Hindawi
Adsorption Science & Technology
Volume 2022, Article ID 7747152, 11 pages
https://doi.org/10.1155/2022/7747152

Research Article

Iminodisuccinic Acid Relieved Cadmium Stress in Rapeseed Leaf by Affecting Cadmium Distribution and Cadmium Chelation with Pectin

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Received 16 March 2022; Revised 22 April 2022; Accepted 8 May 2022; Published 13 June 2022

Academic Editor: Qiaohui Fan

Rapeseed (*Brassica napus* L.) is a nutritious vegetable, while cadmium (Cd) pollution threatens the growth, productivity, and food security of rapeseed. By studying the effects of iminodisuccinic acid (IDS), an easily biodegradable and environmental friendly chelating agent, on Cd distribution at the organ and cellular level, we found IDS promoted dry matter accumulation of rapeseed and increased the contents of photosynthetic pigment in leaves. Inhibited root-shoot Cd transport resulted in higher activity of antioxidant enzymes and decreased hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) accumulation in leaves, which indicated that IDS contributed to alleviating Cd-caused oxidative damage in leaf cells. Additionally, IDS increased Cd subcellular distribution in cell wall (CW), especially in covalently bound pectin (CSP), and relieved Cd toxicity in organelle of leaves. IDS also enhanced demethylation of CSP. The Cd content in CSP, demethylation degree, and pectin methylesterase activity of CSP increased by 37.95%, 13.34%, and 13.16%, respectively, while IDS did not change the contents of different CW components. The improved Cd fixation in leaf CW was mainly attributed to enhance demethylation of covalently bound pectin (CSP) and Cd chelation with CSP.

1. Introduction

Excessive cadmium (Cd) in soil has remarkably affected the annual output of agricultural products due to reducing the production and quality of crops and vegetables [1, 2] which ultimately threatens food security [3, 4]. There are various methods of Cd-contaminated soil remediation, such as inoculation of soil with cadmium-resistant bacterium or biochar-supported microbial cell composite ([5, 6]), combined application of lime and organic matter [7], and phytoremediation of hyperaccumulation [8–10]. Cd absorbed by roots is easily transported to shoots due to its high mobility in plants [11, 12]. Rapeseed (*Brassica napus*) is not only a widespread oil crop [13], but also a popular nutritious vegetable. It is essential to reduce Cd content in the edible part of rapeseed to ensure food safety and enhance plant Cd resistance, which is also an effective strategy to improve rapeseed growth and production [14].

It has been verified extensively in many studies that the subcellular distribution of Cd is critical to Cd resistance in plants [15, 16], and cell wall (CW) as the first barrier can efficiently prevent Cd from entering cells [17–19]. In plants, most of the Cd^{2+} exists in CW [20, 21] by chelating with different CW components (pectin, cellulose, and hemicellulose) [22]. The CW immobilization of Cd, mainly due to chelating with pectin and carboxyl groups (COO-), arises from the demethylation of pectin by the catalysis of pectin methylesterase (PME) [9, 22].

Iminodisuccinic acid (IDS) is synthesized from maleic anhydride, ammonia, and sodium hydroxide. It is regarded as a kind of "green" chelating agent with low toxicity and fast degradation [23]. Previous researches have indicated the effects of some chelating agents, such as ethylenediamine tetraacetic acid (EDTA) [24, 25] and polyaspartic acid (PASP) on plant resistance to Cd [26]. Jing and Wang [27] also reported the stabilizing effect of IDS on metal ions.

Although there is ample evidence that IDS increases the aboveground biomass and decreases Cd accumulation in leaves of plants [28], however, few researchers studied the physiological responses of plants to Cd by IDS application, especially the effects on subcellular Cd distribution and CW components. Therefore, our experiment was set up to (a) study the Cd uptake and transportation in rapeseed by IDS application; (b) investigate how IDS affects the Cd resistance, subcellular Cd reallocation, and Cd chelation with different CW components; and (c) ultimately reveal the internal regulation mechanism underlying IDS improving Cd resistance in leaves of rapeseed.

2. Material and Methods

2.1. Experimental Arrangement and Growth Condition. A pot cultural experiment was set up in a glass greenhouse at Qingdao Agricultural University. The lightly Cd-contaminated soil with pH = 5.58 and total Cd content = 1.24 mg/kg was used in this experiment. The content of organic matter, total nitrogen (N), total phosphorus (P), and total potassium (K) in soil was 34.68, 1.90, 1.29, and 243.42 g/kg soil, respectively. To begin, 0.50 g urea, 0.82 g $Ca(H_2PO_4)2H_2O$, 0.26 g KCl, and 0.10 g H_3BO_3 were mixed thoroughly with 2 kg of soil before being filled into the pot. We set up two different treatments: conventional fertilizer treatment (CK) and conventional fertilizer +0.3% IDS (IDS). Each treatment contained five replicates (five pots), and each pot cultured two rapeseed seedlings. IDS is purchased from Hebei Wozi Environmental Protection Technology Co., Ltd, and the purity of IDS was more than 60%, and the molar mass was 337.1 Da. Fifteen days after *Brassica napus* L. seed germination, uniform seedlings were chosen and transplanted to pots of different treatments and then were cultured in a solar greenhouse for 45 days. All seedlings were randomly arranged to guarantee the condition's uniformity. During the experiment, the field capacity of about 80% was maintained by daily weighing each pot and evaluating water loss.

2.2. Analysis of Plant Dry Matter and Cd Concentration in Plants. After the rapeseed roots and leaves were separated and dried in an oven to constant weight at 75°C, their dry weights were measured and noted. Dried samples were ground to fine powder with a mortar and weighed, and then, all root powder and 0.10 g leaf powder were, respectively, digested in 5 ml concentrated nitric acid (HNO_3) in water bath kettle at 100°C for 2 h. The Cd concentration in the leaves and roots was then measured using inductively coupled plasma mass spectrometry (ICP-MS; PerkinElmer, MA, USA).

2.3. Quantification of Leaf Photosynthetic Pigment. The photosynthetic pigments (including chlorophyll a, b, and carotenoid) of fresh leaves were extracted with 95% ethanol and quantified based on the method of Wu et al. [2]. After extracting for 24 h under the condition of darkness at 25°C, the absorbance was determined by a spectrophotometer (HitachiUV-3100 UV/VIS; TECHCOMP, Shanghai, China) at the wavelengths of 665, 649, and 470 nm. The contents of chlorophyll a, chlorophyll b, and carotenoids were calculated according to corresponding absorbance values and following formulae:

$$\begin{aligned}
\text{chlorophyll a}\,(\text{mg/L}) &= 13.95A_{665} - 6.88A_{649};\\
\text{chlorophyll b}\,(\text{mg/L}) &= 24.96A_{649} - 7.32A_{665};\\
\text{carotenoid}\,(\text{mg/L}) &= (1000A_{470} - 2.05\text{Ca} - 114.8\text{Cb})/245.
\end{aligned} \tag{1}$$

2.4. Measurement of Reactive Oxygen Species (ROS), Antioxidant Enzymes, and Malondialdehyde (MDA) Contents. The superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) were extracted from fresh leaves and determined by spectrophotometry (HitachiUV-3100 UV/VIS; TECHCOMP, Shanghai, China) according to Liu and Liu [29]. The activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and MDA content in leaves was quantified using the methods of Giannopolitis and Ries [30], Reff [31], De Azevedo Neto et al. [32], and Buege and Aust [33], respectively.

2.5. Determination of Cd Contents in Subcellular Components and Different CW Fraction. First, the leaf was processed into three subcellular components (CWs, organelles, and soluble fractions) according to Rathore [34] and Weigel and Jager [35], and then, CWs were further separated into several components: covalently bound pectin (CSP), ion-bound pectin (ISP), cellulose, and hemicellulose based on the method of Hu and Brown [36]. Then, the content of different CW component was measured by corresponding kits (Komin Biotechnology Co., Ltd., Suzhou, China). The Cd contents in the leaf subcellular components and different CW component were quantified by the ICP-MS (PerkinElmer, MA, USA).

2.6. Determination of the Pectin Methylation Degree (DM) and Pectin Methylesterase (PME) Activity. The degree of methylation of the ISP and CSP was determined based on the method of Anthon and Barrett [37]. Briefly, after the solution of pectin extract and NaOH was incubated at 25°C for 0.5 h, the H_2SO_4, Tris-HCl, MBTH, and alcohol oxidase (AO) were added in order. Then, the mixture was incubated at 30°C for 20 min, and ammonium ferric sulfate and sulfaminic acid solution were immediately added to terminate the reaction. Finally, the absorbance of the solution was measured by a spectrophotometer (HitachiUV-3100 UV/VIS; TECHCOMP, Shanghai, China) at the wavelength of 620 nm. The DM was calculated as the demethylation degree = 100 − DM.

The PME activity of fresh leaves was measured using the available commercial kit (PME-2-G, Suzhou Comin Biotechnology Co., Ltd.).

2.7. Data Statistical Analysis. The Student *t* test was applied to data with the Statistical Package for Social Sciences (SPSS ver. 19.0, SPSS Inc.). Different lowercase letters (a, b) indicated significant difference between CK and IDS treatments at the $P < 0.05$ level.

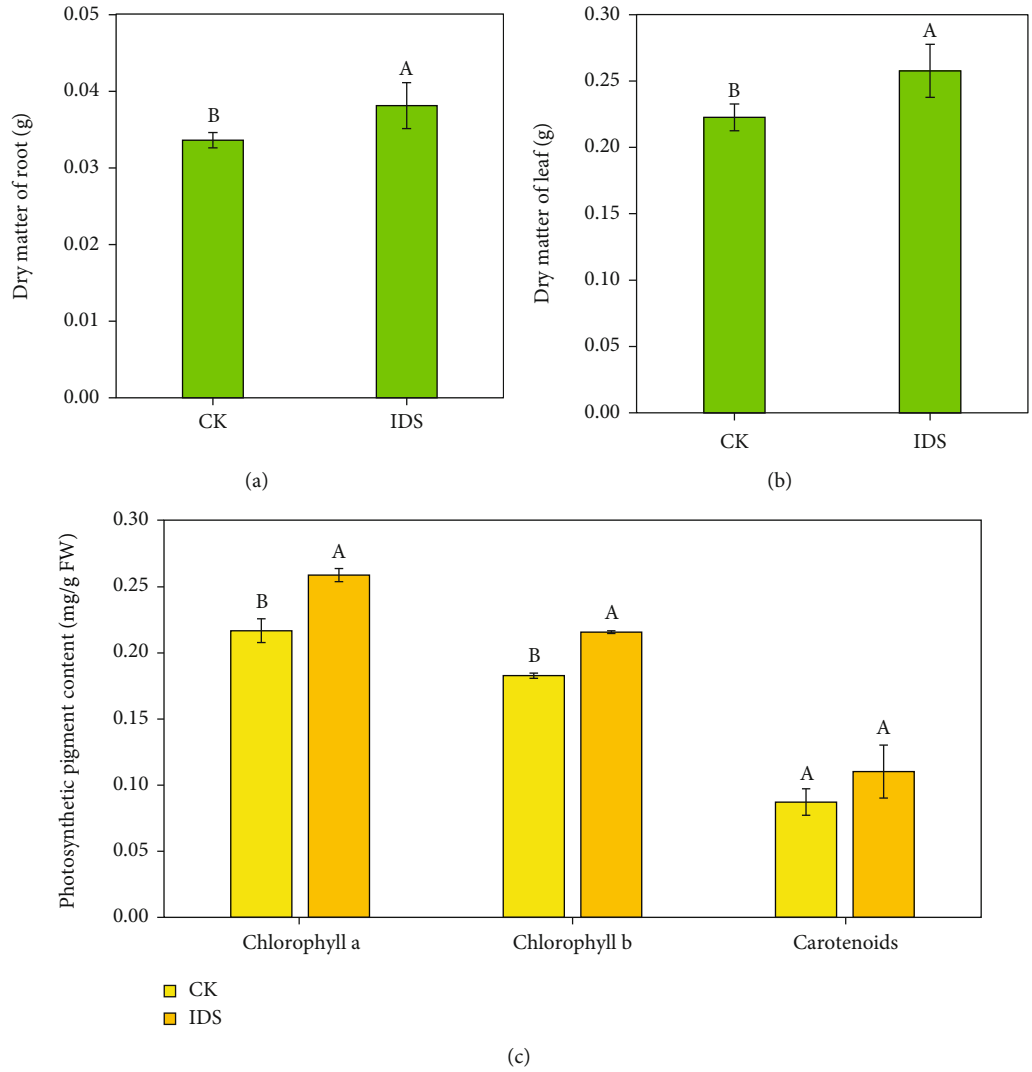


Figure 1: The effects of IDS on dry mass and pigment content in rapeseed under Cd stress. Mean ± SD ($n = 5$). Different uppercase letters (A, B) indicate significant difference between CK and IDS treatment at $P < 0.05$.

Table 1: The effects of IDS on Cd concentration and distribution in rapeseed seedlings. Mean ± SD ($n = 5$). Different capital letter indicates significant difference between these two treatments at $P < 0.05$ level.

Treatment	Cd concentration (μg/g)		Cd accumulation (μg/plant)		Cd distribution (%)		Cd transfer coefficient (%)
	Leaf	Root	Leaf	Root	Leaf	Root	
CK	4.5 ± 0.2A	13.6 ± 0.8A	1.0 ± 0.1A	0.5 ± 0.0A	68.3 ± 2.3A	31.7 ± 1.2B	33.5 ± 2.0A
IDS	3.1 ± 0.1B	14.3 ± 0.4A	0.8 ± 0.1B	0.5 ± 0.0A	59.8 ± 1.9B	40.2 ± 3.1A	21.9 ± 0.9B

Note: Cd transfer coefficient% = Cd concentration in leaves/Cd concentration in roots × 100.

3. Results

3.1. IDS Increased Plant Dry Mass and Photosynthetic Pigment Contents. The results shown in Figure 1(a) and Figure 1(b) suggest that IDS application significantly increased the dry mass of rapeseed roots and leaves, indicating that IDS promoted the growth of rapeseed under Cd stress. In the meantime, our study found increased photosynthetic pigment contents, especially chlorophyll a and chlorophyll b ($P < 0.05$) in rapeseed leaves of IDS treatment (Figure 1(c)).

3.2. IDS Decreased Cd Accumulation and Transportation in Rapeseed Leaves. To identify the effects of IDS on Cd in rapeseed seedlings, we quantified Cd concentration and calculated Cd accumulation in roots and leaves, Cd distribution, and Cd transfer coefficient. Table 1 suggests that IDS application did not affect Cd concentration and

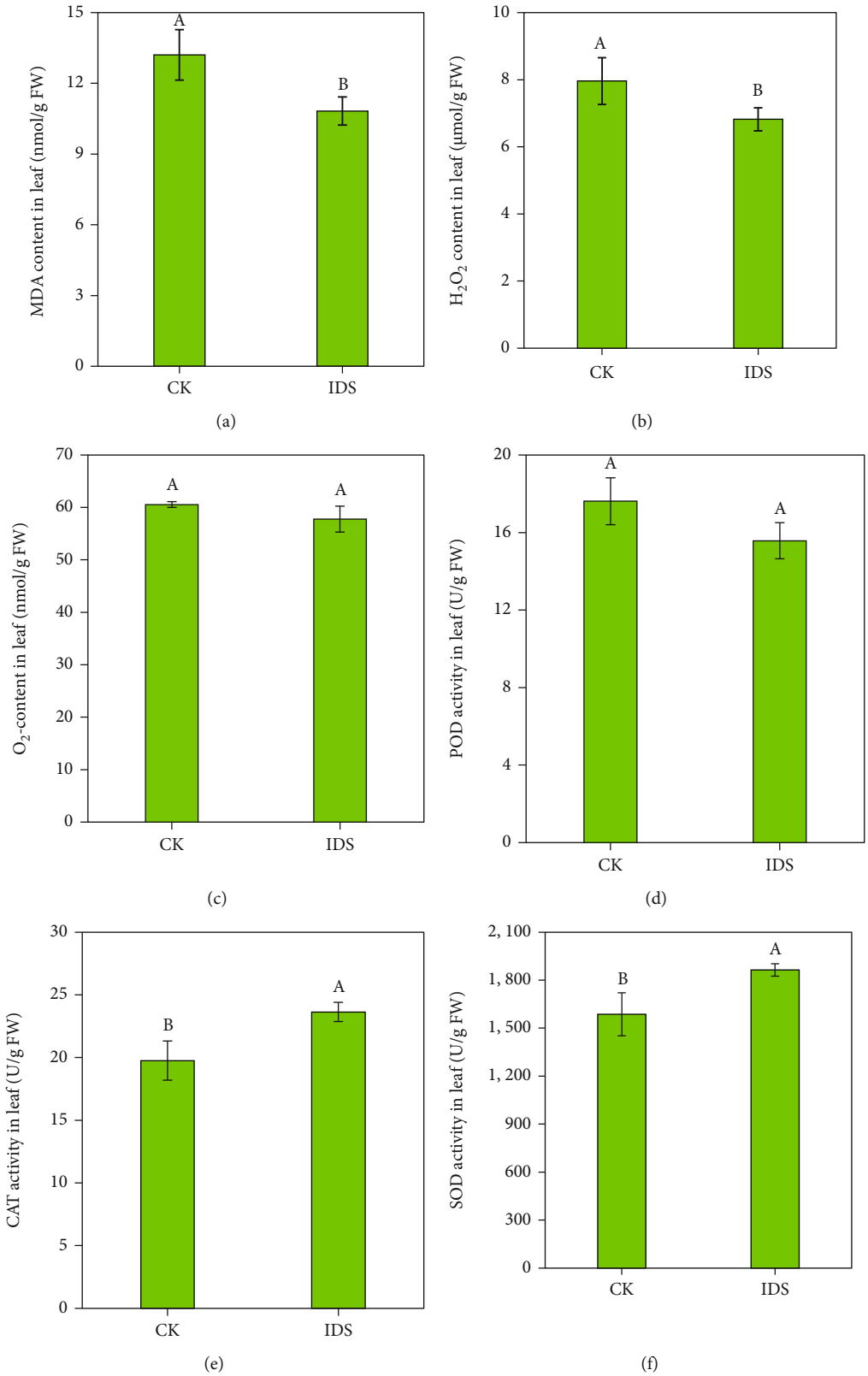


Figure 2: The effects of IDS on MDA, ROS, and antioxidant enzyme system in rapeseed leaves under Cd stress. Mean ± SD ($n = 5$). Different uppercase letters (A, B) indicate significant difference between CK and IDS treatment at $P < 0.05$.

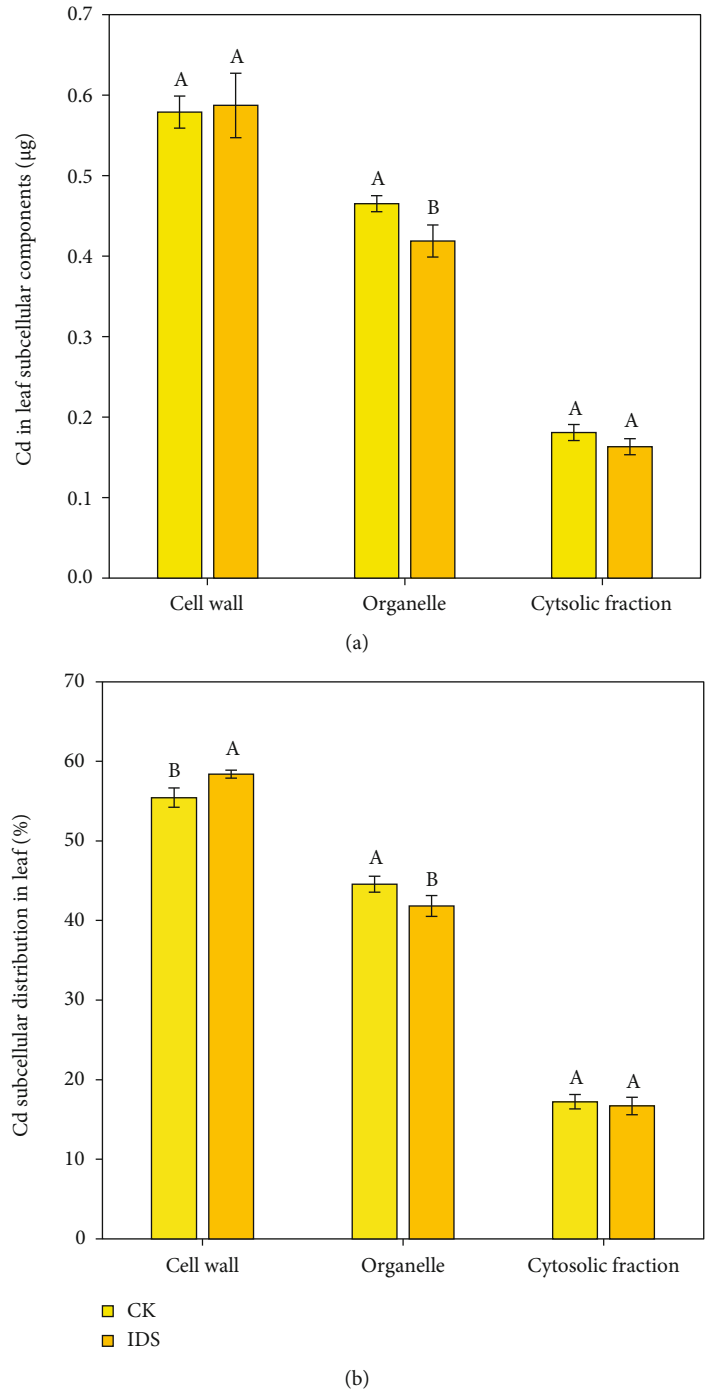
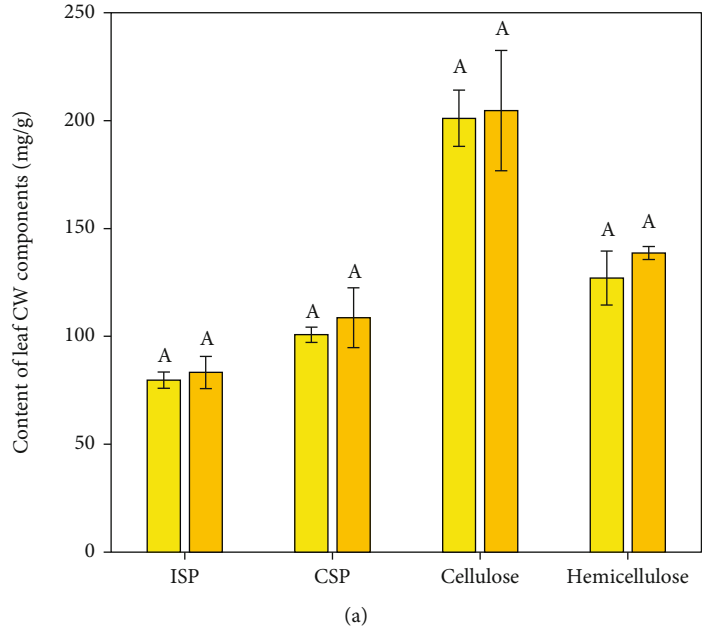


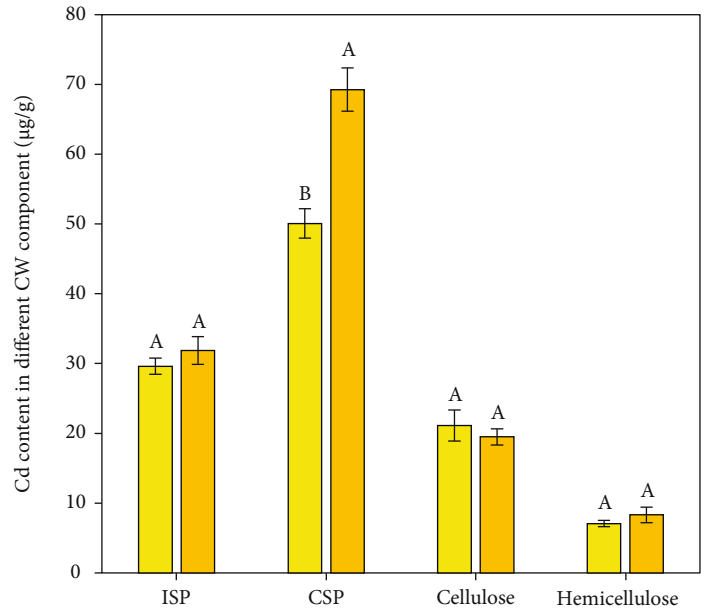
FIGURE 3: The effects of IDS on the subcellular distribution ratio of Cd in rapeseed leaves. Mean ± SD ($n = 5$). Different uppercase letters (A, B) indicate significant difference between CK and IDS treatment at $P < 0.05$.

accumulation in roots, but decreased Cd concentration and accumulation in leaves. Moreover, the Cd distribution ratios in the leaves and roots of IDS treatment were raised and reduced by 12.5% and 26.8%, respectively, compared to that of CK treatment. Cd transfer coefficient can indicate the ability of Cd transport and distribution from roots to the leaves. The decreased Cd transfer coefficient also suggested that IDS significantly reduced the Cd transport from roots to leaves.

3.3. IDS Decreased Oxidative Stress of ROS on Cells in Rapeseed Leaves. Cadmium could induce MDA and ROS accumulation to exacerbate cell membrane liquid peroxidation and oxidative damage to plant cells. According to the results of MDA, ROS content, and antioxidant enzyme activity in leaves, IDS application on rapeseed seedlings planted in Cd-contaminated soil decreased MDA and H_2O_2 accumulation in leaves at a significant level ($P < 0.05$), but had no obvious effect ($P > 0.05$) on $O_2^{\cdot-}$ contents



(a)



(b)

Figure 4: Continued.

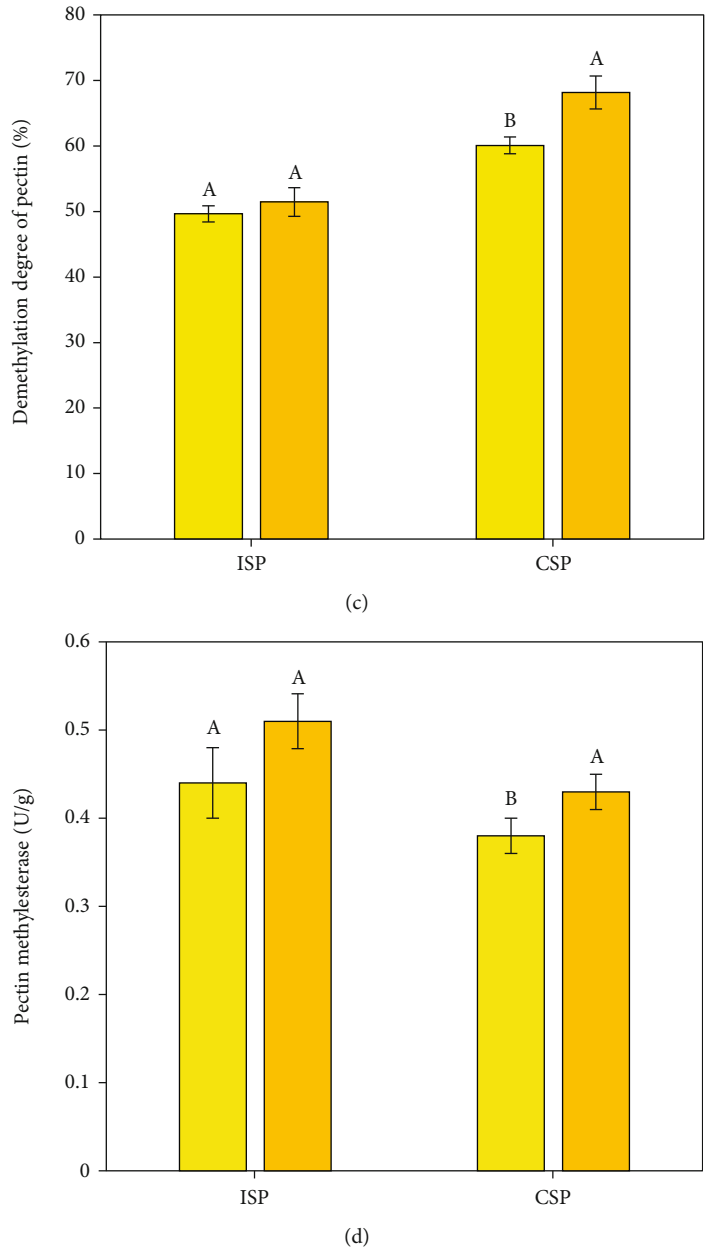


Figure 4: The effects of IDS on CW components, Cd content in CW components, and pectin demethylation in rapeseed leaves. Mean ± SD ($n = 5$). Different uppercase letters (A, B) indicate significant difference between CK and IDS treatment at $P < 0.05$.

(Figures 2(a)–2(c)). Improved activity of CAT and SOD, which is related to the removal of excess ROS in plants, was observed in rapeseed leaves under IDS application (Figures 2(e) and 2(f)). However, there was no significant effect on POD activity (Figure 2(d)).

3.4. IDS Promoted CW Fixation of Cd and Decreased Cd in Organelles in Rapeseed Leaves. To further reveal how IDS affects Cd subcellular distribution, we fractioned different parts of leaves into CWs, organelles, and cytosolic fractions (excluding vacuoles) and measured Cd content in each of these components in rapeseed leaves. IDS application did not affect Cd contents in CWs and cytosolic fractions, while significantly decreased Cd contents in organelles (Figure 3(a)). The Cd distribution ratio in different CW components shown in Figure 3(b) suggests higher Cd distribution in CWs and lower Cd distribution in organelles in leaves treated with IDS, indicating relieving Cd toxicity in organelles. In addition, IDS had no obvious effect on Cd content in cytosolic fractions (Figure 3(a)), indicating that IDS did not affect the vacuolar compartmentalization of Cd.

3.5. IDS Increased Cd Content in CSP by Promoting Pectin Demethylation in Rapeseed Leaves. The Cd in CWs was primarily chelated by various CW components such as cellulose, pectin, and hemicellulose. IDS application to rapeseed under Cd stress did not change the content of CSP, ISP, cellulose, and hemicellulose at a significant level ($P < 0.05$)

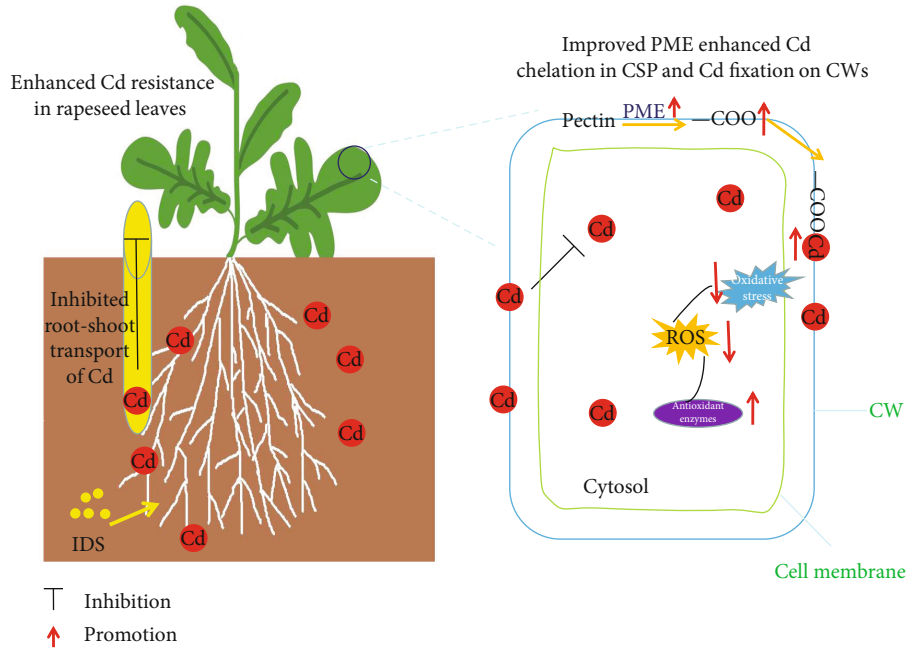


Figure 5: The schematic diagram illustrating the effects of IDS on cell wall components in determining Cd tolerance in leaves of rapeseed.

(Figure 4(a)). Interestingly, Cd contents in CSP were increased from 50.1 μg/g to 69.9 μg/g (Figure 4(b)), indicating that IDS promoted Cd chelation with CSP of the leaf CWs. To identify the ability of pectin to chelate Cd, we determined PME activity and the degree of methyl esterification of pectin, and the results showed that IDS increased PME activity of CSP and promoted demethylation of CSP (Figures 4(c) and 4(d)), thereby improving the carboxyl groups that can bind Cd.

4. Discussions

4.1. IDS Inhibited Cd Transportation from Roots to Shoots in Rapeseed. In plants, water, mineral nutrients, and heavy metals such as Cd absorbed from soil are transported from roots to shoots [38, 39]. In this study, IDS supplication did not significantly affect the mobility of Cd in soil (Table S1) and Cd accumulation in rapeseed roots (Table 1). However, our study indicated that IDS reduced Cd distribution to leaves of rapeseed and significantly decreased Cd transfer coefficient from roots to shoots (Table 1), suggesting that IDS inhibited the root-shoot transportation of Cd and ultimately alleviated Cd stress in the edible part of rapeseed [14], which is conducive to reducing the potential safety hazards of agricultural products.

4.2. IDS Decreased ROS Accumulation and Activated Antioxidant Enzymes in Leaves. Excessive Cd in plants usually increases the MDA content, which may raise membrane lipid peroxidation degree and indirectly aggravate the oxidative stress in cells [2, 18, 40]. ROS accumulation also causes oxidative damage to plants [41]. The presence of heavy metals can not only change the soil enzyme system, such as urease, protease, catalase, phosphatase, and β-glucosidase [42, 43], but also antioxidant enzymes in plants [2]. Higher antioxidant enzyme activity has been widely perceived to reduce ROS accumulation in plants under different stress [44–46]. Many studies have proposed that the activated antioxidant enzyme system plays an essential role in improving plant Cd resistance and acts as ROS scavengers [47]. In the present study, activated activities of CAT and SOD by IDS application contributed to decreased MDA content and reduced H_2O_2 accumulation under Cd toxicity in leaves (Figure 2). However, the significantly decreased Cd concentration in leaves with IDS supply (Table 1) may result in higher antioxidant enzyme activity, lower MDA, and ROS accumulation.

4.3. IDS Enhanced Cd Fixation in Leaf CWs by Increasing the Cd Chelation with CSP. It has been reported that the Cd subcellular reallocation determines Cd resistance in plants [15, 19, 41]. CW fixation and vacuole compartmentalization both play pivotal roles in enhancing plant Cd resistance [9, 16, 48]. 0.3% IDS application had no remarkable effect on Cd sequestration into vacuoles, but promoted Cd fixation in CWs, thereby decreasing Cd stress in organelles (Figure 3). The CW is the first barrier preventing Cd from entering cells, and CW fixation of Cd depends on the chelation of Cd in different CW components [9, 17]. To further investigate which CW component was mainly involved in improving Cd chelation with leaf CWs by IDS treatment, the content of different CW component and their Cd concentration was further analyzed. The results indicated that IDS did not affect the content of ISP, cellulose, CSP, and hemicellulose, while the Cd concentration in CSP was increased by IDS (Figures 4(a) and 4(b)).

Among the several CW components, pectin is the main component in Cd adsorption to CWs, and the pectin is

demethylated by PME and releases COO- that could form complexes with Cd^{2+} [17]. Our study found that IDS did not increase CSP content (Figure 4(b)), but significantly improved the degree of demethylation of CSP by raising PME activity (Figures 4(c) and 4(d)), producing more available groups for Cd binding to CWs and, thereby, improving Cd detoxification [14, 49]. The results indicated that the primary cause of improving Cd chelation with pectin was due to enhanced PME activity of CSP by IDS supply.

In summary, reducing Cd transport from roots to leaves combined with promoting more Cd retention in the CSP of leaf CWs induced by IDS application contributed to alleviating Cd stress in rapeseed leaves (Figure 5).

5. Conclusions

The results demonstrated that IDS significantly increased the dry matter and photosynthetic pigment contents and decreased Cd transfer coefficient from roots to leaves of rapeseed in Cd-polluted soil. In the meantime, decreased Cd in leaves resulted in higher activity of CAT and SOD, which led to less H_2O_2 accumulation and alleviated oxidative damage to cells. Additionally, the higher PME activity and lower demethylation degree of CSP induced by IDS were the main cause of higher chelation of Cd with CSP in leaves, which could be attributed to more distribution of Cd in CWs and less in organelles. Overall, the study results concluded that IDS application contributed to alleviating Cd stress in leaves of rapeseed by reducing Cd transport from roots to leaves combined with promoting more Cd retention in the CSP of leaf CWs. Our study enriches the theoretical basis of IDS improving Cd resistance of rapeseed leaves at a cellular level, and the results suggest that IDS has a good prospect for decreasing Cd in edible crops such as rapeseed.

Data Availability

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure

An earlier version of preprint on the manuscript has been presented as Research Square according to the following link https://www.researchsquare.com/article/rs-1098394/v1.

Conflicts of Interest

The authors declare that they have no conflict of interest financially or otherwise.

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Hui Tian, Zihan Zhu, and Haixing Song. The first draft of the manuscript was written by Xiuwen Wu, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This project is financially supported by the Natural Science Foundation of Shandong Province (ZR202102190565), the Start-up Fund for Introduced Talent of Qingdao Agricultural University (1121013), and the Scientific research project of Xuchang company of Henan Tobacco Company (6602421124).

Supplementary Materials

Supplementary Materials Table S1 Comparisons of soil characteristics between the control (CK) and IDS treatment. Mean ± SD ($n = 5$). Different lowercase indicates significant difference between these two treatments at $P < 0.05$ level. *(Supplementary Materials)*

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