

The Effect of Hydro-Priming and Proline Priming of Lettuce (*Lactuca sativa* L.) Seeds on Germination, Photosynthetic Pigments and Metal Metabolism under Cadmium Stress

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Abstract: Cadmium (Cd) is considered one of the most toxic heavy metals to living organisms, being very persistent in soil and non-biodegradable, thus posing a long-term hazard to plants and humans. In recent years, the application of different molecules at the seed level, known as chemical seed priming, has been studied as a method to improve stress tolerance in plants. In the present study, we tested the effect of hydro-priming and proline priming of lettuce (*Lactuca sativa* L.) seeds on germination, photosynthetic pigments, and metal metabolism under cadmium stress. Plants primed with proline showed better germination under cadmium stress (100% versus 84% for non-primed and hydro-primed seeds). Priming with 20 mM of proline increased the chlorophyll *a* and total chlorophyll contents by 40.8% and 18.6%, respectively, while these parameters decreased in other seedlings under Cd stress. Similarly, 20 mM of proline improved the uptake of Zn and Fe in roots under Cd stress. This indicates that 20 mM of proline treatments may be beneficial for maintaining a normal photosynthetic capacity and mineral uptake under Cd stress, but further metabolomics and transcriptomic data should reveal the exact mechanisms of action.

Keywords: lettuce (*Lactuca sativa* L.); cadmium stress; seed priming; hydro-priming; proline

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1. Introduction

Heavy metal stress is one of the most damaging abiotic stressors because it causes toxicity by targeting important molecules and vital processes in the plant cell [1]. Cadmium (Cd) is considered one of the most toxic heavy metals to living organisms [2] and is usually associated with fertilizer use. It is highly persistent in soil and is not biodegradable, thus posing a long-term hazard to plants and humans. In addition, this metal is highly soluble and mobile in the form of a divalent cation (Cd²⁺) and can easily interfere with plant processes by damaging plant structures and generating reactive oxygen species (ROS) [3]. Even if the plant does not accumulate Cd, its presence in the soil can cause other problems, such as a lower micronutrient uptake (found at higher Cd doses) [4] and micronutrient imbalance [5]. Cd exposure has been found to reduce iron (Fe) and manganese (Mn) uptake [6], which affects plant growth and biomass production.

Lettuce (*Lactuca sativa* L.) is one of the most popular and economically important leafy vegetable crops in the world [7]. It is a low-calorie vegetable but it contains many health-promoting compounds such as carotenoids, phenolic compounds, essential minerals, various vitamins, and dietary fiber [8]. Lettuce has a high ability to accumulate Cd from the soil without showing visible symptoms of metal toxicity, which poses a potential

risk to human health [9]. Therefore, various approaches have been used to reduce cadmium (Cd) toxicity in lettuce. For example, the foliar application of fulvic acid [9], abscisic acid [10], melatonin [11], and gibberellin [12] can significantly mitigate Cd-induced toxic symptoms in lettuce. Higher concentrations of cadmium can reduce lettuce growth, the chlorophyll content, and the photosynthesis rate through the promotion of chlorophyll and carotenoid degradation [12]. Chlorophyll is essential in light absorption processes and electron transfer, indirectly affecting plant growth. The inability of the plant to produce energy by photosynthesis can induce a reduction in other synthetic processes that are energy-driven, resulting in decreased plant growth [13]. All of these processes can affect lettuce yield as well as quality and food security when harvested due to the disruption of plant growth and changes in mineral absorption, often resulting in the bioaccumulation of harmful metals.

In recent years, the application of different molecules at the seed level, known as seed priming, has been investigated as a method to improve stress tolerance in plants [14]. Seed priming is based on controlled seed hydration, which places seeds in a “primed state” that stores this brief pulse and provides for more robust and resilient plants [15]. This process triggers metabolism prior to germination, without the formation of radicles, and involves the activation of enzymes, the buildup of metabolites, and other processes that help shorten the lag time during germination and have other positive effects on seed performance [14]. Priming seeds with silicone [16,17] and salicylic acid [18] has already shown promise as a priming agent for reducing Cd toxicity in lettuce. Proline is an amino acid that is present at much higher concentrations in seeds than in vegetative tissues, suggesting that proline plays an important role in seed metabolism [19]. Proline is involved in plant responses to Cd stress [20], and the exogenous administration of proline through seed priming can increase endogenous proline levels, which can modulate a variety of physiological and biochemical processes, resulting in improved growth [21]. However, the effect of seed priming with proline on the response to cadmium stress in lettuce has not yet been investigated. Priming can result in improvements in the growth and yield of lettuce under cadmium stress, but how priming affects the quality of the crop is still not well understood. In the present study, we investigated the effect of hydro-priming and priming with 10- and 20-mM proline on basic growth parameters of lettuce as well as the absorption and accumulation of significant minerals that can be affected by Cd stress.

2. Materials and Methods

2.1. Priming Treatments

Lettuce (*Lactuca sativa* L.) seeds were purchased from Royal Seeds (Italy). Priming of seeds was performed by immersing seeds in the appropriate solution for 24 h at 4 °C: distilled water (hydro-primed seeds), 10 mM proline, 20 mM proline, and non-primed seeds were used as controls. After 24 h, all seeds were rinsed twice with sterile distilled water and air-dried for 72 h to reach the original moisture content. Seeds were packed in tubes with silica gel to keep moisture low and stored at 4 °C until cultivation.

2.2. Plant Growing Conditions

Primed and non-primed seeds were cultured on growth media containing mineral nutrients (Murashige and Skoog, 1962), 3% (*w/v*) sucrose (Sigma Aldrich, Taufkirchen, Germany) as a carbon source, and 0.8% (*w/v*) agar (Sigma Aldrich, Taufkirchen, Germany) as a gelling agent. Half of the media were spiked with 0.25 mM Cd²⁺ in the form of the nitrate salt Cd(NO₃)₂, and the other half served as a control. For all media, the pH was adjusted to 5.8 with KOH/HCl before the addition of agar (*w/v*; 0.8%). The media were then sterilized in an autoclave and distributed into labeled, sterile Petri dishes under the laminar airflow hood. The media were stored at room temperature for 48 h before culturing to check for contamination. The surface sterilized seeds were cultured using an aseptic

technique under a laminar airflow hood in the prepared media. All cultures were maintained in a growth chamber under light (16 h photoperiod; 2000 lux) from Osram Fluora 18 W at 23 °C (± 2 °C) and 70% humidity. Plants were analyzed after four weeks of culture.

2.3. Determination of Germination Percentage and Seedlings Performance

For seed germination evaluation, five Petri dishes each containing 10 cultivated seeds (three independent replicates were performed for each condition total of 150 seeds per treatment) were grown under controlled conditions. The effect of seed priming on lettuce seed performance under cadmium stress was evaluated after 15 days of cultivation by counting germinated and non-germinated seeds and calculating the percentage of germination.

2.4. Determination of Photosynthetic Pigments

Analysis of photosynthetic pigment content was performed using 80% acetone fresh mass extracts of lettuce shoots as a function of priming and stress conditions. Spectrophotometric determination of absorbance was performed against a blank (80% acetone), and quantification was performed according to the equations of Lichtenthaler and Buschmann [22] and expressed in milligrams of chlorophyll per gram of FW.

2.5. Determination of Mineral Content

Mineral content analysis was performed by flame atomic absorption spectrometry (FAAS) for shoots and roots. The content of Cd, Fe, and Zn was determined after wet digestion and expressed as mg/kg DW. Shoot and root tissues were dried at 60 °C, finely ground, and then digested in a mixture of HNO₃ and 30% H₂O₂. After digestion, the solutions were analyzed for cadmium, iron, and zinc content by FAAS under optimized measurement conditions.

2.6. Translocation Factor

Cadmium concentrations determined for root and shoot samples were used for calculation of translocation factor. The translocation factor (TF) was calculated as the Cd concentration in shoot divided by the Cd concentration in root $TF = C_{shoot}/C_{root}$.

2.7. Statistical Analysis

The cultivation of plants had a randomized design. For all treatments, 10 seeds per Petri dish \times five Petri dishes (50 seeds) were cultivated in MS medium in three independent replicates (150 seeds in total). All results were expressed as mean values (\pm standard deviation; SD) of the three independent replicates. The analysis of variance of parametric data was performed using Factorial ANOVA test with Newman–Keuls test as post hoc analysis. The differences between treatments considering seed priming and cadmium concentration were evaluated at $p < 0.01$. All statistical testing was performed using Statistica 10.0 software (Copyright© StatSoft. Inc. 1984–2011, Tulsa, Oklahoma).

3. Results and Discussion

3.1. Effects of Seed Priming on Germination and Photosynthetic Capacity

To determine the effect of priming on germination under cadmium stress, we evaluated the germination percentage of primed and non-primed seeds grown in a medium containing 0.25 mM Cd and in a control medium. The results are shown in Table 1.

Table 1. Germination percentage of primed and non-primed lettuce seeds grown in 0.25 mM Cd and in control plants (without Cd in the media).

| | Control | 0.25 mM Cd |
|---------------------------|-------------------|-------------------|
| Non-primed | 100% ^a | 84% ^b |
| Hydro-primed | 85% ^c | 85% ^b |
| Primed with 10 mM proline | 90% ^{bc} | 100% ^a |
| Primed with 20 mM proline | 95% ^{ab} | 100% ^a |

Parameters within one column sharing the same letter do not differ significantly according to ANOVA post hoc analysis of variance using Newman–Keuls test at the level of significance $p < 0.01$.

The germination rate was significantly reduced by Cd exposure in unprimed and hydro-primed seeds, while seeds primed with proline showed increased germination under Cd stress. Interestingly, the germination of proline-primed seeds was inhibited under optimal conditions. This phenomenon of inhibited germination and/or growth of primed plants has been previously recorded, and it is known as the Bet Hedge phenomenon [23,24] or bet-hedging. This phenomenon has been correlated to stress memory and primed memory, and it is a result of compromised growth under optimal conditions in exchange for better performance in stressed conditions [24]. Negative effects of Cd on the germination rate in lettuce have been noted previously [25], and some treatments can mitigate Cd stress, such as priming seeds with silicon [17] or, as recorded in our study, using proline as the priming agent. The levels of Cd used in this study have an inhibiting effect on the germination of other species, such as rice [26], while we recorded significant improvements in germination under Cd stress when using proline. The reason for the better tolerance of toxic Cd concentrations in lettuce could be due to the harder seed coat, which prevents Cd penetration. However, a high germination rate does not necessarily mean better seedling performance because, once the seedling emerges from the seed, new defense mechanisms must be activated to cope with Cd stress [25].

Photosynthesis is an important process for plants that is very sensitive to changes in environmental conditions, and different abiotic stressors can severely impair photosynthetic processes, including effects on chlorophyll synthesis. An evaluation of the chlorophyll content can indicate the earliest changes in photosynthetic activities, making it a very effective parameter for tracking the Cd inhibitory effects on photosynthesis for seedlings grown from unprimed and hydro-primed seeds primed with 10 and 20 mM proline under cadmium stress.

The chlorophyll content was significantly reduced by Cd stress in non-primed, hydro-primed, and 10 mM proline-primed plants, while in plants primed with 20 mM proline, the chlorophyll *a* and total chlorophyll contents were significantly higher than in the control plants (Figure 1). Although priming with 20 mM proline had a positive effect on the chlorophyll content, the content of carotenoids in Cd-exposed seedlings decreased in all the analyzed samples. These results are expected because, according to the proteomic analysis by Zhang et al. [13], Cd stress was found to inhibit the expression of key enzymes during chlorophyll synthesis. They also reported that Cd damages the xanthophyll cycle, which may result in a lower total carotenoid content, which was also the case in our study. In green vegetables such as lettuce, the green color is associated with freshness, so a decrease in the chlorophyll content under Cd stress may affect the market value of these plants. According to our results, this can be prevented by priming the seeds with 20 mM proline, which increases the content of chlorophyll *a* and the total chlorophylls. These results are in agreement with those of Ambreen et al. [21], who reported that priming seeds with proline improves the efficiency of photosystem II.

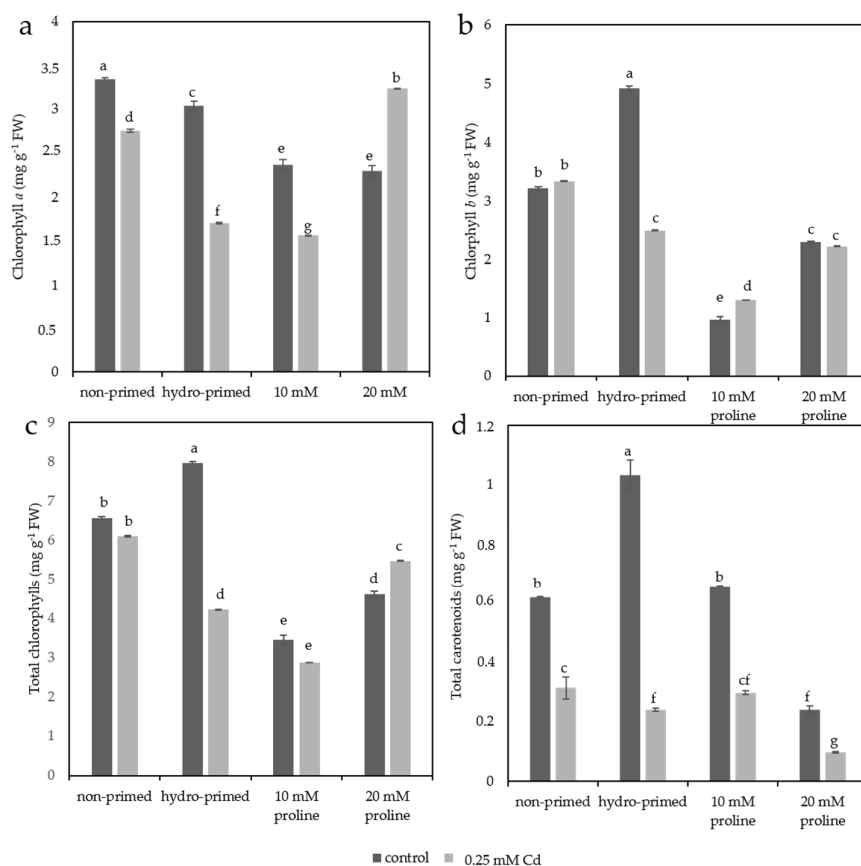


Figure 1. Chlorophyll and carotenoid contents in primed and non-primed plants under Cd stress in relation to control conditions. Columns within one condition sharing the same letter above the column do not differ significantly according to ANOVA post hoc analysis of variance using Newman–Keuls test at the level of significance $p < 0.01$. Asterisk following the letter above columns marks significant decrease/increase in chlorophyll or carotenoid contents under Cd stress conditions in relation to control.

According to Lichtenthaler and Buschmann [22], the ratio of chlorophyll *a* and chlorophyll *b* as well as the ratio of the total chlorophyll and total carotenoids could provide useful information about the adaptation of plants to stress conditions.

The chlorophyll *a/b* ratio decreased significantly in unprimed and 10 mM proline-primed seedlings (Table 2). In hydro-primed seedlings, this ratio remained the same in control seedlings and seedlings grown under Cd, while in seedlings primed with 20 mM proline, this ratio was higher in plants grown under Cd stress than in the control. Under stress conditions, the chlorophyll *a/b* ratio tends to increase, as chlorophyll *a* is reduced more compared to chlorophyll *b* [27], which is evident from our results for the seedlings grown from non-primed seeds. Maintaining more chlorophyll *a* than chlorophyll *b* is critical for survival under severe conditions [27], resulting in an increase in the ratio of chlorophyll *a/b*, as in the case of our seedlings grown from seeds primed with 20 mM proline. Also, similar to our results, Sadeghipour [28] reported that priming seeds with 25 and 50 mM proline increased the chlorophyll level of Cowpea (*Vigna unguiculata* (L.) Walp.) under Cd stress.

Table 2. Chlorophyll *a*/chlorophyll *b* and total chlorophylls/total carotenoids ratio in shoots of primed and non-primed lettuce seedlings under Cd stress and in control.

| | | Chlorophyll <i>a</i> /Chlorophyll <i>b</i> | Total Chlorophylls/ Total Carotenoids |
|---------------------------|------------|---|--|
| Non-primed | control | 1.04 ^d ± 0.01 | 10.64 ^c ± 0.02 |
| | 0.25 mM Cd | 0.83 ^e ± 0.01 | 19.72 ^b ± 2.42 |
| Hydro-primed | control | 0.62 ^f ± 0.01 | 7.74 ^{cd} ± 0.38 |
| | 0.25 mM Cd | 0.68 ^f ± 0.00 | 17.58 ^b ± 0.45 |
| Primed with 10 mM proline | control | 2.49 ^a ± 0.07 | 5.11 ^d ± 0.18 |
| | 0.25 mM Cd | 1.21 ^c ± 0.01 | 9.66 ^c ± 0.19 |
| Primed with 20 mM proline | control | 1.00 ^d ± 0.03 | 19.32 ^b ± 1.36 |
| | 0.25 mM Cd | 1.45 ^b ± 0.00 | 56.59 ^a ± 1.31 |

Parameters within one column sharing the same letter do not differ significantly according to ANOVA post hoc analysis of variance using Newman–Keuls test at the level of significance $p < 0.01$.

3.2. Effect of Seed Priming on Mineral Absorption

The levels of cadmium, iron, and zinc in lettuce roots and shoots grown from primed and non-primed seeds are shown in Table 3.

Table 3. Cd, Fe, and Zn content in shoots and roots of primed and non-primed lettuce under Cd stress and in control.

| | | Cd mg/g dw | | Fe mg/g dw | | Zn mg/g dw | |
|---------------------------|---------------------------|----------------------------|----------------------------|-----------------------------|------------------------------|----------------------------|----------------------------|
| | | Roots | Shoot | Roots | Shoot | Roots | Shoot |
| Non-primed | control | 6.82 ^d ± 4.57 | 1.17 ^f ± 0.84 | 1461.21 ^b ± 8.62 | 122.56 ^c ± 2.81 | 81.97 ^f ± 4.24 | 235.02 ^c ± 1.49 |
| | 0.25 mM Cd | 469.04 ^a ± 0.00 | 174.21 ^d ± 0.96 | 1276.3 ^c ± 10.32 | 32.59 ^g ± 2.24 | 75.86 ^g ± 3.84 | 124.04 ^h ± 1.80 |
| | Hydro-primed | control | 7.35 ^d ± 2.01 | 3.29 ^{ef} ± 0.70 | 1943.18 ^a ± 5.35 | 203.01 ^a ± 1.64 | 151.84 ^a ± 0.50 |
| Hydro-primed | 0.25 mM Cd | 287.44 ^b ± 1.27 | 210.87 ^c ± 2.41 | 1159.58 ^d ± 4.12 | 54.58 ^f ± 0.63 | 135.85 ^b ± 0.44 | 147.91 ^g ± 0.09 |
| | Primed with 10 mM proline | control | 3.06 ^d ± 0.31 | 1.82 ^f ± 0.69 | 330.33 ^h ± 132.59 | 185.88 ^b ± 2.98 | 107.81 ^d ± 3.24 |
| 0.25 mM Cd | | 298.27 ^b ± 5.67 | 223.03 ^b ± 3.51 | 446.78 ^g ± 7.01 | 110.82 ^d ± 0.06 | 87.46 ^e ± 5.12 | 202.33 ^d ± 3.99 |
| Primed with 20 mM proline | | control | 2.24 ^d ± 1.87 | 6.92 ^e ± 1.24 | 531.76 ^f ± 0.37 | 120.80 ^c ± 3.34 | 111.21 ^d ± 0.52 |
| | 0.25 mM Cd | 202.26 ^c ± 3.19 | 235.24 ^a ± 2.49 | 807.46 ^e ± 15.24 | 95.86 ^e ± 1.15 | 129.35 ^c ± 0.54 | 202.19 ^d ± 0.23 |
| | Cd | | | | | | |

Parameters within one column sharing the same letter do not differ significantly according to ANOVA post hoc analysis of variance using Newman–Keuls test at the level of significance $p < 0.01$.

The presence of Cd in the medium resulted in an increased Cd content in the roots and shoots of all plants compared to the control. Compared to the non-primed seedlings, the Cd concentration was lower in the lettuce roots of all the primed variants, with increased translocation and accumulation of Cd in the shoots. Lettuce is known as a plant with high Cd accumulation [29], and this is also evident from our results. Cd transporters in plants are also involved in the transport of essential nutrients such as Zn and Fe [30]. Iron (Fe) is necessary for plant growth, with the redox of Fe²⁺ and Fe³⁺ involved in the plant electron transport chain, photosynthesis, respiration, and chloroplast synthesis [31,32].

When plants are under Cd stress, Cd can impair the uptake and transport of Fe by plants and cause plant iron deficiency chlorosis, which further exacerbates Cd-induced oxidative stress and inhibits plant growth [33,34]. Our results show a decrease in the Fe content in seedlings, roots, and shoots of non-primed and hydro-primed seeds under Cd stress, while priming with proline increased the translocation of Fe as well as Zn.

Non-primed seedlings show an impairment of the translocation of Fe and Zn under Cd stress, with more prominent effects on the transport of Fe (Table 4). It is recorded that the uptake of Fe is not affected, but the loading in the phloem and the translocation from roots are. It is recorded that Cd inhibits Ca-like metals by competition, but its effects on decreased translocation have a deeper cause. Most likely, Cd also decreases transport by chelators through the xylem, which can be connected to Cd effects on signaling pathways and xylem transporters [35]. An increase in Fe translocation in primed plants suggests that proline influences a plant's tolerance of Cd beyond the obvious alleviation of growth-inhibiting effects but also might trigger signaling-related events. There is evidence that correlates Cd uptake with Fe regulated expression of genes for ABC transporters affecting the rate of Fe/Cd uptake and transport [36]. Additionally, for affected Fe translocations, Zn translocation was also reduced in non-primed and hydro-primed seedlings. Zn is an essential micronutrient for many metabolic functions, and the uptake of Cd by plant roots follows the same pathway as that of zinc (Zn) [30]. Therefore, it is not surprising that, according to our results, Cd decreases Zn content in all the samples studied, except in the root samples primed with 20 mM proline. The decreased translocation of Zn affects photosynthesis through its role in the maintenance of Rubisco activase and the activity of chloroplastic Zn-SOD [37]. Increases in Zn translocation in primed plants (especially with 10 mM proline) could be attributed to the amplification of Zn(II)/Cd(II) competition at the plasma membrane, which can result in decreases in Cd uptake and at the same time, increases in the translocation of Cd can be recorded (Chen et al., 2018). Our results confirmed this hypothesis. We recorded a decrease in Cd uptake in proline-primed plants but an increase in the translocation factor; this process was followed by the increased translocation of Zn and Fe as well in Cd stress conditions. Non-primed plants demonstrate a high uptake of Cd and severe impairment of the uptake and translocation of Fe and Zn under Cd stress. Such findings suggest that proline has a different mechanism compared to hydro-priming and that some components of these tolerance-inducing mechanisms are concentration-dependent, considering differences between two proline priming treatments.

Table 4. Translocation factor of primed and non-primed lettuce under Cd stress and in control.

| | | TF (Cd) | TF (Fe) | TF (Zn) |
|---------------------------|------------|---------------------------|--------------------------|---------------------------|
| Non-primed | control | 0.23 ^d ± 0.25 | 0.08 ^b ± 0.00 | 2.87 ^a ± 0.13 |
| | 0.25 mM Cd | 0.37 ^{cd} ± 0.00 | 0.03 ^b ± 0.00 | 1.64 ^b ± 0.06 |
| Hydro-primed | control | 0.45 ^{cd} ± 0.03 | 0.10 ^b ± 0.00 | 1.23 ^b ± 0.01 |
| | 0.25 mM Cd | 0.73 ^{bc} ± 0.01 | 0.05 ^b ± 0.00 | 1.09 ^b ± 0.00 |
| Primed with 10 μM proline | control | 0.61 ^c ± 0.27 | 0.64 ^a ± 0.29 | 2.95 ^a ± 1.28 |
| | 0.25 mM Cd | 0.75 ^{bc} ± 0.01 | 0.25 ^b ± 0.00 | 2.32 ^{ab} ± 0.18 |
| Primed with 20 μM proline | control | 6.76 ^a ± 0.34 | 0.23 ^b ± 0.01 | 2.26 ^{ab} ± 0.02 |
| | 0.25 mM Cd | 1.16 ^b ± 0.01 | 0.12 ^b ± 0.00 | 1.56 ^b ± 0.01 |

Parameters within one column sharing the same letter do not differ significantly according to ANOVA post hoc analysis of variance using Newman–Keuls test at the level of significance $p < 0.01$.

4. Conclusions

Seed priming has been investigated as an effective agent for increasing plant tolerance to stressors, including heavy metals. What has not been investigated is how seed priming affects other minerals and how seed priming is reflected not only in plant growth but also in mineral absorption and metabolism. In this work, we investigated the effects of hydro-priming and proline priming on plant growth and metal metabolism under Cd

stress. The Cd inhibitory effect on lettuce growth has been confirmed, mainly due to the effects on photosynthetic apparatus, but also a significant effect on mineral absorption has been recorded. From the tested priming agents, proline seems to be effective, especially when used in higher concentrations for the maintenance of a normal photosynthetic capacity and Zn and Fe uptakes under Cd stress, but further metabolomics and transcriptomic analysis should confirm these statements. Our results suggest that proline induces changes in seeds, creating a memory that enables plants to better survive Cd stress not only through the alleviation of the growth-impairing effects of Cd but also through the activation of Fe- and Zn-dependent signaling mechanisms affecting the translocation of minerals. However, further in-depth metabolome and transcriptome studies are needed to explain these mechanisms.

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