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Cadmium-Induced Changes in the Accumulation of Sugars and the *PsGolS* Transcript in *Pisum sativum* L.

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Abstract: Cadmium (Cd) is a key stress factor that affects plant development. To examine the influence of Cd stress, we analysed the tissue localisation of polysaccharides (Periodic Acid Schiff reaction), qualitative and quantitative changes in soluble carbohydrates (High-Resolution Gas Chromatography), and the expression of the galactinol synthase (*PsGolS*) and raffinose synthase (*PsRS*) genes in 4-week-old *Pisum sativum* L. 'Pegaz'. The plants were treated with 10, 50, 100, and 200 µM $CdSO_4$ for one week and analysed on the 1st, 7th, and 28th days after Cd application. Pea as an excluder plant accumulated Cd mainly in the roots. Cd induced starch grain storage in the stems and the accumulation of soluble carbohydrates in roots and shoots after 28 days of Cd treatment. In controls, soluble carbohydrate levels decreased during the plant growth. In addition, Cd increased galactinol and raffinose levels, indicating their important role in response to Cd stress in peas. Moreover, the analysis confirmed that the expression of *PsGolS* was induced by Cd. Overall, the results of the distribution of carbohydrates in pea plants, together with the inhibition of seed production by Cd, indicate that plants tend to allocate energy to stress response mechanisms rather than to reproductive processes.

Keywords: galactinol; high-resolution gas chromatography; PAS reaction; raffinose; translocation factor; tolerance index

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1. Introduction

The concentration of cadmium (Cd) in the air, soil, and waters of the Earth is a result of natural and human activities such as industrial processes and agricultural practices [1,2]. The rising levels of this highly toxic heavy metal have led to a global environmental concern, particularly for plants [1,2]. Cd also poses a risk to livestock and human health, as it can accumulate in plants and subsequently be transferred through the food chain. Plants developed strategies for the accumulation and tolerance of heavy metals in response to environmental selection pressures caused by heavy metal pollution. These strategies enable them to play a crucial role in the bioremediation of heavy metal-contaminated environments [3]. The bioconcentration factor (BCF, the root-to-soil ratio of heavy metal) and the translocation factor (TF, the shoot-to-root ratio of heavy metal) are used to determine the level of phytoremediation in plants. Plants with more than one TF and BCF (TF > 1 and BCF > 1) are expected to be used in phytoextraction [4,5]. One study [6] showed that *Pisum sativum* has phytostabilization potential and exhibits Cd excluder behaviour (a BCF > 1

and TF < 1) when grown in hydroponics and treated with 50 μM $CdSO_4$. The excluder plants contribute to environmental sustainability and ecosystem resilience by acting as natural barriers against the harmful effects of heavy metal contamination [7]. The present study was carried out to understand the mechanisms underlying the harmful impact of Cd on plants and to address the ecological and agricultural consequences associated with Cd pollution.

Although Cd has not been shown to be required for metabolic processes, it is effectively absorbed by plants [8] and can affect plant growth and development at all stages [9]. Cd exerts its detrimental effects on plant development through multiple pathways, affecting physiological and biochemical processes [10–17]. One of the primary targets of Cd toxicity is the disruption of essential mineral nutrient uptake and homeostasis [14,18–21]. Consequently, this disrupts critical processes such as photosynthesis, respiration, and cellular metabolism, ultimately impairing plant growth and development [14,22,23]. Studies showed that exposure to Cd can cause a delay in flowering time and, consequently, in grain maturation, a smaller number of fruits or pods per plant, and reductions in their weight and yield [17]. Our previous study indicated that Cd affects the flowering time and the seed development of peas [14]. Interestingly, the inhibitory effect of Cd on photosynthesis and transpiration rate was observed later during the experiment [14]. For this study, we investigated the effect of Cd on changes in carbohydrate profiles over the course of the experiment. In addition, the study was carried out on fully developed but not flowering plants to analyse changes in sugar distribution under Cd stress during the transition from the vegetative to the generative stage of plant development.

Carbohydrates serve as essential energy sources and signalling molecules, and thus their availability and distribution are critical for developmental processes [24]. The Cd-induced alteration of total soluble sugar levels and carbohydrate metabolism was previously described [20,25–30]. The Cd exposure significantly increased soluble and reducing carbohydrates in summer savory (*Satureja hortensis* L.) [31], while decreasing the reducing sugar content in wheat seedlings [20]. Interestingly, Sun et al. [32] showed the pronounced accumulation of galactinol and raffinose family oligosaccharides (RFOs) during Cd stress in *Arabidopsis thaliana*. Here, we analysed the profile and changes of soluble carbohydrates using the high-resolution gas chromatography (HRGC) method, which allowed us to determine detailed information about the composition and concentration of individual sugars. This method overcomes the limitations of some previous techniques and provides more precise and comprehensive data on the presence and levels of specific sugars.

The accumulation of Cd also altered the levels of starch in plants, a source of energy that contributes to the overall growth and development of plants [20,25,33–35]. The previous research [33,34,36] demonstrated that plants respond differently in this regard. Cd-induced changes in the mesophyll cells of cotton leaf included an increase in the number and size of starch grains [34], while in *Ceratophyllum demersum* plants had no effect on starch accumulation [33], and in *Avicennia schaueriana* plants led to a decrease in starch grains [36]. In the present study, we analysed the starch accumulation using the Periodic acid Schiff (PAS) reaction. Bouzon et al. [37] and Simioni et al. [38] showed that this method is suitable not only to demonstrate changes in the number but also the location of starch grains in specific tissues.

In contrast to previous studies, we also analysed the level of soluble carbohydrates not only after the Cd-treatment but also during the Cd-treatment to gain insight into the strategies of pea plants in response to heavy metal contamination. To examine the role of Cd in long-term effects on carbohydrate dynamics, the 4-week plants of *Pisum sativum* L. 'Pegaz' after 1, 7, and 28 days of Cd treatment were analysed. We examined the location of polysaccharides (PAS method) and soluble carbohydrate alterations (using a gas chromatography method, HRGC). Furthermore, the results of the galactinol and raffinose content in the shoots led us to perform an expression analysis of the raffinose synthase (*PsRS)* and galactinol synthase (*PsGolS)* after the Cd treatment to determine the Cd

involvement in this process. This knowledge enhances the understanding of the metabolic adaptations and regulatory networks that underlie plant responses to Cd-induced stress.

2. Materials and Methods

2.1. Plant Material

The seeds of *Pisum sativum* L. 'Pegaz' were seeded in a wet perlite and grown in a glasshouse at 22–26 °C, watered with distilled water, and with ½ MS medium without sucrose [39]. The 4 weeks-old fully grown, but not flowering plants were treated with 10, 50, 100, and 200 µM $CdSO_4$ or water (control) for one week (three times a week—30 mL, 30 mL, and 40 mL, control plants received equal quantities of distilled water, with a total 100 mL of solution per pot—ø 15 cm, each pot with 5 plants) as previously described [14]. The plants were cultivated for another 28 days after treatment. For individual analysis, aboveground (shoots or leaves and stems) and/or underground (roots) parts of plants were collected on the 1st, 7th, and 28th days since the first application of Cd. The experiment was carried out in three replicates.

2.2. Periodic Acid Schiff (PAS) Reaction

The leaves and stems were analysed using a modified method, using periodic acid and Schiff's reagent [40]. The middle pea leaves were collected after 7 days of Cd treatment (control, 50 and 200 µM Cd), and the youngest fully developed leaves and the oldest leaves (not senescence) after 28 days of the experiment (control, 50 and 200 µM Cd). The stems were analysed after 7 and 28 days of Cd treatment (control, 50 and 200 µM Cd). Carnoy's fixed (ethanol/acetic acid, 3:1) pea leaves, stems, and roots were placed in 0.5% periodic acid (at 4 °C) for 12 h, then rinsed with distilled water (30 min, at room temperature, RT) and 70% ethanol (2 × 10 min, RT) and transferred to Schiff's Reagent (Stamar, Dąbrowa Górnicza, Poland) for 12 h, at RT. The tissues were placed in the reducing rinse (1 g potassium iodide, 1 g sodium thiosulfate pentahydrate in 20 mL of H_2O, and 0.5 mL of 20% hydrochloric acid) for 24 h. Before embedding (Spurr, Polysciences Inc.; Warrington, PA, USA) and sectioning (Ultracut R, Leica; Wetzlar, Germany) probes were rinsed with H_2O for 30 min and dehydrated in a series of ethanol. The tissue sections were analysed under an optical microscope (Nikon Eclipse 80i; Melville, NY, USA).

2.3. Soluble Carbohydrate Analysis

The carbohydrate level was analysed in the plants (shoot and root system) 24 h, 7, and 28 days after Cd treatment. The 28 days after Cd application control plants and plants treated with 10 µM $CdSO_4$ produced pods with seeds which were collected separately from the shoots. The freshly collected plant material was frozen in liquid nitrogen and then lyophilised. The dry material was pulverised in a mixer mill (MM 200, Retsch, Verder Group, The Netherlands), and soluble carbohydrates were extracted from the dry flour (40–42 mg for each biological repetition) with 800 µL of 50% ethanol: water (1:1, *v*/*v*), containing 100 µg of xylitol (as an internal standard) at 90 °C for 30 min. After cooling to room temperature, the homogenate was centrifuged at 14,000 rpm (21,000× *g* for 30 min at 4 °C), and the 400 µL of the supernatant was transferred to the ultra-spin filters (0.22 µm pore size) and centrifuged again (for 10 min). A clear filtrate (200 µL) was brought to dryness in a speed vacuum rotary evaporator (JWElectronic, Warszawa, Poland). The dry residues were derivatised with 200 µL of a mixture of TMSI/pyridine (1:1, *v*/*v*, Sigma-Aldrich, St. Louis, MO, USA) at 80 °C for 45 min. The TMS derivatives of carbohydrates were analysed with the high-resolution gas chromatography (HRGC) method on a capillary column (Rtx-1, 15 m length, 0.25 mm diameter, 0.1 µm thickness of 100% dimethyl polysiloxane layer, Restek, Anchem Plus, Warsaw, Poland) in a gas chromatograph (GC2010, Shimadzu, Kyoto, Japan), under conditions described previously [41]. The results were calculated using the internal standard method. Soluble carbohydrates were quantified from the standard curves; the ratios of the area of signals for each known compound to the area of the signal for xylitol, the internal standard, were plotted against known amounts (over the range of 10–250 µg)

of each compound [41]. Standards of xylitol, *myo*-inositol, and soluble carbohydrates were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.4. Gene Expression Analysis

Total RNA was extracted from three biological replicates, each consisting of shoots pooled from at least 5 plants. Plant tissue stored in an ultra-freezer was ground in liquid nitrogen and extracted with modified methods described by Wang et al. [42]. At the same time, RNA extraction buffer (100 mM Tris-HCl pH 9.0, 2% β-mercaptoethanol, 1% SDS) and Tri-Reagent were added to the samples homogenised in liquid nitrogen. The isolated RNA (5 µg) was treated with DNase (DNA-free, Promega, Madison, WI, USA) and the synthesis of cDNA was performed with Superscript II Reverse Transcriptase (Invitrogen, Waltham, MA, USA) at 42 °C for 1 h using an oligo dT primer, according to the manufacturer's protocol. Oligonucleotides for *PsGolS* mRNA (AJ243815: GenBank) were forward 5′CACGAAACTGAAACGTGCAT3′/reverse 5′-TCAGTTAAGCTGCCGAAGGT3′; for *PsRS* mRNA (AJ426475: GenBank): forward 5′GGAACAAACGGACACGAACT3′/reverse 5′AACTGGTCCACCAGAGATGG3′; and for the *EF1α* (X96555: GenBank) an internal standard, forward 5′TTCCCTTCGTTCCCATCTCTG3′/reverse 5′TACAAGCATACCGGGCTTCA3′ [43,44]. Semiquantitative PCR was performed on 2 µL cDNA (equivalent to approximately 0.5 µg starting RNA), 1 µM each primer, 0.2 mM of each dNTP, 2.0 mM $MgCl_2$, GoTaq buffer, and 0.75 U of GoTaq polymerase (Promega) in 30 µL total volume. The following conditions were used for the PCR amplification of *PsGolS* (962 bp) and *PsRS* (1287 bp): initial denaturation at 94 °C (4 min); touchdown cycles [94 °C (30 s), 68 to 61 °C (30 s), 72 °C (60 s)] (one cycle for each temperature) and 25 cycles at 94 °C (30 s), 61 °C (30 s) and 72 °C (60 s) followed by extension at 72 °C (10 min). Amplification of *EF1α* mRNA (236 bp) was carried out with 2 µL of cDNA with the following conditions set for PCR: initial denaturation at 94 °C (4 min); touchdown cycles [94 °C (15 s), 68 to 61 °C (15 s), 72 °C (30 s)] (one cycle for each temperature) and 20 cycles at 94 °C (15 s), 61 °C (15 s) and 72 °C (30 s) followed by extension at 72 °C (5 min). The intensity of bands was evaluated in a gel image analysis system (Gene Tools, Syngene, Cambridge, UK). Expression levels of *PsGolS* and *PsRS* were normalised with *EF* expression.

2.5. Accumulation of Cd Concentration in Pea Plants

After 28 days of Cd treatment, the shoots and roots of the pea were harvested for Cd accumulation analysis. The oven-dried samples were mineralised for 4–5 h in a 3:1 (v/v) mixture of HNO_3 and $HClO_4$ using a temperature step gradient (maximum of 200 °C) (DK 20, VELP Scientifica, Usmate, Italy). Digests were diluted to 25 mL with deionised water. A flame atomic absorption iCE 3000 Series spectrometer (Thermo Fisher, Waltham, MA, USA) was used to measure the Cd content.

2.6. Calculation of the Tolerance Index (TI) and Translocation Factor (TF)

The tolerance index (TI) was calculated to assess the plant's ability to grow in the presence of a specific concentration of Cd [45,46]. TI was calculated as follows:

$$\mathrm{TI} = \frac{\text{Dry weight (DW) of the plants grown in Cd solution}}{\text{Dry weight (DW) of the plants grown in control solution}} \times 100$$

The translocation factor (TF) measures the plant's ability to move accumulated metal from its roots to its aerial portions. The following is how TF was calculated [7,47]:

$$\mathrm{TF} = \frac{\text{C aerial parts}}{\text{C roots}}$$

where "C aerial parts" is the concentration (µg/g DW) of metal in the above-ground tissues and "C roots" is the concentration (µg/g DW) of metal in the roots.

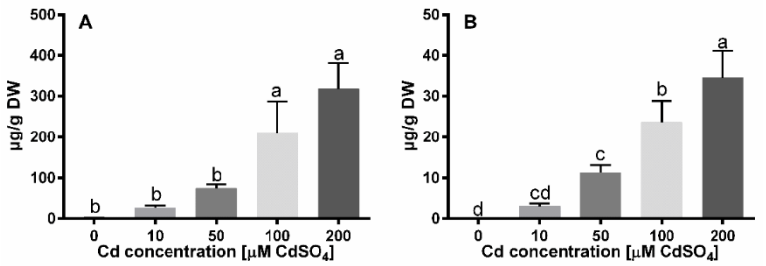
2.7. Statistical Analysis

All data shown in the figures are mean ± standard deviation (SD). The results were subjected to analysis of variance (ANOVA) and Tukey's post-hoc test with the level of significance set at $p < 0.05$ (lower case letters in the figures) or $p < 0.01$ (upper case letters in the figures). STATISTICA (ver. 13.1 Dell Inc., Tulsa, OK, USA) was used for statistical analysis.

3. Results

3.1. Accumulation, Translocation Factor, and Tolerance Index of Cd in Pea Plants

Cd accumulation, TF, and TI in pea plants were measured 28 days after Cd treatment (Figure 1). Roots accumulated more Cd than shoots (Figure 1A,B). Furthermore, the largest amounts of Cd were found in the roots and shoots of plants treated with 100 and 200 µM $CdSO_4$ (Figure 1A,B). Except for plants treated with 50 µM $CdSO_4$, the TF (Figure 1C) was comparable for all Cd treatments. The TF of plants treated with 50 µM $CdSO_4$ was 25.7, 22.9, and 30.5% higher than that of plants treated with 10, 100, and 200 µM $CdSO_4$, respectively. Interestingly, although the TI analysis (Figure 1C) did not reveal significant differences across treatments, the TI values of the plants treated with 10 and 50 µM $CdSO_4$ were greater than the TI values of the plants treated with 100 and 200 µM $CdSO_4$.



C

$CdSO_4$ [µM]	TF [%]	TI [%]
10	0.12 ± 0.03	103.19 ± 1.03
50	0.16 ± 0.04	101.54 ± 8.82
100	0.12 ± 0.05	96.23 ± 18.09
200	0.11 ± 0.01	93.23 ± 0.73

Figure 1. Cd accumulation in roots (**A**) and shoots (**B**), translocation factor, and tolerance index (**C**) of Cd after 28 days in *P. sativum* of the plants treated with Cd (10, 50, 100 and 200 µM $CdSO_4$). Each value is the mean of three replicates ± SD. Different letters represent significant differences ($p < 0.05$).

3.2. PAS-Reactive Elements and Tissue Structure Analysis after Cd Treatment

The localisation and amount of the PAS-reactive elements were analysed during the experiment (Figures 2–6). The PAS reaction resulted in purple staining of starch and the cell wall polysaccharides. Figure 2 shows the cross-sections of the leaves of control, 50, and 200 µM Cd-treated plants 7 days after treatment (Figure 2A–C) and the cross-sections of young leaves (Figure 2D–F) and old leaves (Figure 2G–I) 28 days after treatment. The number of PAS-positive deposits was highest in the leaves 28 days after Cd treatment (Figure 2D–I) followed by those after 7 days of treatment (Figure 2A–C).

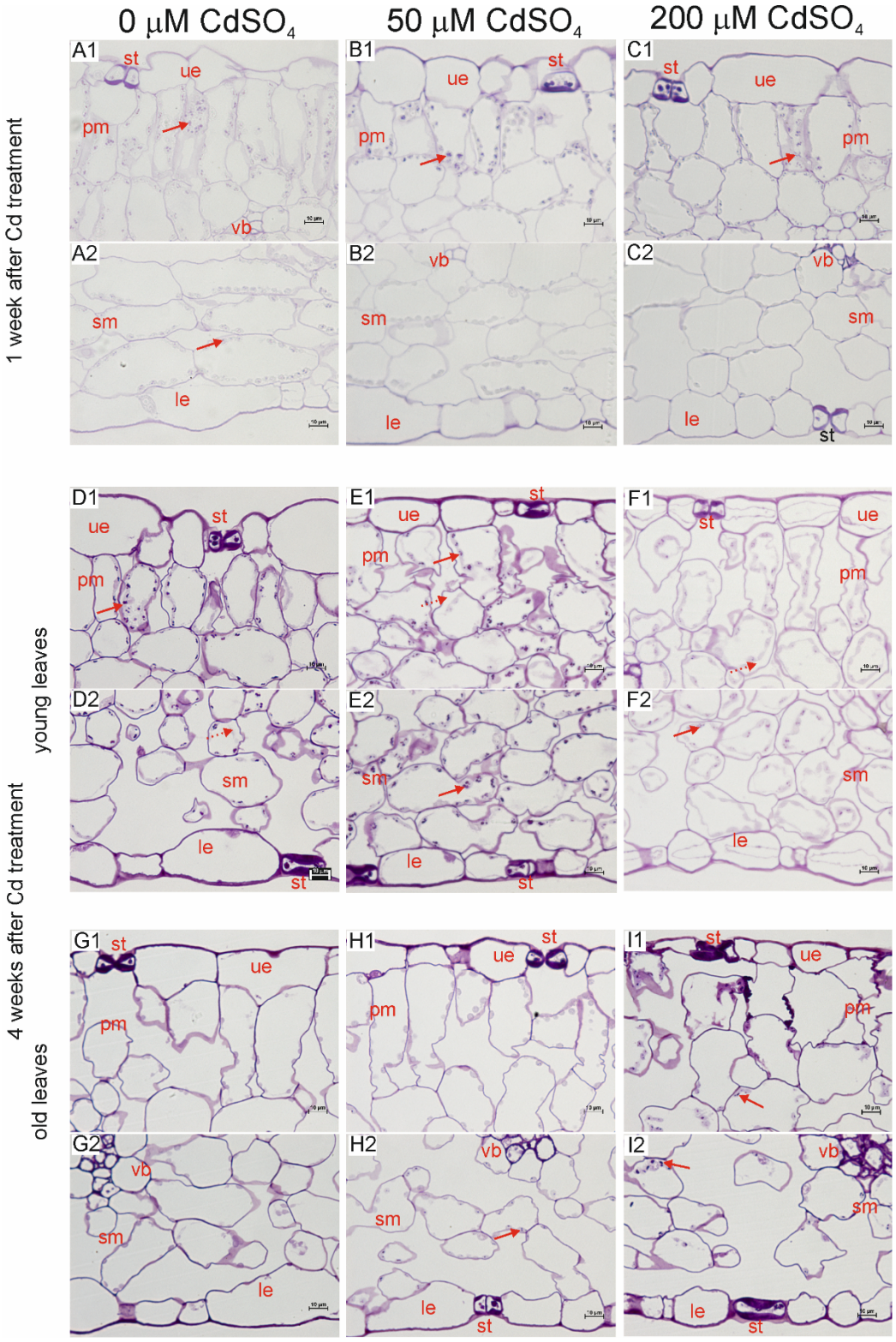


Figure 2. Cross-sections of the leaves of control (**A1**,**A2**,**D1**,**D2**,**G1**,**G2**), 50 (**B1**,**B2**,**E1**,**E2**,**H1**,**H2**) and 200 µM $CdSO_4$ (**C1**,**C2**,**F1**,**F2**,**I1**,**I2**) treated *P. sativum* plants. Images show leaves 7 days after Cd-treatment (**A1**,**A2**,**B1**,**B2**,**C1**,**C2**), young leaves (**D1**,**D2**,**E1**,**E2**,**F1**,**F2**) and older leaves 28 days after Cd treatment. The upper and lower parts of the leaf cross-section are denoted by "1" and "2", respectively. The purple colour shows the PAS-reactive elements, arrows indicate starch grains. The dotted arrows show plasmolysis. Abbreviations: st—stomata, pm—palisade mesophyll, sm—spongy mesophyll, vb—vascular bundle, ue—upper epidermis, le—lower epidermis. Scale bar 10 µm.

The distribution of the starch grains in the leaves and stems of *P. sativum* during the experiment was depicted in Table 1. Many starch grains were observed in the young leaves (Figure 2D,E) but not in the old leaves of the control plants and those treated with 50 µM Cd (Figure 2G,H). In contrast to this, the number of starch grains in the leaf mesophyll of 200 µM Cd-treated plants was higher in old leaves than in young ones (Figure 2I,F, accordingly).

Table 1. Distribution of starch grains in the tissues of *P. sativum*.

		LEAVES			STEM		
		0 µM $CdSO_4$	**50 µM $CdSO_4$**	**200 µM $CdSO_4$**	**0 µM $CdSO_4$**	**50 µM $CdSO_4$**	**200 µM $CdSO_4$**
1 week after Cd-treatment		+	+	+	+	+	–
4 weeks after Cd-treatment	Young leaves	+	+	–	–	+	+
	Old leaves	–	–	+			

"+" starch grains: present, "–" starch grains: low number or absent.

The distribution scheme of PAS-positive cell wall polysaccharides was similar. Moreover, excessive plasmolysis in the cells of the young leaves (Figure 2(F1)—dotted arrows) and alteration of the phloem in the old leaves of 200 µM Cd-treated plants (Figures 2I and 3—arrows, dotted arrows) compared to control plants were observed.

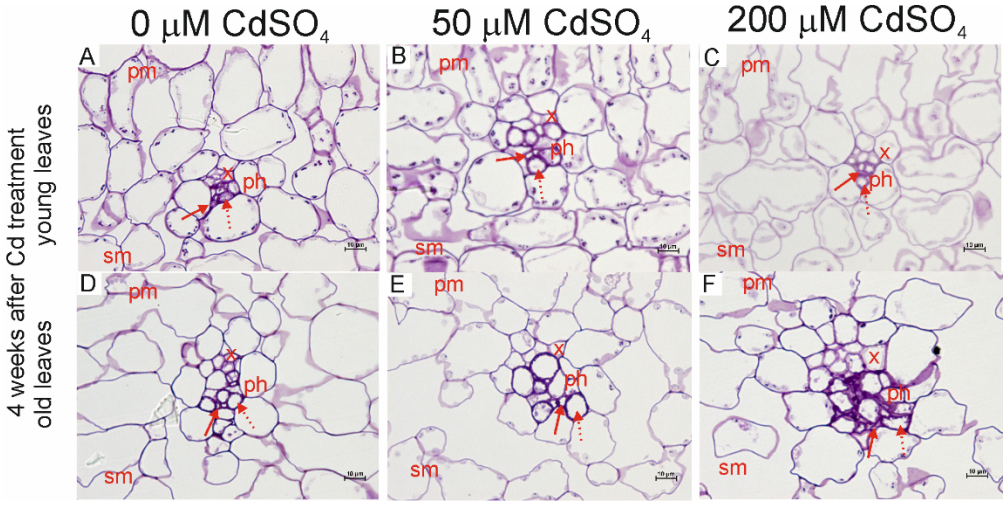


Figure 3. Cross-sections of the leaves of control (**A**,**D**) 50 (**B**,**E**) and 200 µM $CdSO_4$ (**C**,**F**)-treated *P. sativum* plants. Images show a vascular bundle structure (arrow—companion cell, dotted arrow—sieve tube). Abbreviations: x—xylem, ph—phloem, pm—palisade mesophyll, sm—spongy mesophyll. Scale bar 10 µm.

The cell walls of both the sieve tubes (dotted arrows) and companion cells (arrows) of these leaves were folded when compared to the other analysed variants of the experiment (Figure 3).

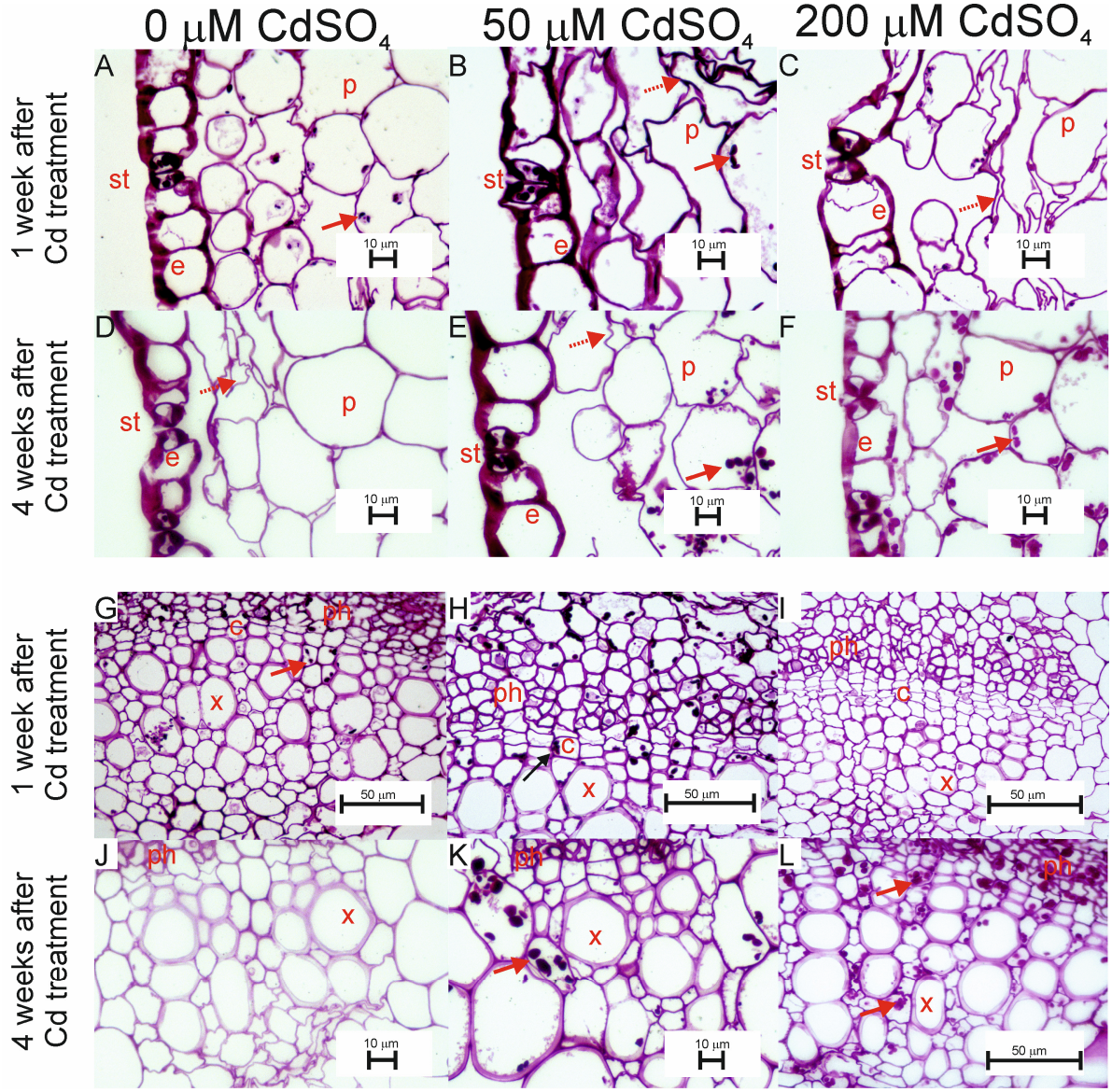


Figure 4. Cross-sections of the epidermis and stem cortex parenchyma (**A**–**F**) and the stem vascular bundle (**G**–**L**) of control (**A**,**D**,**G**,**J**) 50 (**B**,**E**,**H**,**K**) and 200 µM $CdSO_4$ (**C**,**F**,**I**,**L**) treated *P. sativum* plants. Images show tissues of the 7 (**A**–**C**) and the 28 days (**D**–**F**) after Cd-treatment. The purple colour shows PAS-reactive elements, arrows indicate starch grains. Dotted arrows indicate tissue alterations. Abbreviations: c—cambium, e—epidermis, p—stem cortex parenchyma, ph—phloem, st—stomata, x—xylem. Scale bar: 10 µm (**A**–**F**,**J**,**K**) and 50 µm (**G**–**I**,**L**).

After 7 days of 50 and 200 µM Cd treatment, the cells of the stem parenchyma were damaged (Figure 4B,C—dashed arrows). However, after 28 days of treatment, some tissue damage was also observed in the control plants (Figure 4D—dashed arrows). Surprisingly, the location of starch granules in stem tissues varied over the experiment (Figure 4—arrows, Table 1). After 7 days of Cd application, the number of starch granules in the chloroplasts of the stem parenchyma (Figure 4A–F—arrows) and the vascular bundle (Figure 4G–L—arrows) was higher in control plants than in plants treated with 200 µM $CdSO_4$. In contrast, 28 days following Cd treatment, the effect was reversed (Figure 4E,F,K,L—arrows). Interestingly, during the experiment, the same large number of starch grains in the stem cortex parenchyma and the stem vascular bundle parenchyma of 50 µM $CdSO_4$ treated plants was noticed (Figure 4B,E,H,K; Table 1).

3.3. Soluble Carbohydrate Content Analysis in the Roots and Shoots of P. sativum

Seven soluble carbohydrates (fructose, glucose, sucrose, *myo*-inositol, maltose, galactinol, and raffinose) were detected in the shoot system and six in the root system (galactinol was not detected) after HRGC analysis (Figure 5). In the roots of control plants, the content of monosaccharides (fructose and glucose) and *myo*-inositol decreased during 28 days of the experiment, while in shoots temporarily increased (until the seventh day) and later also decreased but remained at a higher level than in the roots (Figure 5). Similar trends in changes in the contents of sucrose and maltose were found in both shoots and roots (Figure 5). The plant treatment with Cd caused a significant change in the content of all identified carbohydrates in both the root and shoot tissues. Generally, Cd increased levels of sugars in both roots and shoots at each time point of the experiment along with the increasing concentration of Cd (in the case of *myo*-inositol, sucrose, and maltose).

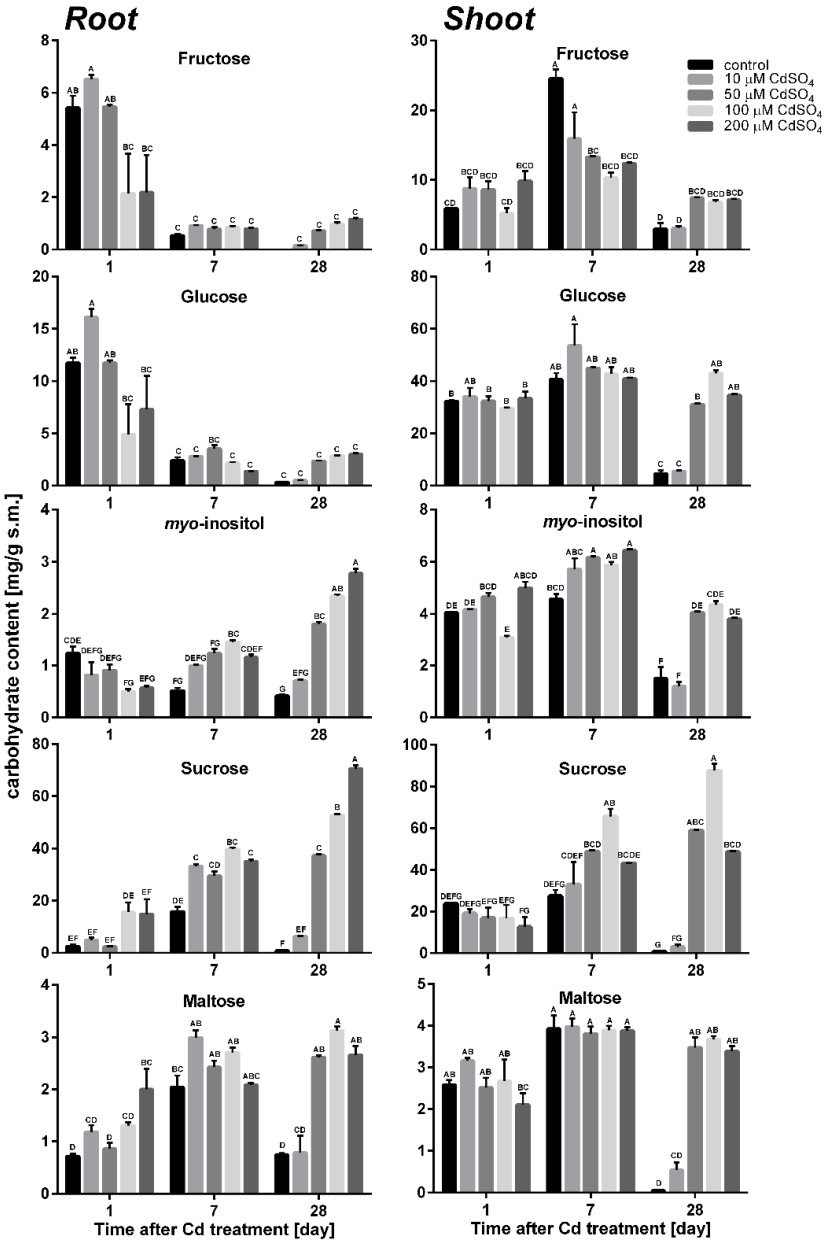


Figure 5. The content of soluble carbohydrates in root and shoot tissues of *P. sativum* plants after 1, 7, and 28 days of treatment with water (control) or cadmium ($CdSO_4$) at a concentration in the range of 10–200 μM. Each value is the mean of three replicates ± SD. Different letters represent significant differences $p < 0.01$.

Moreover, the increasing concentration of Cd had a significant effect on the expression of enzymes responsible for the synthesis of galactinol and raffinose (Figure 6) and the accumulation of both galactinol and raffinose during the first 7 days after plant treatment with Cd.

3.4. Analysis of the Accumulation of the Galactinol and Raffinose and the Expression of the PsGolS and PsRS after Cd-Treatment

Galactinol was not detected in the roots and shoots of control plants and those after one day of Cd treatment (Figure 6). Galactinol was detected solely in Cd-treated shoots, and its content was found to be positively associated with the Cd concentration used during the experiment. After 28 days of Cd treatment, the lowest amount of galactinol was found in the 10 µM Cd-treated shoot while the highest was in the 50, 100, and 200 µM Cd-treated shoots. Similarly to galactinol, raffinose in the roots was exclusively detected in Cd-treated plants, with the largest level of raffinose observed after 7 days of Cd treatment in the 100 µM Cd-treated root and after 28 days of Cd treatment in the 200 µM Cd-treated roots. The raffinose content in the shoot was nearly five times greater than in the root. The raffinose level in the 100 µM Cd-treated shoot was higher after 7 days of Cd treatment than in the 10, 200, 50, and control shoots, respectively. Nevertheless, after 28 days of Cd treatment, the maximum amount of raffinose was observed in the 50, 100, and 200 µM treated shoots (Figure 6A).

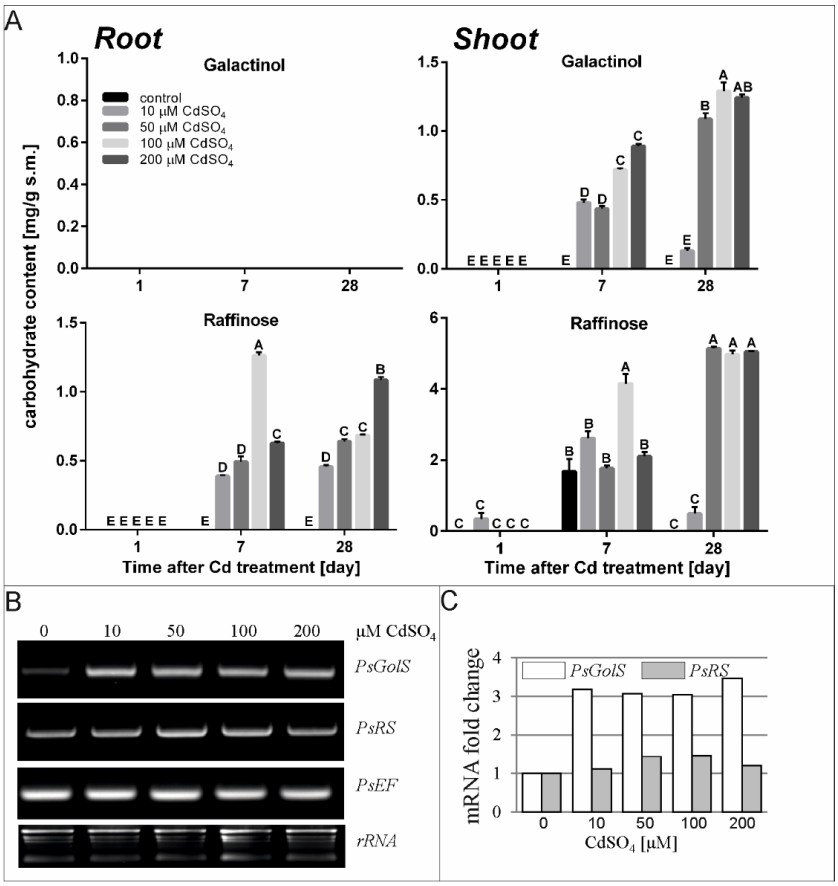


Figure 6. (**A**): HRGC profiles of galactinol and raffinose in the root and shoot after 1, 7, and 28 days of cadmium treatment. Each value is the mean of three replicates ± SD. Different letters represent significant differences $p < 0.01$. (**B**): Accumulation of *PsGolS* and *PsRS* transcripts in the shoots of pea plants after 1 day of Cd treatment. Representative gel of RT PCR for *PsGolS* and *PsRS* (*PsEF* represents a housekeeping control gene; rRNA represents the quality of 1 µg of the extracted RNA). (**C**): *PsGolS* and *PsRS* mRNA fold changes relative to control (0 µM $CdSO_4$).

Following the finding of Cd-dependent changes in raffinose and galactinol levels in pea shoots, we examined the expression of *PsGolS* and *PsRS* after 1 day of Cd treatment (Figure 6B). The results of semiquantitative RT-PCR demonstrated that the relatively low level of *PsGolS* mRNA in the control increased in Cd-treated samples, reaching at least a level threee-fold higher than detected in the control sample. Cd treatment, on the other hand, had a relatively minor effect on *PsRS* expression (Figure 6B,C). Cd stress, 50 and 100 µM, induced a 1,4-fold increase in the level of *PsRS* transcript. These findings agree with the quantities of galactinol and raffinose detected by GC in shoots after 7 days of Cd treatment (Figure 6).

3.5. Soluble Carbohydrate Content Analysis in the Pods and Seeds of P. sativum

The pea pods (Figure 7) and seeds (Figure S1) were analysed after 28 days of Cd treatment. Although all plants produced flowers and pods, only control and 10 µM Cd-treated plants produced seeds that could be analysed using HRGC (Figure S1). The amount of all seven detected soluble carbohydrates (fructose, glucose, sucrose, *myo*-inositol, maltose, raffinose, and galactinol) was higher in the pods of the Cd-treated plants (Figure 7).

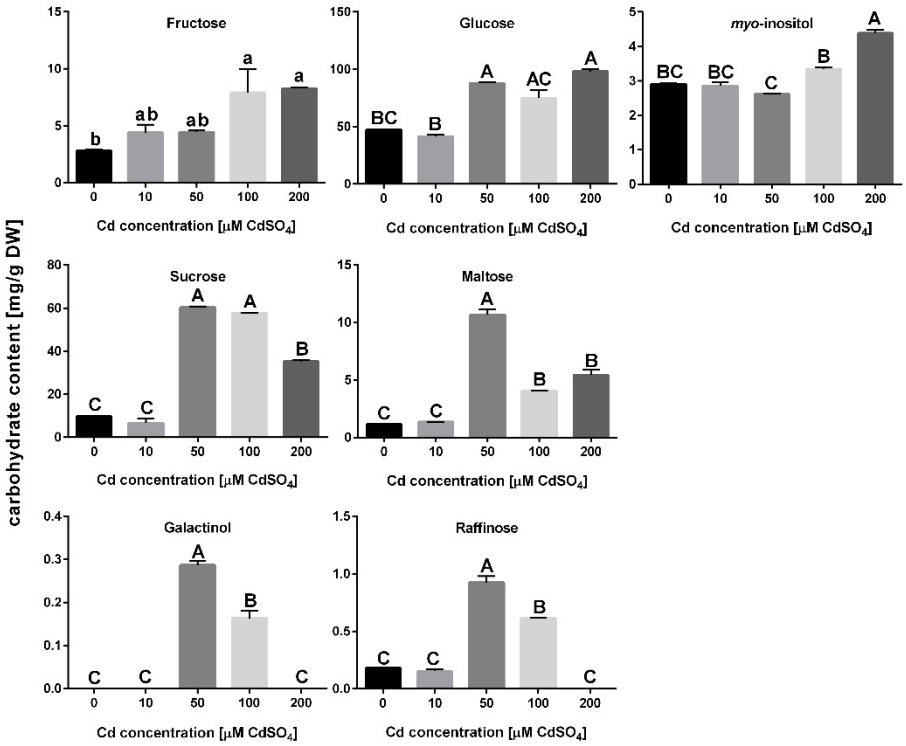


Figure 7. The content of soluble carbohydrates in pods of *P. sativum* plants on the 28th day after cadmium treatment. Each value is the mean of three replicates ± SD. Different letters represent significant differences at $p < 0.05$ (lowercase letters) or $p < 0.01$ (uppercase letters).

The levels of fructose, glucose, and *myo*-inositol increased along with increasing concentrations of $CdSO_4$. Sucrose and maltose amounts were higher in the pod tissues of the 50, 100, and 200 µM $CdSO_4$-treated plants, whereas in pods of control and 10 µM Cd-treated plants they were significantly ($p < 0.01$) lower (Figure 7). Galactinol was not detected in control, 10, and 200 µM Cd-treated pea pods, while raffinose was not detected only in the 200 µM Cd-treated tissues. The significantly highest level of both galactinol and raffinose was observed in the 50 µM Cd-treated pea pods.

The plants treated with 50, 100, and 200 µM Cd did not produce seeds; thus, only seeds of the control plants and plants treated with 10 µM Cd were examined (Figure S1). Nine soluble carbohydrates (fructose, glucose, sucrose, *myo*-inositol, maltose, raffinose, galactinol, stachyose, and verbascose) were detected. Except for the levels of sucrose and

verbascose, which were greater in the seeds of Cd-treated plants, the levels of all identified sugars were identical to one another. In contrast, the seeds of the control plants had larger quantities of *myo*-inositol and raffinose (Figure S1).

4. Discussion

In the presented paper, the PAS staining technique was coupled with HRGC for the precise measurement and profiling of individual monosaccharides, oligosaccharides, and polysaccharides. This integration clarified the complex carbohydrate changes induced by Cd stress, including changes in the sugar content, starch accumulation, and the dynamics of specific carbohydrate compounds, such as galactinol and raffinose. The experiment was conducted 1, 7, and 28 days after the treatment with Cd on pea plants during the transition from the vegetative phase to the generative phase of plant development. During this transition, plants undergo significant changes in their growth and development, shifting from producing leaves and stems to producing flowers and fruits. Our previous studies showed that after the fourth week of growth, pea plants start to produce flowers [14]. The present study confirmed the inhibitory effect of Cd on seed production when plants were treated with 50, 100, and 200 μM $CdSO_4$. As described in Section 3, these plants had accumulated 11.35, 23.68, and 34.57 μg/g of Cd in the DW of the shoots and 73.50, 208.89, and 317.68 μg/g of Cd in the DW of the roots, respectively. This confirms the results of previous studies suggesting most plants show visible symptoms of Cd toxicity when the Cd concentration in the plant tissue reaches 3 to 30 mg/kg, the total Cd concentration in the soil exceeds 8 mg/kg, or the bioavailable Cd concentration becomes >0.001 mg/kg [48]. Furthermore, both the results of Cd concentration and TF (TF < 1) showed that the majority of Cd absorbed by pea plants was stored in the root system. Consistent with prior research, our findings support the classification of pea plants as excluder plants [6]. This suggests that pea plants exhibit a strategy for the accumulation of Cd within the roots or to inhibit the translocation of Cd to the above-ground parts. Interestingly, the analysis of the TI showed that the Cd stress does not have a significant impact on the biomass production of pea plants. However, as previously confirmed, Cd influences the vegetative stage of development by increasing the number of internodes per shoot length and by disturbing the root growth but also affects the transition from the vegetative to the generative stage of the development of pea plants [14].

The present study focused on the effect of Cd on the starch distribution and soluble carbohydrates concentration after 1, 7, and 28 days of Cd treatment in pea plants. Furthermore, we analysed soluble carbohydrates in the seeds and pots of control plants and plants treated with 10 μM $CdSO_4$. We showed that Cd stress had a great impact on starch distribution patterns in leaves and stems. The PAS reaction, which is based on the oxidation of carbohydrates by periodic acid followed by their subsequent reaction with Schiff reagents, allows for the visualisation and quantification of polysaccharides such as starch and cellulose in plants during development or stress [40,49]. To the best of our knowledge, this is the first study of Cd-induced alterations in PAS-positive deposits in higher plants (*P. sativum* leaves and stems). However, this method was used for the analysis of Cd's effect on apical segments of *Hypnea musciformis* [37] and young gametophytes of *Gelidium floridanum* [38]. Similarly to Bouzon et al. [37], we observed accumulation of starch grains in the leaves of control and Cd-treated plants and also in the stems of the control and 50 μM Cd-treated plants after 1 week of the treatment. Moreover, the increased number of starch grains was observed in young leaves of control and 50 μM $CdSO_4$-treated plants 4 weeks after Cd treatment. However, it is important to notice the differences in the starch accumulation in the stems between control and Cd-treated plants after 4 weeks of Cd treatment. This characteristic accumulation of starch in the stems may suggest the disturbance of the translocation of carbon from the stems to flowers and then fruits since these plants did not produce seeds. It was shown that the starch is required for pollen maturation [50]. Verna and Dubey [51] showed no definitive pattern of changes in the starch content in the two rice cultivars, while Biswas and Pal [30] showed a significant accumulation of

starch in three rice cultivars after Cd treatment. Moreover, Cd-induced starch accumulation was also observed in the common reed [52], *Phyllanthus amarus* [53], and *Lemna minor* [54]. Higuchi et al. [52] proposed that the common reed may preferentially allocate absorbed carbon as the carbon source for the synthesis of Cd and α-glucan complexes in its stem, followed by the suppression of Cd transfer to the leaves, which operate as the photosynthetic organ. These responses may allow the common reed to grow successfully even in conditions of severe Cd stress. Starch–sugar interconversion in source and sink tissues plays a substantial physiological role in all plants [24]. The presented results confirmed that the inhibitory effect of Cd stress may be caused by energy allocation. During abiotic stress, plants prioritise energy allocation towards stress response mechanisms rather than reproductive processes [55]. These results and our previous study [14] showed that 50, 100, and 200 µM Cd-treated plants do not produce seeds. This indicates that the shift in resource allocation can affect the supply of sugars to develop flowers and seeds, potentially impacting the seed development and yield.

The accumulation of starch in the stems and changes in soluble carbohydrates in Cd-treated plants suggest that the impact of Cd is critical for the transport of sugars from leaves to flowers and the development of seeds. Cd-induced accumulation or steady concentrations of fructose, glucose, *myo*-inositol, sucrose, and maltose were observed in plants 4 weeks after Cd treatment at concentrations greater than 50 µM $CdSO_4$, while in control plants and plants treated with 10 µM $CdSO_4$, they decreased. This can be a confirmation that, in plants producing flowers and pods, soluble sugars are transferred to the flowers/fruits. The effect of sugar on vegetative development and floral transition has been discussed [56–58]. Previous studies showed Cd-induced increases in the levels of soluble and reducing carbohydrates [31,59] or a decrease in the content of reducing sugars [20,60]. Analysis of the Cd effect on maize varieties suggests that sucrose metabolism may be a secondary Cd response, and that the Cd-sensitive variety used more carbohydrates to defend against Cd stress rather than to support the growth of the Cd-tolerant variety [59]. Moreover, similar to the presented results, the effect of Cd and the accumulation of sucrose or fructose changed during the Cd treatment [61]. Li et al. [61] also suggested that changes in the sucrose metabolism were induced to maintain the osmotic balance in damaged cells and to protect the plant from Cd stress. The decrease in the water content is described as one of the effects of Cd stress on plants [60,62].

Interestingly, the Cd-induced accumulation of galactinol in the shoots and raffinose in the shoots and roots was also observed. Raffinose accumulation after Cd treatment was first noted by Costa and Spitz [26] in in vitro cultured *Lupinus albus*. Sun et al. [32] also observed an increased level of raffinose in two-week-old *Arabidopsis thaliana* seedlings after exposure to Cd. Since then, similarly to our results, Cd-induced expression of genes encoding galactinol synthase (GolS) and raffinose synthase (RS) has also been noted in rice [63]. The involvement of the galactinol synthase gene (*GolS*) in heavy metal stress was reported recently by Ranjan et al. [64]. There are numerous data showing stress-induced expression of *GolS* and/or *RS*. Induction of the expression of the *PsGolS* and *PsRS* genes during dehydration and osmotic stress in pea seedlings was also observed [44,65]. Moreover, Koning et al. [66] showed that the genes *GolS1*, *GolS3*, and *stachyose synthase* were significantly upregulated in the leaves of the *Phaseolus vulgaris* cv. CIAP7247F under drought stress. Transcriptional analysis indicated that most *Citrus sinensis GolS* (*CsGolS*) genes show a stress-inducible expression in response to drought and salt stress treatments, as well as to '*Candidatus* Liberibacter asiaticus' infection [67]. The comprehensive analysis of *RS* and *GolS* gene families in kiwifruit (*Actinidia chinensis* and *Actinidia eriantha*) revealed that abiotic stresses strongly induced *AcGolS1/2/4/8* and *AcRS2/4/8/11* expression [68]. Similarly, the analysis conducted on potato (*Solanum tuberosum* L.) showed that *StGolS4* exhibited significantly high expression levels after PEG-6000, and abscisic acid (ABA) treatments, and under salt stress [69]. Furthermore, it was found that all four isoforms of *CsGolS* in the cucumber genome were also upregulated by salt, drought, cold, and heat [70].

Transcriptome analysis carried out on rice reveals that Cd stress signalling controls the expression of genes in the drought stress signal pathway [63]. Exposure to Cd mediates gene expression by transcription factors such as DREB/CBS, WRKY, HSF, MYB, bHLH, bZIP, ERF, and NAC [63,71]. Most of these transcription factors have been described as upstream regulators of genes that encode *GolS* and *RS* [72]. The presence of putative *cis*-regulatory elements recognised by some of these transcription factors has been noticed in promoter regions of *PsGolS* as well as *PsRS* genes [65]. Nonetheless, the knowledge of the Cd-induced signalling pathway that leads to an increase in galactinol and raffinose levels is still limited. Data show that ABA or reactive oxygen species (ROS) mediate the activity of GolS on an mRNA or protein basis [69,73–76]. The role of ABA in Cd response is not well-established [77]. ABA can induce or reduce the accumulation of Cd and mitigate its toxicity, which can vary among species [61,77]. On the other hand, Cd induces oxidative stress in plants, including ROS production [78]. The role of galactinol and raffinose due to ROS-scavenging ability has been discussed [79,80]. Recently, Salvi et al. [81] also showed that Arabidopsis *CaGolS* transgenic lines, with increasing levels of galactinol and raffinose, accumulate less ROS and are more resistant against various abiotic stresses. Moreover, it has been shown that raffinose could prevent cellular leakage during dehydration by membrane stabilisation and fusion after rehydration [82]. Additionally, the location of raffinose in chloroplast allows it to protect thylakoids and stabilise photosystem II [83,84].

It is important to note that galactinol synthase (GolS) catalyses the first step in the biosynthetic pathway of the raffinose family of oligosaccharides (RFOs), producing galactinol from UDP-galactose and *myo*-inositol. Galactinol plays a crucial role as a major donor of galactosyl residues moieties in the synthesis of raffinose (from sucrose and galactinol) and later on stachyose (from raffinose) and verbascose (from stachyose). RFOs are believed to play a pivotal role in the tolerance to seed desiccation [85]. They are also the predominant transport carbohydrates in some plant families (i.e., Cucurbitaceae), act as signalling molecules after pathogen attack and wounding, and accumulate in vegetative tissues in response to a variety of abiotic stresses [85]. It has been shown that stress-inducible GolS plays a key role in the accumulation of galactinol and raffinose, which may function as osmoprotectants in drought, osmotic, a salt stress tolerance of plants [86,87]. The root and epicotyl of the winter vetch seedlings accumulated elevated amounts of galactinol and raffinose as the osmotic potential was lowered [88]. The elevated levels of galactinol and raffinose could also be the result of the dehydration that often accompanies the Cd stress response in plants [22,62,89].

The effect of Cd was also observed on the distribution of carbohydrates in the seeds and pods of peas. As mentioned earlier only control plants and plants treated with 10 μM $CdSO_4$ produced matured seeds [14]. El-Okkiah et al. [90] also showed a reduced number of pods per plant, fewer seeds per pod, and lower seed weight after Cd treatment of pea plants. The present analysis of soluble carbohydrates in the seeds showed that Cd affected the accumulation of sucrose, *myo*-inositol, raffinose, and verbascose. It is worth noting, that the level of RFOs in pea seeds is comparable to that observed by Gawłowska et al. [91]. Interesting results were noticed in the soluble carbohydrate distribution in pods after Cd treatment. The accumulation of sucrose and glucose in pods of plants treated with 50, 100, and 200 μM $CdSO_4$ was noticeable. Additionally, the highest level of maltose, galactinol, and raffinose but the lowest level of *myo*-inositol was observed in pods of plants treated with 50 μM $CdSO_4$. In contrast to that, the highest level of *myo*-inositol and the lowest level of galactinol and raffinose were observed in pods of the plants treated with 200 μM $CdSO_4$. The pods play an important role as a sink in the allocation of plant resources for the development of seeds [92,93]. In the early stages of seed development, the pod walls act as a temporary reservoir for assimilates, e.g., carbohydrates transported from the leaves/shoots before they are translocated to the developing seeds. In the pod wall, the level of soluble sugars falls during the later stages of development. These are possibly transported into the developing seeds and utilised there for starch synthesis, and thus a pod wall acts as a temporary reservoir of carbohydrates to be later transferred to seeds [94].

The studies carried out by Harvey [95] showed that the proportion of the total ^{14}C fixed by a $^{14}CO_2$-fed leaf which was exported, was related to the ^{14}C-sink capacity of the pods. Out of the total ^{14}C fixed by a leaf, the proportion that was exported within 24 h was related to the ^{14}C-sink capacity of the pods. Four days from anthesis, pea plants retained a substantial amount (51–67%) of the ^{14}C in the fed leaf and exported only a small portion (6–17%) to the pod. However, at 22 days from anthesis, the plants exported a higher average percentage (78%) to the pods and retained less (14–26%) in the fed leaf, indicating a shift towards increased carbon allocation to the pods as they mature [95]. This indicates that the proportion of ^{14}C exported to a pod increased with the plant age. Presented studies show that Cd-induced accumulation of the soluble carbohydrates in the shoots, in comparison to the control plants, where the level of sugars decreased during the experiment. This confirms the inhibitory effect of Cd on the translocation of assimilates to pods, and thus the inhibition of pod and seed development.

5. Conclusions

The presented study showed that cadmium exposure does not deplete soluble carbohydrates but alters carbohydrate distribution, impacting the resource allocation and sugar supply to flowers and fruits, and thus affecting the seed development and yield. The cadmium-induced expression of *PsGolS* confirms that the accumulation of galactinol and raffinose can enhance plant tolerance to heavy metal stress. Overall, the shift in carbohydrate allocation and metabolism under cadmium stress highlights the essential role of carbohydrates in plant defence mechanisms against heavy metal toxicity, demonstrating the complex ways in which plants adapt to and cope with environmental stressors such as cadmium.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14135486/s1, Figure S1. The content of soluble carbohydrates in seeds collected from the control plants and plants treated with 10 μM $CdSO_4$. Each value is the mean of three replicates ± SD. Different letters represent significant differences at $p < 0.05$ (lowercase letters) or $p < 0.01$ (uppercase letters).

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