

Article

Improvement of Cadmium Detoxification Potential and Plant Growth Promotion by Bacterial Endophytes

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Abstract: Cadmium (Cd) is a heavy metal that suppresses plant growth; however, application of endophytic bacteria can increase resistance of plants against Cd, as well as improve plant growth. Two bacterial endophytic strains were isolated from *Solanum nigrum* and were identified as *Serratia* sp. AI001 and *Enterobacter* sp. AI002 by 16S DNA sequencing. Strains AI001 and AI002, tolerated up to 25 mg/mL Cd in broth culture and showed phosphate solubilization potential in Pikovskaya agar medium. AI001 and AI002 produced indole-3-acetic acid, which was confirmed by gas spectrometry-mass chromatography. Brassica plants stressed with 0, 5, 15, and 25 mg/L Cd showed significant decrease in plant growth, chlorophyll content and biomass, and significant increase in Cd dose-dependent electrolyte leakage. Inoculation of strain AI001 or AI002 significantly enhanced the plant growth attributes of shoot length, root length, chlorophyll content, and biomass as compared to those in uninoculated plants. Reduced glutathione contents in plants stressed with different concentrations of Cd also increased with inoculation of AI001 and AI002. The reason of Cd resistance enhancement in plants by inocula could be due to their greater plant growth promoting activities as well as their antioxidative response.

Keywords: endophytic bacteria; indole-3-acetic acid; cadmium accumulation; phosphate solubilization; reduced glutathione.

1. Introduction

Microorganisms living inside the plants tissues without damaging plant cells and tissues are called endophytes. The endophytes make a mutualistic association with the different tissues of the plant, causing positive effects on plant physiology [1]. Endophytes have very significant role in tissue proliferation and differentiation and in growth promotion of plant [2]. In addition, they can protect plants against variety of stressors when plant are exposed to environmental stresses [3]. Moreover, the endophytes are excellent source of biologically active secondary compounds that results a number of numerous chemical such as alkaloids, steroids, flavonoids contents, steroids compounds, terpenoids and phenolic compounds [4]. The role of the endophytes are highly dependent on biotic and abiotic stressors including heavy metal stress [5, 6].

Plants under the Cd stress undergo various chemical changes, which leads to change in cell signaling molecules, osmoregularity, and secondary metabolites [6]. Disruption of membranes including organelle membrane and cell membrane are greatly affected by Cd and other heavy metals and establish electrolytes leakage causing ionic imbalance which trigger the production of reactive

oxygen species (ROS) [7]. The chain reaction of ROS disrupts the metabolic activity of the cell and proteins that act as signaling molecules are denatured and hence lose the functionality [8]. In addition, lipid molecules that are integral part of the cellular membrane are also disrupted by ROS and cause lysis cell. Plant defense system consisted of antioxidant response is generated to cope with ROS and neutralize the damaging effects of the ROS [9].

The bacterial endophytes has been investigated to support the plant growth and development and through an array of metabolites such as phytohormones [10], phosphate solubilization [11], nitrogen fixation, siderophore production and antioxidant production [9]. The endophytes solubilize the phosphate have very significant role in the plant growth and promotion. They turn the phosphate in to readily available form by solubilize it through different organic molecules and plant assimilate this solubilized phosphate [12]. Phytohormones are regulators for plant growth that helps in creating signals to control cell division and are major factor in growth promotion as well as development of the plant [13]. In addition, they also control plant responses to changes in environment, enabling the plant to cope with environmental stresses [14]. Recent research has recognized that certain endophytic microbes, including bacteria, increase growth of the plants by providing plant hormones, indole-3-acetic acid (IAA), cytokinins, and gibberellins [15]. Bacteria in the types *Paenibacillus*, *Agromyces*, *Microbacterium*, *Methylophaga* and *Bacillus* are stated to produce IAA [16]. IAA generated by bacteria, exist in the rhizosphere increases root growth and availability of nutrient because of the larger occupied area of fertile soil that is causing augmented biomass of the plants to be resistance against diseases [14].

We aimed to isolate bacterial endophytes from *S. nigrum* and assesse them against Cd tolerance in plants. The isolates were inoculated on Brassica plants contaminated with different concentrations of Cd to analyze the Cd resistance potential in plants. Moreover, reactive oxygen species, plant growth potential e.g., plant growth attributes, plant biomass under the influence of endophyte treatments were also determined.

2. Results

2.1 Isolation and genotypic characterization of bacterial isolates

Endophytic bacterial strains, AI001 and AI002 were isolated from *S. nigrum* plants. The purified PCR products of 16S rDNA from the two bacterial strains (AI001 and AI002) were sequenced, revealing nucleotide sequence lengths of approximately 1022 bp and 571 bp, respectively. The sequences were aligned with sequences identified by BLAST search of the NCBI database. Strains AI001 and AI002 were identified by the maximum parsimony (MP) method, with 10 reference sequences selected for each strain to construct a phylogenetic tree with 1,000 bootstrap replications. The selected sequences showed the highest query coverage and sequence homology with AI001 and AI002. Results of the BLAST sequence search (Figure 1A) indicated the endophyte isolate AI001 had sequence identity with various strains of *Serratia* sp. and strain AI002 showed sequence identity with various strains of *Enterobacter aerogenes* (Figure 1B). Based on results of sequence homology and the phylogenetic analysis, endophyte isolate AI001 was designated *Serratia* sp. AI001, and the 16S rDNA sequence was deposited in GenBank under accession number KX898586. Isolate AI002, with GenBank accession number KX898961, was identified as *Enterobacter* sp. AI002.

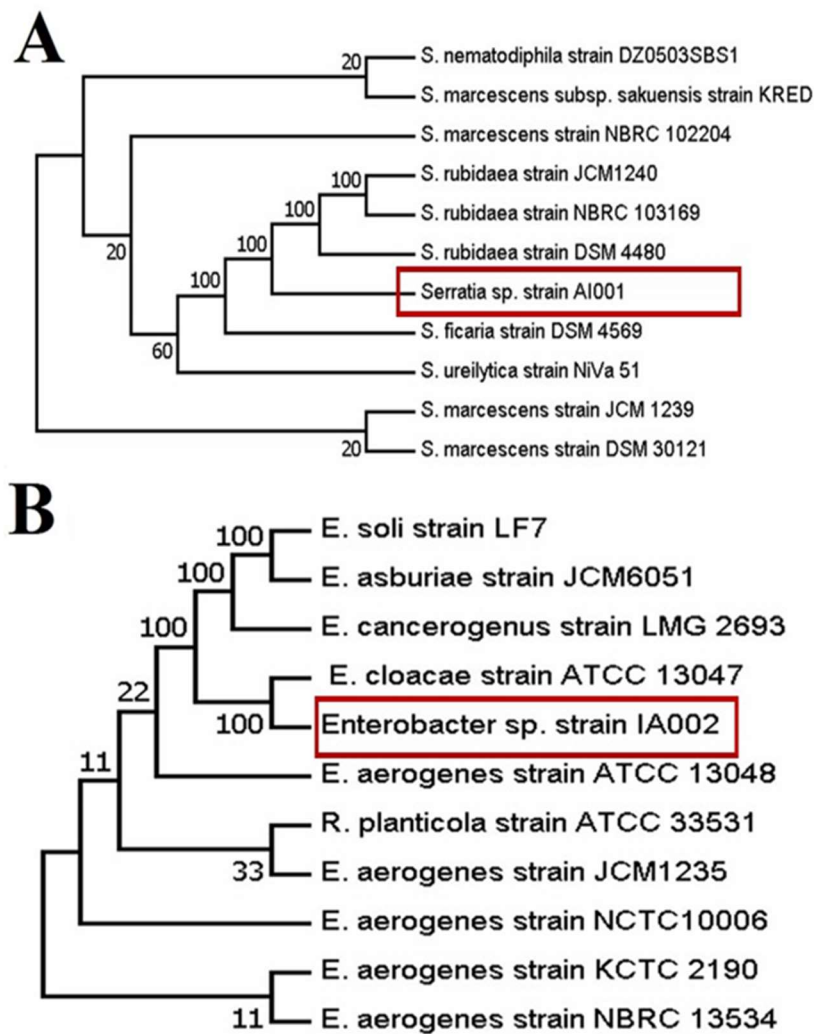


Figure 1. Phylogenetic tree showing the relationships of endophytic isolates with other bacterial species based on an analysis of the partial 16S rRNA. (A) The phylogenetic relationship of AI001 with related bacterial species. (B) Phylogenetic tree of AI002 and other bacterial species.

2.2. Cadmium tolerance

Bacterial isolates AI001 and AI002 were screened against Cd in LB medium containing different concentrations of Cd, i.e., 0, 5, 10, 15, 20, 25, or 30 mg/mL. The results revealed that both strains could tolerate high concentrations of Cd in the culture broth. However, the addition of Cd reduced the growth rate of both strains and prolonged the initial phase of bacterial growth. Although strain AI001 tolerated a Cd concentration as high as 25 mg/mL, the growth pattern revealed that AI001 was tolerant of Cd up to 20 mg/mL and that tolerance dropped sharply at 25 mg/mL (Figure 2A). In addition, growth was negligible at 30 mg/mL Cd, which was considered the MIC. Similarly, strain AI002 was tolerant of Cd concentrations up to 25 mg/mL, and then its growth was reduced to negligible at 30 mg/mL, the Cd MIC (Figure 2B).

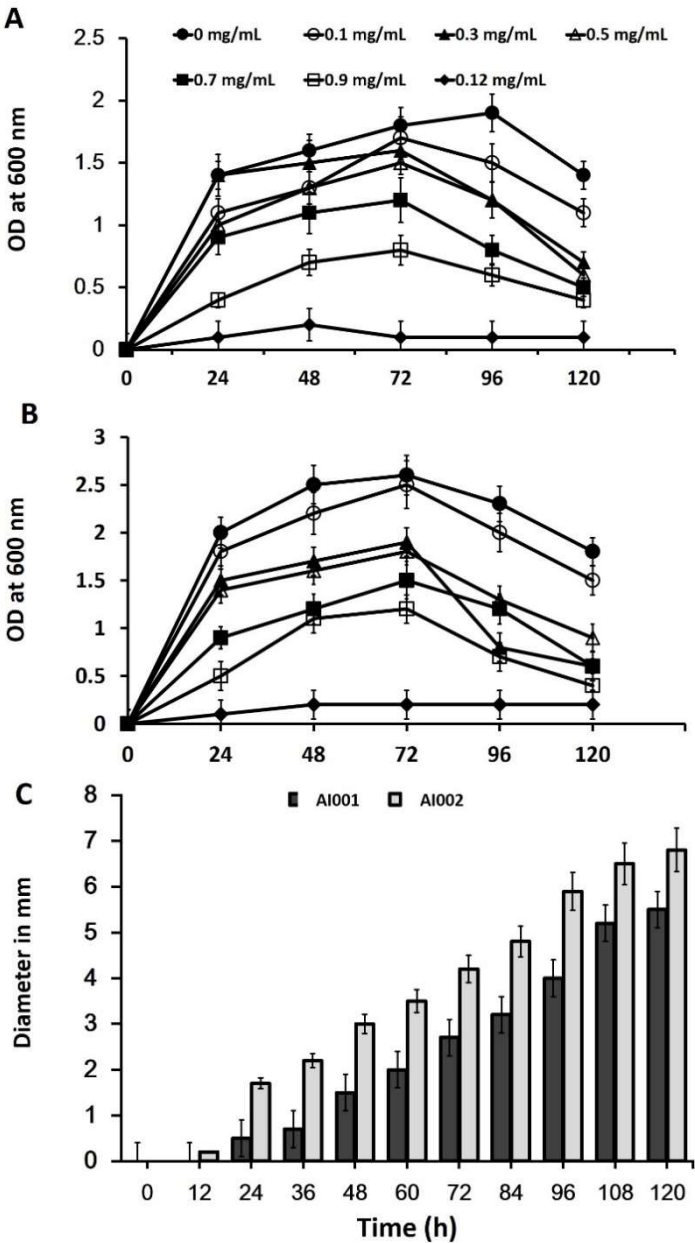


Figure 2. Assessment of the cadmium (Cd) tolerance and Phosphate solubilization of bacterial strain AI001 or AI002 at different concentrations of Cd. (A) Growth curve of AI001 in LB medium supplemented with various concentrations of Cd. (B) Growth curve pattern of AI002 under the Cd stress. (C) Phosphate solubilization by AI001 and AI002 shown on PVK agar plates. Values are averages of three replicates; error bars represent standard deviations.

2.3. Phosphate solubilization

The isolated strains, AI001 and AI002 were cultured on PVK agar plats and the medium was supplemented with $\text{Ca}_3(\text{PO}_4)_2$ to determine their phosphate solubilization strength of the isolates. Assessment of phosphate solubilization of AI001 and AI002 was carried out by visualizing the clear zones on PVK agar plates which were formed by bacterial colonies grown on medium. the clear zones were generated on the PVK agar plates surfaces revealed that AI001 and AI002 were successfully

solubilized the $\text{Ca}_3(\text{PO}_4)_2$. Reading of the diameter of the clear zones were obtained after an interval of 12 h and the experiment duration was 5 days long (Figure 2C). The amount of organic acid secreted by the bacterial strain determine the size of the clear zone the larger the diameter more secretion of organic acid which convert insoluble phosphate into solubilized. The results showed that AI002 produced larger clear zones as compared to AI001 and hence was capable of more phosphate solubilization.

2.4. Assessment of bacterial role in Cd phytoremediation

Plant growth attributes of *Brassica* plants contaminated with various concentrations of Cd, i.e., 0, 5, 15, or 25 mg/mL, and incubated with AI001 and AI002 for 15 days were compared with those of plants contaminated using Cd but uninoculated with AI001 and AI002. Results (Table 1) showed that Cd stress significantly decreased ($P < 0.05$) almost all of the growth of plant in the term of total plant length, chlorophyll contents and biomass, in comparison with those of the control plants. However, plants treated with the bacterial isolates promoted growth of plant in the term of total plant length, chlorophyll contents and biomass and also enhanced biomass in the presence of Cd. Chlorophyll contents of the plants were an important indicator of photosynthesis, which is necessary for the survival of the plant. Cd stress significantly ($P < 0.05$) reduced the chlorophyll contents of plants in comparison with the contents in non-stressed plants. However, inoculation of AI001 and AI002 significantly increased the chlorophyll contents of plants contaminated with Cd and therefore, enhanced biomass of the plant in compression with non-inoculated plants stressed with Cd. Inoculation of strains AI001 and AI002 significantly affected plant growth and development at all concentrations of Cd tested. Strain AI001 was more effective than strain AI002 in promoting the growth of plants not exposed to Cd. However, strain AI002 consistently improved growth of plant in the term of total plant length, chlorophyll contents and biomass as compared to AI001 in plants exposed to all Cd concentrations (5, 15, or 25 mg/mL).

Table 1. Plant growth attributes of Brassica inoculated with bacterial endophytes AI001 or AI002.

Treatment	Endophyte	Shoot length	Root length	Chlorophyll contents (mg/g FW)	Biomass (mg)
Control	Uninoculated	7.5 ± 0.54a	4.40 ± 0.84a	15.44 ± 0.11a	55.77 ± 3.4a
	AI001	9.6 ± 0.52b	6.77 ± 0.73b	20.45 ± 0.13b	75.43 ± 5.6b
	AI002	9.2 ± 0.71b	7.45 ± 0.91c	23.15 ± 0.15c	84.60 ± 6.2c
5 mg	Uninoculated	5.2 ± 0.47a	3.41 ± 0.88a	11.78 ± 0.11a	46.87 ± 3.4a
	AI001	6.3 ± 0.78b	5.18 ± 1.00b	17.45 ± 0.15b	59.48 ± 5.6b
	AI002	6.6 ± 0.85b	6.45 ± 0.79c	20.15 ± 0.14c	74.47 ± 6.2c
15 mg	Uninoculated	4.5 ± 0.42a	2.50 ± 0.84a	09.44 ± 0.17a	33.54 ± 2.9a
	AI001	6.1 ± 0.37b	4.77 ± 0.76b	15.35 ± 0.13b	49.71 ± 6.1b
	AI002	6.3 ± 0.42b	5.55 ± 0.49c	16.17 ± 0.14c	61.58 ± 5.6c
25 mg	Uninoculated	3.1 ± 0.44a	2.20 ± 0.74a	06.44 ± 0.07a	28.85 ± 2.9a
	AI001	5.1 ± 0.54b	4.77 ± 0.83b	12.45 ± 0.19b	38.37 ± 6.1b
	AI002	5.8 ± 0.55b	4.45 ± 0.55b	15.15 ± 0.14c	54.32 ± 8.7c

Values in each column represent the mean ± standard deviation. Values marked with different letters in each column are significantly different (at P < 0.05), as determined by Duncan’s multiple range test. * FW= Fresh weight

2.5. IAA analysis

Indole-3-acetic acid was analyzed using GC-MS-SIM, produced by bacterial strains AI001 and AI002 culture broth. IAA contains has both a carboxylic acid and a hydrophobic moiety in an indole ring, which facilitates purification of IAA through ion exchange and allows analysis by GC-SIM-MS. IAA in the purified extracts of AI001 and AI002 was detected by peaks at 10.15 min (retention time). The retention times of IAA in the extract coincided with the retention time of the standard (10:15 min) by GC, and the diagnostic ions for IAA were m/z 130 for both the extracts and IAA standard in the MS spectra (Figure 3A, B).

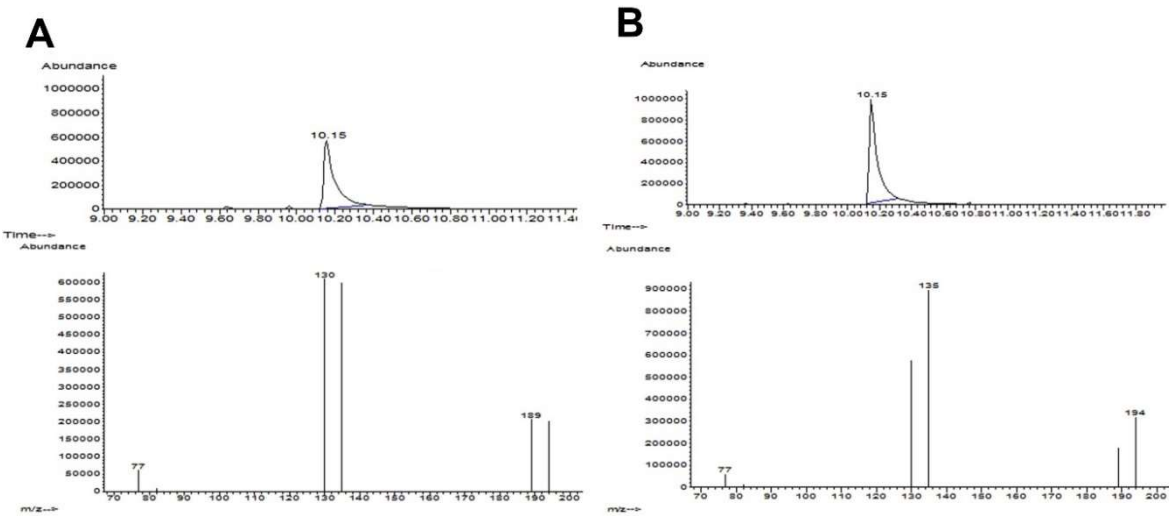


Figure 3. Gas spectrometry-mass chromatography (GC-MS) analysis of indole-3-acetic acid (IAA). The IAA peaks were at appropriate time range set for IAA and spectra of IAA were

dominated by the formation of the indole ion (m/z 130). (A) GC-MS confirmation of IAA in AI001. (B) IAA production in AI002, as determined by GC-MS.

2.6. Effects on lateral roots of the plants

Because IAA has the greatest effects on roots of plants, the roots of plants under Cd stress and after AI001 and AI002 inoculation were a particular focus of this study. Cd stress reduced root length and lateral root growth significantly ($P < 0.05$). Results (Figure 4A) showed that inoculation with AI001 or AI002 significantly increased ($P < 0.05$) lateral roots of the plants in comparison with plants contaminated with Cd and without strains, AI001 and AI002 inoculation. Inoculation of bacterial strains not only significantly ($P < 0.05$) increased root length but also increased the number of lateral roots; hence, root biomass was increased relative to that of the control. Inoculation of AI002 significantly increased ($P < 0.05$) lateral roots of in plants applied with 5 mg/mL or 15 mg/mL of Cd in comparison with plants inoculated with AI001. However, at 25 mg/mL concentration of Cd, the lateral roots of the plants + AI001 and/or AI002 were comparable; however, the numbers of lateral roots in both were higher significantly than non-inoculated control plants.

2.7. Analysis of GSH contents

Cd induces the generation of ROS that disrupt the function of cellular organelles by creating an imbalance in the oxidation/reduction cycle. To rescue cellular function, plant cells, as well as their endophytic microbes, synthesize a wide array of antioxidants to detoxify hazardous heavy metals and regulate cellular responses. Glutathione is a key metal scavenger with great affinity for metals because of its -SH group. In the present study, the GSH contents of plants were measured. The results (Figure 4B) showed that inoculation of strain AI001 or AI002 significantly increased ($P < 0.05$) the GSH contents in plants stressed with various concentrations of Cd, i.e., 5, 15, or 25 mg/mL, compared to the contents in uninoculated plants. The results also revealed that inoculation of strain AI001 sharply increased the GSH contents in plants with a low-to-moderate amount of Cd stress (5–15 mg/mL); however, the increase in GSH slowed at higher concentrations of Cd, although the GSH contents in these plants were still significantly higher than those in uninoculated plants. AI002 consistently increased the GSH contents in plants stressed by all concentrations of Cd (5, 15, or 25 mg/mL). Moreover, plants inoculated with AI002 showed a significant increase in GSH contents as compared to plants inoculated with AI001.

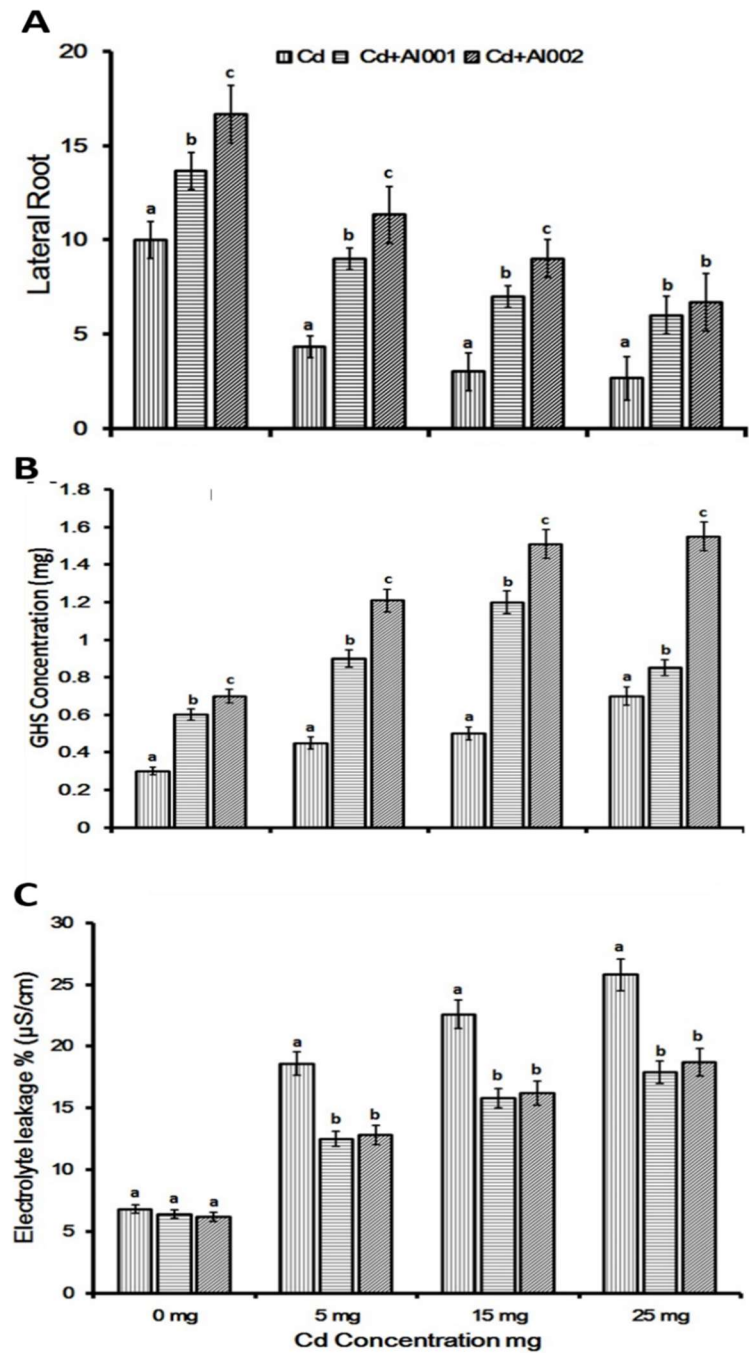


Figure 4. Effects of bacterial inoculation on lateral roots, reactive oxygen species and electrolyte leakage in plants stressed with various concentrations of Cd. (A) Lateral roots were induced by inoculation of AI001 and AI002 (B) Enhancement in reduced glutathione contents in plants with inoculation of AI001 or AI002. (C) Electrolyte leakage decreased with the inoculation of AI001 or AI002. Values are presented as the mean \pm standard deviation; error bars represent standard deviations. Different letters over the error bars indicate significant differences ($P < 0.05$), as estimated by Duncan's multiple range test.

2.7. Electrolyte leakage

The present study revealed that Cd stress damaged the cell membranes of plant tissues, resulting in dose-dependent leakage of ions (electrolytes) from plant tissues. The rate of electrolyte leakage

from plants increased with an increase in Cd concentration. Results (Figure 4C) showed that inoculation with AI001 or AI002 greatly improved the tolerance of plants to Cd stress, and that electrolyte leakage increased with an increase in the concentration of Cd. However, inoculation of the AI001 and AI002 reduced electrolyte leakage in plants exposed to Cd stress.

3. Discussion

A plant body forms a complex ecosystem on its surface as well as in the internal tissues and is inhabited by various bacteria and fungi [18]. These endophytes are important contributors of secondary metabolites, including flavonoids, alkaloids, antibiotics, and anticancer antimicrobial compounds. In addition, these endophytes also promote tolerance to and detoxify heavy metals such as zinc, Cd, lead and arsenic. Outcomes of the Cd stressors to plants has extensively been investigated in agricultural soil as well as in water media (hydroponic) [19]. Soil is consisted of very complex biological, physical and chemical components, which give it a unique structure and texture that determine the bioavailability of heavy metals including Cd, hence provide a safe guard to the roots, and keep the roots from the toxicity of the heavy metals [13]. Unlike soil, agar medium is thought to be a good growth supporter in order to screen the plants without being affected by metal immobilization and get constant controlled growth [20].

The present study was aimed to assess the plant growth-promoting characteristic of endophytes and also their significance in Cd tolerance and detoxification in plants commonly known as phytoremediation. The endophytic bacterial community of *S. nigrum* plants was explored, and several members were isolated and characterized. The bacterial endophytes, isolated from Cd accumulator plant, *S. nigrum* were predictably Cd tolerant because *S. nigrum* is known as a Cd hyperaccumulator [6]. Previous reports have indicated that bacterial endophytes from *S. nigrum* were not only capable of detoxifying Cd but also promoting growth of *S. nigrum* [13]. Based on previous findings, we designed a study to isolate bacterial endophytes from *S. nigrum* and to determine their effects on plants other than *S. nigrum*. Brassica was selected for the experiment because of its tolerance for heavy metals and comparatively short life cycle [21].

Cadmium has been classified as one of the most toxic heavy metal, which has very powerful toxic effects in animal including human being. Toxicity of the Cd also causes chemical and biochemical imbalance and reduce the yields of very important economic crops. Results our study revealed that Cd stress reduces plant growth attributes such as shoot length, root length, and chlorophyll contents, which results in a reduction in plant biomass. In the studies by Sun et al. [18] and Hossain et al. [20], Cd stress has been reported to reduce biomass in a number of plant species due to the degradation of chlorophyll, which results in the inhibition of the photosynthetic electron transport system. Gallego et al. [22] found that Cd stress reduced biomass and growth in a variety of plant species. These adverse effects on plant growth and biomass seem to result, at least in part, because of the degradation of chlorophyll, which inhibits photosynthesis and photosynthetic electron transport [20]. Some microbes, including plant-associated microbes, have the ability to survive and thrive in environments with heavy metals by detoxifying them through accumulating, binding, and/or immobilizing these metals [23]. In addition, Haferburg and Kothe [24], reported that endophytic microbe including bacteria enhance the Cd transport and through ion exchange through surface of the cell membrane in roots of the plants in which heavy metals were immobilized and detoxified. Furthermore, Cd resistant bacterial, *Rahnella* sp. isolated from *Polygonum pubescens*

promoted growth in plants and enhanced the Cd tolerance in *P. pubescens* [21]. A number of studies have reported that plant growth promoting bacteria e.g., *Pseudomonas* sp. RJ10, *Xanthomonas* sp. RJ3, *Bacillus* sp. RJ31, *Azomonas* sp. RJ4, and *Bacillus* sp. RJ16, showed a great heavy metals detoxification. They also showed great tolerance in combination with plant growth, development and enhancement of biomass on roots and shoots *Brassica juncea* and *B. napus* [25-27]. Kartik et al. [28] also reported bacterial strains such as *Stenotrophomonas* sp., *Providencia* spp., *Bacillus* spp., and *Morganella* sp. which showed better plant growth promoting activities and greater Cd resistance and detoxification potential in phytoremediation.

Promotion of plant growth by IAA are consequences of the promotion of cell division, cell elongation, and regulation of gene expression. Combined production of IAA by both plants and endophytic bacteria have multiple roles such as cell elongation, cell division, adventitious and lateral roots generation [29]. In addition, IAA has also an efficient role in biosynthesis of 1-aminocyclopropane-1-carboxylate (ACC) synthesis [6]. Similarly Kahn et al. [13] discovered endophytic strain *Serratia* sp. RSC-14, promoted growth attributes of the plants such as root and shoot length, biomass and chlorophyll contents. The RSC-14 was also characterized for enhancement of phytoremediation of Cd in plants. *Sphingomonas* sp. isolated from *Sedum alfredii*, produced IAA and showed growth promotion in plants and heavy metal detoxification Chen et al. [30]. Similarly results our study indicated that IAA production by both AI001 and AI002 was influenced by addition of tryptophan in culture broth. Our results were had great similarity with results of Ma et al. [27], when they measured IAA production by *Phyllobacterium myrsinacearum* RC6b in culture medium supplemented with tryptophan.

Nutrients, along with phytohormones are equally important in order to promote growth of the plants, exposed to any biotic and/or abiotic stress [13]. These nutrients not only enhance plant growth but also provide tolerance against biological and non-biological hazards [6]. Mobility of essential nutrients including solubilized phosphate is greatly confiscated by high concentration of toxic heavy metal such as Cd in soil. However, endophytic bacteria can withstand the loss of nutrients by synthesizing compounds which solubilize the phosphate present in the soil as insolubilized phosphate [31]. Endophytic isolates, AI001 and AI002 were used in the present study and the results revealed that the application of the strains enhanced the phosphate solubilization, in the medium detected by clear zone in the plates. Khan et al. [13] also characterized the *Serratia* sp. for phosphate solubility and phytoremediation of Cd, and he also reported that the application of the bacterium increased the chlorophyll contents, root and shoot length and total biomass of the plants. Enhancement of these plant attributes is directly related to biomass production, which facilitates the removal of more contaminants from the soil.

GSH is a major antioxidant, produced by fungi, animals, plants and also by bacteria. It has capability to prevent injury of intracellular cellular organelles and released ROS such as free radicals, lipid peroxides and peroxides [6]. Results present study indicated that GSH production augmented with the inoculation of AI001 and AI002 in Cd-stressed plants. The results indicated that generation of GSH by the applied endophytes had an important role in the detoxification of ROS [13]. Wang et al. [32] and Sun et al. [18], studied sample results when they applied bacterial isolates on reported in *B. juncea* and *S. nigrum* respectively, which showed that bacterial inoculation increased the GSH content of plants, reducing Cd toxicity and promoting plant growth.

Moreover, inoculation of AI001 and AI002 significantly reduced electrolyte leakage in the leaves of plants exposed to Cd stress. These results indicated that inoculation reduced the oxidative stress in tissues of these plants, supporting the suggestion that bacterial strains provide protection against oxidative damage to plants under Cd stress [6, 13]. In summary, the results of this study indicate that bacterial strains AI001 and AI002 have different approaches to promoting Cd tolerance such as phytohormone synthesis, phosphate solubilization, and antioxidant production to enhance plant growth and reduce the antioxidant response. Any one or all of these characteristics of the endophytic isolates may be responsible for plant growth promotion.

4. Materials and Methods

4.1. Isolation of bacterial endophytes from *Solanum nigrum*

S. nigrum plant was cut into small pieces from each section e.g., shoot, leaves and stem using sterilized surgical blade. The small pieces were surface sterilized using 0.8% sodium hypochlorite (NaClO) for 30 s followed by 75% ethanol for 1 min. the surface sterilized samples were treated with distilled water to remove all the traces of disinfectants. Eventually the stems, leaves as well as roots were incubated in LB agar plates (tryptone, 1%; difco yeast extract, 0.5%; difco agar, 1.5%; and NaCl, 1%) to get the colonies of endophytic bacteria. The plates were developed at 37°C and the liquid culture was stored in 50% glycerol at -80°C.

4.2. Cadmium tolerance of bacterial endophytes

Cadmium tolerance of the bacterial isolate was determined by following the method described by Khan et al. [16]. The LB media with Cd concentration 0 mg to 30 mg/mL with concentration increase of 5 mg/mL was prepared and bacteria were grown in these media incubated at 37°C for 3 days. The Cd tolerant bacteria were selected for further analysis and the minimal inhibitory concentrations (MIC) were determined at which microbial growth was completely inhibited. The Cd concentrations required for the inhibition of bacteria were determined in various concentrations of Cd from 0 mg/mL to 30 mg/mL the cultures were inoculated with bacterial endophytes, and growth outlines were checked using calculating the optical densities (OD_{600nm}) of cultures every 24 h using a spectrophotometer.

4.3 Phosphate solubilization

Phosphate solubilization of the AI001 and AI002 were determined using methodology of Ullah et al. [14]. The bacteria were grown in media having contents (NH₄)₂SO₄, 5 g/L; Ca₃(PO₄)₂, 10 g/L; 0.5 g/L; KCl, 0.2 g/L; NaCl, 0.2 g/L; MnSO₄·H₂O, 0.2 mg/L; glucose, MgSO₄·7H₂O, 0.1 g/L; FeSO₄·7H₂O, 0.2 mg/L and agar, 15 g/L; yeast extract, 0.5 g/L (Pikovskaya; PVK) agar plates. The plated having microbes were incubated for the interval of 168 h and afterward diameters of halo obtaining from phosphate solubilization were quantified. The mean values and standard deviations of the three repeats were presented in error bars.

4.4. Plants bio-assay using bacterial endophytes

Bioassay was conducted on Brassica plants to estimate the value of bacterial endophytes in the term of Cd detoxification and plant growth promotion. Brassica seeds at the amount of 200 hundreds were treated with 75% ethanol, surface sterilization along with NaClO (0.8%) for 1 min treatment. The 7–10 washing of the seeds were conducted using deionized distilled water to remove NaClO

traces from the surfaces. Seeds were placed on wet autoclaved filter paper on the petri-plates for germination and was kept moist by adding distilled water after interval of 12 h. Germinated seed (equally) were selected and taken to sterile pots filled with 0.8% agar up to 50 mL. Three replicates of 10 pots per replicate each were used in single set of experimental trail. The controlled environment of temperature and humidity 28°C; day night duration 16 h/8 h respectively with 1600 lx of light and 60% relative humidity of growth chamber was provided for the growth of plants. Plants were inoculated with various concentration of Cd from 0 to 30 mg/mL with concentration difference of 10 mg/mL at the time of seeding.

The isolates were grown in media broth of LB and was allowed to grow for 72 h at 30°C on an rpm of 200 in a shaker. The broths were centrifuged using tabletop centrifuge at 10,000 ×g for 10 min, and the supernatant was thrown away and pellets taken for further use. The pellets were washed and dissolved in autoclaved distilled water with adjusted value of CFU was attained 108 cfu. The prepared inocula were used to treat the seedling grown in 0.8% agar water medium with different concentrations Cd. The treatment was made at two leaves stages of the seedling in grown in chamber of controlled environment. The consequences of Cd on plants were estimated in comparison with untreated control (0 mg/mL). The plant growth attributes including biomass, root and shoot length and in addition lateral roots and chlorophyll contents were analyzed after harvesting experiment. The lateral roots were of most attention due to determination of IAA effects on plants, which were produced by microbes. The experiment was repeated three times.

4.5. Gas spectrometry-mass chromatography (GC-MS) analysis of IAA

The analysis of IAA by GC-MS carried out using procedure mentioned by Ullah et al. [14]. Isolates were cultured in LB (50 mL) and incubated at 30°C for 72 h. the culture broth was centrifuged for 10 min at 10,000 ×g and supernatant was retained. The supernatant pH 2.8 ± 2 was adjusted using 1 N HCl and the 3 volume of ethyl acetate was used to extract using solvent solvent extract. The top layer of the extract was obtained and then liquid part was dried by evaporation using evaporator-having vacuum called rotary. Dehydrated extract was mixed with 10% MeOH (2 mL). The methanol contents of suspension was again dried by stream oxygen free N₂ gas. Ethereal diazomethane (1.5 mL) was used to dissolve the dried residues was dried by N₂ gas stream to evaporate the diazomethane contents. In sample was dissolved in ethyl acetate and was injected in GC-MS after filtration. The GC-MS was run in selected ion monitoring (SIM). The experiment was performed three times and mean values were used in results.

4.6. Reduced glutathione (GSH) assessment

Spectrophotometer was used to estimate the reduced glutathione (GSH) in the plant samples treated with different concentrations of Cd along with bacterial consortia at 412 nm. The estimation of GSH, 100 mg of plant samples were ground to homogenization with 5%-trichloroacetic acid (3 mL) in ice-cold mortar and pestle. The mixture was then centrifuged to centrifugation at 10,000 ×g for 15 min. the cell free supernatant was filtered and then was poured into fresh tubes for the GSH analysis. Culture supernatants at the concentration of 1 mL was mixed with Ellman's reagent (0.5 mL) and 150 mM phosphate (NaH₂PO₄) buffer (2.8 mL) having a pH 7.0. The mixture was put at 30°C for 5 min and the absorbance was noticed at 412 nm using spectrophotometer (Shimadzu, UV-1800, Japan). The GSH contents was estimated in comparison with values of a standard curve.

4.7. Electrolyte leakage

Many components are actively involved to sustain life and keep the cell viable in adverse environmental conditions. Cell membrane of plants also provides biochemical and physiological strength to tissues. The electrolytes leakages was determined in percent using leave of the sample plants. Almost 10 fresh leaves were cut into pieces after five rinses were merged into water and was incubated for 5 h at 25°C. The electrolyte leakage of the leaves incubated at 25°C were recorded, known as electric conductivity (EC1) 5 h using a conductivity meter. Afterward the leaves was then boiled for 25 min in water bath to denature the tissues of the leaf and get discharge the electrolytes. The 2nd electrolyte conductivity, (EC2) was estimated after 25 min of denaturation. The leakages of the leaves were recorded by putting the values in formula: $E = (EC1/EC2) \times 100$. The experiment was triplicated to get mean values to present in results.

4.8. Extraction of DNA for PCR analysis

Total genomic DNA of bacterial isolates were extracted using standard genomic DNA extraction protocol [17]. The PCR was used to amplify the DNA through universal primers targeting the 16S rDNA, 27F (5'-AGA GTT TGA TC(AC) TGG CTC AG-3') and 1492R (5'-CGG (CT)TA CCT TGT TAC GAC TT-3'). The PCR conditions were optimized for 30 cycles and each cycle was optimized as 97°C; 1 min, 54°C; 30 s, 72°C; 1 min. the initial denaturation and final extension were 98°C and 72°C for 5 min each respectively. The 16S rDNA sequences was submitted to NCBI for accession number.

4.9. Statistical analysis

For data in each treatment, at least three plants were analyzed from thee independent experiments conducted in completely randomized design. The data (means±SD) of thee independent repeats was statistical assessed using MS office (Excel 2016). Duncan's multiple range test (DMRT) was performed using SAS, (SAS 9.1, USA) to get significant differences between treatments and controls.

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