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Exogenous Salicylic Acid Modifies Cell Wall Lignification, Total Phenolic Content, PAL-Activity in Wheat (*Triticum aestivum* L.) and Buckwheat (*Fagopyrum esculentum* Moench) Plants under Cadmium Chloride Impact

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Abstract: Salicylic acid (SA) is considered one of the most important plant signaling molecules involved in abiotic and biotic stress tolerance. Application of 0,05 mM SA enhanced plants' tolerance to cadmium stress by modulating levels of several metabolites, including components of antioxidative defense, osmolytes, secondary metabolites, and metal-chelating compounds. Seeds of bread wheat (*Triticum aestivum* L.) and buckwheat (*Fagopyrum esculentum* Moench) were presoaked in 0,05 mM SA/or distilled water for 5 h, with the following germination in a dark thermostat for 3 d. Uniform seedlings were planted into +Cd / or -Cd-containing substrate and grown for 16/8 h light/dark. It was revealed that seed pretreatment with SA alone caused a 3-5-fold decrease in phenylalanine ammonia-lyase (PAL) activity and total phenolic content compared to the control water treatment. Seed pretreatment with SA alleviated the negative impact of Cd on plant growth, PAL activity, and total phenolic content. These results indicate that salicylate treatment alleviates Cd toxicity by modifying lignin synthesis and accumulation of flavonoids in wheat and buckwheat plants. Protective effects were more pronounced for buckwheat with the studied variables' high total phenolic content.

Keywords: salicylic acid; lignin; phenolic compounds; phenylalanine ammonia-lyase; cadmium chloride**.**

1. Introduction

Salicylic acid (SA) belongs to the class of phenolic compounds [1] and plays a key role both in a plant's immunity [2-4] and responses to abiotic stresses [5,6].

One of the serious problems of agricultural production worldwide and in Ukraine is soil heavy metal pollution [7-9]; it affects food safety and marketability, crop yields, and environmental health of soil organisms [10,11]. Cadmium toxicity has a high impact on plants and consequently affects the ecosystem, where plants form an integral component [7,12,13].

Current research data support the possible mediatory role of SA in protecting physiological and biochemical processes under cadmium toxicity. There are reports that SA

increases the distribution ratio of Cd in lignin due to the formation of chelate complexes with phenolic compounds and precedes the processes of changes in the enzymatic activity of PAL [14].

Bread wheat (*Triticum aestivum* L.) is an important crop worldwide and a major staple crop in Ukraine [15-17], whereas b. Buckwheat (*Fagopyrum esculentum* Moench) belongs to pseudocereal food crops and healthy herbs [18], emerging as an alternative for traditional food sources due to its high nutrient value and productivity [19-22]. The main buckwheat producers are China, Ukraine, Kazakhstan, but generally, it is consumed or traded locally [3,23]. Several authors showed that exogenous SA seed pretreatment alleviated the negative effect of Cd on crop plants' growth parameters [20] and chlorophyll level [24]. Application of 0,05 mM SA to wheat seeds also decreased the extent of the negative Cd effect, as revealed by a decline in the level of phenolic content [25-27].

The presented work aimed to study the influence of seed pretreatment with exogenous SA on PAL activity, the total content of phenolic compounds, and lignin distribution under cadmium chloride impact on bread wheat and buckwheat plants.

2. Materials and Methods

2.1. Experimental design and treatments.

Wheat plants (*Triticum aestivum* L.) var. Podolyanka (Institute of Plant Physiology and Genetics of National Academy of Science of Ukraine, V.M. Remeslo Myronivka Institute of Wheat of NAAS, https://sops.gov.ua/reestr-sortiv-roslin) and buckwheat (*Fagopyrum esculentum* Moench.) var. Rubra (State Agrarian and Engineering University in Podilia) were used for laboratory vegetative experiments. The seeds were sterilized in 1% potassium permanganate solution for 20 min. Their surface was rinsed with water at least twice. Then, the seeds were soaked for 5 h in 0,05mM SA (Sphera Sim, Lviv, Ukraine) solution or distilled water (control). The optimal concentration of SA was determined experimentally, based on the previous data [28,29] and literature reports [30,31].

2.2. Seed germination and growing plants.

The seeds of the selected varieties were germinated on filter paper in Petri dishes at 23°C for 3 days in a dark thermostat. Uniform seedlings were transferred into pots filled with sterile sand supplemented with Hoagland's nutrient solution (Hoagland and Arnon, 1950) [32] and grown under controlled conditions (16/8-h photoperiod) for 3 weeks. Cadmium was applied as cadmium chloride (CdCl) salt (Sphera Sim, Lviv, Ukraine) in the dose of 25 ppm of the substrate. Control plants were grown without Cd and SA treatments; experimental plants were grown from seeds treated with SA +/- Cd in the substrate. The content of phenolic compounds and PAL activity were determined on the 7^{th}, $14^{th,}$ and 21^{st} days of plant growth.

2.3. Phenylalanine ammonia-lyase activity assay.

Phenylalanine ammonia-lyase (PAL, L-Phenylalanine Ammonia-Lyase EC 4.3.1.24) activity was determined spectrophotometrically by the modified Zucker method [33,34]. Samples of 0,5 g plant tissue were homogenized manually in 2 ml of 25 mM borate buffer (pH 8,8) containing 23 µL mercaptoethanol (Sphera Sim, Lviv, Ukraine). The homogenates were centrifuged for 20 min at 8000 g, and the supernatant was used for the enzymatic assay. PAL

assay mixture contained 1 ml of the supernatant, 1 ml of borate buffer, 1 ml of 12 mM L-phenylalanine (Sphera Sim, Lviv, Ukraine). The resulting mixture was incubated at 37 °C for 1 hour; the reaction was stopped by 15% trichloroacetic acid. Absorbance at 274 nm was measured using a spectrophotometer (ULAB 101, China). PAL activity was expressed in mM of cinnamic acid per gram of protein. Protein was determined by the Bradford method [35].

2.4. Determination of total phenolics.

The total content of phenolic compounds in the plant shoots and roots was determined by the modified Folin-Denis′ method [36,37]. The reaction mixture was obtained by mixing 0.5 ml of ethanol extract, 0.5 ml of 10% Folin-Denis′ reagent (Sphera Sim, Lviv, Ukraine), and 1 ml of 7.5% $NaHCO_3$, with the following incubation at room temperature for 60 min and absorbance readings at 725-730 nm with ULAB 101 Spectrophotometer. Gallic acid (GA) (Sphera Sim, Lviv, Ukraine) standard solutions were prepared using the same procedure.

2.5. Flavonoids estimation.

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 [34,38].

2.6. Lignin visualization.

We used the Wiesner reaction based on phloroglucinol condensation with cinnamic aldehydes (coniferyl aldehyde) in an acidic environment and developed a cherry red-colored product [39]. A thin stem section obtained with a razor after washing in the distilled water was placed on a glass slide and stained with 5 % phloroglucinol in alcohol (Sphera Sim, Lviv, Ukraine), then treated with 25 % H_2SO_4. After 10 min, the sections were examined under a light microscope (XS-2610 Led MICROmed, Ukraine) [40].

2.7. Statistical analyses.

Each experiment was performed in five replications. The means 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).

3. Results and Discussion

The accumulation of secondary metabolites is activated by abiotic stresses, signal molecules, or elicitors in various plants [41]. In particular, the production of secondary metabolites can be promoted by salicylic acid. Exogenous SA could stimulate the production of endogenous SA and phenolic compounds in plants, thus helping the treated plants to reduce

the load of abiotic stress [42]. Many studies have documented the role of other phytohormones in enhancing the level of some classes of phenolic compounds [43-46]. Some reports showed the effects of jasmonic acid, chitosan, and salicylic acid (SA) on the accumulation of phenolic compounds in germinated buckwheat. However, in this case, treatment with SA in concentrations of 50, 100, and 150 mg/L did not affect the production of phenolic compounds in buckwheat plants [47].

There are a lot of data that SA influences the activity of phenylalanine ammonia-lyase (PAL), the key enzyme of phenolic compounds biosynthesis in many plants, in particular, saffron leaves (*Crocus sativus* L.) [48], barley shoots (*Hordeum vulgare* L.) [49], *Vitis vinifera* L. fruits [50], wheat (*Triticum aestivum* L.) roots and shoots [51] and buckwheat (*Fagopyrum esculentum* Moench.) plants [47]. A peak of PAL activity was observed in *Matricaria chamomilla* plants under heavy metal (Ni and Cd) stress with the application of SA [46,52]. SA affects cell wall biosynthesis, namely lignification in *Brachypodium distachyon* [53] and rice (*Oryza sativa* L.) [54]. Previously, we have demonstrated that SA plays a regulatory role at the stage of phenolic compounds quantity, which means that SA can both increase or decrease the concentration of total phenolic compounds in plant tissues [29]. We have studied the influence of SA seed pretreatment on wheat and buckwheat tolerance to the subsequent treatment with cadmium chloride (25 ppm) in laboratory vegetative experiments.

Phenylalanine ammonium-lyase (PAL), a key enzyme in the phenylpropanoid pathway, is involved in the defense responses of plant cells [55,56]. Synthesis of phenolic compounds and their accumulation under stress conditions may be associated with increased activity of PAL [57]. Therefore, PAL has been generally recognized as a marker of environmental stress in different plant species [58]. PAL activity shows significant changes for a short period in plant tissues [34,59]. There are data that salt stress increased dynamic changes of PAL activity in plant seedlings [60].

On the 7th day of the experiment, we observed a significant, 5-fold increment in PAL activity for both wheat and buckwheat under 0,05 mM SA treatment alone. In contrast, it decreased on the 14th and 21st days of the experiment (Figures 1, 2). Exceptions were noted for buckwheat root tissues, where PAL activity remained high at all experimental stages. Wheat plants also had significantly higher PAL activity in roots than in shoots. The results confirm that roots are the main place of phenolic synthesis and localization. Our suggestion is supported by similar results by Colak et al. for wheat (*T. aestivum*) plants [61].

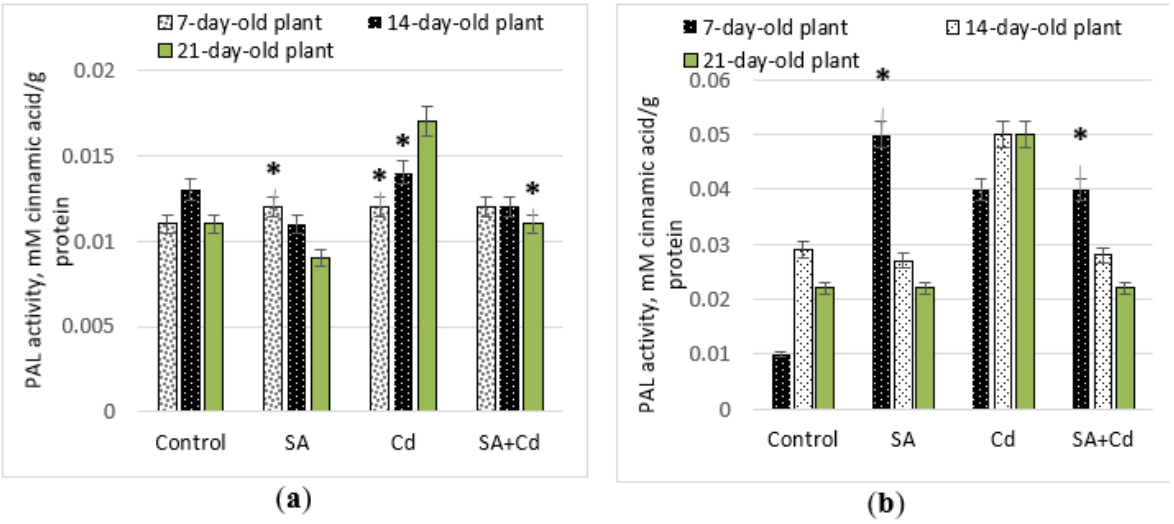

Figure 1. Effect of $CdCl_2$ (25 ppm) and seed pretreatment with 0.05 mM SA on phenylalanine ammonia-lyase (PAL) activity in wheat (a)-roots, (b) -shoots; laboratory experiment. * – P<0.05.

On the 7th day of the experiment, under $CdCl_2$ impact in wheat treated with SA, we observed a 4-fold increase in PAL activity in shoot tissues; PAL activity decreased on the next time points (Figure 1b). In the roots, we observed a linear increase of the enzymatic activity, with a slight increase on the 7th and 14th days and 0,6 times on the 21st day under cadmium stress compared to control (Figure 1a). We assume that exogenously applied SA positively affects PAL activity in wheat (Figure 1). The obtained data confirm data on early changes in PAL activity under stress conditions [62].

Buckwheat plants in control demonstrated a comparatively high PAL activity in the roots. Under $CdCl_2$ influence, there was a 2-fold increase in enzyme activity (Figure 2). A prolonged cadmium impact caused a higher enzyme activity. In the shoots, an increase of PAL activity was observed on the 7th day of cadmium stress, whereas on the 21st day, we observed a 3-fold decrease in this activity. Seed pretreatment with SA increased PAL activity thus, we suppose that it can, at least partially, be explained by common pathways of synthesis of SA and other phenols.

Our results revealed changes in PAL enzymatic activity without a distinct time- or age dependence in the untreated control plants. On the other hand, PAL activity depended on cadmium chloride and SA application. When comparing wheat and buckwheat plants, the higher PAL activity in buckwheat was noted (Figure 2).

As the published data suggest, early changes in PAL activity are characteristic of plant tissues [62]. In particular, a short-term increase in the activity of PAL and peroxidase enzymes, which activate the protective mechanisms of wheat plants, was shown by Feduraev [63]. PAL activity also changes under the influence of biogenic factors, such as chitosan and salicylic acid [27].

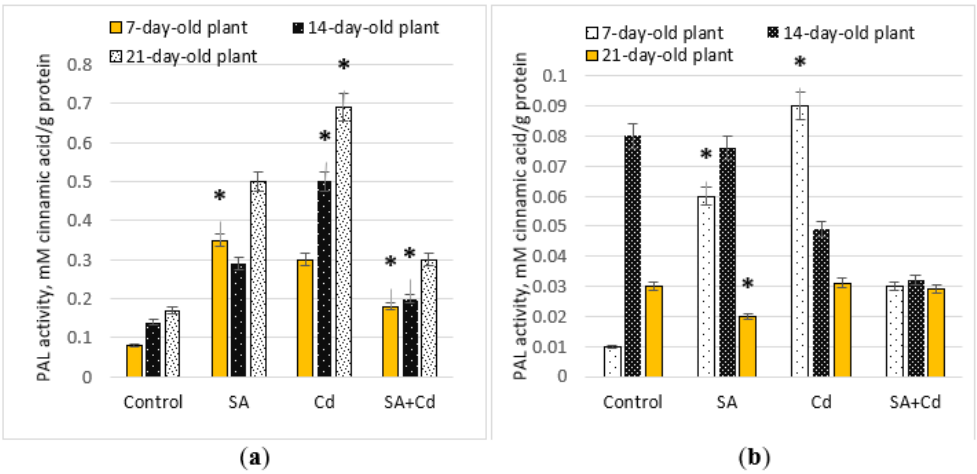

Figure 2. Effect of $CdCl_2$ (25 ppm) and seed pretreatment with 0.05 mM SA on phenylalanine ammonia-lyase (PAL) activity in buckwheat (a)-roots, (b)-shoots; laboratory experiment. * – $P<0.05$.

According to the published data, changes in PAL activity led to different changes in total phenolic compound content [2,64]. Phenolics are important for natural plant metabolism and development; they participate in cell wall development, photosynthesis, respiration, plant-to-plant allelopathic interactions, defense against pathogens and herbivores [64]. Plants also produce them in response to biotic or abiotic stresses [2].

Total phenolic compounds in the control wheat plants were accumulated predominantly in the root tissues, whereas, in the shoots, their concentration decreased during the experiment (Figure 3). SA alone induced a decrease in the content of phenolics in both the roots and shoots (Figure 3). Roots, the first organs exposed to heavy metals, are considered their main sources

within the plant organism [65]. It is assumed that a high content of phenolic compounds in roots and its relatively low content in shoots may serve as a protective mechanism and a logical redistribution of phenolics observed under heavy metal stress [45,59].

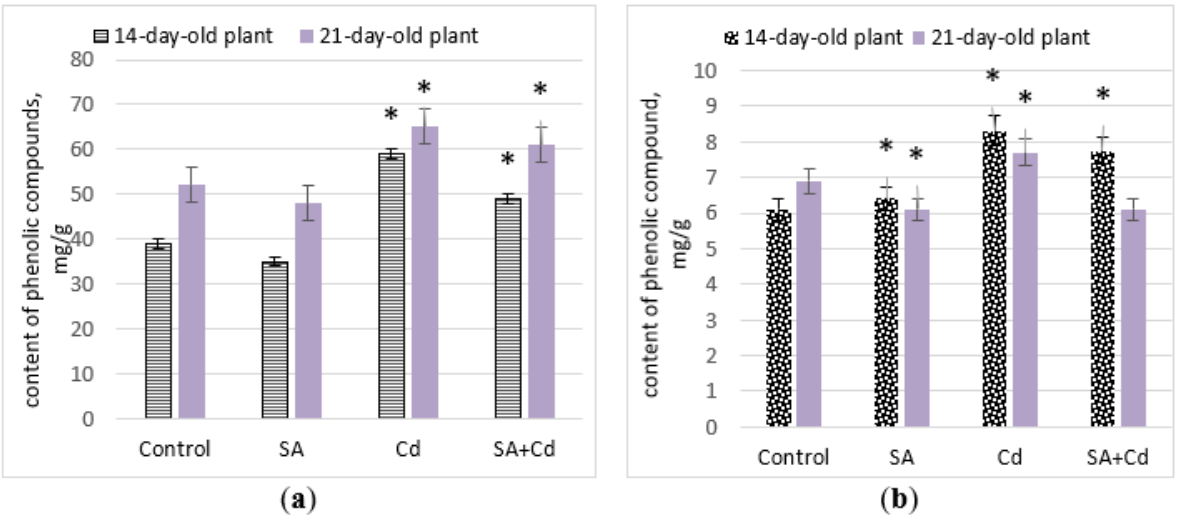

Figure 3. Effect of $CdCl_2$ (25 ppm) and seed pretreatment with 0.05 mM SA on total phenolic content in wheat (a)-roots, (b)-shoots, laboratory experiment. * – P<0.05.

The obtained results are consistent with the published data, where in most cases, the root tissue is the site of synthesis and localization of phenolic compounds [45,63,66,67]. A significant accumulation of phenolic compounds is reported for *Fagopyrum esculentum* Moench., *Kandelia obovata* L., *Lactuca sativa* L., and *Hypericum perforatum* L. plants under heavy metal stress [44,62,68,69].

Under the influence of Cd, a 1,2-1,5-fold increase in total phenolic concentration in the wheat organs was observed (Figure 3). The prolonged action of CdCl was followed by an increase in total phenolic compounds concentrations in the roots, which is consistent with their protective functions and the ability to form chelate complexes with heavy metals [70].

In the buckwheat plants, the concentration of total phenolics was higher than that in the wheat plants (Figure 4). Cadmium increased phenolic compounds by 1,5 times at both time points – the 14th and 21st days of the experiment. SA alone also increased their accumulation, but in the presence of CdCl, the concentration of phenolics was lower than in the separate cases with Cd or SA (Figure 4). The presented results confirm the involvement of SA in plant stress responses to cadmium.

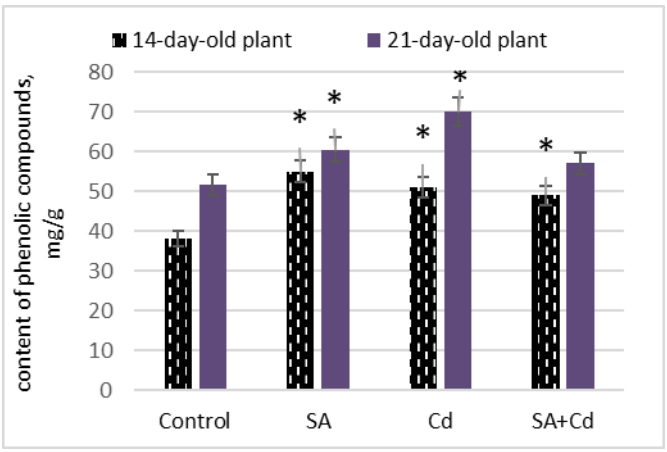

Figure 4. Effect of seed pretreatment with 0.05 mM SA on phenolic content in buckwheat shoots under $CdCl_2$ (25 ppm) impact, laboratory experiment. * – P<0.05.

An increase in PAL enzymatic activity leads to growth in phenolic compounds, which results from increased lignification of cell walls [63]. The key compounds involved and precursors of lignin formation are flavonoids. Therefore, the growth of concentration of these compounds under the action of cadmium is rarely observed [71,72]. Flavonoids possess antioxidant properties; they produce a protective effect and perform a barrier function due to lignin formation [72].

The main function of flavonoids is to protect plants from various exogenous influences. Any biotic and abiotic stressors can intensify the biosynthesis of flavonoids in different anatomical parts of the plant [73]. The antioxidant properties of these compounds and their barrier function are realized through the formation of tannins and lignin [71]. An insignificant increase in these compounds was caused by processes of lignification of cell walls of buckwheat [68] and wheat [66,67] plants.

Exogenous SA induced lignin formation, especially in buckwheat cell walls, both on the 14th and 21st days of plant growth (Figures 5,6). The results show a difference in lignin accumulation in the area of the metaxylem of the above-ground part of the plant depending on the growing conditions, particularly the differences in lignin accumulation in the shoots under SA and Cd influence. It should be noted that different parts of the plant – leaf, stem, node – were selected to detect lignin. In the control variants of longitudinal sections, lignin formation was not observed (Figure 5).

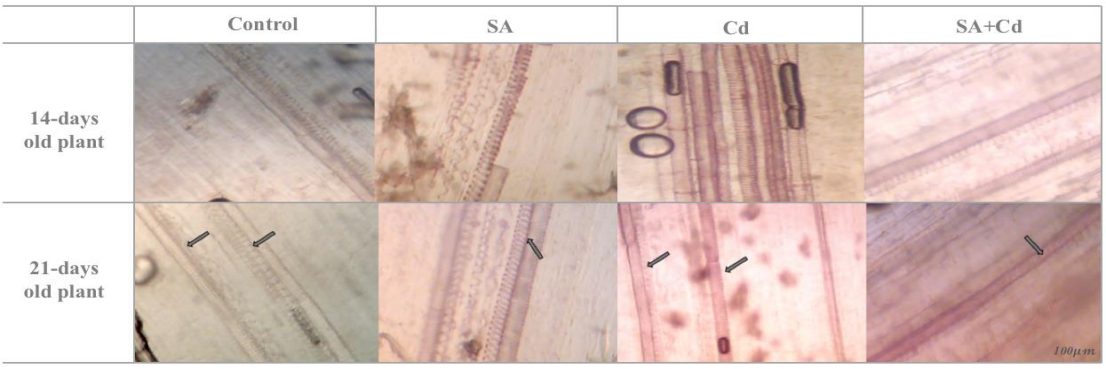

Figure 5. Localization of lignin with the Wiesner reaction (cherry red color, black arrows) in shoots (longitudinal sections) of 14-, 21-day buckwheat (*Fagopyrum esculentum* Moench, Rubra var.) plants under the action of cadmium chloride and salicylic acid (laboratory pot experiment, seed pretreatment with 0,05mM SA, dose of $CdCl_2$ - 25 ppm).

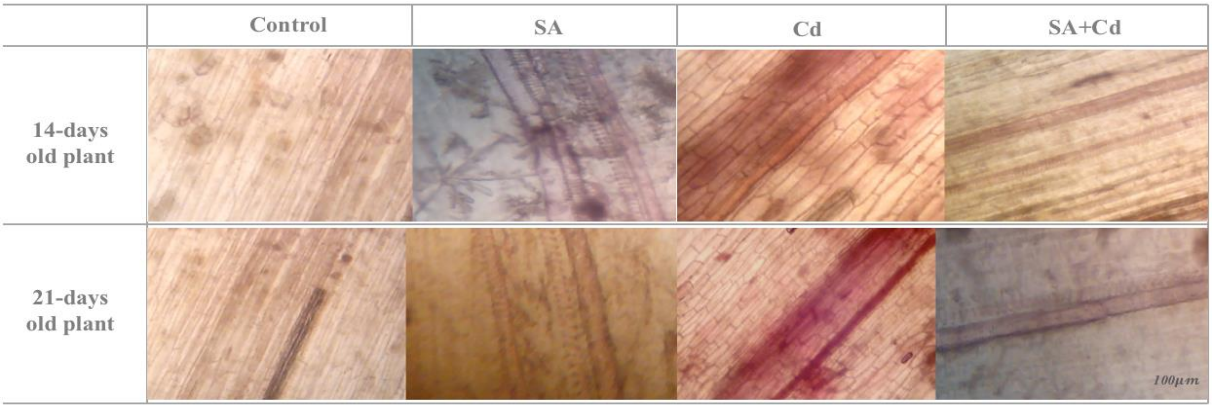

Figure 6. Localization of lignin with the Wiesner reaction (cherry red color, black arrows) in shoots (longitudinal sections) of 14-, 21-day wheat (*Triticum aestivum* L., Podolanka var.) plants under the action of cadmium chloride and salicylic acid (laboratory pot experiment, seed pretreatment with 0,05mM SA, dose of $CdCl_2$ - 25 ppm).

An active form of lignin is associated with an excessive accumulation of heavy metal ions in the environment [74]. Different tissues of one plant may differ in the composition of lignin, which may result from different expressions of lignin biosynthesis genes. SA, as a substance of phenolic origin, also influenced the process of lignification in buckwheat plants (Figure 5); similar results obtained on mutants and transgenic plants prove the metabolic plasticity of lignin biosynthesis [75-77].

Experimental time points did not show a significant difference in the accumulation of this compound in control. However, the accumulation of lignin depends on the conditions of plant growth. Control groups of plants accumulated significantly less lignin compared to plants that grew under cadmium stress or under the action of salicylic acid. The process of lignification is active in all variants of the research, except control, with a predominance in buckwheat plants caused by the varietal specificity. Lignin production has a temporal dependence that increases on the 21st day of plant growth. Salicylic acid also affects the lignification of the cell wall process.

Salicylic acid promoted a local increase in flavonoids at the early stages of growth with their further decrease on the 21st day of the experiment (Table 1).

Table 1. Effect of $CdCl_2$ and SA on total flavonoids content in buckwheat (*Fagopyrum esculentum* Moench) organs, mg/g (laboratory pot experiment, seed pretreatment with 0,05mM SA, 25 ppm $CdCl_2$ in the substrate). * – P<0.05.

Variant	Roots		Shoots	
	14 days	21 days	14 days	21 days
Control	0,046±0,002	0,024±0,004	0,285±0,004	0,163±0,001
SA	0,107±0,006*	0,026±0,004	0,429±0,005*	0,178±0,002
Cd	0,035±0,003	0,054±0,002*	0,203±0,003	0,204±0,004*
SA+Cd	0,033±0,003	0,032±0,003	0,318±0,004*	0,123±0,002

At the early stages of the growth of seedlings, high flavonoids concentrations were detected in the shoots, with no significant values in the roots. The increase in flavonoids concentration in plant organs under the application of salicylate is probably due to the common path of synthesis for these compounds, as in the case of the total phenolic level. The decrease in the content of flavonoids on the 21st day of plant growth might be caused by their decay and be associated with an increased level of other classes of phenolic compounds, such as lignin. The effect of cadmium ions caused a decrease in the content of flavonoids in the roots on the 14th day, while a significant increase in their content compared to control was observed on the 21st day. The content of these compounds under the action of cadmium chloride in the shoots did not change during the experiment (Table 1).

Thus, an increase in total phenolic compounds under the impact of cadmium chloride has been revealed both for buckwheat and wheat plants in laboratory experiments. Seed pretreatment with 0.05 mM SA modulated the level of phenolic compounds in plant organs under cadmium stress. SA also affected the activity of PAL, the key enzyme of phenolic metabolism. All experimental variants with wheat plants, except for the combined SA and $CdCl_2$ action, demonstrated an increase in total phenolic content and PAL activity, in contrast to control values. These data were subjected to correlation analysis; the correlation index was 0.7-0.9, indicating a strong direct dependence between the studied parameters (Figure 7a). In the wheat roots, the correlation between the content of phenolic compounds and PAL activity is average, its indicator ranging within 0.75-0.8 units, which demonstrates the direct impact (Figure 7b). In buckwheat shoots, the connection between phenolic compounds and PAL enzymatic activity is not present; data confirmed by correlation analysis, index ranged from -

0.3 to 0.05 (Figure 7c). It may be due to a high basic content of phenolic compounds in this buckwheat variety and/or involve other enzymes responsible for synthesizing phenolic compounds.

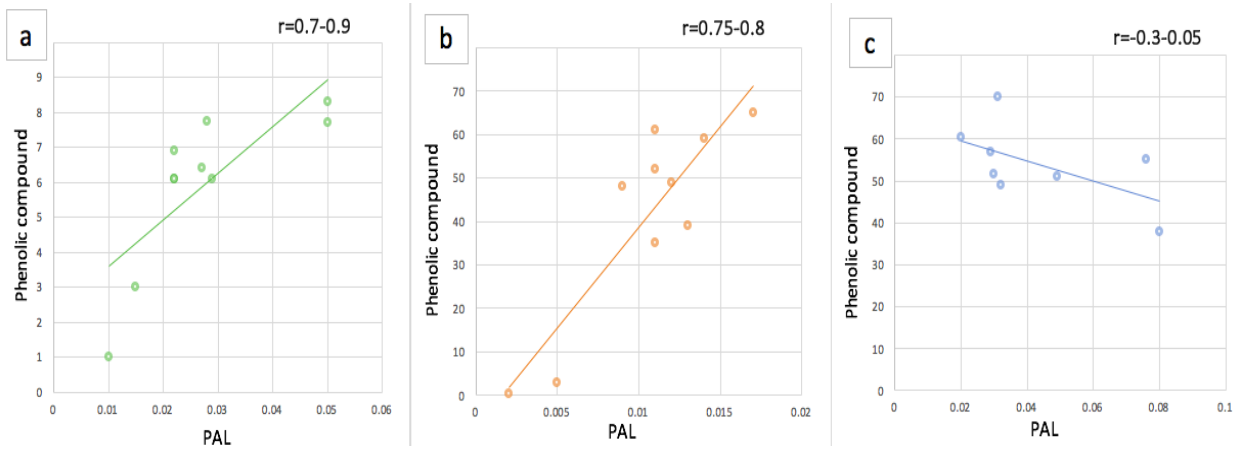

Figure 7. Correlation among the measured parameters - total phenolic content and phenylalanine ammonia-lyase (PAL) in wheat (*Triticum aestivum* L.) a- shoots, b- roots, and buckwheat (*Fagopyrum esculentum* Moench) c- shoots.

However, the present/non-present correlation between phenolic compounds and PAL activity does not exclude the positive and regulatory effects of SA on these separate parameters. The obtained results indicate that lignin formation correlated with flavonoids content. Moreover, cadmium and SA acid increase lignin formation without any changes in flavonoid content.

4. Conclusions

The results of this study will be useful for cultivating plants on Cd-contaminated soils. The application of exogenous SA can be recommended for the pretreatment of wheat and buckwheat seeds to alleviate cadmium toxicity. A pre-sowing seed treatment with SA affects the concentration of phenolic compounds in shoots, modifies PAL activity, and decreases the lignification of cell walls in wheat while increasing it in buckwheat plants if compared to the lignification under the action of cadmium chloride without SA. This assumption is supported by the data on a significant decline in Cd accumulation in SA-pretreated plants, especially in the shoots. The protective role of SA in cadmium-stressed plants is unambiguous. It can be related to antioxidant process regulation, enhanced PAL activity, a decrease in the content of total phenolic compounds, lignin formation in leave tissues, and an increased lignification in root tissues. The present study's findings can be useful for the safe cultivation of pre-treated plants with the optimal concentration of SA on Cd-contaminated soils.

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Conflicts of Interest

The authors declare no conflict of interest.

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