Impacts of the Inoculation of *Piriformospora indica* on Photosynthesis, Osmoregulatory Substances, and Antioxidant Enzymes of Alfalfa Seedlings under Cadmium Stress

Bingqian Liu 1, Chunchun An 1, Shuying Jiao 2, Fengyuan Jia 1, Ruilin Liu 1, Qicong Wu 1 and Zhi Dong 1,*

1 College of Forestry, Mountain Tai Forest Ecosystem Research Station of State Forestry Administration, Shandong Agricultural University, Tai’an 271018, China
2 College of Resources and Environment, Shandong Agricultural University, Tai’an 271018, China
* Correspondence: nmgdz@163.com

Abstract: With the random discharging of industrial and agricultural wastewater, a large amount of cadmium (Cd) has accumulated in the soil, which seriously affects the growth of crops and people’s food safety. In this study, alfalfa was used as the material for studying the effects of the inoculation of *Piriformospora indica* (*P. indica*) on photosynthesis, osmoregulatory substances, and antioxidant enzymes of alfalfa seedlings at different Cd concentrations (0, 5, 10, 30, 50, and 100 mg/L) through hydroponic experiments. The results showed that with the increase in Cd concentration, the chlorophyll content, net photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (Gs) of alfalfa all decreased gradually, while the intercellular CO₂ concentration (Ci) decreased at first and then increased. However, compared with non-inoculated control plants, the inoculation of *P. indica* improved the photosynthesis (41.97%) of alfalfa under Cd stress, increased the chlorophyll content (43.70%), and significantly increased the contents of proline (29.86%), soluble proteins (38.54%), and antioxidant enzyme activities. It was concluded that *P. indica* alleviates the negative effects of Cd on alfalfa plants to some extent. This is because *P. indica* can resist Cd stress and improve plant growth in cadmium-contaminated agricultural soil by alleviating membrane peroxidation damage, regulating osmotic regulatory substances, and enhancing enzyme activity to improve the antioxidant defense system. Thus, *P. indica* can be considered a biological fertilizer for improving plant growth and physiology in soils contaminated with cadmium.

Keywords: *Piriformospora indica*; cadmium stress; photosynthesis; *Medicago sativa* L.

1. Introduction

Along with the rapid development of agriculture and industry, the pollution of soil by cadmium (Cd) is becoming increasingly serious [1]. In cadmium-contaminated soils, the Cd accumulates in various crops due to the high mobility of agricultural soil-crop systems, severely inhibiting plant growth [2]. Plants show symptoms of poisoning when Cd is in excess, such as reduction in growth, chlorosis, and necrosis [3,4]. The element causes peroxidative damage to plants by inducing the production of reactive oxygen radicals. Cd also causes photosynthetic damage [5], affects protein metabolism [6], and induces the activity of antioxidant enzymes, mostly causing an imbalance. Similarly, high rates of nonenzymatic antioxidants such as proline are induced by Cd [7]. Therefore, taking effective measures to improve the tolerance of crops and reduce the uptake and accumulation of pollutants is an important issue of concern for agriculture based in polluted areas.

Alfalfa (*Medicago sativa* L.) has a strong resistance to Cd; therefore it has been applied as a phytoremediation measure to cadmium-contaminated soil [8,9]. Alfalfa is a legume plant that is popular primarily as good feed for poultry and livestock, with a high nutritional value. Improving the quality of alfalfa could then greatly improve the quality of meat, eggs, and milk of livestock, which is conducive to the development of animal husbandry.
Under Cd stress, plants, on the one hand, trigger various mechanisms to operate in a coordinated manner when dealing with heavy metals [10]; on the other hand, they can build contacts with many beneficial external and internal soil microorganisms to improve their growth [11–13]. It has been recently documented that symbiotic systems of plants and endophytic fungi can promote plant growth, improve plant yield, and improve resistance to various abiotic and biotic stresses [14,15]. Among them, *Piriformospora indica*, also known as *Serendipita indica* (*P. indica*)—a plant root endophytic fungus—is similar to arbuscular mycorrhizal fungi (AMF) in many morphologies, as it can reduce the bioaccumulation of Cd and promote the growth of plants in cadmium-contaminated soil [16]. At present, much evidence has supported the symbiotic systems between *P. indica* and plants in enhancing the ability of these plants to resist stress under adversity [17]. A large number of studies have shown that *P. indica* can strengthen the process of heavy metal transport and enrichment in plants and root stabilization to reduce the degree of heavy metal pollution in soil [18,19]. It can also enhance the expression of antioxidant system components and stress-related genes [20–22] and induce plant stress resistance [23–25]. *P. indica* has been reported to enhance the activity of antioxidant enzyme systems in barley [26], corn [27], and banyan [28]. In addition, under both mycorrhizal association and stresses, the content of proline involved in the resistance to oxidative stress of cells also increased [29]. In functional and growth-promotion aspects, *P. indica* can promote plant growth [30], and the absorption and utilization of nutrients, while improving the plants’ nutritional value [28]. This effect has been seen in corn [27] and wheat [31]. Inoculation of *P. indica* increased chlorophyll content and tolerance of alfalfa in the Phenanthrene and Cadmium co-contaminated soil [16]. Moreover, *P. indica* can also alleviate the inhibition of maize photosynthesis by heavy metals to some extent [32]. Therefore, this study hypothesized that inoculation of *P. indica* could improve the resistance of alfalfa to Cd stress and promote growth.

Because of the multiple beneficial effects of *P. indica* on plants, this fungus has important agronomic and ecological applications [33]. However, fewer studies have been conducted using this technique to remediate Cd contamination in alfalfa growth. Photosynthesis involves a variety of components and is the basis of all green plant and microorganism growth, being one of the most important physiological processes of plant growth. Moreover, Cd causes photosynthesis to be vulnerable to reduced pigment content and photosynthetic efficiency [34,35]. Any damage caused by Cd at any level will seriously affect the overall photosynthetic capacity. In addition, plants can improve their antioxidant capacity by regulating enzyme and non-enzyme substances to resist damage under stress [36–38]. Thus, another objective of our study was to investigate the possible mechanisms of interactions between *P. indica* and plants to resist Cd contamination. Studying the photosynthetic characteristics and antioxidant capacity of plants is an effective way to study the mechanism of plants’ adaptations to their living environment. Considering the high agronomic potential of *P. indica*, this study was carried out to investigate the effects of *P. indica* on photosynthesis, the contents of osmotic regulatory substances, and the activity of antioxidant enzymes of POD, CAT, and SOD in alfalfa under different Cd stress levels.

2. Materials and Method

2.1. Preparation of Endophytic Fungi

The *P. indica* was inoculated on potato dextrose agar (PDA) for one week at 25 °C and activated on a new PDA tablet at 25 °C for one week. Next, culture solution (200 mL) was added to each conical flask, and 8–10 small pieces of 1 cm³ solid medium containing mycelia were put into the conical flask, sealed with sealing film, and then placed into a shaker to avoid light culture. The temperature was set at 25 °C and the rotation speed was 160 r/min. Finally, 3 g of *P. indica* mycelia was weighed and 200 mL water was added into the juicer, this was pressed and crushed until no floccule mycelia could be seen, then fixed capacity to 1 L and mixed evenly as a 3 g/L suspension of *P. indica* for use.
2.2. Plant Material and Experimental Design

Alfalfa with full granules was selected, soaked in 3 g/L sodium hypochlorite for 20 min, and washed and disinfected in 70% ethanol for 20 min. After rinsing well with deionized water, the seeds were evenly sown into the planting grooves and covered with vermiculite. After germination and growth of the seeds for one month, transplanting of the seedlings was carried out. Selected healthy and consistent seedlings were put into water to slowly rinse off the vermiculite, and then they were fixed with cotton to transfer the water containing a 1/2 Hoagland nutrient solution. After one week of hydroponic growth, when the plants were fully adapted to the hydroponic environment, the hydroponic solution of the inoculated plants was replaced with the 3 g/L suspensions of \( P. \) indica containing the same nutritional components; the non-inoculated plants were not treated. The water was changed and the suspension of \( P. \) indica was replenished every 2–3 days.

After 25 days of \( P. \) indica colonization in the plants, the seedlings with and without \( P. \) indica colonization in the roots were transferred to clean water, supplemented with a nutrient solution, and then the standard samples of heavy metals were added; the plants without aggravated metals were used as the blank control. After seven days, the experiment was completed and samples were collected after measuring their growth and photosynthetic parameters to determine other physiological indicators. This experiment was conducted with a completely randomized design and required 12 inoculated and non-inoculated treatments with six concentrations of Cd stress treatments (0 mg L\(^{-1}\), 5 mg L\(^{-1}\), 10 mg L\(^{-1}\), 30 mg L\(^{-1}\), 50 mg L\(^{-1}\), and 100 mg L\(^{-1}\)) were set according to the pre-experiment and the related literature. Each experiment was repeated three times.

2.3. Estimation of Growth Parameters

After seven days of Cd stress, three plants from each treatment were selected for the measurement of their growth indexes, the plant heights were measured with a ruler, and the number of leaves was recorded. After the gas exchange parameters were measured, three samples from each treatment were collected and dried in an oven at 75 °C prior to recording biomass.

2.4. Gaseous Exchange Measurement

Net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO2 concentration (Ci), and transpiration rate (Tr) were measured using a portable photosynthetic system from 9:00 a.m. to 11:00 a.m. under natural sunlight after seven days of Cd stress (LI-6800, USA). All measurements were made at a relative humidity of 55%, light intensity of 1000 µmol m\(^{-2}\) s\(^{-1}\), and leaf temperature of 30 °C. The CO₂ concentration was maintained at 400 µmol m\(^{-2}\) s\(^{-1}\) inside the leaf chamber. Three healthy leaves with intact middle and upper structures were selected for testing; each leaf was measured three times and the average value was taken [39].

2.5. Chlorophyll Content (Chl)

The chlorophyll content of plants was measured after their extraction from leaf samples (0.1 g) with ethanol (95 %) as extraction solvent. The leaves were placed in the dark for 24 h until the leaves turned white. The absorbance value of the extracted chloroplast pigment was measured at the wavelengths of 665 nm and 649 nm [40].

2.6. Soluble Protein (SP) and Proline Concentrations (Pro)

The soluble protein concentration was measured using Bradford’s (1976) method. After pretreatment, 200 µL of the sample was absorbed and the absorbance was measured at a 595 nm wavelength for the microplate analyzer. Proline concentration was measured according to the method of Bates et al. (1973) [41].
2.7. Antioxidant Enzyme Activity

Fresh leaves (0.2 g) were weighed and homogenized in a 50 mM potassium phosphate buffer (2 mL). It was then transferred to a centrifuge tube and 3 mL of phosphate buffer was added. The supernatant was collected after centrifugation at 4 °C for 20 min at 12,000 rpm to determine the enzyme activity. Determination of superoxide dismutase (SOD) activity was performed by nitrogen blue tetrazolium (NBT) photochemical reduction method [39]. The peroxidase (POD) activity was assayed by guaiacol colorimetric method [42]. The catalase (CAT) activity was measured according to UV absorption method [43].

2.8. Statistical Analysis

All data were subjected to one-way ANOVA using SPSS statistical software (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation (SD), and Duncan's multiple range tests at the 0.05 % level were used to compare the significant differences among treatments. Drawing was performed using GraphPad Prism 8.

3. Results

3.1. Number of Leaves, Plant Height, and Biomass

The biomass of alfalfa can be used to reflect the plant's tolerance to pollutants. Biomass decreased under Cd stress, while inoculation with P. indica significantly improved biomass compared with those of the non-inoculated plants. In addition, the number of leaves and plant height were increased compared to that in the control (Table 1).

<table>
<thead>
<tr>
<th>Cd (mg L⁻¹)</th>
<th>Treatments</th>
<th>Number of Leaves (Pieces)</th>
<th>Plant Height (cm)</th>
<th>Biomass (g/Plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>non-inoculated</td>
<td>12.00 ± 3.00 ab</td>
<td>22.83 ± 2.02 ab</td>
<td>0.39 ± 0.00 a</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>12.67 ± 2.08 A</td>
<td>25.20 ± 2.61 A</td>
<td>0.39 ± 0.01 B</td>
</tr>
<tr>
<td>5</td>
<td>non-inoculated</td>
<td>14.00 ± 1.00 a</td>
<td>24.30 ± 2.79 a</td>
<td>0.40 ± 0.01 a</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>15.67 ± 4.04 A</td>
<td>26.00 ± 6.00 A</td>
<td>0.40 ± 0.01 B</td>
</tr>
<tr>
<td>10</td>
<td>non-inoculated</td>
<td>10.00 ± 1.73 b</td>
<td>23.13 ± 1.11 ab</td>
<td>0.37 ± 0.01 b</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>11.67 ± 2.89 A</td>
<td>24.33 ± 3.06 A</td>
<td>0.44 ± 0.01 A ***</td>
</tr>
<tr>
<td>30</td>
<td>non-inoculated</td>
<td>9.67 ± 1.53 b</td>
<td>22.50 ± 3.91 ab</td>
<td>0.36 ± 0.00 bc</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>11.67 ± 3.21 A</td>
<td>23.00 ± 1.00 A</td>
<td>0.38 ± 0.00 C ***</td>
</tr>
<tr>
<td>50</td>
<td>non-inoculated</td>
<td>9.00 ± 1.00 b</td>
<td>22.00 ± 1.73 ab</td>
<td>0.35 ± 0.01 bc</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>11.00 ± 3.61 A</td>
<td>22.50 ± 1.48 A</td>
<td>0.37 ± 0.01 CD</td>
</tr>
<tr>
<td>100</td>
<td>non-inoculated</td>
<td>8.67 ± 1.15 b</td>
<td>18.27 ± 2.00 b</td>
<td>0.34 ± 0.01 c</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>10.00 ± 1.73 A</td>
<td>20.80 ± 4.85 A</td>
<td>0.35 ± 0.01 D</td>
</tr>
</tbody>
</table>

Values in this table refer to mean ± SD (n = 3). Different lowercase letters indicate significant (p < 0.05) differences between different concentrations of Cd treatments without inoculation, while capital letters indicate differences between treatments with inoculation. Asterisks indicate a significant (p < 0.05) difference between P. indica treatments within the same Cd concentrations; ns—not significant; *** p < 0.001.

3.2. Chlorophyll Content

The chlorophyll content is an important indicator of leaves' photosynthetic capacity and plants' health status. In non-inoculated plants, chlorophyll content in the leaves slightly increased to 5 mg/L Cd. The presence of P. indica significantly (p < 0.05) increased the contents of chlorophyll between 0 and 30 mg/L Cd in alfalfa leaves compared to non-inoculated plants, which increased 16.945%, 43.69%, 46.56%, and 43.34%, respectively. With the increase in Cd concentration, the improvement effect of P. indica on chlorophyll gradually decreased (Figure 1).
Figure 1. Effects of Cd stress and inoculation of *P. indica* on chlorophyll content of alfalfa. Means followed by the same letter are not significantly different (*p* < 0.05) according to LSD test (*n* = 6). Different lowercase letters indicate significant (*p* < 0.05) differences between different concentrations of Cd treatments without inoculation, while capital letters indicate differences between treatments with inoculation. Asterisks indicate a significant (*p* < 0.05) difference between *P. indica* treatments within the same Cd concentrations; ns—not significant; *p* < 0.05; **p* < 0.01; ***p* < 0.001.

3.3. Leaf Gas Exchange

Photosynthesis is the basis of plant growth and material accumulation, which is of great significance to plant growth and development. Under no-cadmium conditions, *P. indica* inoculation of alfalfa plants raised the leaves’ net photosynthesis, transpiration rate, intercellular CO₂ concentration, and net photosynthesis (Table 2). The transpiration rate and net photosynthesis in both inoculated and non-inoculated plants gradually decreased with the increase in Cd concentration, with inoculated plants performing higher than non-inoculated plants (Table 2). The net photosynthetic rates of inoculated and uninoculated plants differed significantly at low- and medium-concentrations (5–30 mg/L) (*p* < 0.01), while Tr and Ci differed significantly at medium and high Cd concentrations (*p* < 0.05) (Table 2). With the increase in Cd concentration, the CO₂ concentration between inoculated and non-connected plants showed a trend of first decreasing and then rising, reaching the lowest value at a concentration of 30 mg/L; at high concentrations, the difference between inoculation and missed plants was significant (*p* < 0.01) (Table 2).

Table 2. Effects of Cd stress and inoculation of *P. indica* on the leaf gas exchange of alfalfa.

<table>
<thead>
<tr>
<th>Cd (mg L⁻¹)</th>
<th>Treatments</th>
<th>Pn (µmol m⁻² s⁻¹)</th>
<th>Tr (mmol m⁻² s⁻¹)</th>
<th>Ci (µmol mol⁻¹)</th>
<th>Gs (mmol m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>non-inoculated</td>
<td>8.10 ± 1.41 a</td>
<td>2.39 ± 0.41 a</td>
<td>275.44 ± 38.32 b</td>
<td>0.12 ± 0.02 b</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>9.89 ± 0.64 A</td>
<td>4.69 ± 1.22 A *</td>
<td>315.30 ± 13.35 A</td>
<td>0.23 ± 0.06 A *</td>
</tr>
<tr>
<td>5</td>
<td>non-inoculated</td>
<td>5.42 ± 0.28 b</td>
<td>3.00 ± 0.08 b</td>
<td>296.69 ± 33.24 a</td>
<td>0.15 ± 0.00 a</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>9.97 ± 1.26 A **</td>
<td>4.14 ± 2.27 A</td>
<td>328.73 ± 7.48 AB</td>
<td>0.21 ± 0.12 A</td>
</tr>
<tr>
<td>10</td>
<td>non-inoculated</td>
<td>5.74 ± 0.34 b</td>
<td>2.84 ± 0.06 b</td>
<td>287.76 ± 7.94 a</td>
<td>0.14 ± 0.00 a</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>8.15 ± 0.16 B ***</td>
<td>2.56 ± 0.18 B</td>
<td>322.64 ± 2.66 BC **</td>
<td>0.13 ± 0.01 AB</td>
</tr>
<tr>
<td>30</td>
<td>non-inoculated</td>
<td>2.26 ± 0.03 c</td>
<td>0.59 ± 0.24 c</td>
<td>226.32 ± 5.14 c</td>
<td>0.03 ± 0.01 c</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>6.12 ± 0.77 C **</td>
<td>1.26 ± 0.14 C *</td>
<td>234.55 ± 4.68 C</td>
<td>0.06 ± 0.01 BC *</td>
</tr>
<tr>
<td>50</td>
<td>non-inoculated</td>
<td>2.51 ± 0.34 c</td>
<td>0.17 ± 0.01 c</td>
<td>251.49 ± 4.26 d</td>
<td>0.01 ± 0.01 d</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>2.08 ± 0.05 D ***</td>
<td>0.73 ± 0.02 D ***</td>
<td>329.46 ± 0.17 C ***</td>
<td>0.01 ± 0.01 BC</td>
</tr>
<tr>
<td>100</td>
<td>non-inoculated</td>
<td>1.01 ± 0.30 d</td>
<td>0.12 ± 0.07 d</td>
<td>268.87 ± 5.00 d</td>
<td>0.01 ± 0.01 d</td>
</tr>
<tr>
<td></td>
<td>inoculated</td>
<td>1.28 ± 0.12 D *</td>
<td>0.72 ± 0.22 D *</td>
<td>341.54 ± 10.70 C ***</td>
<td>0.04 ± 0.01 C *</td>
</tr>
</tbody>
</table>

Values in this table refer to mean ± SD (*n* = 3). Different lowercase letters indicate significant (*p* < 0.05) differences between different concentrations of Cd treatments without inoculation, while capital letters indicate differences between treatments with inoculation. Asterisks indicate a significant (*p* < 0.05) difference between *P. indica* treatments within the same Cd concentrations; ns—not significant; *p* < 0.05; **p* < 0.01; ***p* < 0.001.

3.4. Proline and Soluble Protein Concentrations

There was no significant difference in proline content between the inoculated and non-inoculated plants at a 0 mg/L Cd concentration. The proline content of alfalfa increased with the increase in Cd concentration generally, and, at the same Cd concentration, the proline content of inoculated alfalfa plants was significantly higher than that of the
non-inoculated plants, which increased by 24.42%, 29.86%, 13.24%, 12.13%, and 17.25%, respectively (Figure 2A). In non-inoculated plants, the contents of soluble sugar increased slightly at 0–10 mg/L Cd concentrations and then decreased gradually with the increase in Cd concentrations, reaching their highest at a 10 mg/L concentration. Inoculation with P. indica increased the soluble protein content but significantly differed only at 0 and 30–100 mg/L Cd concentrations (p < 0.001). In the inoculated plants, there was no significant difference in the soluble sugar content at different Cd concentrations (Figure 2B).

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Effects of Cd stress and inoculation of *P. indica* on osmotic substance concentrations of alfalfa. (A) Proline; (B) Soluble protein. Means followed by the same letter are not significantly different (p < 0.05) according to LSD test. Different lowercase letters indicate significant (p < 0.05) differences between different concentrations of Cd treatments without inoculation, while capital letters indicate differences between treatments with inoculation. Asterisk indicates a significant (p < 0.05) difference between *P. indica* treatments within the same Cd concentrations; ns—not significant; ***p < 0.001.

### 3.5. Antioxidant Parameters of the Leaves

In non-inoculated plants at 5 mg/L Cd, a significant increase in POD activity compared to that of 0 mg/L Cd was observed. However, between the 10 mg/L and 100 mg/L Cd treatments, the POD activity of both inoculated and non-inoculated plants significantly decreased compared with that under a 0 mg/L Cd concentration. The POD activity was significantly increased by inoculation of *P. indica* at 0 mg/L and 10 to 100 mg/L concentrations of Cd (p < 0.01) (Figure 3A). In non-inoculated plants, CAT activity in the leaves significantly increased between 0 mg/L to 50 mg/L Cd, whereas it decreased at 100 mg/L Cd. The CAT activity was significantly increased by inoculation of *P. indica* at different concentrations of Cd. The activity increased by 57.14%, 24%, 53.57%, 19.51%, 24.49%, and 46.51%, respectively (Figure 3B). With the increase in Cd concentration, the activity of SOD first increased and then decreased, and the highest activity was found at 30 mg/L Cd. Except for the treatment with 30 mg/L Cd concentration, the leaf SOD activity of inoculated plants was significantly increased compared with that of non-inoculated plants, increasing by 32.16%, 155.74%, 54.98%, 87.03%, and 29.32%, respectively (Figure 3C).
In non-inoculated plants at 5 mg/L Cd, a significant increase in POD activity compared to that under a 0 mg/L Cd concentration. The POD activity of both inoculated and non-inoculated plants was significantly increased compared with that of non-inoculated plants at 5 mg/L Cd, whereas it decreased at 100 mg/L Cd. Except for the treatment with 30 mg/L Cd concentration, the leaf SOD activity was significantly increased by inoculation of *P. indica* at 5 mg/L Cd, a significant increase in POD activity compared to that of 0 mg/L Cd was observed. However, the SOD activity decreased significantly compared to that under a 0 mg/L Cd concentration. The POD activity of both inoculated and non-inoculated plants increased with the increase in Cd concentrations; ns—not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

3.6. Effects of *P. indica* on Alfalfa Growth by Affecting Photosynthesis, The Content of Osmotic Regulatory Substances, and Activity of Antioxidant Enzymes

We predicted the direct and indirect effects of *P. indica* on alfalfa growth by affecting photosynthesis, the osmoregulatory system, and the antioxidant system by using a structural equation model (Figure 4). Path analysis indicated that SP, Pn, Chl, SOD, and Pro were the direct factors affecting plant growth. Among them, SP, Pn, Chl, and SOD had positive effects on alfalfa growth (standardized coefficient = 0.26, 0.11, 0.62, and 0.18, respectively), while Pro had negative effects (standardized coefficient = −0.34).
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Promoted the growth of alfalfa to varying degrees. It significantly alleviated the inhibitory effect of cadmium on plant growth at low Cd concentrations but this protective effect was not significant at high Cd concentrations and could not maintain normal plant growth. According to the results of this study, it is closely related to the decrease in photosynthetic rate and antioxidant enzyme activity in plants under high Cd concentration. Moreover, the better growth performance of inoculated plants may also be attributed to the fact that the P. indica and plant co-living system can enhance the extraction of soil water and expand the absorption range of water through epitaxial hyphae [44].

The photosynthetic efficiency of plants is closely related to growth and higher photosynthetic efficiency helps plants accumulate more photosynthetic products, which in turn promotes the growth and development of plants [45]. The results indicate that different concentrations of Cd inhibited the net photosynthetic rate (Pn) of alfalfa because of the high sensitivity of photosynthesis to the environment and heavy metals [34,46]. Previous studies have found that the factors for the decrease in plant photosynthetic rate include stomatal limitation and non-stomatal limitation factors [47]. When Ci and Gs change in the same direction, the decrease in the photosynthetic rate is caused by the decrease in stomatal conductance; otherwise, it is caused by non-stomatal factors [48]. The results showed that the Gs and Ci of alfalfa decreased under low and medium Cd concentrations, indicating that the decrease in photosynthesis at this time was the result of stomatal limitations, which may be because the decrease in Gs reduced the uptake of photosynthetic substrate CO₂ by plant cells and that led to the increase in Ci. This results in a decrease in Pn, however, inoculation of P. indica increased the Pn and gas exchange efficiency in...
alfalfa leaves. This may be because the addition of *P. indica* increased the Gs and Tr of plants and made more external CO2 enter the plants, so as to provide more raw materials for photosynthesis, improve the net photosynthetic rate of host plants, and enhance the stress resistance. Previous studies already discovered that the inoculation of fungi can increase the photosynthetic efficiency of host plants [46]. However, with the increase in Cd concentration, Pn, Tr, and Gs decreased and Ci concentration increased in the two groups of treatments, indicating that the decrease in photosynthesis at high Cd concentrations was caused by non-stomatal limiting factors. This may be after Cd\(^{2+}\) enters plant cells when the structure and function of chloroplasts are damaged [49], which inhibits chlorophyll synthetase activity [50] resulting in a reduction in chlorophyll content [34], and thus the photosynthetic rate decreases [51,52].

This was also verified in the present study. In this study, the Pn and Chl of non-inoculated plants significantly decreased with increasing Cd concentrations. This is because photosynthesis is susceptible to a decrease in pigment content and photosynthetic efficiency caused by Cd [6]. In a way, inhibition of photosynthesis can be attributed to reduced chlorophyll biosynthesis [53]. This study further confirmed that *P. indica* has a protective effect on chlorophyll, which can significantly alleviate the degradation of chlorophyll in alfalfa under Cd stress (Figure 1). This is because the accumulation of chlorophyll facilitates the capture and utilization of light energy by leaves and maintains PSII activity [45,54], thus increasing the rate of photosynthesis to cope with Cd stress in alfalfa. However, this effect is significant at low Cd concentrations and decreases with increasing Cd concentration (Figure 4).

Some studies have shown that under the duress of adversity, the production rate of \(H_2O_2\) and \(O_2^-\) in plant leaves increases [55], which is transformed into reactive oxygen species through a series of reactions and then causes a series of damage to plant organisms, cell membranes, and enzymes [38,51,56]. The accumulation of osmotic regulatory substances and the improvement in antioxidant capacity are two important mechanisms for plants to survive under stress. As a cytosolic osmotic agent, enzyme and cell structure protective agent, and free radical scavenger [57] it can remove the reactive oxygen species produced by plants under adverse environmental conditions [58]. In this present study, the concentration of proline in plant leaves increased with the increase in Cd concentration (Figure 2). This was an adaptive regulation of alfalfa to adverse external conditions, which was consistent with previous findings [59]. In this study, the proline content of inoculated and non-inoculated alfalfa increased in response to Cd, suggesting a similar stress response. Nevertheless, the proline content of inoculated plants showed a significant increase in response to Cd compared to non-inoculated plants, suggesting that proline may have a role in mitigating Cd toxicity. Furthermore, free proline has great hydrophilicity and its increase helps cells or tissues retain water [60] and improve the enzymatic activities of SOD, POD, and CAT [61]. At the same time, as a carbohydrate source, it can prevent the peroxidation of membrane lipids and proteins by reactive oxygen species [58]. Under stress conditions, plants can increase the synthesis of soluble proteins, affect the osmoregulation of plant somatic membrane, participate in the metabolism of plant cells, and directly participate in the process of adaptation to stress. In this study, the soluble protein content of uninoculated plants increased first and then decreased, indicating that plant biofilms were seriously damaged under high Cd stress. Compared with non-inoculation plants, *P. indica* increased the content of soluble protein, and this effect was particularly significant under high Cd stress, indicating that inoculation of *P. indica* could reduce the damage of Cd to plant biofilm and improve the survival ability of plants under stress by increasing the content of soluble proteins. Osmotic regulation can also maintain photosynthesis, photochemical activity, and photosynthetic rate. Cd leads to a decrease in Pn, while osmotic adjustment can maintain certain Gs in plant leaves by maintaining turgor pressure, which is conducive to maintaining a high level of Ci content in leaves, so as to avoid or reduce the photoinhibition effect on leaves [62].
Cd limits photosynthesis and growth by slowing the activity of various enzymes [63]. This study shows that the activity of CAT increased with the increase in Cd concentration, while the activity of SOD and POD increased first and then decreased (Figure 3). The results indicate that cadmium-induced oxidative stress in alfalfa leaves is consistent with the effect of Cd on the antioxidant system of alfalfa in previous studies [64,65]. This may be due to the excessive accumulation of reactive oxygen species [66–68], which results in damage to cell membrane structure [69] and increased membrane permeability [70]. At this time, SOD, CAT, and POD, as important enzymes to remove reactive oxygen species in plants, can remove excess reactive oxygen species by changing their activities [71]. SOD can dismutate $\text{O}_2^{-}$ into $\text{O}_2$ and $\text{H}_2\text{O}_2$, while POD and CAT catalyze $\text{H}_2\text{O}_2$ to form $\text{H}_2\text{O}$. Under low Cd concentrations, the POD activity increased significantly, while SOD and CAT did not change significantly, indicating that alfalfa regulated the antioxidant system mainly by increasing the activity of POD under low Cd concentrations. At medium concentrations of Cd, the POD activity continued to decrease, while the activities of CAT and SOD increased and the increasing trend of SOD was significantly higher than that of CAT, indicating that alfalfa mainly regulated antioxidants by increasing the activities of CAT and SOD under medium Cd concentrations. At high concentrations of Cd, SOD is inactivated, alfalfa regulates antioxidants mainly by increasing CAT activity but this regulatory effect also decreases. However, the activities of CAT, SOD, and POD increased to different degrees after inoculation with *P. indica* (Figure 3). These results suggest that *P. indica* can resist Cd stress and enhance the antioxidant defense system by increasing the activity of plant antioxidant enzymes and ultimately improving the photosynthetic efficiency of alfalfa seedlings, promoting plant growth, and enhancing the ability of plants to tolerate Cd. This conclusion is consistent with the previous findings of *P. indica* in alleviating oxidative damage and growth in tomatoes [72], *solanumn* [73], wheat [31], cabbage [36], banyan [28], stevia [74], and medicinal plant Bacopa monniera [75] under stress.

5. Conclusions

The heavy metal Cd has a promoting effect at 5 mg/L concentration and a certain inhibitory effect on the growth of alfalfa at 10–100 mg/L concentrations with a significant toxic stimulating effect. *P. indica* increases the content of chlorophyll in the alfalfa leaves, improves the photosynthetic efficiency of alfalfa, produces more photosynthetic products, maintains the plant’s basic metabolism and defensive ability, and promotes the growth of plants. The symbiotic system of *P. indica* and plants promoted the accumulation of osmotic regulatory substances (SP and Pro) and improved the antioxidant capacity (SOD, POD, and CAT) under Cd stress to reduce the membrane peroxidation damage. Finally, *P. indica* effectively promoted the growth of alfalfa and photosynthesis and increased the viability of alfalfa under Cd stress. The results of this study add to the knowledge that *P. indica* improves plant tolerance under Cd contamination. It proves that *P. indica* has greater potential for application as a biofertilizer. However, further observational studies on the effects of *P. indica* on plant systems’ performance and yield are needed for an effective introduction to the sustainable management of agroecosystems.

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