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Inoculation with a bacterial consortium alleviates the effect of cadmium overdose in soybean plants

Research Article

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Abstract: Inoculating plants that have inefficient antioxidant systems with plant-associated bacteria allows them to overcome heavy metal intoxication. We monitored protein oxidation, the activity of plant defense system enzymes, and the phenolics content in soybean (*Glycine max* L.) during a prolonged exposure to cadmium (Cd). The assistance of the bacterial consortium reduced the bioavailability of Cd in a soil containing 10 times the metal's Standard Maximum Value (SMV). This reduced the accumulation of Cd in the soybeans' roots and seeds. At 100 SMV, bacterial inoculation resulted in increased Cd bioavailability, which enhanced cadmium uptake by the soybean plants. At both Cd concentrations, oxidative stress was more prolonged in the soybean's roots than its leaves. In cadmium-polluted soil, glutathion peroxidase activity changed more rapidly in the roots of plants when they had been inoculated. Inhibition of the peroxidases' activities strengthened the activity of glutathione-S-transferase; increased the phenolics content in plant roots; and alleviated stress in inoculated soybean plants compared to untreated plants. The bacterial consortium may be recommended for a plant protection at 10 SMV Cd in the soil, and for phytostabilization at 100 SMV.

Keywords: *Bacterial consortium • Soybeans • Cadmium • Oxidative stress • Glutathione-S-transferase (GST) • Guaiacol peroxidases (GPX) • Soluble phenolics*

1. Introduction

Cadmium is among the ten most hazardous substances listed by the American Agency for Toxic Substances and Disease [1]. This heavy metal (HM) is readily translocated to plants, resulting in reduced productivity and human intoxication [1]. Plants employ two different methods of cadmium detoxification: *via* phytochelatins, which bind metal cations and compartmentalize them in vacuoles [2]; and by their efflux *via* a cadmium transporter, in the presence of glutathione and ATP [3,4]. Li *et al.* [5] suggested that the secretion of organic acids is a functional metal resistance mechanism that chelates metal ions extracellularly, reducing their uptake and subsequent impact on physiological processes in roots. Zhang *et al.* [6] suggested that Cd tolerance in plants relied on species-specific, as well as more general, defense systems.

Unlike iron and copper, cadmium is not redox-reactive; its toxicity is mediated by blocking sulfhydryl groups in glutathione and proteins, leading to an inhibition of their activity or disruption of their structure. Furthermore, Cd is known to displace Zn and Fe ions from metalloproteins, inactivating them and releasing free Fe that can catalyse the generation of reactive oxygen species (ROS) *via* the Fenton reaction [7,8]. In addition, hydrogen peroxide, superoxide radicals, hydroxyl radicals and nitric oxide radicals could be indirectly generated [9]. ROS are highly reactive, and cause oxidative stress in plants, accompanied by the

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free-radical oxidation (FRO) of lipids, proteins, acid polysaccharides, and nucleic acids in the plant cell [10,11]. As a consequence, tissues injured by oxidative stress generally contain increased concentrations of carbonylated proteins and malondialdehyde, and show increased ethylene production. On the other hand, ROS – mainly H_2O_2 and O^{2-} – act as important signal transduction molecules [12]. Indeed, the ROS network is essential to mediate resistance to multiple stresses in plants [13], integrating different signals that originate from different cellular compartments during abiotic stress [14]. Aside from toxicity mediated by oxidative stress, cadmium also causes a deleterious effect by deactivating DNA repair activity [15].

Regulation of intracellular ROS concentration is controlled by the three-level antioxidant defense system. Firstly, superoxide dismutase (SOD), catalase and peroxidases scavenge ROS. Subsequently, glutathione peroxidase (GPX) and glutathione-S-transferase (GST) neutralize the products of peroxide oxidation of lipids that are associated with development of FRO. In addition, GST deactivates the toxic products of FRO, and the damaged cell components. Additional metabolites, such as ascorbate, tocopherol, *etc.*, are employed to adjust the ROS level in cells [16]. Studies reported either an increase or decline in antioxidant enzyme activity dependent on the plant species, plant organ, and metal concentration [17-21]. It was assumed that under moderate stress conditions, a plant responded by increasing the antioxidant enzymes' activities, but under extreme toxicity, a general failure of the metabolism caused its attenuation. Activation of SOD and inhibition of the GPX, catalase and ascorbate peroxidase (APX) activities (as a result of blocking their SH-groups) results in H_2O_2 accumulation and causes an oxidative burst, which restores catalase, APX and GST activities.

The toxicity of HM to plants might be relieved by the use of microorganisms [12,22-26]. Because HM are more abundant in microbial habitats, microbes have evolved several mechanisms to tolerate their presence. These mechanisms include the formation and sequestration of HM in complexes; reduction of a metal to a less toxic species; induction of the oxidative stress response; resistance to membrane perturbation; and the direct efflux of a metal [4,27]. Specific responses to cadmium in bacteria include the reduction of oxyanions to non-toxic elemental ions; detoxification of Cd with cysteine by CdS-cluster formation; and efflux pumps [28].

Van Loon [29] summarizes various mechanisms by which microbes directly impact plant development, which include the induction of systemic tolerance to abiotic stressors [30]. Our previous study on the French marigold (*Tagetes patula* L.), grown on a rocky substrate with an excess of HM, demonstrated the protective effects of a Consortium of Rationally Assembled Bacterial Species (CRABS) against HM-induced damage [23]. The current study aims to define whether this consortium of bacteria promotes restoration of an oxidant-antioxidant homeostasis – and thus an improved crop yield – in soybean, under varying cadmium concentrations.

2. Experimental Procedures

2.1 Bacteria and media

The bacterial consortium, which was applied to seeds pre-sowing, consisted of *Pseudomonas* sp. IMBG163, *Pseudomonas aureofaciens* IMBG164, *Paenibacillus* sp. IMBG156, *Klebsiella oxytoca* IMBG26, *Pantoea agglomerans* IMBG56, *Bradyrhizobium japonicum* IMBG172, and *Stenotrophomonas maltophilia* IMBG147. Bacteria were grown in the following nutrient media for 18–24 hours at 28°C: *Paenibacillus* sp. in M9 [31]; pseudomonads in KB [32]; other cultures in LB [31]. Threshold concentrations of heavy metals were determined from 10 ml of broth once the bacterial consortium had begun to grow. Overnight cultures were inoculated into liquid media containing filter-sterilized salts ($CdSO_4$, $CuSO_4$, $ZnSO_4$ – all from Sigma-Aldrich, USA). The optical density of populations was monitored with NanoDrop ND-1000 (NanoDrop Technologies, USA) at 620 nm. Measurements were only recorded when bacterial growth was visible.

2.2 Plot experiments

Experiments were conducted in 2007 at the Erastivska Research Station, Institute of Grain Growing, Ukrainian Academy of Agrarian Sciences (48°51" N, 33°17" E) in a grain-fallow crop rotation. The pH of soil (chernozem) was 6.5–7.0; its constituents included: organic matter, 3.8 weight %; N, 143 mg/kg soil; P, 88 mg/kg soil; K, 143 mg/kg soil; Zn, 38.8 mg/kg soil; Mn, 473.0 mg/kg soil; Cu, 12.5 mg/kg soil; Co, 8.0 mg/kg soil; Fe, 835.0 mg/kg of the soil. The temperature exceeded 10°C on 172 days. The total precipitation was 280 mm. The Podil'ska 416 cultivar of soybean (*Glycine max* (L.) were used. Cadmium was added in the form of 0.1 N water solution of $CdSO_4$, in order to obtain 10 and 100 SMV – corresponding to 30 and 300 mg/kg soil, respectively. Soybeans were inoculated with the bacterial consortium prior to seeding. This was prepared by mixing suspensions of each bacterial strain in equal proportions, at a titre of 10^9 CFU/ml, before a further 100× dilution. Each the inoculated and control seeds were sown in five randomly located 1×1 m plots. Plant materials (root, leaf and seed tissues) were collected at

five periods points in the plants' development: under at the point of forming pseudo-leaf formation; the formation of the first, and of the second, true leaves; during flowering; and at bean inception (stages 1-5).

2.3 Biochemical analyses

The protein carbonyl content in plant biomass was determined using the methods described in [33]; the soluble phenolics content as described in [34]; the activity of guaiacol peroxidase (GPX, EC 1.11.1.7) as described in [35]; and the activity of glutathione-S-transferase (EC 2.5.1.18) as described in [36].

2.4 Protein carbonyl content

Plant sample (500 mg FW) was ground with 5 ml of 10% trichloracetic acid (TCA) and centrifuged at 10 000 g for 10 min. Precipitated proteins were incubated with equal volume of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl for 1 h at room temperature and centrifuged at 10 000 g for 10 min. The resulting pellet was washed with a 1:1 mixture of ethanol-ethylacetate (three times) and dissolved in 8 M urea at 100°C. The absorption was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) at 370 nm. Protein carbonyl content was expressed in mmol/mg protein assuming a molar extinction coefficient of 21 000.

2.5 Phenolic compounds

Plant material (1.0 g) was homogenized in 10 ml of 80% methanol and agitated for 15 min at 70°C. 1 ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin-Ciocalteu reagent, and the solution was kept at 25°C. After 3 min, 1 ml of a saturated solution of Na_2CO_3 and 1 ml of distilled water were added, and the reaction mixture was incubated for 1 h at 25°C. The absorption of the resultant blue color was measured using NanoDrop ND-1000 spectrophotometer at 725 nm. The total soluble phenolics content was calculated by comparison with a standard curve obtained from a Folin-Ciocalteu reaction with phenol. Results were expressed as phenol equivalents in µg/g of fresh weight.

2.6 Plant extraction

500 mg fresh weight (FW) of plant sample was ground with 5 ml of ice-cold extraction buffer, which consisted of 50 mM phosphate buffer (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.2% insoluble polyvinylpyrrolidone. The homogenate was centrifuged at 10 000 g for 20 min at 4°C. The supernatant was used to determine enzyme activity [35].

2.7 Guaiacol peroxidase

Peroxidase activity was determined at 30°C by direct spectrophotometry. The reaction mixture consisted of 0.05 ml plant extract, 0.25 ml of 7 mM guaiacol, 0.25 ml of 6 mM H_2O_2, and 1.45 ml of 100 mM phosphate buffer (pH 7.4). The absorption at 470 nm was measured after 1 min. The enzyme activity was expressed in mmol oxidized guaiacol per mg protein per min, assuming a molar extinction coefficient of 5.6. The protein content was determined using the method of Lowry [37] using BSA for calibration.

2.8 Glutathione-S-transferase

Glutathione-S-transferase activity was determined at 30°C by direct spectrophotometry. The reaction mixture consisted of 0.03 ml plant extract, 0.2 ml of 10 mM GSH, 0.02 ml of 0.1 M 1-chloro-2,4-dinitrobenzene and 1.75 ml of 100 mM phosphate buffer (pH 6.5). The absorption at 340 nm was measured after 1 min. The enzyme activity was expressed in nmol 2,4-dinitrophenyl-S-glutathione per mg protein per min, assuming a molar extinction coefficient of 9.6. Protein content was determined by the method of Lowry [37] using BSA for calibration.

2.9 Cadmium elemental analyses

Analyses in soil and plant material were performed using flame atomic adsorption spectrophotometry. Dried soil samples of 2 and 5 g were extracted with 1 N HCl and ammonium acetate buffer (pH 4.8), respectively (in proportion 1:10 m/v) and filtered through filter paper. Dried plant material (1 g) was wet ashed in a mixture of nitric acid (65% m/v) and hydrogen peroxide (50% m/v) until the solution turned clear. After liquid evaporation, the total sample volume was made up to 40 ml with 10% HNO_3. Bacterial samples were prepared in the same manner. Bacterial cells were harvested by centrifugation, a pellet was dried, weighed and wet ashed. The metal amount was then measured using the atomic absorption spectrometer (Selmi, Ukraine) equipped with a deuterium lamp for a background correction.

2.10 Statistical analysis

The significance of differences between means were analyzed using Student's t-test ($P<0.05$).

Strain	Concentration, мМ		
	Zn	Cu	Cd
Paenibacillus sp. IMBG156	0.5	0.5	0.1
Klebsiella oxytoca IMBG26	0.5	1.5	0.1
Pseudomonas sp. IMBG163	0.1	1.0	0.05
Pantoea agglomerans IMBG56	0.1	0.5	0.05
P. aureofaciens IMBG164	1.0	1.5	0.1
Stenotrophomonas maltophilia IMBG147	1.0	3.0	1.0

Table 1. Threshold concentrations of heavy metals when bacteria are viable, мМ.

3. Results and Discussion

3.1 Effect of bacterial inoculation on morpho-physiological parameters under elevated cadmium concentrations

The consortium of bacteria described above were assembled with the aim to grow cover crops in industrial zones without the application of agrochemicals. Bacteria were evaluated by various parameters, such as the biomobilization of nutrition-essential elements (*Paenibacillus* sp. IMBG156, *Stenotrophomonas maltophilia* IMBG147), nitrogen fixation (*Klebsiella oxytoca* IMBG26), induction of systemic tolerance to environmental stressors (*Pseudomonas* sp. IMBG163), and protection from diseases by competitiveness and antagonism (*Pantoea agglomerans* IMBG56, *P. aureofaciens* IMBG164). The consortium of bacteria was tolerant to HM excess in a rocky substrate [38]. In this study, the resistance of individual strains to cadmium (along with zinc and copper) was examined. The most Cd-tolerant bacterium was *S. maltophilia* IMBG147; other bacteria were 2–3-fold less tolerant (Table 1). The species that were less Cd-tolerant (*Paenibacillus* sp. IMBG156 and *P. agglomerans* IMBG56) were the most abundant in the plant rhizosphere and rhizoplane on cadmium-polluted plots (data not shown).

Soybeans are known to accumulate heavy metals, so are a suitable model plant in which to study cadmium uptake and accumulation. In this study, at the end of soybeans' vegetative growth, the content of acid-soluble Cd in non-inoculated plants was 30- and 118-fold higher under 10 and 100 SMV, respectively; the exchangeable Cd content was 29- and 183-fold higher compared to an uncontaminated control (Table 2). A high Cd availability in the soil raised the uptake and accumulation of Cd in soybean organs: in leaves, by 4.0 and 37.0 times at 10 and 100 SMV, respectively; in roots, by 3.8 and 39.0 times; and in beans, by 5.6 and 29.0 times. Inoculation had the opposite effect on cadmium bioavailability and accumulation by plants at different Cd concentrations. At 10 SMV Cd, bacteria lowered the mobile cadmium forms by 1.6–2 times, and reduced the accumulation of cadmium in roots and beans; contrarily, Cd levels rose in leaves (Table 2). At 100 SMV Cd, bacterial inoculation resulted in increased cadmium bioavailability, which enhanced the uptake of cadmium into roots, leaves, and beans. Cadmium concentration in roots was as high as that observed in tissues of hyperaccumulating plants.

The cadmium content in soybean seeds exceeded SMV in soybean plants grown on relatively clean plots to which no Cd was added. A similar observation was reported in [20] when, despite a relatively low content in the soil, Cd was translocated to soybean pods and seeds; the latter had Cd concentrations 3–4 times above the limit set by the Codex Alimentarius Commission. There were significant differences in the cadmium content of soybean seeds grown in each type of soil in Japan; the cadmium level in seeds varied from 0.46 to 12.68 mg/kg across 18 soybean cultivars [39]. In our case, bacterial inoculation of soybeans diminished the effect of cadmium accumulation in seeds at 10 SMV Cd, and it is possible that manipulation with the CRABS structure will allow crop plants grown on polluted lands to assimilate essential ions without taking up toxic ions.

Cadmium added to soil	Variant	Cadmium content in a soil extract, mg/kg dry weight		Cadmium content in a plant organ, mg/kg dry weight		
		*Acid-soluble form	** Ion-exchangeable form	Leaves	Roots	Seeds***
None	Control	0.140	0.060	0.356	1.949	0.351
	Bacterial consortium	0.090	0.030	0.433	0.745	0.203
30 mg/kg	Control	4.300	1.750	1.469	7.351	1.969
	Bacterial consortium	2.000	1.100	2.094	6.566	1.316
300 mg/kg	Control	16.500	11.000	13.229	76.065	10.325
	Bacterial consortium	20.500	13.000	18.398	126.823	12.401

Table 2. Effect of the bacterial consortium on cadmium content in the soil and plant biomass, mg/kg DW.

** extracted with 1 N HCl in ratio 1:10 (w/v)*
*** extracted with ammonium-acetate buffer (pH 4.8) in ratio 1:10 (w/v)*
**** Ukrainian Standard Maximum Value in grain is 0.20 mg/kg DW.*

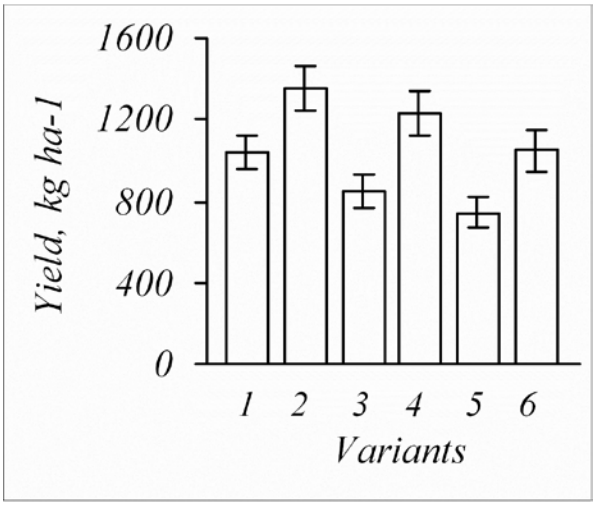

Figure 1. A crop yield of soy at cadmium contamination. Variants: 1, 3, and 5 – control on clean soil, at 10 and 100 SMV of cadmium, respectively; 2, 4, and 6 – bacterial consortium on clean soil, at 10 and 100 SMV of cadmium, respectively.

High cadmium concentration affected plant morphology. Increased soil contamination level resulted in a reduction of both plant biomass and the number of soy root nodules produced. At 100 SMV Cd, shoot height decreased by 20%, whilst the number of nodules reduced by 44%. Crop yield fell by 19–29% compared to the control (Figure 1). Plant development lagged 5–7 days behind control plants, and destructive leaf chlorosis occurred at the third developmental stage. The pre-sowing treatment of soybean seed with CRABS improved germination efficiency by up to 80% in all Cd-contaminated trials, promoted the development of the root system (data not shown) and increased plant biomass and seed output in both contaminated and uncontaminated soil, compared to non-inoculated variants (see Figure 1).

Inoculation exhibited opposite effects on cadmium accumulation in the soybean plant organs at different levels of contamination. At 10 SMV Cd, uptake was diminished (except in the leaves); at 100 SMV. This may have relevance to the effect when at lower concentrations of cadmium in the soil bacteria were able to reduce its bioavailability.

3.2 Effects of bacterial inoculation on protein oxidation under elevated cadmium concentrations

Within the physiologically active plant cells, there is a balance between oxygen activation and oxygen deactivation, so the amount of ROS remains at a safe level. In clean soil (without Cd added), interaction between the bacterial consortium and the soybean plants generated a statistically insignificant increase in protein peroxide oxidation. During plant growth, this equilibrated at 10–18% above the level in the control. At the bean ripening stage, it rose to 43% above control, indicating the development of a weak oxidative stress (Figure 2A). At 10 and 100 SMV Cd, protein oxidation was more pronounced in the soybean roots compared to the control (by 34 and 58%, respectively), and this correlated with the dose of metal added. However, after the appearance of true leaves, the symptoms of the oxidative stress became more strongly expressed under lower concentrations of Cd in the soil; at 100 SMV the difference with the control diminished. Consequently, at a higher cadmium concentration the alarm stage was completed at the first stage of plant development; however, at a lower Cd content the plant responded more slowly, and maximal protein oxidation was only observed at the second developmental stage. Soybeans treated with bacteria adapted more rapidly to the stress caused by cadmium. At the first developmental stage, mechanisms of the H_2O_2 formation were initiated, as indirectly indicated by the 1.6-fold increase of cell protein oxidation (Figure 2A). This parameter varied in a very narrow range during plant growth, and demonstrated the equilibration of oxidant-antioxidant reactions in the soybean roots.

The content of aldehyde and ketone protein derivatives in the young soybean leaves did not vary significantly over time, while in the bacterial treatment content was increased from the beginning of the third stage; at the end of the vegetative stage a 3-fold difference was observed (Figure 2B).

3.3 Effect of bacterial inoculation on the activity of free guaiacol peroxidases and glutathione-S-transferase

The oxidation level of proteins in cells is determined by the efficiency of the antioxidant systems, which perform two roles. Firstly, they neutralize excessive ROS, preventing FRO of cellular macromolecules. One of the most active components of this system are unspecific peroxidases that oxidize phenolic substrates such as guaiacol. Secondly, glutathione-S-transferase detoxifies FRO products, under the assistance of reduced glutathione (GSH), an important component for the redox balance of the cell. Plants have a basic level of antioxidant enzyme activity that rises over time, as a result of progressive protein oxidation as the organism develops and senesces. Exposure to cadmium provoked pronounced responses of plant antioxidant systems.

Antioxidant changes were more pronounced in plant roots, which Cd targeted first. In soybean roots, GPX activity increased sharply by a factor of two after the appearance of the first true leaf pair; during flowering it decreased to its initial level, and it began to rise again as beans ripened (Figure 3A). An analogous increase of unspecific GPX in response to Cd has been observed in pine roots [19], barley [40], and coontail [41]. In plant leaves the GPX activity did not change by a biologically significant amount, but at 100 SMV Cd, at the flowering

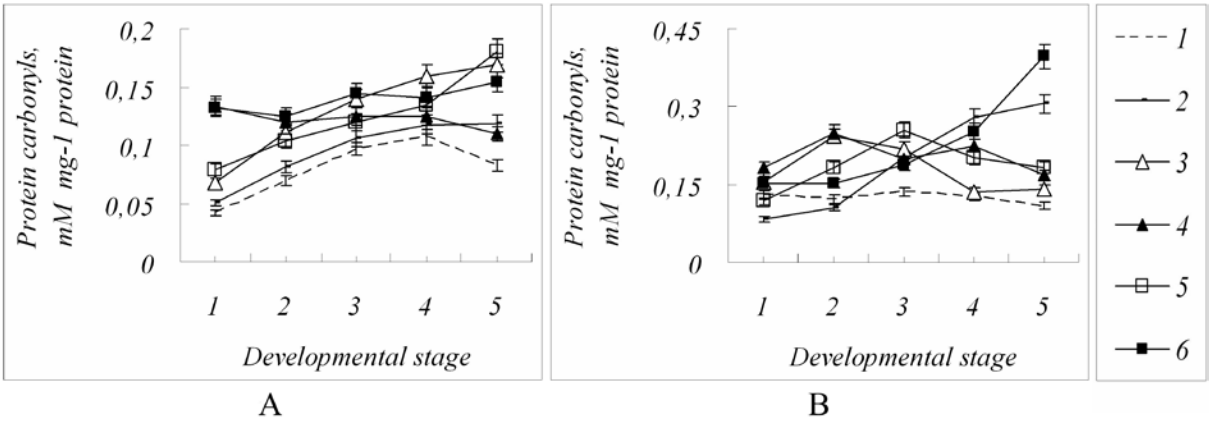

Figure 2. Effect of the bacterial inoculation on protein carbonyl content in the soybean roots (A) and leaves (B) at cadmium contamination: control on the provisionally clean soil (1), at 10 (3), and 100 SMV (5); the bacterial consortium on the provisionally clean soil (2), at 10 (4) and 100 SMV (6). Developmental stage: 1, pseudo-leaves; 2, first true leaves; 3, second true leaves; 4, flowering; 5, bean inception.

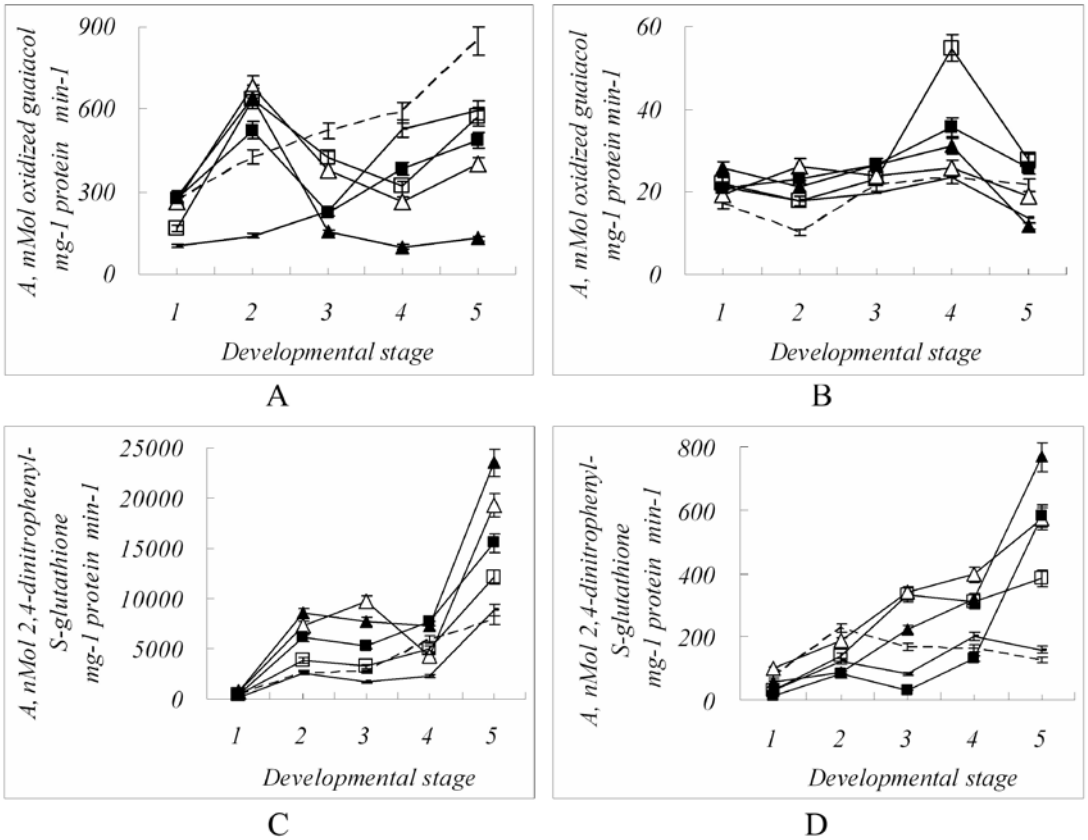

Figure 3. Effect of the bacterial consortium on the activities of free guaiacol peroxidases (A, B) and glutathione-S-transferase (C, D) of the soybean roots (A, C) and leaves (B, D) at cadmium contamination: control in the provisionally clean soil (1), at 10 (3) and 100 SMV of cadmium (5); the bacterial consortium in the provisionally clean soil (2), at 10 (4) and 100 SMV (6).

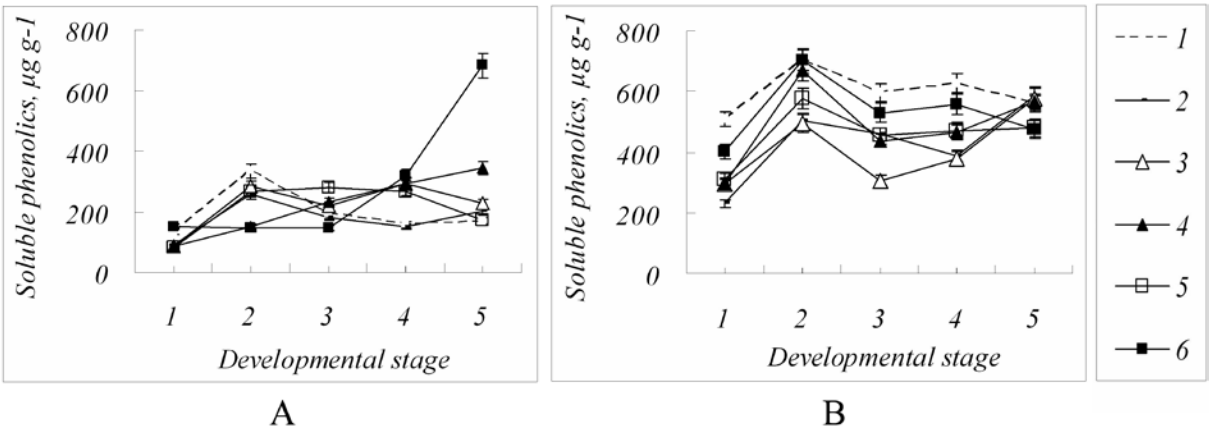

Figure 4. Effect of the bacterial inoculation on a soluble phenolics content in the soybean roots (A) and leaves (B) at cadmium contamination: control in the provisionally clean soil (1), at 10 (3) and 100 SMV of cadmium (5); the bacterial consortium in the provisionally clean soil (2), at 10 (4) and 100 SMV (6).

stage, there was a peak which exceeded the control 1.5-fold (Figure 3B). Inhibition of the GPX activity as a result of soybeans' inoculation probably resulted in an increase of H_2O_2, and an oxidative burst activated the next stage of antioxidant defense where glutathione-S-transferase became involved. The treatment of plants with bacteria accelerated the acquisition of cadmium-resistance. At the third developmental stage, at 10 and 100 SMV respectively, treated plants demonstrated a 2.5- and 1.9-fold inhibition of GPX activity compared to untreated plants.

Commonly, plants respond to Cd exposure by increasing glutathione consumption in order to produce more phytochelatin [42]. Depletion of GSH may favor the accumulation of ROS and disturb developmental processes. GST is the enzyme that catalyses the conjugation of reduced glutathione *via* the sulfhydryl group to electrophilic centers on a wide variety of substrates, including peroxidised lipids. GST, as well as GPX, was activated in the soybean after formation of the first true leaf pair (Figure 3C). GST activity grew linearly in the soybean leaves, and increased exponentially in the soybean roots at the cadmium-contaminated areas throughout of a plant's vegetative stage (Figure 3C-3D). It should be noted that at 10 SMV Cd, the GST activity in roots was higher than at 100 SMV, probably because GPX inhibition was more prolonged.

Our analysis of the data presented in Figure 3 leads us to conclude that during the initial stages of plant growth, under high concentrations of cadmium in the substrate, the level of protein peroxide oxidation depends on the action of the antioxidant enzymes, such as free guaiacol peroxidases and glutathione-S-transferases. An increased GPX activity is compensated for by a corresponding decrease in GST activity and *vice versa*. Because of the inhibition of peroxidase activity, bacteria appear to enhance a signal during the oxidative burst that triggers plant defense mechanisms.

3.4 Effect of bacterial inoculation on the content of soluble phenolic compounds (PC)

The biosynthesis and oxidation of PC is one of the first plant responses to any stress. The enhanced biosynthesis of phenolics in the roots acts as a defense against pathogens [43]. In the soybean roots, PC content rose when true leaves first appeared, coincident with the promotion of plant growth, and with root branching. The inoculation of soybeans with bacteria diminished the content of these compounds, balancing oxidative processes (Figure 4A). We observed a difference in the phenolics content of roots of inoculated and non-inoculated plants grown in contaminated soil. Although the PC content of the roots of non-inoculated plants was below the control level until the second growth stage, the subsequent decrease in their biosynthesis only occurred at the fifth stage, which could indicate that phenolics participate in the detoxification or binding of cadmium ions in the roots. The bacterial treatment of soybean plants grown at high concentrations of cadmium reduced the level of PC accumulation during the first stages of plant growth. Similar results have been observed when Cd-treatment resulted in increased concentrations of soluble phenolics in non-mycorrhizal roots, but not in mycorrhizal roots [12]; mycorrhyzal fungi buffered Cd stress. However, beginning from the third stage at 10 SMV and the fourth stage at 100 SMV, the PC content increased; probably in connection with the completion of the alarm stage and a transition to a cadmium-tolerance stage. The increase in the roots' PC content was more obvious at high cadmium concentrations. The intracellular cadmium level probably reached a

threshold and triggered detoxification mechanisms. The change in PC content in the leaves of both inoculated and non-inoculated plants was very small compared to the roots (Figure 4B). At the same time, the content of phenolics in the leaves and roots was approximately in the same range. Other mechanisms, such as antioxidant enzymes (peroxidases and glutathione-S-transferases) and phytochelatins, probably played a major role in overcoming stress in soybean plants. It well established that peroxidases catalyze the oxidative polymerization of phenylpropanoid compounds, resulting in the lignification of the cell wall, and thus a reduction of its permeability. No clear correlation between the phenolics content and the guaiacol peroxidase activity of soybeans was observed. It is possible that polyphenoloxidases are involved in the phenolics metabolism of soybean plants.

Our general conclusion is that the bacterial consortium alleviated the negative effect of cadmium on plant growth at 10 SMV Cd in the soil. There are reports in literature about preventive role of bacteria *Ochrobacterium intermedium, Brevibacillus, Kluyvera ascorbata* in accumulation of HM by plants [44-46]. In our case, this may have relevance to the different mechanisms. Model bacteria promoted the acquisition of cadmium tolerance in soybean plants, by enhancing the antioxidant systems. The oxidative burst, reflected by parameters such as the increased concentration of carbonylated proteins and the increase of GPX and GST activity, triggered the plant defense system. A similar mechanism of HM tolerance was observed when this CRABS was used for growing French marigolds in anorthosite under an excess of HM and with a low bioavailability of plant-essential macro- and microelements [25]. The inoculation of plants with metal-resistant, plant-growth promoting rhizobacteria containing 1-aminocyclopropane-1-carboxylate (ACC) deaminase reduced the ethylene emission whilst increasing the tolerance index of the seedlings against Cd in contaminated soil [22,34]. On the other hand, at lower cadmium concentrations, bacteria were able to reduce Cd bioavailability. One putative mechanism for this behaviour is phosphorus mobilization, binding Cd to P and resulting in insoluble cadmium phosphate formation. The action of bacteria to prevent Cd accumulation was observed in our previous study on the naturally HM-contaminated soil in Dniproperovsk region, at the level of 1–5 SMV, in the 1 km industrial zone [26]. Moreover, it is known that the rhizosphere microbes can protect plants against the toxic effects of Cd by facilitating the uptake of Fe^{3+} [47,48]. Our previous study exhibited the CRABS' high ability to liberate Fe^{3+} from substrates with a low nutrient availability [25], and we cannot preclude the involvement of this mechanism in plant defense. The manipulation of the microbial community structure will allow a balance to be struck between the increased uptake of plant-essential ions, and the prevention of the uptake of toxic ones. Bacteria could also help plants that naturally possess weak antioxidant systems.

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