Instituto Tecnológico y de Estudios Superiores de Monterrey

Campus Monterrey

School of Engineering and Sciences

Microbial inoculation of *Tagetes erecta* in a phytoremediation system to enhance arsenic and cadmium removal from polluted soils

A thesis presented by

Arantza-Sánchez-Jiménez

Submitted to the
School of Engineering and Sciences
in partial fulfillment of the requirements for the degree of

Master of Science

In

Biotechnology

Dedication

To my mother, Silvia Jiménez Márquez, who has always given me unconditional love and support to accomplish my goals. I owe you everything.

To my boyfriend, Rodolfo Galván Rodríguez, who has always encouraged me to do my best in research, and has always provided me with motivation and adequate gear to fulfill my work. You have always believed in me, and entitled me as the best student.

This wouldn't have been possible without you. You were my main motivation for pushing through this work.

Acknowledgements

I would like to express my deepest gratitude to all those who have been side by side with me: my committee members and main advisor, additional professors that have collaborated to this work with their valuable advises, my friends and, of course, my beloved family.

Thank you Dr. Grisel Fierros Romero, Dr. Mauricio Nahuam Chávez Avilés, Dr. Juan Ignacio Valiente Banuet, Dr. Erika Kothe, and Dr. Hiram Medrano Roldán; for providing me of an example of great researchers.

Thank you, Genaro Ruiz, for all your support, and for welcoming me into your lab in times of need and desperation.

Thanks to all of my professors, for teaching me and making me fall in love with science.

To my friends, for going on an adventure with me to retrieve samples, and for lightening the dark days.

Thanks to my family, for always understanding the late-night hours of work and stress, and for recognizing my efforts.

And also, special thanks to my alma mater, Tecnológico de Monterrey, for providing me of a full scholarship to keep on with my studies; and to CONACYT, for covering my research along with living expenses.

From the bottom of my heart, thank you all.

Microbial inoculation of *Tagetes erecta* in a phytoremediation system to enhance arsenic and cadmium removal from polluted soils

by

Arantza-Sánchez-Jiménez

Abstract

In the presented research, a phytoremediation system was established by the inoculation of heavy metal resistant microorganisms isolated from the Mexican volcanic area of Los Azufres, Michoacán into the Mexican ornamental flower *Tagetes erecta*; with the final purpose of enhancing their individual bioremediation capacities when combined for their application in *ex situ* bioremediation of soils polluted with arsenic and cadmium. The methodology consisted of three main steps: the microbial isolation and characterization, the *in vitro* system establishment between the isolated microbes and the selected plant species to evaluate their interaction, and the creation of microcosms to evaluate their potential for *ex situ* bioremediation use. The results suggest that our isolated microbes, determined as LA1 & LA2, are heavy metal resistant, with a respective MIC of 1800 ppm of As and 250 ppm of As and Cd each; and that LA2 favors the development of *Tagetes erecta* in a medium supplemented with these heavy metals and enhances the plant's tolerance. This signifies an intriguing option to use as a phytoremediation system for *ex situ* bioremediation of soils polluted with arsenic and cadmium.

List of Figures

List of Tables

Contents

Chapter 1

Introduction

It is impressive to say that it has been almost over a century since special concern about pollution caused by anthropogenic activities and its hopeless consequences was brought about to the modern world. Nonetheless, the impacts derived from the unbalance of these activities are still threatening the environmental fate of the coming years.

Having this priority in mind, not only is it important to seize the growing environmental corruption, but it is also essential to amend what has already been defiled. Numerous efforts have been implemented to diminish the outcome of the corresponding excess of xenobiotic substances mending within nature.

A wide spectrum of contaminants already forms part of what we call environment, and while the interest to flip this around continues to grow, new clean up technologies wager promising solutions to this issue. However, while some agents can be easily treated, some others excel by their outstanding permanence on the environment, which is the case of heavy metals.

Special concerns about the non-degradability of heavy metals explain their urge to be treated, particularly lead, mercury, cadmium, and arsenic [24]. Their environmental accumulation leads to an extended dispersion through water and soil, compromising the life of organisms by the eventual integration of said elements into the trophic chain, not mentioning the transcendental diseases caused by them, and the damage that they cause to not only humans, but to plants and animals as well.

Compared to traditional methods of attention to treat said components, “living” remediation is rather chosen in the form of biotechnology, where bioremediation strategies are a clue to neutralize heavy metals’ hazardous effects, among other contaminants, providing a cost-effective, permanent, safe and less laborious solution [22].

Bioremediation is therefore defined as the elimination of contaminants using biological systems by their break down, transformation or degradation; technology that perfectly suits remediation purposes. Bioremediation can be performed in an *ex situ* and *in situ* modality; where *ex situ* remediation consists of the transportation of contaminated matter to another site for further treatment, and *in situ* remediation implies the procedures to be performed in the place of origin [25].

Microorganisms (comprehending bacteria, yeast, fungi, and even archaeon) and plants are the living tools that act as catalysts [30] to remediate industrial wastes such as heavy metals, pesticides, toxic chemical fertilizers, among others [53],

meaning that microorganisms are able to take up and transform heavy metals, as well as suited plants to remove and restrain metals from the ground.

Biotechnology and its derivative disciplines including Bio and Phytoremediation open up a series of expectations to restore polluted environments, where the combination of both microorganisms and plants is an approach to ensure a more efficient clean up [9].

On this research thesis, a phytoremediation system composed by microorganisms isolated from the Mexican volcanic area of Los Azufres, Michoacán, and the Mexican ornamental flower *Tagetes erecta,* is established to remediate soils polluted by arsenic and cadmium in a potential *ex situ* performance.

Chapter 2

Theoretical Framework

Heavy metals and soil pollution

Heavy metals are non-biodegradable components of the earth that are characterized by having a high density (above 5 $\boldsymbol{g / cm^3}$) and a high atomic weight (ranging between 63.5 and 200.6 $\boldsymbol{g/mol}$) [47]. They represent 53 of 90 naturally occurring elements, and are distinguished by a high electrical conductivity, malleability, and a transition activity caused by their incomplete d orbitals [3] that allows them to form complex compounds with cells and biomolecules [36], meaning a bioaccumulative potential that threatens all the components of an ecosystem.

Their origin implies a variety of sources, including their natural liberation from the earth's crust by erosion, volcanic activity, and meteorization; as well as an anthropogenic cause that incorporates battery production, mining, explosive manufacturing, use of pesticides, phosphate fertilizers, sewage irrigation, steel and electroplating industries, textiles and wood preservation, among others [35][4].

While some of these elements could be required in minimal quantities by some organisms as trace and micronutrients (Co, Cu, Fe, Cr, Mn, Mo, Ni, V and Zn), others are not needed for biological functions; yet, they could be accumulated, and are considered as the most hazardous elements (Pb, Cd, Hg and As) [9][39].

A heavy metal's toxicity is given by their solubility and oxidation state; where acidity contributes to the metal's bioavailability, with the exception of As, Mo, Se, and Cr, which are more available in alkaline soils [14]. Regarding their oxidation state, heavy metals can exist in multiple forms, shown in Table 1.

Table 1. Oxidation states of heavy metals

Heavy metal	Available oxidation states
Vanadium	$V^{-}, V^{1+}, V^{2+}, V^{3+}, V^{4+}, V^{5+}$
Chromium	$Cr^{2-}, Cr^{-}, Cr^{1+}, Cr^{2+}, Cr^{3+}, Cr^{4+}, Cr^{5+}, Cr^{6+}$
Manganese	$Mn^{3-}, Mn^{2-}, Mn^{-}, Mn^{1+}, Mn^{2+}, Mn^{3+}, Mn^{4+}, Mn^{5+}, Mn^{6+}, Mn^{7+}$
Iron	$Fe^{2-}, Fe^{-}, Fe^{1+}, Fe^{2+}, Fe^{3+}, Fe^{4+}, Fe^{5+}, Fe^{6+}, Fe^{7+}, Fe^{8+}$
Cobalt	$Co^{-}, Co^{1+}, Co^{2+}, Co^{3+}, Co^{4+}, Co^{5+}$
Nickel	$Ni^{-}, Ni^{1+}, Ni^{2+}, Ni^{3+}, Ni^{4+}$
Copper	Cu^{2+}, Cu^{+}
Zinc	Zn^{2+}, Zn^{+}
Arsenic	$As^{3-}, As^{2+}, As^{3+}, As^{5+}$
Silver	$Ag^{3+}, Ag^{4+}Ag^{2+}, Ag^{+}$
Cadmium	Cd^{2+}, Cd^{+}
Mercury	Hg^{4+}, Hg^{2+}, Hg^{+}
Lead	$Pb^{4-}, Pb^{2+}, Pb^{4+}$

[37]

Whenever there is a high concentration of heavy metal ions present in an organism or environment, we speak of a risk scenario where toxic effects start to show. Potentially Toxic Elements (PTE) in Mexico include Pb, Cd, Zn, As, Se and Hg [44] nevertheless, in Mexican territory, there are a variety of elements that are regulated not to overpass established concentrations in soil. Said legislation is shown in Table 2, where the maximum permissible concentrations of heavy metals are specified according to the soil use.

Table 2. Maximum permissible concentration of heavy metals in soil, established by the Official Mexican Nom NOM-147-SEMARNAT/SSA1-2004; according to the soil use

Pollutant	Agricultural/residential/commercial use (mg/kg)	Industrial use (mg/kg)
Arsenic	22	260
Barium	5400	67000
Beryllium	150	1900
Cadmium	37	450
Hexavalent Chromium	280	510
Mercury	23	310
Nickel	1600	20000
Silver	390	5100
Lead	400	800
Selenium	390	5100
Thallium	5.2	67
Vanadium	78	1000

[37]

Soils and sediment systems imply a complex composition whose quality is determined by the quantity of heavy metals present in them. Some of the consequences that derive from an overdose of heavy metals constituting the ground cause an alteration in the integrity of physicochemical and biological composition of the soil, its nutrimental reduction, pH variation and acidification, reduction in microbial activity and microbial displacement, soil vulnerability towards erosion and desiccation, and furthermore, promotion and development of severe diseases and ailments in all humans, animals, and plants; including the

bioaccumulation of these elements in living organisms and their biomagnification in the trophic chain [44].

These elements are endured in different ways, depending on the organism they face. Beginning with human vulnerability, heavy metals are conferred with an intriguing toxicity due to their bioavailability and their lipid solubility. Major affections are caused by dermal contact, ingestion or inhalation, wherein absorption of heavy metals induces the transport of these elements and distributes them through body tissues, persisting in organs such as bones, liver, or kidneys [25].

From a vegetative point of view, metal ions are known to damage cell membranes, inhibit enzymatic and photosynthetic activities, and drive the generation of reactive oxygen species in plants. These stress effects make plants more susceptible to climate change, and the plant ‘s productivity is compromised [41].

Molecular affections resulting from heavy metal exposure consist on their attachment to the binding sites of proteins; removing other molecules and causing cellular malfunction [25]. In biological systems, heavy metals affect organelles like cellular membrane, mitochondria, lysosomes, endoplasmic reticulum, nucleus, and enzymes related to detoxification metabolism and cell damage repair; and also, intervene with DNA structure [14].

The gravity of heavy metal exposure is clear, implicating long-term consequences in multiple aspects. In this sense, human health is mainly threatened by lead, mercury, and certainly, cadmium and arsenic.

Arsenic and cadmium

Highlighting the overriding elements of concern and their fatalities, arsenic is a cardiovascular and neurobehavioral disorder trigger [25]. Ailments related to As exposure include intoxication (surprisingly ordinary), damage in the central nervous system, liver, skin and cancer. The most common way of intoxication by As is through dietary consumption or ingestion of contaminated water that overpasses a concentration of 10 $\boldsymbol{\mu g/L}$, established by the World Health organization [54:55]; being As (V) and As (III) rapidly absorbed in the animal digestive tissue.

As (V) has the capacity to associate with oxides formed by Fe, Mn, and Al; and has a high reactivity with thiol groups in metabolic proteins and enzymes that causally create ROS; and replaces phosphate ions, acquiring the access into the cellular channels [11].

Naturally, As is associated with other elements like S, Mn, Fe, Co, Ni, Pb, and Sn. Similar to Cd, this element is considered as a group 1 carcinogenic by the OMS [28:29], mainly demonstrating liver and skin cancer.

Countries reported by a high level of contamination in terms of arsenic concentration and potential risk in Latin America include Argentina, Chile, Mexico, Guatemala, Nicaragua, Perú, Bolivia, Brazil and Uruguay [11].

On the other hand, cadmium exposure derives into lung and stomach cancer, osteoporosis, chromosomal aberrations and damage; as well as liver, kidney, lungs, and heart dysfunction [25]. Being a highly soluble element makes of cadmium an extremely toxic metal [43], where it has been determined that the minimum quantity of Cd to derive into tragic effects in human health is of solely 2 mg.

The presence of cadmium in the environmental ground originates from products such as paintings, inks, polymers, batteries, glass, alloys, coatings, and mining tailings; and is generally associated with other metals such as Zn and Pb, forming sulfides and oxides [44].

Cadmium is a non-essential metal for cellular enzyme activities; however, both arsenic and cadmium are extremely reactive and toxic at high concentrations [33]. This element interacts with cellular functional groups substituting other metals that have cellular functions, for instance, Zn; and has a high affinity to sulfide groups [44].

Cadmium contaminated soil is considered a global concern since it can be easily absorbed by plants and eventually by humans via the food chain [30]; and speaking of damage caused in plants due to their exposure to metals like arsenic and cadmium, the first one causes growth reduction and alteration of Ca, K, P, and Mn concentrations in the plants; and the second one causes photosynthesis and transpiration inhibition, as well as chlorophyll inhibition; and modification in Mn, Ca and K concentrations [3].

Other parameters implied in physiological responses that occur when plants are exposed to heavy metals are the seedling survival, the biomass of the plant, shoot and root growth, pollen tube growth, and other cellular responses like mitosis and cytokinesis inhibition, as well as inhibition of carbon and nitrogen fixation, among others [5].

Though heavy metals appear to be complex pollutants to handle, “living” remediation is an attractive method to mend soils contaminated by them, and furthermore, the combination of both microorganisms and plants is an approach to ensure a more efficient clean-up [9].

The literature proposes a transformation ratio, by microorganisms alone, of 27% for Cr, 20% for Co, 31% for Cd, 22% for Pb, 7% for Ni, 5% for Zinc and about 18% for As & Cd [29]. If an additional setting is built where these microorganisms team up with the proper plants, a powerful combination is set on board, but understanding their individual capacities first is in order.

Heavy metal resistant microorganisms and tolerant plants

Exposure to contaminants and heavy metals provides resistance and adaptation on behalf of microbes by the natural selection and pressure to survive [29]; and on the other hand, heavy metal pollution can lead to phytotoxic effects over plants, forcing them to develop tolerant genotypes [5].

All plants respond to heavy metal exposure, but depending on the nature of the species and the magnitude of the element, the response will be different [5].

A clear difference between tolerant and non-tolerant plant species is their ability to thrive, survive, and reproduce in metal-contaminated substrates. Tolerance can be presented in different ways, for instance, in multiple metal tolerance and co-tolerance, where plants could possess a low level tolerance to a metal that is not present in the immediate environment (since metalliferous environments are contaminated by multiple elements in potentially toxic concentration). This has also been observed in microorganisms; and the reason could be embedded in a gene flow effect between plants grown alongside other tolerant ones [5]. However, tolerance, being a specific and inheritable characteristic, can also be an inducible factor; and being that way, tolerance could also be lost.

Some mechanisms involved in metal detoxification in plants consist on plant cell wall binding, active transport of ions into cell vacuoles, intracellular complexation with peptide ligands such as phytochelatins (PCs) and metallothioneins (MTs), and sequestration of metal-siderophore complexes in root apoplasm or soil [31]. Others consist of exudate production, one of the most critical strategies in plants to tolerate high metal concentrations [31], especially low molecular weight organic acids (LMWOAs) like citric, oxalic, malic, and succinic acid [32], to detoxify As, Cd & Pb [34].

As to microbes, although the intracellular concentration of metal ions can sometimes be controlled by protein families in the cells, such as the ABC (uptake and efflux of metal ions.), P-type ATPases (bidirectional transport of metal ions), CDF efflux proteins (Cation diffusor facilitator driven by chemiosmotic forces), or transenvelope proteins (Transport proteins constituted by Outer Membrane Factors OMF, MFP Membrane Fusion Protein family, and RND Resistance, Nodulation, and cell Division); their effect on living organisms can be counterproductive [36].

Related to arsenic, microbial populations that own these mechanisms of tolerance and resistance are embedded in the *ars* operon, identified by metagenomics [29]. Arsenic bioremediation mechanisms found in microorganisms consist of 4 main processes: methylation, demethylation, oxidation, and reduction. Soluble forms of As, like As (V) & As(III) are interiorized in the cells by channels present in glycophorins since they resemble phosphate channels [11]. Arsenate reduction or detoxification consists of the conversion of As(V) into As(III), expelled from the cell.

Being arsenite more toxic than arsenate, the transformation is caused by the arsenite oxidase enzyme, catalyzing As(III) into As (V) [11].

Speaking of Cd, this metal ion is also introduced by cellular channels like ABC transporters, RND proteins like the Czc system which is mainly a zinc exporter and Ncc which is a nickel exporter, and accumulated by the magnesium, calcium, and manganese uptake systems [36]. Damage caused by cadmium in a cellular point of view is summarized with its capacity to bind with thiol groups, to denature proteins, dame cell membrane, and to intervene with zinc and calcium metabolisms [36].

Pollutants could be interpreted as natural selection agents, since they generate metabolic interaction mechanisms with them, found in plants, yeast and prokaryotes. Sites that present high levels of heavy metals cause *in situ* resistance and microbial adaptation due to natural selection and survival pressure towards contaminants, heavy metals and metalloids [29]; therefore, the search of functional microorganisms with bioremediation properties begins in polluted sites and extreme environments.

Mexican Volcanic Areas & Los Azufres, Michoacán.

Volcanic areas are attached to hydro and geothermal fields, representing extreme environments that trigger adaptive processes like pollutant resistance due to the liberation of microelements like magnesium, boron, manganese, vanadium, polyphenol, and other components rich in heavy metals that cause precipitation, sediment and accumulation of these elements [14].

There are numerous volcanic areas located in America; and speaking of Mexico, a variety of volcanic areas are also numbered. Mountains and plains, as well as volcanoes, are important life zones for diverse organisms [16]. Biotic areas of Mexico have received recent attention because of its wide and diverse environmental richness [16].

Los Azufres, Michoacán (19º46′51.7″N and 100º39′23.6″W), is a hydrotermal spring system in the Mexican Volcanic Axis and part of the Mexican Volcanic Belt, which crosses Mexico from East to West. This volcanic center is located 200 km northwest of Mexico City and presents active mineralized geothermal systems, fumaroles, and boiling mud pools [7]. This area underlies a minimum age of 10.2 $\pm$ 0.6 million years [16].

Microbes retrieved from geothermal sediments provide a research field involving the study if links between physico-chemical conditions and microbial diversity and activity in geothermal sites, as well as the discovery of new bio catalyzers for remediation bioprocesses [7].

Long recognized for its thermal manifestations, the geothermal potential has offered further study opportunities recently. Being a volcanic and thermal

environment, genetic wealth related to evolutive adaptations to withstand conditions like heavy metal resistance is potential.

Bioremediation and Phytoremediation

Whereas some agents can be easily treated, some pollutants cannot be degraded, which is the case with heavy metals. Though heavy metals cannot be fully removed, particular organisms contribute to their neutralization in order to reduce their harmful effect on the environment [39]. Compared to traditional methods of remediation implying a physic-chemical mix of procedures, the use of "living" remediation is rather chosen.

Bioremediation is therefore defined as the elimination of contaminants using biological systems by their break down, transformation or degradation. Microorganisms (comprehending bacteria, yeast, fungi, and even archaeon) and plants are the imperative tools required to remediate industrial wastes such as heavy metals, pesticides, toxic chemical fertilizers, among others [38], due to their resemblance as biological catalysts in a bioremediation system [52] arranged by suitable components to fix contaminated environments, meaning microorganisms that are able to take up and transform heavy metals, as well as suited plants to remove and restrain metals from the ground [38].

Microbial bioremediation emphasizes on the clean-up of organic and inorganic contaminants by their reduction, removal, degradation, transformation, detoxification, or immobilization of contaminants [12]; where microorganisms are utilized as biosorbents of heavy metals and metalloids for the decontamination of heavy metal polluted sites employing yeasts, fungi, algae, bacteria by their functional genes, enzymes, metabolic routes and intracellular processes.

Budding from this biotechnological subject, phytoremediation and more elaborated cleansing techniques also take place to this purpose. The upgrowth of bioremediation techniques utilizing plants with microbes is the outcome of the pursuit for alternative methods with clean-up purposes that has lead to the development of bioaugmentation or rhizoremediation [27].

The bioaugmentation method improves the degradation and enhances the transformation rate of xenobiotics by the insertion of specific microorganisms [27]; and on the other hand, when phytoremediation and bioaugmentation combine, rhizoremediation takes place. During this process, exudates derived from plant roots can improve efficiency of phytoremediation [38] and spread the bacteria through the soil. Substantial degradation of pollutants is due to the microbes living in the rhizosphere, dominated by Gram-negative rods such as *Pseudomonas* spp [27].

Heavy metal tolerance in plants is importantly influenced by the root tissue since it is able to regulate absorption from the rhizosphere, and therefore, the sequestration and translocation of metals to aerial parts [50]. Additional to this,

plants should be able to mitigate oxidative stress associated to elevated levels of heavy metals [12]. Plants provide numerous ways of opposing contaminants, as it shown in Table 3.

Table 3. Bioremediation mechanisms in plants

Mechanism	Description	Reference
Phytoaccumulation or Phytoextraction	Pollutant take up in plant biomass, taken from the soil through the roots into upper plant components.	
Phytofiltration	Represents three types of filtration in plants: Rhizofiltration (use of roots), blastofiltration (use of seedlings), and caulofiltration (use of excised plant shoots).	[9] [30]
Rhizofiltration	Elimination of toxic substances from groundwater through root filtration by terrestrial and aquatic plants.	[8]
Phytostimulation	Use of exudates from plant roots to stimulate microbial activity and enhance it.	
Phytostabilization	Immobilization of metal by plants, reducing their bioavailability; turning them into less harmful and preventing their spread in the environment.	
Phytovolatization	Pollutants taken from the soil are transformed into volatile forms and transpired into the atmosphere, mainly Hg & Se.	
Phytodegradation	Breakdown of organic contaminants into non-hazardous forms by plant enzymes.	
Detoxification	Involves processes such as adsorption, chelation, transformation, and inactivation of metals.	

However, not all plants can be used for phytoremediation processes, but there is a preference for hyperaccumulators.

A hyperaccumulator plant species is characterized by their rapid growth, extensive root system, high biomass production, and by remaining healthy even when achieving high metal concentrations, enough to maintain a population [51][30].

In order to call a plant a hyperaccumulator, it needs to fulfill the following aspects: accumulating capacity of 10 000 mg/kg for Zn and Mn, 1000 mg/kg for Co, Cu, Ni, As, and Se, and 100 mg/kg for Cd; a translocation factor greater than 1, meaning the ratio of metal concentrations in shoots to roots; and a bioconcentration factor greater than 1, meaning the ratio of metal concentration in plants to soil [43].

Although the use of ornamental plants as phytoremediation agents against heavy metal soil pollution is a recent case of study, these plants in particular offer additional benefits to bioremediation purposes including a reduced food chain contamination, revenue generation, and a landscape embellishment [17].

Tagetes erecta

Although hyperaccumulating plants are often targeted for phytoremediation use, the use of ornamental flowers is a field of expansion to fulfill this purpose.

Belonging to the Asteraceae family*, Tagetes erecta*, commonly known as Mexican or Aztec marigold, is a native to Central America ornamental plant whose flowers are extensively grown, particularly from June to November, and that represent a spicy cultural signature to Mexican culture; meaning one of the most important commercially grown flowers in the country, being Mexico City, Puebla, Hidalgo, Guerrero, Michoacán, Tlaxcala, San Luis Potosí, Morelos, Oaxaca, Ciudad de México, and Durango the 10 Mexican entities with highest production of *Tagetes erecta* [45].

The Asteraceae family is one of the largest flowering plant families around the world [30], also cultivated in countries like India, Pakistan, and China [6].

A delighting benefit of using *Tagetes erecta* with bioremediation purposes includes the embellishment of the landscape with a hidden benefit [17,47]; although the major innovation in applying this ornamental plant for phytoremediation purposes is the establishment of a remarkable system composed by endemic species and microbes.

Mexican marigold is featured with a rapid growth, adequate biomass, and extensive root system; making it an attractive candidate to be used in phytoremediation [8], not mentioning the background it carries.

Background

Although marigold has been used mainly as an ornamental flower in the Mexican culture, the *Tagetes* genus has been utilized with interests involving its natural pigment, biologically active compounds, natural oils [10], fungicide properties and antimicrobial activity [22], nematicidal and insecticidal activities [30], along with its antioxidant properties [6].

However, a new interest has risen when considering its rapid growth, vigorous root system, and its capacity to proliferate in poor soils: the phytoremediation interest guided towards soil remediation of organic and inorganic substances [10].

Tagetes erecta has demonstrated to be a potential hyperaccumulator due to its high bioaccumulation and tolerance mechanisms to heavy metals such as Chromium at concentrations up to 0.12 mmol/L of Cr (III) [10], Cadmium at 50 mg/kg with a remarkable accumulation in above ground tissues and great root to shoot translocation with a high extraction coefficient [6] as well as zinc (Zn) and lead (Pb) removal capacities [49]. Additional to these metals, the effect of the symbiosis between *Tagetes erecta* and *Glomus intraradices* in the uptake of Copper (II) has also been evaluated, leading to a conclusion where mycorrhizal

colonization resulted in a root tissue Cu accumulation, proposing this plant species as a potential phytostabilizator of Cu in contaminated soils [8].

Compared to Guinea grass (*Panicum maximum*), cosmos (*Cosmos sulphureus*), and sunflower (*Helianthus annuus*), *Tagetes erecta* was demonstrated to have the highest cadmium accumulation in the total plant [43].

Even in Mexico, marigold is explored in the engineering field with the purpose of cleaning gray water for its reuse.

In terms of symbiotic microbial relationships, *Tagetes erecta* has been supplemented with Efficient Microorganism consortiums in the shape of organic compost bioaugmentation to increase its growth and promote the use of organic farming with means of sustaining soil health. Said consortiums involved *Candida tropicalis, Phanerochaete chrysosporium, Streptomyces globisporous, Lactobacillus* sp., and photosynthetic bacteria [47].

Although the research field involving *Tagetes erecta* is ancient and extensive, the exploration of this ornamental flower with microbes isolated from Mexican extreme environments has not yet taken off.

Chapter 3

Justification

The environmental accumulation of non-degradable pollutants such as heavy metals compromises the life of the organisms, particularly by elements such as arsenic and cadmium, representing two of the most hazardous elements comprised in this group.

Compared to traditional methods of remediation, the use of "living" remediation is rather chosen; where the use of suitable plants and microbes is gaining popularity to neutralize heavy-metal hazard in order to reduce their harmful effect on the environment, providing an eco-friendly, cost-effective, and safe solution.

The adequate combination of microorganisms and plants can enhance the removal of heavy metals from polluted soils, enabling a multiple remediation and approaching a more efficient clean up; goal that is pursued on this research.

Chapter 4

Hypothesis

Microorganisms isolated from Los Azufres, Michoacán can enhance bioremediation of heavy metals when combined with hyperaccumulating plants such as *Tagetes erecta* in a Phytoremediation System.

Chapter 5

Objectives

General objective

To establish a successful phytoremediation system composed by microorganisms isolated from volcanic areas such as Los Azufres, Michoacán and the hyperaccumulating plant *Tagetes erecta* to enhance the removal of arsenic and cadmium from polluted soils.

Particular objectives

- To Identify and/or discover heavy metal resistant microorganisms isolated from the Mexican volcanic area, Los Azufres, Michoacán.
- To describe a favorable Plant-Microbe interaction and understand this relationship.
- To determine the system's capacity to remove arsenic and cadmium.
- To evaluate the potential of the system for *ex situ* remediation purposes.

Chapter 6

Materials and Methods

Microbial Isolation

Soil samples were retrieved from hot spots that contained a high mineral concentration at Los Azufres, Michoacán (19º46′51.7″N and 100º39′23.6″W).

To begin with the selection of heavy metal resistant microorganisms, solutions of 20 000 ppm were prepared for each metal, by dissolving 1 g of metallic salts, cadmium sulfate for cadmium (MEYER) and sodium (meta) arsenite for arsenic (Aldrich), in 50 mL of distilled water. Once homogenized, the solutions were sterilized by filtration using 0.2 µm syringe filters. With the preparation of these solutions, further supplementation of arsenic and cadmium at specific concentrations was easily manipulated by using the dilution equation (1), where c_1 stands for Concentration 1, c_2 stands for Concentration 2, v_1 stands for Volume 1, and v_2 stands for Volume 2.

$$c_1 * v_1 = c_2 * v_2 \quad (1)$$

Samples were grown by adding 0.5 g of each soil sample into 20 mL of nutritious broth, supplemented with arsenic and cadmium at 50 ppm each in order to start with the selection of heavy metal resistant microorganisms. The samples were cultured at 27ºC for 7 days.

Once growth was perceived, aliquots of 100 µL were transferred into nutritious agar plates and distributed with a sterile “L” stick, with an increased concentration of arsenic and cadmium, raised to the double. This transference allowed the observation of morphological differences between the microorganisms that grew from the samples and facilitated the isolation of microorganisms into different plates.

Microbial Characterization

Minimum Inhibitory Concentration (MIC) Determination

When a strain succeeded to grow in nutritious medium supplemented with arsenic and cadmium, the concentrations were increased to the double so that Petri dish plates (Isolates) containing microorganisms reached concentrations of 50, 100, 200, and then 250 for arsenic and cadmium; and 400, 800, 1600, and even 1800 ppm for just arsenic.

If a microorganism failed to grow, and others overcame its concentration, said microorganism was discarded, obtaining two strains designated as the most

resistant ones, nominated as LA1 (Los Azufres 1) and LA2 (Los Azufres 2), with a respective MIC of 1800 ppm of As and 250 ppm of As & Cd.

Microscopic Evaluation

With the purpose of characterizing our microorganisms of interest they were both observed under an OLYMPUS BX51 microscope, where it was determined that LA1 corresponds to a Gram-negative bacterium, and LA2 corresponds to a pink yeast, probably *Rhodotorula* sp. This assumption would be subsequently evaluated.

Microbial growth

A kinetic-like evaluation was performed to evaluate the growth behavior of LA1 and LA2 at different concentrations of arsenic and cadmium. The main purpose of this procedure was to determine the optimum point at which the microorganisms could be inoculated into our selected plant species.

A microbial loop was used to collect each microorganism's biomass from plates, and afterwards, inoculated into Falcon tubes containing 20 mL of fresh nutritious broth. The tubes were incubated at 27ºC for 24 h.

To begin with the readings, a 1 mL aliquot from the 24 h microbial growth was added to 10 mL of fresh nutritious broth. This was carried out for each treatment to be evaluated, shown in Table 4 as follows:

Table 4. Microbial growth experiment summarized

Strain	Treatment to be evaluated
LA1	0 ppm of As 200 ppm of As 400 ppm of As
LA2	0 ppm of As and Cd 50 ppm of As and Cd 100 ppm of As and Cd

Each reading was taken every 3 h for a total period of 60 h. Samples were read in 96 well plates in a BioRad xMark Spectrophotometer using a wavelength of 600 nm.

DNA Extraction

With the objective of identifying our selected microorganisms in a molecular aspect, DNA was extracted to be stored for further characterization.

Two methods were used, one based on the Invitrogen "Pure Link Mini Kit" by Thermo Fisher Scientific extraction corresponding to the Gram-negative section,

and an adaptation where the lysis step was carried out with glass beads. Both are described underneath:

Invitrogen Pure Link Mini Kit by Thermo Fisher Scientific

Microbial biomass was taken with a loop and suspended in 180 µL of Genomic Digestion Buffer. 20 µL of proteinase K were added and agitated in vortex for lysis. The suspension was incubated at 55ºC for 45 minutes.

After incubation, 20 µL of RNAse A were added and vortexed. The suspension was incubated for 2 minutes at room temperature and 200 µL of Genomic Lysis/Binding Buffer were added and vortexed. Then, 200 µL of 96% ethanol were added and vortexed for 5 seconds.

To proceed with DNA binding, 640 µL of the lysate were transferred to a PureLink Spin Column. The suspension was centrifuged at 10 000 rpm for 1 minute at room temperature and the effluent was discarded to continue.

For the washing step, 500 µL of Wash Buffer 1 were added to the column and centrifuged at 10 000 rpm for 1 minute at room temperature. The effluent was discarded and 500 µL of Wash Buffer 2 were added to the column and centrifuged at maximum speed for 3 minutes at room temperature.

For DNA resuspension, the column was placed inside a 1 mL Eppendorf tube and 25 µL of sterile Milli Q water were added to the column and incubated at room temperature for 1 minute. The tube was centrifuged at maximum speed for 1 minute at room temperature. The last two steps were repeated to obtain 50 µL of DNA.

Finally, the extracted DNA was stored at -4 ºC.

Adaptation to glass beads lysis

For this adaptation method, the difference was placed at the lysis step, were instead of using a lytic enzyme, this breakage step was carried out with beads. 800 µL of liquid microbial growth were added to a 1 mL Eppendorf tube. The tube was centrifuged at 10 000 rpm for 3 minutes. The supernatant was discarded and the pellet was resuspended in 800 µL of Milli Q sterile water.

About 40 mg of sterile glass beads were added to the tube and vortexed for 3 minutes. The suspension was centrifuged at 10 000 rpm for 1 minute and the supernatant was retrieved for the next steps.

The steps corresponding to the binding of the DNA, washing, DNA resuspension and storage were carried out the same way as described in the PureLink Mini Kit protocol.

With the intention of identifying LA1 and LA2, the successfully extracted DNA samples were meant to be amplified using 16S for the bacterium LA1 and ITS for the yeast LA2 for further sequencing.

Tagetes erecta's tolerance screening towards arsenic and cadmium

Seed disinfection and stratification

Commercial seeds were purchased from Rancho Los Molinos.

Tagetes erecta's seeds disinfection process consisted on mixing the seeds with 70% ethanol for 5 minutes. Afterwards, the ethanol was discarded and replaced with a solution consisting of 2% of hypochlorite and 0.1% detergent in sterile water, agitated for 15 minutes.

The disinfection solution was rinsed out with 5 washing steps of 15 minutes each, using sterile water to wash out all of the solution.

After the last wash, the seeds were stored in sterile water at 4ºC for at least 3 days for stratification.

Medium preparation and tolerance screening

The medium used for the *in vitro* experiment consisted on plantMedia Murashige Skoog (MS) Basal Medium at 0.2X, Gold Biotechnology Phytoagar at 3 g/L (semi-solid), and sucrose supplementation at 30 g/L.

Seedlings were germinated *in vitro* in the previously described medium, with a photoperiod of 16 h daily, at a temperature of 25ºC.

After 7 days, the seeds were transferred to fresh medium supplemented with heavy metals, arsenic and cadmium at 30 and 40 ppm respectively, to verify that the selected plant species would be useful for the bioremediation of a real scenario that overpass the limits established by the Official Mexican Nom NOM-147-SEMARNAT/SSA1-2004.

Plant-microbe interaction system *in vitro*

Seedlings were grown *in vitro*, according to the previously established conditions. After 7 days of growth, they were transferred to fresh medium supplemented with heavy metals and/or microorganisms. Arsenic, cadmium and the strains LA1 and LA2 were added while the medium remained warm, at a temperature of 40ºC.

The microbes were inoculated at a concentration of $\mathbf{1x10^6}$ cells/mL, determined with a Neubauer chamber. The treatments are summarized in Table 5.

Table 5. Plant-microbe interaction system *in vitro,* experiment summarized

Treatment	Strain	Heavy metal supplementation	Number of Replicates
Control	None	None	X3
Arsenic	None	As at 30 ppm	
Cadmium	None	Cd at 30 ppm	
Arsenic & Cadmium	None	As & Cd at 30 & 40 ppm respectively	
LA1	Inoculation with LA1	None	
		As at 30 ppm	
		Cd at 40 ppm	
		As & Cd at 30 & 40 ppm respectively	
LA2	Inoculation with LA2	None	
		As at 30 ppm	
		Cd at 40 ppm	
		As & Cd at 30 & 40 ppm respectively	
LA1 & LA2	Inoculation with LA1 & LA2	None	
		As at 30 ppm	
		Cd at 40 ppm	
		As & Cd at 30 and 40 ppm respectively	

Number of leaves, shoot length, primary root length, number of secondary roots, fresh weight of aerial and root parts, and dry weight of aerial and root parts were measured for further analysis.

Microcosms establishment

Tagetes erecta seeds were germinated *in vitro* in the previously presented medium, and after 10 days (once secondary roots were developed) seedlings were transferred to a germination tray, filled with sterilized Peat moss. The seedlings were left to grow for 30 days and they were irrigated with drinking water.

Fertilization was carried out using Ultrasol (15-30-15, 1% S, 1% MgO and Micronutrients) in a dilution of 1 gr/L, once every third day; and Bayfolan (9% Nitrogen, 7% P2O5, 6% KO and Microelements) in a dilution of 5 ml/L, once a week by spraying the leaves.

To proceed with the microcosms establishment, pots were filled with 40 g of non-inoculated or inoculated substrate (Peat moss), according to the treatment. The bioaugmented pots contained $\mathbf{1}\boldsymbol{x}\mathbf{10}^{\mathbf{6}}$ cells/mg.

Once the seedlings had a considerable size, they were transferred into pots containing heavy metals and/or the isolated strains, as summarized in Table 6.

Table 6. Microcosms establishment, experiment summarized

Treatment	Strain	Heavy metal supplementation	Number of Replicates
Control	None	None	X3
Arsenic	None	As at 30 ppm	
Arsenic & Cadmium	None	As & Cd at 30 and 40 ppm respectively	
LA1	Inoculation with LA1	As at 30 ppm	
LA2	Inoculation with LA2	As & Cd at 30 and 40 ppm respectively	
LA1 & LA2	Inoculation with LA1 & LA2	As & Cd at 30 and 40 ppm respectively	

The treatments were established according to what was observed in the plant-microbe interaction experiment carried out *in vitro*.

Heavy metal uptake measurements

The most efficient treatment observed in the Microcosm experiment was selected for Inductively Coupled Plasma Mass Spectrometry (ICP) measurement of initial and final concentration of As and Cd in both the substrate and plant tissue, making of this test a destructive one, where the whole plant was analyzed.

ICP analysis is a highly sensitive technique that can determine concentrations of trace and major elements that consists on a spectrometric reading where the sample is vaporized as a fine aerosol of droplets by a nebulizer that aspirates argon along the sample after ionization. This technique is adequate to measure a range of chemical elements for the analysis of metal samples [28].

The labs that carried out the ICP analysis were SIASA Querétaro and the Instituto Tecnológico de Durango, México.

Chapter 7

Results and Discussion

Microbial Isolation

The selected microorganisms were labeled as LA1 & LA2, shown next:

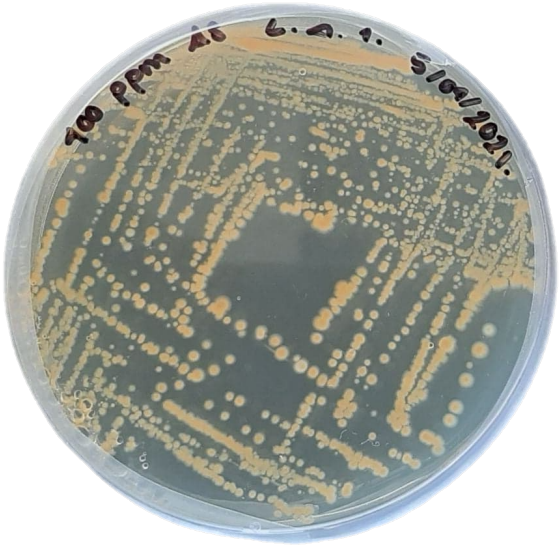

Fig 1. LA1. Orange bacteria labeled as LA1, grown in Nutritious Agar at 27ºC for 72 h.

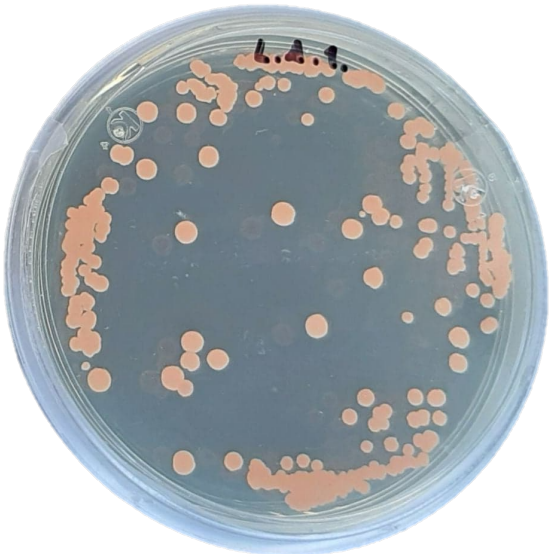

Fig 2. LA2. Pink yeast labeled as LA2, grown in PDA at 27ºC for 72 h.

As it is shown in Fig. 2., the pink yeast nominated as LA2 presents a pigmented morphology. This characteristic resembles the *Rhodotorula* sp., a yeast belonging

to the Basidiomycota division. Its macroscopic morphology consists on coral to pink colonies that reach a mature growth in 4 days. A representative image is provided in Fig. 3.

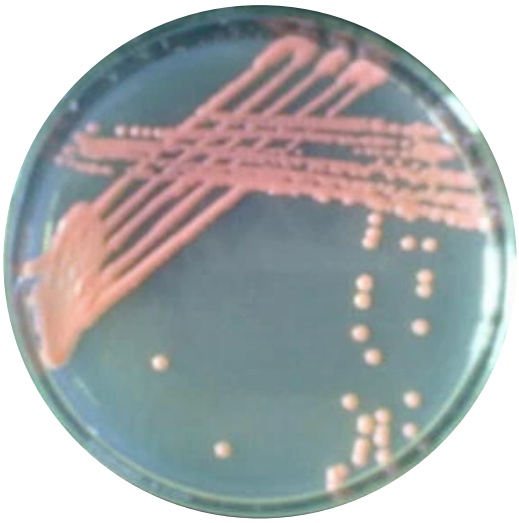

Fig. 3. *Rhodotorula sp.* Representative image of LA2 & *Rhodotorula* sp. resemblance [1].

Microbial Characterization

Minimum Inhibitory Concentration (MIC) determination

As described in Chapter 3, the Minimum Inhibitory Concentration (MIC) was determined by streaking the corresponding microorganism in plates at growing concentrations of As & Cd until it failed to grow. The orange bacteria LA1 proved to be resistant to As up to a concentration of 400, 800, 1200, and even 1800 ppm; and the pink yeast LA2 proved to be resistant to As & Cd up to a concentration of 50, 100, 150, 200, and 250 ppm each.

Fig. 4 and 5. MIC determination experiment for LA1. Orange bacteria grown in Nutritious medium at 27ºC for 72 h; where LA1 succeeded to grow at concentrations of 400, 800, 1200, and 1800 ppm of As.

Fig. 6 and 7. MIC determination experiment for LA2. Pink yeast grown in PDA at 27ºC for 48 h, where LA2 succeeded to grow at 50, 100, 200, 225, and 250 ppm of As and Cd.

Since microbes isolated from volcanic and geothermal areas have developed heavy-metal resistance due to their linkage with heavy-metal biogeochemical cycles, addressing the microbial richness of said environments is in order. It is important to keep in mind that metals like arsenic and cadmium are present in volcanic and geothermal springs. The arsenic resistance system of some microorganisms may also provide Cd tolerance; where the *ars* operon and *cadC* are the main systems involved in said resistance [40].

The identification of heavy-metal resistant strains is an extending field to obtain new biological tools towards Bioremediation. For instance, Pupolo *et al.* also isolated a new strain of *Geobacillus stearothermophilus* from the hydrothermally active zone of the Campi Flegrei volcano in Naples, Italy. Curiously, this strain was also resistant to arsenic and cadmium with a resistance of 1.9 mM of As(III), 117 mM of As(V), and 0.90 mM of Cd(II); among other metals like Co, Cr, Cu, Hg, Ni & V [40].

An additional intriguing research is the one developed by Brito *et al.*, where a microbial isolation of sulfur and sulfate reducer microbes was carried out from Los Azufres, Michoacán; resulting in the identification of *Rhodobacter, Acidithiobacillus, Thiomonas, Desulfurella and Thermodesulfobium* genera [7]. Therefore, it would

be possible that the isolated bacterium corresponds to one of this genus, and own a sulfur oxidizing activity; however, this suspicion still has to be addressed.

Microscopic Evaluation

Gram staining was executed for the orange bacterium, LA1, to begin with its characterization and to propose a DNA extraction protocol. It was determined that LA1 was a Gram-negative bacterium.

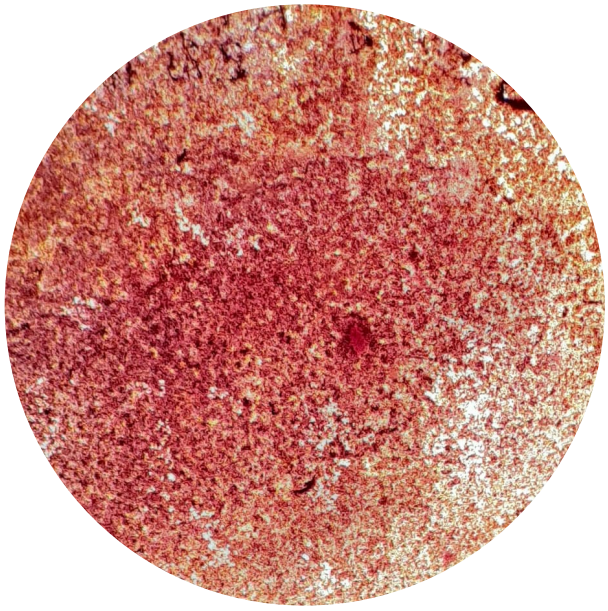

Fig. 8. Gram-negative staining of LA1. The observed microbial sample was taken from a Petri dish, grown for 48 h at 27ºC.

Gram-negative bacteria are model microorganisms used in laboratory experiments because of their fast growth, along with an extensively reported metal resistance [48]. The importance of Gram-negative bacteria use in bioremediation lies on their hidden mechanisms to avoid heavy metal toxicity. Gram-negative bacterial cell wall limits their movement due to the phospholipids, lipoproteins, lipopolysaccharides, and other proteins present in their outer membrane, where the phosphorylated groups located in the membrane also represent an absorbance region. This characteristic provides metal-binding capacity and triggering of heavy metal resistance [15].

Some Gram-negative examples of this nature include *Marinobacter* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Azotobacter* sp., *Enterobacter* sp., *Cupriavidus metallidurans* [48*].* Having this on mind, the impressive arsenic resistance and MIC of 1800 ppm of As belonging to the bacterium nominated as LA1 could be partly explained by the nature of its cell wall.

On the other hand, LA2 was observed under the microscope to verify that, in fact, the pink culture was not a bacterium, but yeast. Yeast are predominant microbial

species in polluted environments; and *Rhodotorula* has been reported as highly resistant to metal ions like Hg, Pb, and Cu; being its biofilm an efficient factor for heavy metal removal, ranging from 91.71 to 95.39% [18]. Since its characteristics agree, it is suggested that LA2 belongs to *Rhodotorula* sp.

Microbial biofilms provide tolerance for metal ions as well, being *Rhodotorula* an exopolysaccharide producer [25]. Other yeasts used in heavy metal bioremediation include *S. cerevisiae, Cryptococcus laurentii, and Candida tropicalis*.

Microbial growth

After carrying out the growth kinetics and analyzing the results, it was observed that multiple thresholds were shown, indicating different log or exponential stages. For instance, LA1 presents log phases at 3, 12, and 36 hours. As to LA2, log phases are perceived at 12, 33, and 45 hours. This could imply a diauxic behavior, an adaptation step that occurs when change in the medium conditions is resented; however, the first recognized exponential phases were considered as an optimum moment of microbial inoculation.

The growth behavior of both microbes is presented in Fig. 9.

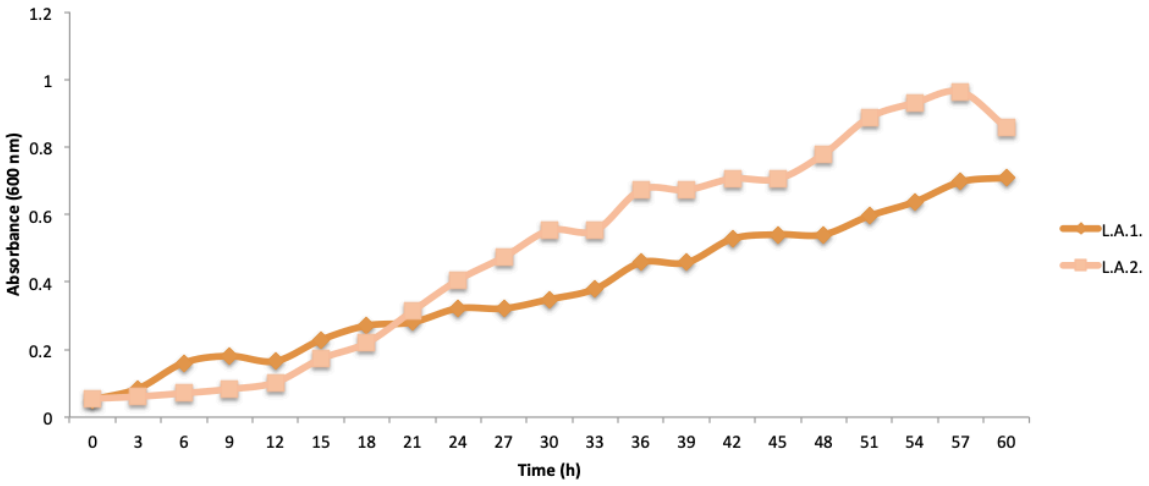

Fig. 9. Microbial Growth of LA1 & LA2. Multiple log stages are perceived. LA1 shows log phases at 3, 12, and 36 hours; while LA2 shows log phases at 12, 33, and 45 hours.

The moment of microbial inoculation is of great importance, since this moment determines the metabolites and processes that the microbe will provide to the system. The lag phase, previous to the log or exponential phase, is a moment of adaptation to the medium, where genes are activated by triggering elements, such as presence of heavy metals. After this phase, the exponential growth occurs when cells are already adapted.

It has been reported that gene expression is dependent of microbial growth rate [26]; therefore, heavy metal resistance genes are activated at this stage. As Rolfe *et al*. titled their article, “Lag Phase is a Distinct Growth Phase That Prepares Bacteria for Exponential Growth and Involves Transient Metal Accumulation”. According to these authors, *Salmonella enterica* has the ability to accumulate iron, calcium, and manganese during lag phase; but other metals like cobalt, nickel, and sodium are accumulated in distinct growth phases. Having this on mind, it is important to carry out inoculation of heavy metal resistant microorganisms at their early stage of growth [42].

DNA Extraction

Two DNA extraction protocols were performed: the Purelink Mini Kit protocol and a glass bead adaptation, obtaining the following data, read by a Nanodrop.

Table 7. Nanodrop readings from DNA extraction protocols, summarized

Strain	Protocol	$ng/\mu l$	A260/A280	A260/A230
LA1	Purelink protocol stored in Elution Buffer	12,1	1,99	1,13
LA1	Purelink protocol stored in Milli Q water	376,7	1,44	0,58
LA1	Glass bead adaptation	3,9	-7,34	-3,47
LA2	Purelink protocol stored in Elution Buffer	24,3	1,96	1,15
LA2	Purelink protocol stored in Milli Q water	18,2	2,01	0,54
LA2	Glass bead adaptation	5.8	1,86	1,10

Considering the desirable parameters of DNA concentration, A260/A280 & A260/A230, the most adequate protocol to proceed with DNA extraction methods for further amplification and molecular identification of LA1 & LA2, was the Invitrogen Purelink Mini Kit by Thermofisher Scientific extraction protocol. Samples retrieved with this method were selected for PCR amplification.

The bacterial strain, LA1, was successfully amplified using 16S rRNA, following the conditions presented in Table 8.

Table 8. PCR conditions for bacterial 16S amplification of LA1

Step	Temperature	Time
Initial Denaturation	95ºC	5 mins
Denaturing	94 ºC	1 min
Annealing	55 ºC	0:30 sec
Extension	72 ºC	2 mins, 34X
Final extension	72 ºC	10 min
	4 ºC	hold

A 1% agarose gel Electrophoresis was prepared to verify a successful amplification; where well number 8 represents the amplified LA1 DNA sample, and wells number 1 and 9 represent a 1 kb Invitrogen ladder by Thermo Fisher Scientific.

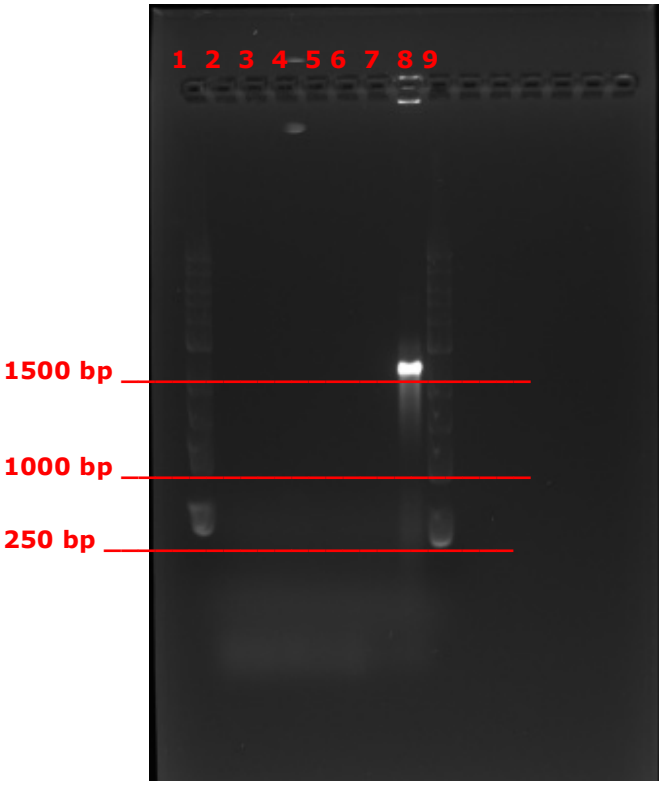

Fig. 10. Electrophoresis gel showing LA1 amplification of bacterial 16S. The amplified band is located near the 1500 bp size, agreeing with the size of 16S.

On the other hand, the pink yeast LA2 could not be amplified. It is suggested that for further experiments, the oligonucleotides belonging to the ITS region must be replaced. Also, it is highly recommended to continue with the identification by sequencing of LA1.

Plant-microbe interaction system *in vitro*

The results of this experiment are summarized in the following figures.

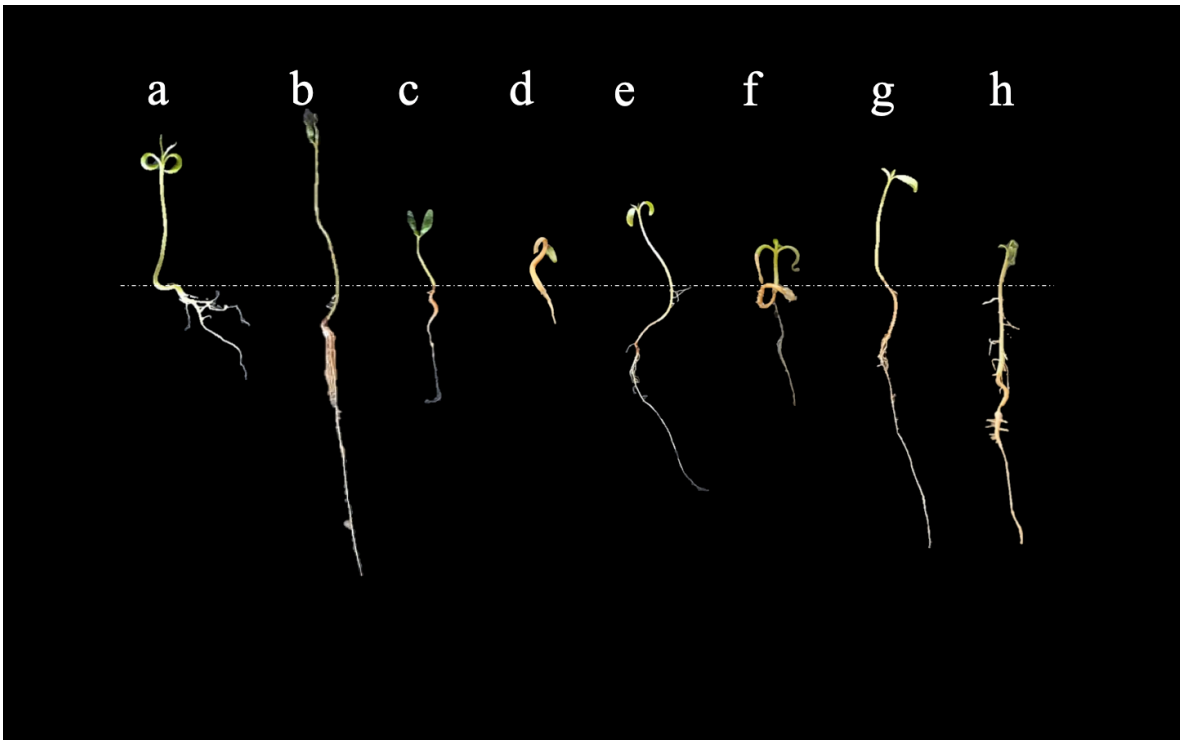

Fig. 11. Representative growth of *Tagetes erecta* inoculated with LA1. Treatments are shown as follows: a) Control, b) Inoculation of LA1, c) As, d) As inoculated by LA1, e) Cd, f) Cd inoculated by LA1, g) As + Cd, h) As + Cd inoculated by LA1.

The main highlight, product of this figure, is that the presence of heavy metals in the inoculated treatments induces a reduction in growth, for instance, in panel d and f. This behavior, where As affects vegetal growth, is reported as mentioned by Acosta-Álvarez.

It is important to keep in mind the perceivable damage caused in plants due to their exposure to metals like arsenic and cadmium, are growth reduction and alteration of Ca, K, P and Mn concentrations in the plants for the first one; and photosynthesis and transpiration inhibition, as well as chlorophyll inhibition; and modification in Mn, Ca and K concentrations for the second one [23]. The growth inhibition could have also been caused by a nutritional blockage.

The overall observation of the panel leads to the conclusion that LA1 alone is not an adequate microbe to be inoculated in a tolerant plant for bioremediation purposes, since is intervenes with its development in presence of heavy metals like As and Cd.

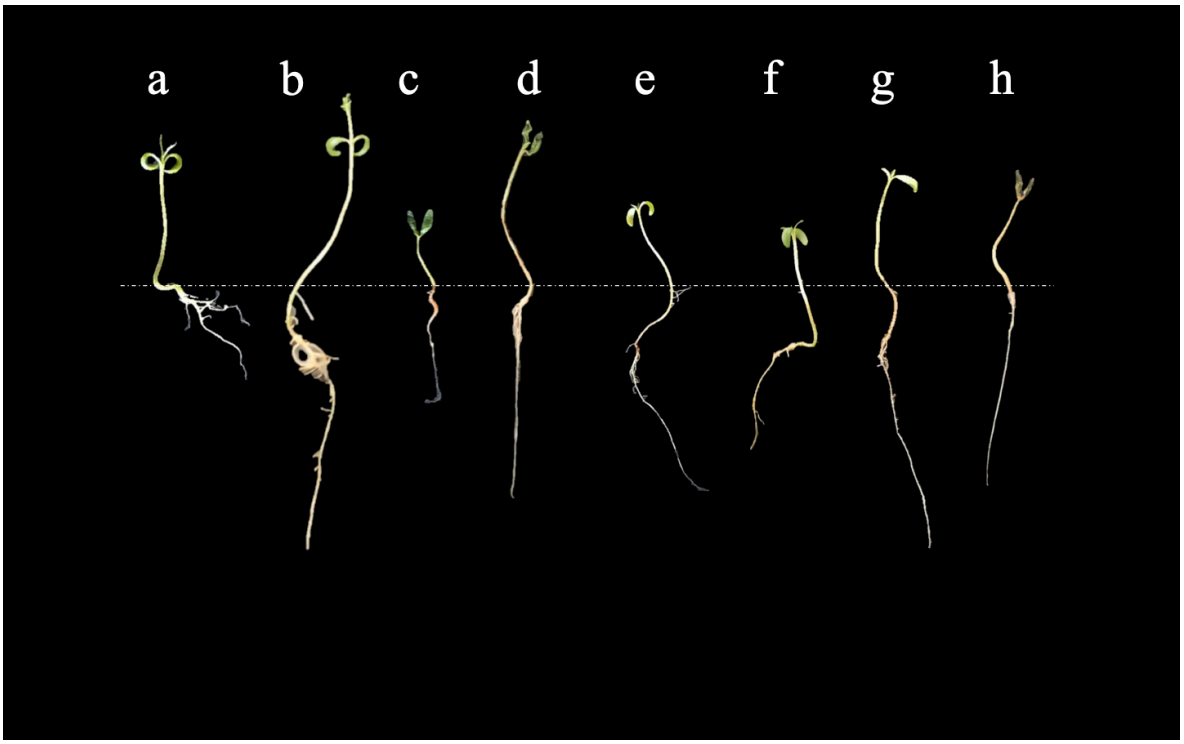

Fig. 12. Representative growth of *Tagetes erecta* inoculated with LA2. Treatments are shown as follows: a) Control, b) Inoculation of LA2, c) As, d) As inoculated by LA2, e) Cd, f) Cd inoculated by LA2, g) As + Cd, h) As + Cd inoculated by LA2.

In this second figure, the treatments involving the pink yeast, LA2, are summarized. What jumps to sight is that there is amelioration in shoot and root growth compared to the treatments where the yeast is not present. For instance, treatments b and d show an evident overcome compared with their counterparts a and c.

Related to this aspect, *Rhodotorula* is reported as a plant-growth promoting microorganism, and is resistant to Cd and As [21], up to a concentration of 100 mg/L for Cd [19]. Metal resistant *Rhodotorula mucilaginosa* strain isolated from industrial water showed effects over reduced/oxidized glutathione when heavy metals were present in a concentration of 100 mg/L. Gluthathione increased with CdCl2 (18.43±3.34) and NaAsO2 (14.76±2.14), compared to the control. Therefore, *Tagetes erecta* might be having an enhanced effect in metal chelation when inoculated with LA2.

This genus also has also been reported as a high IAA-producing yeast with a potential to be used as a biocontrol [20]. The production of exopolysaccharides favors the survival of microorganisms in presence if exogenous components like heavy metals.

Considering these beneficial aspects of As & Cd reported in *Rhodotorula* sp., and its plant-growth promoting activity, it is suggested that LA2 continues to agree in

this sense and is considered as an adequate microorganism to be inoculated in tolerant plants to enhance their individual characteristics of heavy metal resistance and bioremediation purposes.

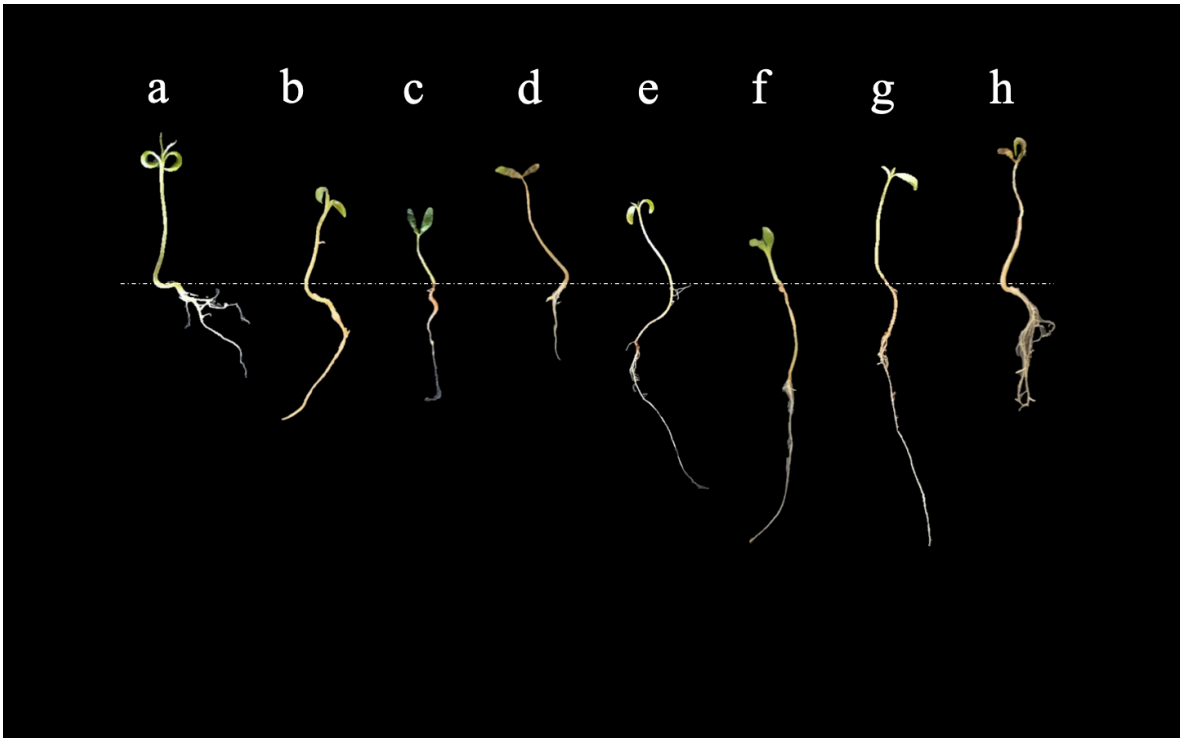

Figure 13. Representative growth of *Tagetes erecta* inoculated with LA1 & LA2. Treatments are shown as follows: a) Control, b) Inoculation of both microorganisms, c) As, d) As inoculated by both microorganisms, e) Cd, f) Cd inoculated by both microorganisms, g) As + Cd, h) As + Cd inoculated by both microorganisms.

Finally, Fig. 13 summarizes the treatments involving the inoculation of both microorganisms. The presence of both LA1 & LA2 do not seem to block or inhibit vegetal growth; however, it does not seen to enhance it either. The inoculated treatments are similar to their non-inoculated counterparts.

It could be proposed that the combination of the microbes has a resilient behavior over the plant, where the detrimental effects shown by inoculation of LA1 alone or the beneficial effects shown by inoculation of LA2 are not repeated when combined, but offset.

Another assumption would describe LA2 with its biocontrol activity, where LA1 seems to be displaced, and therefore, the detrimental effects it causes are not shown.

Either way, from the 3 previously presented figures, LA2 seems to be the best candidate for phytoremediation purposes.

Statistical analysis of plant-microbe interaction system *in vitro*.

Growth parameters including root length, number of secondary roots, shoot length, and number of leaves, were measured for each established treatment for an *in vitro* interaction analysis between *Tagetes erecta* and LA1 & LA2.

A Tuckey Statistical Analysis with a confidence level of 95%, error rate of comparison of 5, and n=3; was executed using Minitab version 19.2020.1.0 for each parameter mentioned above. Means that do not share a letter are significantly different; if letters are not shown, statistical difference is not given. In addition to this, figures were created using GraphPad Prism Version 9.1.2 (225), where the median value ± SE and letters belonging to Tuckey's test are shown.

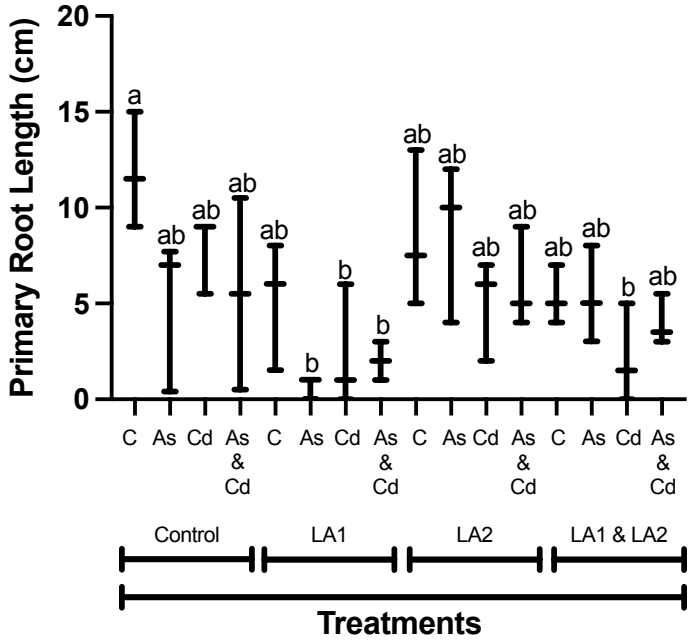

Figure 14. Statistical analysis corresponding to primary root length. All treatments are similar to the control, with the exception of LA1 in the presence of any metal and their combination, and LA1 & LA2 with Cd.

As expected, LA2 shares a group where root elongation is statistically similar. This could be explained by the background that yeast like *Rhodotorula* present related to plant growth promotion, more specifically, root elongation and auxin production.

Data included in Group b show a significantly lower Mean compared to the Control, and considering the Control as a healthy state for *Tagetes erecta* seedlings, it is concluded that the remaining treatments can thrive in their respective conditions. However, a more robust analysis is suggested with more replicates.

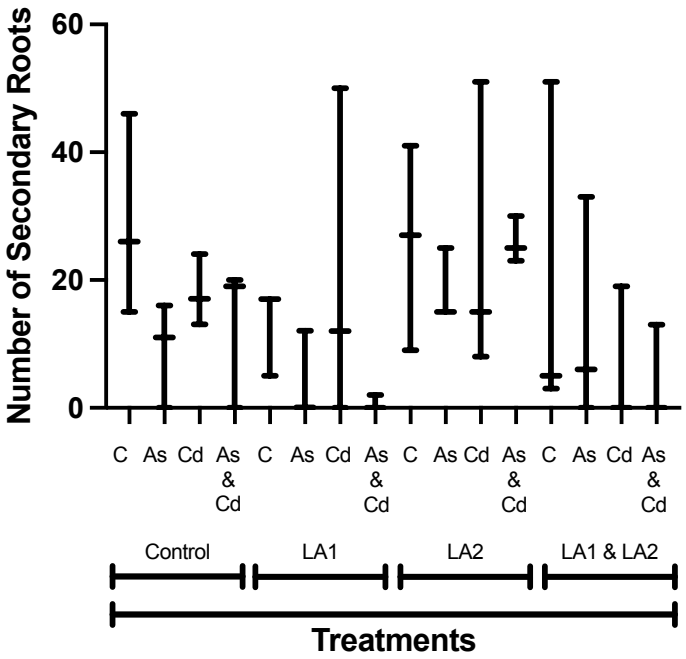

Figure 15. Statistical Analysis corresponding to number of secondary roots. According to Tuckey's test, there is no significant difference between treatments.

Since significant means are not recognized between the treatments, it cannot be inferred that any of the treatments' components are responsible for secondary root development or its inhibition. However, data shown in LA2 Control inoculation is similar to the overall Control; therefore, a healthy root system is observed with yeast inoculation.

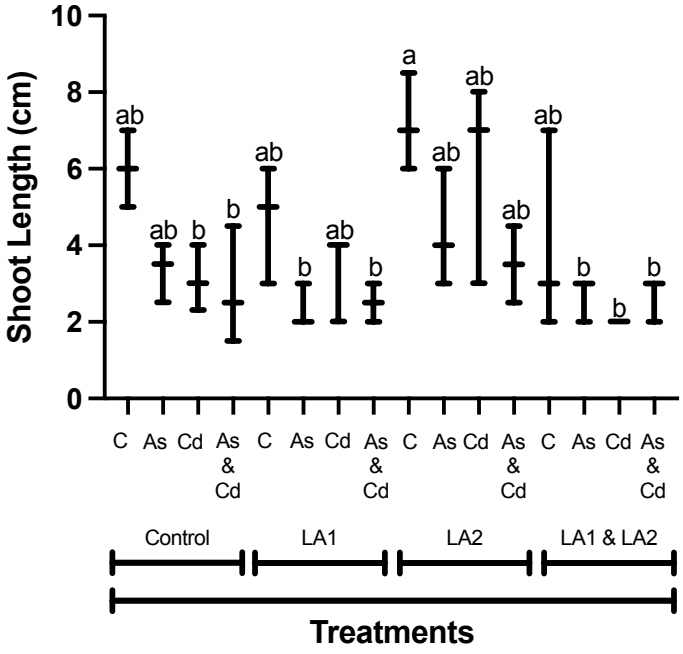

Figure 16. Statistical analysis corresponding to shoot length. The best treatment according to shoot length is the microbial inoculation of LA2.

Shoot elongation by microbial inoculation of LA2 overpasses the healthy state of Control; therefore, there is an evident promotion of this parameter caused by the pink yeast LA2.

On the other hand, the considered “worst” treatments include inoculation of both microorganisms in the presence of any metal and their combination; and inoculation of LA1 in the presence of As and both metals; as well as controls of Cd and both metals.

The remaining treatments are similar to the best treatment, LA2 inoculation. Again, the background provided by *Rhodotorula* agrees with the observed data.

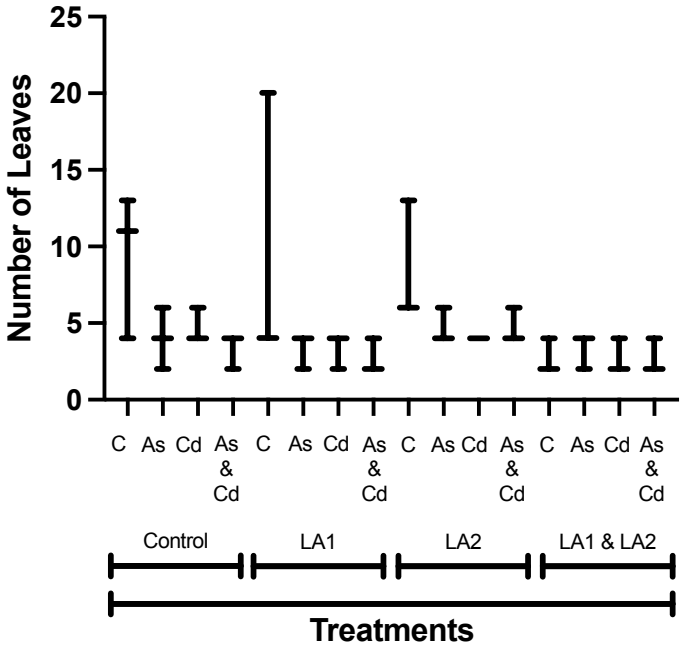

Figure 17. Statistical analysis corresponding to number of leaves. There is no aggrupation, therefore, the treatments do not influence the development of leaves.

Once more, there is no statistical difference of means between treatments; therefore, it cannot be inferred that any of the treatments' components are the responsible of *de novo* leaf development or its inhibition.

This could be due to the early stage at which seedlings are analyzed, stage where cotyledons have just opened.

Microcosms establishment

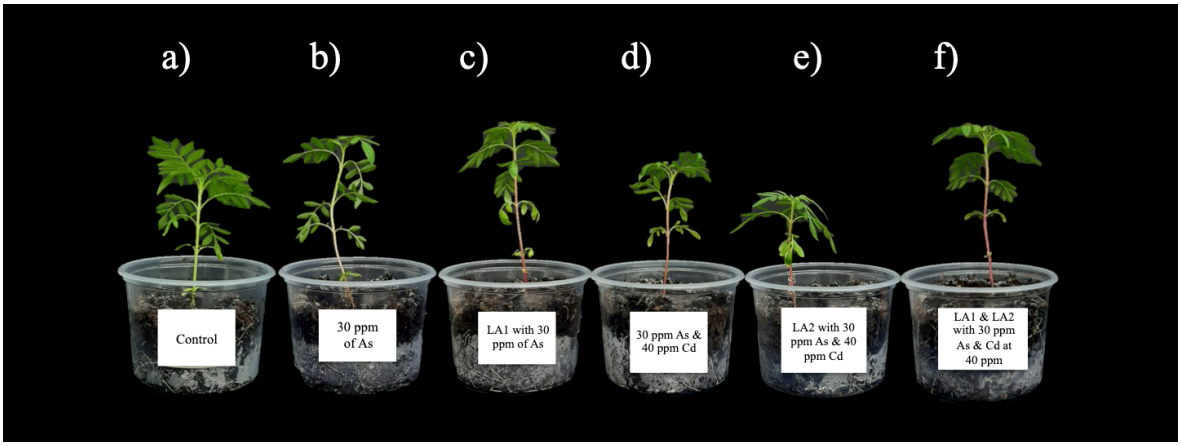

Fig.18. **Representative Microcosms establishment of *Tagetes erecta* and the selected microbes LA1 & LA2.** a) Control; b) 30 ppm As; c) Inoculation of LA1 with 30 ppm As; d) 30 ppm As & 40 ppm Cd; e) Inoculation of LA2 with 30 ppm As & 40 ppm Cd; f) Inoculation of LA1 & LA2 with 30 ppm As & 40 ppm.

The replicated treatments in microcosms showed a different behavior than the *in vitro* interaction between *Tagetes erecta* and LA1 & LA2. Changes given from initial time (T0) and after 18 days of transplant (Tf) are summarized in Table 9.

Table 9. Summary of the change registered in microcosms after 18 days of transference

Treatment	Shoot Growth Average (cm)	*De novo* Leaf Number Average	Survival (%)
Control	9.5 ± 0.86	69.33 ± 7.21	100
30 ppm As	7.16 ± 6.44	42 ± 37.04	66.66
LA1 with 30 ppm As	8.83 ± 1.25	62 ± 12.48	100
30 ppm As & 40 ppm Cd	7.83 ± 7	52.66 ± 46.30	66.66
LA2 with 30 ppm As & 40 ppm Cd	6.5 ± 5.89	42.33 ± 36.66	33.33
LA1 & LA2 with 30 ppm As & 40 ppm Cd	10.83 ± 1.89	71.66 ± 5.5	100

According to the data presented in Table 9, the best treatment corresponds to LA1 & LA2 with 30 ppm As & 40 pm Cd (panel f); where the Shoot Growth Average, *de novo* leaf number average, and percentage of Survival are the highest values between all treatments; even better than the Control. However, for ICP analysis, the treatments that presented a survival rate of 100% were selected.

The intention of this experiment was to measure the reduction of arsenic and cadmium in the substrate, and the accumulation of the metals in *Tagetes erecta* when inoculated with LA1 & LA2.

Different performance could have been expected since the plants used for microcosms establishment were older than the ones used for the *in vitro* interaction experiment. Also, the matrix in which the microbes and the plants develop is different, and the pot experiment is performed in open air.

Heavy metal uptake measurements

Once the microcosms reached day 18 of the experiment, they were sent to SIASA Lab in Querétaro, México, to be analyzed by ICP. The obtained results are shown in Table 9.

Table 10. Treatments and samples sent for ICP Analysis.

Sample	Treatment	As Reading (mg/kg)	Cd Reading (mg/kg)
Substrate	Control	0.687	0.072
Vegetal Tissue	Control	2.935	0.270
Substrate at T0	Arsenic and Cadmium	17.741	17.395
Substrate at T0	As & Cd + LA1 & LA2	6.603	27.987
Vegetal Tissue at Tf	As & Cd + LA1 & LA2	< 0.0005011	< 0.0002506

The obtained results do not agree with the prepared microcosms. Control samples were expected not to present As & Cd at all; and the treatments containing As & Cd + LA1 & LA2 were expected to present the higher concentrations.

It is possible that the samples were not homogenized correctly, or that the presence of As & Cd in Control experiment were due to fertilization. However, fertilization was carried out equally for all seedlings and plants.

Since the results are out of order and comprehension, the rest of the microcosms and replicates will be sent for ICP analysis to the Instituto Tecnológico de Durango. Conclusions will be drawn then.

Statistical analysis of microcosms establishment.

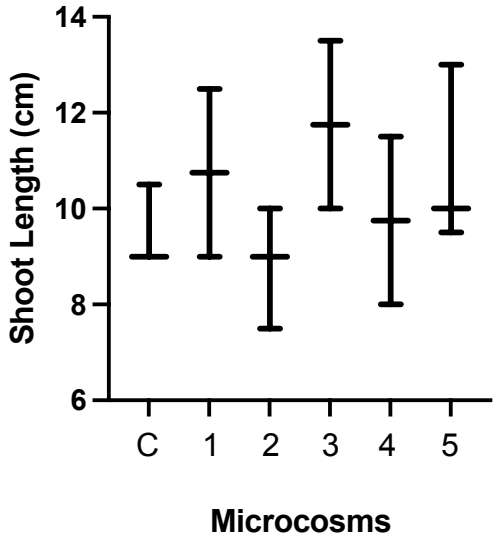

Fig.19. **Statistical Analysis of microcosms shoot length development.** Median values of shoot length ± SE, n=3 are shown. C corresponds to Control; 1) 30 ppm As; 2) Inoculation of LA1 with 30 ppm As; 3) 30 ppm As & 40 ppm Cd; 4) Inoculation of LA2 with 30 ppm As & 40 ppm Cd; 5) Inoculation of LA1 & LA2 with 30 ppm As & 40 ppm.

Statistical differences by aggrupation is not registered, however, as shown in Table 10, treatments overpass control development in terms of shoot. It could be concluded that treatments present a better development compared with the control when sown in pots. Open air interactions might benefit heavy-metal tolerance and PGPMs development.

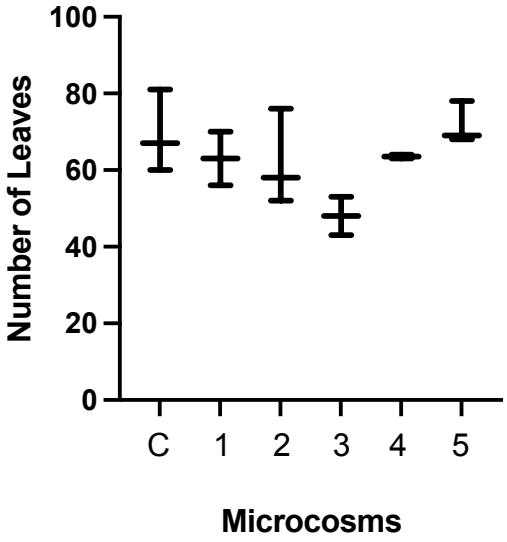

Fig.20. **Statistical Analysis of microcosms *de novo* leaf development.** Median values of shoot length ± SE, n=3 are shown. C corresponds to Control; 1) 30 ppm As; 2) Inoculation of LA1 with 30 ppm As; 3) 30 ppm As & 40 ppm Cd; 4) Inoculation of LA2 with 30 ppm As & 40 ppm Cd; 5) Inoculation of LA1 & LA2 with 30 ppm As & 40 ppm.

De novo leaf development does not seem to be different between treatments. This means that leaf development did not stop in the presence of metals and/or microbes; therefore, any treatment can thrive when sown in pots.

However, once more, data shown in Table 10 could point out the treatment involving LA1 & LA2 in the presence of both metals as the better treatment, overpassing measurements related to controls. However, it is recommended to repeat experiments with a higher number of replicates to draw conclusions.

Chapter 8

Conclusions

With the previously presented data it can be concluded that the selected plant species, *Tagetes erecta*, and the microbial strain isolated from Los Azufres Michoacán, LA2, are great candidates to use in a phytoremediation system alone or combined with LA1; since they present a beneficial or non detrimental interaction when in presence of heavy metals like arsenic and cadmium.

Chapter 9

Perspectives

It is highly suggested to continue with the molecular identification and sequencing processes for both LA1 and LA2 to exploit the microbial diversity from extreme environments like Mexican volcanic areas; and explore their application with bioremediation purposes.

In addition, an incorporating experiment to this thesis would cover an *ex situ* bioremediation procedure utilizing this system in order to analyze the feasibility of its application in real contaminated soils.

Furthermore, it is also encouraged to try this phytoremediation system with other heavy metals and different type of pollutants.

Finally, the possible escalation of the phytoremediation process in agricultural/residential/commercial type of soil would be ideal.

Appendix A

Abbreviations and acronyms

Table A.1 Abbreviations

	Description
LA1	**L**os **A**zufres **1**
LA2	**L**os **A**zufres **2**
MIC	**M**inimum **I**nhibitory **C**oncentration
ICP	**I**nductively **C**oupled **P**lasma
ROS	**R**eactive **O**xygen **S**pecies
PTE	**P**otentially **T**oxic **E**lements

Table A.2 Acronyms

	Description
As	Arsenic
Cd	Cadmium
Cr	Chromium
Hg	Mercury
Ni	Nickel
Au	Silver
Pb	Lead
Zn	Zinc
Mo	Molybdenum
Se	Selenium
Al	Aluminum
Fe	Iron
Ca	Calcium
K	Potassium
Mn	Manganese
Mg	Magnesium
S	Sulfur
Co	Cobalt
Sn	Stannum
Ppm	Parts per million
T0	Initial Time
Tf	Final Time

Bibliography

[1] Abdul Salam, J., Lakshmi, V., Das, D. *et al.* Biodegradation of lindane using a novel yeast strain, *Rhodotorula* sp. VITJzN03 isolated from agricultural soil. *World J Microbiol Biotechnol* **29,** 475–487 (2013). https://doi.org/10.1007/s11274-012-1201-4

[2] Abhilash, P. C*., et al. "*Plant-Microbe Interactions: Novel Applications for Exploitation in Multipurpose Remediation Technologies." *Trends Biotechnol* 30.8 (2012): 416-20. Print.

[3] Acosta-Álvarez, M. Determinación de metales pesados en suelos agrícolas del Valle del Mezquital, Hgo. Universidad Autónoma del Estado de Hidalgo. (2007).

[4] Ayangbenro, A. S., and O. O. Babalola. "A New Strategy for Heavy Metal Polluted Environments: A Review of Microbial Biosorbents." *Int J Environ Res Public Health* 14.1 (2017). Print.

[5] Baker, A. J. M., and P. L. Walker. "Physiological Responses of Plants to Heavy Metals and the Quantification of Tolerance and Toxicity." *Chemical Speciation & Bioavailability* 1.1 (1989): 7-17. Print.

[6] Bashir, S., and A. H. Gilani. "Studies on the Antioxidant and Analgesic Activities of Aztec Marigold (*Tagetes Erecta*) Flowers." *Phytother Res* 22.12 (2008): 1692-4. Print.

[7] Brito, E. M*., et al.* "Microbial Diversity in Los Azufres Geothermal Field (Michoacan, Mexico) and Isolation of Representative Sulfate and Sulfur Reducers." *Extremophiles* 18.2 (2014): 385-98. Print.

[8] Castillo, O S *et al.* "The effect of the symbiosis between *Tagetes erecta* L. (marigold) and *Glomus intraradices* in the uptake of Copper(II) and its implications for phytoremediation." *New biotechnology* vol. 29,1 (2011): 156-64. doi:10.1016/j.nbt.2011.05.009

[9] Chibuike, G. U., and S. C. Obiora. "Heavy Metal Polluted Soils: Effect on Plants and Bioremediation Methods." *Applied and Environmental Soil Science* 2014 (2014): 752708. Print.

[10] Coelho, L., Bastos, A., Pinho, P., Souza, G., Carvalho, J., Coehlo, V., Oliveira, C., Domingues, R., and Faquin, V. "Marigold (*Tagetes erecta*): The Potential Value in the Phytoremediation of Chromium", *Pedosphere*, Volume 27, Issue 3 (2017): 559-568, ISSN 1002-0160,https://doi.org/10.1016/S1002-0160(17)60351-5.

[11] Corsini, A., Cavalca, L., Crippa, L., Zaccheo, P., Andreoni, "Impact of glucose on microbial community of a soil containing pyrite cinders: Role of bacteria in arsenic mobilization under submerged condition." (2010). Soil Biol Biochem. 42:699-707

[12] Das, M., and Subodh Maiti. "Metal Mine Waste and Phytoremediation: A Review." *Asian J. Water Environ. Pollut.* 4 (2007): 169-76. Print.

[13] De la Peña-Cerda, V. "Evaluación de la Concentración de Plomo y Cadmio en Suelo Superficial de Parques y Plazas Públicas, en tres municipios del área metropolitana de Monterrey, Nuevo León, México". Universidad Autónoma de Nuevo León. (2014).

[14] Fiallos-Morales, M. “Cuantificación de metales pesados y calidad microbiológica de frutas y vegetales que se expenden en el Mercado mayorista de la ciudad de Ambato”. Universidad Técnica de Ambato. Facultad de Ciencia e Ingeniería en Alimentos. Ambato-Ecuador. (2017).
[15] Giovanella, Patricia *et al.* “Metal resistance mechanisms in Gram-negative bacteria and their potential to remove Hg in the presence of other metals.” *Ecotoxicology and environmental safety* vol. 140 (2017): 162-169. doi:10.1016/j.ecoenv.2017.02.010
[16] Goldman, E. A., and R. T. Moore. "The Biotic Provinces of Mexico." *J Mammal* 26 (1945): 347-60. Print.
[17] Goswami, S., and S. Das. "Screening of Cadmium and Copper Phytoremediation Ability of *Tagetes Erecta*, Using Biochemical Parameters and Scanning Electron Microscopy-Energy-Dispersive X-Ray Microanalysis." *Environ Toxicol Chem* 36.9 (2017): 2533-42. Print.
[18] Grujić, S., Vasić, S., Radojević, I. *et al.* Comparison of the *Rhodotorula mucilaginosa* Biofilm and Planktonic Culture on Heavy Metal Susceptibility and Removal Potential. *Water Air Soil Pollut* **228,** 73 (2017). https://doi.org/10.1007/s11270-017-3259-y
[19] Hongli, Yuan, *et al*. "Mechanism of Cadmium Resistance and Adsorption of a Yeast Strain *Rhodotorula* Sp. Y11." *Science in China, Series D: Earth Sciences* 48 (2005): 262-68. Print.
[20] Ignatova, Lyudmila V *et al*. “Plant growth-promoting and antifungal activity of yeasts from dark chestnut soil.” *Microbiological research* vol. 175 (2015): 78-83. doi:10.1016/j.micres.2015.03.008
[21] Ilyas, Sidra *et al*. “Proteomic analysis of an environmental isolate of Rhodotorula mucilaginosa after arsenic and cadmium challenge: Identification of a protein expression signature for heavy metal exposure.” *Journal of proteomics* vol. 141 (2016): 47-56. doi:10.1016/j.jprot.2016.04.012
[22] Jain, Reena, *et al*. "In Vitro Anti Bacterial Potential of Different Extracts of *Tagetes Erecta* and *Tagetes Patula*." *J Nat Sci Res* 2 (2013). Print.
[23] Jaishankar, Monisha *et al*. “Toxicity, mechanism and health effects of some heavy metals.” *Interdisciplinary toxicology* vol. 7,2 (2014): 60-72. doi:10.2478/intox-2014-0009
[24] Jarup, L. "Hazards of Heavy Metal Contamination." *Br Med Bull* 68 (2003): 167-82. Print.
[25] Kapahi, M., and S. Sachdeva. "Bioremediation Options for Heavy Metal Pollution." *J Health Pollut* 9.24 (2019): 191203. Print.
[26] Klumpp, Stefan *et al*. “Growth rate-dependent global effects on gene expression in bacteria.” *Cell* vol. 139,7 (2009): 1366-75. doi:10.1016/j.cell.2009.12.001
[27] Kuiper, I., *et al.* "Rhizoremediation: A Beneficial Plant-Microbe Interaction." *Mol Plant Microbe Interact* 17.1 (2004): 6-15. Print.
[28] Laboratory Testing Inc. ICP Analysis. Retrieved from: https://www.labtesting.com/services/materials-testing/chemical-analysis/icp-analysis/

[29] Latorre-Ochoa, S.M. “Búsqueda de genes de Resistencia a arsenic en el metagenoma microbiano de la Sabana de Bogotá”. Universidad Nacional de Colombia (2014).
[30] Liu, Z., W. Chen, and X. He. "Evaluation of Hyperaccumulation Potentials to Cadmium (Cd) in Six Ornamental Species (Compositae)." *Int J Phytoremediation* 20.14 (2018): 1464-69. Print.
[31] Ma, Y., *et al*. "Biochemical and Molecular Mechanisms of Plant-Microbe-Metal Interactions: Relevance for Phytoremediation." *Front Plant Sci* 7 (2016): 918. Print.
[32] Meier, S., *et al.* "Influence of Copper on Root Exudate Patterns in Some Metallophytes and Agricultural Plants." *Ecotoxicology and Environmental Safety* 75 (2012): 8-15. Print.
[33] Mendoza-Cozatl, D. G., *et al*. "Long-Distance Transport, Vacuolar Sequestration, Tolerance, and Transcriptional Responses Induced by Cadmium and Arsenic." *Curr Opin Plant Biol* 14.5 (2011): 554-62. Print.
[34] Mleczek, M., *et al.* "Influence of Ca/Mg Ratio on Phytoextraction Properties of *Salix Viminalis* I. The Effectiveness of Cd, Cu, Pb, and Zn Bioaccumulation and Plant Growth." *Int J Phytoremediation* 14.1 (2012): 75-88. Print.
[35] Morais S, Costa FG, Pereira ML. Heavy metals and human health. In: Oosthuizen J, editor. *Environmental health – emerging issues and practice.* 2012. pp. 227–246. [InTech]
[36] Nies, D. H. "Microbial Heavy-Metal Resistance." *Appl Microbiol Biotechnol* 51.6 (1999): 730-50. Print.
[37] Official Mexican Nom NOM-147-SEMARNAT/SSA1-2004. http://www.profepa.gob.mx/innovaportal/file/1392/1/nom-147-semarnat_ssa1-2004.pdf
[38] Phieler, René, *et al.* "Phytoremediation Using Microbially Mediated Metal Accumulation in Sorghum Bicolor." *Environmental Science and Pollution Research* 22.24 (2015): 19408-16. Print.
[39] Pratush, A., A. Kumar, and Z. Hu. "Adverse Effect of Heavy Metals (as, Pb, Hg, and Cr) on Health and Their Bioremediation Strategies: A Review." *Int Microbiol* 21.3 (2018): 97-106. Print.
[40] Puopolo, Rosanna *et al.* “Identification of a New Heavy-Metal-Resistant Strain of *Geobacillus stearothermophilus* Isolated from a Hydrothermally Active Volcanic Area in Southern Italy.” *International journal of environmental research and public health* vol. 17,8 2678. 14 Apr. 2020, doi:10.3390/ijerph17082678
[41] Rajkumar, M., *et al.* "Perspectives of Plant-Associated Microbes in Heavy Metal Phytoremediation." *Biotechnol Adv* 30.6 (2012): 1562-74. Print.
[42] Rolfe, Matthew D., et al. "Lag Phase Is a Distinct Growth Phase That Prepares Bacteria for Exponential Growth and Involves Transient Metal Accumulation." *Journal of Bacteriology* 194.3 (2012): 686-701. Print.
[43] Rungruang, Nonglak, Sandhya Babel, and Preeda Parkpian. "Screening of Potential Hyperaccumulator for Cadmium from Contaminated Soil." *Desalination and Water Treatment - DESALIN WATER TREAT* 32 (2011): 19-26. Print.

[44] Sánchez-Contreras, J.C. Metodologías analíticas para la determinación de metales tóxicos en muestras de interés ambiental. 2010. Universidad Nacional de Colombia. Master's Thesis.
[45] SEDAPA. Oaxaca entre los 10 estados de mayor producción de cempasúchil. Retrieved from: https://www.oaxaca.gob.mx/sedapa/oaxaca-entre-los-10-estados-de-mayor-produccion-de-cempasuchil/#:~:text=Oaxaca%20entre%20los%2010%20estados%20de%20mayor%20producción%20de%20cempasúchil,-3&text=El%20Estado%20de%20México%2C%20Puebla,adornan%20los%20altares%20de%20muertos.
[46] Shadman, Seyedeh Malahat, *et al*. "Chapter 8 - Aptamer-Based Electrochemical Biosensors." *Electrochemical Biosensors*. Ed. Ensafi, Ali A.: Elsevier, 2019. 213-51. Print.
[47] Sharma, Anamika, *et al.* "Efficient Microorganism Compost Benefits Plant Growth and Improves Soil Health in Calendula and Marigold." *Horticultural Plant Journal* 3.2 (2017): 67-72. Print.
[48] Singh R.P., Anwar M.N., Singh D., Bahuguna V., Manchanda G., Yang Y. (2020) Deciphering the Key Factors for Heavy Metal Resistance in Gram-Negative Bacteria. In: Singh R., Manchanda G., Maurya I., Wei Y. (eds) Microbial Versatility in Varied Environments. Springer, Singapore. https://doi.org/10.1007/978-981-15-3028-9_7
[49] Sinhal, V. K., A. Srivastava, and V. P. Singh. "Edta and Citric Acid Mediated Phytoextraction of Zn, Cu, Pb and Cd through Marigold (*Tagetes Erecta*)." *J Environ Biol* 31.3 (2010): 255-9. Print.
[50] SOARES, CLÁUDIO ROBERTO FONSÊCA SOUSA, *et al*. "Acúmulo E Distribuição De Metais Pesados Nas Raízes, Caule E Folhas De Mudas De Árvores Em Solo Contaminado Por Rejeitos De Indústria De Zinco." *Revista Brasileira de Fisiologia Vegetal* 13 (2001): 302-15. Print.
[51] van der Ent, A., Baker, A. J. M., Reeves, R. D., Pollard, A. J., & Schat, H. “Hyperaccumulators of metal and metalloid trace elements: facts and fiction.” *Plant and Soil*, (2013): 319-334. https://doi.org/10.1007/s11104-012-1287-3
[52] van Dillewijn, P., *et al*. "Bioremediation, a Broad Perspective." *Microb Biotechnol* 2.2 (2009): 125-7. Print.
[53] Verma, Jay P., and Durgesh K. Jaiswal. "Book Review: Advances in Biodegradation and Bioremediation of Industrial Waste." *Frontiers in Microbiology* 6.1555 (2016). Print.
[54] WHO & IARC.WORLD HEALTH ORGANIZTION and INTERNATIONAL AGENCY FOR RESEARCH ON CANCER. “IARC Monographs on the evaluation of carcinogenic risks to humans”. Volume 23. Some Metals and Metallic Compounds (1998).
[55] WHO & IARC.WORLD HEALTH ORGANIZTION and INTERNATIONAL AGENCY FOR RESEARCH ON CANCER. “IARC Monographs on the evaluation of carcinogenic risks to humans”. Volume 84. Some Drinking-water Desinfectants and Contaminants, including Arsenic (2004)

[56] Xu, LW., Wang, GY. & Shi, YP. “Chemical constituents from *Tagetes erecta* flowers”. Chem Nat Compd 47, 281–283 (2011). https://doi.org/10.1007/s10600-011-9905-5

Additional Work

Published papers

Review article in Revision, Submitted to SN Applied Sciences in April 19th, 2021.

Review
Bio and Phytoremediation: Plants and microbes to the rescue of heavy metal polluted soils.

Sánchez-Jiménez Arantza1, Medrano-Roldán Hiram2, Kothe Erika3, Chávez-Avilés Mauricio Nahuam4, Valiente-Banuet Juan I.1, Fierros-Romero Grisel1*

Abstract

Hyperaccumulating plants and heavy metal resistant microbes own mechanisms embedded in their metabolism, proteins, and genes that confer them with "superpowers" that allow them to assimilate heavy metals in order to amend polluted soils; and when combined in a symbiotic system, these super characteristics could complement each other and be enhanced to overpower the exposure to toxic environments. A variety of benefits have been registered from symbiotic relationships, including plants teaming up with microbes to cope down with non-biodegradable elements such as heavy metals; but a manipulated interaction might signify a greater insight towards the application of bioremediation systems. These manipulations could consist of genetic engineering and/or additional supplementation of molecules and microbes. In the present study, a modernized connection between plants and microbes involving their controlled management is summarized in a visionary display.

Keywords: Bioremediation, phytoremediation, heavy metals, PGPMs.

Presentations in International Congresses

Appreciating the opportunities I had to share my research topic and results with groups of common interests, the Congresses I participated in are listed below.

- Oral presentation titled "Plant-microbe interaction systems to enhance bioremediation of heavy-metal polluted environments", executed in the 2nd International Congress of NanoBioEngineering CINBI, organized by the Universidad Autónoma de Nuevo León, on October 30th, 2020.

- Presentation of the poster titled "Plant-microbe interaction system to enhance bioremediation of heavy-metal polluted environments", given in the 51st Congress of Research and Development CID51, organized by the Instituto Tecnológico y de Estudios Superiores de Monterrey on February 2021.

This document was typed in using Microsoft Word by Arantza Sánchez Jiménez.