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FITORREMEDIACION DE SUELOS CONTAMINADOS CON Cd Y Zn MEDIANTE EL USO DE *Lupinus uncinatus* Schldl.

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ا لرحمان بسم الله
الرحيم

BISMILLAH-IR-
RAHMAN-IR-
RAHIM

CON EL NOMBRE
DE ALLAH

EL
MISERICORDIOSO,
EL COMPASIVO

IN THE NAME OF
ALLAH

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SUMMARY

Remediation of sites contaminated with toxic metals is challenging. Large areas of land in many countries have been contaminated by cadmium and zinc due to a wide range of pollution sources. Phytoremediation offers the benefits of being *in situ*, low cost and environmentally sustainable. *Lupinus* species is starting to generate interest for phytoremediation of soils showing intermediate metal pollution. The aim of this research was to explore the accumulating behavior and tolerance of Mexican native *Lupinus uncinatus* Schldl. towards increasing concentrations of Zn (0, 200, 400, 600 mg Zn kg^{-1} soil) and Cd (0, 9, 18, 27 mg Cd kg^{-1}soil) as $ZnCl_2$ and $CdCl_2.2_{1/2}H_2O$ respectively in soil (pot study) and hydroponic solution (0, 30, 40, and 50 µM Zn and 0, 3, 4, and 5 µM Cd). The pot soil was incubated with the same Zn and Cd treatments to study the DTPA extractable fractions of the two metals at different intervals (1, 5, 15, 25, 60, 90 days).

The results of pot trial with Zn revealed that Zn reduced root dry weight while increased overall plant dry matter (DM).The tissue Zn concentrations of leaves, stems and roots at 600 mg Zn kg^{-1} treatment were 4063, 14771, and 10569 mg Zn kg^{-1} DM. The shoot:root Zn ratios were 1.17, 1.63 and 1.78 for 200, 400 and 600 mg Zn kg^{-1} treatments respectively. More than 64% of the total Zn uptake was translocated to the shoots at 600 mg Zn kg^{-1}.

The findings of pot trial with Cd demonstrated that *L. uncinatus* suffered significant dry matter loss as well inhibition of plant height and number of leaves in response to imposed Cd stress. The plant was able to accumulate 713, 343 and 197 mg Cd kg^{-1} DM in the roots, stems and leaves for 27 mg Cd kg^{-1} treatment respectively. The shoot:root Cd ratio was < 1 indicating a poor root to shoot translocation.

The results from soil incubation experiment showed that maximum DTPA extractable contents of both the metals were found at day 01 after incubation.

The variation in these contents was non significant from 15th day till the end of the incubation period suggesting a strong metal sorption.

In the hydroponic solution study, at the highest doses of Zn and Cd (50 µM and 5 µM respectively), the amounts of metal accumulated in roots, stems and leaves were 1289, 1918, and 1132 mg Zn kg^{-1} DM and 2467, 227, and 164 mg Cd kg^{-1} DM respectively. The shoot:root Zn ratios obtained for 50, 40, 30 µM treatments were 2.36:1, 2.28:1 and 2.32:1 respectively while the same ratio in case of Cd remained < 1 for the three Cd treatments. No significant effect on plant dry biomass was observed in both the cases. Significant changes in plant mineral composition were found, however, concentrations were generally above the deficiency levels.

The results from this research suggest that *L. uncinatus* has the potential to grow in contaminated soils under studied edaphic conditions and may be used as an alternative species for phytoremediation and revegetation of Zn and Cd contaminated soils respectively.

RESUMEN

La remediación de sitios contaminados con metales tóxicos es un gran reto. Grandes extensiones de tierra en muchos países se encuentran contaminadas con cadmio y zinc, debido a diversas fuentes de contaminación. La fitoremediación es una técnica que se aplica in situ con las ventajas de un bajo costo y de ser sustentable para el ambiente. Las especies del genero *Lupinus* han despertado recientemente gran interés para su uso en la fitoremediación de suelos contaminados con estos metales.

El objetivo de esta investigación fue explorar la acumulación y tolerancia de *Lupinus uncinatus* Schldl., especie nativa de México, aplicando al suelo concentraciones crecientes de Zn (0, 200, 400, y 600 mg Zn kg^{-1} suelo) y Cd (0, 9, 18, 27 mg Cd kg^{-1} suelo) en forma de $ZnCl_2$ y $CdCl_2.2_{1/2}H_2O$, respectivamente. Se condujo un experimento utilizando una solución hidropónica aplicando para Zn (0, 30, 40, y 50 µM) y para Cd (0, 3, 4, y 5 µM). Finalmente, se incubo el suelo (en macetas) con los mismos tratamientos del Zn y Cd para estudiar las fracciones extraídas con DTPA de ambos metales a diferentes intervalos (1, 5, 15, 25, 60, 90 dias).

Los resultados del experimento en macetas mostraron que el Zn disminuyó el peso seco de la raíz mientras que aumentó la materia seca total de la planta. Las concentraciones del Zn en los tejidos vegetales de hoja, tallo y raiz, fueron de 4063, 14771, y 10569 mg Zn kg^{-1} materia seca para el tratamiento en que se aplicaron 600 mg Zn kg^{-1} suelo. La proporción del Zn en la parte aérea y raíz fue de 1.17, 1.63 y 1.78 para los tratamientos 200, 400 y 600 mg Zn kg^{-1} respectivamente. Más de 64% del Zn absorbido fue traslocado a la parte aérea al aplicar 600 mg Zn kg^{-1}.

Para el caso del Cd, se observo una perdida significativa de materia seca, así como inhibición de la altura de la planta y el número de hojas en respuesta al estrés del Cd. La planta acumuló 713, 343 y 197 mg Cd kg^{-1} materia seca en las

raíces, tallos y hojas respectivamente para el tratamiento 27 mg Cd kg^{-1} suelo. La proporción del Cd en la parte aérea y raíz fue < 1 lo cual indica una pobre traslocación del metal hacia la parte aérea.

La incubación de suelo mostró que el contenido máximo extraíble con DTPA de ambos metales fue el día 01, después de la incubación. La variación en este contenido no fue significativa a partir del día 15 hasta el final del período de la incubación, lo que sugiere una sorción fuerte del metal.

En el estudio con la solución hidropónica, las concentraciones del Zn y Cd acumuladas en raíces, tallos y hojas fueron de 1289, 1918, y 1132 mg Zn kg^{-1} materia seca y de 2467, 227, y 164 mg Cd kg^{-1}materia seca para las dosis 50 µM Zn y 5 µM Cd respectivamente. Las proporciones del Zn en la parte aérea y raíz obtenidas para los tratamientos 50, 40, 30 µM Zn fueron 2.36 **:** 1, 2.28 **:** 1 y 2.32 **:** 1 respectivamente. En el caso del Cd, las proporciones fueron < 1 para los tres tratamientos. La materia seca no tuvo efecto significativo para ambos metales. También se observaron variaciones significativas en composición mineral de la planta, sin embargo las concentraciones de elementos esenciales se encontraban dentro del límite de suficiencia.

Los resultados de esta investigación sugieren *que L. uncinatus* tiene el potencial de crecer en suelos contaminados con metales, bajo las condiciones edáficas estudiadas, mostrando que esta especie es una buena alternativa para la fitotoremediacion en el caso del Zn y para la revegetación de suelos contaminados con Cd.

I. INTRODUCCION

El desarrollo creciente de las actividades agrícolas, industriales y urbanas ha originado numerosos problemas ambientales por la presencia de metales pesados y compuestos orgánicos tóxicos en el suelo (McGrath, 1987; Weissenhorn et al., 1995; Mulligan et al*.,* 2000; Bhattacharya et al*.,* 2002). Grandes extensiones de tierra se encuentran contaminadas por el insumo antropogénico de metales pesados (Klang-Westin y Eriksson, 2003). La preocupación relacionada con los posibles efectos de metales pesados sobre la salud humana y su acumulación en los suelos y plantas se ha incrementado en años recientes (Ceiśliński et al*.,* 1998). Las actividades antropogénicas tales como la minería y fundición de metales, el vertido de los residuos municipales, procesos de fabricación, la disposición de las pilas usadas y operaciones agrícolas como la adición de fertilizantes inorgánicos y plaguicidas son las principales fuentes de contaminación ambiental (Cui et al*.,* 2004). La contaminación edáfica influye directamente en la salud humana debido a que los metales tienen un potencial excelente de transferencia ecológica (Bermea et al*.,* 2002).

La amplia circulación de metales pesados en los suelos, el agua y el aire y su inevitable transferencia a la cadena alimenticia siguen siendo un tema ambiental importante el cual involucra riesgos desconocidos para las generaciones futuras (Nriagu y Pacyna, 1988).

El Zinc es un elemento esencial para los procesos metabólicos de las plantas y los animales. Sin embargo, también se puede acumular en concentraciones tóxicas en el ambiente (Lock y Janssen, 2001). Es un constituyente natural de los ciclos biogeoquímicos de la tierra y es requerido por las plantas en cantidades trazas.

Las actividades humanas tales como la minería, la producción de agroquímicos y el uso agrícola de lodos residuales pueden originar cantidades excesivas de Zn en suelos (Kabata-Pendias y Pendias, 1992; Kiesken, 1995). Adicionalmente las concentraciones de Zn encontradas en los suelos normalmente exceden a las requeridas como nutriente y pueden causar fitotoxicidad o daño a los microorganismos del suelo (Zhao et al., 2003).

El Cadmio es un metal altamente tóxico y se ha clasificado en el septimo lugar de los primeros 20 compuestos tóxicos principalmente por su influencia negativa en los sistemas enzimáticos celulares (Sanita et al., 1999; Al-Khedhairy et al., 2001). La contaminación de Cd en los suelos agrícolas no origina peligro al crecimiento vegetal, sin embargo, debido a la facilidad con la que se puede transferir del suelo a la cadena alimenticia humana, se considera un peligro importante para la salud humana. El tiempo de residencia de Cd en suelos es de miles de años (Alloway, 1995).

Por fenómenos tales como la minería y la fundición de metales, la producción industrial y el trànsito vehicular, los suelos agrícolas pueden estar contaminados con Cd asi como por el uso agrícola de lodos residuales, fertilizantes fosfóricos, y materiales encalantes (Alloway, 1995; Fergusson, 1990; Kabata-Pendias y Pendias, 1992; Adriano, 2001). El Cadmio es uno de los metales traza más móviles en el suelo y normalmente se transfiere significativamente a las plantas.

Su biodisponibilidad en suelos es generalmente alta en comparación a otros metales debido a su alta solubilidad y la predominancia de enlaces de energía baja con la fracción sólida del suelo (Alloway, 1995; Gérard et al., 2000; McLaughlin, 2000).

Las técnicas empleadas actualmente para controlar la contaminación del suelo incluyen la excavación y el entierro del suelo contaminado así como el tratamiento químico o microbiano del suelo contaminado con residuos orgánicos.

La mayoría de ellas son costosas y requieren mucha inversión de energía especialmente para los suelos contaminados con metales.

La Fitorremediación de metales pesados se considera cada vez con mayor relevancia como una solución rentable y menos agresiva para el ambiente con el fin de remediar los suelos contaminados con metales pesados (Chaney, 1983; Salt et al., 1998; Chaney et al., 2000; McGrath et al*.,* 2000). Esta es una técnica novedosa para limpiar los suelos contaminados usando plantas la cual ofrece los beneficios de ser efectuada *in-situ*, tener bajo costo y ser sustentable para el ambiente (Salt et al., 1998; Long et al., 2002). La investigación básica y aplicada han demostrado claramente que algunas especies vegetales poseen el potencial genético de remover, degradar, metabolizar y/o inmovilizar una amplia cantidad de los contaminantes (Chaney et al., 1997).

Las plantas que se usan para extraer metales pesados tienen que ser las más apropiadas para una contaminación especifica. Deben incluir tolerancia a metales pesados específicos, adaptación a las condiciones edáficas y climáticas, habilidad de absorción y acumulación de metales y adaptación espacial de las raíces a la distribución de contaminación.

Las especies del genero *Lupinus* poseen características ecofisiológicas que incluyen una capacidad de solublizar y absorber los elementos del suelo a través de un sistema radical extensivo además de tener relativamente alta tolerancia a ciertos factores limitantes ambientales, exceso de los nitratos y el calcio, baja temperatura de la raíz (Zornoza et al., 2002), exceso de cal (Tang y Robson, 1993), y salinidad (Fernández-Pascual et al., 1996). También se conoce que algunas especies de *Lupinus* pueden acumular Mn y Al (Reay y Waugh, 1981). Recientemente se ha observado tambien la acumulación de Cd y Hg en las raíces y las partes aéreas (Carpena et al., 2001; Vera et al., 2002). Ximenez-Embun et al., (2002) han reportado que *Lupinus* tiene la capacidad de limpiar las aguas contaminadas acumulando los metales pesados como Hg, Cd, y Cr.

Zornoza et al., (2002) demostraron que *Lupinus* tiene alta capacidad de retener Cd en las paredes celulares. Todas estas características, hacen de esta especie una candidata excelente para la fitorremediación de los sistemas contaminados (Pastor et al., 2003).

Actualmente, a nivel mundial muchos estudios generalmente han estado relacionados con *Lupinus albus*. Sin embargo en México a pesar de la gran diversidad de especies vegetales, existen pocos estudios sobre el potencial de especies silvestres que puedan desarrollarse en suelos contaminados con metales pesados. Por lo que es importante buscar especies nativas aptas para suelos contaminados, las cuales sean útiles para sanear y recuperar esos espacios yermos y peligrosos con el fin de que las generaciones actuales y futuras puedan vivir con menos riesgos de salud.

En consideración a lo anterior, el objetivo de esta investigación fue explorar la respuesta del crecimiento de *Lupinus uncinatus* Schldl. en presencia de metales pesados, así como también su acumulación y distribución en los diferentes órganos de la planta, tanto en macetas como en solución hidropónica, agregando diferentes concentraciones de Zn y Cd en ambos medios.

Por lo tanto, esta tesis se presenta en forma de cuatro artículos, cada uno de los cuales describe un experimento.

El primero discute el efecto del Zn sobre crecimiento vegetal, sobreviviencia y tolerancia de la planta asi como hiperacumulacion y distribucion del metal en los diferentes órganos en macetas; el segundo analiza los efectos del Cd sobre crecimiento y materia seca y acumulacion y transporte del mismo en la planta en macetas; el tercero reporta las fracciones biodisponibles del Zn y Cd a lo largo de diferentes intervalos en suelo usado para los experimentos de macetas y el cuarto estudia el crecimiento vegetal y comportamiento nutrimental como indice de respuesta de la planta al estrés metalico usando solucion hidroponica como sustrato. Al final los resultados se han descutido de manera general.

El suelo utilizado en esta investigacion fue colectado de San Pablo Ixayoc, Municipio de Texcoco, Estado de Mexico y las semillas de *L. uncinatus* fueron recolectadas del mismo sitio y de San Francisco, Municipio de Malinalco, Estado de Mexico (ver Mapa, Figura 1).

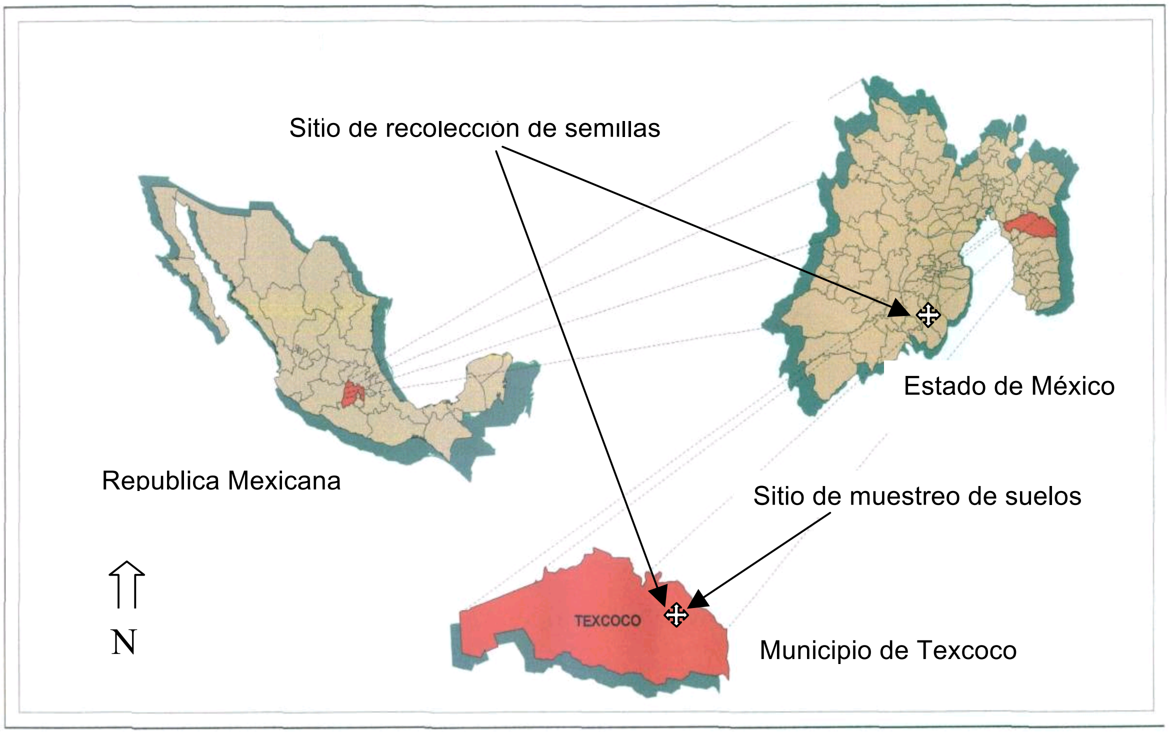

Figura 1. Localización de sitios de muestreo de suelos y semillas.

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II. PHYTOEXTRACTION OF ZINC USING MEXICAN NATIVE *Lupinus uncinatus* Schldl.

Key words: *Lupinus uncinatus* Schldl., Phytoextraction, Phytoremediation, Zinc

ABSTRACT

One of the aims of phytoremediation is the screening of potential plant species capable of tolerating and accumulating high amounts of metals in their harvestable parts. *Lupinus* species are starting to generate interest for the phytoremediation of soils having intermediate metal pollution. Among these metals, Zn causes major phytotoxicity problems and is common in polluted soils all around the world. A pot experiment was conducted in greenhouse to evaluate Zn hyperaccumulation potential of Mexican native *Lupinus uncinatus* Schldl. For this purpose the effects of different Zn supply levels on plant dry matter yield, survival, metal tolerance and Zn accumulation and distribution in roots, stem and leaves were investigated. Zn was added as $ZnCl_2$ at the rate of 0, 200, 400 and 600 mg Zn kg^{-1} soil (with four replicates) to four week old Lupin plants growing in pots.

Statistical analyses of data revealed significant effects of Zn on root dry weight, Zn uptake in roots, stems and leaves and shoot:root Zn ratio. Root dry weight was significantly reduced. However, a significant increase in overall plant dry matter yield was obtained as compared to control.

Progressive Zn supply levels produced clear toxicity symptoms with the increasing interaction time between metal and the plants. Metal tolerance was

reduced with rising stress of Zn contamination. The amount of Zn accumulated in stems reached 9632 and 14771 mg kg^{-1} at Zn supply levels of 400 and 600 mg kg^{-1} respectively.

The shoot: root Zn ratio was greater than one and more than 64% of total Zn uptake by *Lupinus* was translocated to the shoots at 600 mg Zn kg^{-1} treatment. These results indicate that *L. uncinatus* has a considerable potential of Zn hyperaccumulation and efficient translocation to its harvestable parts which makes it a phytoremediation option to be further explored.

INTRODUCTION

The growing development of agricultural, industrial and urban activities has given rise to a number of environmental problems because of the presence of heavy metals and toxic organic compounds in soil (McGrath, 1987; Weissenhorn et al., 1995; Mulligan et al., 2000; Bhattacharya et al., 2002). Large areas of land throughout the world are affected by anthropogenic input of heavy metals (Klang-Westin and Eriksson, 2003). Concern over the possible health and ecosystem effects of heavy metals in soils and accumulation in plants has increased in recent years (Ceiśliński et al., 1998). Human activities such as mining, smelting, dumping of municipal wastes, manufacturing processes, disposal of used batteries and agricultural operations like addition of inorganic fertilizers, pesticides, etc. are the main sources of environmental pollution (Cui et al., 2004). The environmental pollution of soil directly influences human health since they have excellent ecological transference potential (Bermea et al., 2002). The greatly increased circulation of toxic metals through the soils, water and air and their inevitable transfer to the food chain remains an important environmental issue which entails some unknown health risks for future generations (Nriagu and Pacyna, 1988).

Zinc is an essential element in plant and animal metabolic processes but it can also accumulate to toxic concentrations in the environment (Lock and Janssen, 2001). Zn is a natural constituent of the earth biogeochemical cycles and is required by plants in trace amounts. As a result of human activities such as metal mining, the use of agrochemicals, and the agricultural use of sewage sludge, Zn can be present in soils and growth media in excessive amounts, raising issues of plant tolerance to high levels of Zn (Kabata- Pendias and Pendias, 1992; Kiesken, 1995). Concentrations of Zn found in contaminated soils frequently exceed those required as nutrients, and may cause phytotoxicity or injury to soil microorganisms (Zhao et al., 2003).

Phytoextraction of heavy metals is increasingly being considered as an environmentally friendly, cost effective solution to remediate soils contaminated by heavy metals (Chaney, 1983; Salt et al., 1998 Chaney et al., 2000; MacGrath et al., 2000;). Phytoremediation is a novel technique to clean up contaminated soils using green plants, which offers the benefits of being in-situ, low cost, and environmentally sustainable (Salt et al., 1998; Long et al., 2002). Fundamental and applied research have unequivocally demonstrated that selected plant species possess the genetic potential to remove, degrade, metabolize or immobilize a wide range of contaminants (Chaney et al., 1997).

Plants that are used to extract heavy metals from contaminated soils have to be the most suitable for the given contamination. It includes tolerance to specific heavy metals, adaptation to soil and climatic characteristics, heavy metal uptake capability and spatial fitting of roots to pollution distribution.

Lupinus species possess ecophysiological characteristics including an ability to solubilise and absorb elements from the soil through the extensive root system, as well as considerable tolerance toward certain limiting environmental factors (Minchin et al., 1992; De Lorenzo et al., 1993; Fernandez-Pascual et al., 1996). It is also known that *Lupinus* can accumulate Mn and Al (Reay and

Waugh, 1981) and recently Carpena et al. (2001) and Vera et al. (2002) have observed the accumulation of other metals (Cd, Hg) in the shoots and roots of *Lupinus*. Ximenez–Embun et al. (2001) while evaluating *Lupinus albus* L. as accumulator of heavy metals (Cd, Pb, and Cr) from waste waters reported the ability of Lupin plants to clean up water. Zornoza et al. (2002) have demonstrated that *Lupinus albus* has a high capacity of Cd retention by cell walls and thiol groups. All facts put together make this species an excellent candidate for the phytoremediation of polluted systems (Pastor et al., 2003).

Nevertheless, many studies generally correspond to hydroponic culture systems and literature related to the growth of this species in polluted soils is scarce. We therefore decided to explore the potential of this species by examining the plant growth response functions and Zn uptake and distribution by this species when grown in pots at varied Zn supply levels.

MATERIALS AND METHODS

A pot experiment was conducted in greenhouse to study zinc hyperaccumulation potential of *L. uncinatus.* The soil was taken from the top 25 cm layer of a crop field at San Pablo Ixayoc, State of Mexico, Mexico (Figure 1 Chapter 1) which was analysed for Zn content and other physicochemical properties (Table 1). The seeds of *L. uncinatus* were also collected from the same site.

The seeds were sown at a rate of 8 pot^{-1} in June 2005 and thinned to leave 3 seedlings in each pot at germination which occurred after 5-7 days. The pots were filled with 5 Kg of above mentioned soil. Zn was added in the form of $ZnCl_2$ at the rate of 0, 200, 400, and 600 mg kg^{-1} after four weeks of plant growth. The pots were placed in a completely randomized design in an unlit greenhouse under normal conditions of temperature and humidity. The average day and night temperatures ranged between 25-29 and 8-11^{0}C respectively. The pots were randomized twice afterwards during the course of experiment. Four replicates were prepared per treatment (200, 400 and 600 mg kg^{-1}) and control. The pots were watered with distilled water as and when necessary.

The growth rate of *L. uncinatus* was evaluated at each soil Zn level in terms of plant biomass, plant survival under Zn stress and tolerance as well as the effect of the metal doses on the accumulation, distribution and transport of Zn within the plant. After two weeks of the application of Zn doses, the plants were collected, separating them into roots, stems and leaves and were rapidly and profusely but carefully washed with water under pressure and rinsed in several recipients containing deionized water. This was followed by drying in an oven at 70^0 C for 72 hours to estimate dry weight. The dried material was ground and 500 mg of these plant parts were tested with nitroperchloric acid and concentrations of Zn in different plant parts were determined by atomic absorption spectrophotometry. Data were subjected to one-way ANOVA analysis followed by a Tukey test for multiple comparison of means (significance level $P < 0.05$). All the statistical tests were performed using SAS version 9.1 (SAS, 2000).

RESULTS AND DISCUSSION

Plant growth and dry matter yield

The dry matter yield of *L. uncinatus* plants after two weeks exposure to increasing Zn levels is presented in Table 2.The roots which are in close and direct contact with the heavy metal and are one of the primary sites of toxic action showed a significant dry matter decrease with increasing Zn treatments as might be expected. For the 600 mg kg^{-1} treatment this reduction reached 57% when compared to control plants (0 mg Zn kg^{-1}). Adverse effects of heavy metals on root growth are well documented. For example Przymisinski and Wozny (1985) observed slowing down of mitotic rate of root cells of *Lupinus leuteus* as a response to high lead concentrations. Moreover the elasticity of cell walls is so much reduced by lead or cadmium that under mechanical stress they may break (Barcelo et al., 1986). Inhibition of root elongation of *Picea abies* seedlings grown in nutrient solutions containing Zn was reported by Godbold and Hüttermann (1985).

The dry matter yield of shoot material per pot ranged from 1.90 to 3.36g (Table 2). A significant increase in the shoot (stem + leaves) dry matter was obtained as compared to control and the total plant biomass also followed the same pattern. Many researchers have reported a similar increase in plant biomass in response to various treatments of different heavy metals like Mn, Cu, (Kidd et al., 2004) , Cd (Vassilev et al., 2004, Yang et al., 2004), etc.

Toxicity symptoms and plant survival under Zn stress

The Lupin plants were exposed to Zn contamination one month after emergence when the young plants were well established with functioning root system and photosynthetic apparatus. From the fourth day onwards after the application of Zn treatments, the plants started exhibiting toxicity symptoms. The most conspicuous and wide spread symptom of toxicity was bronzing of affected leaves accompanied by tip necrosis and inward curling, starting with the younger leaves but progressing down the plant with time. Some of these leaves eventually became entirely necrotic and dropped off, leaving plants with leafless sections of stem.

The toxicity symptoms became more severe with increasing Zn levels and exposure time. The 600 mg Zn kg^{-1} treatment was the first to show these symptoms (4th day after the application of Zn treatments) where the leaf wilting and fall off was severe followed by plant death. While in rest of the traetments, the plants were able to survive.

In spite of a low survival rate of plants in the presence of 600 mg Zn kg^{-1} treatment, a stimulatory effect on plant growth was observed as regards the plant dry matter accumulation (Table 2) as well as high metal tolerance indices were demonstrated by the plant (Figure 1). Pastor et al (2003) reported that the growth of *Lupinus albus* L. was severely affected from 300 mg Zn kg^{-1} onwards in a decalcified calcic luvisol. They further found that 500 mg Zn kg^{-1} was barely tolerated and growth ceased completely at 700 mg Zn kg^{-1} after 12 weeks of plant growth in the contaminated soil.

Metal Tolerance

After two weeks of growth in Zn contaminated soil, the ability of *Lupinus* to tolerate Zn stress was assessed using the index of tolerance (IT). The latter is calculated as the mean weight of a plant grown in the presence of a metal divided by the mean weight of a control plant, expressed as percentage.

Figure 1 depicts the tolerance of Lupin plants to grow in Zn contaminated soil. An index of tolerance of 50%, which means 50% of optimum growth, is considered to be the minimum desired biomass production for plants growing in a metal contaminated site (Ximenez-Embun et al., 2002).

The Zn treatments appear to have a stimulatory effect on the dry matter yield of the plant as the metal indices for Zn 200, Zn 400 and Zn 600 are 146%, 134% and 150% respectively. Metal tolerance may result from two basic strategies: metal exclusion and metal detoxification (Baker, 1981). The excluders prevent metal uptake into roots avoiding translocation and accumulation in shoots (De Vos et al., 1991). In contrast, hyperaccumulators absorb high levels of metals in cells. For Zn several hyperaccumulator species have been identified.

For Zn tolerance, plants have been reported to have five putative mechanisms for regulation of cytoplasmic Zn: 1) Low uptake across the plasma membrane 2) Sequestration in a sub cellular organelle 3) Precipitation as insoluble salts 4) Complexation to low molecular weight organic ligands and 5) active extrusion across the plasma membrane into the apoplast (Brune et al., 1994; Adriano, 2001).

High metal tolerance indices observed in this study indicate that this species has a considerable potential for utilization in Zn phytoextraction. However there is evidence suggesting that biochemical mechanisms that confer hyperaccumulation properties are both species specific and metal specific. Understanding physiology and biochemistry of metal tolerance in Lupin plants is needed in order to harness optimally the phytoextraction potential of this species.

Plant accumulation and transport of Zn

Another problem to be faced in phytoremediation, along with tolerance, is related to metal accumulation and distribution in plants, especially concerning the translocation of metals from roots to shoots. In spite of the short interaction time between plant and contaminated soil of two weeks, considerably elevated Zn concentrations were found in roots, stem and leaves of Lupin plants.

As Figure 2 depicts, the concentration of Zn in all the plant parts generally increased in parallel with the rising levels of Zn contamination in the growing medium indicating a substantial hyperaccumulation potential of this species under conditions of the present study as also previously reported by Ximenez-Embun et al. (2002) and Pastor et al. (2003) in case of *L. albus*. Zn concentrations in stem were 3720 , 9632, and14771 mg Zn kg^{-1} while the Zn concentrations in leaves were found to be 3090, 3938 and 4063 mg Zn kg^{-1} for 200, 400 and 600 mg Zn kg^{-1} treatments respectively. Roots of the lupin plants exhibited Zn accumulation of 5818, 8301 and 10569 mg Zn kg^{-1} for 200, 400 and 600 mg Zn kg^{-1} treatments.

These results demonstrate that *L. uncinatus* plants are able to accumulate Zn in environments with slightly acidic pH (as was the case in this study) or pH near to neutrality, which is one of the preferred environments of this species

(Lopez-Bellido and Fuentes, 1986). These results agree with the findings of Pastor et al (2003) who reported a Zn uptake of 3605 mg Zn kg^{-1} by *Lupinus albus* L. grown in an acidic soil contaminated with 300 mg Zn kg^{-1}. Similarly Lupin plants have also been reported to accumulate other heavy metals. An uptake of 4900 mg kg^{-1}, 2300 mg kg^{-1}, 400 mg kg^{-1} and 200 mg kg^{-1} of Cd, Hg, Pb and Cr respectively by *L. albus* has been reported in contaminated sand by Ximenez-Embun *et al.* (2002). A good growth rate of *Lupinus albus* L. was observed by Pastor et al. (2003) when cultivated in vermiculite pots with pH 6.7 and a high level of Zn. Under these conditions 1400 mg kg^{-1} of this element were detected in shoot and 4100 mg kg^{-1} in roots in only three weeks of treatment. Hence, based on the results of present study and keeping in view the findings of other researchers, *Lupinus* species seems to offer promising prospects for metal phytoremediation.

Another important and noteworthy aspect of Zn accumulation is the substantially high concentrations of Zn in shoots of Lupin plants as compared with those of the root system which is clear from shoot: root ratios approaching 1.78 in case of 600 mg Zn kg^{-1} treatment (Table 3). The obtained results demonstrated that shoot: root Zn distribution of these plants grown in pots have ratios >1 showing efficient translocation of Zn from roots to shoots which is a recognized characteristic of the hyperaccumulator plants. The mobility of Zn increased with increasing addition of the metal in soil as reflected by progressively increasing shoot: root ratios (Table 3).

Lupin plants have also showed an excellent transport potential from roots to shoots in case of Hg hyperaccumulation (Ximenez-Embun et al., 2002) thereby indicating an underlying mechanism of cellular transport of heavy metals within the plant. In addition to more efficient uptake, it is possible that the plant roots may increase the availability of heavy metals and hence increase the pool of metal available for uptake (Knight et al., 1997). In general Zn efficient genotypes typically contain lower Zn concentrations in roots and higher concentrations in young leaf blades (e.g oilseed rape) indicating a better transport and utilization of Zn (Adriano, 2001).

The mechanism of Zinc uptake and the effect of different soil factors on plant uptake of Zn are important in trying to optimize the ability of Lupin plants to extract metals. However, the mechanism by which Lupins take up and translocate Zn remains poorly characterized due to scarce literature available on this subject. In this study, the accumulating behaviour shown by *L. uncinatus* imparts it a potential value as phytoremediator of contaminated areas with slightly acidic or neutral soils.

Metal Distribution within the plant at different Zn supply levels

The distribution of Zn in the leaves, stem and roots differed among the Zn treatments. The Zn concentration in the shoots increased while that in the roots decreased with increasing Zn levels from 200 to 600 mg Zn kg^{-1} (Figure 3). At Zn level 200 mg Zn kg^{-1} 54% and 46% of total Zn uptake was allocated in shoot and root respectively while at 600 mg Zn kg^{-1} the distribution among shoot and root was 64% and 36% respectively. These results indicate that 1) at higher Zn concentrations, shoot tends to accumulate the major part of metal uptake which coincides with Pastor et al. (2003); 2) With the increasing Zn contamination the changing distribution of metal within the plant seems to be one of the other plant response mechanisms against the imposed metal stress.

CONCLUSIONS

The presented data indicate that in spite of showing toxicity symptoms in response to varied Zn supply levels, Lupin plants have shown a considerable potential for Zn phytoextraction which was evident from high metal tolerance indices, shoot:root ratios and increased dry biomass showed by the plant under Zn stress. The root growth response to two week exposure to Zn contamination was a significant dry weight reduction; however the total plant biomass witnessed a stimulatory effect when compared to control plants.

The high concentrations of Zn accumulation in different plant organs coupled with efficient translocation to shoot confirmed that *L. uncinatus* offers a promising future for phytoremediation of soils contaminated with Zinc. Future research must be done in open field conditions to verify the hyperaccumulation potential of this species as the objective of this study was to determine the response of Mexican native *L. uncinatus* to high Zn concentrations and their pattern of metal accumulation.

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Table 1. Selected physical and chemical characteristics of pot soil.

pH	6.29
E.C	0.16 dSm^{-1}
Organic Matter	1.19 %
Soil Texture	Sand (34%); Silt (32%); Clay (34%)
Bulk Density	1.13 Mgm^{3}
Total Zn content	65 mg kg^{-1}

Table 2. Dry matter yield of *L. uncinatus* as affected by two weeks exposure to different Zn supply levels. Numbers in the same column followed by different letters differ significantly (P< 0.05) according to Tukey test.

Zn supply levels	**Dry matter yield (g pot^{-1})**		
(mg kg^{-1})	**Root**	**Shoot**	**Plant**
0	0.56 a	1.90 b	2.46 b
200	0.31 b	3.29 a	3.60 a
400	0.30 bc	2.99 a	3.29 a
600	0.32 c	3.36 a	3.68 c

Table 3. Shoot:Root Zn concentration ratios in *L. uncinatus* grown in Zn contaminated soil.

Zn supply levels (mg kg^{-1})	**Shoot:Root Zn Ratio**
0	1.06
200	1.17
400	1.63
600	1.78

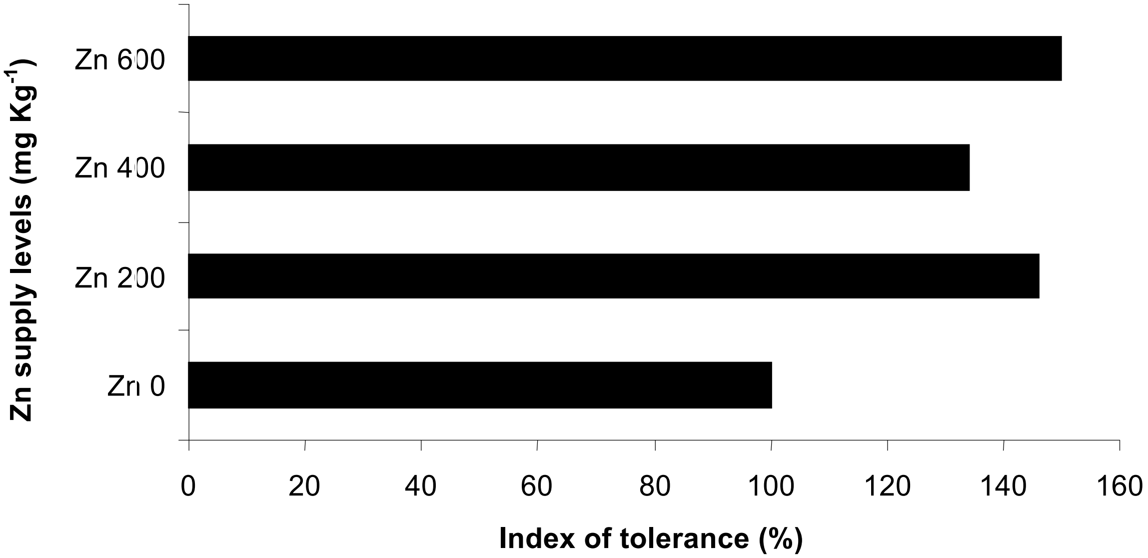

Figure 1. Tolerance index for *L. uncinatus* after a two week growing period in a zinc contaminated soil. (Zn 0=control, Zn 200= 200 mg Zn kg^{-1}, Zn 400= 400 mg Zn kg^{-1}, Zn 600= 600 mg Zn kg^{-1}).

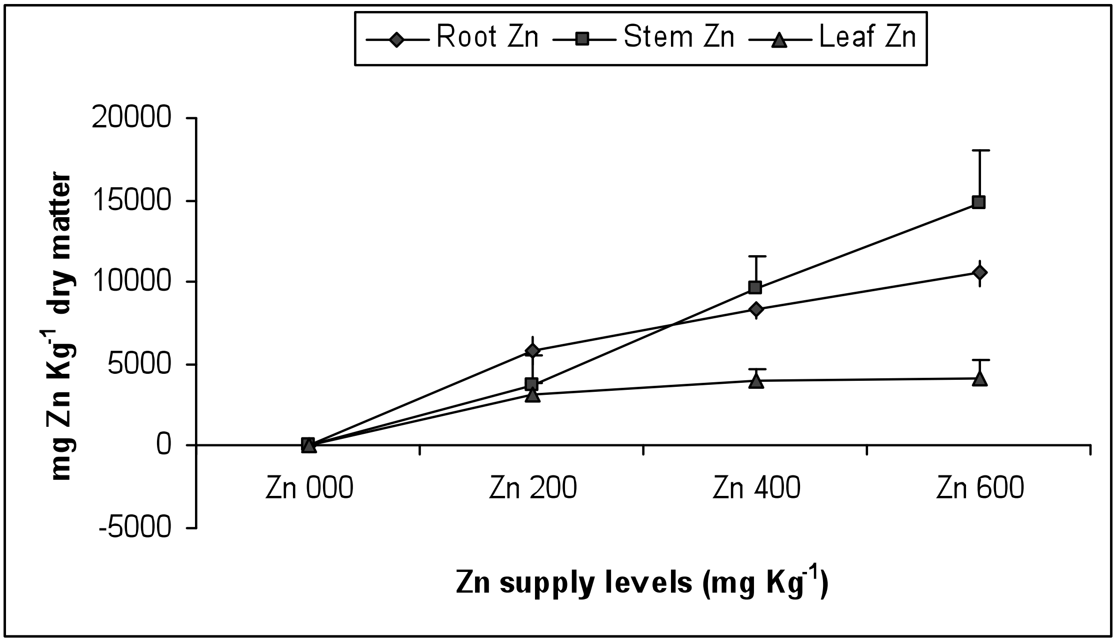

Figure 2. Zn uptake by roots, stems and leaves of *L. uncinatus* exposed to different Zn supply levels for two weeks. Vertical bars represent ±SE (*n=4*).

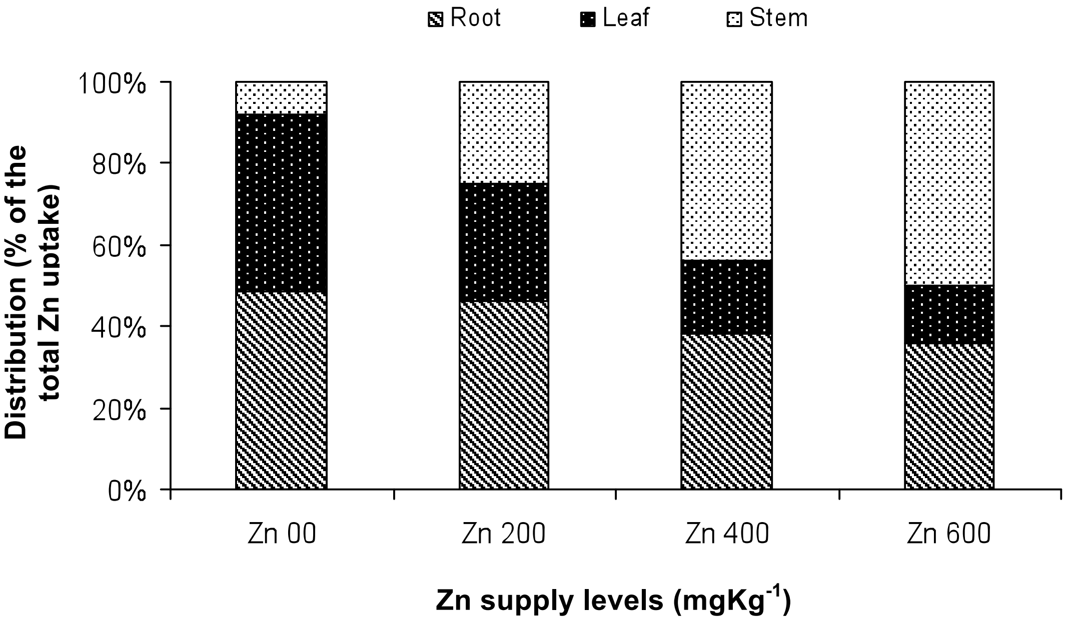

Figure 3. Zn distribution in various plant parts of *L. uncinatus* grown at different Zn supply levels for two weeks.

III. CADMIUM TOLERANCE AND PHYTOREMEDIATION BY *Lupinus uncinatus* Schldl.

Key words: Cd, Hyperaccumulation, *Lupinus uncinatus* Schldl., Metal Tolerance, Phytoextraction.

ABSTRACT

Remediation of sites contaminated with toxic metals is challenging. Large areas of land in many countries have been contaminated by cadmium due to a wide range of pollution sources. Phytoremediation offers the benefits of being *in situ*, low cost and environmentally sustainable. *Lupinus* species is starting to generate interest for phytoremediation of soils showing intermediate metal pollution. The aim of this study was to explore the accumulating behavior and tolerance of *Lupinus uncinatus* Schldl. towards increasing Cd concentrations in soil. For this purpose the effects of different Cd concentrations on plant growth, survival, metal tolerance, Cd accumulation and distribution in various plant organs were investigated. An 18 week pot trial was performed under greenhouse conditions. Cd was added as $CdCl_2.2_{1/2}H_2O$ at the rate of 0, 3, 6 and 9 mg Cd kg^{-1} soil at three different occasions (after 4th, 12th and 15th week of plant growth) with four replicates. The total Cd concentrations added, thus, reached to 9, 18 and 27 mg Cd kg^{-1}. Cd inhibited plant height and number of leaves and induced a significant change in dry matter yield of roots, stems and leaves. Metal tolerance Indices of 88, 82 and 49% were obtained for 9, 18 and 27 mg Cd kg^{-1} treatments. The maximal shoot Cd concentration (stem$_+$leaves) of 540 mg Cd kg^{-1} dry matter was found at 27 mg Cd kg^{-1} treatment.

The poor translocation of Cd from roots to shoot was evident from shoot:root ratios <1. The present work is the first report about the growth performance of *L. uncinatus* under Cd stress, its degree of tolerance and pattern of Cd accumulation in response to varying Cd levels in soil. All these results support the use of *L. uncinatus* for phytostabilization and revegetation of soils polluted with Cd.

INTRODUCTION

The wide spread heavy metal contamination poses long-term risks to environmental quality and sustainable food production (Weckx and Clijsters, 1996). Techniques currently employed to control soil contamination include excavation and burial of toxic metal-contaminated soil, or microbial or chemical treatment of organic polluted soil. Most of them are expensive and labor intensive, especially for the cleanup of metal-contaminated soil. Thus, phytoremediation, a technique using plants to remove contamination from soil, has become a topical research field in the last decade as it is safe and potentially cheap compared to traditional remediation techniques (Cunningham et al., 1996; Chaney et al*.,* 1997).

Cadmium is a highly toxic metal and has been ranked number 7 among the top 20 toxins mainly due to its negative influence on the enzymatic systems of cells (Sanita et al., 1999; Al-Khedhairy et al., 2001). Cadmium contamination in agricultural soils is unlikely to effect plant growth, however, as Cd is easily transferred to human food chain from the soils Cd contamination is a great threat to human health. These effects limit the marketing of agricultural products and reduce the profitability of the agricultural industry. Cadmium is residual in soil for thousands of years (Alloway, 1995).

Besides mining, smelting, industrial production and traffic, our agricultural soils could be contaminated with Cd through utilization of sewage sludge, phosphate fertilizers and liming materials (Alloway, 1990; Fergusson, 1990; Kabata-Pendias and Pendias, 1992; Adriano, 2001). Cadmium is one of the most mobile trace elements in soils and is often significantly taken up by plants. Its bioavailability in soils is high compared to other metals as a result of its high solubility and the predominance of low energy bonds to the soil solid phase (Alloway, 1995; Gérard et al., 2000; McLaughlin, 2000).

Some reports about Cd tolerance of *Lupinus albus* can be found in literature (Zornoza et al., 2002, Ximenez-Embun et al.,2002, Page et al., 2006). However no information about the response of *L. uncinatus* to metal stress is available.The aim of this study was, therefore, to determine the growth performance of Mexican native. *L. uncinatus* under Cd stress, their degree of tolerance to varying Cd supply levels in soil and their pattern of metal accumulation. These results would thus establish the potential of this species for the phytoremediation of Cd contaminated soils.

MATERIALS AND METHODS

A pot experiment was conducted in greenhouse to study the Cd phytoremediation potential of *L. uncinatus.* Soil was taken from the top 25 cm layer of a crop field at San Pablo Ixayoc, State of Mexico, Mexico (Figure 1 Chapter 1), air dried, weighed and analyzed for its total Cd content and other physical and chemical properties (Table 1).

Plastic pots 15 cm in diameter were filled with 5 kg of soil. Seeds of *L. uncinatus* collected from the same site as soil, were sown on 27th June 2005. Germination occurred after 5-7 days. One week after germination plants were thinned to 3 plants per pot. After four weeks of growth (4-08-2005) the plants were treated with cadmium in the form of cadmium chloride ($CdCl_{2.}\ 2._{1/2}\ H_2O$) at the rate of 0, 3, 6, and 9 mg kg^{-1} dissolved in 15 mL of deionzed water.

The pots were placed in a randomized design in greenhouse under normal conditions of temperature and humidity. The average day and night temperatures ranged between 25-29 and 8-11^{0}C respectively. The pots were re-randomized three times in the duration of the experiment. Four replicates were prepared per treatment (3, 6 and 9 mg kg^{-1}) and control. The pots were watered with distilled water every 2-3 days to maintain 70% of field capacity. The plants were applied with two successive Cd doses at different intervals (Table 2). Plant growth was measured in terms of plant height and number of leaves at each Cd level, and dry weight as well as the content and distribution of Cd in the roots, stem and leaves were determined at the conclusion of the experiment.

After three weeks of the last Cd dose (10-11-2005, a total of 18 weeks of plant growth), the plants were collected, separating them into roots, stems and leaves and were rapidly and profusely but gently washed with water and rinsed in several recipients containing deionized water. This was followed by drying in an oven at 70^0 C for 72 hours to estimate dry weight. The dried material was ground and 500 mg of these plant parts were treated with nitroperchloric acid. Concentrations of Cd in roots, stem and leaves were determined by atomic absorption spectrophotometry. Data were subjected to one-way ANOVA analysis followed by a Tukey test for multiple comparison of means (Significance level $P < 0.05$). All the statistical tests were performed using SAS version 9.1 (SAS, 2000).

RESULTS AND DISCUSSION

Plant height and number of leaves

Effect of external Cd levels on plant height and number of leaves is shown in Table 3. A reduction in plant height was observed with increasing Cd levels which was although, statistically significant only for 27 mg Cd kg^{-1} treatment when compared with control. The other two Cd levels (9 mg Cd kg^{-1} and 18 mg Cd kg^{-1}) did not affect significantly plant height as compared to the control.

A significant decrease in number of leaves was observed among various Cd levels. This decrease was progressive with the rising levels of Cd. After 14 weeks of cadmium stress the reduction in number of leaves as compared to control in 27 mg Cd kg^{-1} treatment was 31%. A similar reduction in plant height and leaf production as compared to control plants was reported by Pastor et al. (2003) in case of *Lupinus alb*us L. grown in an acid soil in the presence of varied Zn supply levels. The reduction in plant growth (plant height and number of leaves) observed in our experiment coincides with the appearance of visible toxicity symptoms in 27 mg Cd kg^{-1} treatment (69% leaf loss with respect to the control) and the magnitude increased with the increasing Cd levels. The symptoms included bronzing of leaves and inward curling resulting in leaf drop in some cases (Figure 1).

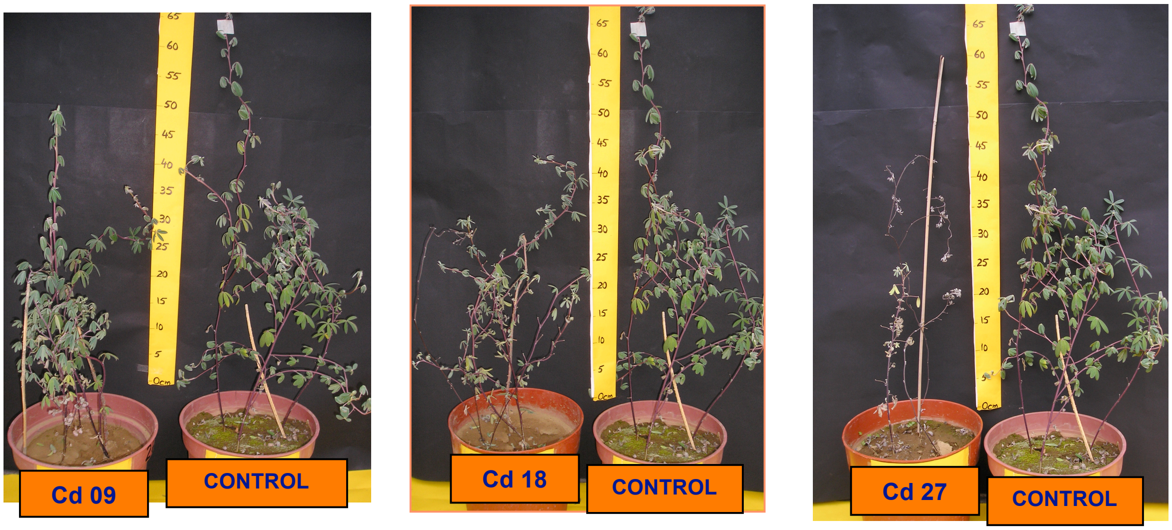

Figure 1. Toxicity symptoms exhibited by *L. uncinatus* in response to various Cd levels imposed.

It is noteworthy that these symptoms appeared after the application of third Cd dose which implies that the plant was tolerant to Cd levels up to 18 mg Cd kg^{-1}.Other heavy metals have also been reported to affect the plant growth. Kidd et al. (2004) found that increment in plant height and leaf production was completely arrested in five populations of *Cistus ladanifer* in response to 250 µM Ni and 500 µM Co.

Dry matter yield as affected by Cd supply levels

Dry matter yield of roots, stem and leaves of *L. uncinatus*. exposed to different Cd levels is given in Table 4. A significant reduction in root, stem and leaf dry matter was observed in response to various Cd treatments imposed. This decrease in dry matter may be attributed to reduced plant height and leaf loss (Table 1). Zornoza et al (2002) while growing *L. albus* L cv Multolupa hydroponically in the presence of 0, 18 and 45 µM Cd noted a 38 and 15% reduction in shoot and root dry weights respectively for 45 µM Cd treatment as compared to the control.

Metal tolerance

After 14 weeks of plant growth in soil contaminated progressively with varied Cd levels, the ability of *L. uncinatus*. to tolerate Cd stress was assessed using index of tolerance (IT). The latter is calculated as the mean weight of a plant grown in the presence of a metal divided by the mean weight of a control plant, expressed as percentage. Figure 2 depicts the tolerance of *L. uncinatus* grown in Cd contaminated soil. An index of tolerance of 50%, which means 50% of optimum growth, is considered to be the minimum desired biomass production for plants growing in a metal contaminated site (Ximenez-Embun et al., 2002).

The presence of 9 and 18 mg Cd kg^{-1} levels did not affect considerably the metal tolerance of the plant with indices 88% and 82% respectively, while a considerable decrease in metal tolerance can be noticed in case of 27 mg Cd kg^{-1} treatment. The highest level affected the plant growth of *L. uncinatus* as also evident from significant reduction in other growth parameters like plant height and number of leaves (Table 3), as well as appearance of visible toxicity symptoms. Ximenez-Embun et al (2002) reported a metal tolerance index close to 100% for *L. albus* when grown for 4 weeks in sand contaminated with 50 mg L^{-1} of Cd $(NO_3)_2$ solution. However this difference in plant response may be attributed to difference in metal exposure period, plant genotype, metal species or growing medium.

Cd accumulation and transport in plant organs

The Cd concentration in the roots stems and leaves of *L. uncinatus* supplied with varied Cd levels have been shown in figure 3. This concentration in all the plant organs was significantly higher than the control treatment. A linear increase in Cd accumulation, irrespective of the plant organ, was found with the rising levels of Cd in the growing medium. After 14 weeks of exposure to varied Cd levels, Cd constituted 109, 29 and 50 mg kg^{-1} dry matter in roots, stem and leaves respectively for 9 mg kg^{-1} treatments. The Cd accumulation for 18 mg kg^{-1} treatments was 208, 81 and 68 mg Cd kg^{-1} dry matter in roots, stem and leaves respectively. The range of values for Cd concentration in roots, stem and leaves of *L. uncinatus* for the highest Cd level (27 mg kg^{-1}) was 713, 343 and 197 mg kg^{-1} dry matter respectively. The shoots (stem+leaves) of *L. uncinatus* accumulated 79, 149 and 540 mg Cd kg^{-1} for the lowest, medium and the highest Cd level respectively.

Cd hyperaccumulation is defined as plant species capable of accumulating more than 100 mg Cd kg^{-1} dry matter in the shoot dry weight (Baker et al., 2000).

In most plant species, Cd concentration is generally lower than 3 mg Cd kg^{-1}, but may reach 20 mg kg^{-1} or more in the Cd enriched soils.

A plant concentration of > 100 mg kg^{-1} may be regarded as exceptional, even on a Cd-contaminated soil (Reeves and Bakers, 2000). A Cd accumulation of 4900 mg kg^{-1} in the shoots of *L. albus* was reported by Ximenez-Embun et al (2002) when grown in sand contaminated with 50 mg L^{-1} of Cd $(NO_3)_2$ solution.

Plant species differ greatly in their ability to take up and transport Cd within the plant. Difference in Cd accumulation capacity and localization appear to be major factors in determining plant tolerance to Cd exposure (Obata and Umebayashi, 1993). The different mobility of metals through the plant can be related to the shoot/root ratio (Shahandeh and Hossner, 2000). Table 5 shows the shoot:root Cd concentration ratios. The said ratio in non-Cd treated plants was >1, while in plants treated with varied Cd levels the ratio remained < 1.

The ratio was <1 irrespective of the Cd level, being the lowest (0.71) for 18 mg Cd kg^{-1} (Table 5). These results indicate that the majority of Cd was accumulated in the roots, particularly for the highest Cd level, suggesting a strong Cd retention during its long-distance transport from roots to shoots which might be a plant mechanism to tolerate the metal stress (Zornoza et al, 2002). The same results have been reported for *L.* albus (Ximenez-Embun et al, 2002; Zornoza et al, 2002) and other crops e.g *Betula Pendula* (Gussarsson, 1994), *Phaseolus vulgaris* L., *Oryza sativa* L., *Brassica oleracea* L. and *Zea maiz* L. (Guo and Marschner et al., 1995). The restricted Cd transport from roots to shoots reduces the Cd concentration in grains much more than in leaves, and consequently lupin seeds should contain only very low levels of Cd, which is an advantage for seed consumption (Page et al., 2006a).

In view of the results obtained in this study, *L. uncinatus* can be used to remediate polluted soils by mechanisms other than phytoextraction. The prompt restoration of a dense vegetation cover is the most useful and widespread method to stabilise contaminated areas like mine wastes and to reduce effects of metal pollution (Bargagli, 1998). The amounts of Cd extracted by *L. uncinatus* are insufficient to use this species for phytoextraction. The excluder behaviour of *L. uncinatus* for Cd lead to higher concentration of this element in roots. These results suggest that, this species can be utilized for the phytostabilization and revegetaion of the Cd contaminated soils.

Cadmium distribution in different plant organs

The distribution of Cd in the leaves, stems and roots of *L. uncinatus* subjected to different Cd levels is presented in figure 4. No change in pattern of Cd distribution in different plant organs was noted in response to varied Cd levels with respect to control. The results indicate that most of the Cd taken up by the plant was retained in roots (root Cd made up 58% of the total Cd uptake by the plant, irrespective of Cd level). Cd concentration decreased in the order: roots>stems>leaves, however no significant difference was found in the Cd content of stems and leaves. The gradient of Cd concentration across the plant indicates poor Cd translocation from roots to shoots.The strong retention of Cd observed in the roots of *L. uncinatus* is in agreement with the previous results (Römer et al., 2000, 2002; Ximenez-Embun et al, 2002; Zornoza et al, 2002; Page, et al., 2006 a,b; Vazquez et al., 2006). Cadmium being a nonessential and pollutant heavy metal found in different concentrations in soil (Sauvé et al., 2000; Lugon-Moulin et al., 2004) is recognised as a toxic compound at the root level and, as it is not needed in the shoot, the plant sequesters it in the roots to avoid damage to the shoot. Moreover distribution between roots and shoots differs with plant species, rooting medium and period of exposure. Some environmental factors such as Cd concentration in the medium, ambient temperature, and light-intensity can affect the distribution of the metal between the shoots and roots (Chino and Baba, 1981).

CONCLUSIONS

The treatment of Cd-polluted soils is a priority keeping in view the high mobility and ecotoxicity of this trace element. However, few techniques combine efficiency, soil preservation and low cost. Phytoremediation may be an effective and efficient solution to these requirements. In the present study the period of plant growth under metal stress was 14 weeks and toxicity symptoms were only seen after the third metal dose in the last three weeks (after a total of 11 week Cd exposure) of the plant growth. Metal tolerance indices obtained indicate tolerance of the plant species for Cd levels 9 mg Cd kg^{-1} and 18 mg Cd kg^{-1}. The roots of *L. uncinatus*. absorbed substantial quantities of Cd especially when this metal occurred at high concentration in soil (27 mg Cd kg^{-1}). Keeping in view all these considerations, it may be concluded that this species can be used in the phytostabilization of Cd by immobilizing it, mainly in roots during each growing season. The Cd tolerance coupled with the ability of *L. uncinatus* to grow under various environmental stresses, nitrate excess, low root temperature, detopping, lime excess and salinity make this species an excellent candidate to form an important part of plant material for phytoremediation research.

The present investigation was the first attempt to determine the growth performance of Mexican native *L. uncinatus*. under Cd stress, their degree of tolerance to varying Cd supply levels in soil and their pattern of metal accumulation. Further research, however, is vital for comprehensive understanding of the mechanisms involved in Lupin plant response functions to Cd stress.

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Table 1. Selected physical and chemical properties of pot soil.

pH	6.29
E.C.	0.16 dSm^{-1}
Organic Matter	1.19%
Soil Texture	Sand (34%); Silt (32%); Clay (34%)
Bulk Density	1.05 gcm^{-3}
Total Cd content	0.16 mg kg^{-1}

Table 2. Different intervals of cadmium addition to pot soil.

	Dates of application of Cd doses			
	4-08-2005	29-09-2005	20-10-2005	Total Cd Concentration (mg kg^{-1})
Cadmium Dose (mg kg^{-1})	3	3	3	9
	6	6	6	18
	9	9	9	27

Table 3. Effect of different Cd supply levels on plant height and number of leaves of *L. uncinatus.*

Cd levels (mg kg^{-1})	**Plant height (cm)**	**Number of leaves (number)**
0	66.25 a	32 a
9	64.50 a	18 ab
18	60.25 a	18 ab
27	29.42 b	10 b

Table 4. Dry matter yield of roots, stem and leaves of *L. uncinatus* exposed to different Cd supply levels for 14 weeks.

Cd supply levels	**Dry matter yield (g pot^{-1})**			
(mg kg^{-1})	**Root**	**Stem**	**Leaf**	**Plant**
0	2.12 a	4.13 a	2.70 a	8.95 a
9	1.97 ab	3.29 b	2.08 ab	7.34 b
18	1.45 b	2.66 c	1.56 bc	5.67 c
27	0.74 c	1.57 d	0.98 c	3.29 d

Table 5. Shoot:Root Cd concentration ratios of *L. uncinatus* subjected to varied Cd supply levels.

Cd supply levels (mg kg^{-1})	**Shoot: Root Cd Ratio**
0	1.2
9	0.72
18	0.71
27	0.75

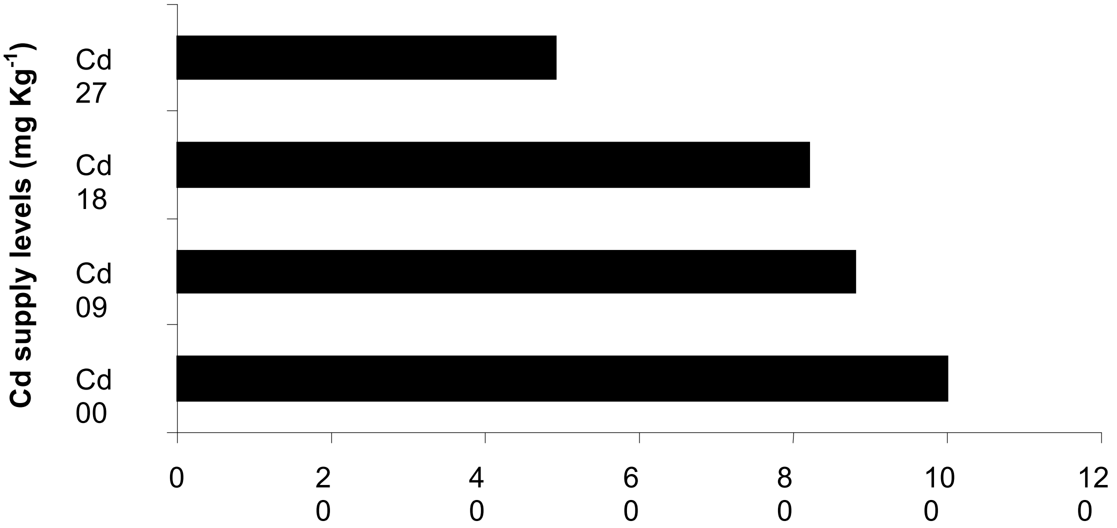

Figure 2. Tolerance index for *L. uncinatus* after a 14 week growing period in a soil contaminated progressively with Cd. (Cd 0= control, Cd 9=9 mg Cd kg^{-1}, Cd 18=18 mg Cd kg^{-1}, Cd 27=27 mg Cd kg^{-1}).

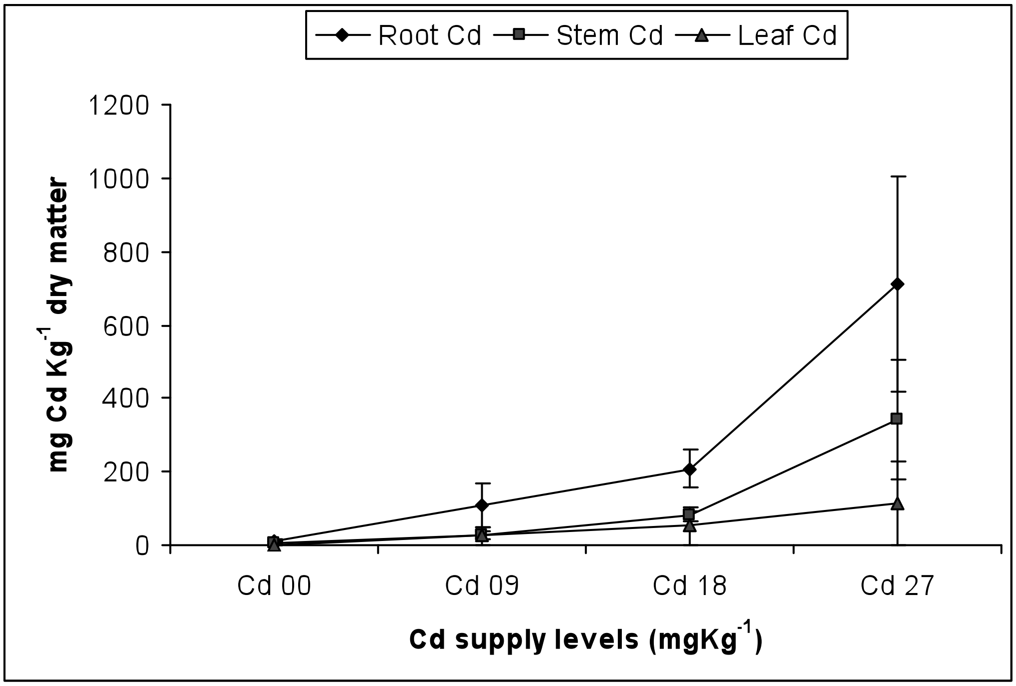

Figure 3. Cd uptake by roots, stem and leaves of *L. uncinatus* exposed to different Cd supply levels for 14 weeks. Vertical bars represent ±SE (*n=4*).

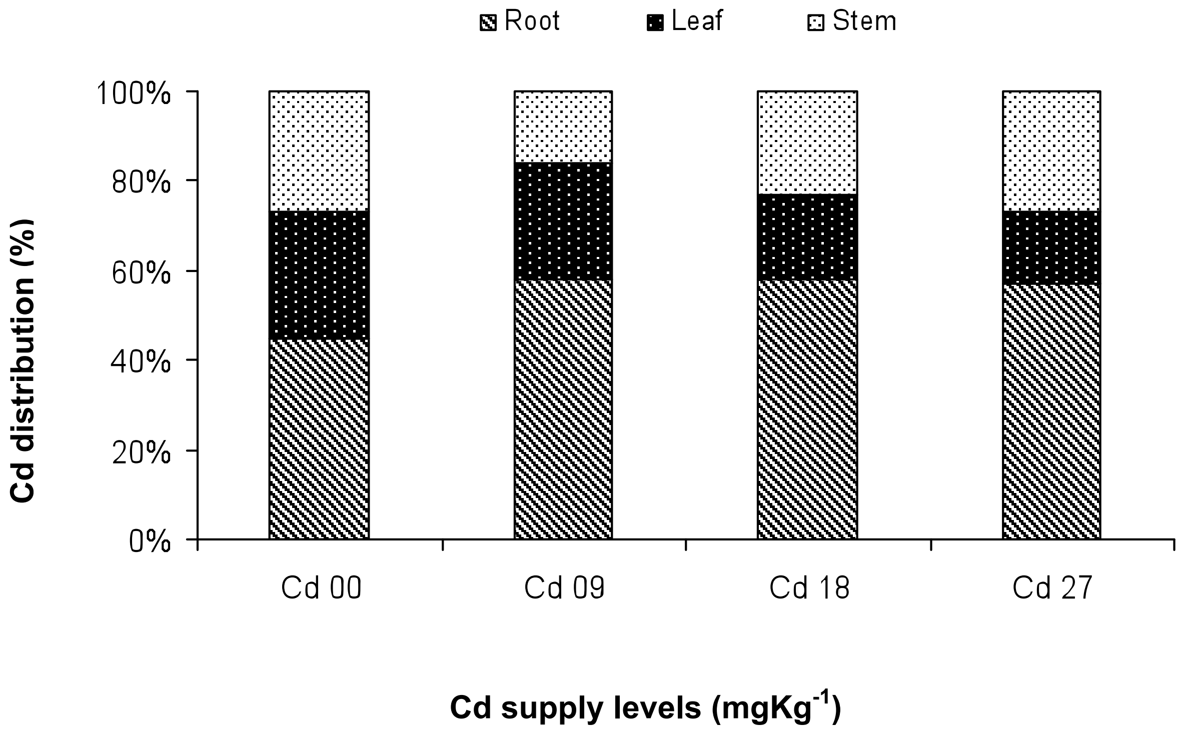

Figure 4. Cd distribution in various plant organs of *L. uncinatus* after 14 weeks of growth at different Cd levels.

IV. CONTAMINATION TIME EFFECT ON PLANT AVAILABLE FRACTIONS OF CADMIUM AND ZINC IN A MEXICAN CLAY LOAM SOIL.

Key words: DTPA extractable Zn, Cadmium, adsorption, plant available heavy metals.

ABSTRACT

Knowledge of plant available fractions of heavy metals in soil can assist directing phytoremediation efforts for contaminated soils. For this reason different doses of $ZnCl_2$ (0, 200, 400 and 600 mg kg^{-1}) and $CdCl_2$. 2 $\frac{1}{2}$ H_2O (0, 9, 18 and 27 mg kg^{-1}) were applied to an uncontaminated slightly acidic clay loam soil from State of Mexico, Mexico, incubated under ambient temperature and humidity for 90 days. Both the metals were extracted with a DTPA-TEA-$CaCl_2$ mixture after 1, 5, 15, 25, 60, and 90 days and analyzed by atomic absorption spectrophotometry. The results showed that DTPA extractable contents of Zn and Cd followed a decreasing trend with increase in incubation time. Maximum contents were found at day 01 in all the treatments. After 15 days of incubation, the variation in extractable contents was non-significant. The rapid adsorption of the metals might be due to elevated clay content (34%) of the incubated soil.

INTRODUCTION

An accurate description of the complex interactions of heavy metals in soils is a prerequisite to predicting their behavior in the vadose zone. Specifically, to predict the fate of heavy metals in soils, one must account for retention and release reactions of the various species in the soil environment.

Heavy metals in soils can be involved in a series of complex chemical and biological interactions including oxidation-reduction, precipitation and dissolution, volatilization and surface and solution phase complexation (Selim and Amacher, 2001).

In the soil heavy metals may occur 1) on ion exchange sites, 2) be incorporated into or on the surface of crystalline or non-crystalline inorganic precipitates, 3) be incorporated into organic compounds, or 4) be in soil solution. Most researchers have recognized that heavy metals are sparingly soluble and occur predominantly in a sorbed state or as a part of insoluble organic or inorganic compounds. Due to low solubility of the heavy metals in soils, their movement in soil profile has been considered usually small (Amaral Sobrinho et al.,1998). The soil acts as a reservoir of heavy metals and also serves as a filter protecting the underground water against these metals thus contributing significantly to reduction of environmental contamination risks (Clarice de Oliveira et al., 2003).

Zinc in soil that is water soluble and adsorbed on exchange sites of colloidal materials is considered to be phytoavailable. It is a consensus that phytoavailable Zn in soil can best be predicted by the use of extractants that remove only a fraction of the total amount. Despite its environmental and financial importance there is no agreement in the literature as to which extractant most accurately estimates the phytoavailability of trace metals in soils (Menzies et al., 2007). More recently, the DTPA-extractable Zn has gained more attention as a diagnostic tool in the Zn nutrition of plants. Whether the soil is sludged (Bidwell and Dowdy, 1987; Bell et al., 1991) or just ordinarily mineral arable soil (Singh et al., 1987.,Dang et al., 1993), the DTPA is the extractant of choice. While a number of researchers have advocated the use of DTPA as an apropriate extractant (Sukkariyah et al., 2005, Keller et al., 2005; Nascimento, 2006, Zhang et al., 2006) the others do not find it very useful for evaluating the bioavailability of metals (Basar, 2005; Feng et al., 2005; Ortiz and Alcaniz, 2006, Menzies, et al., 2007).

Ample literature on extractants used to measure phytoavailable Zn (e.g., salt solutions, acids, bases, and chelates) exists. The needs vary by country, crop, and soil type. A soil test using one of the extractants is done to 1) determine if the Zn level is sufficient and the soil requires no fertilization with Zn, and 2) determine if the Zn level is excessive and will cause phytotoxicity, as in the case of sludged soils or soils heavily contaminated. In the latter case, management strategies should be part of any operational scheme, including adjustment of soil pH, adding amendments or selecting tolerant plant species (Adriano, 2001). Extractable Zn has been positively correlated with total Zn, organic matter, clay content, and cation exchange capacity (Follet and Lindsay, 1970) and inversely correlated with free $CaCO_3$, soil pH, and base saturation (Adriano, 2001).

On the other hand, special attention should be paid to Cd pollution in the soil-plant system, due to its mobility in soil and the small concentration at which its toxic effect begins to show (Zornoza, et al., 2002). In most soils, more than 99% of the Cd content is associated with the solid phase and less than 1% is found in the soil solution. As Cd leachability and availability to plants conceptually are related to Cd in the soil solution and to pools of solid Cd that can be made available through the soil solution, it is mandatory to understand and quantify the solid phase Cd and the reactions and processes that govern the distribution of Cd between the solid phase and the soil solution. Adsorption is considered to be the dominating process governing Cd distribution between soil solid phases and the soil solution. The term adsorption accounts for the many different processes that may actively bind Cd to the solid phases of soils. Desorption describes the reverse process that releases Cd from the solid phases into the soil solution. The relationship between these two processes determines the distribution of Cd between the solid phases of the soil and the soil solution. Several factors related to the characteristics of solute can affect the actual distribution of Cd in the soil such as pH, cationic composition, competing trace metals and organic and inorganic ligands (Christensen and Huang, 1999).

Since the bioavailability of free metal in soil solution is regulated by the adsorption processes in organic and inorganic soil fractions, this study was aimed at understanding phytoavailability of soil Zn and Cd in terms of their DTPA extractable contents in response to various doses of these metals applied to the incubated soil as influenced by time intervals.

MATERIALS AND METHODS

An unpolluted soil sample was obtained from the top 25 cm layer of a crop field at San Pablo Ixayoc, State of Mexico, Mexico (19^0 23' 43''and 19^0 28' 37'' North and between 98^0 42' 51 '' and 98^0 48' 12'' West; Figure 1, Chapter 1).The soil was air dried, ground and passed through a 2 mm sieve and analyzed for its total Cd and Zn content and other physical and chemical properties (Table 1). The experiment was undertaken in April-June, 2006.

Unicel containers with 7 cm diameter were filled with 500 g of soil and incubated at ambient temperature and humidity. The temperature oscillated between 25 and 35 °C during the course of experiment. The moisture level was maintained at 70% of soil water holding capacity and deficient water was replenished by weighing the containers every 2-3 days. Distilled water was used for this purpose. The experimental units were arranged in a randomized design with four treatments for each metal and three replications. The containers were re-randomized five times in the duration of the experiment. Zinc was applied in the form of $ZnCl_2$ at the rate of 0, 200, 400 and 600 mg kg^{-1} while Cd in the form of $CdCl_{2.}\ 2_{1/2}\ H_2O$ at the rate of 0, 9, 18 and 27 mg kg^{-1}. The selection of the doses was based on the fact that in a previous study the same doses were applied to the soils used in this experiment for assessing Zn and Cd phytoextraction potential of *Lupinus uncinatus* Schldl. Zinc and cadmium were extracted with 0.005M DTPA, 0.1 M TEA and 0.1 M $CaCl_2$ (Soil analysis-Hand book of reference methods, 2000) in all the samples after 1, 5, 15, 25, 60 and 90 days of soil incubation with the metals. The concentrations of the two metals in the extract were analyzed using atomic absorption spectrophotometry (Perkin-Elmer, 3110). The results were expressed on oven dry weight basis. Data were subjected to one-way ANOVA followed by a Tukey test for multiple comparison of means (Significance level $P < 0.05$). All the statistical tests were performed using SAS version 9.1 (SAS, 2000).

RESULTS AND DISCUSSION

After incubation of different applied doses of $ZnCl_2$ for 1, 5, 15, 25, 60, and 90 days, the DTPA extractable Zn contents obtained are given in Figure 1. Generally DTPA extractable Zn decreased with increase in incubation time. The magnitude of DTPA extractable Zn was found proportional to the dose of metal applied to the soil. Maximum content was found at day 01 in all the treatments, followed by significant decrease at day 05 and day 15 while during the rest of the incubation period the difference in the DTPA Zn was non-significant statistically ($p<0.05$). These results suggest that Zn was sorbed by soil constituents rapidly as mentioned by Adriano (2001) who found that usual amounts of Zn applied were adsorbed almost completely within a short period of time. A similar decrease in DTPA extractable Zn was reported by Kandpal et al. (2004) who studied effect of metal spiking and incubation on sequentially extractable pools of various heavy metals. Romero et al. (2007) observed that adsorption and coprecipitation on Fe-precipitates played an important role in the mobilization and attenuation of Zn, Cd, Cu and As within the abondoned lead/zinc mine tailings in central-southern Mexico. Sorption of Zn in soils is an important factor governing Zn concentrations in soil solution and Zn bioavailability to plants. Clay minerals, hydrous oxides, carbonates, pH, cation exchange capacity, organic matter and soil type are known to affect soil sorption of added Zn.

Since the incubated soil had a high clay content (Table 1) and consequently high density of OH groups, favoring specific adsorption of Zn through covalent bonds (Clarice de Oliveira et al., 2003). While studying the effects of soil properties on Zn adsorption, Shuman (1985) found that soils with high silicate clay content or organic matter possess high Zn adsorption ability as compared to sandy soils with low organic matter content.

Simple organic compounds such as amino acids and phosphoric acid as well as low molecular weight fulvic acid are efficient complexing and chelating agents of Zn, favoring its high mobility (Alloway, 1995), while the incubated soil in this study with low organic matter (Table 1) facilitated Zn adsorption.

While observing DTPA extractable Cd contents during the period of incubation (Table 2), a similar significant reduction in extractable Cd content was observed at day 05 followed by non-significant decreases until the end of the incubation period. Adsorption is the operating mechanism of the reaction of Cd at low concentrations with soils. Using loamy sand and sandy loam soils, sorption of Cd was demonstrated as a fast process where > 95% of the sorption took place within the first 10 minutes and equilibrium was attained within an hour (Christensen, 1984).The presented data highlights one important fact that the plant available Cd content was highest during first five days after its application to the incubated soil. However a continuous decrease in DTPA extractable Cd was observed throughout the incubation period thus showing persistent soil sorption of the metal. Adriano (2001) enlists several factors which may influence the degree by which Cd is adsorbed on soil surfaces. Some of them are pH, ionic strength, exchangeable cations, competing trace metals, organic and inorganic ligands and natural properties of soil components. Gray and Maclaren (2006) described that the importance of soil properties such as soil pH, total carbon and oxalate extractable Fe and Al oxides varied between heavy metals such as Ni, Cu, Cr, Pb, Cd and Zn. Metal solubility decreases in soil as the $CaCO_3$ content and soil pH increases while organic matter promotes the availability of metals by supplying complexing agents that interfere with the fixation of these metals (Mani and Kumar, 2005). Iron and manganese oxides and clay minerals like gibbsite, chlorite, smectite and goethite are the soil components with greater effect in the reduction of heavy metal availability (Vega et al., 2004). The results obtained in the present study provide valuable information on metal availability in contaminated soils and offer an indication of the potential risk a metal may pose to a given soil environment, along with providing a basis for developing soil quality guidelines for the prevention, investigation and clean-up of soil metal contamination.

CONCLUSIONS

The accumulation of heavy metals in plants is related to concentration and chemical fractions of the metals in soils. Understanding chemical fractions and availabilities of the metals in soils is necessary for their management. The sorption-desorption behaviour of Cd and Zn assist prediction of their biovailability and toxicity in the subsurface environment. The results from this study provided an insight into the plant available fraction of Cd and Zn over a 90 days incubation period and made a quantitative assessment of their soil mobility possible. Both the metals had maximum plant available content within 24 hours of their application to the soil which decreased abruptly after this period. Hence these findings will be relevant to future research efforts on exploring the phytoremediation potential of some native plant species for such soils.

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Parameter	Value	Method
pH	6.29	2:1 Water
E.C.	0.16 dSm^{-1}	Conductivity meter
Organic Matter	1.19%	Walkley and Black
Particle size Distribution/ Soil textural class	Sand (34%) Silt (32%) Clay (34%)/clay loam	Hydrometer method.
Bulk Density	1.05 gcm^{-3}	
Total Cd content	0.16 mg kg^{-1}	AAS*
Total Zn content	65 mg kg^{-1}	AAS*

*Atomic Absorption Spectrophotometry.

Table 1. Some physical and chemical ´properties of incubated soil.

Treatment (mg Cd kg^{-1})	Days after incubation					
	1	5	15	25	60	90
00	0.40 a (0.06)	0.41a (0.06)	0.38 a (0.09)	0.43 a (0.06)	0.34 a (0.05)	0.43 a (0.05)
09	7.33 a (0.57)	3.66 b (1.15)	2.33 bc (0.57)	2.33 bc (0.57)	1.16 c (0.76)	0.76 c (0.25)
18	16.6 a (2.51)	7.0 b (2.00)	3.7 bc (1.20)	3.0 bc (1.00)	2.3 c (0.60)	1.5 c (0.40)
27	31.0 a (3.00)	10.3 b (2.10)	6.7 bc (1.50)	6.0 bc (1.00)	4.3 c (1.50)	3.0 c (0.20)

Table 2. DTPA extractable Cd at various time intervals in response to different doses of Cd applied to incubated soil. Means in the same row followed by different letters differ significantly ($P<0.05$) according to Tukey test. Values within parenthesis represent standard deviation (n=3).

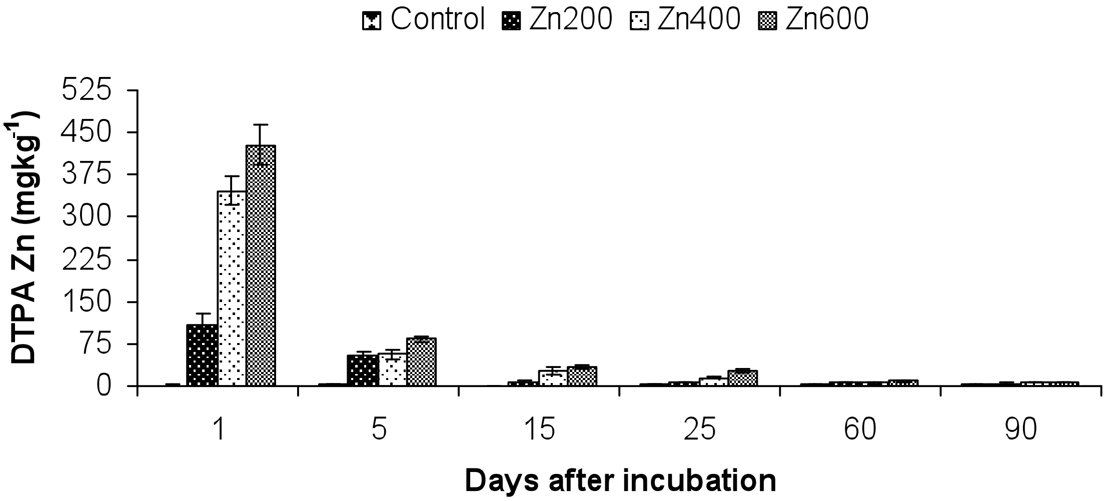

Figure 1. Effect of various Zn doses on DTPA extractable Zn as influenced by time intervals.

V. ZINC AND CADMIUM UPTAKE BY *Lupinus uncinatus* Schldl. GROWN IN NUTRIENT SOLUTION

Key words: Lupin plants, Hydroponics, Heavy metals, Phytoextraction.

ABSTRACT

The effects of Zn and Cd on the growth, mineral composition (P, K, Ca, Mg. Fe, Zn, Mn and Cu) and metal accumulation by *Lupinus uncinatus* Schldl. were investigated in a hydroponic experiment. Plants were exposed to increasing concentrations of Zn (0, 30, 40, and 50 µM) and Cd (0, 3, 4, and 5 µM) for one week. The species showed different patterns of metal accumulation and distribution in the plant parts suggesting different mechanisms of metal tolerance for each metal. Significantly higher concentrations of both metals were accumulated in the harvestable biomass as compared to control. At the highest doses of Zn and Cd , the amounts of respective metal accumulated in roots, stems and leaves were 1289, 1918, and 1132 mgkg^{-1}dry matter and 2467, 227, and 164 mgkg^{-1}dry matter respectively. The shoot:root Zn ratios obtained for 50, 40, 30 µM treatments were 2.36:1, 2.28:1 and 2.32:1 respectively while the same ratio in case of Cd remained < 1 for the three Cd treatments. No significant effect on plant dry biomass was observed in both the cases. Significant changes in plant mineral composition were found, however, concentrations were generally above the deficiency levels.

INTRODUCTION

Heavy metals and metalloids are an increasing environmental problem worldwide. Some industrial activities and agricultural practices increase their level in the substrate, and the possible introduction of these elements in the food chain is an increasing human health concern (Cakmak et al.,2000). Unlike organic compounds, metals cannot be degraded, and the cleanup usually requires their removal. However this energy intensive approach can be prohibitively expensive. In addition, the metal removing process often employs stringent physicochemical agents which can dramatically inhibit soil fertility with subsequent negative impacts on the ecosystem. Phytoremediation has been proposed as a cost effective, environmental-friendly alternative technology (Lasat, 2002).

Heavy metal-tolerant plants belonging to genus *Alyssum*, *Thalspi* or *Silene* have been identified for a long time (Brooks, 1998) and their use for phytoextraction purpose has been recommended (Schwartz et al., 2003; Zhao et al., 2003). Most hyperaccumulators, however, are difficult to manage and have a shallow root system, and their interest is, therefore, limited in the case of deep contamination (Keller et al., 2003).

The use of deep rooting *Lupinus* species is of particular interest in this context. These plants have shown a relatively high tolerance to various environmental stresses, nitrate excess, low root temperature, detopping (De Lorenzo et al., 1993; Ianetta et al.,1993), lime excess (Tang and Robson, 1993), and salinity (Fernandez-Pascual, et al., 1996). Some *Lupinus* species are able to accumulate Zn (Pastor et al., 2003) Cd (Brennan and Bolland, 2003), Mg, Al (Reay and Waugh, 1981), Hg (Vera et al., 2002), and Pb and Cr (Ximenez-Embun et al., 2001). However to the best of our knowledge, no data are available concerning metal accumulation in *L. uncinatus.*.

The first step to assess the potential interest of a plant species for phytoextraction is to quantify, in a controlled environment, the mean level of toxic metal accumulation in relation to the growth rate. The present study deals with Zn and Cd, two elements sharing numerous similar chemical properties that are often present concomitantly in polluted areas. Accumulation of these elements was quantified in *L. uncinatus* Schldl. Plants were exposed to a nutrient solution with varying treatments of Zn or Cd to determine the specific toxicity of these elements in relation to their level of accumulation. Moreover, the effect of these metals on nutrient concentration and distribution of Zn and Cd in various plant parts were studied.

MATERIALS AND METHODS

Plant material and growth conditions

A nutrient solution study was conducted to define patterns of Zn and Cd uptake by *L. uncinatus* at varying solution concentrations of Zn and Cd. Seeds were collected from the plants growing naturally on the mountainside in San Francisco, State of Mexico, Mexico (Figure 1, Chapter 1). The study was conducted in a greenhouse where the average day and night temperatures were 33^0C and 10^0 C respectively.

Seeds were placed in plastic pots where they germinated after 4-6 days. The plants were allowed to grow in pots for 6 weeks before they were transplanted to each 2 liter plastic buckets containing the nutrient solution of the following composition.

Compound	Concentration
KH_2PO_4, mM	3.24
KNO_3, mM	0.64
NH_4NO_3, mM	6.87
$Ca.Cl_2.2H_2O$, mM	3.75
$MgSO_4.7H_2O$, mM	2.07
$H_3BO_{3,}$ µM	10
$MnCl_2.4H_2O$, µM	3
$ZnSO_{4.}7H_2O_,$ µM	1
$CuSO_4.5H_2O_,$ µM	0.3
FeEDTA (10% Fe), mg	50

Plants were maintained in the growth medium for an additional one week before treatments were initiated. Four plants were placed into each of the 2-L plastic buckets for the experiment. The buckets were continuously aerated with the help of an air pump and in a completely randomized designed with five replications. Buckets were filled with the previously described solution minus Zn and Cd. Four treatments each of Zn and Cd were then applied separately as $ZnCl_2$ and $CdCl_2$. $2_{1/2}$ H_2O respectively. Zinc and cadmium treatments were 0, 30, 40, and 50 µM Zn and 0, 3, 4, and 5 µM Cd.respectively. Normal concentrations of zinc for nutrient solutions range between 0.75 µM and 1 µM (Brown et al., 1995; Lutts et al., 2004). In most uncontaminated soils water soluble Zn and Cd are < I µM (Brown et al., 1995). The Zn treatments were thus based on this criterion of basal Zn concentration while those of the Cd were assigned keeping in view Zn treatments as reference. Solution pH was maintained near 6.0. All plants were equally replicated among all the treatments. Deionized water was added to buckets to maintain solution volume daily. The solution was replaced completely every week.

Harvest

Eight days after application of treatments, all the plants were harvested in all the treatments. At harvest, roots, stems, and leaves of each plant were separated and weighed for fresh weight determination. Roots were rinsed in ionized water and then gently blotted dry. Dry weight was determined after 5 days of incubation in an oven at 70 ^{0}C.

Sample analysis

For each sample, digestion of dry matter (approximately 50 mg) was accomplished at 80 ^{0}C with nitroperchloric acid. The concentrations of P and K were determined by flame photometry while Ca, Mg, Zn, Fe, Cu, Mn and Cd were determined through atomic absorption spectrophotometry. In Zn treatments due to insufficient plant dry matter 3 replicates of all the plant organs were analyzed for elemental composition while in case of Cd it happened only in case of root samples.

Statistical treatment

Data obtained from each type of organ were analyzed by a two–way analysis of variance performed with the model procedure of SAS version 9.1 (SAS, 2000). If the F value indicated significant differences ($P<0.05$), mean differences were compared according to the Tukey Studentised Range (HSD) test.

RESULTS AND DISCUSSION

Plant survival and Visual toxicity symptoms

Two days after the initiation of treatments, the lower leaves of the plants receiving 50 µM Zn and 5 µM Cd treatments started exhibiting leaf wilting. The leaves also had necrotic spots. The most conspicuous and widespread symptom of toxicity was bronzing of affected leaves accompanied by tip necrosis and inward curling, starting with the older leaves but progressing up the plant with time.

The toxicity symptoms became more severe with increasing Zn and Cd levels and exposure time. The 50 µM Zn and 5 µM Cd treatments were the first to show these symptoms where the leaf wilting and fall of leaves was severe followed by plant death. At the end of one week exposure to different metal doses, all the plants died in 50 µM Zn and 5 µM Cd, while in 40 µM Zn and 4 µM Cd the plants survived but showed signs of toxicity. In the lowest dose of both Zn and Cd the toxicity symptoms were minimum with some purpling of older leaves.

Concentration of Zinc and Cadmium in plant tissues

After one week of exposure to various doses of Zn, concentrations of this metal accumulated in different plant organs by *L. uncinatus* are given in Figure.1**.** Generally significantly higher concentrations accumulated in various Zn treatments as compared to control. Although a considerable amount of Zn was accumulated in roots, accumulation of this element was significantly higher in aerial parts (stem+leaves). Having grown one week in the presence of 50 µM Zn, Zn constituted 1289, 1918, and 1132 mgkg^{-1}dry matter in roots, stems and leaves respectively. For the 40 µM Zn treatment amounts of Zn accumulated in roots, stems, and leaves were 1128, 1690, 885 mgkg^{-1}dry matter respectively. While the lowest Zn treatment (30 μM Zn) yielded 737, 1051, and 661 mg Zn kg^{-1} dry matter in roots, stems, and leaves respectively. Although the tissue Zn concentration increased with the Zn content of the growth medium, no significant difference was found in 50 μM Zn and 40 μM Zn treatments.

Cd concentrations in plants followed different pattern than Zn concentration (Figure 2). The major portion of Cd accumulated was retained by roots. Root Cd concentrations were 175, 2467, 2045, and 1424 $mgkg^{-1}$ for 0, 5, 4, and 3 μM Cd. All the treatments showed significantly higher Cd accumulation in various organs of the plant as compared to the control ($P < 0.05$). In view of the results obtained in this study Cd uptake by the Lupin plants was a function of the Cd concentration in the growth medium. A consensus exists that over a certain range, there is a positive, almost curvilinear, correlation between the levels of Cd in the medium and the resulting Cd concentration in the plant tissues (Adriano, 2001).

Zn and Cd translocated

Total Zn and Cd translocated to shoot (stem+leaves) tissue (Concentration of metal in dry matter x dry weight) was calculated**.** The maximum amount of Cd translocated to shoot, 0.4 mg $Cdpot^{-1}$(Figure 3), by *L. uncinatus*. was found in case of 5 μM Cd treatment which represented 60% more as compared to control. However there was no significant difference in the amount of Cd translocated among various treatments with added Cd. Cadmium is rather readily translocated throughout the plant following its uptake by the roots. Distribution between roots and shoots differs with plant species, rooting medium and time of treatment (Adriano, 2001).

In case of Zn (Figure. 4**)** the amount translocated to harvestable biomass was much greater when compared to the control plants. For example the plants exposed to 50 μM Zn translocated 252% more Zn to the shoots as compared to control. The magnitude of Zn translocation increased with the increasing Zn solution concentration. These results suggest that this species can be a candidate for Zn phytoextraction.

Shoot/root partitioning of Zinc and Cadmium

Shoot:root ratios of metals are < 1 when non tolerant or indicator species are grown in contaminated soils (Peterson, 1983; Baker and Walker,1990). With regard to shoot:root Zn ratio, *L. uncinatus* exhibited ratios >1 in all the treatments (Table. 1). These results indicate that with increasing Zn concentration in solution, the ability of the plant to translocate Zn to its aerial parts decreased. However, the fact that it remained >1 shows hyperaccumulation potential of this species for Zn.

This pattern of uptake was not followed by *L. uncinatus* in case of Cd (Table 2). The shoot:root Cd ratios remained <1 in all the treatments where Cd was added to the nutrient solution. However in both the metals, the partitioning between shoot and root was lower than control which suggest that exposure to heavy metal concentrations tend to decrease the ability of the plant to transport the same to its aerial parts. In case of Cd, lower uptake in shoot may also be a mechanism to avoid Cd toxicity as also concluded by Zornoza et al., (2002). In general, roots contain at least twice the Cd concentration found in shoots (Koeppe, 1977). Chino and Baba (1981) while studying Cd uptake by rice observed that some environmental factors such as Cd concentration in the medium, ambient temperature, and light intensity can affect the distribution of the metal between the roots and the shoots. The findings from the present study also point towards the fact that the specialized mechanisms responsible for Zn uptake in *L. uncinatus* does not control Cd uptake. The specificity of metal uptake mechanism has also been reported by Brown et al. (1995) for *Thlaspi caerulescens.* Although the present experiment did not examine uptake of Zn and Cd when supplied concomitantly, different accumulation patterns for the two metals from solution to root cell plasma may suggest that different mechanisms are involved for each metal.

Zn and Cd distribution in various plant parts

Separation of the plant tissue of *L. uncinatus*. into leaves, stems and roots and subsequent analysis showed that distribution of Zn and Cd in plants parts was consistent across the treatments. Significantly higher concentrations of Zn were accumulated in stem tissues than leaf or root irrespective of the treatment (Figure 5). It appears that stem accumulation of Zn is one of the plant response mechanisms to imposed Zn stress as we also observed enhanced stem Zn concentration in pot experiment with the same species (Chapter 2). Changed partitioning of metals in various organs of the plant as a response to metal stress has been reported by other authors as well (Brown et al., 1995, Pastor et al, 2003).

Cadmium concentrations in leaf and stem tissue were significantly lower than for roots in all the Cd treatments (Figure 6). The pattern of Cd distribution between root, stem and leaves remained consistent over the range of treatments where Cd was applied to the growth medium. However, control showed different trend of Cd distribution in various plant organs. This may suggest that the observed ´pattern of Cd partitioning is a plant strategy to counter toxicity as also hinted by Zornoza et al., (2002).

Heavy metal accumulation and mineral nutrition

Zn treatments

No significant effect on P concentrations was recorded in response to various Zn doses irrespective of the plant part (Table 3). Phosphorus concentrations in plant aerial parts ranged from 0.3-0.5% of dry matter which is P requirement for optimal growth during vegetative stage of growth according to Marschner (1995). These results, thus, indicate that the P nutrition requirements of the plants were adequately met. The exposure of *L. uncinatus*. to 30, 40,and 50 µM Zn did not significantly affect stem and leaf K concentrations. A significant reduction in root K, however, was observed in the Zn treatments as compared to control which caused a significant increase in shoot:root varying from 1.7:1 (control) to 4.9:1 (30 µM Zn), 4.3:1 (40 µM Zn) and 4.9:1 (50 µM Zn). These results are in agreement with the findings of kidd et al (2004) who reported an increase in transport of K to shoot tissue of *Cistus ladanifer* when grown in a nutrient solution in presence of various heavy metals such as Cd, Cr, Mn and Pb. They attributed this increase to the maintenance of a charge balance between organic acid production and metal complexation in the leaf cells.

As also reported by Bernal and MacGrath (1994), Zn treatments in this experiment significantly increased shoot Ca, Mg and Mn compared with the roots showing transport of these elements from the root system to the aerial parts. This is also clear form the higher shoot:root ratios for both the elements (Table 4). Tolra et al., (1996) reported the same pattern of enhanced Ca and mg shoot:root ratios for *T. caerulescens* when grown in nutrient solution at various solution Zn concentrations ranging from 100-1500 µM. The ability of a plant to maintain the concentrations of essential elements within the range considered adequate for growth in normal plants, has been termed as an accompanied trait along with phytoextraction of metals (Tolra et al., 1996).

Fe content of leaves was reduced as compared to control however the effect was not significant statistically (Table 3). Lutts et al., (2004) reported reduced Fe concentrations in stem of *Atriplex halimus* L. in response to 0.1mM Zn in solution culture. However they did not note any inhibition in iron uptake in spite of a reduced translocation from roots to shoots. Tolra et al (1996) found no effect on shoot Fe in *T. caerulescens* plants exposed to 750, 1000 and 1500 µM Zn in nutrient solution. Zn doses significantly increased leaf Cu content while the stem Cu concentrations were reduced as compared to control.

Cd treatments

Cd treatments did not produce any significant effect on P, Ca and Mn concentrations irrespective of the plant part (Table 5). Phosphorus requirement for optimal growth is in the range of 0.3-0.5% of the plant dry matter during the vegetative stage of growth (Marschner, 1995). Shoot P concentrations in this study were in the range of 0.75-0.8% of dry matter which shows that *L. uncinatus* did not suffer from P deficiency.

In some non-tolerant species, exposure to heavy metals, such as Cu, results in disruption of the root plasma membrane (Ernst et al., 2000). After only a short period this can lead to K^+-leakage, and a reduction in root K concentration. In the present research, the root concentration of K was significantly reduced after exposure to 4 and 5 µM Cd which could have resulted due to K^+ leakage from the disrupted root plasma membrane. Shoot:root K increased from 5.79:1 (control) to 6.53:1 (4 µM Cd) and 9.79:1 (5 µM Cd) (Table 6) which clearly shows that *L. uncinatus* maintained and increased K transport to shoot at Cd levels imposed in this study. Potassium is a dominant cation in ionic charge balance in cells. Plants can produce increased concentrations of organic acids to complex foliar metals such as Zn and Cd (Marschner, 1995) and the increased transport of K to the shoots observed in this investigation could be in order to maintain a charge balance between acid production and metal complexation in the leaf cells (Kidd et al., 2004).

Cd treatments increased Zn concentration in root and leaf tissue while decreased its content in stem as compared to control. Shoot:root Zn, thus, decreased from 1.38 (control) to 0.87, 0.89 and 0.81 in 3, 4 and 5 µM Cd treatments respectively (Table 6) which suggest an antagonistic effect of Cd on Zn uptake–transport processes, since both may be transported by a similar carrier mechanism (Bernal and MacGrath, 1994). A similar increase in root and leaf Zn as a response to 3 week exposure to 0.1 mM Cd in nutrient solution has been reported by Lutts, et al. (2004) in *Atriplex halimus.* Moreover, Cd and Zn appear to compete for certain organic ligands *in vivo*. Because Cd competes with Zn in forming protein complexes, a negative association between the two can be expected (Adriano, 2001).

Leaf Mg increased significantly as compared to control as a result of Cd treatments which indicated that Cd increased Mg transport to the shoot. Root Cu decreased significantly at 3 µM Cd while increased at 4 µM Cd and no significant difference as compared to control observed at 5 µM Cd. Stem Cu decreased at 3 µM and 4 µM Cd but increased at 5 µM Cd.

High levels of Fe were retained in the roots, indicating that Fe transport to the shoots was reduced (Table 6). This reduction in Fe translocation to the shoot appears to be a common feature in plants after exposure to heavy metals. Bernal and McGrath (1994) reported a similar retention of Fe in the roots of *Alyssum murale,* a nickel hyperaccumulator when grown in a nutrient solution at 120 and 8.8 µM Zn and Cd respectively. Liu et al., (2000) also found that exposure of mung bean plants to 5 µM of Cd, Cu, and Ni produced the same effect on Fe transport. In another experiment by Ebbs and Kochian (1997) 100 µM Zn reduced shoot Fe in three *Brassica* species grown in hydroponic solution.

Fodor et al. (1996) demonstrated that in cucumber (*Cucumis sativus* L.) a strong inhibition of photosynthesis may result from Fe deficiency in Cd-treated plants. While 10 µM Cd reduced by more than 90% the leaf Fe concentration of *Cucumber*, Lutts et al., (2004) found that 0.1 mM Cd had no effect at all on the leaf Fe concentration of *Atriplex halimus* .

Plant fresh weight and dry matter yield as affected by heavy metals

No significant effect on plant fresh weight and dry matter yield was observed as a result of one week exposure to varying Cd and Zn solution concentrations. It may indicate that the metal concentrations used in this study were not high enough to affect the biomass production of *L. uncinatus*. Another explanation for the sustained biomass yield could be the shorter exposure period and the growth stage of the plant (6 weeks old) when the meal stress was imposed. However it is of vital importance to carry out further studies to investigate the mechanisms involved in Zn hyperaccumulation by this species. *Lupinus* represents a huge diversity of species the majority of which is still to be investigated. The native wild species of this genera conserve immense genetic potential to be harnessed. *L. uncinatus* is one of the native Lupinus species and the purpose of the present experiment was to explore its potential for heavy metals hyperaccumulation and tolerance.

CONCLUSIONS

The objective of the present study was to carry out a preliminary test to determine the relative performance of *L. uncinatus*, their degree of tolerance to various concentrations of bioavailable Zn and Cd, and their patterns of metal accumulation. The hydroponics allows for these plant responses to be quantified in a rapid and simple manner. The shoot:root Zn ratios obtained in this experiment without any dry matter loss show that *L. uncinatus* has the potential to hyperaccumlate Zn. The toxicity symptoms were only seen at the highest Zn treatment. However in case of Cd the behavior of the plant was different. Although it tolerated 3 and 4 µM Cd treatments without any significant loss of dry matter yield, the bulk of the metal accumulated in the roots and the pattern of some essential nutrients such as K, Mg, Zn, Fe, and Cu was altered.

This suggests that this species may tolerate Cd without its hyperaccumulation and shows exclusion mechanism of Cd tolerance. It must be emphasized here that although the hydroponic technique can provide a useful investigative tool, it does not accurately reflect the growth or patterns of metal uptake that would occur in the complex physicochemical and biological environment of soils. Further research, therefore, in the field conditions is necessary to confirm the trends obtained in this study.

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Table 1. Shoot:Root Zn concentration ratios of *L. uncinatus* after one week exposure to various Zn concentrations in nutrient solution.

Zn treatment (μM)	Shoot:Root Zn Ratio
0	4:1
50	2.36:1
40	2.28:1
30	2.32:1

Table 2. Shoot:Root Cd concentration ratios of *L. uncinatus* after one week exposure to various Cd concentrations in nutrient solution.

Cd treatment (μM)	Shoot:Root Cd Ratio
0	1.48
5	0.15
4	0.18
3	0.23

Table 3. Mineral nutrient distribution within leaf, stem and root tissues of *L. uncinatus* grown in nutrient solution with varying Zn concentrations. For given elements means followed by the same letter across treatments and within a plant part are not significantly different at ($P<0.05$). (ND= not determined).

Treatment	Plant	P	K	Ca	Mg	Fe	Mn	Cu
Zn (μM)	**organ**	**--------------------gkg^{-1}----------------------**				**----------------mgkg^{-1}----------------**		
00	**Leaf**	3.8 a	34.58 a	13.12 a	1.87 a	4366.3 a	2135.3 a	103.3 b
	Stem	5.78 a	27.30 a	5.77 b	5.92 b	ND	431.7 a	593.7 a
	Root	5.35 a	36.20 a	8.61 a	7.10 b	ND	4104 a	ND
50	**Leaf**	2.80 a	35.83 a	13.76 a	2.82 a	3561.3 a	2287.0 a	115.6 ab
	Stem	4.07 a	41.44 a	10.94 a	7.53 ab	ND	637.7 a	581.7 a
	Root	4.96 a	15.93 a	7.48 a	7.71 ab	ND	1435.0 b	ND
40	**Leaf**	3.08 a	34.43 a	12.40 a	2.58 a	3424.3 a	2165.0 a	121.6 ab
	Stem	3.91 a	42.30 a	10.60 a	8.36 a	ND	562.3 a	338.3 ab
	Root	4.17 a	17.74 b	6.21 a	8.24 a	ND	1071.0 c	ND
30	**Leaf**	3.25 a	35.87 a	11.81 a	2.54 a	3827.3 a	2085.0 a	135.0 a
	Stem	5.33 a	35.23 a	11.26 a	8.86 a	ND	630.0 a	308.3 b
	Root	3.93 a	14.31 b	6.75 a	5.56 c	ND	650.0 d	ND

Table 4. Shoot:Root concentration ratios of some nutrients as affected by one week exposure of *L. uncinatus* to various Zn concentrations in nutrient solution.

Zn treatment (μM)	K	Ca	Mg	Mn
0	1.7:1	2.2:1	1.1:1	0.62:1
50	4.9:1	3.3:1	1.34:1	2.03:1
40	4.3:1	3.7:1	1.32:1	2.54:1
30	4.9:1	3.4:1	2.1:1	4.17:1

Table 5. Mineral nutrient distribution within leaf, stem and root tissues of *L. uncinatus* grown in nutrient solution with varying Cd concentrations. For given elements means followed by the same letter across treatments and within a plant part are not significantly different at (P<0.05).

Treatment	**Plant**	**P**	**K**	**Ca**	**Mg**	**Zn**	**Fe**	**Mn**	**Cu**
Cd (µM)	**organ**	**---------------------- gkg^{-1}------------------------**				**------------------------- mgkg^{-1}---------------------------**			
0	**Leaf**	3.48 a	34.26 a	12.70 a	2.64 b	405.2 b	2940.6 a	1574 a	163.6 a
	Stem	4.59 a	40.84 a	8.00 a	6.76 a	725.6 a	1537.4 a	438.4 a	488.0 a
	Root	5.72 a	12.97 ab	7.15 a	5.14 ab	814.0 c	1735.7 c	1143.3 a	977.3 ab
5	**Leaf**	3.31 a	35.09 a	11.46 a	4.96 a	667.6 a	2703.6 a	1924 a	152.4 a
	Stem	4.24 a	39.18 a	7.05 a	5.27 a	570.8 ab	1408.4 a	465.2 a	472.8 ab
	Root	4.74 a	7.58 b	7.46 a	4.62 b	1533 a	3532.0 a	1299.3 a	945.0 ab
4	**Leaf**	3.08 a	33.38 a	12.95 a	4.0 ab	599.2 ab	2967.8 a	1840 a	223.4 a
	Stem	4.60 a	38.50 a	6.86 a	5.13 a	543.2 ab	1594.2 a	502.8 a	282.2 c
	Root	4.55 a	11.00 ab	10.09 a	5.04 ab	1282 b	3181 ab	1383.3 a	1086.0 a
3	**Leaf**	3.30 a	34.32 a	12.12 a	4.91 a	682.8 a	2640.6 a	1718 a	197.2 a
	Stem	4.43 a	44.18 a	7.92 a	6.11 a	411.2 b	1285.4 a	586.8 a	343.8 bc
	Root	4.64 a	14.25 a	9.42 a	6.03 a	1250 b	2818.7 b	1202.7 a	635.0 b

Table 6. Shoot:Root concentration ratios of some nutrients as affected by one week exposure of *L. uncinatus* to various Cd concentrations in nutrient solution.

Cd treatment (µM)	**K**	**Mg**	**Zn**	**Fe**	**Cu**
0	5.79:1	1.82:1	1.38:1	2.58:1	0.70:1
5	9.79:1	2.21:1	0.81:1	1.16:1	0.70:1
4	6.53:1	1.81:1	0.89:1	1.43:1	0.46:1
3	5.50:1	1.82:1	0.87:1	1.39:1	0.85:1

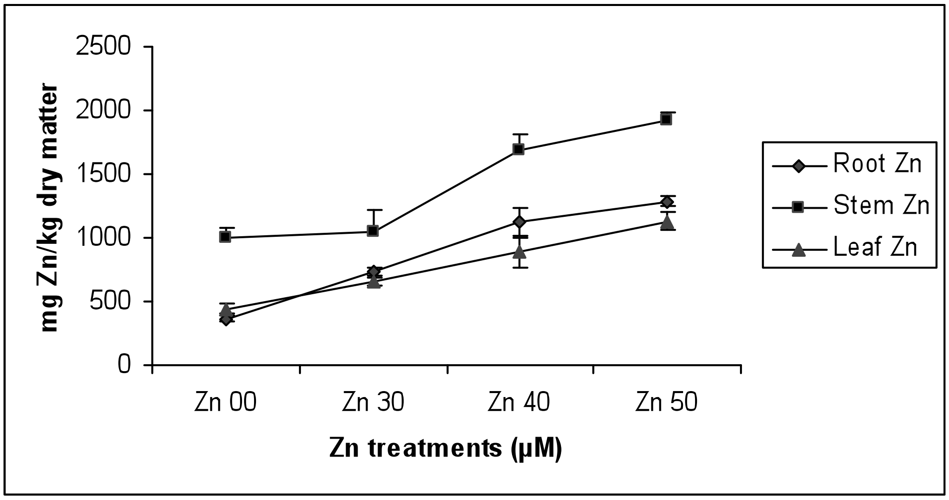

Figure 1. Zinc concentration in root, stem, and leaf *of L. uncinatus* grown in nutrient solution for one week with varying Zn concentrations. Bars represent standard deviations of values averaged across the treatments.

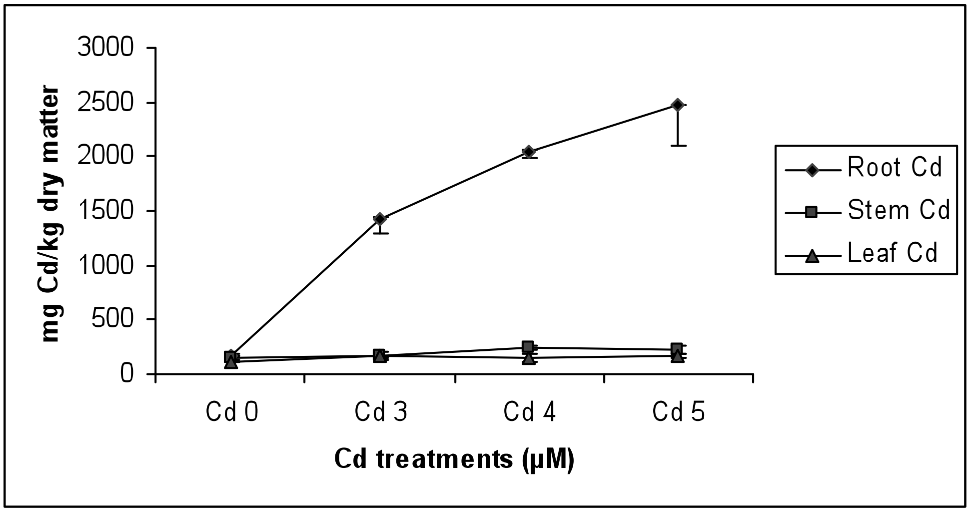

Figure 2. Cadmium concentrations in root, stem, and leaf *of L. uncinatus* grown in nutrient solution for one week with varying Cd concentrations. Bars represent standard deviations of values averaged across the treatments.

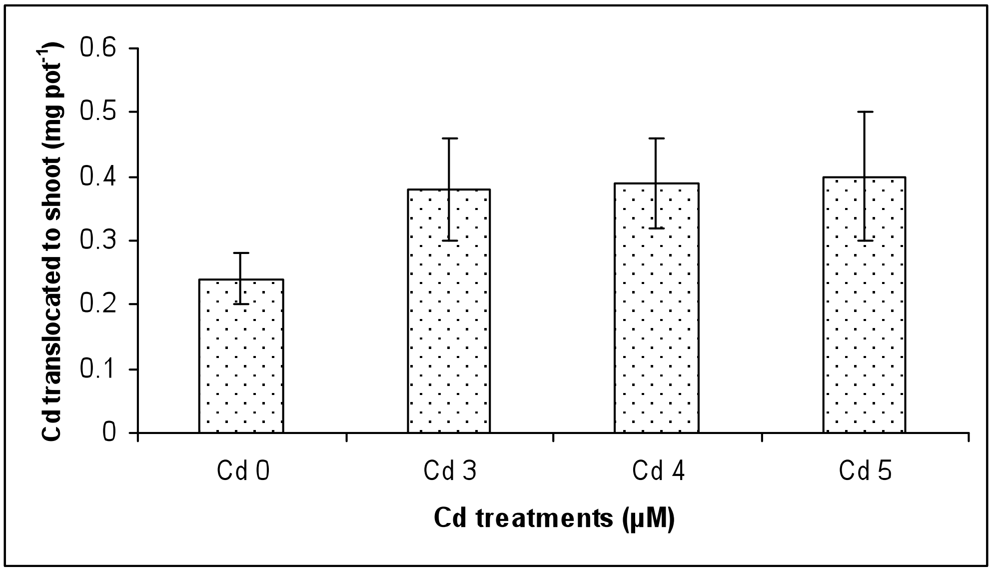

Figure 3. Cadmium translocated to harvestable biomass by *L. uncinatus* grown in nutrient solution at various Cd levels for one week. Bars represent standard deviations of values averaged across treatments

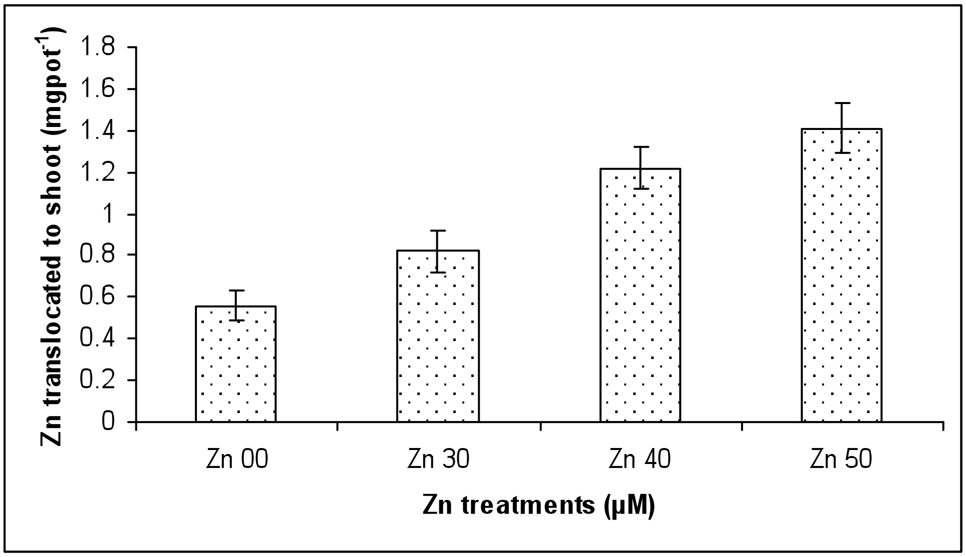

Figure 4. Zinc translocated to harvestable biomass by *L. uncinatus* grown in nutrient solution at various Zn levels for one week. Bars represent standard deviations of values averaged across treatments.

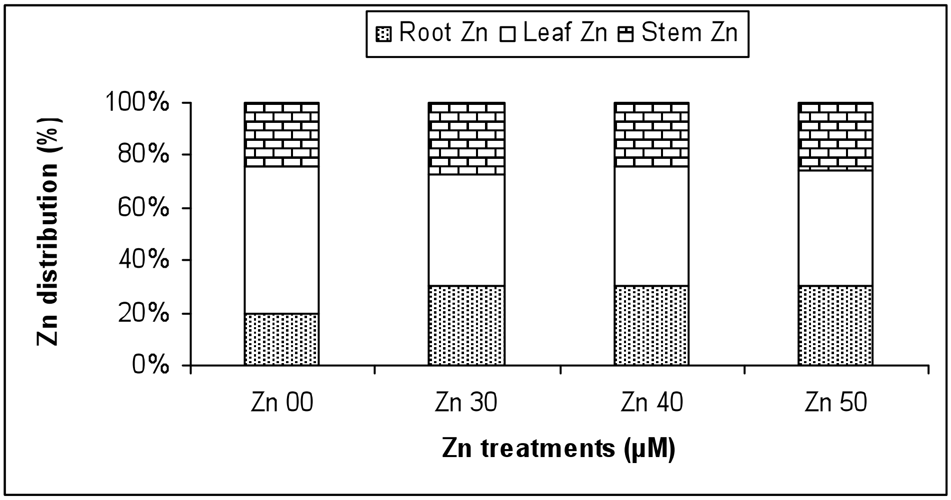

Figure 5. Zn distribution in various plant organs of *L. uncinatus* after one week of growth in nutrient solution at different Zn levels.

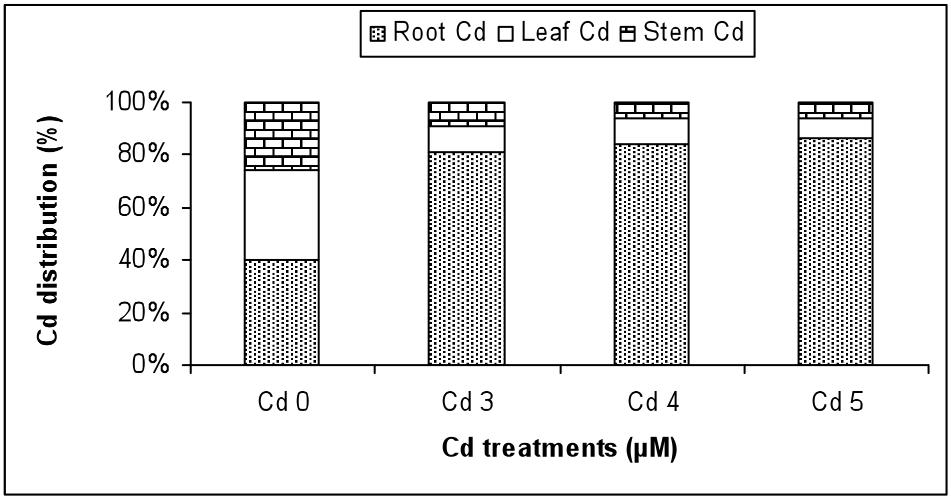

Figure 6. Cd distribution in various plant organs of *L. uncinatus* after one week of growth in nutrient solution at different Cd levels.

VI. DISCUSIÓN GENERAL

La fitoremediacion es el uso de plantas para extraer o estabilizar los contaminantes. Actualmente existe la necesidad de buscar especies vegetales nativas que tengan la capacidad de extraer altas concentraciones de metales tóxicos, mayor distribución, adaptación a múltiples factores adversos ambientales y biomasa cosechable. El objetivo del presente trabajo fue evaluar el potencial de fitoremediación de suelos contaminados con Zn y Cd mediante el uso de *L. uncinatus,* una especie nativa de los bosques de México. La evaluación del potencial de una especie vegetal para la fitoremediación requiere, como primer paso, cuantificar el nivel promedio de la acumulación del metal en un ambiente controlado en relación a la tasa de su crecimiento, con esta finalidad se efectuaron cuatro experimentos para determinar la toxicidad especifica de Zn y Cd en relación a su nivel de acumulación en el tejido vegetal y se observó el efecto de estos metales sobre la concentración de elementos esenciales y su distribución en diferentes órganos de la planta. En dos primeros experimentos se utilizo el suelo no contaminado y plantas de *Lupinus*, aplicando diferentes tratamientos de Zn y Cd. En el tercer experimento se utilizo una solución hidropónica con diferentes concentraciones de metales para cuantificar el nivel de acumulación de estos metales en la planta. En el último experimento se incubaron las mismas concentraciones de Zn y Cd en el suelo, para explorar su comportamiento sin plantas de *Lupinus;* extrayéndose los contenidos de Zn y Cd con DTPA a 1, 5, 15, 25, 60 y 90 días después de la incubación.

El estudio de incubación mostró que la mayor disponibilidad de los metales fue en las primeras 24 horas, la disponibilidad disminuyó significativamente 5 días después de la incubación.

Las fracciones de Zn y Cd extraídas por DTPA no mostraron diferencias significativas después de 15 días de incubación hasta la conclusión del estudio a los 90 días (Figura 1, Tabla 2, Capitulo IV).

En el estudio de invernadero se encontró que *L. uncinatus* acumuló hasta 540 mgCd kg^{-1} materia seca en el tratamiento 27 mg Cd kg^{-1}suelo. La mayor parte del Cd fue retenida en las raíces cuando la planta se desarrollo en suelo (Tabla 5, Capitulo III). Un patron similar de acumulación se observo cuando se aplicaron diferentes niveles de Cd a las plantas de *Lupinus* en solución hidropónica (Tabla 2, Capitulo V). Esto parece indicar que la mayor acumulación del Cd en las raíces puede ser un mecanismo de tolerancia de exclusión del metal.

La concentración de Cd en la planta desminuyó en ambos estudios (invernadero y solución hidropónica) de la siguiente manera: raíz>tallo>hoja. El gradiente de concentración del Cd indica una retención fuerte del mismo durante su transporte desde las raíces hacia la parte aérea de planta. Lo anterior coincide con lo reportado por Zornoza et al. (2002) y Ximenez-Embun et al. (2002) quienes encontraron efectos similares de retención de Cd en las raíces de *Lupinus albus* L. en respuesta a 18 y 45 µM Cd en solución hidropónica y 50 mg Cd L^{-1} en arena contaminada, respectivamente. Una tendencia similar de acumulación ha sido reportada para otros cultivos como *Betula pendula* (Gussarsson, 1994), *Phaseolus vulgaris* L.*, Oryza sativa* L., *Brassica oleracea* L. y *Zea maiz* L. (Guo y Marschner, 1995).

En el experimento con Zn en suelo, la mayor parte del metal fue acumulada en la parte aérea. El estudio en solución hidropónica también confirmó la tendencia de mayor proporción de acumulación de Zn en la parte aérea. Los índices de tolerancia (peso seco promedio de la planta en presencia del metal/peso seco promedio de la planta testigo) fueron altos, con índices de 146, 134 y 150% en los tratamientos con 200, 400 y 600 mg Zn kg^{-1} suelo, respectivamente (Figura 1, Capitulo II). La materia seca aumentó en los tratamientos con Zn en comparación al testigo y la proporción del Zn en la parte aérea fue significativamente mayor que el testigo.

Los tratamientos de Zn aumentaron la proporción de absorción de algunos elementos esenciales como K, Ca, Mg y Mn (Tabla 4, Capitulo V) lo cual pudo haber ayudado a la planta a incrementar su resistencia al estrés metálico. En el estudio utilizando suelo, se aumentó la producción de materia seca mientras que en la solución nutritiva *Lupinus* pudo mantener la producción de materia seca sin un efecto del metal.

Todo lo anterior sugiere que *L. uncinatus*. tiene la capacidad de extraer zinc en su biomasa cosechable, lo cual le confiere ser una especie idonea a utilizarse en fitoremediación de suelos contaminados con zinc.

VII. RECOMENDACIONES GENERALES

La presente investigación aporto evidencia de que *L. uncinatus* tiene el potencial de establecerse en las condiciones edáficas de contaminación estudiada.

Se propone el uso de *L. uncinatus* como alternativa para fitorecuperacion de suelos contaminados con zinc y para revegetar y fitoestabilizar suelos con niveles excesivos del cadmio.

Para asegurar el éxito de la fitoremediación mediante el uso de *L. uncinatus* es necesario enfocar aspectos relevantes de esta especie como el estudio de la base molecular de su tolerancia a los metales, sus mecanismos de absorción, mejoramiento genético, y diferentes estrategias para incrementar su producción agronómica.

Se deben también efectuarse estudios con rangos mas amplios de Zn y Cd en condiciones de campo para verificar los patrones potenciales de acumulación y tolerancia de *L. uncinatus,* aumentando el periodo de exposición al metal que cubra todo el ciclo vegetativo de la planta.

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