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ORIGINAL PAPER

Study on cellular changes and potential endotrophy of wheat roots due to colonization of Chromium reducing bacteria

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Abstract Chromium is a highly toxic metal for all living organisms. Industrial use of chromium has resulted in serious widespread pollution. Biological treatment (bioremediation) has proven to be a cost-effective option for cleanup of metal-contaminated sites. Several bacteria and plant species are able to tolerate high levels of chromium compounds that can be used for cleanup. An experiment was designed to study the colonization behavior of two indigenous Cr(VI)-reducing bacterial strain *Pseudomonas aeruginosa* Rb-1 and *Ochrobactrum intermedium* Rb-2 that were grown in wheat system amended with and without Cr(VI). Hydroponically grown wheat seedlings were co-inoculated with bacterial cultures to study the root colonization potential by fluorescent and electron microscopy. Bacterial inoculation caused significant increase in the growth of seedlings under Cr(VI) stress. Fluorescent microscopy showed good colonization potential of both bacterial strains with roots of inoculated seedlings. Electron micrographs revealed that Rb-1 tended to accumulate in the form of clusters, while Rb-2 preferred to be attach in groups of two or three cells to the root surface of inoculated seedlings. Chromium stress led to the elongation of bacterial rods along with uneven cell surface due to wrapping of cells in mucilaginous material. Cr(VI) stress also resulted in the damaging of plant root surface. Hence, few cells of Rb-2 entered the damaged root cortex cells and appeared as endophytes. Excessive production of fibrillar material by both bacteria under chromium stress could clearly be observed. Both strains displayed auxin production and Cr(VI) reduction ability, showing promise for bioremediation purposes.

Keywords Cr(VI) reducing bacteria · Artificial association · Electron microscopy · *Triticum aestivum*

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Introduction

Soluble hexavalent chromium is an environmental contaminant, widely recognized to act as a carcinogen and mutagen to humans and other living organisms (World Health Association 1993). The environmental fate of chromium is dependent on its oxidation state. Cr(III) compounds are less toxic, less water-soluble under neutral pH and unable to cross cell membranes, while Cr(VI) is readily bioavailable due to its high solubility. This makes reduction of Cr(VI)–Cr(III), a good method for soil and water cleanup (US EPA 1998).

The use of ecosystem services implies the simultaneous use of plants and its associated bacteria for cleanup of heavy metal-contaminated soil in phytoremediation (Glick 2010). These biological methods are economical and sustainable with fewer side effects compared to conventional methods (Chukwuma et al. 2012). Plant supported remediation of heavy metal-polluted environment is a rising field which includes roots of different plant and their associated microbes for the metal removal (Faisal and Hasnain 2003). Some organisms have developed strategies to deal with elevated concentrations of heavy metals, including bacteria and plants (Rajkumar et al. 2005; Congeevaram et al. 2007). Resistance mechanisms displayed by microorganisms and probably by plants include

biosorption, diminished accumulation, precipitation, reduction of Cr(VI)–Cr(III) and chromate efflux (Cervantes et al. 2001; Srinath et al. 2002). Recruitment of these systems is essential for biotechnological purposes and can be used as tools in bioremediation of Cr pollution (Cheung and Gu 2007). The cosmopolitan occurrence of indigenous microbes having the ability to reduce and tolerate Cr(VI) in the biosphere led to the idea of potential use of these microbes for in situ bioremediation of Cr(VI)-contaminated sites (Camargo et al. 2003; Middleton et al. 2003).

Bacteria present on the plant roots can be chemotactically attracted by the exudates secreted from the roots (Yao and Allen 2006). Root exudates are a heterogeneous mixture of compounds, such as amino acids, enzymes, carbohydrates, mucilage, simple sugars and other organic acids, that are released from the roots. These compounds are good nutrient sources for bacteria (Yaryura et al. 2008). Root exudate acts as potent chemotactic stimuli and alleviates the bacterial motility (Molina et al. 2003). Microbial populations are thus more likely to be abundant in actively growing areas of roots such as root tips because of release of more nutrients from these areas. Chemotaxis and biofilm formation are mainly responsible for the efficient root colonization along with the active involvement of many factors like different cellular components for membrane proteins and outer sheaths (Dowine 2010; Timmusk et al. 2011).

Plant colonization by plant growth-promoting rhizobacteria is one of the key factors in plant growth promotion. Rhizobacteria may stimulate plant growth by producing phytohormones, escalating the availability and uptake of nutrients, decreasing heavy metal toxicity and antagonizing plant pathogens (Gholami et al. 2009). Plant root colonization is a relationship, in which both the plant and the bacteria get benefits from each other. Bacteria and plants are equally responsible for the choice of their partner via production of chemical signals. The bacteria that exist close to the plant root under various abiotic stresses such as heavy metals, salinity, pH have evolved different mechanisms to lessen them (Glick 2010). In bacterial plant association, the plant and the microbe are in direct contact with each other synthesizing a wide range of naturally important compounds, which are required for the initiation and maintenance of colonization (Bais et al. 2006; Rai et al. 2000). Auxin, which is mainly indole-3-acetic acid (IAA), is produced by many bacteria growing in association with plant roots (Tsavkelova et al. 2005; Dimkpa et al. 2009). Increment in the length of inoculated seedling is due to enhanced auxin production as auxin-producing microbe is more efficient in colonization of plant roots (Ahmed et al. 2010). Bacteria can bring about the auxin biosynthesis by tryptophan-dependent as well as tryptophan-independent pathway (Shahab et al. 2009).

Various microscopic techniques have been applied to study the bacterial association with plant roots. Fluorescent and electron microscopy are commonly used in microbial ecology for direct observation of microbial distribution on the plant root surface. Electron microscopy is described as one of the best techniques for ultrastructural studies of microbes at higher resolution (Li et al. 2004). Microscopy is a very good tool for studying plant root colonization. Fluorescent microscopy is commonly used in microbial ecology for studying the microbial association on the plant root surface. Cr(VI)-reducing bacteria, the bacterial strain *O. intermedium* Rb-2, was isolated, and found to have good Cr(VI)-reducing ability (Batool et al. 2012). As part of evaluating the suitability for bioremediation of two indigenous Cr(VI)-reducing bacterial strains, the colonization potential and possible endophytic nature of these strains were studied using electron microscopy as a tool to exploit cellular changes during colonization.

Materials and methods

Bacterial strains and growth conditions

Pseudomonas aeruginosa Rb-1 (FJ70126) and *Ochrobactrum intermedium* Rb-2 (FJ870125) are gram-negative Cr(VI)-reducing bacterial strains previously isolated from tannery effluent. They were obtained from bacterial stock cultures of the Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan. Both strains are able to tolerate high levels of K_2CrO_4, i.e., 40 mg l^{-1} on Luria–Bertani (LB) agar and 25 mg l^{-1} on Luria–Bertani (LB) broth. These strains efficiently reduce a significant amount of Cr(VI). They were typically cultured on Luria–Bertani (LB) agar (pH 7.0) supplemented with 1,000 μg ml^{-1} of Cr(VI) at 37 °C.

Seedling length

Two-week-old wheat *Triticum aestivum* var. Inqlab-91 seedlings were harvested, and the length was measured from top of the shoot to the tip of the longest root in centimeters.

Bacterial Cr(VI) reduction and estimation of auxin

To study the involvement of bacterial strains with wheat seedlings growing in Cr(VI), residual Cr(VI) was estimated in the culture medium after incubation with wheat plants. At the end of the experiment, 100 μl of samples from each treatment was taken, and the amount of Cr(VI) reduced was determined spectrophotometrically at 540 nm (Clesceri et al. 1998). Experiments were performed in triplicate.

In order to determine the possible role of the bacterial auxin in an increment of the seedling length, auxin content was estimated. The Salkowski colorimetric technique was used for the estimation of auxin (Glickmann and Dessaux 1995). Cultures were harvested by centrifugation at 5,000*g* for 20 min. Salkowski reagent was added to the supernatant in a ratio of 2:1 (v/v). Concentrations of auxin-like substances were estimated by measuring absorbance at 530 nm after 30 min in the dark at room temperature against a control containing 1 ml culture medium and 2 ml Salkowski reagent.

Bacterial colonization of plant roots

Triticum aestivum var. Inqlab-91 seeds were obtained from Pakistan Agriculture Research Council—PARC. Firstly, seeds of wheat were surface sterilized with 0.1 % $HgCl_2$ and then aseptically transferred to petri dishes containing Whatman filter paper No. 1. The filter paper was moistened with autoclaved distilled water. Plates were kept in the dark for 3 days. The germination process was followed daily. After germination, plates were incubated under light (16:8 day–light, 200 μE m^2 S^{-1}) for 4 days. Roots of seven-day-old seedlings of wheat were suspended in 50-ml tubes containing 25 ml of Hoagland's nutrient solution supplemented with 0 and 350 μg ml^{-1} of hexavalent chromium. The amount of Cr(VI) was adjusted to a level that will stress the plant, but allow it to grow. One microliter of bacterial inoculum was added to suspended seedling roots in Hoagland's nutrient solution. Incubation of bacteria and wheat seedlings was carried out at 25 °C (16:8 day–night, 200 μE m^2 S^{-1}). After 7 days of incubation, the seedlings were harvested and roots were excised.

Plant roots with bacterial colonization were cut into short pieces of 5–10 mm. A small piece of plant root was aseptically transferred to the clean glass slide, flooded with acridine orange (1 %) solution for 5 min and then washed. Oven-dried slides were observed under the Leica DMLS fluorescent microscope (LiecaWetzlar GmbH, Germany) with blue filter.

Electron microscopy for the assessment of endophytic behavior

Plant roots with bacterial colonization were cut into small pieces of 5–10 mm. Samples were prepared for scanning and transmission electron microscopy as described by Lounatmaa (1985) and observed using field-emission scanning (JEOL JSM-6335F) and transmission electron microscope (JEOL JEM-1200EX), operated at 60 kV.

Statistical analysis

Data was statistically analyzed using SPSS personal (version 16, SPSS Inc, Chicago). Analysis of variance (ANOVA) was performed, and mean was separated using Duncan's multiple range test ($P \leq 0.05$).

Result and discussion

The main hypothesis was that the colonization of chromium reducing bacteria will cause cellular changes in the plant. Endotrophy of wheat roots was demonstrated in this study using electron microscopy. Both bacterial strains found to be auxin producers exhibited excellent colonization potential of roots of wheat seedlings in the presence as well as in the absence of Cr(VI) stress.

To show interactions of bacteria with plants in polluted conditions is cumbersome since, the conditions have to be carefully adjusted. The plant has to be able to grow, but clearly be stressed to reveal the effects of the pollutant both on the plant and the bacteria, individually and together. Direct bacterial effects on the plant can be detected as growth of the plant, but that does not tell the nature of interaction.

Seedling length

Addition of 350 μg ml^{-1} hexavalent chromium caused significant stress on wheat seedlings. Seedling length was reduced 37.5 % compared with control, showing well-adjusted experimental conditions. Generally, seedling length was again increased by bacterial inoculation. *O. intermedium* Rb-2 caused a higher increase in length (33.7 %) of wheat seedlings than Rb-1 (26.2 %) under K_2CrO_4 stresses (Table 1). Faisal and Hasnain (2005) also reported that the increment in the growth parameters of sunflower was due to bacterial inoculation in 300 μg ml^{-1} of Cr(VI). Significant increment in seedling length by the inoculation of Rb-2 revealed that there is a relationship

Table 1 Effect of bacterial inoculation on the length (cm) of *Triticum aestivum* var. Inqlab-91 seedlings at 0 and 350 μg ml^{-1} of K_2CrO_4 concentrations

Treatment	Control	Rb-1	Rb-2
Cr(0)	18.41 ± 0.66 (a)	21.73 ± 0.28 (b)	23.39 ± 0.15 (c)
Cr(VI)	11.50 ± 0.25 (a)	23.23 ± 0.45 (b)	24.61 ± 0.37 (c)

Mean of three independent experiments ± standard error of the mean. Different letter(s) in parenthesis indicates significant difference between treatments using Duncan's multiple range test ($P \leq 0.05$)

between the endophytic colonization pattern and growth improvement of wheat. Many bacterial species are found to promote plant growth under heavy metal-stress conditions (Sabri and Hasnain 1997; Mukhopadhyay and Aery 2000). Quantitative difference in production of root exudates by the crop determines the degree of bacterial colonization of the root (Hassen and Labuschagne 2010).

Bacterial Cr(VI) reduction and auxin content

Both strains showed significant Cr(VI) reduction potential in conjunction with wheat plants. *O. intermedium* Rb-2 proved to be more efficient Cr(VI) reducer than Rb-1. It showed almost 96 % reduction of Cr(VI)–Cr(III), thus decreasing the amount of the Cr(VI) in the surrounding medium, whereas *P. aeruginosa* Rb-1 reduced 76 % of harmful Cr(VI) to less-toxic Cr(III) compared to inoculum-free control treatment (Fig. 1a). The stronger reduction capacity was then accompanied by higher biochemical activity observed through measured parameters.

Both bacterial strains proved to be auxin producers, but Rb-2 showed more significant production of auxin (23.3 and 38.9 μg ml^{-1}) in chromium-free as well as chromium-supplemented media. *P. aeruginosa* Rb-1 also showed higher auxin production (28.9 μg ml^{-1}) in chromium-supplemented media than in chromium-free media (Fig. 1b). We showed that both bacterial strains Rb-1 and Rb-2 produced IAA in tryptophan-supplemented media. On the other hand, we found that auxin was present already in the culture medium, in which bacteria were grown along with plant roots without the addition of tryptophan. This may be due to the presence of tryptophan in root exudates. Ahmed et al. (2010) reported root exudates as a source of tryptophan for plant-associated bacteria. Previously, enhanced auxin production under chromium stress has been described by Faisal and Hasnain (2005). Auxin production conferred by both studied strains showed their plant growth-promoting ability.

Bacterial colonization

Both bacterial strains Rb-1 and Rb-2 colonized wheat seedlings which are shown by fluorescent microscopy, but they exhibited a degree of variation in their ability. Both the strains showed an association with the roots in chromium-free as well as chromium-supplemented medium

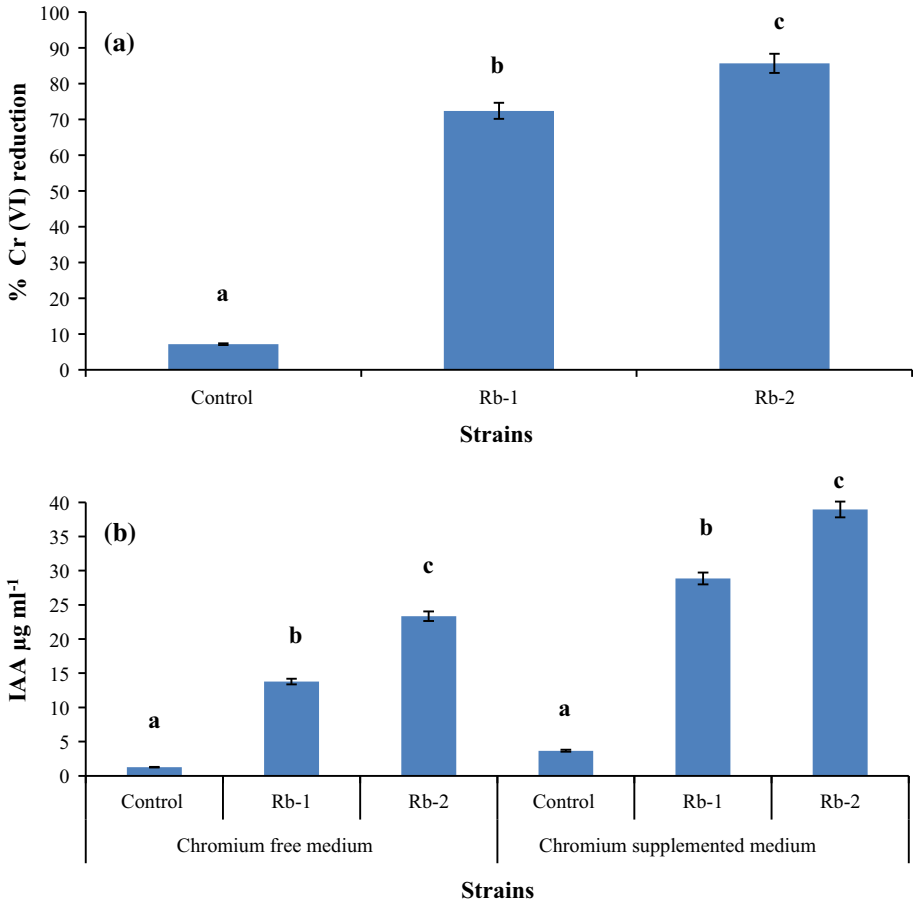

Fig. 1 **a** Estimation of Cr(VI) reduced by bacterial isolates *P. aeruginosa* Rb1 and *O. intermedium* Rb2 after co-cultivation with wheat seedlings at initial concentration of 350 μg ml^{-1} of K_2CrO_4. **b** Estimation of auxin in Hoagland's solution after co-cultivation of bacterial isolates *P. aeruginosa* Rb1 and *O. intermedium* Rb2 with wheat seedlings. Mean of three independent measurements along with standard deviation. *Different letters bold* at the *top* of the *bar* indicate significant differences between treatments using Duncan's multiple range test ($P \leq 0.05$)

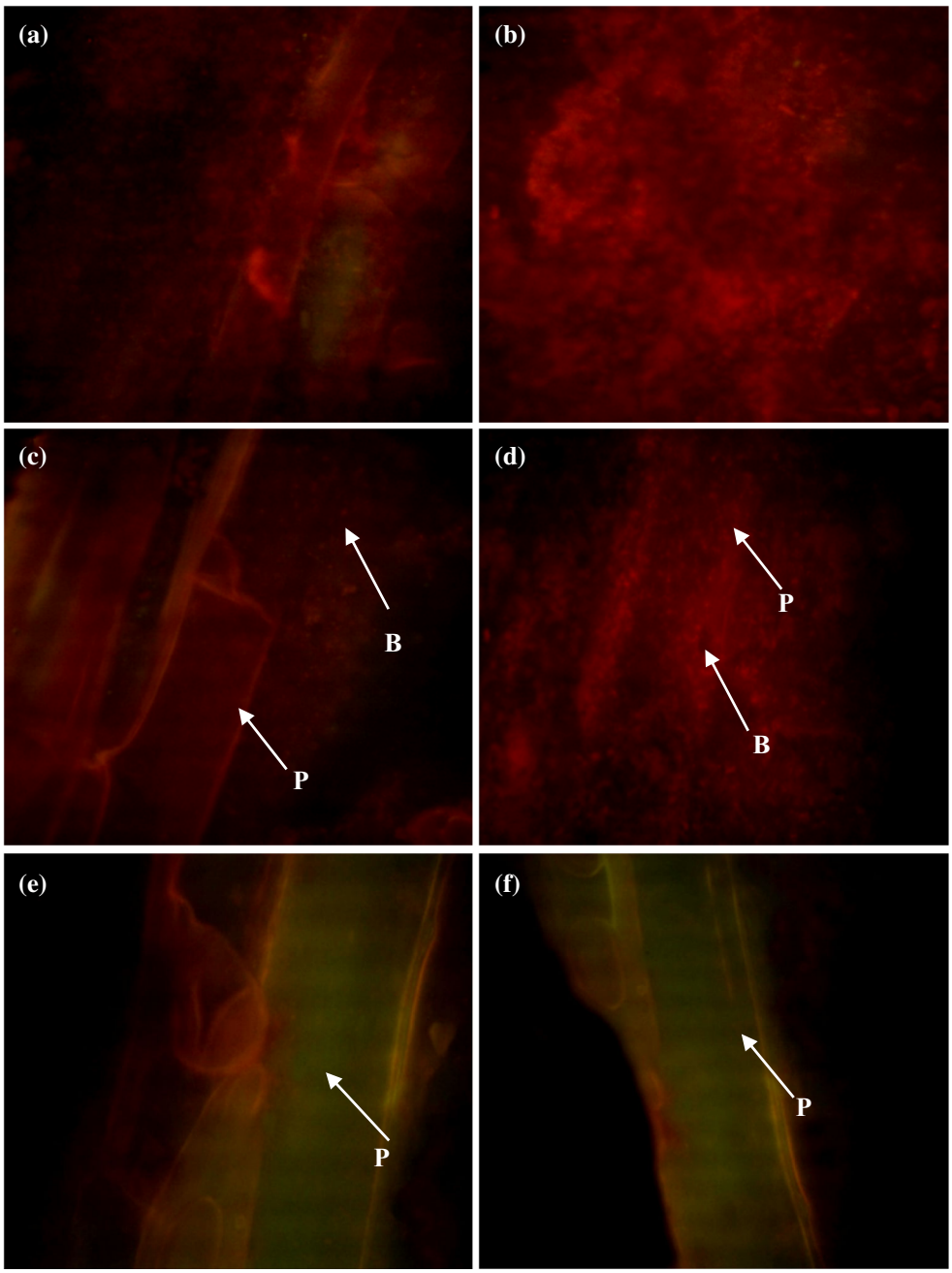

Fig. 2 Fluorescent microscopy of root sample colonized by bacterial isolates. **a** *O. intermedium* Rb-2 spreading on root surface in the absence of chromium. **b** *O. intermedium* Rb-2 growing and spreading on root surface under chromium stress. **c** *P. aeruginosa* Rb-1 colonizing under chromium-free conditions. **d** *P. aeruginosa* Rb-1 growing on root surface under chromium stress. **e** Control root sample without any inoculation in the chromium-free conditions. **f** Control root sample in the presence of chromium stress (*P* plant root cell, *B* bacterial cells)

(Fig. 2a–d). *O. intermedium* Rb-2 showed significantly higher colonization potential with the roots than *P. aeruginosa* Rb-1. The associations with the roots were enhanced under chromium stress showing an even distribution of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 throughout the root surface of wheat seedlings. More dense colonization behavior was exhibited by *O. intermedium* Rb-2 on the root surface compared to *P. aeruginosa* Rb-1 after 7 days of incubation. Strain Rb-2 preferred to grow in the form of bacterial assemblies rather than in the solitary form (Fig. 2a–d). Seedling roots grown with bacteria in chromium-supplemented media exhibited more bacterial associations compared to chromium-free media. No bacteria were detected on the root surface of non-inoculated

control under stress as well as stress-free conditions (Fig. 2e–f). The presence of a large number of bacterial cells in areas with the mucilaginous material showed an interaction between plant and bacteria. Root exudates play a significant role in controlling the bacterial population on the plant root surface, and the bacterial growth rate is directly linked to their ability to utilize the growth substrates present in the surrounding environment (Greer-Phillips et al. 2004). Chemotaxis is accompanied by the attachment of bacterial cells on the plant root surface. Attached bacterial cells in our study produced mucilaginous material probably exo-polysaccharides which improves bacterial attachment to the root surface by acting as a glue (Sala et al. 2008). These processes will eventually lead to the formation of biofilm on the root surface (Morris and Monier 2003; Santaella et al. 2008).

Electron microscopy for the assessment of endophytic behavior

For the assessment of cellular changes during chromium stress and endophytic behavior of selected bacterial strains, electron microscopy was performed. Scanning electron microscopy (SEM) revealed surface colonization both by *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2. The root surface of non-inoculated wheat seedling appeared smooth and intact, whereas chromium stress led to the distortion of epidermal root surface of non-inoculated seedling (Fig. 3a–b). Inoculated seedlings showed uneven root surface both in the presence as well as in the absence of chromium stress. Scanning electron micrographs revealed the accumulation of *P. aeruginosa* Rb-1 in the form of clusters, whereas *O. intermedium* Rb-2 preferred to be attached as two to three cells on the root surface of inoculated seedlings (Fig. 3c–d). In general, the plant root surface appeared smooth, but bacterial colonization resulted in major cell damage and formation of extensive network of microfibril secreted by growing bacterial cells in our study. This can be explained by that as bacterial attachment needs rough surface, bacteria first converted the smooth root surface to the uneven surface by hydrolytic enzymes establishing physical contact. A prior study described the hydrolytic action of bacterial enzymes secreted by rhizobacteria on the root surface of morning glory (Kim and Kremer 2005). Bacterial cells in our study showed an association on the plant root surface with fibrillar material, most likely exo-polysaccharides. Conflicting views prevail regarding the nature and origin of this mucilaginous material, as it may be of bacterial or plant origin (Santaella et al. 2008). SEM and TEM analyses of *O. intermedium* Rb-2 showed clear production of this fibrillar material from the bacterial cell (Fig. 3e–h). This fibrillar material may be involved in the attachment of bacterial cells to root surface. Microfibril production was found to be higher under chromium stress than without chromium. The possible role of microfibrils in anchoring the bacterial cells to plant surface and entrapping more bacteria has been discussed (De Jong et al. 2009; Subramaniam et al. 2009).

Chromium stress led to the elongation of bacterial rods along with uneven cell surface due to wrapping of cells in mucilaginous material. *O. intermedium* Rb-2 was more efficient in the production of mucilage (Fig. 3e–f). The mucilage produced got entangled in the damaged epidermis to the root surface forming peculiar structures (Fig. 3h). Rapid multiplication of these ensnared bacteria may lead to the establishment of more dense bacterial population on the plant root surface. Enhanced production of exo-polysaccharide by bacteria under heavy metal stress is one of the mechanisms to alleviate the toxic effects of heavy metal stress on living organisms. On the one hand, exo-polysaccharides produced by bacteria facilitate their attachment to plant roots, and on the other hand, it immobilizes the heavy metals such as chromium. Both bacteria that colonized the plant roots of wheat reduced the highly mobile and toxic Cr(VI) into less-toxic and less-mobile Cr(III), thus eventually reducing its accessibility to plant roots as proposed by Wani et al. 2009.

Transmission electron microscopy of non-inoculated seedling showed the absence of detectable bacteria on the root surface, while inoculated seedlings exhibited the even distribution of large bacterial population on the root surface. Rb-1 exhibited association only with the surface of plant roots (Fig. 3d). Damage of the root's surface was, however, observed in the roots of wheat seedlings grown under Cr(VI) stress. Consequently, few cells of *O. intermedium* Rb-2 showed penetration into the damaged root cortex cells. The occasional intracellular presence of Rb-2 under chromium-stress conditions is probably due to the toxicity of Cr(VI) to wheat roots and a consequence of possible hydrolytic action of bacterial enzymes (Dimkpa et al. 2009). This intracellular penetration may be one of the mechanisms of bacteria to protect themselves from hazardous effects of hexavalent chromium (Khan et al. 2009). Bacterial inoculations alleviate the adverse effects of chromium salt on seedling length of wheat as compared to non-inoculated treatments (Table 1).

Cells of bacterial strain Rb-2 appeared as long rods, while some of them were smaller ones (Fig. 4e). Bacterization with *O. intermedium* Rb-2 resulted in partial dissolution of plasma membrane and cell wall under chromium-stress as well as chromium-free conditions. The excessive production of fibrillar material by bacteria under chromium stress could clearly also be observed in TEM analysis (Fig. 4d–e). The establishment of microcolonies by *O. intermedium* Rb-2 on the root surface of wheat seedlings can easily be observed in Fig. 3g. Souissi et al. (1997)

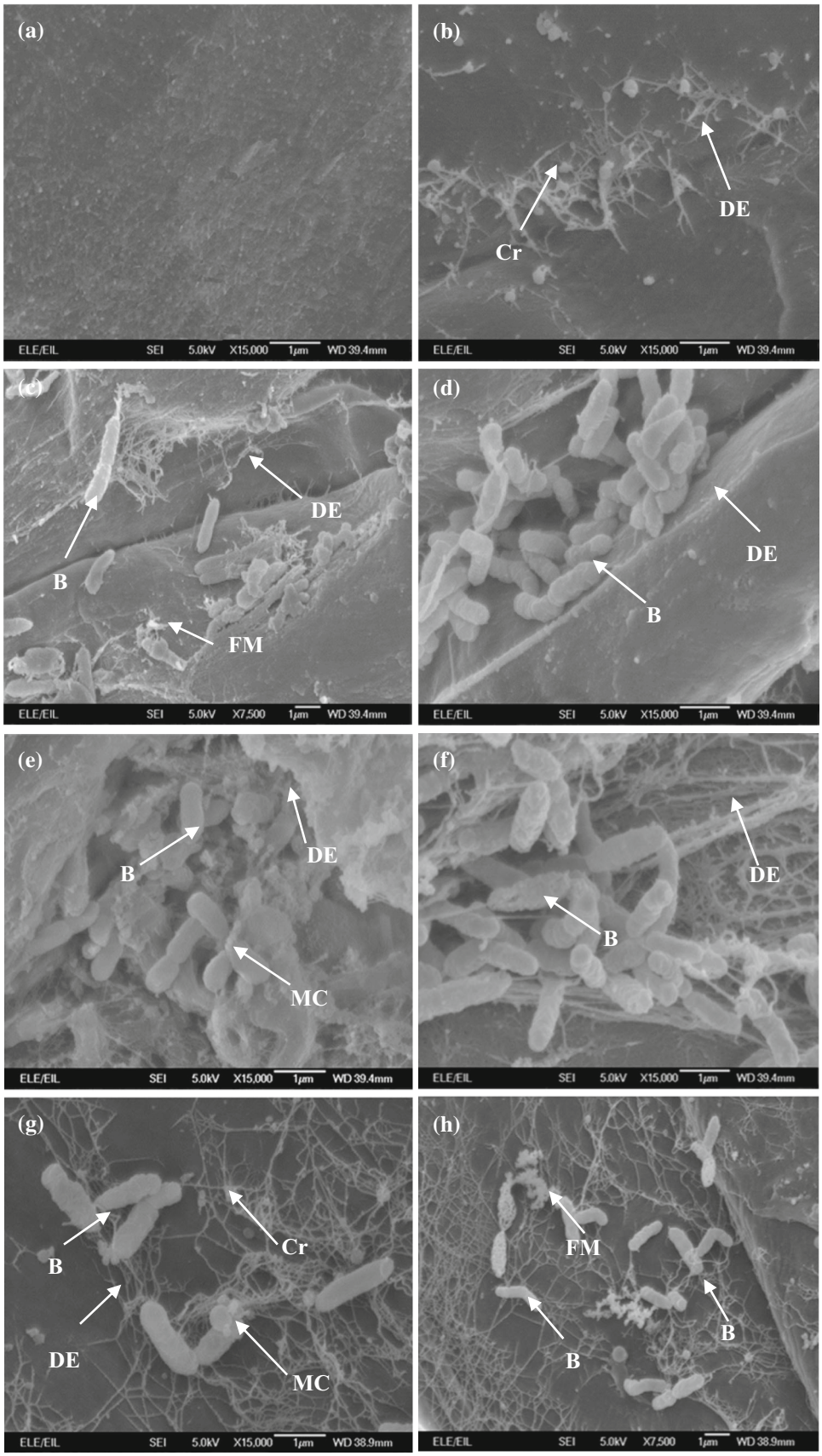

Fig. 3 Scanning electron microscopy of root sample **a** without inoculation in the absence of chromium stress and **b** without inoculation in the presence of chromium stress. **c** Colonized by Rb-2 in chromium-free conditions demonstrating cell arrangement. **d** Colonized by Rb-1 in chromium-free conditions showing group arrangement of cells. **e** Colonized by *O. intermedium* Rb-2 in the presence of chromium stress demonstrating intracellular penetration through damaged epidermis. **f** Colonized by *P. aeruginosa* Rb-1 in the presence of chromium stress showing group arrangement. **g** Colonized by *O. intermedium* Rb-2 showing microcolony formation. **h** Colonized by *O. intermedium* Rb-2 exhibiting microfibril production (*MC* microcolony, *FM* fibrillar material, *DE* damaged root epidermis, *B* bacteria, *Cr* chromium (VI) particles)

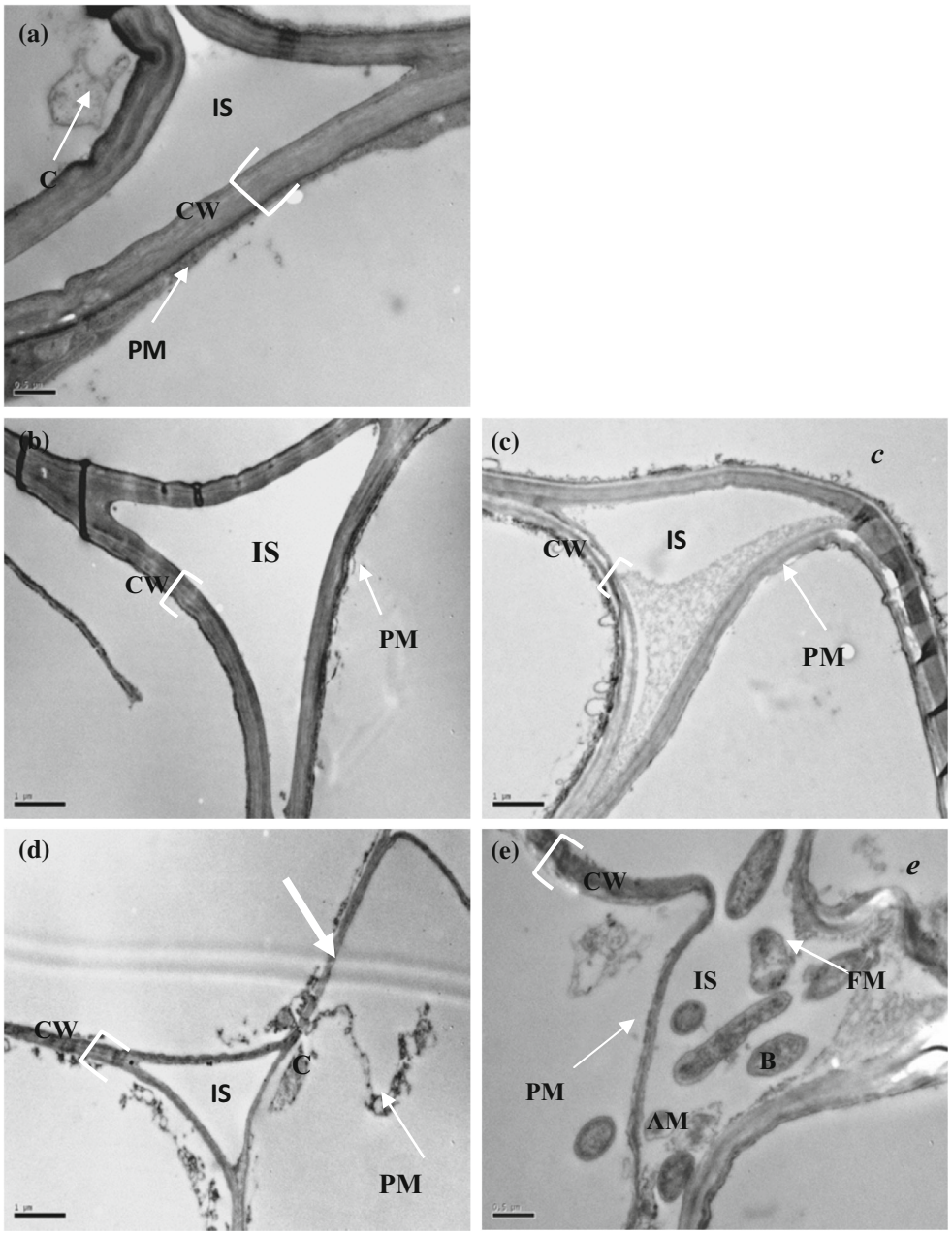

Fig. 4 Transmission electron microscopy of wheat roots. **a** Control root sample without any inoculation showing no bacteria in intracellular space. **b** Inoculated with *P. aeruginosa* Rb-1 in chromium-free conditions exhibiting the absence of bacteria in intracellular space. **c** Inoculated with *P. aeruginosa* Rb-1 in the presence of chromium stress. **d** Colonized by *O. intermedium* Rb-2 in the absence of chromium stress. **e** Colonized by *O. intermedium* Rb-2 in the presence of chromium stress (*AM* amorphous material, *CW* cell wall, *FM* fibrillar material, *IS* intracellular space, *PM* plasma membrane; dissolution of plasma membrane and cell wall is indicated by *arrowheads*)

indicated that these sites are ideal for the bacterial replication following penetration. Kim and Kremer (2005) proposed that effective colonization of plant root surface by the inoculated bacteria led to the formation of microcolonies.

For practical applications, the immobilization of microorganisms on surfaces in treatment systems could increase biomass loading, leading to higher rates of metal transformation. Plants in phytoremediation are used as bacterial carriers of potent bacteria to remediate polluted sites, and the associations of Rb-1 and Rb-2 with plant roots may open up new possibilities for their use in bioremediation of metal pollution. Utilization of Cr(VI)-reducing bacteria with root colonization potential is a cost-

effective option for the remediation of chromium-contaminated environment.

Conclusion

It can be concluded that *O. intermedium* Rb-2 performed more efficiently in colonizing the wheat roots compared to *P. aeruginosa* Rb-1 and exhibited better stimulation of seedling growth along with the Cr(VI) reduction under chromium-free as well as chromium-supplemented conditions. The endophytic nature of this strain was also shown. Both the chromium reducing bacterial strains lowered the toxicity of Cr(VI) by reducing it into the less-toxic Cr(III). These findings highlight the role of these Cr(VI)-reducing bacterial strains along with wheat plants in the reclamation of metal-polluted environment.

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