https://dx.doi.org/10.22161/ijhaf.2.1.4 *ISSN: 2456-8635*

Effects of Cd on Microbial Properties, Enzymatic Activities, Soil pH and Salinity in The Rhizosphere of *Medicago sativa*

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Abstract*—Plants have mechanisms for accumulation, tolerance or alleviation of high levels of heavy metals in contaminated soil**. Some contaminants can be absorbed by the plants and are then broken down by plant enzymes. The objective of this particular study was measure the potential activities of three enzymes (dehydrogenase, protease and phosphatase) in the alfalfa rhizospheres under the stress at different concentrations of cadmium salts, and inoculated different biofertilizers strains of S. meliloti and coinoculated with Trichoderma strains. As well as pH, NaCl, $CaCO_3$ tolerance and antibiotic resistance were investigated. The results show that the growth rate of sinorhizobial strains decreased with increasing of NaCl and $CaCO_3$ concentration. Sinorhizobial strains grew in environments of pH ranged between5.5-7.5. There was variable response to antibiotic of all sinorhizobial strains. As well it was clearly showed that Cdso4 reduced the activity of phosphatase. Trichoderma harzianum stimulate the enzyme activity more than Trichoderma viride.*

***Keywords*— Alfalfa, Heavy metals, pH condition, Rhizobioremediation.**

I. INTRODUCTION

One of the major concerns in the world is the pollution and contamination of the soil. Heavy metal contamination of soil due to industrialization and other humane activities has become an environmental problem with consequent problems for the human population. High concentrations of heavy metals in soil have a selective effect on plant population. These results in a low diversity of species in different trophic levels and the heavy metals can remain in soil for a long time. Tolerance is the capacity of plant or microorganisms to live and adapt to elevated heavy metal concentrations is the soil. Alfalfa is temperate forage frequently exposed to low water availability, N-deficient soils and high temperature conditions. Photosynthesis supplies organic carbon to nodules, where it is used by the nitrogenase enzyme in the bacteroid inside nodules as a source of energy and reducing power to fix N_2 (Azcón-Bieto *et al*. 2000). The products of N_2-fixation, either amides or ureids, are exported to the plant via the xylem (Schubert *et al*. 1995; Walsh 1995). Factors that increase photosynthesis increase N_2-fixation, while factors decreasing photosynthesis tend to decrease N_2-fixation. This coupling results in the regulation of nitrogenase activity in plants by photosynthesis (C supply), N availability (N source strength) and N demand (N sink strength). Many authors have reported the relationship between solubility of heavy metals in sludge amended soil and soil pH. The rhizosphere is a micro-environment with characters that differ markedly from the bulk soil as a result of the biological activity of roots and colonization by microorganisms. These differences are caused by various including changes in soil properties, type and amount of fertilizer applied and plant species (El-Motaium and Badawy 2000). Soto *et al*. (2004) mentioned that soil acidification is one of the environmental factors that more strongly hampers the establishment of an effective symbiotic interaction between rhizobia and leguminous plants. *S. meliloti* and the acid-tolerant *Rhizobium* sp. strain LPU83are able to nodulate alfalfa plants at pH 5.6 but both exhibit a delayed nodulation and a reduction in the number of elicited nodules. However, the addition of Ca at low pH does affect neither nod gene expression in alfalfa-nodulating rhizobia (*S. meliloti* or strain LPU83) nor the quality of nod gene inducers exudated by alfalfa plant, in contrast to what has been reported previously. Progressive soil acidification is a worldwide problem with important economic consequences for countries which rely on alfalfa production for cattle nutrition (Von-Uexküll and Mutert 1995, Del Papa *et al*. 1999) the poor performance of alfalfa plants in moderately acid soils (pH 5.5-6.5) is mainly due to the effects of low pH on the establishment of the symbiosis between this legume and the N-fixing bacteria *S. meliloti* (Graham 1992; Glenn and Dilworth 1994; Zahran 1999). Low pH causes a reduction in the rate of nodulation as well as in the number of nodules elicited by the bacteria. Soto *et al*. (2004) stated that three stages of the *Rhizobium*-legume

https://dx.doi.org/10.22161/ijhaf.2.1.4 *ISSN: 2456-8635*

symbiosis have been reported to be affected by Ca and pH, which possibly account for the dependence of alfalfa nodulation for these factors: growth of the bacteria, rhizobial attachment to roots and the induction of nod gene expression. *S. meliloti* strains are extremely sensitive to acidic pH, and will grow only above pH 5.5(Graham 1992; Glenn and Dilworth 1994; Zahran 1999). In low pH conditions, the growth rate of *S. meliloti* strains is increased by concentrations of Ca in the mill molar range, whereas no such increase is observed at neutral pH (Howieson *et al*. 1992; Reeves *et al*. 1993). Heavy metals constitute a potential hazard for water, soils and sediments. It has been shown that heavy metal at certain concentrations can have long-term toxic effects within ecosystems (Majer *et al*. 2002) and have a clear negative influence on biologically mediated soil processes (Lee *et al*. 2002). It is generally accepted that accumulated heavy metal reduce the amount of soil microbial biomass (Brookes and McGrath 1984; Chander *et al*. 1995) and various enzyme activities, leading to a decrease in the functional diversity in the soil ecosystem (Kandeler *et al* . 1996). However, metal exposure may also lead to the development of metal tolerant microbial population (Ellis *et al*. 2003). Due to their relation to soil functionality, the soil microbial population and activity have been proposed as useful indicators of soil improvement and soil degradation (Pankhurst *et al*. 1995; Dick *et al*. 1996). In addition, soil enzyme activities are considered as sensitive and early indicators of both natural and anthropogenic disturbances (Giller *et al*. 1998). The most important soil factors influencing plant Cd accumulation are soil pH and Cd concentration. Soil Cd is distributed between a number of pools or fractions of which only the Cd in soil solution is thought to be directly available for uptake by plants. Soil pH is the principal factor governing the concentration of Cd in the soil solution. Cd adsorption to soil particles is greater in neutral or alkaline soil than in acidic ones and this leads to increased Cd levels in the soil solution. As a consequence, plant uptake of Cd decreases as soil pH increases. (Shore and Douben 1994; Eisler 1985).

This investigation was done to show the influence of *Trichoderma* strain application and different concentrations of Cd salt to the sandy brown forest soil and competitive ability of laboratory selected (heavy metal and antibiotics) stains of *S. meliloti* isolated from different habitats to nodulate alfalfa.

II. MATERIALS AND METHODS

2.1 Plant and soil sample

Alfalfa a seed was used with sandy brown soil, the preparation was carried out as it mentioned by (Shwerif, 2018). The experiments were carried out in pots with soil contaminated by cadmium salts, with three replicates. The Cd salts ($CdCl_2$ and $CdSO_4$ with different concentrations) were applied to soil before the plantation and plant inoculation.

2.2 Preparation of inocula and selection of strains

The selection and inocula of different biofertilizers strains of *S. meliloti* and coinoculated of *Trichoderma* strains (*T. viride* and *T. harzianum)* were prepared as described by (Shwerif, 2018).

2.3 Ecophysiological selection of S. *meliloti* strains

2.3.1 Antibiotic resistance

Sinorhizobium strains were challenged by 10 antibiotics of Bacto-sensitivty discs to determine their antibiotic resistance. This method was suitable to measure the antibiotic resistance as the basis for selecting a diverse sub-set of effective strains for further investigations. The investigation was carried out as it mentioned by Bayoumi *et al*. (1988a). the antibiotics used and their concentrations (µ ml^{-1}) were amoxicillin (A 25), ampicillin sulphate (AM 20), chloramphenicol (CH 30), clindamycin (CL 2), gentamicin (G 20), naladixic acid (NA 30), neomycin (NE 30), vancomycin (V 50), tobramycin(TO 10), and tetracyclin (T 50). Each Sinorhizobium strain was streaked on YMA plates and subjected to five antibiotic discs. The plates were incubated for 24h at 28°C. The test was carried out in triplicates. The results were indicated by (+) and (-) for resistance and sensitive, respectively.

2.3.2 NaCl tolerance:

Applying the experimental protocol mentioned by Bayoumi *et al*. (1995a, 1995b) and Bayoumi and Kecskés (1998), the sinorhizobial strains, which form nodules on alfalfa roots plus the two standard strains (GHR-94, GH-130) were screened for NaCl tolerance. YMB (100 ml) in 250ml Erlenmeyer flasks was supplemented with various levels of NaCl (0, 0.01, 0.05, 0.1, 0.2, 0.4, and 0.8% w/v) and sterilized at 121°C for 20 min. Media were inoculated with 1 ml (about 10^6 cfu of sinorhizobial cells) of a 1-day-old culture of the tested sinorhizobial isolates. The flasks were incubated at 28°C with constant shaking at 150 rev min^{-1} for one day. After incubation, the relative growth rate of each bacterial strain to the NaCl tolerance. The standard YMA of Vincent (1970) with 0.01% w/v NaCl was used as a control treatment.

2.3.3 Survival of rhizobia nodulating lupin under varying levels of $CaCO_3$

An YMB medium containing different levels of $CaCO_3$ (0-9 % w/v) was used to investigate $CaCO_3$ tolerance sinorhizobial strains. By modifying the experimental

https://dx.doi.org/10.22161/ijhaf.2.1.4 *ISSN: 2456-8635*

protocol mentioned by Bayoumi *et al*. (1995a, 1995b), Erlenmeyer flasks (250 ml) which contained 100ml of YMB medium were supplemented with 0, 0.15, 0.3, 0.45 and 0.6 % w/v $CaCO_3$ l^{-1} and were inoculated with 1 ml (about $1x10^6$ cfu of sinorhizobial cells) of a bacterial suspension. Inoculated flasks were incubated in rotary shaker (150 rev min^{-1}) at 28°C for one day. The growth rate of sinorhizobial strains was estimated by (+) and (-) for tolerance and sensitive, respectively of the investigated strains to the $CaCO_3$ tolerance. The standard YMA of Vincent (1970) with 0.3 % w/v $CaCO_3$ was used as a control treatment.

2.3.4 Survival of rhizobial isolates at various pH values

Modified method of Bayoumi *et al*. (1995a, 1995b) was applied in the following investigation. An YMB medium was prepared and sterilized at 121°C for 20 min. the pH of the medium (6.8) was modified to represent the required values of 4.5, 5.5, 7.5, 8.5 and 10 by 1 NHCl or NaOH. Measurements of pH occurred at the start of the experiment. Erlenmeyer flasks contained 100 ml of YMB medium adjusted to the required pH level were inoculated with 1 ml ($1x10^6$ sinorhizobial cfu) as a standard inoculum. Flasks were kept on a rotary incubator shaker (150 rev min^{-1}) at 28°C for one day. The sinorhizobial growth rate was determined by (+) and (-) for tolerance and sensitive, respectively of the investigated strains to the range of pH as well as by a plate count technique using YMA with Congo-red. Strains tolerant to higher levels of acidity or alkalinity were selected.

2.4 Enzymatic potential activities in alfalfa cadmium treated rhizosphere and inoculated by *S. meliloti* and *Trichoderma* strains.

Most enzymes are also contained in plant roots. Therefore, all visible plant fragments were carefully removed from the soil sample prior to the enzyme assays. The following enzymes were determined:

2.4.1 Dehydrogenase activity: Enzymes activity was assessed in 1g of soil at 60% of its water field capacity, exposed to 0.2 ml of 4 % 2-P-iodophenyl-3-p-nitrophenyl-5-phenyl-tetrazolium chloride in sterile distilled water for 20h at 22°C in darkness. The iodonitrotetrazolium formazan (INTF) formed was extracted with 10ml of a mixture of 1:1.5 ethylene chloride/ acetone by shaking vigorously for 1min and filtration through a Whatman no.5 filter paper. INTF was measured spectrophotometrically at 490nm (García *et al*. 1993). Dehydrogenase activity is expressed as µg of INTF per gram dry soil.

2.4.2 Protease activity on N-α-benzoyl-L-argininamide (Protease- BBA)

Two ml of phosphate buffer (pH7) and 0.5ml of 0.05M N-α-benzoyl-L-arginamide (BAA) substrate were added to 0.5 g soil sample. The mixture was incubated at 37°C for min and then diluted to 10 ml with sterile distilled water. The release of ammonium was measured in the same way as for urease (Nannipieri *et al*. 1980). Protease activity is expressed as µmol of NH_4^+ -N released per gram dry soil per hour.

2.4.3 Phosphatase activity

Two ml of 0.1 M maleate buffer (pH 6.5) and 0.5 ml 0f 0.115 M *p*-nitrophenyl phosphate (PNP) were added to 0.5g of soil sample and incubated at 37°C for 90 min. the reaction was stopped by cooling to 2°C for 15min, then 0.5 ml of M $CaCl_2$ and 2 ml of 0.5 M NaOH were added and the mixture was centrifuged to 4000rpm for 5 min. the reaction product was filtrated and the filtrate (p-nitrophenol) was analysed colorimertically at 398nm (Tabatabai and Bermner 1969). Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH. Measurement of PNP absorbance was at 398 nm. Phosphatase activity is expressed as µmol of PNP per gram dry soil and incubation time (hour).

III. RESULTS

3.1 Selection of *S. meliloti* strains

Antibiotic resistance, tolerance to NaCl, CaCO3 and growth rate at different pH were able to reduced and characterize the strains to four strains that are furthermore, tolerant to investigated enzymes activity.

3.2 Antibiotic resistance

In this study, and because of the number of antibiotics used, a great diversity among the strains in their sensitivity was found, making this investigation useful for distinguishing among all tested strains. The presented study showed that S. *meliloti* strains (Table 1) were resistant to chloramphenicol (CH). Nadalixic acid had the lowest effect on the growth of the tested strains except GHR-94, GH-130, GHF-162, GHF-1141, GHF-230, GHF-281, GHF-2130, GHF-353, GHF-3111 and GHF-3153. All strains were sensitive to tetracyclin (50µg/ml), neomycin (30µg/ml) and tobramycin (10µg/ml). The strains were sensitive to amoxicillin (25µg/ml) but the following strains GHR-94, GH-130, GHF-162, GHF-1141, GHF-230, GHF-281, GHF-2130, GHF-353, GHF-3111 and GHF-3153 were resistant to ampicillin at 20 µg/ml.

https://dx.doi.org/10.22161/ijhaf.2.1.4 *ISSN: 2456-8635*

Table.1: Antibiotic resistance of the selected strains

Strains	Antibiotic (µg/ml)									
	A (25)	AM (20)	CH (30)	CL (2)	G (20)	NA (30)	NE (30)	V (50)	TO (10)	T (50)
GHR-94	-	+	+	-	-	+	-	+	+	-
GH-130	-	+	+	-	-	+	-	+	+	-
GHF-131	-	-	+	-	-	+	-	+	+	-
GHF-162	-	+	+	-	-	+	-	+	+	-
GHF-1120	-	-	+	-	-	+	-	+	+	-
GHF-1141	-	+	+	-	-	+	-	+	+	-
GHF-214	-	-	+	-	-	+	-	+	+	-
GHF-230	-	+	+	-	-	+	-	+	+	-
GHF-243	-	-	+	-	-	+	-	+	+	-
GHF-270	-	-	+	-	-	+	-	+	+	-
GHF-281	-	+	+	-	-	+	-	+	+	-
GHF-290	-	-	+	-	-	+	-	+	+	-
GHF-2100	-	-	+	-	-	+	-	+	+	-
GHF-2130	-	+	+	-	-	+	-	+	+	-
GHF-321	-	-	+	-	-	+	-	+	+	-
GHF-353	-	+	+	-	-	+	-	+	+	-
GHF-372	-	-	+	-	-	+	-	+	+	-
GHF-3111	-	+	+	-	-	+	-	+	+	-
GHF-3150	-	-	+	-	-	+	-	+	+	-
GHF-3153	-	+	+	-	-	+	-	+	+	-

3.3 NaCl Tolerance

All the strains were able to withstand 0.1% NaCl whereas 0.4 and 0.8 % NaCl inhibited growth. According to the growth of the strains at 0.2 %, the strains were divided into two groups, the first group of GH-130, GHF-162, GHF-1141, GHF-230, GHF-281, GHF-2130, GHF-353, GHF-3111, GHF-3150 and GHF-3153 were able to tolerate the salt concentration, while the strains of the second group failed to tolerate more than 0.1% NaCl (Table 2). This indicates that sinorhizobial strains isolates from non-cultivated field and without previous treatments (sandy brown forest soil) generally have a high tolerance strains to salinity stress.

The results show that the sinorhizobial strains isolates tested are variable in their response to higher NaCl concentration.

3.4 $CaCO_3$ Tolerance

The results illustrated that the strains showed reasonable growth rates at different concentrations of $CaCO_3$, and this responded differently to different concentrations of $CaCO_3$ is due to the variation of the strains. The growth Rate of the strains was tested under different levels of $CaCO_3$ (0-0.75% w/v) in YMB medium (Table 3).

The growth rate showed a decrease with increasing $CaCO_3$ levels. Interaction between sinorhizobial strains and $CaCO_3$ levels affected the growth significantly. All strains showed the highest numbers of colonies up to 0.3 % $CaCO_3$ except GHR-94, GH-130, GHF-162, GHF-1141, GHF-230, GHF281, GHF-2100, GHF-2130, GHF-353, GHF-3111 and GHF-3153 that can grow at 0.45% $CaCO_3$. The activation of $CaCO_3$ on the growth of sinorhizobial at low concentration may be due to its buffering effect on the YMB medium. At higher $CaCO_3$ concentrations the growth decreased with increased concentration.

Table.2: Response of the Sinorhizobium strains to NaCl salt stress NaCl salt stress

Strains	Growth at different NaCl concentration (% in w/v)						
	0	**0.01**	**0.05**	**0.1**	**0.2**	**0.4**	**0.8**
GHR-94	+	+	+	+	-	-	-
GH-130	+	+	+	+	+	-	-
GHF-131	+	+	+	+	-	-	-
GHF-162	+	+	+	+	+	-	-

https://dx.doi.org/10.22161/ijhaf.2.1.4 *ISSN: 2456-8635*

Strains	Growth at different NaCl concentration (% in w/v)						
	0	0.01	0.05	0.1	0.2	0.4	0.8
GHF-1120	+	+	+	+	-	-	-
GHF-1141	+	+	+	+	+	-	-
GHF-214	+	+	+	+	-	-	-
GHF-230	+	+	+	+	+	-	-
GHF-243	+	+	+	+	-	-	-
GHF-270	+	+	+	+	-	-	-
GHF-281	+	+	+	+	+	-	-
GHF-290	+	+	+	+	-	-	-
GHF-2100	+	+	+	+	-	-	-
GHF-2130	+	+	+	+	+	-	-
GHF-321	+	+	+	+	-	-	-
GHF-353	+	+	+	+	+	-	-
GHF-372	+	+	+	+	-	-	-
GHF-3111	+	+	+	+	+	-	-
GHF-3150	+	+	+	+	+	-	-
GHF-3153	+	+	+	+	+	-	-

Table.3: Growth of the strains in YMB amended with different concentrations of $CaCO_3$

Strains	Growth at different $CaCO_3$ concentration (% in w/v)					
	0	0. 15	0.3	0.45	0.6	0.75
GHR-94	+	+	+	+	-	-
GH-130	+	+	+	+	-	-
GHF-131	+	+	+	-	-	-
GHF-162	+	+	+	+	-	-
GHF-1120	+	+	+	-	-	-
GHF-1141	+	+	+	+	-	-
GHF-214	+	+	+	-	-	-
GHF-230	+	+	+	+	-	-
GHF-243	+	+	+	-	-	-
GHF-270	+	+	+	-	-	-
GHF-281	+	+	+	+	-	-
GHF-290	+	+	+	-	-	-
GHF-2100	+	+	+	+	-	-
GHF-2130	+	+	+	+	-	-
GHF-321	+	+	+	-	-	-
GHF-353	+	+	+	+	-	-
GHF-372	+	+	+	-	-	-
GHF-3111	+	+	+	+	-	-
GHF-3150	+	+	+	+	-	-
GHF-3153	+	+	+	+	-	-

3.5 PH tolerance

The strains were characterized with respect to their growth response to ph. The results indicated that all strains grew in the environments of pH ranged between 5.5 and 7.5. The results illustrated that the strains of GHR-94, GH-130, GHF-162, GHF-1141, GHF-230, GHF-281, GHF-2100, GHF-2130, GHF-353, GHF-3111 and GHF-3153 tolerant to extremes of low and high pH since they grew over a range of pH from 4 and below 9 (Table 4). The response of sinorhizobial strains to varying pH showed a second order polynomial response with an optimum between pH 5 and 7.

https://dx.doi.org/10.22161/ijhaf.2.1.4 *ISSN: 2456-8635*

Table.4: Growth of the strains in YMB with different pH values.

Strains	Growth at different pH					
	4.5	5.5	6.8	7.5	8.5	10
GHR-94	+	+	+	+	+	-
GH-130	+	+	+	+	+	-
GHF-131	-	+	+	+	-	-
GHF-162	+	+	+	+	+	-
GHF-1120	-	+	+	+	-	-
GHF-1141	+	+	+	+	+	-
GHF-214	-	+	+	+	-	-
GHF-230	+	+	+	+	+	-
GHF-243	-	+	+	+	-	-
GHF-270	-	+	+	+	-	-
GHF-281	+	+	+	+	+	-
GHF-290	-	+	+	+	-	-
GHF-2100	+	+	+	+	+	-
GHF-2130	+	+	+	+	+	-
GHF-321	-	+	+	+	-	-
GHF-353	+	+	+	+	+	-
GHF-372	-	+	+	+	-	-
GHF-3111	+	+	+	+	+	-
GHF-3150	+	+	+	+	+	-
GHF-3153	+	+	+	+	+	-

The data from the present study showed varied degrees of antibiotic resistance, pH, NaCl and $CaCO_3$ tolerance and identified some strains with have better growth characteristics compared with the other strains. For example strains GH-130, GHF-162, GHF-1141, GHF-230, GHF-281, GHF-2130, GHF-353, GHF-3111 and GHF-3150 and GHF-3153 tolerated NaCl up to 0.2% and strains GHR-94, GH-130, GHF-162, GHF-1141, GHF-230, GHF-281, GHF-2100, GHF-2130, GHF-353, GHF-3111 and GHF-3153 tolerated $CaCO_3$ up to 0.45%. There was significant growth in pH tolerance among the sinorhizobial strains, where some strains showed their response to grow in acidic and alkaline environments, these strains are GHR-94, GH-130, GHF-162, GHF-1141, GHF-230, GHF-281, GHF-2100, GHF-2130, GHF-353, GHF-3111 and GHF-3153.

3.6 Effect of Cd on potential enzyme activities

Three soil enzymes work as soil bio-indicators were studied under the effect of Cd pollution (Table 5). Generally, it was found that the potential enzyme activities was maximal with soil inoculation with *Trichoderma harzianum* more than with *Trichoderma viride* and in soil polluted with cadmium chloride more than cadmium sulphate at dose 60mg cd more than 120mg Cd. Table 5 demonstrates the interaction between the microbial inoculation in the rhizosphere of alfalfa grew in polluted soil with either 60 mg Cd or 120 mg Cd in the form of $CdCl_2$ or $CdSO_4$ and the potential dehydrogenase, protease and phosphatase as bioindicators of soil pollution. The results of the present investigations showed that dehydrogenase was maximal at the treatment of plant rhizosphere with the combination of GHF-3153 in presence of *Trichoderma harzianum* strain in the soil polluted with 60 mg Cd in the form of $CdCl_2$, GHF-281 in the presence of *Trichoderma harzianum* strain in the soil polluted with 120 mg Cd in the form of $CdSO_4$ and GHF-281 in the presence of *Trichoderma harzianum* strain in the soil polluted with 120 mg Cd in the form of $CdSO_4$. The lowest activities of the investigated enzymes were at the rhizosphere of the plant grown in control soil treated with 120 mg $CdSO_4$/ kg.

The potential activity of protease was at maximum when the plant grew in soil treated with 60 mg or 120 mg $CdCl_2$/kg and the rhizosphere was inoculated with GHF-3153 in the presence of *Trichoderma harzianum* strain or inoculated with GHF-281 in soil rhizosphere treated with 60 mg $CdCl_2$/kg. in the soil treatment with $CdSO_4$, maximum activities were found at the combinations of GHF-3153 or GHF-281 in the presence of *Trichoderma harzianum* strain at the soil polluted with 60mg Cd, GHF-281 in presence of *Trichoderma harzianum* strain at the soil polluted with 120mg Cd and GHF-3153 in presence of *Trichoderma harzianum* strain at the soil polluted with 120 mg Cd. Regarding to the activities of the soil bioindicator phosphatase, it was found that maximal

https://dx.doi.org/10.22161/ijhaf.2.1.4 *ISSN: 2456-8635*

activities in rhizosphere polluted with CdCl2 were measured at the combinations of GHF-3153 or GHF-281 in the presence of *Trichoderma harzianum* strain at the soil polluted with 60 mg Cd and GHF-3153 in the presence of *Trichoderma harzianum* strain at the soil polluted with120 mg Cd. The lowest activity was recorded at the plant rhizosphere polluted with 120 mg $CdSO_4$ without microbial inoculation. Significant differences were measured at *P= 0.05* compared with the control treatments.

Table.5: Effect of Cd on the potential enzyme activities in Medicago sativa grown in Cd amended soil and inoculated with Sinorhizobium meliloti in presence or in absence of Trichoderma strains.

Treatments	**Dehydrogenase (µg INTF/g soil)**		**Protease (µmol NH^+_4-N/g soil/h)**		**Phosphatase (µmol PNP/g soil/h)**	
	T.harzianum	***T. viride***	***T.harzianum***	***T. viride***	***T.harzianum***	***T. viride***
Control	71.2	70.4	1.42	1.39	56.3	52.4
Control+60mg $CdCl_2$	87.3	78.8	1.56	1.45	79.2	77.2
Control+120mg $CdCl_2$	92.6	90.7	1.86a	1.73	99.1a	98.9a
GHR-94+60mg $CdCl_2$	135.8a	130.6a	1.97a	1.95a	117.6a	102.6a
GHR-94+120mg $CdCl_2$	117.1a	108.1	1.94a	1.79a	104.7a	99.6a
GHF-162+60mg $CdCl_2$	147.3a	137.5a	2.08a	1.97a	117.4a	106.1a
GHF-162+120mg $CdCl_2$	128.5a	105.4	1.97a	1.84a	106.1a	104.3a
GHF-281+60mg $CdCl_2$	**169a**	**154.5a**	**2.4a**	**2.28a**	**155.7a**	**132.3a**
GHF-281+120mg $CdCl_2$	157.9a	149.9a	2.09a	1.96a	133.9a	124.6a
GHF-3153+60mg $CdCl_2$	**187.7a**	**184.3a**	**2.97a**	**2.69a**	**177.6a**	**157.5a**
GHF-3153+120mg $CdCl_2$	164.9a	157.1a	2.56a	2.45a	154.5a	139.6a
Control+60mg $CdSO_4$	67.4	58.4	1.23	1.13	59.8	57.1
Control+120mg $CdSO_4$	56.6	51.7	1.16	1.13	49.9	48.4
GHR-94+60mg $CdSO_4$	115.8a	110.7a	1.66	1.56	102.6a	92.6a
GHR-94+120mg $CdSO_4$	98.1	98.1	1.44	1.38	94.7a	92.6a
GHF-162+60mg $CdSO_4$	133.3a	125.5a	2.08a	1.97a	117.4a	106.1a
GHF-162+120mg $CdSO_4$	122.5a	100.1	1.77	1.64	100.3a	94.9a
GHF-281+60mg $CdSO_4$	**147a**	**144.5a**	**2.33a**	**2.22a**	**135.7a**	**122.3a**
GHF-281+120mg $CdSO_4$	134.9a	131.9a	2.19a	2.16a	131.9a	120.6a
GHF-3153+60mg $CdSO_4$	**178.7a**	**174.2a**	**2.37a**	**2.49a**	**157.6a**	**146.3a**
GHF-3153+120mg $CdSO_4$	154.9a	147.1a	2.22a	2.15a	134.9a	129.2a
***LSD* (*P*=0.05)**	42.8	39.6	0.38	0.36	37.2	33.3

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

IV. DISSCUSION

The potential for the rhizobium-legume symbiotic association in acidic soil or metal contaminated soils to fix N2 depends upon the capacity to maintain an adequate population of ineffective bacteria in soil. Giller *et al.* (1998) studied the effects of heavy metals from biosolids on the population and N_2- fixing potential of *R. leguminosarum bv. trifolii*, under two PH regimes. They found few significant effects of biosolids-borne heavy metals on plants, N_2-fixation and number of rhizobia at the concentrations of metals studied, as long as the soil pH was maintained near 6, where reduction in rhizobial number and plant parameters was observed, the decrease was attributed primarily to low soil pH and/ or heavy metal toxicity from biosolids. Our result showed that the best grew of all strains was in pH ranged between 5.5-7.5. Measurements of soil enzyme activities have been used as an indicator of the effect of such soil manipulation (Naseby and Lynch, 1998) and may be useful for gaining a better understanding of the nature of the perturbations caused to ecosystem function after microbial inoculations. Soil enzyme activities have also been used as an indicator of carbon leakage from roots (Naseby and Lynch 1998, Naseby et al. 1999). The increased enzyme activities found in the rhizosphere of mycorrhizal plants indicate an increase in C and nutrient leakage from roots. So, the changes detected in the present study suggest a direct effect of AM colonization, as well as an indirect effect through changes in microbial composition in rhizosphere soil. The increased esterase activity in some *P.*

https://dx.doi.org/10.22161/ijhaf.2.1.4 *ISSN: 2456-8635*

fluorescens inoculated plants could be related to the metabolic versatility of this microbial group (Bolton *et al*. 1993). The P cycle enzyme activities are inversely related to P availability (Tadano *et al*. 1993) and when P is a limiting nutrient its demand increases, resulting in an increase in phosphatase activity, as occurred in natural AMF- colonized rhizospheres. Treatments which decrease the available phosphate cause an overall increase in phosphatase activity (Azcón and Barea 1997). Camprubi et al. (1995) found higher population of chitinase producers in the rhizosphere of non-mycorrhizal plants, an effect which was not corroborated in the present study. The increase in chitinase activity, caused by mycorrhization and inoculation of Trichoderma could be related to biological control activities. Trehalase activity, a symbiotic determinant was expected to be higher in mycorrhizal treatments (Mellor, 1992), but this was found only in plants inoculated with *G. deserticola* and *G. mosseae*, probably due to the better colonizing ability of these strains.

V. CONCLUSION

In conclusion, the results demonstrate that selection of adapted nodule bacterial strains under stress condition in culture is possible. Therefore, if there is a significant correlation between the strain performance under stress in pure culture and strain behavior under symbiotic conditions, pure culture evaluation may be a useful tool in the search for sinorhizobial strains better suited for soil environments where high NaCl, pH and CaCO3 constitute a limitation for symbiotic N2-fixation. However, field experiments are needed to determine the survival of the rhizobia strains that perform best under stress conditions in the laboratory.

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