

# The Role of Ni and Cd-Resistant Rhizobacteria in Promotion of Rice Seedlings Growth and Alleviation Combined Phytotoxicity of Ni and Cd

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## Research Article

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# Abstract

The heavy metal compound pollution is becoming a serious problem in the environmental management. In the present study, we focused on the combined pollution of nickel (Ni) and cadmium (Cd) and isolated three Ni and Cd-resistant rhizobacteria (strains Y3, Y4 and Y5) with important PGP traits. According to 16S rDNA sequence homology, strains Y3, Y4 and Y5 were identified as *Pseudomonas* sp., *Chryseobacterium* sp. and *Enterobacter* sp., respectively. Then the effects of strains Y3, Y4 and Y5 on rice growth under the combined stress of Ni and Cd were examined. The results indicated that strains Y3, Y4 and Y5 enhanced the seed germination, biomass production and chlorophyll accumulation of rice seedling under compound stress of Ni and Cd. These strains also conferred rice tolerance to Ni and Cd by increasing antioxidant enzyme activity in seedlings. Furthermore, comparing among three strains, it was found that strain Y3 with stronger ability of IAA production, EPS production and ACC deaminase activity had the best promotion effect on rice growth under the combined stress of Ni and Cd. In addition, strain Y5 had better IAA production capacity and a better effect on the growth of rice roots than strain Y4 under Ni and Cd stress, which indicated that IAA production might play a particularly critical role in root growth under the stress of Ni and Cd.

## 1. Introduction

The heavy metal compound pollution is becoming a serious problem in the environmental management. Heavy metals are basically non-biodegradable, which may accumulate in the soil thus exerting a lasting damage on the plant growth[\*]. Cadmium (Cd) and nickel (Ni) are two typical heavy metals. Cd usually comes from the power stations, mining and metal-working industries (Abbas et al. 2014), which interferes in the absorption, transportation and use of certain elements by plants. It can affect the permeability of the plasma membrane, inducing lipid peroxidation and abnormal chloroplast metabolism (Nagajyoti et al. 2010). Excess Ni can interfere with the lipid composition and H-ATPase activity of the plasma membrane in plants (Nagajyoti et al. 2010). In China, Cd and Ni polluted most in farmland, with the polluted areas account for 7.0% and 4.8%, respectively ([http://www.gov.cn/foot/2014-04/17/content\\_2661768.htm](http://www.gov.cn/foot/2014-04/17/content_2661768.htm)). The soil fertility will be degenerated by the combined pollution of Cd and Ni, leading to a decline of the crop production.. The management of compound pollution of Ni and Cd merits a particular attention.

Due to the negative effects on crops, numerous methods are studied to alleviate the phytotoxicity and enhance crop growth under Ni and Cd pollution. Among all of the methods, the biological management can suppress the negative effect on the plants prominently, especially the method of bacteria management. The bacteria affecting the plant growth positively are called as plant growth promoting rhizobacteria (PGPR), which can improve plant growth through direct or indirect mechanisms: (a) PGPR can produce indoleacetic acid (IAA), an important hormone for stimulating plant growth (Etesami, Maheshwari 2018). (b) PGPR can produce aminocyclopropane-1-carboxylate (ACC) deaminase that cleave the ACC (ethylene precursor) and reduce the ethylene production of stressed plants to avoid plant growth inhibition or death (Glick 2014). (c) PGPR can induce the activity of plant antioxidant enzymes

and protect plant cells from oxidative damage (Paul, Lade 2014). (d) PGPR can produce siderophores, which could bind and complex Fe(III) to help plants obtain iron from the environment. (e) PGPR can dissolve insoluble inorganic phosphates and help plants cope with their phosphorus needs (Ferreira et al. 2019).

The bacteria affecting the plant growth positively are called as plant growth promoting rhizobacteria (PGPR), which can improve plant growth through direct or indirect mechanisms. Heavy metal resistant PGPR is of the great demand for agriculture and phytoremediation. It helps plants to avoid metal-induced phytotoxicity and promote plant growth as well. Inoculation with *Bacillus licheniformis* NCCP-59 improved the seed germination and biochemical characteristics of plants under Ni stress (Jamil et al. 2014). It was also found that inoculation of PGPR enhanced morphological characters and alleviated Cd-induced oxidative stress in seedlings (Pramanik et al. 2018, Zhu et al. 2019). However, few studies focused on the compound pollution of Ni and Cd which is becoming a severe problem to the crop production. In addition, the different effects of the strains with different PGP traits on plant growth under compound pollution of Ni and Cd were also unexplored.

Rice is one of the most consumed cereals in the human diet in the world (Jamil et al. 2014). However, the production of rice is severely affected by Ni and Cd stress, which is a significant reason for the loss of agricultural lands and rice reduction. Therefore, it is important to alleviate the adverse effects of salt stress on rice.

The aim of the present study was to examine the role of the isolated bacteria *Pseudomonas* sp. strain Y3, *Chryseobacterium* sp. strain Y4 and *Enterobacter* sp. strain Y5 in promoting rice growth and enhancing rice tolerance to Ni and Cd. In addition, we also compared and analyzed the different promoting effects of strains Y3, Y4 and Y5 with different PGP traits on rice growth under Ni and Cd stress.

## 2. Materials And Methods

### 2.1 Screening and identification of isolates

#### 2.1.1 Rhizobacterial screening and isolation

Rhizosphere soil samples were collected in the field of Tianjin University (39° 06' 33.24" N, 117° 10' 18.9" E). It used to be an electromechanical factory and the field is contaminated with heavy metal. So, we tried to screen for heavy metal-tolerant strains. The analysis of soil properties was supplemented as Table S1.

To screen isolated with heavy metal tolerance, soil bacterial suspension was plated in Dworkin and Foster (DF) medium supplemented with 5 mM ACC (instead of  $(\text{NH}_4)_2\text{SO}_4$ ) and 50  $\mu\text{g}/\text{mL}$   $\text{Ni}^{2+}$  ( $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ) and 100  $\mu\text{g}/\text{mL}$   $\text{Cd}^{2+}$  ( $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ ). After incubating at 28°C for 72 hours, the isolates were then purified by the dilution streak plate method repeated by cycles to have a pure culture of single colony.

## 2.1.2 Effects of Ni-Cd compound pollution on the growth of strains

Minimum inhibitory concentration (MIC) is an important indicator to show the tolerance to heavy metal. To determine the MIC, the isolates grew in LB plates and broth containing different concentrations of Cd or Ni. of Ni or Cd was determined when the isolates failed to growth in the plates after 7 days.

The growth curve could reflect the growth of isolates under different conditions. To show the effects of Cd and Ni on strains more intuitively, the strains were grown in LB broth with Ni 20 µg/mL and Cd 40 µg/mL under 28°C and 160 rpm. Another group of LB broth without heavy metals was used as control. The OD<sub>600</sub> of strains were measured at intervals of every 3 hours up to 60 hours.

## 2.1.3 Bacterial identification

After genomic DNA extraction, the 16S rDNA was amplified by using universal primers 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3']. The amplified 16S rDNA PCR products were sequenced by Genewiz (Suzhou, China). The sequencing results were spliced and cut by DNASTAR software to obtain the 16S rDNA gene sequences. Then we compared the 16S rDNA sequences with the entire GenBank nucleotide database using BLAST algorithm at NCBI database. The distance-based phylogenetic tree was made based on neighbor-joining (NJ) method using MEGA X software. The statistical significance of branch points was calculated by 1,000 bootstrap values.

## 2.2 Plant growth promoting (PGP) properties of the selected isolates

Plant growth promoting rhizobacteria possessed with different PGP properties. The quantitative and qualitative analyses of PGP properties could effectively indicate the effects of the strains on plant growth promotion. Thus, the bacterial strains were further analyzed for *in vitro* PGP properties like production of IAA, siderophore, EPS, ammonia and ACC deaminase activity as described earlier.

### 2.2.1 IAA

To estimate IAA production, the bacterial cultures grew in LB broth supplemented with 0.5% L-tryptophan. After 36 hours, the cultured suspension was centrifuged and the supernatant (2 mL) was mixed with 2 mL of Salkowski reagent. Then the OD value was measured at 530 nm (Bric et al. 1991).

### 2.2.2 ACC deaminase activity

ACC deaminase activity was also tested. The isolates were inoculated in DF liquid medium supplemented with 5 mM ACC (instead of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>). After centrifugation, the precipitate was further processed and the ACC deaminase activity was measured by estimating the amount of α-keto butyrate released from ACC degradation (Penrose, Glick 2003). The ACC deaminase activity was expressed as the amount of α-

ketobutyric acid produced per milligram of protein per hour. The protein was estimated as the description in the previous report (Bradford 1976).

## 2.2.3 Phosphate solubilizing

To determine the phosphate dissolving ability, bacterial isolates were grown in Pikovskaya's broth and incubated. After centrifugation, the supernatant (500  $\mu$ L) was then mixed with the same volume of 10% w/v trichloroacetic acid and 4 mL reagents (3 M  $H_2SO_4$ , 2.5% w/v ammonium molybdate, 10% w/v ascorbic acid and distilled water in the ratio of 1: 1: 1: 2). The OD value was measured at 820 nm and the concentration of dissolved phosphate was determined by  $KH_2PO_4$  standard curve (Mitra et al. 2018a).

## 2.2.4 Siderophores

The siderophores production by bacterial strains were analyzed as described by Payne (1994). The bacterial solution was inoculated into minimal medium (MM) medium (iron-deficient medium) and after centrifugation, 0.5 mL of CAS analysis solution was added to 0.5 mL of supernatant. The absorbance at 700 nm was measured as a blank and the liquid medium plus CAS analysis solution plus 5-sulfosalicylic acid as a reference.

## 2.2.5 Exopolysaccharide (EPS)

In short, freshly grown strain cultures inoculated in nutrient broth (containing (g/L):  $Na_3C_6H_5O_7$  (3), KCl (2),  $MgSO_4 \cdot 7H_2O$  (20),  $MnCl_2 \cdot 4H_2O$  (0.036),  $FeSO_4 \cdot 7H_2O$  (0.05), casein amino acid (7.5)) were incubated (Vimal et al. 2019). The supernatant after centrifuging was added with three volumes of cold ethanol dropwise with stirring. After incubating at 4°C overnight, the precipitate was dried to determine the weight.

## 2.2.6 Ammonia ( $NH_3$ )

For  $NH_3$  production, the freshly grown culture was inoculated into 10 mL of peptone solution (10 g/L) in each test tube for 48 hours. Adding Nessler reagent (0.5 mL) to each test tube and the development of brown to yellow was a positive test for  $NH_3$  production (Ahmad et al. 2008).

## 2.2.7 Biofilm

As description in the previous report (Hassan et al. 2011), in a test tube, the isolates were inoculated in 10 mL of trypsin soy broth (TSB) containing 1% glucose at 28°C and 160 rpm for 24 hours. After incubation, the test tube was emptied and then stained with crystal violet (0.1%). If the membrane is visible on the wall and bottom of the tube, biofilm formation is considered positive.

## 2.3 Pot experiment

Then, the pot experiment was conducted and the strains were inoculated in rice under different treatment. Various physiological indicators of plants are determined to explore the effects of strains on rice growth under Ni and Cd stress.

## **2.3.1 Determination of treatment concentration**

To determine the treatment concentration of Ni and Cd compound pollution, a preliminary experiment for confirming  $EC_{25}/EC_{50}$  (effective concentration at which 25%/50% germination was inhibited) value of single metal was conducted. Then, the method of He (2020) was used to determine the combined concentration of Ni and Cd.

## **2.3.2 Germination experiment**

The germination rate of seeds is one of the important indexes in response to the effects of stress on plants. To explore the effects of heavy metals and inoculated strains on rice seed germination, the sterilized rice seeds were placed in plates and treated as the above groups. The concentrations of different bacterial culture were OD = 0.8. The rice seeds germinated in a dark environment at 30°C, and the germination rates were calculated after 5 days.

## **2.3.3 Seedling inoculation**

When the seedlings grew to 3 cm, the seedlings with uniform growth status were selected and moved to pots, 5 plants per pot. The inoculation was carried out according to the above treatment groups (OD = 0.8). All treatment groups were under the conditions of 25°C, 4,000 Lux illumination intensity and 60% relative humidity with light exposure for 12 hours and darkness for 12 hours.

## **2.3.4 Determination of morphological parameters and biomass**

To quantify the growth status of plants, the morphological parameters and biomass are measured after 30 days. The shoot height and root length of the seedling were measured and recorded. After the roots and shoots of the seedlings were separated with a scalpel, the fresh weights were measured. Then the roots and shoots were put at a constant temperature of 115°C in the incubator for 2 hours and dried to constant weight at 70°C to determine the dry weight.

## **2.3.5 Chlorophyll content**

Chlorophyll is the main pigment for photosynthesis of plants, which directly affects the anabolism of plants. The chlorophyll in the seedlings was extracted with 95% ethanol in the dark for the extraction of pigment. After centrifugation, the absorbance of supernatant was measured at 665 and 649 nm to estimate the chlorophyll contents.

## **2.3.6 Determination of antioxidant enzyme activity**

Antioxidant enzyme is an important antioxidative defense mechanism in plant, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX). To determine the activity of antioxidant enzyme, the enzyme extract was extracted by the method of Zhang (2009).

To determine the activity of SOD, 3 mL reaction mixture (as described by Giannopolitis (1977)) was mixed with 0.1 mL of enzyme extract. The mixture was placed under two fluorescent lamps of 4,000 Lux illumination intensity. The reaction lasted for 10 minutes. The reaction without light and the reaction without enzyme extract were used as calibration standards. The absorbance was measured at 560 nm.

CAT activity was determined by monitoring the decomposition of  $\text{H}_2\text{O}_2$  at 240 nm (Aebi 1984). 0.1 mL enzyme extract was added to the reaction mixture (3 mL) consisting of 100 mM phosphate buffer (pH 7.0), 0.1  $\mu\text{M}$  EDTA and 0.1%  $\text{H}_2\text{O}_2$ . The reaction was initiated by adding enzyme extract. The decrease in  $\text{H}_2\text{O}_2$  level was determined by measuring the absorbance at 240 nm.

The activity of POX was based on the oxidation of guaiacol by using hydrogen peroxide. The reaction was started by adding 20  $\mu\text{L}$  of enzyme extract to 3 mL reaction mixture (as described by Zhang (2009)). The absorbance was measured at 470 nm when the reaction started and was measured again after 5 minutes.

### **2.3.7 Determination of malondialdehyde (MDA) and proline content**

The content of MDA is a manifestation of the degree of peroxidation of plant cell membranes. MDA concentration was quantified by 2-thiobarbituric acid (TBA) reaction (Heath, Packer 1968). 1 mL of 0.6% TBA was added to 1 mL of enzyme extract. After incubating in a boiling bath for 30 minutes, it was transferred to an ice bath. After centrifugation, the absorbance at 450, 532 and 600 nm was measured to estimate the MDA concentration.

Proline is one of the components of plant protein and accumulates in plant under stress condition. Accumulated proline plays an important role in plants. To determine the content of proline, fresh seedlings (0.5 g) were homogenized in 3 mL of 5% (w/v) sulfosalicylic acid. After centrifugation, the supernatant was treated with ninhydrin and glacial acetic acid (1: 1, v/v). The mixture was boiled at 100°C for 30 minutes and then was placed on ice for 5 minutes. The reaction mixture was extracted with an equal volume of toluene. The toluene-containing chromophore was extracted and the absorbance was measured at 520 nm (Bates 1973).

### **2.3.8 Histochemical staining and determination of $\text{H}_2\text{O}_2$ and $\text{O}_2^-$**

$\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  are two forms of ROS, which accumulate in plant under stress and cause oxidative damage and cell death. For qualitative analysis of the accumulation of  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  in rice seedlings, histochemical staining with 3,3-diaminobenzidine (DAB) and NBT was conducted as described by Ji (2020). The quantitative analysis of  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  was determined by commercial kits (T01076 and T01123, Leagene, Beijing, China).

### **2.3.9 Determination of Ni and Cd content in seedling**

Finally, the accumulation of heavy metals in plants was also considered. After drying, the seedlings were digested with  $\text{HNO}_3$  and  $\text{HClO}_4$ . Then the concentration of Ni and Cd was determined by atomic absorption spectrometry (AAS) (Yang et al. 2009).

## 2.4 Statistical analysis

All experiments done in triplicates and data were analyzed using the SPSS 20.0 statistical software package and summarized as the means  $\pm$  standard errors (SE). Differences between groups were analyzed by one-way (ANOVA) followed by the Duncan's test. Different small letter alphabets above the graphs indicate significance at  $p < 0.05$  between any two groups compared while similar letters indicate no significant result. Cluster analysis was conducted by the online software MetaboAnalyst 4.0 (<http://www.metaboanalyst.ca>).

## 3. Results

### 3.1 Isolation and identification of strains

After screening, the isolates Y3, Y4 and Y5 were with ACC deaminase activity and Ni-Cd resistance. The colonies and the results of Gram staining of strains Y3, Y4 and Y5 were as shown in Fig. S1. The 16S rDNA analysis revealed that the strains Y3, Y4 and Y5 indicated 98–100% similarity with the known sequences in GenBank and belonged to different genera of *Pseudomonas* sp., *Chryseobacterium* sp. and *Enterobacter* sp., respectively. To determine the relationship between the strains with other species, the phylogenetic tree based on 16S rDNA was constructed as shown in Fig. S2. The sequences of strains Y3, Y4 and Y5 were deposited at NCBI with accession numbers MT672755, MT672756 and MT672757, respectively. Finally, strains Y3, Y4 and Y5 were deposited to China General Microbiological Culture Collection Center (CGMCC), Beijing, China, with accession number 17926, 16183 and 14118, respectively.

### 3.2 Effect of Ni and Cd on growth of the strains

The MIC of Ni and Cd to Y3, Y4 and Y5 were Ni 300  $\mu\text{g}/\text{mL}$  or Cd 200  $\mu\text{g}/\text{mL}$ , Ni 500  $\mu\text{g}/\text{mL}$  or Cd 300  $\mu\text{g}/\text{mL}$  and Ni 500  $\mu\text{g}/\text{mL}$  or Cd 400  $\mu\text{g}/\text{mL}$ , respectively. As shown in Fig. S3-S5, strain Y3 was more sensitive to individual and combined toxicity of Ni and Cd than Y4 and Y5. As shown in Fig.S5, after 15 hours, the growth curve of Y3 grown in the medium containing Ni and Cd was always lower than that grown in the medium without Ni and Cd. After 60 h, the growth of Y3 reduced by 28% under Ni and Cd stress. In contrast, the growth of Y4 and Y5 was hardly affected by Ni and Cd stress. Under the combined stress of Ni and Cd, the growth curves of Y4 and Y5 showed no significant decrease compared with the control.

### 3.3 PGP traits of the isolates

The quantitative analyses of PGP traits showed that the selected strains possessed the ability of IAA production, ACC deaminase, phosphate solubilization, siderophores production and EPS development.

In the absence of Ni and Cd, the IAA production of Y3, Y4 and Y5 was 16, 15 and 14 µg/ml, respectively. In addition, the IAA production of strains Y3 and Y5 treated with Ni and Cd did not show the significant declines (Fig. 1a). As shown in Fig. 1b, without adding Ni and Cd, the ACC deaminase activity of strains Y3, Y4 and Y5 was 24, 19 and 22 ng α-keto butyrate/mg protein/h, respectively. Under the combined stress of Ni and Cd, the ACC deaminase activity of strain Y3 was also the highest. In the absence and presence of Ni and Cd, the phosphate solubilization of strain Y4 was the highest with 74 ppm and 61 ppm, followed by Y3 with 58 ppm and 46 ppm and Y5 with 57 ppm and 45 ppm (Fig. 1c). The siderophore units of strains Y4, Y3 and Y5 were 85, 78 and 56 % without Ni and Cd, compared with that of 75, 64 and 43% under Ni and Cd stress (Fig. 1d). Without Ni and Cd, the EPS production of Y3, Y4 and Y5 was 0.036, 0.034 and 0.032 g/mL, respectively, which was also above 0.032 g/mL under Ni and Cd stress (Fig. S6). In the presence or absence of Ni and Cd, all three strains could produce NH<sub>3</sub>, which is an important PGP mechanism that indirectly influences plants growth. Similarly, strains Y3, Y4 and Y5 were all effective biofilm formers in both conditions.

## **3.4 Plant growth promoting effects of selected strains**

### **3.4.1 Rice seed germination**

The EC<sub>25</sub> and EC<sub>50</sub> values were 20 µg/mL and 50 µg/mL for Ni<sup>2+</sup>, and were 60 µg/mL and 100 µg/mL for Cd<sup>2+</sup>, respectively. As shown in Fig. S7, a combined toxicity assay was carried by joining 20 µg/mL (EC<sub>25</sub>) Ni<sup>2+</sup> and gradient concentrations 25 (EC<sub>10</sub>), 40, 50, 60 (EC<sub>25</sub>), 70, 80, 90 and 100 µg/mL (EC<sub>50</sub>) of Cd<sup>2+</sup>. The results showed that the compound concentration that inhibited 50% germination rate was Ni 20 µg/mL and Cd 40 µg/mL (Fig. S7). So, this combined concentration was the treatment concentration for subsequent experiments.

As shown in Fig. S8, the germination rate of rice seed was about 70% without any treatments and it reduced to 38% after the treatment of Ni and Cd. The germination rate of rice seeds after inoculation showed no significant increase without Ni and Cd. In contrast, the rice seed germination rate was significantly enhanced by inoculation of strains under Ni and Cd stress. The germination rate of rice seeds increased by 43%, 54% and 36% after inoculation of strains Y3, Y4 and Y5 under Ni and Cd stress.

### **3.4.2 Effect of strains on seedling growth**

The inoculation of three strains had positive effects on promoting plant growth (Fig. 2). In the absence of Ni and Cd, the growth of rice seedlings was promoted significantly after inoculation with strains. For example, the root length increased by 32, 36, 14% after inoculation with strains Y3, Y4 and Y5. As shown in Table 1, Ni and Cd stress significantly ( $p < 0.05$ ) inhibited the biomass production of rice seedlings. For example, the shoot fresh weight of rice decreased by 30% after treated with Ni and Cd. Under the treatment of Ni and Cd, the biomass production was observed to be significantly ( $p < 0.05$ ) enhanced after inoculation of the strains. The shoot height of rice seedlings inoculated with strains Y3, Y4 and Y5 increased by 47, 41 and 34%; the root length increased by 56, 47 and 47%. The shoot fresh weight increased by 35, 19 and 18% while almost all the increase in root fresh weight exceeded 30%. The

increase in shoot dry weight and root dry weight was also more than 30%. In general, inoculation with Y4 had the best promoting effects on the biomass production of rice in the absence of Ni and Cd while inoculation with Y3 had the best promoting effects under combined stress of Ni and Cd.

Chlorophyll-a and chlorophyll-b are the basis of photosynthesis. After Ni and Cd treatment, the contents of chlorophyll a, chlorophyll b and total chlorophyll all decreased by more than 30% (Table 2). The estimation of the chlorophyll content suggested an increase in the pigment by 70% in strains-inoculated seedlings than the uninoculated seedlings under metal stress. Also, it was found that the content of chlorophyll-b in the rice seedlings inoculated with Y3 and Y4 under the heavy metal treatment was even higher than that without strain inoculation and heavy metal treatment.

### **3.4.3 Effect of selected strains on antioxidants**

Antioxidant capacity plays an important role in tolerance and better growth under Ni and Cd stress. As shown in Fig. 3, SOD, POX and CAT activity in rice increased by 53, 47 and 28% under Ni and Cd stress, respectively, compared with that without Ni and Cd treatment. In addition, these activities of antioxidant enzymes in seedlings were further enhanced with inoculated strains (Fig. 3a-c), with inoculation with strain Y3 showing the most increase. Under the combined stress of Ni and Cd, the SOD, POX and CAT activity in rice inoculated with strains Y3 increased by 24, 28 and 37% compared with that non-inoculation treatment. The results indicated that inoculation with Y3 had the best effects on increasing antioxidant enzyme activity in seedlings under Ni and Cd combined stress.

### **3.4.4 Effect of strains on ROS accumulation in rice seedlings**

As shown in Fig. 4, with Ni and Cd treatment, DAB and NBT staining were clearly showed in rice leaves, which indicated more  $H_2O_2$  and  $O_2^-$  accumulation. But less DAB and NBT staining were observed in inoculated rice seedlings under Ni and Cd stress, which indicated that strains Y3, Y4 and Y5 significantly reduced the level of ROS in rice seedlings under the combined pollution of Ni and Cd. The results of quantitative analysis of ROS also showed the similar conclusion (Fig. 5a-b). Under the combined stress of Ni and Cd, the  $H_2O_2$  content in the rice seedlings inoculated with Y3, Y4 and Y5 significantly decreased by 19%, 16% and 13%. The content of  $O_2^-$  showed the similar trend that reduced by 17%, 14% and 13%. The results showed that inoculation with Y3 had the best effects on alleviating the oxidative damage of Ni and Cd combined stress. In addition, under the non-polluting conditions, there was no significant differences in ROS content between inoculated and uninoculated rice plants.

Under Ni and Cd stress, the MDA content increased by about 1.5 times compared with control. The results showed that the MDA contents of the strains-inoculated groups were significantly ( $p < 0.05$ ) reduced contrast with un-inoculated group (Fig. 5c). In this study, the three strains lowered the proline accumulation in rice seedlings by 21, 19 and 15% under Ni and Cd stress (Fig. 5d).

### **3.4.5 Effect of strains on Ni and Cd accumulation**

As shown in Fig.S9, there was no statistically significant difference in heavy metal content between inoculated and uninoculated rice plants. The results indicated that the application of rhizobacteria did not increase the uptake of Ni and Cd by the rice plant.

### 3.4.6 PCA and cluster analysis

As shown in Fig. S10, the cluster analysis of all growth and antioxidant indexes in all samples (including control and heavy metal treated seedlings that with and without inoculation) revealed that the samples that un-treated and treated by Ni and Cd were clustered into two major clusters. The treatment of Ni and Cd in rice seedlings could down-regulate the growth indexes and up-regulate the activities of antioxidant enzymes. However, inoculation of three strains could alleviate the adverse effects of combined phytotoxicity of Ni and Cd on rice growth.

## 4. Discussion

Various PGPR genera were reported for their dual characteristics of heavy metal tolerance and plant growth promotion. *Pseudomonas*, *Chryseobacterium* and *Enterobacter* in this study were also with PGP characters to enhance plant growth under heavy metal stress. It was reported that inoculation with *Pseudomonas putida* favored the healthy growth of *Eruca sativa* under Cd stress (Kamran et al. 2015). In another study, the biomass of maize inoculated with *Chryseobacterium humi* increased by more than half in soils contaminated with Cd (Moreira et al. 2014). The *Enterobacter* sp. strain EG16 was proved to tolerate high external Cd concentrations and produce IAA to promote plant growth (Chen et al. 2016). All three PGPR genera were with significant growth promoting character and huge application potential. However, most reports did not focus on combined stress of Ni and Cd. In this study, three genera strains were comprehensively analyzed and the PGP traits in the presence or absence of Ni and Cd were determined. The different effects of the strains with different PGP traits on rice growth under compound stress of Ni and Cd were also explored.

IAA an important hormone and it is known to be involved in cell division, cell expansion, tissue differentiation and resistance to stress conditions (Dourado et al. 2013). Heavy metal may disrupt the synthesis mechanism of IAA, leading to senescence and cell death (Mitra et al. 2018b). In our study, strains Y3, Y4 and Y5 could produce IAA and stimulate rice growth under Ni and Cd stress (Fig. 1a). The results were consistent with the study of Dourado (2013). Plants produce large amounts of ethylene when under stress while high concentrations of ethylene can cause plant growth inhibition or death. The study of Ghosh (2018) and Gupta (2019) confirmed that ACC deaminase producing strains reduced ethylene production in plants under abiotic stress. Other studies (Ahmad et al. 2014, Islam et al. 2014) considered that the concentration of ethylene in the plant could be reduced for that ACC deaminase decreased the content of ACC. In this study, the strains Y3, Y4 and Y5 were all ACC deaminase producers (Fig. 1b) and the inoculation of the strains might reduce Ni and Cd stress sensitivity to plants by inhibited ethylene synthesis. Most phosphorus in the soil is insoluble and cannot support plant growth. The absorption of phosphate under heavy metal stress is further reduced (Aw et al. 2019). The strains Y3, Y4 and Y5 could dissolve phosphate and increase the absorption of phosphate by plants (Fig. 1c). PGPR

with siderophores could inhibit the movement of toxic ions and maintain the ionic balance (Hashem et al. 2019). Strains Y3 and Y4 possessed the potent ability to produce siderophores and promote the growth of rice seedlings (Fig. 1d). In the previous study, *Bacterium Enterobacter* sp. promote plant growth due to its Cd-induced siderophore production (Chen et al. 2016). However, in our study, the production of siderophores in three strains decreased under the combined stress of Ni and Cd. This might be due to the combined stress and different concentrations of heavy metals. Bacteria colonized on the root surface with EPS could act as a protective barrier against stress. In our study, strains Y3, Y4 and Y5 could produce EPS in the absence of Ni and Cd (Fig. S5). In addition, the production of EPS in three strains hardly decreased under the combined stress of Ni and Cd. The results were consistent with the previous research (Mukherjee et al. 2019). The biofilm of rhizobacteria could protect plants from abiotic stress (Ansari, Ahmad 2019). In this study, the three biofilm forming strains Y3, Y4 and Y5 contributed to the increase of the root length and shoot height of rice under Ni and Cd stress, which was similar to the previous research (Itusha et al. 2019).

The study of PGPR under compound heavy metal stress is important in developing new biotechnologies of plant growth promotion. However, despite many heavy metal-resistant strains reported, there were few of them focusing on compound pollution of Ni and Cd. In this study, three potent PGPRs were applied in rice seedling under compound stress of Ni and Cd. The root-shoot length and biomasses were directly related to plant growth, which increased significantly after inoculated with strains Y3, Y4 and Y5 in the absence and presence of Ni and Cd. The results proved that the inoculated strains mitigated the negative effects of Ni and Cd and alleviated the toxicity to the growth of rice seedlings (Table 1). The biosynthesis of chlorophyll was impeded by heavy metal stress (Pramanik et al. 2017). In our study, the content of chlorophyll decreased under compound stress of Ni and Cd, which was alleviated by inoculating strains Y3, Y4 and Y5 (Table 2). Similar results were observed in tomato under Cd stress (Khanna et al. 2019). Overall, the rice inoculated with strains Y3, Y4 and Y5 were all showed better growth features compared with the un-inoculated groups, including the increase of biomass and chlorophyll content. The results indicated the remarkable effects of these three strains on promoting rice seedling growth under Ni and Cd stress.

ROS are produced continuously by various metabolic pathways in different cellular compartments such as chloroplast, mitochondria and peroxisomes (Gill, Tuteja 2010). ROS molecules are scavenged by various antioxidative defense mechanisms. SOD can mediate the detoxification of superoxide radicals by catalyzing the dismutation of superoxide anions to hydrogen peroxide ( $H_2O_2$ ), which is further neutralized to water and oxygen by the enzyme CAT and POX (Mitra et al. 2018b). Heavy metal stress may perturb the equilibrium between the production and the scavenging of ROS, and increase lipid peroxidation via ROS generation (Gill, Tuteja 2010). In the present study, under Ni and Cd stress, the elevated levels of  $H_2O_2$  and  $O_2^-$  in the rice seedlings might cause the oxidative injury to rice plant, and the elevated levels of MDA showed the possible consequence of the damage of membrane lipid (Fig. 5). Inoculating of rhizobacteria positively impacted antioxidant enzyme activity in rice seedlings under the compound stress of Ni and Cd (Fig. 5) and decrease the contents of  $H_2O_2$ ,  $O_2^-$  and MDA in plants significantly. The

results indicated that bacterial inoculation helped the plant to reduce the ROS and MDA contents by increasing the antioxidant enzyme activity. Proline is an important osmotic substance that can participate in maintaining tissue moisture content. In this study, the accumulation of proline increased significantly in rice seedling with the treatment of Ni and Cd compared to control, however, this accumulation declined in the rice seedlings inoculated with strains (Fig. 5). Other studies had similar conclusions that PGPR reduced the negative consequences of oxidative stress caused by heavy metal toxicity through enhancing the activity of ROS scavenging enzymatic antioxidants and reducing ROS accumulation (Pramanik et al. 2017, Hashem et al. 2016, Shah et al. 2001, Wan et al. 2012).

As mentioned above, many studies showed that PGP traits of strains played important roles in plants under heavy metal stress. For example, it was reported that *Flavobacterium* sp. strain 5P-3 with ACC deaminase activity improved growth of plant *B. juncea* in the presence of Cd (Belimov et al. 2005). The previous study revealed the siderophore production contributed to plant growth promotion. Another report indicated that *Halomonas* sp. Exo1, an efficient producer of EPS, showed enhanced rice growth promotion under combined stress of salt and arsenic (Mukherjee et al. 2019). In this study, we found that the different PGP traits of strains might be associated with the different effects on rice growth in the absence and presence of Ni and Cd. For example, strain Y4 possessed with better ability of phosphate solubilization and siderophores production compared with strains Y3 and Y5 (Fig. 1). In the absence of Ni and Cd, inoculation of Y4 had the best promoting effects on rice growth, which indicated the important roles of phosphate solubilization and siderophores production in promoting plant growth (Table 1). In the other hand, strain Y5 possessed with better ability of IAA production compared with Y4 and inoculation with Y5 showed better promoting effects on root growth under the combined stress of Ni and Cd. The results indicated that IAA production was of vital importance in root growth under Ni and Cd stress. In addition, strain Y3 possessed with better ability of IAA production, EPS production and ACC deaminase activity compared with strains Y4 and Y5 (Fig. 1). Under the combined stress of Ni and Cd, inoculation with Y3 had the best effects on enhancing the tolerance to Ni and Cd and alleviating oxidative damage of rice seedlings. The results showed that IAA production, EPS production and ACC deaminase activity might be more important for promoting plant growth under the combined stress of Ni and Cd.

## 5. Conclusion

In the present study, *Pseudomonas* sp. strain Y3, *Chryseobacterium* sp. strain Y4. and *Enterobacter* sp. strain Y5 were isolated from rice rhizosphere soil. These strains were resistant to Ni and Cd and possessed with important PGP traits like IAA production, ACC deaminase activity, phosphate solubilizing ability, siderophores production and EPS development. In this study, the rice inoculated with strains Y3, Y4 and Y5 showed better growth features in comparison with non-inoculation rice plants in the absence and presence of Ni and Cd. The study also revealed that inoculation with strains Y3, Y4 and Y5 increased antioxidant enzyme activity and reduced oxidative damage under the combined stress of Ni and Cd. Therefore, the strains could be treated as the effective plant growth promoters in Ni and Cd-contaminated field. In addition, by analyzing the PGP traits of strains and their effects on plant growth, we found that IAA production, EPS production and ACC deaminase activity played more important roles in promoting

plant growth under compound pollution of Ni and Cd. Among them, IAA production might be critical in root growth under Ni and Cd stress.

## Declarations

## Acknowledgements

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## Tables

**Table 1 Plant growth promoting effects of strains on rice seedling**

Treatment groups		Shoot height (cm)	Root length (cm)	Shoot fresh weight (mg)	Root fresh weight (mg)	Shoot dry weight (mg)	Root dry weight (mg)
Control	N	18.35 ± 0.34 <sup>d</sup>	9.06 ± 0.42 <sup>b</sup>	64.0 ± 3.0 <sup>e</sup>	9.2 ± 0.3 <sup>cd</sup>	25.5 ± 0.3 <sup>c</sup>	5.7 ± 0.3 <sup>c</sup>
	Y3	24.16 ± 1.62 <sup>a</sup>	12.62 ± 0.73 <sup>a</sup>	117.7 ± 4.4 <sup>b</sup>	11.0 ± 0.7 <sup>ab</sup>	38.1 ± 1.3 <sup>a</sup>	6.8 ± 0.4 <sup>ab</sup>
	Y4	24.98 ± 0.34 <sup>a</sup>	12.62 ± 0.06 <sup>a</sup>	140.3 ± 3.1 <sup>a</sup>	11.7 ± 0.5 <sup>a</sup>	39.6 ± 0.7 <sup>a</sup>	7.3 ± 0.3 <sup>a</sup>
	Y5	20.91 ± 0.32 <sup>bc</sup>	12.98 ± 0.48 <sup>a</sup>	91.7 ± 1.9 <sup>c</sup>	10.1 ± 1.1 <sup>bc</sup>	33.4 ± 0.8 <sup>b</sup>	6.5 ± 0.6 <sup>b</sup>
Ni20 Cd40	N	15.14 ± 0.96 <sup>e</sup>	5.17 ± 0.48 <sup>d</sup>	48.8 ± 2.2 <sup>f</sup>	6.0 ± 0.4 <sup>f</sup>	17.8 ± 0.4 <sup>d</sup>	3.7 ± 0.2 <sup>e</sup>
	Y3	22.30 ± 0.68 <sup>b</sup>	8.06 ± 0.54 <sup>c</sup>	71.1 ± 5.0 <sup>d</sup>	8.2 ± 0.3 <sup>de</sup>	25.9 ± 1.2 <sup>c</sup>	5.2 ± 0.1 <sup>cd</sup>
	Y4	18.35 ± 0.34 <sup>d</sup>	9.06 ± 0.42 <sup>b</sup>	062.3 ± 3.1 <sup>e</sup>	7.7 ± 0.5 <sup>e</sup>	25.6 ± 1.4 <sup>c</sup>	5.7 ± 0.3 <sup>c</sup>
	Y5	24.16 ± 1.62 <sup>a</sup>	12.62 ± 0.73 <sup>a</sup>	62.2 ± 1.4 <sup>e</sup>	8.4 ± 0.5 <sup>de</sup>	25.1 ± 0.9 <sup>c</sup>	6.8 ± 0.4 <sup>ab</sup>

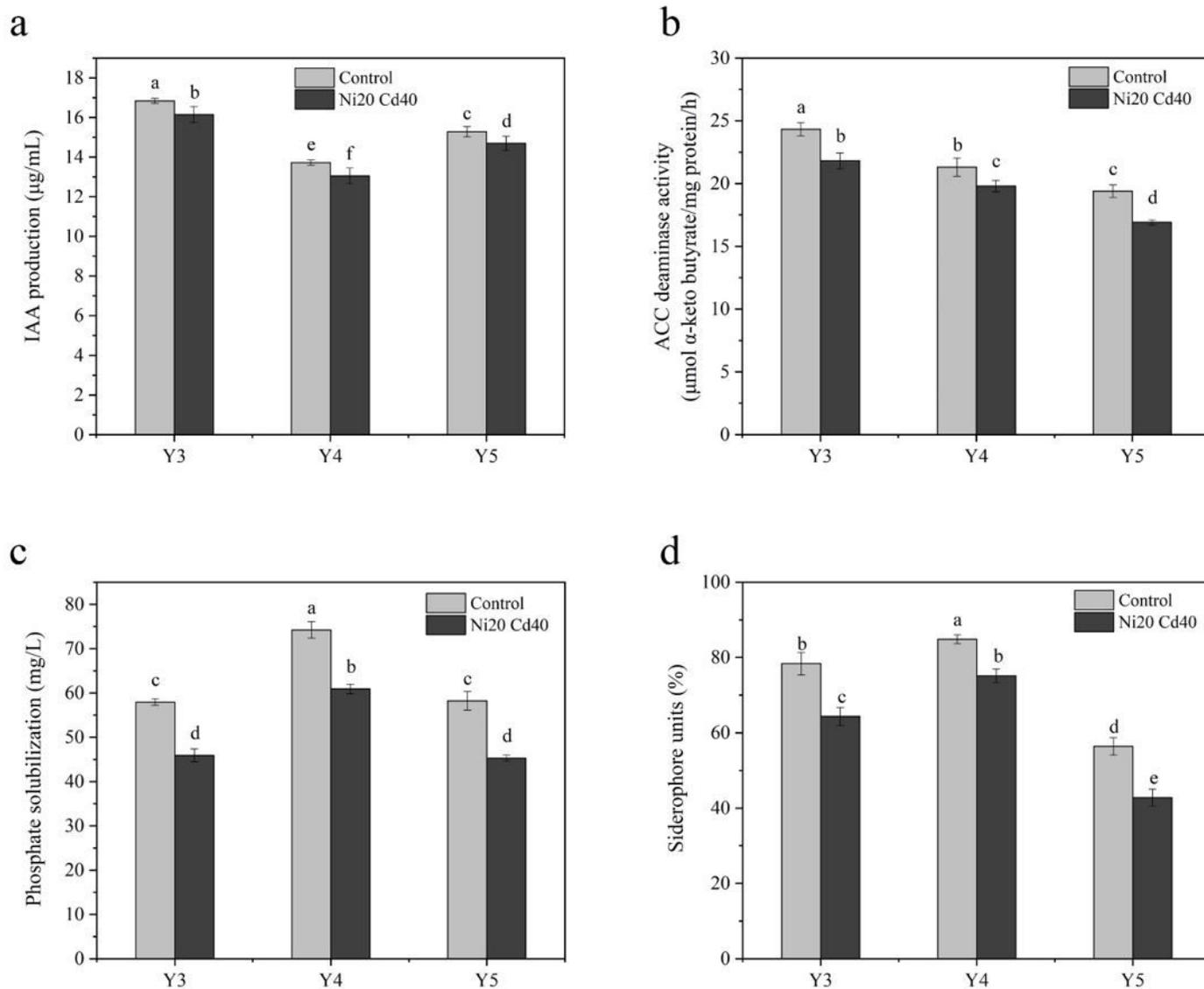
Values are the means  $\pm$  standard errors of three replicates. Different letters show statistically significant differences ( $p < 0.05$ ) among all treatment groups in growth parameters (Control: treated with water, N: non-inoculation).

**Table 2 Effect of the strains on the content of chlorophyll a, chlorophyll b and total chlorophyll.**

Treatment groups		Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Total chlorophyll (mg/g FW)
Control	N	1.1343 $\pm$ 0.0210 <sup>f</sup>	0.3576 $\pm$ 0.0221 <sup>e</sup>	1.4920 $\pm$ 0.0016 <sup>g</sup>
	Y3	1.6840 $\pm$ 0.0168 <sup>a</sup>	0.5411 $\pm$ 0.0121 <sup>a</sup>	2.2251 $\pm$ 0.0178 <sup>a</sup>
	Y4	1.5730 $\pm$ 0.0102 <sup>b</sup>	0.4951 $\pm$ 0.0131 <sup>b</sup>	2.0681 $\pm$ 0.0096 <sup>b</sup>
	Y5	1.2866 $\pm$ 0.0188 <sup>c</sup>	0.3999 $\pm$ 0.0107 <sup>d</sup>	1.6865 $\pm$ 0.0090 <sup>e</sup>
Ni20 Cd40	N	0.6630 $\pm$ 0.0214 <sup>g</sup>	0.3011 $\pm$ 0.0147 <sup>f</sup>	0.9641 $\pm$ 0.0111 <sup>h</sup>
	Y3	1.3143 $\pm$ 0.0282 <sup>c</sup>	0.5110 $\pm$ 0.0077 <sup>b</sup>	1.8253 $\pm$ 0.0217 <sup>c</sup>
	Y4	1.2352 $\pm$ 0.0058 <sup>d</sup>	0.5109 $\pm$ 0.0029 <sup>b</sup>	1.7461 $\pm$ 0.0081 <sup>d</sup>
	Y5	1.19270 $\pm$ 0.0059 <sup>e</sup>	0.4394 $\pm$ 0.0079 <sup>c</sup>	1.6321 $\pm$ 0.0107 <sup>f</sup>

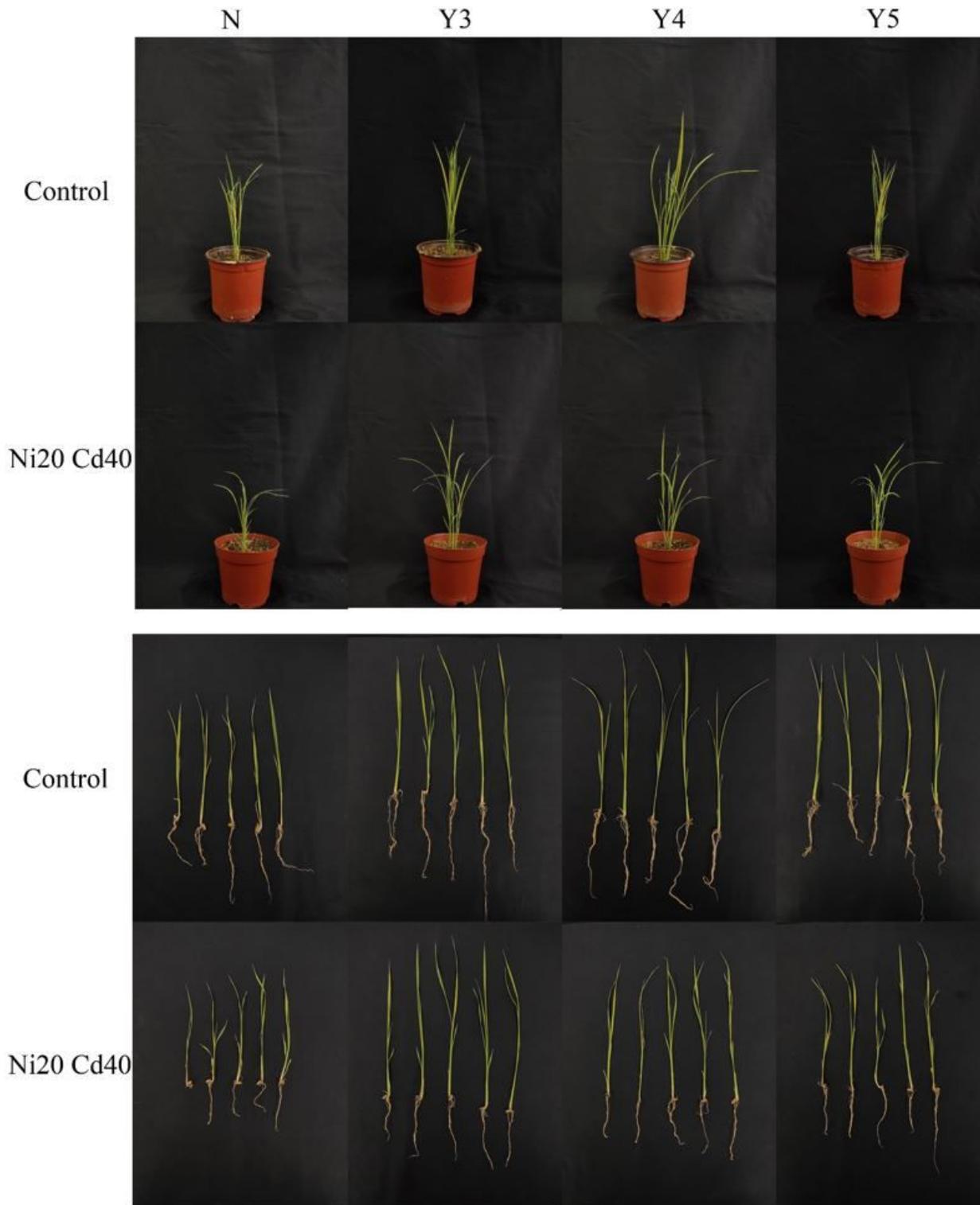
Values are the means  $\pm$  standard errors of three replicates. Different letters show statistically significant differences ( $p < 0.05$ ) among all treatment groups in chlorophyll content (Control: treated with water, N: non-inoculation).

## Figures



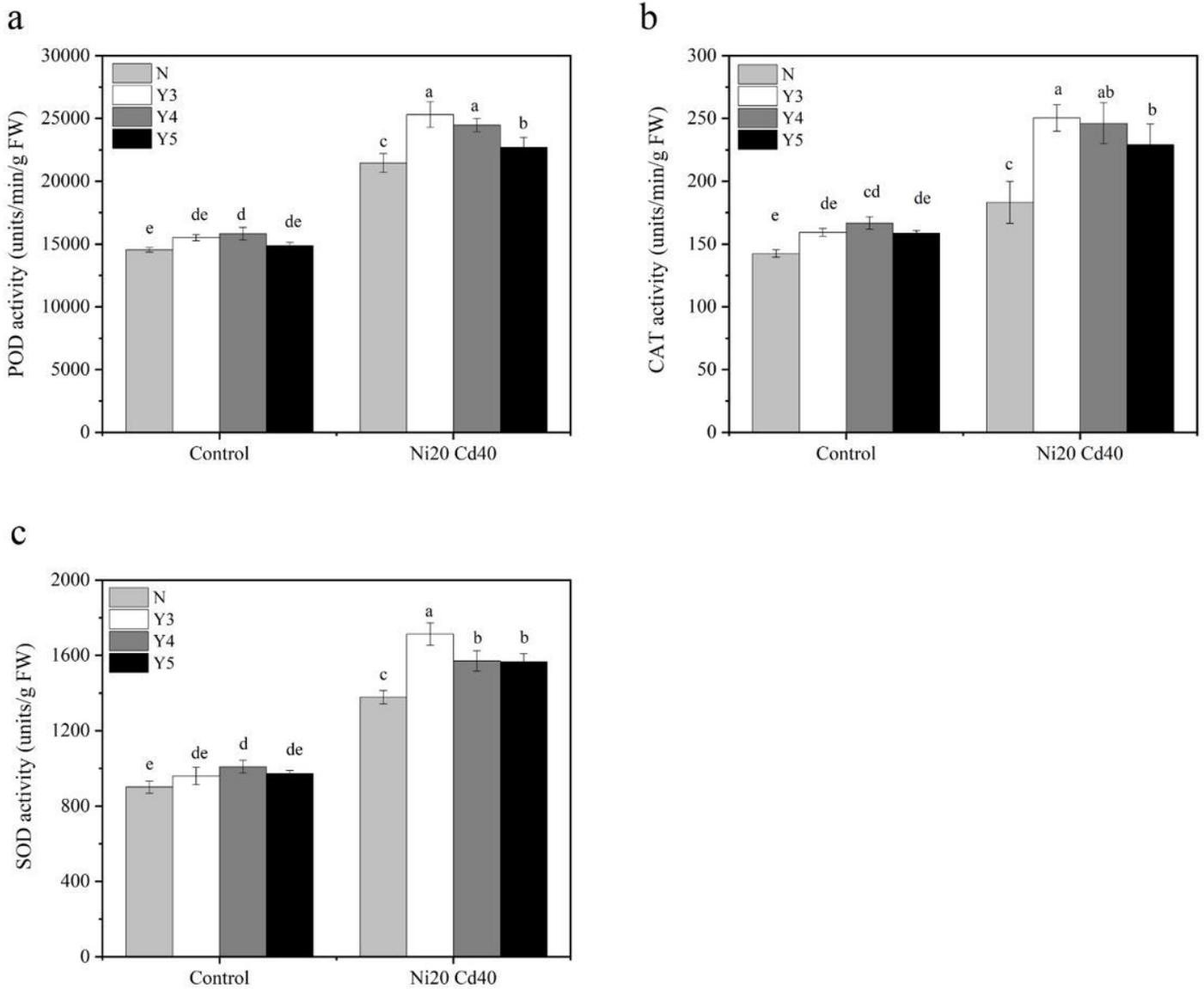
**Figure 1**

The quantitative estimation of different PGPR characteristics of strains Y3, Y4 and Y5: IAA production (a), ACC deaminase (b), phosphate solubilization (c) and siderophore (d). Values are the means  $\pm$  standard errors of three replicates. Error bars depict standard errors and different letters above the bars show statistically significant different values ( $p < 0.05$ ) according to ANOVA followed by the Duncan's test.



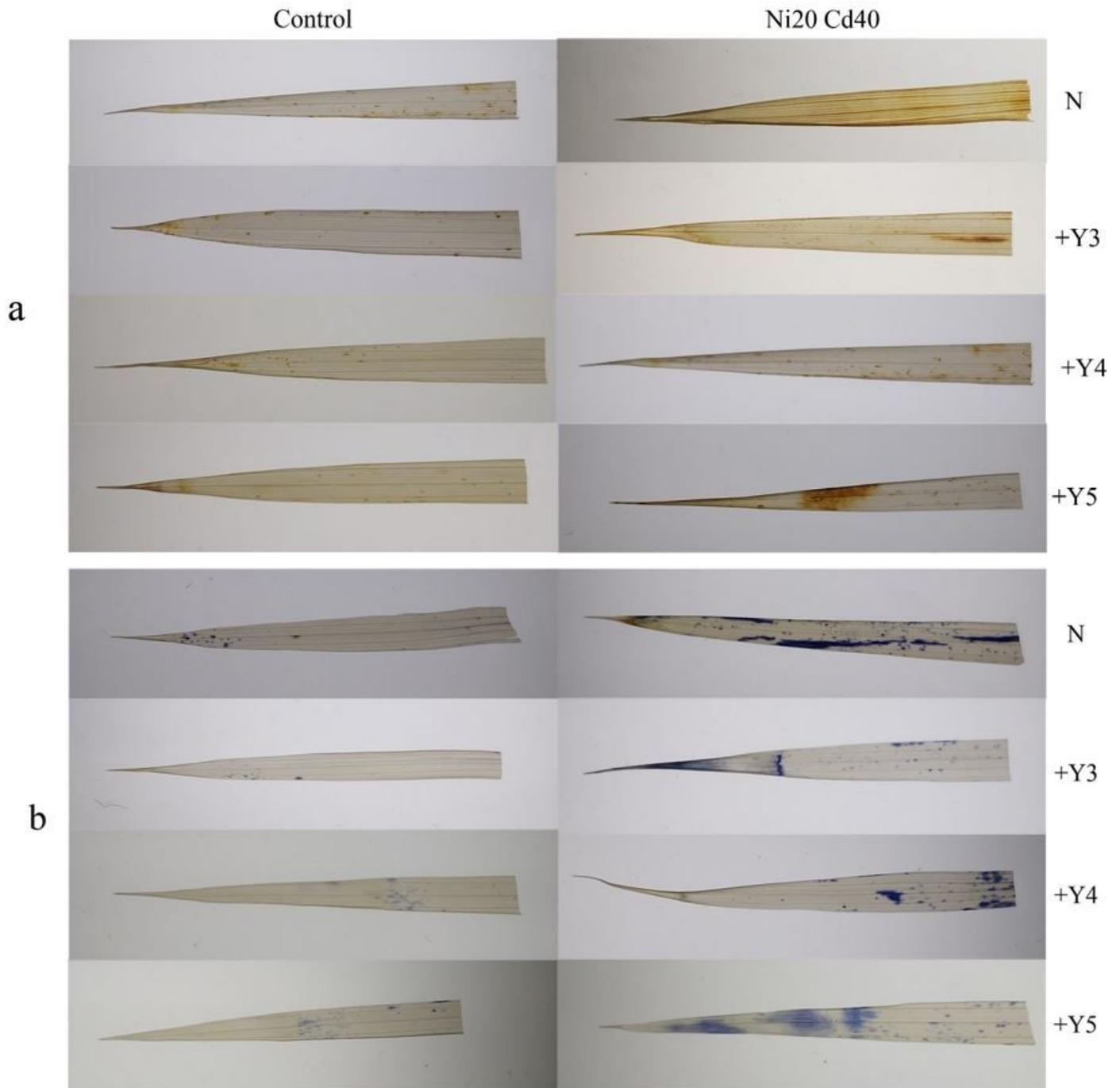
**Figure 2**

Photograph of rice seedlings in different treatment groups (Control: treated with water, N: non-inoculation).



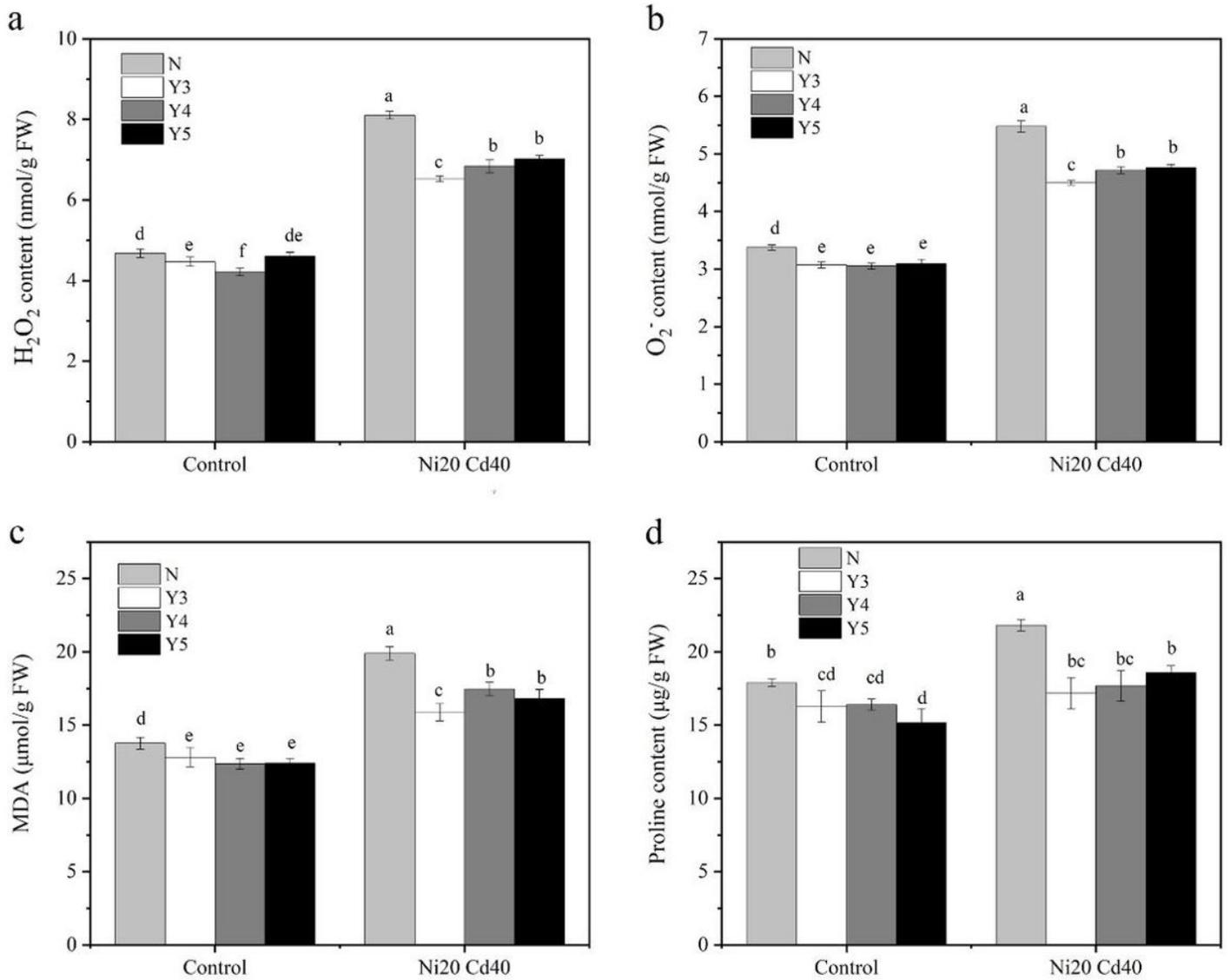
**Figure 3**

Effect of strains on Ni and Cd stress induced - POX activity (a), Catalase activity (b), SOD activity(c), (Control: treated with water, N: non-inoculation). Values are the means  $\pm$  standard errors of three replicates. Error bars depict standard errors and different letters above the bars show statistically significant different values ( $p < 0.05$ ) according to ANOVA followed by the Duncan's test.



**Figure 4**

The H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> accumulation in rice seedlings: DAB staining (a), NBT staining (b) (Control: treated with water, N: non-inoculation).



**Figure 5**

H<sub>2</sub>O<sub>2</sub> content (a) and O<sub>2</sub><sup>-</sup> content (b) MDA content (c) and proline content (d) (Control: treated with water, N: non-inoculation). Values are the means ± standard errors of three replicates. Error bars depict standard errors and different letters above the bars show statistically significant different values (p < 0.05) according to ANOVA followed by the Duncan's test.

## Supplementary Files

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