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Bacteria isolated from the chloragogenous tissue of the earthworm *Aporrectodea molleri*: molecular identification and characterization of their potential to promote the growth (PGP) and the phytoremediation ability of *Spinacia oleracea* cultivated under heavy metal stress

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Introduction

Earthworms (class *Oligochaeta*) comprise 90% of the soil's biomass (Brown and Doube 2004), contributing to the decomposition of organic matter, recycling of nutrients, and infiltration of water. Through their feeding and burrowing habits, earthworms create habitats for other organisms, demonstrating their role as ecosystem engineers and essential components of soil biodiversity (Edwards 2004; Bohlen 2017). Earthworms have a direct and significant impact on the soil microbiota. They can either digest microorganisms, thereby reducing microbial biomass, particularly that of fungi, or they can select or stimulate the growth of other microorganisms that aid them in the digestion of organic matter (Medina-Sauza et al. 2019). In contrast, earthworms' vital activity ensures the transport and distribution of microorganisms throughout the soil (Kuzyakov and Blagodatskaya 2015; Wani et al. 2017).

Throughout the last few decades, numerous studies have been conducted on the microorganisms associated with earthworms, specifically the symbiotic bacteria, either permanently or temporarily. The digestive tract of earthworms is the most thoroughly investigated organ; the bacteria inhabiting it appear to be related to the specific diet of each earthworm species, and their relative abundance is affected by the quality and availability of food resources (Drake and Horn 2007; Wani et al. 2017). The presence of earthworm-specific microbial symbionts outside of the digestive tract have been confirmed in nephridia. This is a monophyletic branch of the genus *Acidovorax*, and its proteolytic activity may be involved in the uptake of peptides and amino acids (Schramm et al. 2003; Davidson et al. 2013; Viana et al. 2018). Due to the fact that the majority of earthworm-isolated bacteria are metabolically more active and possess a variety of advantageous properties, interest in these organisms continues to increase (Singh et al. 2015). Numerous studies have documented the ability of earthworm gut isolates to function as plant growth-promoting bacteria (PGP), remove heavy metals, and/or degrade pollutants (Verma et al. 2011; Mudziwapasi et al. 2016; Singh et al. 2016; Biswas et al. 2018; Banerjee et al. 2019). In addition, earthworms possess chloragogenous tissue, a layer of cells surrounding the digestive tract that is in constant contact with the coelomic fluid, which is in constant contact with the external environment. The chloragogenous tissue is composed of chloragocytes, which are glycogen and lipid storage cells capable of accumulating additional metabolites (Fischer 1978; Fischer and Molnár 1992; Roubalová et al. 2015). They can neutralize heavy metals and function as cation exchangers and ion regulators in these processes (Vogel and Seifert 1992; Roubalová et al. 2018). The location of the chloragogenous tissue may have supported the growth of bacteria from the external environment that had resisted the immune effect of the coelomic fluid or outgrown the intestinal wall. It would be intriguing to investigate the potential presence of a bacterial community in chloragogenous tissue, since no studies have been conducted in this area previously.

Besides, heavy metal contamination of soils has become a major environmental problem in the present era, negatively impacting the health of plants, animals, and humans worldwide (Bhatti et al. 2018). This pollution is the result of both natural and anthropogenic processes, such as the excessive use of agricultural chemicals, wastewater irrigation, mining, and industrial waste discharge (Etesami 2018; Ahemad 2019). In addition, the total concentration of metals in the environment does not always reflect their biological toxicity or bioavailability, as factors such as pH, soil organic matter, soil texture, and nutrients influence their retention and mobility, making it difficult to assess metal risk accurately (Xu et al. 2012). Heavy metals in the soil environment cannot be mineralized or broken down into less toxic forms, unlike other contaminants. Multiple conventional physical-chemical methods have been employed. However, these treatments have limited utility due to their high cost, extensive labor requirements, irreversible alterations to soil structure, and disruption of soil microbiota (RoyChowdhury et al. 2018). Finding cost-effective, long-lasting, and environmentally responsible solutions for heavy metal remediation is a top priority.

Phytoremediation is an ecofriendly technique that uses plants to remove metals from the soil, either by absorbing and retaining the metals in their roots (Phytostabilization) or by transporting the metals to aerial tissues (phytoextraction). Few plant species are able to survive in heavy metal-contaminated soils. Some, due to their metal-extracting properties, can absorb heavy metals and accumulate them to unnaturally high levels (Adeoye et al. 2022). However, since the majority of these hyperaccumulators are slow-growing and have low biomass, it is tempting to boost the phytoremediation potential of non-hyperaccumulating plants that achieve substantial biomass even under stressful conditions (Mishra et al. 2017). Combining phytoremediation with soil bioaugmentation utilizing metal-tolerant bacteria capable of promoting plant growth has resulted in a potentially effective strategy for enhancing phytoremediation efficiency.

As an integral part of the soil, bacteria are capable of forming beneficial relationships with the vast majority of terrestrial plants. Through a number of processes, such as metal biosorption, bioaccumulation, precipitation, complexation, reduction, and oxidation, they can protect plants from the harmful effects of heavy metals (Ma et al. 2016; Etesami 2018). By influencing the mobility and availability of heavy metals, these bacteria benefit plants through a number of direct and indirect processes. Others are capable of producing organic acids and siderophores, thereby increasing the availability of nutrients and heavy metals. In addition, they can promote plant growth by modulating phytohormone levels and enhancing nutrient uptake (Chen et al. 2014; Ma et al. 2016). Upon soil inoculation, bacteria can either increase or decrease metal bioavailability, resulting in accelerated phytoremediation (increased heavy metal mobilization and phytoextraction) in metal-bearing soils or decreased heavy metal uptake (increased heavy metal immobilization) by plants (Ngugi et al. 2021). However, the factors that modulate the interaction between plants and bacteria and metal uptake remain unknown. The search for these multi-beneficial isolates with intriguing properties has extended beyond rhizospheric soil or mining regions to the most prevalent soil invertebrate biomass - earthworms. In fact, earthworms can survive in soils contaminated with heavy metals because they bind the metals in their chloragogenous tissue with organometallic compounds (Roubalová et al. 2018). Numerous researchers have shown that numerous earthworm PGP isolates can tolerate and remove multiple heavy metals in vitro (Biswas et al., 2018 ; Banerjee et al., 2019 ; Šrut et al., 2019 ; Liu et al., 2020 b ; Sun et al., 2020). However, few studies have examined their bioremediation capabilities in complex contaminated environments such as soil.

Spinach (*Spinacia oleracea* L.) is a widely cultivated and consumed leafy vegetable known for its high mineral content, including vitamin C, calcium, iron, phosphorus, sodium, and potassium (Ferreira et al. 2018). Due to this plant's ability to resist and accumulate heavy metals when grown in a contaminated environment, its edibility has been called into question (Salaskar et al., 2011 ; Suthar et al., 2013 ; Yin et al., 2016 ; Eid et al., 2017 ; Zafar-ul-Hye et al., 2020). In spite of this, the phytoremediation capacity of spinach grown in metal-bearing, highly cadmium-contaminated soil, as well as the potential use of multi-beneficial bacteria to enhance its capabilities, have been inadequately studied. Therefore, it is necessary to investigate the physiological response of spinach to various heavy metals, as well as the mechanisms influencing their uptake, translocation, and accumulation.

Subsequently, the overall objective of this thesis was to isolate and characterize the living bacterial community at the level of the chloragogenous tissue of the earthworm and to investigate their potential use as bioremediation agents to aid in the phytoremediation of heavy metal-contaminated soils.

Specific objectives :

- Identification of the most abundant species of earthworms at the level of the water dam in the region of Akrach, Rabat, Morocco.
- Study of the role of earthworm extracts on the growth of yeasts.
- Isolation and identification of aerobic bacteria from the chloragogenous tissue of earthworms.
- Characterization of isolated strains: plant growth promoting traits and resistance to heavy metals.
- Study of the effect of coating spinach seeds with bacterial isolates, on germination and growth of seedlings grown under metal stress.
- Investigation of the potential of the isolated bacteria to mobilize metals from the soil, to improve the bio-physical-chemical properties of the soil, to improve the growth of *Spinacia oleracea* L. grown in a metal-bearing soil (highly contaminated by cadmium), and to improve its phytoremediation potential.

Materials and Methods / Results and discussion

Adopted methodologies and detailed results are included in the attached articles.

General discussion and perspectives

As the most abundant invertebrate biomass in the soil, earthworms have demonstrated their ability to participate in almost all soil biogeochemical cycles in a positive manner (Edwards et al., 1996 ; Edwards, 2004 ; Bohlen, 2017), making them an attractive subject for researchers to investigate their interactions with soil components and microbial communities. The sampling site for earthworms was chosen near the water dam in the Akrach neighborhood of Rabat because earthworms thrive in temperate regions with sufficient moisture and organic matter. Specimens of earthworms were collected by excavating to a depth of 20 to 30 centimeters and transporting them to the laboratory, where the most abundant species were chosen for further study.

In contrast to morphological identification, molecular identification was performed successfully using four genes (COI, 16S rRNA, ND1, and 28S rRNA) and the earthworm specimen was identified as *Aporrectodea molleri*, demonstrating the efficacy of molecular methods in identification because all the four genes produced concordant results. Indeed, *Aporrectodea molleri* is a common species of earthworm in Mediterranean climates; its presence has been previously documented in Morocco, Algeria, Portugal, Spain, France, and Mexico (Trigo et al., 1988 ; Baha, 1997 ; Monroy et al., 2003 ; Pérez-Losada et al., 2012 ; Bazri, 2013 ; Reynolds et al., 2019). In contrast, little is known about Aporrectodea molleri in terms of its ecological role and microbial interactions. In addition, it would be interesting to map the different species of earthworms found in various regions of Morocco, as no such research has been conducted previously.

In our laboratory, we conducted a preliminary investigation into the impact of earthworm extracts (crude and cutaneous excretions) on a variety of soil microorganisms, with a particular focus on yeast growth. The results indicated that the crude earthworm extracts at a concentration of 3.75 g L^{-1} promoted significantly yeast growth (for both tested species) compared to the universal culture medium Sabouraud Dextrose Agar, which served as the growth control. Comparable results were obtained from extracts of fasted earthworms and freshly harvested earthworms, indicating that the nutrients and growth substances in the extracts are derived primarily from the worms' intrinsic composition and not from their intestinal contents. Dry earthworm powder has previously been reported to contain 60 to 70 percent protein, 8 to 20 percent carbohydrates, 7 to 10 percent lipids, 2 to 3 percent minerals, various vitamins, and rhizogenic indolic compounds, all of which represent a source of nutrients for soil microorganisms at optimal concentrations (Edwards, 1985 ; El Harti et al., 2001 ; Sogbesan et al., 2008 ; Anitha et al., 2012 ; Köse et al., 2017). These preliminary studies paved the way for the development of a culture medium that could optimize the cultivation and growth of soil microorganisms. In contrast to crude extracts, even at low concentrations of 0.06 g L^{-1}, cutaneous excretions inhibited the growth of both yeast species tested. In fact, cutaneous excretions consist of all fluids produced by the earthworm's surface, including mucus, coelomic fluid, and urine

(Salmon, 2001 ; Santocki et al., 2016 ; Raouane et al., 2020 ; Chelkha et al., 2021). They contain mucopolysaccharides and proteins that act as bactericides, reducing the number of microorganisms on the earthworm's surface and protecting it from microorganism invasion (Cooper et al., 2003 ; Wang et al., 2011). Using the activity of living coelomocytes, other studies have confirmed the inhibitory effect of earthworm coelomic fluid on the growth of various plant pathogenic microorganisms (Ueda et al., 2008 ; Plavšin et al., 2017 ; Ečimović et al., 2021). However, the fact that these excretions were sterilized at 121°C suggests that the molecule(s) responsible for this inhibitory effect are most likely derived from the humoral activity responsible for worm immunity and possess a thermostable property; its purification, characterization, identification, and proper application would be fascinating to investigate.

In recent years, the majority of bacteria isolated from the digestive tracts and castings of earthworms have been the subject of extensive research. However, no research has been conducted on the chloragogenous tissue, a layer of cells that surrounds the digestive tract. In this present study, we isolated and identified, for the first time, 15 bacterial strains associated with the chloragogenous tissue of *Aporrectodea molleri*. These strains were separated into two major phyla (*Firmicutes* and *Proteobacteria*) and four taxa (*Aeromonadaceae*, *Enterobacteriaceae*, *Pseudomonadaceae*, and *Bacillaceae*). The isolated strains were phylogenetically classified similarly to the majority of previous studies on bacterial communities. Nevertheless, the isolation of only bacteria belonging to the taxa *Firmicutes* and *Gammaproteobacteria* may be due to an inhibitory effect of the earthworm's coelomic fluid (Byzov et al. 2009 ; Singh et al. 2016 ; Wani et al. 2017) and/or the selected culture conditions. Consequently, a potential metagenomic study would shed additional light on this bacterial community.

Functional characterization of the isolated strains revealed that they possess a diverse enzyme profile, indicating that they are metabolically active. Previous studies confirmed that bacteria isolated from earthworms produced several enzymes, which may be involved in the decomposition of complex organic materials and natural polymers, during the digestion process, while coexisting in transient or permanent symbiosis with their host (Byzov et al. 2009, 2015 ; Samson et al. 2020). Moreover, several studies have established the efficacy of earthworm isolates due to their numerous beneficial properties (Biswas et al., 2018 ; Banerjee et al., 2019 ; Sun et al., 2020), which was consistent with our study, as three of the fifteen bacterial isolates were able to simultaneously produce IAA and siderophore, fix atmospheric nitrogen and produce ammonia, and solubilize phosphate and potassium. Five strains displayed at least four PGP characteristics simultaneously. The correlation between siderophore-producing strains and metal tolerance was intriguing as all siderophore-producing bacterial isolates could withstand high concentrations of Zn, Cu, Cd, and Ni. This correlation is supported by the fact that siderophores are involved in metal transport and sequestration without allowing the metals to cross the cell membrane (Rajkumar et al. 2010 ; Khan et al. 2018 ; Kramer et al. 2020). This resulted in the selection of five siderophore-producing bacteria (*Bacillus sp.* TC4, *B. circulans* TC7, *Pseudomonas sp*. TC33, *B. subtilis* TC34, and *Terribacillus sp.* TC45) for *in vivo* studies.

Environmental pollution has become a major problem, contributing to a rise in catastrophic climate events and wreaking havoc on ecosystems around the world, including agroecosystems (Ebert and Engels 2020). This is due to the fact that plants are continuously exposed to numerous stresses, including abiotic stresses (such as heavy metals). Metal stresses significantly reduce global plant yields and can also negatively impact seed emergence uniformity, vigor, and ultimately crop yield. In addition, the occurrence of these extreme climates will have a significant impact on crop quality and quantity, putting global food security at risk (Shahzad et al. 2021). In light of these considerations, the majority of current methods involve a seed pretreatment procedure that is environmentally friendly, economical, and requires less land to produce the same quantity of grain (Reddy 2012). In this study, the seed coating method was chosen to assess the effects of five bacterial isolates on seed germination and seedling development under normal and abnormal conditions (due to heavy metals). When grown in normal and highly cadmium-contaminated soil, *Spinacia oleracea* seeds coated with one of our five bacterial strains had significantly increased germination rates, growth, and seedling biomass. Numerous heavy metal-tolerant PGPBs, such as *Pseudomonas*, *Bacillus*, *Methylobacterium*, and *Streptomyces*, have been demonstrated to mitigate heavy metal toxicity when used as a seed inoculant, thereby promoting root growth by increasing root surface area and nutrient uptake by young seedlings (Prajapati et al., 2020 ; Chakraborti et al., 2022).

In the past few decades, the development of industrialization and urbanization has led to an increase in the amount of heavy metals in the environment, causing worldwide concern. Heavy metals, unlike other contaminants, cannot be degraded by biological or physical processes and persist in the soil for an extended period of time, posing a long-term threat to the environment. In order to prevent heavy metals from entering the terrestrial, atmospheric, and aquatic environments, and to mitigate soil contamination, corrective measures are required. To date, numerous strategies for restoring heavy metal-contaminated soils have been developed (Kumar et al. 2021). There is a growing need to develop cost-effective, efficient, and environmentally friendly remediation technologies to recover heavy metals-contaminated soils (RoyChowdhury et al. 2018). The present study utilized a combination of two bioremediation strategies, phytoremediation aided by plant growth-promoting bacteria in the presence of heavy metals, to bioremediate a soil heavily contaminated with cadmium as well as with moderate concentrations of zinc, copper, nickel, and manganese. *Spinacia oleracea* responded favorably to the presence or absence of the inoculant in a soil contaminated with heavy metals. The growth and chlorophyll content of *Spinacia oleracea* plants grown under conditions of high metal toxicity, primarily due to cadmium, were enhanced by soil inoculation with the five selected bacteria. While exposed to these toxic conditions, the bacterial isolates significantly increased soil enzyme activity and nitrogen-phosphorus-potassium content, which is likely attributable to their *in vitro*-proven PGP characteristics, which in general mitigated metal stress. Numerous studies have reported that PGP bacteria under Cd stress stimulate plant growth (Castro et al., 2015 ; Wiangkham et al., 2018 ; Ma et al., 2020 ; Liu et al., 2022). By producing ACC deaminase, siderophores and hydrocyanide (HCN), IAA, and nutrient solubilization, these bacteria can affect plant growth. Shilev et al., (2020), Wang et al., (2020 b) et Renu et al., (2021), observed similar positive effects of bacterial inoculation on plant protection to metal exposure in spinach inoculated with PGPB in the presence of Cd. *Bacillus circulans* TC7 was able to promote plant growth parameters (spinach length and biomass) while minimizing metal bioaccumulation, indicating its potential use as a plant growth promoter under heavy metal stress with high cadmium contamination. In contrast, soil amendment with *Bacillus subtilis* TC34 and *Pseudomonas sp.* TC33 improved metal mobility and increased metal bioaccumulation (except for Ni) in spinach tissues, as well as translocation and accumulation of cadmium at the shoot level, while decreasing their toxicity. However, the interaction between the plant and the bacterial inoculant in a metal-contaminated environment remains complex, as additional research is required to determine whether the bacteria will increase or decrease the accumulation of heavy metals by plants. Additional analyses, such as the determination of proline accumulation, lipid peroxidation, and antioxidant activity, would have been of interest. Furthermore, determining the plant response to soil inoculation with PGP bacteria by analyzing systemic gene resistance induction and metabolic changes would have aid in the comprehension of the process.

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