

Role of *Trichoderma* and *Sinorhizobium* Strains for Improving Growth and Nutritional Status of Alfalfa under Cd Stress

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Abstract— The plant rhizosphere is a major soil ecological environment for plant- microbe interactions involving colonization of different microorganisms in and around the roots of the growing plant. Plants can be used in the remediation of soils contaminated with heavy metals. The objective of this study was determine the relationship between the effect of Cd on the symbiotic model of *Sinorhizobium meliloti* – *Medicago sativa* and the application of *Trichoderma* sp. on the nutritional status as well as biochemical characterization of the sandy brown forest soil. The effects of biofertilizer *Sinorhizobium* and coinoculants *Trichoderma* strains on growth, chlorophyll and N, P and K content of alfalfa growing in soil polluted by cadmium were investigated. The results indicate that the presence of the saprobe fungi *Trichoderma harzianum* further enhanced shoot dry weight, N, P and K content of *Sinorhizobium meliloti*-alfalfa symbiotic model. The co-inoculation of alfalfa with *T. harzianum* was more effective for Cd uptake. The effects of the bio-multiple inoculants on the growth of alfalfa were stimulated the colonization of *Sinorhizobium* strains in the rhizosphere, promoted the nodulation potential and increased the dry organic matter. *Sinorhizobium meliloti* interacts with alfalfa as a model for rhizobioremediation and *Trichoderma* strains interact with this model as nodule promoters as well as a partner in the process of cleaning the plant rhizosphere from cadmium metal.

Keywords— Alfalfa (*Medicago sativa*), heavy metals, *Sinorhizobium*, *Trichoderma* fungi.

I. INTRODUCTION

Soil are reservoirs for heavy metals generated by industrial activities e.g. metal finishing, paint pigment and battery manufacturing, leather tanning, mining activities, municipal waste water sludge, urban composts, pesticides, phosphate fertilizer, or from atmospheric depositions (Ariano, 1986, Kabata-Pendias, 1992). Metalliferous or industrial soil, which is heavily enriched with toxic metals, can support the growth of

specific plant species called metallophytes, which have long attracted the interest of botanist and are now considered as potential tools for phytoremediation (phytostabilization or phytoextraction). Some of these hypertolerant plants also have the ability to accumulate high concentration of metals in their tissues, (Boularbah *et al.*, 2006). Approximately 450 plant species have been classified as hyperaccumulators of heavy metals, (Baker *et al.*, 1994; Morel *et al.*, 1997).

The health hazards associated with soil contamination with trace elements having toxic effects together with high cost of removal and replacement of polluted soil have prompted to develop alternative and cheaper technologies to recover the degraded land. Current research in this area now includes plants to remediate polluted soils and to facilitate improvement of soil structure, the innovative technique being known as phytoremediation (Brooks, 1998). The possibilities of using such plant species which are easily growing in different climates, and using their biomass in non-food industries, can make them ideal plants for phytoremediation purposes (Linger *et al.*, 2002; Khan, 2003). Phytoremediation must be considered as a long-term strategy (Cunningham *et al.*, 1995).

Since 1904, when the term rhizosphere was first coined by Hiltner (1904), rhizosphere processes of plants have been widely investigated, however, little attention has been paid to the microbial community of rhizospheres of plants growing on metal contaminated sites. Soil microorganisms, including plant root associated free-living as well as symbiotic rhizobacteria and mycorrhizal fungi in particular, are integral part of the rhizosphere biota. The overall result of plant-rhizosphere microbe interactions is a higher microbial density and their metabolic activity in the rhizosphere, even in metal contaminated soils (Vander, 1998). Plants also influence the structure of microbial communities through the release of root exudates (Grayston and Campbell, 1996, Grayston *et al.*, 1996; Grayston *et al.*, 1998; Kozdroj and Van Elsas., 2000) and by providing surfaces for

colonization. However, it is not understood how specific plants increase the remediation of contaminated soil (Kirk *et al.*, 2005).

Alfalfa is the most important forage crop in the world. The seed germination and vigor condition of alfalfa seedling are characteristics that determine plant establishment and initial crop development. Many leguminous plants and particularly alfalfa have extensive soil-root system extending as deep as 2m. Rhizosphere or soil-root systems limit the diffusion of solutes in the soil. Hence the plant can serve both as a vehicle for limiting the spread of solutes and by promoting the mineralization of the toxic compound. The general feasibility of rhizosphere approaches to remediation has been previously demonstrated (Anderson and Coat 1994). Frequently, alfalfa development is hampered by environmental conditions, such as metal deficiencies and metal toxicity, as well as by diseases. Previous investigations have shown that common root-colonizing bacteria are plant growth-promoting microorganisms that can improve plant development under a variety of environmental conditions (Reynders and Vlassik 1982, Kapulnik *et al.* 1985, Sarig *et al.* 1988), however, the known mechanisms by which they promote plant growth are quite variable. Some of them promote plant growth mediated by their production of plant regulators (Mordukhava *et al.* 1991). Previous studies demonstrated that alfalfa plants have the ability to geminate and grow in montmorillonite clay individually contaminated with 80 mg/kg of Cd, Cu and Ni and 160 mg/kg of Zn (Peralta-Videa *et al.* 2002). Based on that information, the following research was designed to determine if a correlation exists between the heavy metal tolerance and the growth stage of alfalfa plants. The rhizosphere also affects the availability of heavy metals. Early studies have indicated that there is higher concentration of exchangeable and carbonate bound Cr, Ni, Zn, Cu, Pb and Cd in the rhizosphere than in bulk soil (Wang *et al.*, 2002). Root –induced changes in the rhizosphere are important factors controlling nutrient dynamics in this zone and thus the mineral nutrition of plants. On the other hand, nutrient dynamics also influence the rhizosphere environment (Stratton *et al.*, 2001). Rhizobia (e.g. *Azorhizobium*, *Rhizobium* and *Sinorhizobium* species) are soil bacteria able to develop N₂-fixing symbiosis with legume plants. *S. meliloti* is a Gram-negative bacterium that can live as a saprophyte in soil or as N-fixing symbiont inside root nodule cells of alfalfa. Fewer studies are the activities of nodulating bacteria in the rhizosphere of the host plants that are important for host infection. These processes include communication between bacteria (Gray *et al.*, 1996), competition for access to infection sites on root (Tripplett and Sadowsky, 1992), gene

transfer (Sullivan and Ronson, 1998) and the growth in the rhizosphere (Robleto *et al.*, 1998).

II. MATERIALS AND METHODS

2.1 Microorganisms: in addition to the new isolates of *Sinorhizobium meliloti* strains (isolated from different root-nodules on root of alfalfa, which grown in 50% sewage sludge amended brown forest soil), two standard strains of *S. meliloti* (heavy metal-tolerant strain GHR94 and salt-tolerant strain GH130) and *Trichoderma* strains (*T. harzianum* (111-2b), *T. viride* (131-2) and *T. koningii* (Cu-3)) isolated from heavy metal contaminated soil Nagyhörcsök, Gyöngyös and Eger, Hungary respectively were obtained from the Gene bank of Culture Collection of the former Research Group of Environmental Microbiology of Hungarian Academy of Sciences.

2.2 Cultural media and test plant: all medial constituents were purchased from Difco Laboratories, Sigma, or Merck Co. Experiments employed the following sterilized media were used for maintained the tested microbial strains as slant cultures as well as carrying out the investigations: Medium for *Sinorhizobium* : (1) Yeast extract mannitol agar: *Sinorhizobium meliloti* isolates were isolated and maintained on yeast extract mannitol agar (YMA) described by Kleczkowska *et al.*, (1968). (2) Yeast extracts mannitol broth: YMA medium without agar (YMB).

Medium for *Trichoderma*: (1) *Trichoderma*- selective medium: *Trichoderma* strains were maintained on *Trichoderma*- selective medium described by Askew and Laing, (1993). (2) Sucrose-yeast extract broth (SYB): for preparation of bioinoculant and to study the sensitivity of the *Trichoderma* strains to Cd salts. Different concentrations of Cd salts were added to SYB medium described by Altomare *et al.*, (1999). Alfalfa (*Medicago sativa* L.) seeds of Hungarian origin.

2.3 Soil sample collection and preparation: the experiments were carried out in pots with soil contaminated by cadmium salts (Cd 11.02 mg kg⁻¹). The soil sample used for the present study was collected from a non-cultivated field of the experimental station at Szent István University, Hungary. The soil of sampling area was sandy brown forest of general properties. The soil sample were collected in plastic bags and transferred to the laboratory. The sample was sieved through a 2 mm mesh to remove plant debris and soil fauna and was stored at cool room for plant-microbe interaction.

2.4 Cadmium salt: cadmium chloride (CdCl₂ · 6H₂O) and cadmium sulphate (3CdSO₄ · 8H₂O) obtained from (Reanal Chemical Company Ltd), with following concentrations used: *In vitro*: 0, 10, 20, 40, 80 and 160 μM. *In vivo*: 0, 10, 20, 40, 80 and 160 mg kg⁻¹ soil.

2.5 Isolation, maintenance and growth of *Sinorhizobium meliloti*:

Routine techniques for further studies of *Rhizobium* are described by Vincent, (1970) were used. Comparatively, the isolates were selected according to the intense pink coloration of the colonies. Sub-culturing occurred only once during the experiment and single colonies were selected and each isolate was restreaked to purify (Somesegaran and Hoben., 1994). The bacterial isolates were stored on YMA slant tubes for further investigation. In screw-capped tubes containing YMA with CaCO_3 (3g l^{-1}), the strains were streaked and maintained at 4°C .

2.6 Screening isolates for nodulation:

A test of ability of the 55 isolates to form nodules on alfalfa root is the ultimate criterion of authenticity as *Sinorhizobium*. According to Jansen and Strijdom., (1982) that competitive ability as determined in autoclaved soils correlated with the ability to infect plants rapidly. The selection of the strains were depended on the intensity of nodule formation on the root (occupation rate in comparison with control strains), and the presence of leghemoglobin formation inside the root- nodule (the pink color) in which the concentration of leghemoglobin within the nodule bears a direct relationship to the amount of molecular N_2 -fixed (Jordan and Garrard., 1951).

2.7 Preparation of inoculate: under complete aseptically conditions, the inoculate of tested *Sinorhizobium meliloti* strains were prepared by growing each microorganism of tested bacterial cultures in 250ml Erlenmeyer flask containing 50 ml of YMB medium. The flasks were incubated at 28°C for 2days on a rotary shaker operating at 150 rpm. The bacterial cells from 10 ml cultures were centrifuged ($12000 \times$ for 4 min) and washed twice in phosphate buffered saline and re-suspended in 10ml of phosphate buffered. This is the final bacterial suspension which was used as inoculum throughout the following investigations.

Preparation of sinorhizobial inocula for *in vitro*: All *Sinorhizobium* inocula used in *in vitro* experiments had a cell capacity of 1×10^6 cell ml^{-1} of saline solution (0.85g NaCl salt dissolved in sterile distilled water and completed to 100ml), this was calibrated by haemocytometer. It was prepared by washing three slant cultures of a 24h old culture, each with 5 ml of sterile 0.85% NaCl solution. The uniform distribution of the cells in the solution was obtained by shaking machine (Bayoumi, 1987). For *in vitro* experiments, *Trichoderma* strains a colony disc of active mycelium of fresh growth colony with 5 mm in diameter was placed carefully in the 250ml Erlenmeyer flask containing 50ml of SYB medium. The flasks were incubated at 28°C for 5 days on a rotary shaker operating at 50 rpm. But, *in vivo* for soil-plant inoculation, conidial suspension was prepared by

washing the growth colony grown on agar surface with saline solution and the conidial number per ml (1×10^4) was calibrated by haemocytometer.

2.8 Ecophysiological selection of *S. meliloti* strains: originally, the total number of isolates was 55, and by the help of nodulation test, the number of root nodule forming strains was reduced to 18 during one month.

2.9 Determination the growth rate of microbial strain under Cd stress

For *Sinorhizobium* strains: two cadmium salts were subjected to establish their effects on the growth rate of 8 *S. meliloti* strains in YMB medium using microfermentor technique (Bayoumi *et al.*, 1995a and 1995b) at six concentration (0, 10, 20, 40, 80 and 160mg l^{-1}) of each salt. Using spectrophotometer (DR-2000 model) under different conditions at wavelength 550nm and compared with the rate of growth in control culture.

For *Trichoderma* strains, a colony disc of active mycelium of fresh growth colony with 5mm in diameter was placed carefully in the center of in 250 ml Erlenmeyer flask containing 50 ml of SYB medium contaminated with two cadmium salts at different concentrations (0, 10, 20, 40, 80 and 160mg l^{-1}). The flasks were incubated at 28°C for 5 days on a rotary shaker operating at 50 rpm.

2.10 Phytoremediation of Cd polluted soil:

The soil remediation ability of the plant-bacteria symbiosis was tested using a sterilized soil with or without Cd contamination, which was supplied as CdCl_2 or CdSO_4 solution to obtain a Cd concentration of 0, 60, and 120mg kg^{-1} dry weight soil. The pots contained a mixture of each 4 kg of soil and approximately 1×10^6 cells of sinorhizobial strain per seedling. Six seedlings of alfalfa, which were three days old after germination (*M. sativa* seeds were surface-sterilized, sown 0.7% agar plate and incubated for three days in the dark at 25°C), were transferred to one pot containing one of Cd concentration mentioned above in addition to one of *Trichoderma* strains and grown under light (16h photoperiod per a day) at $25\text{-}28^\circ\text{C}$ for 8 weeks. Shoots, roots and nodules were harvested from 8 weeks-old plants. Cd concentration was measured directly from the soluble fraction using AAS.

2.11 Monitoring the biological N_2 -fixation under Cd stress:

The experiment was designed to detect the interactions between the two cadmium salts amendment and the biological N_2 -fixation in brown forest soil. The study was conducted in pot experiment predicated to different concentrations of cadmium doses. The cadmium salts were applied to the soil before the plantation and plant inoculation. Generally, the following experiments of predicated the impacts of cadmium on the plant-microbe

interactions in addition to coinoculate the system with *Trichoderma* strains (H1-2b *T. harzianum*, 131-2 *T. viride*) were carried out using one Hungarian cultivar of alfalfa, three new isolated strains of *S. meliloti* (GHF-162, GHF-281 and GHF-3153), one standard strain (GHR-94) and two cadmium salts at three concentrations (0, 60 and 120 mg kg⁻¹ soil) and performed in the greenhouse.

Seeds were selected for healthy and uniformity without any injury, and surface sterilized with 70 % ethanol followed by acidified 0.2 % HgCl₂ for 5 minutes, and thoroughly washed in several changes of sterile distilled water. Seeds were then soaked for 8h at room temperature in sterile distilled water (soaking water was changed every 2 h) and then seeds were germinated on sterile moistened filter paper in large petri dish for 72h in the dark at 28°C according to Franco and Vincent, (1976). Sterile water was added to the germinated seeds when required. Under a septic conditions, eight seedlings were transplanted into each pot and covered with a layer of approximately 2cm of sterile soil. Pots were cellophane covered. Seven days after transplanting, pots were thinned to four seedlings pot⁻¹. Seedling rhizosphere was inoculated with 10 ml of a suspension of biofertilizer *Sinorhizobium* inoculum (GHR-94, GHF-162, GHF-281, or GHF- 3153) prepared as follows: each one of the five biofertilizer *S. meliloti* strains was grown in YMB for 48h at 28°C, to give a final cell concentration 1x10⁶ cell capacity ml⁻¹, using hemocytometer for calibration. Seedlings were watered with sterile tap water when required, and the plants grown under natural illumination 14h photoperiod at around 28±2°C according to Ta and Faris, (1988). Consequently, the inoculated plant seedlings were coinoculated with one of the following *Trichoderma harzianum* (H1-2b) or *Trichoderma viride* (131-2).

The experiment was conducted in three replicate for each of treatment. Seedlings were watered with sterile distilled water when required. The plants were grown under natural illumination (16h photoperiod) at around 26-18±2°C. The experiment was conducted for 8 weeks, and then plants were carefully uprooted, and washed several times in tap water for farther investigations.

2.12 Plant growth parameters:

Data recorded/ plant as follows: Height of plant shoot in cm, number of root nodules. Dry weight of plants biomass and nodules were determined after oven dried at 75°C to a constant weight and the values were expressed

as g plant⁻¹ and mg root nodules plant⁻¹. Total N-content (mg plant⁻¹) was measured using micro-Kjeldahl method as a criterion of N₂-fixation (Burris, 1974). Cd concentrations were measured after digestion of the air-dried plant samples with HNO₃ + H₂O₂, followed by inductively coupled plasma atomic emission spectrometry (ICP- AES), as described by Mikanova *et al.*, (2001).

2.13 Measurement of chlorophyll and total N, P and K contents:

The chlorophyll a and b of alfalfa leaves was extracted with 80% (v:v) acetone at 8 weeks after transplanting and estimated by a procedure as described by Lichtenthaler, (1987). The sample size for this measurement was a 5 mm diameter leaf. The total N, P and K contents in shoots of alfalfa plants were analyzed as described by Mingorance, (2002) using a microwave system after digestion of samples with H₂SO₄+ H₂O₂. Total N and P were determined by colorimetry using automatic air-segment continuous flow analysis and K was analyzed by flame photometry.

2.14 Statistical analyses

Values are mean of three to eight replicates. All data were subjected to one-way analysis of variance. Data were processed by analysis of variance (ANOVA) and Fisher's protected least significant differences (LSD) when appropriate (Sokal and Rohlf 1981).

III. RESULT

3.1 SELECTION OF *S. MELILOTI* STRAINS

Using different microbiological assays and the growth on various growth media and pure cultures, the number of the isolates was reduced. Therefore, a final 18 out of 55 bacterial isolates were collected from different root-nodules of various plant samples and then this number of isolates was reduced by the help of plant inoculation to become 8 according to the faster and early development of root nodules on the roots of alfalfa plants.

3.1.1 Performance of nodulation formation and plant dry weight

Table 1 shows the differences between the strains on the competitive ability in the nodule formation (nodule number per plant). When the strains have the ability to form nodule, it means that they are able to survive and multiply in the environment under normal conditions (without stressed factors). These results gave an account on the mode of the strains to colonize the rhizosphere and the roots of the host plant.

Table 1. Plant dry weight and number of nodules per plant as criteria for the ability to form effective N₂-fixing nodules with the host plant (authentication).

| Inoculation with isolates | Plant dry matter (mg) | Number of nodules per plant |
|---------------------------|-----------------------|-----------------------------|
| Uninoculated plant | 153 | 0 |
| Standard: GHR-94 | 231 | 10 |
| Standard: GH-130 | 254 | 10 |
| GHF-131 | 241 | 10 |
| GHF-162 | 412 | 14 |
| GHF-1120 | 225 | 9 |
| GHF-1141 | 228 | 9 |
| GHF-214 | 244 | 10 |
| GHF-230 | 312 | 13 |
| GHF-243 | 267 | 10 |
| GHF-270 | 281 | 11 |
| GHF-281 | 457 | 14 |
| GHF-290 | 288 | 11 |
| GHF-2100 | 315 | 13 |
| GHF-2130 | 309 | 12 |
| GHF-321 | 297 | 11 |
| GHF-353 | 326 | 13 |
| GHF-372 | 317 | 13 |
| GHF-3111 | 303 | 12 |
| GHF-3150 | 331 | 13 |
| GHF-3153 | 461 | 14 |

3.2 In vitro tolerance of inoculant strains to Cd salts

3.2.1 For sinorhizobial biofertilizer inoculants:

The effect of cadmium in chloride and sulfate forms on the relative growth rates of the selected *Sinorhizobium* strains are presented in Fig 1 & 2. The percentage relative growth of sinorhizobial strains was obtained in comparing with the control. Results clearly demonstrate that the addition of cadmium affected growth conditions differentially with the concentrations of the salt. It was found that by increasing the Cd concentration in the

growth medium the growth rate of the strains decreased. But, GHR-94, GHF-162, GHF-281 and GHF-3153 strains of *Sinorhizobium* were the most tolerant against the concentrations of CdCl₂ (Fig 1). Similar results were obtained in case of treated the growth medium with CdSO₄ (Fig 2). This figure shows that the relative growth rates of the strains were more inhibited by concentrations of the salt investigated. Comparatively, it was found that the effect of sulfate form of the cadmium salt was more toxic to the *Sinorhizobium* strains.

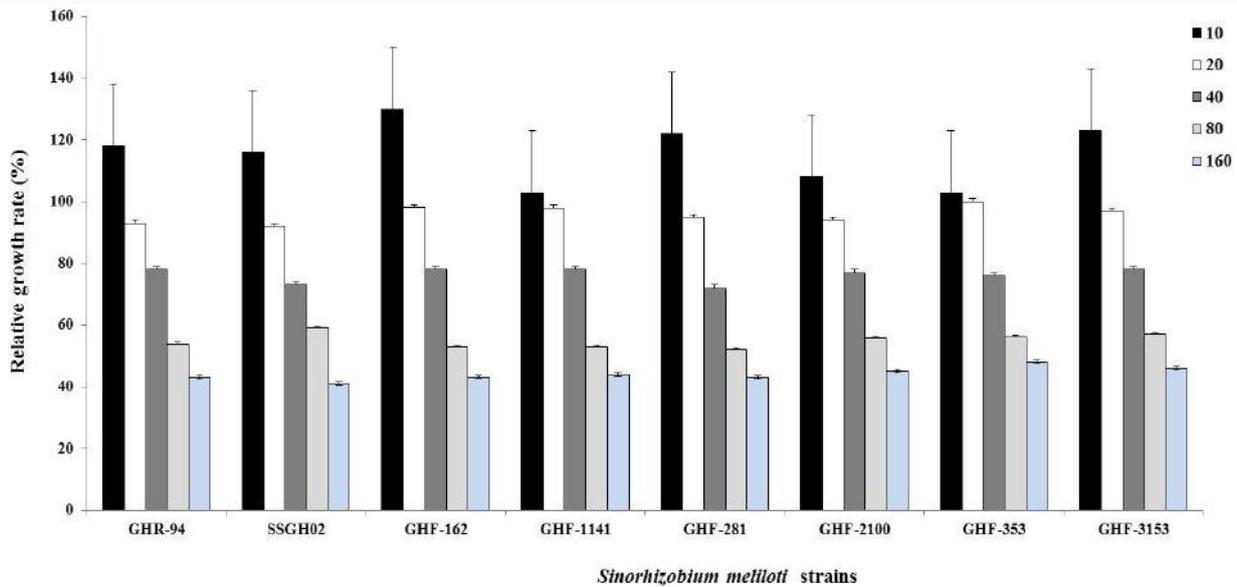


Fig. 1: Effect of cadmium chloride concentrations on the relative growth rate *Sinorhizobium meliloti* strains.

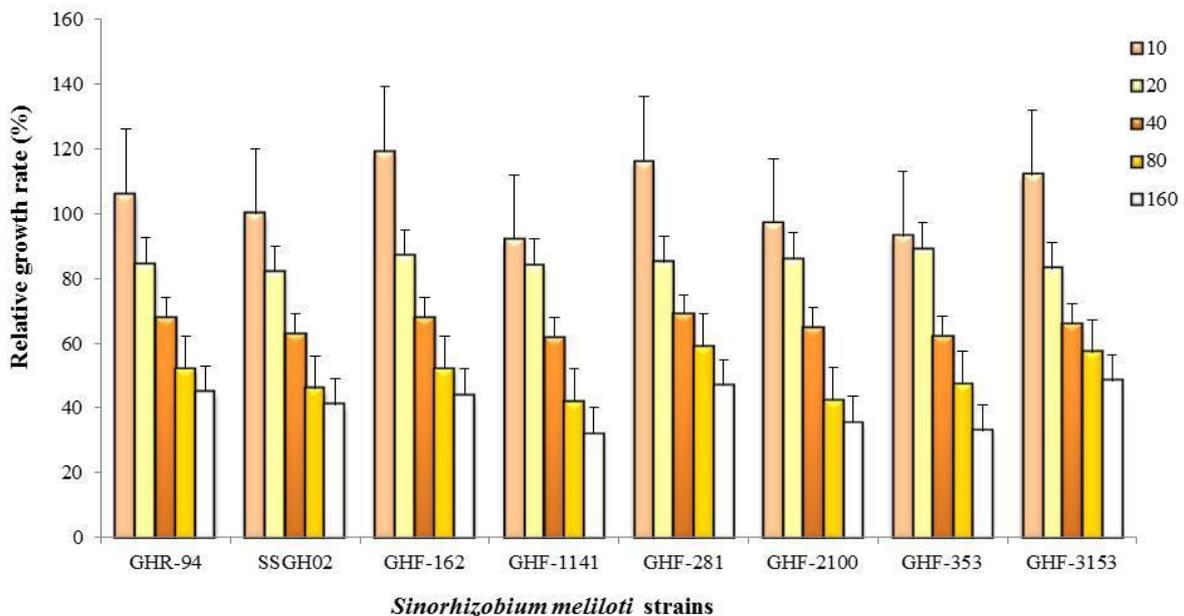


Fig. 2: Effect of cadmium sulfate concentrations on the relative growth rate *Sinorhizobium meliloti* strains

3.2.2 For Trichoderma coinoculants:

In SY broth medium, effect of different concentrations (0, 10, 20, 40, 80 and 160 mg^l⁻¹) of Cd salts were tested and the results showed that the *Trichoderma* strains were tolerated the low concentrations in term of dry weight of fungal colony. But by increasing the concentrations the dry weight of fungal colony was decreased. Fig 3 shows that no significant differences among the strains toward CdCl₂ concentrations, and the *T. harizanum* (H1-2b) was

more tolerant strain and Cu-3 of *T. koningii* was more sensitive to the CdCl₂. Fig 4 demonstrates that *T. harizanum* was the most tolerant strain to CdSO₄ than *T. viride* and *T. koningii*. Comparatively, the results of this investigation showed that CdSO₄ had more toxic effects on the colony dry weight of the three *Trichoderma* strains than CdCl₂.

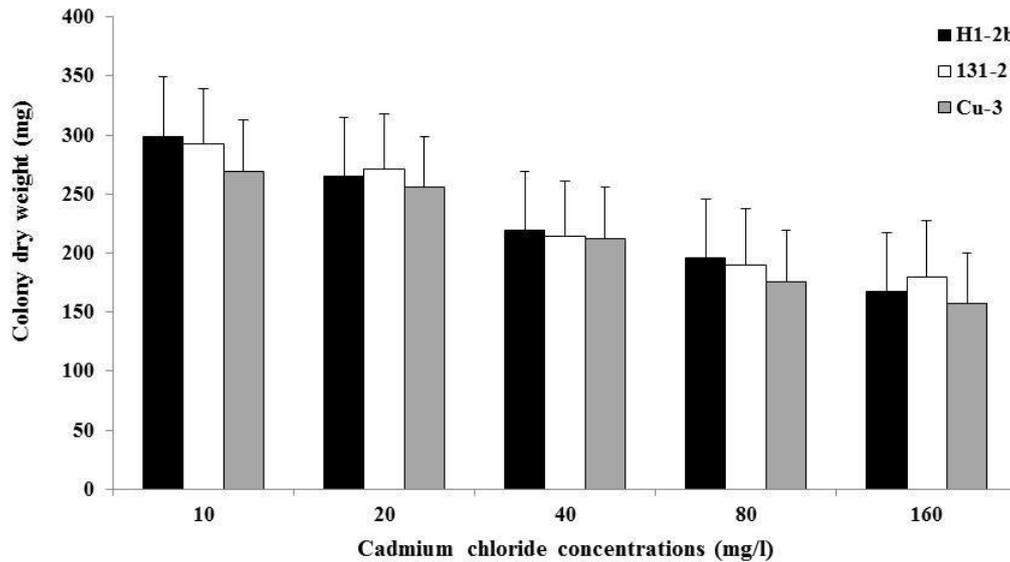


Fig. 3: Effect of cadmium chloride on the colonial dry weight of *Trichoderma* strains

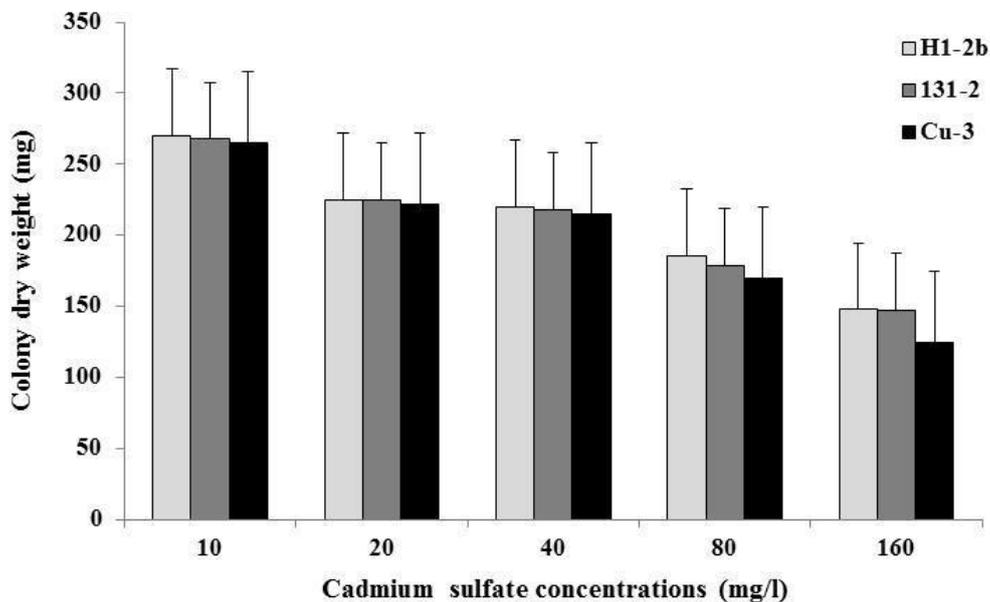


Fig. 4: Effect of cadmium sulfate on the colonial dry weight of *Trichoderma* strains

3.3 In vivo experiments

3.3.1 Effect of Cd on plant biomass

Generally, the addition of *Trichoderma* strains to the Cd-treated soil as co-inoculating agent improved the plant biomass (shoot and root) of the plant inoculated by four *S. meliloti* strains. The results for the effect of cadmium on the biomass of the alfalfa plants with the inoculants and coinoculants are present in Table 2. All plants were grown even in the presence of the highest concentration (120mg Cd/kg soil). In all cases of the Cd, the biomass per plant varied directly with the metal concentrations applied to the soil and depended on the biofertilizer and coinoculants strain. There were significant differences between the biomass throughout the all plants and the highest concentration of Cd applied to the soil. Moreover,

that biomass which was measured at 60mg/kg was significantly increased compared with those at 120mg/kg. The results of this study showed that the coinoculation with *T.harzianum* gave more nodule numbers compared with those coinoculated with *T. viride* strains especially when the plant inoculated by GHF-3153 followed by GHF-281, GHF-162 and GHR-94. It was noted that the R/S ratios were high in all cases which were coinoculated with *Trichoderma* strains and this gave evidence that the *Trichoderma* strains are proved to be plant growth promoting agents. Previously, in the present work, it was

found that the two strains of *Trichoderma* are proved also to be nodule promoting agents due to the increasing the nodule numbers compared with those grown on the roots of plants only inoculated with the biofertilizers. The

results indicated that the soil amended with CdSO₄ had more toxic effect on the investigated parameters of shoot and root dry weight and R/S ratio than CdCl₂.

Table.2: Shoot and root dry weight of *Medicago sativa* grown in Cd-contaminated soil at concentrations 60 and 120 mg/kg soil and inoculated with *Sinorhizobium meliloti* in presence or in absence of *Trichoderma harzianum* and *Trichoderma viride* strains

| Treatments | Shoot dry weight (g) | | Root dry weight (g) | | R/S ratio | |
|----------------------------------|----------------------|------------------|---------------------|------------------|--------------------|------------------|
| | <i>T.harzianum</i> | <i>T. viride</i> | <i>T.harzianum</i> | <i>T. viride</i> | <i>T.harzianum</i> | <i>T. viride</i> |
| Control | 1.76 | 1.72 | 0.42 | 0.41 | 0.21 | 0.21 |
| Control+60mg CdCl ₂ | 2.02 | 1.95 | 0.55a | 0.54a | 0.3a | 0.28 |
| Control+120mg CdCl ₂ | 1.96 | 1.94 | 0.59 | 0.57a | 0.32a | 0.3a |
| GHR-94+60mg CdCl ₂ | 2.24a | 2.16a | 0.67a | 0.61a | 0.4a | 0.38a |
| GHR-94+120mg CdCl ₂ | 2.11a | 2.03a | 0.61a | 0.62a | 0.35a | 0.29a |
| GHF-162+60mg CdCl ₂ | 2.25a | 2.34a | 0.66a | 0.64a | 0.41a | 0.33a |
| GHF-162+120mg CdCl ₂ | 2.08a | 2.02 | 0.69a | 0.63aa | 0.4a | 0.38a |
| GHF-281+60mg CdCl ₂ | 2.68a | 2.61a | 0.76a | 0.72a | 0.45a | 0.41a |
| GHF-281+120mg CdCl ₂ | 2.58a | 2.49a | 0.74a | 0.64a | 0.48a | 0.45a |
| GHF-3153+60mg CdCl ₂ | 2.77a | 2.68a | 0.81a | 0.70a | 0.49a | 0.48a |
| GHF-3153+120mg CdCl ₂ | 2.72a | 2.64a | 0.77a | 0.69a | 0.49a | 0.48a |
| Control+60mg CdSO ₄ | 1.85 | 1.81 | 0.52 | 0.5 | 0.26 | 0.26 |
| Control+120mg CdSO ₄ | 1.76 | 1.74 | 0.49 | 0.47 | 0.27 | 0.26 |
| GHR-94+60mg CdSO ₄ | 1.95 | 1.86 | 0.57a | 0.51a | 0.3a | 0.28 |
| GHR-94+120mg CdSO ₄ | 1.91 | 1.83 | 0.53a | 0.52a | 0.27 | 0.26 |
| GHF-162+60mg CdSO ₄ | 1.95 | 1.94 | 0.56a | 0.54a | 0.31a | 0.30a |
| GHF-162+120mg CdSO ₄ | 1.98 | 1.92 | 0.59a | 0.53a | 0.34a | 0.32a |
| GHF-281+60mg CdSO ₄ | 2.08a | 2.01 | 0.66a | 0.65a | 0.35a | 0.33a |
| GHF-281+120mg CdSO ₄ | 2.25a | 2.22a | 0.71a | 0.65a | 0.38a | 0.35a |
| GHF-3153+60mg CdSO ₄ | 2.47a | 2.38a | 0.82a | 0.77a | 0.39a | 0.38a |
| GHF-3153+120mg CdSO ₄ | 2.62a | 2.54a | 0.67a | 0.61a | 0.42a | 0.42a |
| LSD (P=0.05) | 0.32 | 0.31 | 0.11 | 0.09 | 0.08 | 0.08 |

Column values followed by the (a) are significantly different with control according to Fisher's LSD test ($P=0.05$).

3.3.2 Effect of Cd on nodulation

Generally, the addition of *Trichoderma* strains to the Cd-treated soil as co-inoculating agent improved the nodulation potent of the four *S. meliloti* strains. The data for the effect of cadmium on the nodulation potential of alfalfa plants with the inoculants are present in Table 3. All plants were nodulated even in the presence of the highest concentration (120mg Cd/kg soil), but with a few number of nodules. In all cases of the Cd, the number of nodules per plant varied directly with the metal concentrations applied to the soil. There were significant differences between the number of nodules throughout the all plants and the highest concentration of Cd applied to the soil. Moreover, that number of root nodules which was counted at 60 mg/kg was significantly increased compared with those at 120 mg/kg.

The results of this study showed that the coinoculation with *T.harzianum* gave more nodule numbers compared with those coinoculated with *T. viride* strain especially when the plant inoculated by GHF-3153 followed by GHF-281, GHF-162 and GHR-94. Similarly, it was found that the nodule dry weight was decreased in plants inoculated with the biofertilizers compared with those of inoculated with biofertilizers and coinoculated with *Trichoderma* strains. The root nodule biomass on the roots of plants coinoculated with *T.harzianum* was higher than those coinoculated with *T. viride* strain in presence of any of biofertilizers.

The data in Table 3 demonstrates that the best nodule number and nodule dry weight was found at the combination of plant grown in soil amended with 60 mg Cd (CdCl₂)/kg inoculated with *Sinorhizobium meliloti* strain GHF-3135 and coinoculated with *T.harzianum*

strain. While the lowest nodule number and nodule dry weight was found at the combination of plant grown in soil amended with 120 mg Cd (CdSO₄)/kg inoculated

with *Sinorhizobium meliloti* strain GHR-94 and coinoculated with *Trichoderma viride* strains.

Table. 3: Number of nodules and nodules dry weight of *Medicago sativa* grown in Cd-contaminated soil inoculated with *Sinorhizobium meliloti* in presence or in absence of *Trichoderma* strains.

| Treatments | Number of nodules/plant | | Nodules dry weight (mg) | |
|----------------------------------|-------------------------|------------------|-------------------------|------------------|
| | <i>T.harzianum</i> | <i>T. viride</i> | <i>T.harzianum</i> | <i>T. viride</i> |
| Control | 0 | 0 | 0 | 0 |
| Control+60mg CdCl ₂ | 0 | 0 | 0 | 0 |
| Control+120mg CdCl ₂ | 0 | 0 | 0 | 0 |
| GHR-94+60mg CdCl ₂ | 32 | 29 | 121 | 118 |
| GHR-94+120mg CdCl ₂ | 26 | 21 | 99 | 78 |
| GHF-162+60mg CdCl ₂ | 41a | 34 | 143 | 174a |
| GHF-162+120mg CdCl ₂ | 34 | 29 | 175a | 122 |
| GHF-281+60mg CdCl ₂ | 54a | 42a | 211a | 171a |
| GHF-281+120mg CdCl ₂ | 43a | 33 | 176a | 134 |
| GHF-3153+60mg CdCl ₂ | 65a | 51a | 224a | 199a |
| GHF-3153+120mg CdCl ₂ | 51a | 39a | 196a | 162a |
| GHR-94+60mg CdSO ₄ | 28 | 25 | 116 | 98 |
| GHR-94+120mg CdSO ₄ | 23 | 19 | 92 | 81 |
| GHF-162+60mg CdSO ₄ | 37a | 31 | 129 | 122 |
| GHF-162+120mg CdSO ₄ | 31 | 26 | 123 | 102 |
| GHF-281+60mg CdSO ₄ | 49a | 38a | 196a | 155a |
| GHF-281+120mg CdSO ₄ | 39 | 30 | 156 | 121 |
| GHF-3153+60mg CdSO ₄ | 58a | 46a | 248a | 201a |
| GHF-3153+120mg CdSO ₄ | 51a | 39a | 202a | 162a |
| LSD (P=0.05) | 15.5 | 14.8 | 74.8 | 62.5 |

Column values followed by the (a) are significantly different with control according to Fisher's LSD test ($P=0.05$).

Comparatively, the results showed high significant differences (at $P=0.05$) in the plant inoculated by either GHF-3153 or GHF-281 in the presence of *Trichoderma harzianum* strains and grew in soil polluted with 60 mg Cd/kg.

3.3.3 Effect of Cd on total chlorophylls content

The results of the present study showed that the addition of *Trichoderma* strains to the Cd amended soil as co-inoculating agent improved the chlorophyll content in the plant leaves especially those inoculated with the four *S. meliloti* strains compared with the control plants. The data for the effect of cadmium on the chlorophyll content of the alfalfa plants with the inoculants are present in Fig 5. All plants were grown even in the presence of the highest concentration (120 mg Cd/kg soil), and the leaves were more healthy than those of control pots or even better than those of only inoculated by biofertilizers or

coinoculated with *Trichoderma* strains only. In all cases of the Cd, the chlorophyll content per plant varied directly with the metal concentrations applied to the soil as well as with the microbial inoculation. There were significant differences between the chlorophyll content throughout the all plants and the highest concentration of Cd applied to the soil. Moreover, that chlorophyll content at 60 mg/kg was significantly increased compared with those at 120mg/kg.

The results of this study showed that the coinoculation with *T. harzianum* increased the chlorophyll content compared with those coinoculated with *T. viride* strain especially when the plant inoculated by GHR-94 followed by GHF-3153, GHF-281 and GHF-162. The lowest chlorophyll content was at control plant grown in untreated soil and control plant grown in soil amended with 120mg Cd/kg.

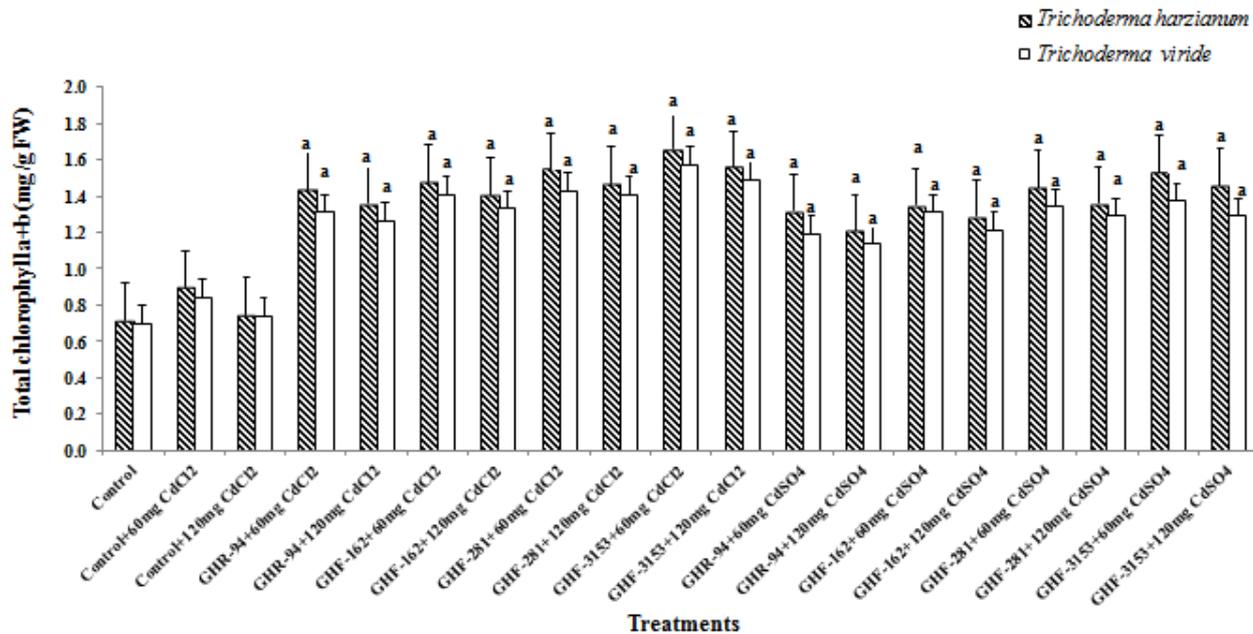


Fig. 5: Total chlorophyll of *Medicago sativa* grown in Cd-contaminated soil inoculated with *Sinorhizobium meliloti* in presence or in absence of *Trichoderma* strains.

3.3.4 Effect of Cd on shoot N, P and K content

Generally, it was found that CdCl₂ more stimulated the bioaccumulation of N, P and K in plant shoot more than those plants grew in soil polluted with CdSO₄. Table 4 shows that the all parameters of the investigations recorded in CdCl₂ soil pollution were higher in N, P and K content in plant shoot than those measured in soil polluted with CdSO₄. Also, the higher investigated

parameters were registered in plants inoculated with GHF-3153 or GHF-281 and coinoculated with *T.harzianum* strain and grew in 60mg Cd/ kg polluted soil. The results of the present study showed that even in CdSO₄ or in CdCl₂ polluted soils, plant nutritional status give significant differences under most of the treatments at P=0.05 compared with the control.

Table. 4: Shoot N, P and K content of *Medicago sativa* grown in Cd contaminated soil and inoculated with *sinorhizobium meliloti* in presence or in absence of *Trichoderma* strains.

| Treatments | N content (mg/plant) | | P (mg) | | K (mg) | |
|----------------------------------|----------------------|------------------|--------------------|------------------|--------------------|------------------|
| | <i>T.harzianum</i> | <i>T. viride</i> | <i>T.harzianum</i> | <i>T. viride</i> | <i>T.harzianum</i> | <i>T. viride</i> |
| Control | 114 | 97 | 31 | 29 | 42 | 38 |
| Control+60mg CdCl ₂ | 125 | 121 | 37 | 33 | 46 | 43 |
| Control+120mg CdCl ₂ | 146 | 137 | 45a | 43a | 55a | 52a |
| GHR-94+60mg CdCl ₂ | 187a | 174a | 52a | 46a | 61a | 58a |
| GHR-94+120mg CdCl ₂ | 177a | 165a | 45a | 44a | 56a | 54a |
| GHF-162+60mg CdCl ₂ | 201a | 189a | 66a | 61a | 67a | 62a |
| GHF-162+120mg CdCl ₂ | 187a | 179a | 52a | 49a | 62a | 59a |
| GHF-281+60mg CdCl ₂ | 235a | 224a | 74a | 69a | 76a | 71a |
| GHF-281+120mg CdCl ₂ | 211a | 208a | 54a | 51a | 59 | 55a |
| GHF-3153+60mg CdCl ₂ | 254a | 247a | 82a | 76a | 82a | 74a |
| GHF-3153+120mg CdCl ₂ | 239a | 232a | 56a | 52a | 66a | 63a |
| Control+60mg CdSO ₄ | 115a | 111 | 32 | 30 | 41 | 39 |
| Control+120mg CdSO ₄ | 137 | 131 | 41 | 39 | 35 | 31 |
| GHR-94+60mg CdSO ₄ | 167a | 154a | 52a | 42 | 51a | 48a |
| GHR-94+120mg CdSO ₄ | 155 | 147a | 44 | 41 | 50 | 47a |
| GHF-162+60mg CdSO ₄ | 181a | 179a | 56a | 51a | 57a | 52a |
| GHF-162+120mg CdSO ₄ | 176a | 165a | 53a | 45a | 52a | 49a |

| Treatments | N content (mg/plant) | | P (mg) | | K (mg) | |
|----------------------------------|----------------------|------------------|--------------------|------------------|--------------------|------------------|
| | <i>T.harzianum</i> | <i>T. viride</i> | <i>T.harzianum</i> | <i>T. viride</i> | <i>T.harzianum</i> | <i>T. viride</i> |
| GHF-281+60mg CdSO ₄ | 224a | 218a | 61a | 56a | 72a | 70a |
| GHF-281+120mg CdSO ₄ | 219a | 206a | 56a | 53a | 69a | 65a |
| GHF-3153+60mg CdSO ₄ | 244a | 232a | 72a | 66a | 72a | 64a |
| GHF-3153+120mg CdSO ₄ | 229a | 221 | 65a | 64a | 68a | 61a |
| LSD (P=0.05) | 43.5 | 43.6 | 13.4 | 12.6 | 12.3 | 11.5 |

Column values followed by the (a) are significantly different with control according to Fisher's LSD test (P = 0.05).

3.3.5 Rhizobioremediation and Cd accumulation in shoot:

Fig 6 demonstrates the Cd uptake by plant shoot throughout the interactions between the rhizomicroorganisms and the absorption surfaces of the plant root. The present study showed that the Cd uptake by plant roots with the association with the biofertilizer *Sinorhizobium* and *Trichoderma* as coinoculating agents was higher in soil polluted with CdSO₄ than in soil polluted with CdCl₂. The more Cd accumulation in the shoot of the alfalfa plant was more in plant roots coinoculate with *Trichoderma harzianum* than those coinoculated with *Trichoderma viride*. Also, the amount of Cd detected in the shoot of the plant was more in those plants grown in the soil polluted with 120 mg Cd/kg than in soil polluted with 60 mg Cd/kg. The roots inoculated with GHF-3153 and GHF-281 has the ability to absorb Cd in high polluted soil better than the soil polluted with

low dose of Cd. From the Fig 6 below, the results can summarized as following: Highest Cd accumulation in alfalfa shoot is found the case of: (a) Inoculating the plant roots with GHF-3153 or by GHF-281 and coinoculating with *Trichoderma harzianum* and grow in soil polluted with 120 mg Cd (CdSO₄)/kg. (b) Inoculating the plant roots with GHF-3153 or by GHF-281 and coinoculating with *Trichoderma harzianum* and grows in soil polluted with 120 mg Cd (CdCl₂)/kg. Lowest Cd accumulation in alfalfa shoot is found the case of: (a) Control and (b) Plant grown in soil of low Cd concentration. The use of term rhizobioremediation her can be replace the term phytoremediation because that the complete system of cooperation is interacted together to tolerate the system against the free Cd ions and allow some of them to be bioabsorbed and bioaccumulated in the plant parts as well as accumulated in the cells of *Sinorhizobium* or *Trichoderma*.

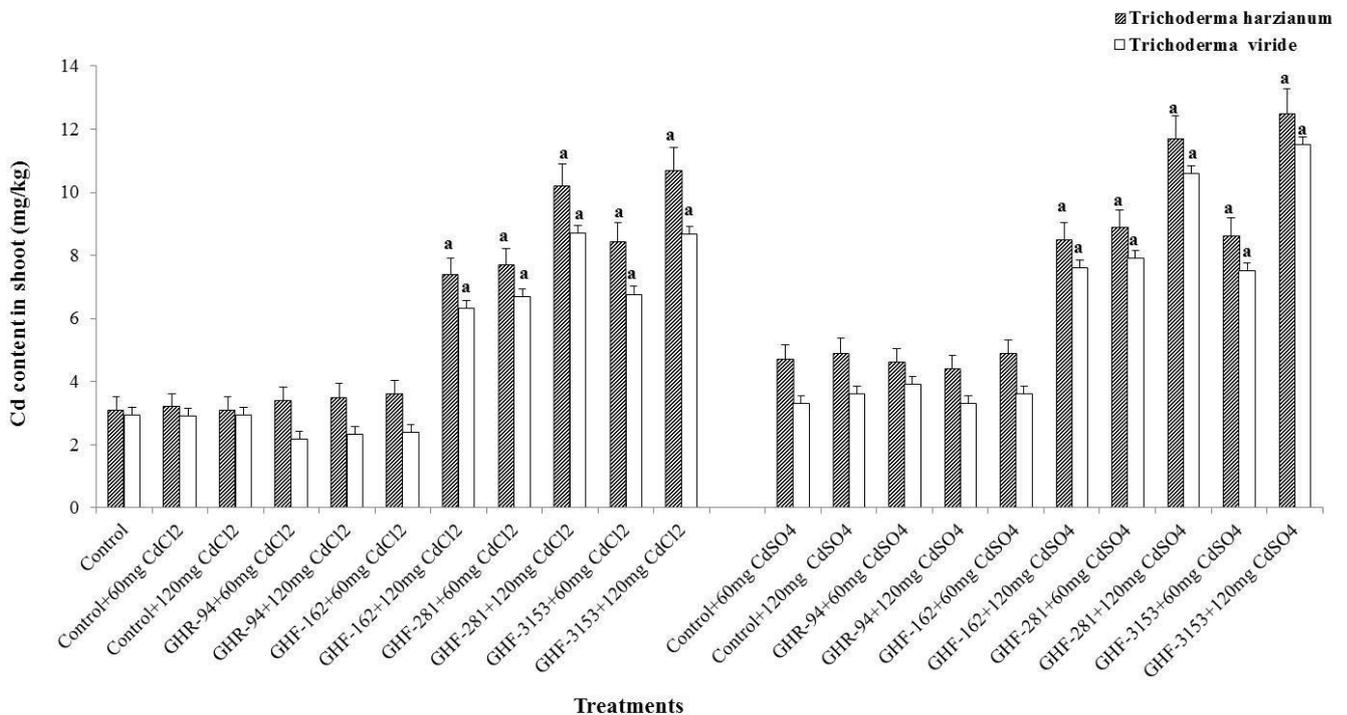


Fig. 6: Shoot Cd content of *Medicago sativa* grown in Cd amended soil and inoculated with *Sinorhizobium meliloti* in presence or in absence of *Trichoderma* strains. Bar values indicate by (a) are significantly different with control according to Fisher's LSD test (P= 0.05).

IV. DISCUSSION

Morrissey *et al.*, (2004) reported that one major challenge for the twenty-first century will be the production of sufficient food-the United Nations Population Fund estimates that the global human population may well reach 10 billion by 2050. This means increasing agricultural productivity of food crops, as plants form the basis of every food chain. The introduction of metal pollutants in various forms in the environment can pose a severe threat to the ecological system due to their negative impact on most life forms (Jaiswal and Malik 2000, Gavrielsea 2004). Although, some amounts of heavy metals are required by all life forms, however, there is a threshold limit to this requirement (Cervantes and Corona 1994). At high concentrations, heavy metal ions react to form toxic compounds in cells (Nies 1999, Choudhury and Spain 2003). Another major problem with metals is their persistence as they tend to persist indefinitely in the food chain (Gupta *et al.* 2000, Aleem *et al.*, 2003). The conventional treatment procedures used for removal of metals are uneconomical (Say *et al.*, 2001). Therefore, there is a need to develop rapid, economical and environmentally benign technology for the removal of metals from industrial effluents. There are certain microorganisms, which can survive in high concentrations of metals and have the potential to accumulate different metals. This is achieved by the virtue of covalent interaction of metal at cell surface or within the cell by different processes (Gadd and White 1993, Bhanoori and Venkateswerlu, 2000). These microbes can be of immense significance in the clean-up of heavy metals from the environment.

Naár and Biró (2006) established that the rate of Cd pollution significantly correlated with the frequency of four of six *Trichoderma* spp. presumably, the Cd tolerance of the isolates from differently contaminated plots is similar. This fact was supported by an earlier study, in which Cd tolerance of *T. harzianum*, *T. virens*, *T. viride* isolates, originating from soils with different Cd pollution levels were studied. The authors said that no considerable difference was found in the minimum inhibitory concentration for *in vitro* growth rate on Cd containing medium. Our *in vitro* results showed that the *Trichoderma* strains can tolerate the different concentration of Cd in broth medium. Harman *et al.* (2004) stated that *Trichoderma* spp. Are free –living fungi that are common in soil and root ecosystems. Root colonization by *Trichoderma* spp. also frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients. Kleifeld and Chet (1992) mentioned that the fungus *T. harzianum* which was applied to pathogen-free soil induced an increase in emergence of

seedlings, plant height, leaf area and dry weight. The results of the present study confirm the remarks and we are in agreement with the authors regarding to the increase of the plant dry weight. Bayoumi *et al.*, (1995b) studied the effects of different abiotic factors (acidity, salinity, nitrate and temperature) on growth rate of root-nodule bacteria (*Rhizobium* and *Bradyrhizobium*) strains were investigated *in vitro*. Strains isolated from *Vicia faba* L., *Coronilla varia* L. and *Lupinus albus* L. exhibited a large variation in tolerance of the above mentioned factors. These bacteria should be screened under stimulated conditions for enhanced survival before selection to be used for commercial inoculant production. Linear correlation matrix data were useful to find the appropriate concentrations for the selection of the tolerant strains. This conclusion can be applied to the data obtained during the experimental records. The statistical examinations carried out on the collected results confirm also that conclusion of Bayoumi *et al.*, (1995b). Alfredo *et al.*, (2006) hypothesized that the remediation measures would reduce heavy metal solubility, increase soil fertility and enhance soil microbial functionality. Also they hypothesized that the addition of different amendments and the development of a root system might induce shifts in the microbial community structure among the different treatments. We are in agreement with these concepts with the regard to the increases of nutritional status in the alfalfa biofertilized and coinoculated by functional microbial groups. Naár *et al.*, (2002) mentioned that the rate and direction of correlations, however, varied with the type of heavy metals when pot experiment was carried out to test the relation between two beneficial microorganisms in Cd, Zn and Ni polluted soil. The recorded results are in agreement with these conclusions respecting the mode of action of the microbial functions on the plant production and the nutrient content in the plants. The most remarkable result of this study is the survival of *Sinorhizobium* strains in the heavy metal contaminated soil which appear to be sensitive to high concentrations of applied cadmium salts.

These results were in agreement with the conclusion of Pálágyi *et al.*, (2004) mentioned that the success of a sinorhizobial inoculant in the soil depends to a large extent on its capacity to compete against indigenous strains. GSM03, a *S. meliloti* strain with enhanced competitiveness for nodule occupancy, was recently established in soil amended with heavy metals containing high doses of Cd, Cu and Pb ion concentrations. The results allowed us to differentiate between alterations in the microbial community apparently caused by inoculation and by the rhizosphere effect induced by the alfalfa plants and by the environment. Only moderate inoculation-dependent effects could be detected, while the

alfalfa plants appeared to have a much stronger influence on the microbial community. There are increasing evidence of adverse effects on microbial processes related to nutrient cycling in these types of soils. Applications of organic fertilizers with low concentrations of heavy metals with improve soil fertility in reclaimed soils. Furthermore, the legume-root nodule symbiosis can be used as an effective parameter for ecotoxicological evaluation of contaminated soils, Smith, (1997). However, in our experiment these factors were similar for all treatments.

Our results clearly demonstrate that small concentrations of Cd in soil cannot cause reductions in the number of rhizobia and indirectly in the nodule number too. These results are in agreement with Giller *et al.*, (1993). Al-Kahal *et al.*, (2001) mentioned that metal uptake by grains seemed to be directly related to the concentration of heavy metals and was greater in the case of an individual metal added separately than in combination. The results of the present study support this conclusion, but our results showed that the accumulation of Cd in the plant shoot was depended on the microbial inoculation and the amount of Cd present in the contaminated soil.

V. CONCLUSION

The results indicate that *Medicago sativa* is suitable to grow and rehabilitate Cd-polluted soils when inoculated specifically with *Sinorhizobium meliloti* tolerant to cadmium salts at various concentrations. Some other soil microorganisms like and *Trichoderma* fungi can further improve the chances to recover these contaminated sites and bring them back into cultivation. The association of *Medicago sativa* with Cd-resistant sinorhizobial strains can further help to improve the resistance of alfalfa to Cd. It can be assumed that such legumes will also support the N nutrition of the *Medicago sativa* provided that an effective *Sinorhizobim-Medicago* association can be established under Cd-polluted conditions.

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