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Soil bioaugmentation with *Cyberlindnera fabianii* diminish phytotoxic effects of chromium (VI) on *Phaseolus vulgaris* L.

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- ✓ leaves

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Abstract

Bioremediation is the most promising and cost effective technology widely used now to clean up soils containing organic or inorganic contaminants. In this study, the objective was to evaluate the capacity of *Cyberlindnera fabianii* yeast strain to remove Cr(VI) from soil and to enhance growth parameter of bean plants (*Phaseolus vulgaris L.*). We showed that both dead and live biomass of *C. fabianii* introduced in soil microcosm contaminated by 40 mg.Kg^{-1} of Cr(VI) removed more than 50% and 60% Cr(VI), respectively. Toxicity of chromium to seed culture of bean was evaluated. The results showed that yeast inoculations improved different growth parameters under Cr(VI) stress and probability value ($P < 0.0001$) demonstrated significance difference between treatments. Scanning electron microscopy (SEM) analysis revealed that Cr(VI) treatment severely damages guard cells on surface leaves of plant, increases number and induces different types of trichomes. Much improvement in the leaves morphology such as decrease in stomata and trichomes density was observed in inoculated plants which confirmed the effectiveness of *C. fabianii* to diminish phytoavailability of toxic chromium from soils.

1. Introduction

Many soils have been contaminated with several heavy metals mostly from mining wastes and industrial discharges. One of the most common polluting metals is chromium, arising from discharged effluents from leather tanning, electroplating, alloy preparation [1]. Because of its widespread use and its adverse impact on the environment, much attention has been on chromium from national and international organizations [2]. Chromium commonly exists as Cr(III) and Cr(Vl), Cr(III) is considered as an essential micronutrient for the maintenance of normal glucose, cholesterol and fatty acid metabolism. In contrast, Cr(VI) is highly toxic, more mobile subject to biological uptake, and is teratogenic and carcinogenic [3]. Hence, several approaches have been taken, to limit its mobility and permeability. Conventional approaches (e.g. land-filling, recycling, pyrolysis and incineration) to the remediation of contaminated sites are inefficient and costly and can also lead to the formation of toxic intermediates [4]. Thus, biological management of pollution (Biotransformation and biosorption) which utilize the potential of microorganisms to transform or to adsorb heavy metal is preferable to conventional systems for their better efficiency [5], and because in general, microorganisms degrade numerous environmental pollutants without producing toxic intermediates [6]. Several microorganisms are now known that reduced Cr(VI) into Cr(III) which in turn is less toxic [7].

In a previous work we showed that *C. fabianii* isolated from chromium contaminated site had a significant potential adsorption of Cr(VI) in liquid medium. In this study we evaluated the possibility to use *C. fabianii* for obtaining the reduction and immobilization of chromium in contaminated soils, and its effect on the germination, growth and leaf morphology of *Phaseolus vulgaris L.* under Cr(VI) stresses.

2. Experimental

2.1. Micro-organism and growth conditions

Strain of *C. fabianii* HE650139 used in the present work has been isolated from chromium contaminated site located in Oued sebou, Fez (Morocco) [8]. The yeast was able to tolerate high concentrations of Cr(VI) in solid medium YPG and also exhibits multiple metal (Ni, Zn, Hg, Pb, Co, Cu, Hg) tolerance. Stock culture of the strain was maintained on YPG solid medium (2% glucose, 2% peptone, 1% yeast extract and 2% agar) and sub-cultured at monthly intervals. The liquid culture medium was prepared by mixing 2% glucose, 0.2% peptone and 0.2% yeast extract. The growth temperature for HE650139 was 30 °C.

A stock solution of chromium was prepared by dissolving potassium dichromate ($K_2Cr_2O_7$) in distilled water and diluted to get the desired concentration.

2.2. Soil microcosm assay

Neutral soil samples were taken from an uncontaminated site far from any industrial activity. The soil was kept 36 h at room temperature to allow water to equilibrate in the soil [9]. After drying, the soil was sieved to recover the fraction below 2 mm. For the soil microcosm (SM) assay, Petri dishes were filled with 35 g of soil and steam-sterilized (three successive sterilizations, at 121 °C for 1 h each). Solution of Cr(VI) was added to the soil up to a final concentration of 40 $mg.kg^{-1}$, and the soil humidity was adjusted to 100% with the culture of yeast isolate (Sterilized SM were inoculated withC. fabianiipre-grown in YPG to a final concentration of 2 $g.L^{-1}$. SM not inoculated, was used as control. All assays were performed in triplicate.

The dishes were incubated at 30 °C, and a soil sample was taking after 1-8 days. The Cr(VI) soil content was determined by diphenylcarbazide method after alkaline digestion of the soil [10].

2.3. Germination and inoculation of bean seeds in soil microcosms

To evaluate the effect of bioremediation of Cr(VI) on germination and growth of bean (P. vulgaris),bean seeds were surface disinfected with 75% alcohol for 30 s, washed several times with distilled water and germinated in Petri dishes (6 seeds/Petri dish) filled with contaminated and inoculated soil as described previously and left to germinate for 4 d at 30 °C. After germination, the number of germinated seeds was counted. Seedlings of bean were subsequently transplanted into different pots filled with 125 g of soil contaminated artificially with Cr(VI) solution at different concentrations (10, 20, 30 and 60 $mg.kg^{-1}$ soil) and inoculated by live and dead cultures of C. fabianii. SM not inoculated, was used as control. The plants were grown in a climate chamber at 30 °C during daytime and 20 °C during the night with 14 h of light alternating with 10 h of darkness. The pots were watered every day with deionized water as needed. After one month, the seedlings were harvested and root length, shoot length and fresh weight of seedlings were measured. Three replicates per treatment are made.

2.4. Observation of foliar structures

Observations of foliar structures, number and morphology of stomata and trichomes in *P. vulgaris* leaves tissues were examined using Quanta 200 FEI scanning electron microscope.

2.5. Statistical analysis

All the treatments were conducted in triplicates and the data presented in the tables are mean ± SEM (Standard Error of Mean) of three replicates. The data were analysed statistically by analysis of variance (ANOVA) and Fisher's LSD test was performed to determine significant differences and to compare the differences between treatments. Statistical analyses were carried out using R (version 2.15.2).

3. Results and discussion

3.1. Chromium removal by C. fabianiiin soil microcosm

Cr(VI) analysis of soil indicate that microcosm inoculated with *C. fabianii* achieved significant removal of Cr(VI) and very low removal of chromate was shown in sterile un-inoculated control (Table 1). In control soil, chromate was reduced by 5% after 8 day, while in the soil inoculated with dead and live biomass of yeast, percentage removal of chromate was 53.5 % and 61 %, respectively.

Table 1: Percent reduction of chromium by dead and live culture of C. fabianii in a sterile microcosm soil artificially contaminated with 40 mg Cr(VI) Kg-1 of soil.

Treatment	Residual Cr(VI) in soil After 8 days (mg kg^{-1})	Cr(VI) removal in soil After 8 days (%)
Control	37.51 ± 0.78^{a}	06.22 ± 1.95
Dead biomass	17.64 ± 0.53^{b}	55.90 ± 1.32
Live biomass	15.13 ± 0.26^{c}	62.17 ± 0.65

Results indicated that chromate reduction in the soil is closely related to the microbial activity and suggested that the live and dead biomass were able to reduce the toxicity of Cr (VI) in the soil and its availability to plants, via its reduction to the less toxic and less mobile trivalent form and/or via the adsorption/accumulation in yeast cells as previously shown [8]. The present study shows that *C. fabianii* biomass is an effective microbial biomass for chromium removal from soil as compared with other fungal species such as *A. niger* and *Gloeophyllum sepiarium* [11; 12]. It was demonstrated that 75% of chromium was significantly removed in soil contaminated by 250 ppm of chromate at 15 day in presence of A. niger. While in the case of *G. sepiarium* 94% of Cr(VI) was reduced after 6 months with an initial Cr(VI) concentration of 3.4 $mg.g^{-1}$of soil.
Furthermore, previous studies have shown thatC. fabianiipossess the ability of reducing Cr(VI) in aqueous solution and may offer an alternative method for Cr(VI) removal from wastewater [8]. Current study demonstrates that this strain can be a candidate microorganism for the development of an appropriate soil remediation method.

3.2. Seedling germination bioassay test

Seed cultures of *P. vulgaris* were used as bioindicators to confirm the effective decrease of bioavailable Cr(VI) in the SM bioaugmented with dead and live culture of *C. fabianii*. The growth of the plants on SM was evaluated assessing the morphology and % of germination, root length, leaf length and total biomass of seedling (Table 2, Table 3) and Analysis of variance (ANOVA) was carried out.From the statistical analysis, it has been inferred that different growth parameters of bean seedlings varies significantly ($P < 0.0001$) among the different treatments. Data shown in Table 2 depicted that the percentage of seed germination decreased monotonically with increasing Cr(VI) concentration. As compared to control (distilled water), about 44.50 %, 61.11 %, 72.33 %, 89.00 % of reduction was revealed at seed germination under 10, 20, 30 and 60 $mg.kg^{-1}$ of Cr(VI), respectively.

Table 2*:* *Effect* of inoculation of live and dead culture of chromium resistant yeast on germination of bean seeds at various concentration of chromate solution.

Yeast biomass	Cr(VI) treatment (mg kg^{-1})	Seed germination (%)		Seed germination inhibition (%)	
		Cr(VI) (control)	Cr(VI) + yeast	Cr(VI) (control)	Cr(VI) + yeast
Dead biomass	10	55.55 ± 5.5	100.00	44.50	00.00
	30	27.66 ± 5.5	55.5 ± 5.5	72.33	44.50
Live biomass	20	38.88 ± 5.5	61.11 ± 5.5	61.11	38.88
	60	11.00 ± 5.5	50.00 ± 5.5	89.00	50.00
Control (distilled water)		100.00		00.00	

Highly toxic effect of Cr(VI) on seedling growth and a significant deterioration in fresh weight, root and leaf length was also found as compared to control ($P < 0.0001$) (Table 3). Similar observation was reported by Kitashiba et al. [13] and Hu et al. [14] in Arabidopsis thaliana. Phenotypic alterations most frequently observed are: growth reduction (root and leaf length), smaller leaves and dark green leaves which that may be due to an

increase in chlorophyll or anthocyanin [15, 16]. Kastori et al. [17] also observed that heavy metal toxicity hampered cell division decrease turgor pressure of plant cells. The reduction of growth parameters may be due to reduction in presence of Cr(VI) of metabolic activity by decreasing nutrient uptake and photosynthetic abilities as suggested by Vazquez et al. [18] and Zeid [19]. Interestingly, the addition of yeast biomass improved germination when compared to non-inoculated control (Table 2). Almost 44.50 %, 22.23 %, 27.83 % and 39.00 % of stimulation in seed germination was observed under 10, 20, 30 and 60 $mg.kg^{-1}$ of Cr(VI), respectively.

Table 3*: Effect* of inoculation with live and dead culture of C. fabianii on shoot length, root length and fresh weight of bean seedlings at various concentrations of chromate solution. (a, b, c, d, e: significant groups Fisher's LSD test).

Yeast biomass	Cr(VI) treatment ($mg\ kg^{-1}$)	Shoot length (cm)		Root length (cm)		Fresh weight (g)	
		Cr(VI) (control)	Cr(VI) + yeast	Cr(VI) (control)	Cr(VI) + yeast	Cr(VI) (control)	Cr(VI) + yeast
Dead biomass	10	6.03 ± 0.14d	13.51 ± 0.2b	10.78 ± 0.15b	11.41 ± 0.21ab	6.34 ± 0.42d	15.81 ± 0.12b
	30	2.38 ± 0.24e	10.35 ± 0.24c	1.99 ± 0.10c	11.47 ± 0.25ab	2.17 ± 0.25e	10.90 ± 0.37c
Live biomass	20	4.60 ± 0.2c	11.10 ± 0.56b	2.57 ± 0.28c	11.34 ± 0.18a	2.57 ± 0.29c	11.67 ± 0.68b
	60	1.77 ± 0.88d	11.11 ± 0.39b	1.33 ± 0.67c	09.68 ± 0.35b	1.27 ± 0.63c	11.14 ± 0.74b
Control (distilled water)		14.56 ± 0.3a		12.01 ± 0.03a		18.58 ± 0.19a	

Root and shoot length of bean seedlings were also significantly increased when grown in soil supplemented with yeast ($P < 0.0001$) (Table 3). From this result it's appearing that addition of *C. fabianii* biomass induced better growth and was advantageous for combating toxicological effects and stimulating growth of bean seedlings by reducing the availability of Cr(VI) to plants. Our results are in agreement with the finding of several authors. Faisal and Hasnain [2] demonstrated that majority of bacterial inoculation increased different growth parameters and caused a decrease in chromate uptake into seedlings as compared to their respective non-inoculated control. Srivastava et al. [11] also reported that inoculation of soil microcosm by *A. niger* increased the seed germination and seedling length under chromate stress.

3.3. Stomata and trichomes as biomonitor of environmental contamination

Leaf stomatal density and leaf trichome density reflects Cr(VI) toxicity on plant (Azmat et al. 2009). The leaf surface of bean was studied using scanning electron microscopy. Paracytic stomata and the types of trichomes observed on the leaf are described (Fig. 1).

Results show that under control conditions where no yeast inoculation was applied, Cr(VI) affected the structure of leaves and their morphology showed variables effects (Fig. 1b, Fig 1c). As compared to leaves of natural plant (untreated control) (Fig. 1a), an increase in the density of stomata and trichomes with simultaneous reduction in the size of the guard cells on the surface of leaves were observed (Fig. 1b). Results also showed that different types of trichomes were being produced by plant on the leaf surface under Cr(VI) stress: (i) small, capitate glandular trichomes; (ii) non-glandular, multicellular, simple hairs; (Fig. 1c), whereas the epidermal cells of control plant did not show any trichome cells in the epidermis (Fig. 1a). Inoculation of *C. fabianii* under Cr(Vl) stress resulted in an improvement in the morphology of guard cells with diminution in the number of stomata and trichomes. As shown in Figure 1d, disappear of glandular trichomes was revealed and only non-glandular trichomes was appeared at a very low density. These results confirme our suggestion about the role of this yeast to decrease bioavailability of Cr(VI) and minimize the toxic effects of this metal.

Foliar morphological variability in bean plant is adaptive. It may be due to self defense system developing in plants under stress condition which provided the support to the plant for their survival in contaminated environment. We can suggest that increase in number of stomata and trichomes may be compassionate to the plant to release the excesses of Cr in the environment to protect the plants from the toxicity of Cr(VI). Azmat et al. [20] indicated that the metal toxicity commonly reduced the absorption of CO_2 due to the reduction of leaf surface area whereas an increase in number of stomata may be helpful to absorb the CO_2 in stress condition for the production of glucose molecule. Han

et al. [21] and Vazquez et al. [18] also reported that the exposure of plants to Cr(VI) causes a change of structure on the surface of leaves and a growing number of trichomes which can be storage areas for metals. Similar findings were also observed by Faisal and Hasnain [2] where Cr(VI) severely damaged the guard cells of sunflower seedlings, they also demonstrated that the inoculation of bacterial strains under Cr(VI) stress resulted on the development in the morphology of stomata.

Based on these results we can conclude that biomass of *C. fabianii* reduce the bioavailability of toxic Cr(VI) to the bean plant and promoted its growth under chromium stress.

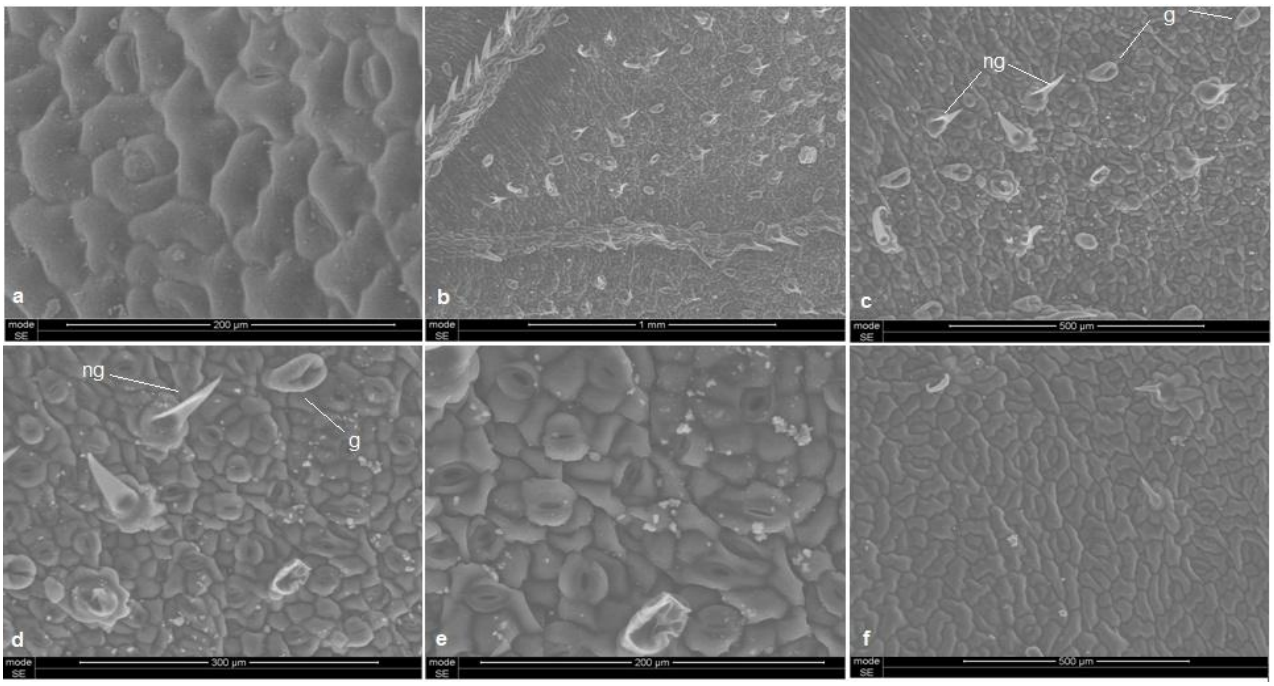

Figure 1: SEM micrographs showing leaf surface of *Phaseolus vulgaris* plants growing at natural condition, under Cr(VI) stress and with yeast inoculation under Cr(VI) stress.(a) leaf surface of natural plant (control) and stomatal cells morphology. Scale Bar = 200 µm; (b) effect of chromate salt on the leaf of plants (density and distribution of trichomes and stomata on leaf surface). Scale Bar = 1 mm; (c,d) details of the two trichomes types on leaves of *P. vulgaris*. (ng) non- glandular trichome, and (g) glandular trichome. Scale Bar = 500, 300 µm; (e) density of stomata and detail of guard cells morphology (damage of guard cells). Scale Bar = 200 µm; (f) Effect of Yeast strains on stomatal morphology and trichomes (few number of trichomes and improvement of guard cells morphology). Scale Bar = 500 µm.

Conclusion

The study has reported that *C. fabianii* yeast strain showed high capacity to remove Cr(VI) from soils. Serious reduction in different growth parameters of bean plant (seed germination, root and shoot length, and fresh weight) were observed under Cr(VI) stress, but seedlings inoculated with dead and live culture of yeast showed much improvement. Analysis of variance (ANOVA) on different growth parameters exhibited significant difference among the treatments. SEM analysis reveals that Cr(VI) application also severely damages plant cells/tissues especially guard cells while biomass of yeast improves leaves structures.

This study demonstrate that *C. fabianii* can be efficient in removing Cr(VI) from the soil and decreasing the bioaviability of this metal to plants, providing thus an interesting option for the remediation of chromate.

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