.** Effect of seed pretreatment with 0.05 mM SA on rutin content in wheat shoots, laboratory experiment. Mean values ± SD of three independent experiments are presented. * – statistically significant differences non-pretreatment vs. pretreatment groups, $p < 0.05$

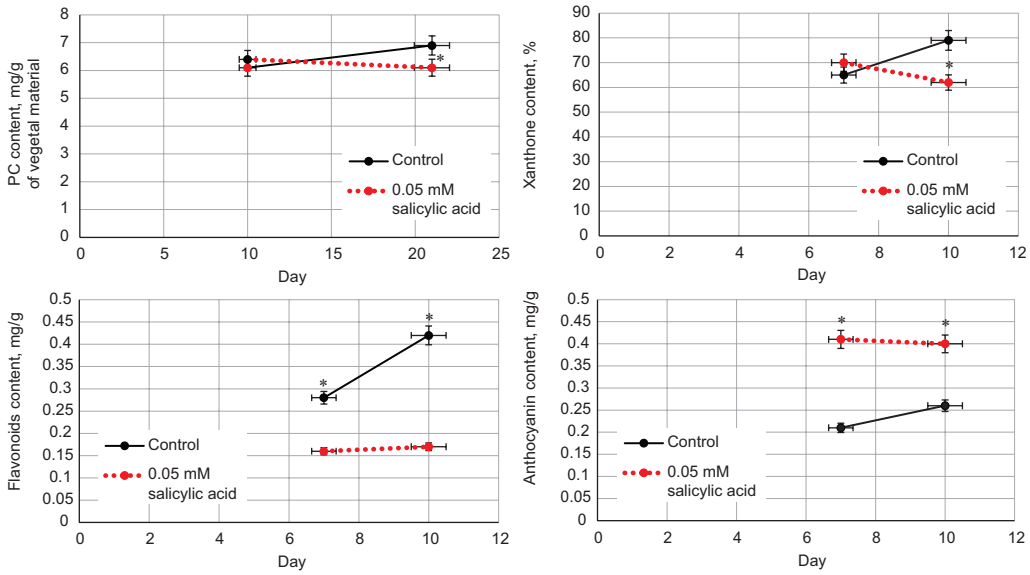


**Fig. 4.** Effect of seed pretreatment with 0.05 mM salicylic acid (SA) on PC (phenolic content), flavonoids, xanthone, anthocyanin content in wheat shoots, laboratory experiment. Mean values ± SD of three independent experiments is presented. * – statistically significant differences non-pretreatment vs. pretreatment groups, $p< 0.05$

As can be seen from the scheme (**Fig. 2**), the synthesis of phenolic compounds is much more complex and may shift towards the synthesis of anthocyanins, for example, or other phenolic compounds or antioxidants.

Therefore, we decided to conduct a correlation analysis with different classes of phenolic compounds and include ascorbic acid. The correlation between the ascorbic acid content and rutin in the wheat shoots is average, with an indicator ranging within 0.94 units, demonstrating the direct impact. There is an average direct interaction between indicators of the content of phenolic compounds and xanthones, ascorbic acid, and rutin with anthocyanins, where the correlation index in both groups is 0.55–0.69. Interestingly, we obtained an inverse average interaction between flavonoids and ascorbic acid (r = 0.62). As mentioned above, rutin is a representative of flavonoids, and we got a solid direct correlation between ascorbic acid and rutin. We believe that an inverse correlation between flavonoids and ascorbic acid occurs due to the diverse composition of this class, where the definition of flavonoid content most often refers to its most common representative, quercetin, etc. However, this still requires a detailed and comprehensive study since we determined the total content of flavonoids and rutin content separately.

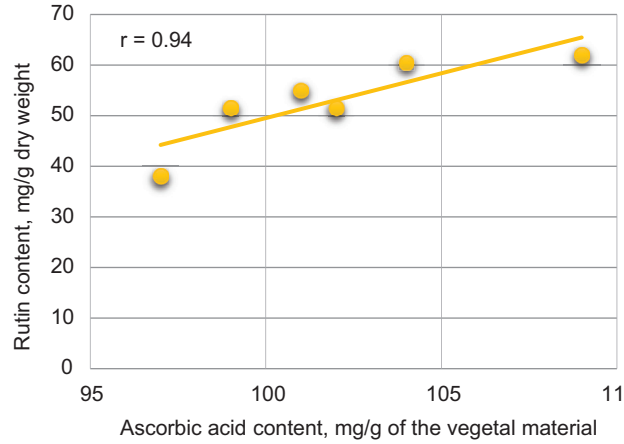


**Fig. 5.** Correlation among the measured parameters – ascorbic acid and rutin content in wheat (*Triticum aestivum* L.) shoots, r = 0.94 direct strong correlation

There is an interrelation between exogenous SA, phenolic compounds, and ascorbic acid. Ascorbic acid, as noted in the study by Smirnoff *et al.* (2000), has functions in photosynthesis as an enzyme cofactor (including the synthesis of ethylene, gibberellins, and anthocyanins). Metabolic pathways are not fully revealed, but our data also confirm the correlation between these compounds (**Fig. 5**). The correlation between ascorbic acid and anthocyanins has been studied and confirmed (Farr, Giusti, 2018). When exposed to intense light, ascorbate and anthocyanins act as photoprotectors. They accumulate in the exact temporal and quantitative equivalent in *Arabidopsis* leaves, which indicates a potential relationship between them. The ascorbate-deficient mutants *vtc1, vtc2,* and *vtc3* accumulated less anthocyanin than *wild-type* (WT) during HL acclimation (Smirnoff, 2000; Page, 2012). Correlation analysis showed an average of 0.55 units between ascorbic acid and anthocyanins. The expected correlation between flavonoids and ascorbic acid was not obtained. Pathways of synthesis of anthocyanins and flavonols have a common precursor, 6,8-dihydroxykaempferol, which is converted

into flavonols through dihydroquercetin (5,7,3′,4′-flavanonol), or 6,8-dihydroxymyricetin, forms anthocyanins (**Fig. 2**, see **Table**).

The fact that there is a connection between ascorbic acid and phenolic compounds has been proven. Gaafar *et al.* 2020 showed that ascorbic acid foliar spray enhanced photosynthetic pigments (chlorophyll and carotenoids), carbonic anhydrase activity, antioxidant activities (2,2-diphenyl-1-picrylhydrazyl free radical activity scavenging activity and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay), growth and seed yield while regulating enzymatic antioxidants (peroxidase), secondary metabolites (phenolic, flavonoids, and tannins), malondialdehyde (MDA) in common bean (*Phaseolus vulgaris* L.) plants water productivity (Gaafar, 2020). It has been found that when combined with flavonoids, ascorbic acid prevents oxidative processes more effectively and inhibits the formation of free radicals.

**Correlation among the measured parameters – ascorbic acid, rutin, PC (phenolic content), flavonoids, xanthone, anthocyanin content in wheat (*Triticum aestivum* L.) shoots. * – certainty p <0.05, ** – p <0.01**

| Studied compounds | Ascorbic acid | Rutin | PC | Flavonoids | Xanthone | Anthocyanin |
|---|---|---|---|---|---|---|
| Ascorbic acid | 1 | | | | | |
| Rutin | 0.935** | 1 | | | | |
| PC | -0.229 | 0.122 | 1 | | | |
| Flavonoids | -0.623 | -0.411 | 0.734* | 1 | | |
| Xanthone | -0.105 | 0.121 | 0.451 | -0.107 | 1 | |
| Anthocyanin | 0,556 | 0.692* | 0.475 | 0.302 | -0.281 | 1 |

Ascorbate, a crucial component, is required by several enzymes as a cofactor, at least *in vitro*. Most of these enzymes are 2-oxoglutarate- and Fe(II)-dependent oxygenases, where ascorbate acts as a reductant to maintain the iron as Fe(II) (Smirnoff & Wheeler, 2000). This role of ascorbate is particularly notable in plants, where it is involved in various processes, including prolyl hydroxylase (EC 1.14.11.2), which catalyzes posttranslational hydroxylation of prolyl residues, notably in the cell wall hydroxyproline-rich glycoproteins (HRGPs) (Rempfer *et al.*, 2024). Several enzymes of this type are also crucial to flavonoid and alkaloid biosynthesis, including anthocyanidin synthase (EC 1.14.11.19), flavonone 3-hydroxylase (1.14.11.9), flavonol synthase (EC 1.14.11.23) and flavone synthase 1 (EC 1.14.20.5), and alkaloid oxygenasesn (EC 1.14.20). This is a complex schematic presentation of the intricate biosynthesis of salicylic acid and flavonoids, and the potential role of ascorbic acid. SA and flavonoids are synthesized from phenylalanine *via* cinnamic acid, an intermediate in the shikimic acid pathway. Flavonols, a specific subgroup of flavonoids, are primarily synthesized from dihydroflavonols, such as dihydroxy-kaempferol, dihydroxy-quercetin, and dihydroxy-myricetin, adding another layer of complexity to the process (Smirnoff, 2000). More data is needed on ascorbic acid, which acts as a cofactor of enzymes and participates in synthesizing anthocyanins and other phenolic compounds. The probable effect of SA on ascorbic acid is also marked with a dashed line (**Fig. 2**). Our data indicate that

the pre-sowing treatment of SA seeds can increase ascorbic acid content. Ascorbate is present in all subcellular compartments, including the apoplast (cell wall), chloroplasts, cytosol, vacuoles, mitochondria, and peroxisomes (Hasanuzzaman *et al.*, 2020). Both SA and ascorbic acid are protected against reactive oxygen species (ROS) (Zandi & Schnug, 2022; Poór, 2020). It has also been proposed that SA and analog benzothiadiazole, a signaling molecule involved in pathogen defense, can directly inhibit catalase and ascorbate peroxidase (Poór, 2020).

## CONCLUSION

The treatment of seeds with SA caused an increase in the content of ascorbic acid and phenolic components in the shoots of wheat plants. A positive correlation was established between the accumulation of ascorbate and phenolic compounds in the studied organs. Our hypothesis on the role of SA and ascorbate in the synthesis of phenolic compounds is supported by our correlation analysis, which indicates an indirect influence of salicylic acid on the content of rutin and ascorbic acid. The synthesis pathway of anthocyanins, potentially involving ascorbic acid as a cofactor, is a particularly intriguing area for further exploration. Our research reveals a decrease in the content of most phenolic compounds, except for anthocyanins, suggesting potential mutual reinforcement or shared metabolic pathways. These findings raise important questions about the complexity of these processes, emphasizing the need for further research.

## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Animal Rights:** This article does not contain any studies with animal subjects performed by any of the authors.

## AUTHOR CONTRIBUTIONS

Conceptualization, [M.K.; Y.K.]; methodology, [M.K.; Y.K.]; validation, [M.K.; Y.K.]; formal analysis, [M.K.; Y.K.].; investigation, [M.K.; Y.K.]; resources, [M.K.; Y.K.]; data curation, [M.K.; Y.K.]; writing – original draft preparation, [M.K.; Y.K.]; writing – review and editing, [M.K.; Y.K.]; visualization, [M.K.; Y.K.]; supervision, [M.K.; Y.K.]; project administration, [M.K.; Y.K.].

All authors have read and agreed to the published version of the manuscript.

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## ВПЛИВ САЛІЦИЛОВОЇ КИСЛОТИ НА ВМІСТ АСКОРБІНОВОЇ КИСЛОТИ І ФЕНОЛЬНИХ СПОЛУК У РОСЛИНАХ ПШЕНИЦІ

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**Обґрунтування.** Саліцилова кислота є важливим фітогормоном рослин, який впливає на різні функції, такі як старіння, дихання та стійкість до стресу. Незважаючи на численні дослідження ролі саліцилової кислоти за стресових умов, її вплив на рослинний організм за нормальних умов залишається менш вивченим. У цьому дослідженні вивчали вплив саліцилової кислоти на біосинтез важливих біохімічних

сполук, таких як аскорбінова кислота, рутин та інші фенольні сполуки в пшениці (*Triticum aestivum* L.), щоб з'ясувати можле застосування цього фітогормону в сільському господарстві.

**Матеріали та методи.** Сорт пшениці "Подолянка" обробляли саліциловою кислотою у концентрації 0,05 мМ і пророщували в контрольованих умовах. Біохімічні аналізи проводили на 7-му, 10-ту та 20-ту добу росту в пагонах рослин із використанням спектрофотометричного методу визначення аскорбінової кислоти, рутину, загальної кількості фенольних сполук, антоціанів, флавоноїдів і ксантонів. Метод визначення рутину включав хроматографію на пластинах зі силікагелем.

**Результати й обговорення.** Обробка насіння саліциловою кислотою достовірно підвищувала вміст аскорбінової кислоти у рослинах пшениці на всіх етапах дослідження. Також було встановлено збільшення вмісту рутину на ранній фазі росту. Однак вміст інших фенольних сполук, таких як ксантони, внаслідок обробки саліциловою кислотою знижувався. Цікаво, що вміст антоціанів за дії саліцилової кислоти зростав, що свідчить про складну взаємодію в біосинтетичних шляхах під впливом фітогормону. Дослідження також виявило кореляції між різними фенольними сполуками, що вказує на взаємопов'язані метаболічні шляхи.

**Висновок.** Саліцилова кислота посилює біосинтез аскорбінової кислоти, а також деяких фенольних сполук, зокрема, рутину, в рослинах пшениці. Це дає змогу використовувати її у сільськогосподарській практиці, спрямованій на підвищення стійкості й харчової цінності рослин. Диференційований вплив саліцилату на різні фенольні сполуки підкреслює складність біохімічних шляхів рослин і необхідність подальших досліджень, щоби повністю зрозуміти ці взаємодії та можливість їхнього практичного застосування.

***Ключові слова:*** *Triticum aestivum* L., саліцилова кислота, фенольні сполуки, аскорбінова кислота, рутин, антоціани, ксантони, флавоноїди

| Received / Одержано | Revision / Доопрацьовано | Accepted / Прийнято | Published / Опубліковано |
|---|---|---|---|
| 28 March, 2024 | 30 May, 2024 | 05 June, 2024 | 26 June, 2024 |