Effect of llama (*Lama glama*) manure and *Trichoderma* strain T1R3 on arsenic uptake by Swiss chard and broad bean crops. A strategy to minimize health risks

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Abstract

The town of Pastos Chicos (Jujuy-Argentina), presents arsenic (As) concentrations in soil (49 mgAs kg^{-1}) and water (1.44 mgAs L^{-1}) significantly above the maximum allowable limits set by National Laws N° 24,585 and N° 24,051. This study aimed to evaluate the effect of llama manure (*Lama glama*) and *Trichoderma* strain T1R3 on As uptake and toxicity in Swiss chard (*Beta vulgaris* var. *cicla*) and broad bean (*Vicia faba* L.) crops, while assessing potential human health risks. Results indicated that *Trichoderma* strain T1R3 inoculation stimulated broad bean plant growth by reducing As stress. Swiss chard crops treated with 5% manure and a manure/ *Trichoderma* T1R3 combination reduced As absorption from 32.46 to 64.02% in roots, and from 35.2 to 44.5% in leaves. Broad bean crops inoculated with *Trichoderma* T1R3 showed a significant mitigation of toxicant accumulation in the leaves (67.42%). Also, the manure/ *Trichoderma* T1R3 combination reduced As accumulation (57.46%) in broad bean roots. The efficacy of llama manure and of the llama manure/ *Trichoderma* T1R3 combination in reducing health hazards that derive from As intake by consuming chard leaves was also reflected in Hazard Quotient < 1 values. Although Cancer Risk values decreased considerably, these showed there was a considerable carcinogenic risk for humans consuming chard leaves. These observations reveal that adding llama manure and *Trichoderma* T1R3 might mitigate As uptake by crops, thus reducing human health risks. This study advanced our understanding the complex llama manure/ *Trichoderma* strain interactions in As-contaminated soils, which are imperative for developing the effective mitigations strategies.

Highlights

- *Trichoderma* strain stimulated broad bean plant growth by reducing As stress.
- Llama manure and a *Trichoderma* reduced As absorption in chard roots and leaves.
- Addition of llama manure increased soil fertility and available phosphorus content.
- Llama manure and *Trichoderma* reduced As accumulation (57.46%) in bean roots.
- The llama manure and *Trichoderma* combination reduced As health hazard.

1. Introduction

Arsenic (As) is a hazardous element, which represents a significant risk to human health when introduced into the food chain, skin exposure, and inhalation (Farooq et al. 2016). Prolonged exposure to As by consuming water or food that contains certain As levels causes serious toxic effects, including skin lesions (pigmentation and keratosis) and lung and bladder cancer (Mandal et al. 2019). Therefore, immobilizing As in contaminated soils may constitute an effective strategy to reduce its accumulation in plants and food (Arco-Lázaro et al. 2016). Actions to mitigate As toxicity and mobility in plants involve the use of organic amendments, and the inoculation of soil with beneficial microorganisms, such as bacteria and ectomycorrhizal fungi (Luo et al. 2016; Nawab et al. 2018). In particular, the genus *Trichoderma* tolerates heavy metals, and using it to immobilize and remove As constitutes an eco-friendly

and cost-effective bioremediation technique to clean up soils contaminated with heavy metals (Govarathanan et al. 2018; Govarathanan et al. 2019a). *Trichoderma* species are of particular interest, as they can survive under a range of environmental conditions and stimulate plant growth and development, by improving water and nutrient absorption from the soil (Tripathi et al. 2017). Several *Trichoderma* strains are well known for their ability to ameliorate abiotic stress in plants by inducing physiological protection, enhancing antioxidant capacity, and endowing plants with resistance to high temperature, high salinity and drought conditions (Khoshmanzar et al. 2019; Poveda 2021). An important function is bioremediation, where *Trichoderma* strains can remediate As-contaminated environments by triggering the transformation of As species through reduction, oxidation, methylation, demethylation, and cellular sequestration (Li et al. 2021). Also, these fungi induce a higher root biomass production, and increase tolerance to As-mediated stress, reducing As accumulation in plants (Caporale et al. 2014). For example, Tripathi et al. (2015) reported that inoculation with *Trichoderma reesei* reduced As toxicity in chickpea plants grown in soils contaminated with 100 mg kg⁻¹ As, mitigating As concentration in edible part plants. Recently, it has been shown that inoculation of soil with beneficial microorganisms, such as *Trichoderma asperellum* SM-12F1, caused As methylation in the soil (Su et al. 2017). This loss of As contents may be due to the volatilization of methylated As, for example, as methylarsonic acid (MMA), and dimethylarsinic acid (DMA), which reduces As phytotoxicity and improves plant growth (Zhang et al. 2018).

Organic amendments have been used by many researchers to immobilize toxic metals in contaminated soils (Nawab et al. 2016; Govarathanan et al. 2019b). For example, Joardar and Kawai (2014) showed that application of pig manure (1, 2, and 3%) with high phosphorus concentrations reduces As concentration (39, 52 and 66%, respectively) in edible parts of Japanese mustard spinach irrigated with As-contaminated water. As phosphorus and As are chemical analogs that are taken up by plants through the same transporters, a high phosphorus content in plant cells can lead to increased competition with As (V) through different biochemical processes (Niazi et al. 2017). Also, Urunmatsoma et al. (2010) demonstrated that the addition of cow dung in chromated copper arsenate contaminated soil decreased Cr, Cu and As mobility and uptake in maize (*Zea mays*). Gadepalle et al. (2008) and Liu et al. (2009) reported that the addition of compost to soils resulted in As complexation/adsorption with the humic substances, decreasing its mobility and phyto-availability in the soils, which made revegetation possible in sites contaminated with As.

The Puna region (Jujuy-Argentina) is a high plateau which rises over 3500 meters above sea level. The soil in this area is arid, and As contamination has a geogenic source. The climate is characterized by a wide temperature range, intense solar radiation, constant winds, low average temperatures and scarce rainfall, which occurs mainly in the summer months, while usually reaching zero levels in winter (PIP 2016). The region is considered a nucleus of biological diversity, whose preservation and protection is in the hands of small producers, who keep traditional small-scale agricultural practices, based on only their knowledge and inputs (PIP 2016). Local studies are mainly focused on Swiss chard and broad beans, as they are viewed as traditional regional crops that contribute to a balanced diet rich in nutrients, such as

proteins, vitamins, amino acids, and minerals, among others. These crops are produced in areas with high As contents in water and soil, exceeding the allowable limits established by the National Law N° 24,051 (0.10 mgAs L⁻¹ in irrigation water), and by National Law N° 24,585 (20 mgAs kg⁻¹ in agricultural soil) (Yañez et al. 2018 and 2019). Vegetables are generally highly sensitive to metal stress, and become a source of As poisoning for humans who grow and consume them. This has caused interest amongst scientists, who are exploring some sustainable and eco-friendly option for remediation and restoration of As-contaminated soils (Mehmood et al. 2017). Therefore, adding organic amendments and beneficial fungi to soil could reduce As accumulation in crops grown in areas polluted with this toxicant, diminishing its translocation to edible plant parts, and thus making agriculture safer and more sustainable.

This study aimed to evaluate the effect of llama manure (*Lama glama*) and *Trichoderma* strain T1R3 on As uptake and toxicity in Swiss chard (*Beta vulgaris* var. *cicla*) and broad bean (*Vicia faba* L.) crops, as well as assessing potential risks to health of humans consuming these vegetables.

2. Materials And Methods

2.1 Arsenic contaminated soil, llama manure and irrigation water sampling

Surface soil samples (at 0–20 cm depth) were collected from Pastos Chicos (23°45'58.8" S- 66°26'14.0" W), in the Susques-Puna Region (Jujuy-Argentina), whereas llama manure samples were collected from a pen near Pastos Chicos (23°38'39.1" S - 66°25'07.6" W). The soil and manure samples were air-dried, sieved (2 mm) and then homogenized prior to determination of pH and organic matter by potentiometry and wet digestion employing the Walkley-Black method, respectively (Nelson and Sommers 1982). Electrical conductivity was measured using the saturation extract method. Other determinations were total nitrogen, according to Bremner and Mulvaney (1982); bioavailable phosphorus content, by means of Bray and Kurtz's method (1945); and sodium and potassium complexes by flame photometry (Eaton et al. 2005). The soil and llama manure were processed for total As quantification using acid digestion according to USEPA method 3050 B (1996). Pulverized samples (0.5 g) were transferred to Teflon beakers, where 10 mL of 50% HNO₃ was added. The solutions were heated on a hot plate at 95°C±5 with a watch glass for 2 h, until they evaporated (without boiling) to about 5 mL. Subsequently, 3 mL of hydrogen peroxide (30% H₂O₂) was added, and the solutions were again heated until the sample suffered no further changes in its appearance. Finally, 10 mL of concentrated HCl was added to the solutions, followed by hot plate heating (95°C±5) for 15 min. After digestion, total As concentration was determined with a hydride generation-atomic absorption spectrometer (HG-AAS), methodology described below. Irrigation water was collected from Río Pastos Chicos (Susques) (23°42'031.300S - 66°26'042.300 W). The following parameters were analyzed, among others: temperature, pH (potentiometry), electrical conductivity (conductivity meter), magnesium and calcium by complexometric volumetric titration as quantified with EDTA (ethylenediaminetetraacetic acid), sodium and potassium complexes as measured

by flame photometry, and carbonates, bicarbonates, chlorides, and sulfates as determined by neutralization titration. Total As in water was quantified using a HG-AAS (Yañez et al. 2018).

Soil, water, and llama manure were collected in a single sampling to avoid variations in physical-chemical properties and As content.

2.2 Microorganism and culture conditions

The fungus used was As tolerant *Trichoderma* strain T1R3 previously selected by Yañez et al. (2017). To obtain an appropriate inoculum (spore suspensions), strain T1R3 was pre-cultivated on potato dextrose agar (PDA) medium, and incubated for 7 days at 28 ° C. Subsequently, 10 mL of sterile 0.85% NaCl was added to release the chlamydospores from the surface of the medium. The filtered suspension was vigorously stirred and subsequently centrifuged at 9000xg, for 10 min. The supernatant was discarded, and the precipitated chlamydospores were re-suspended in sterile 0.85% NaCl (10^7 log units/mL CFU).

2.3 Aqueous extract of llama manure

An aqueous manure extract was prepared by finely disintegrating the manure in a mortar, and sieving it with a 2 mm pore size mesh to remove stones, plastic, paper, among other elements. Afterwards, a suspension was made in 5% distilled water, and stirred for 12 h at 150 rpm. This aqueous suspension was then filtered to remove the non-solubilized material, and the resulting extract was kept cold until its use.

2.4 Arsenic uptake mitigation experiments

The broad bean seeds used in the trial belonged to the Agua Dulce variety and were provided by the “Pro Huerta” program of Ministerio de Desarrollo Social de la Nación Argentina, whereas the Swiss chard (*Beta vulgaris* var. *cicla*) seeds were supplied by the company Emeral Seeds-USA.

The chard seeds were germinated in multipot trays and transferred to black polyethylene bags, each containing 1 kg of soil. Each broad bean seed was kept in a bag too, and sown directly into 3 kg of soil. The chard and broad bean tests lasted 60 and 180 days, respectively. The crops were grown in soil typical of Pastos Chicos and irrigation water came from the local river (Yañez et al. 2018). Constant volumes of irrigation waters were added to each pot of the experiment, in order to maintain the soil moisture at

70% of the field capacity, avoiding any phenomenon of leaching. Different pot experiments were carried out in a greenhouse at ambient temperature, with a natural light and darkness regime. The seeds were surface sterilized and dipped in a *Trichoderma* T1R3 chlamydospores suspension inoculum, at a CFU of 10^7 log units/mL. One month after cultivating chards, and two months after cultivating broad beans, these were again inoculated with 1 mL of the spore suspension, and with 10 mL of the aqueous llama manure extract. In this study, As uptake mitigation experiments had a completely randomized design, with a total of 60 pots, including the amended and control soils, was prepared in triplicate.

The treatments were as follows:

- 1) Chard seedlings and broad bean seeds in soil with As, irrigated with As-contaminated water (control).
- 2) Chard seedlings and broad bean seeds in soil with As, irrigated with As-contaminated water and applied with 5% (w/w) llama manure.
- 3) Chard seedlings and broad bean seeds in soil with As, irrigated with As-contaminated water and applied with a *Trichoderma* T1R3 spore suspension.
- 4) Chard seedlings and broad bean seeds in soil with As, irrigated with As-contaminated water and application of 5% llama manure (w/w) and a *Trichoderma* T1R3 spore suspension.

Salinity reduces plant growth, development, yield and seed quality when the concentration the salts reaches 4 dS m⁻¹ (Acosta-Motos et al. 2017). Thus, the 5% llama manure was chosen according to electrical conductivity of 3.42 dS m⁻¹ in the manure-soil combination.

For dry weight and As content analysis, roots and aerial parts different plant parts were oven dried at 70°C for 72 h. Samples had been dried following the As quantification methodology (as described below), but the dry weight values were turned into wet weight, so as to reveal the potential health risks brought about by consuming produce from these crops. The As concentration in wet weight was determined considering a humidity of 90.6%, according to the following equation: wet weight concentration = dry weight concentration × (1 - % humidity) (Costa et al. 2003).

Arsenic mobility in the chard and broad bean crops was evaluated by estimating translocation factors (TF) according to following equation:

$$TF = \frac{AsT \text{ aerial}}{AsT \text{ root}}$$

Where, AsT aerial and AsT root represent the total As concentration in the aerial and root plant (Sharma et al. 2020).

2.5 Non-carcinogenic health risks

The health risk represented by the intake of As through consumption of Swiss chard was assessed in terms of the hazard quotient (HQ), which was calculated with the following Eq. (the corresponding parameters are explained in Table 1):

$$HQ = \frac{EF \times ED \times FIR \times C}{RfD \times BW \times AT} \times 10^{-3}$$

2.6 Cancer risk assessment

The Cancer Risk (CR) which derives from consuming Swiss chard was determined using the following equation, as described by Shahid et al. (2017) (its parameters are explained in Table 1).

$$CR = \frac{C \times EF \times ED \times FIR \times CSF}{BW \times AT} \times 10^{-3}$$

A CR lower than 10^{-6} is considered to be negligible, one between 10^{-6} and 10^{-4} is generally considered acceptable, whereas a CR above 10^{-4} is deemed unacceptable, with a high potential for causing cancer (Muñoz et al. 2017).

2.7 Total arsenic determination

For the analysis of total As, 1 g dried sample of plant was digested in

a mixture of the mineralizing agent, 20% w/v magnesium nitrate [$Mg(NO_3)_2$] and 2% w/v magnesium oxide (MgO). Then, 5 mL of 50% v/v nitric acid (HNO_3) was added to promote organic matter oxidation, and the sample was heated on a hot

plate at 90°C. Finally, the preparation was muffled at 550°C for 24 h until white ash was formed. This was resuspended in 10% v/v hydrochloric acid (HCl) to measure total As. The suspension was subjected to a prereduction step with a solution of potassium iodide/ascorbic acid. Arsine (AsH_3) was then formed by reaction with a solution of sodium borohydride (BH_4Na) in an alkaline medium, together with a solution of HCl as the source of hydrogen ions using a HG-AAS (PerkinElmer AAnalyst 100, interfaced with the FIAS 400 hydride generator). The detection limit of the method was $0.1 \mu gAs L^{-1}$ and quantification limit was $0.3 \mu gAs L^{-1}$, with a linear response of up to $5 \mu gAs L^{-1}$ ($r = 0.9996$). Relative error amounted to 10%, and equipment sensitivity was checked with external certified standards ($0.1 \mu gAs L^{-1}$, Certipur-Merck).

2.8 Statistical analysis

The results are expressed as mean values with standard deviations (SDs) as a measure of dispersion (means $\pm$ SD). The differences between individual means were compared by one-way analysis of variance (ANOVA). When significant differences were found,

Duncan's post-test was used to separate the effects among treatments. Tests were considered significantly different at $p < 0.05$. These statistical analyses were performed using professional versions of Infostat software.

3. Results And Discussion

3.1 Soil, llama manure and irrigation water characterization

The physicochemical properties of the soil, llama manure, and irrigation water used in this study are presented in Table 2. The soil from Pastos Chicos presented a sandy-loam texture. As reported by Mehmood et al. (2017), a higher sand amount in the soil contributes to low As retention, which represents a greater amount of As available for the crops. Considering its electrical conductivity value ($1.98 dS m^{-1}$), the soil had the characteristics of an alkaline and slightly saline type, according to Richards' criteria

(1982). The soil presented a moderately alkaline pH (8.3), 5.12% organic matter content, and a high proportion of phosphorus (72.8 mg kg⁻¹). It also had a total As concentration of 49 mg kg⁻¹, exceeding the maximum As concentration level of 20 mg kg⁻¹ recommended by National Law N° 24,585 for agricultural soils.

The llama manure presented a slightly alkaline pH (7.6), and a content of organic matter (23%) which represents a good quality organic fertilizer (Chan et al. 2016). According to Richards (1982), the electrical conductivity (12.76 dS m⁻¹) of the saturation extract indicated a manure with high salinity. In addition, the manure revealed high contents of nitrogen (1.6%), phosphorus (418 mg kg⁻¹), and potassium (23.20 cmol_c kg⁻¹), important nutrients for plant growth and development (Shrivastav et al. 2020). The chemical analysis of the llama manure showed a total As concentration of 13.3 mg kg⁻¹, this reflects that water and vegetation are a source of As transfer in the food chain.

The water had a moderately alkaline pH (8.25), and an electrical conductivity of 2.58 dS/m, which indicates very high salinity according to Richards (1954). A sodium absorption ratio (SAR) of 9.92 was found, which represents a high risk of soil salinization (Yañez et al., 2018). In addition, water analysis showed a total As content of 1.44 mg L⁻¹, which was 14-fold higher than the maximum limit allowed for irrigation water (National Law N° 24,051).

3.2 Growth of Swiss chard and broad bean crops exposed to arsenic, llama manure and *Trichoderma* T1R3

Dry biomass is a critical parameter for assessing As effects on crop growth (Niazi et al. 2017). The results showed that in the chard crops treated with llama manure alone, the total dry biomass of plants was significantly lower than the control (Fig. 1). In the T1R3 strain treatment and in those with the llama manure/ *Trichoderma* T1R3 combination no produced significant differences among them and the control (Fig. 1). The As content in llama manure (13 mg kg⁻¹), the soil (49 mg kg⁻¹), and irrigation water (1.44 mg L⁻¹) negatively influenced plant growth and led to a lower biomass production. It is well known that As has negative effects on plant metabolic functions, reducing growth, and changing nutrient balance and assimilation (Mirza et al. 2016). Besides, it can interfere with photosynthetic activity, metabolic processes, and water absorption (Gusman et al. 2013). Additionally, it causes oxidative stress and lipid peroxidation due to the overproduction of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide, and hydroxyl radicals (Tripathi et al. 2017). For example, Tripathi et al. (2013) showed that As stress negatively affected chickpea (*Cicer arietinum* L.) germination (25.9%), and stem length (15%) and diameter (30%) in comparison to the control treatment. Also, they registered a significant decrease in root growth, which also affected lateral roots (58%), and in root dry weight (66%) and length (49%). In addition, the high calcium content in the Pastos Chicos soil may reduce phosphate availability to plants due to the possible formation of Ca-phosphate precipitates (Mehmood et al. 2017). According to the soil texture (sandy loam), phosphorus contents added through the llama manure amendments could have displaced As ions adsorb onto sand particles, thus increasing As uptake and

reducing plant growth (Anawar et al. 2018). Similarly, Klaber and Barker (2014) demonstrated that growth of rice cutgrass (*Leersia oryzoides* Sw.) and tall fescue (*Festuca arundinacea* Schreb.) was not enhanced by phosphorus fertilization.

The broad bean crops inoculated with *Trichoderma* T1R3 strain showed significant differences respect to the control (Fig. 1). The fungus application considerably stimulated broad bean plant growth, and possibly increased phosphorus and nitrogen uptake and the production of auxin and siderophores, ameliorating the adverse effects of As toxicity (Zhang et al. 2018; Khoshmanzar et al. 2019). These results are in accordance with those reported by Caporale et al. (2014), who published the beneficial effects of *Trichoderma harzianum* strain T22 and *Trichoderma atroviride* strain P1 on lettuce growth. These authors also reported a reduction of As toxicity when the plants were irrigated with As-contaminated water (5 and 10 mg L⁻¹). In this sense, Anawar et al. (2018) revealed that irrigating with As-rich water may change the As-phosphorus balance in the soil solution, causing the mobilization and availability of phosphorus for the plant nutrition. Furthermore, Tripathi et al. (2017) suggested that As is methylated in soils that have been inoculated with *Trichoderma*, and this could alleviate As stress in chickpea. Besides, as mentioned before, broad bean and chard crops growth might be determined by the synergistic relationships among the *Trichoderma* T1R3 strain, changes in As availability, and the physiological responses of the crops.

3.3 Influence of llama manure and *Trichoderma* strain T1R3 on arsenic absorption by Swiss chard and broad bean crops

In this study, the results showed that the application of llama manure and llama manure/ *Trichoderma* T1R3 combination, significantly reduced As concentration in chard roots and leaves. The *Trichoderma* strain application, significantly decreases the As concentration in chard root, compared to the controls (Table 3).

In the broad bean crops with llama manure amendments and the addition of llama manure/ *Trichoderma* T1R3 combination, significantly reduced the As content in bean roots. Also, in bean leaves, the addition of llama manure and *Trichoderma* T1R3 inoculation significantly lowered As concentration, as compared with the control (Table 4). In contrast, *Trichoderma* strain led to the highest As accumulation in broad bean roots, with concentrations significantly higher in comparison to the other treatments (Table 4).

The lower As absorption in chard and broad bean (roots and leaves), can be attributed to the combined effect of microbial activity, As adsorption to soil particles and organic material, such as manure. Adsorption is the first process that takes place when As are in contact with a soil, affecting processes such as leaching, bioavailability or toxicity (Morillo and Villaverde, 2017). This strategy leads to a reduced availability of As, which in turns improves plant growth (Mehmood et al. 2017; Nawab et al. 2018). In this sense, Mehmood et al. (2017) reported that in the (sandy loam) soil in Narwala, contaminated with As (0, 40, 80, 120 mg kg⁻¹), the addition of compost (2.5%) decreases As concentration in maize shoot, significantly improving shoot dry biomass. In addition, Nawab et al. (2018) demonstrated that applying

5% farmyard manure to the soil showed the highest reduction in As bioaccumulation in pea (21 to 37%) and chili (18 to 36%), respect to the control treatments. Also, *Trichoderma* induced As methylation in soil could be the reason for less As uptake in *Trichoderma* inoculated plants. This loss of As could be due to volatilization of methylated As, such as in the form of trimethylarsine (TMA) or trimethylarsine oxide (TMAO) (Wang et al. 2015; Tripathi et al. 2017). Lower root uptake of methylated As species has been reported by several studies (Mishra et al. 2016 and 2017).

Furthermore, organic matter decomposition results in a release of simple aliphatic acids, sugar acids, amino acids, phenols, phosphates and carbonate minerals, which act as adsorption sites for As ions. This reduces their mobility in the soil solution, mitigating As toxicity hazards and making the toxicant unavailable for plants (Mehmood et al. 2017; Nawab et al. 2018; Mandal et al. 2019). The presence of phosphorus in the soil (72.8 mg kg^{-1}) and in the llama manure (418 mg kg^{-1}) may have positive effects on plant growth and reduce As uptake. This is possibly due to the fact that in plant cells, phosphorus can compete with As (As(V)) in different important biochemical processes, where As substitutes for phosphorus (Niazi et al. 2017).

The pH of the soil plays an important role in the As bioavailability, and its subsequent bioaccumulation in plants (Nawab et al. 2018). In the present study, the alkaline pH of the soil and water (Table 2) could have contributed to As coprecipitation with sulfate or calcium, reducing its availability (Natasha et al. 2020). According to Chaoua et al. (2019), in alkaline pH soils, the concentrations of metal ions drop due to an increased surface oxide charge, or on account of either processes of precipitation of metal hydroxides, or of formation of insoluble organic complexes.

In addition, calcium is generally known to reduce As accumulation in plants by forming stable Ca-arsenate precipitates (Hassan et al. 2014). In this study, high calcium concentrations in the soil (8.8 meq L^{-1}) and in the irrigation water (4 meq L^{-1}) could form stable precipitates, such as calcium arsenate, and reduce As uptake by crops. This was observed by Liu et al. (2014), reported that applying CaO_2 to the soil significantly reduced As accumulation in celery shoots. Similarly, Shahid et al. (2017) reported that the application of Ca (1, 5 and 10 mM) significantly reduced As transfer to spinach aerial parts.

In this study, the high levels of As ($590.83 \text{ mg kg}^{-1}$) accumulated in broad bean roots (*Trichoderma* strain treatment) could be attributed to the fact that *Trichoderma* T1R3 released organic acids, such as gluconic acid, fumaric acid, and citric acid, which decreased soil pH and caused the dissolution of phosphate and As, among other compounds, thus resulting in a greater bioavailability of the toxicant and of nutrients in the rhizosphere (Stewart et al. 2014; Anawar et al. 2018). In the two crops studied in this work, the roots were the organs with the highest accumulated As, probably attributed to the toxicant was compartmentalized in root vacuoles, gets complexed with sulfhydryl ($-\text{SH}$) groups of peptides, such as γ -glutamylcysteine, glutathione and phytochelatins (Mishra et al. 2016 and 2017). These phenomena were observed in crops such as rice, tomato, beans, chard and lettuce (Caporale et al. 2013; Pigna et al. 2013; Yañez et al. 2018 and 2019). The results presented in this work are consistent with previous studies by Kumwimba et al. (2013), these authors reported values of $534.06 \text{ mg kg}^{-1}$ As in roots of hydroponic

lettuce crops, showed that the average As concentration in roots was 19–26 times higher than in shoots. Also, studies conducted by Babu et al. (2014), who inoculated a metal-polluted mining ground with *Trichoderma virens* chlamydospores, and grew corn plants to evaluate the mobility of toxicants, found that the fungus significantly increased As accumulation in corn roots (31%), compared with plants grown in soils without inoculation.

In our study, broad bean crops did not develop pods with seeds, so As could not be quantified. The salinity of llama manure (12.76 dS m^{-1}), the soil (1.98 dS m^{-1}), and the irrigation water (2.58 dS m^{-1}) could have negatively affected crop development. Saline medium has several adverse effects on plant growth, as a result of a low osmotic potential of the soil solution (osmotic stress), specific ion effects, nutritional imbalance, or a combination of these factors (Rafiq et al. 2017; Parvez et al. 2020). Moreover, chlorosis symptoms were observed in the leaves, which were later affected by foliar necrosis.

3.4 Arsenic translocation factors in Swiss chard and broad bean crops applied with llama manure and *Trichoderma* T1R3

The ability of plants to mobilize As from roots to leaves was calculated as a translocation factor (TF). Due to high accumulation of As in plant roots, As translocation from root to leaves was low ($\text{TF} < 1$) in all As treatments (Table 5). According to Sharma et al. (2020), a $\text{TF} < 1$ indicates poor As translocation from roots to the aerial parts. It could be observed that $\text{TF} = 0.01$ value for broad bean crop treated with *Trichoderma* strain showed a considerably lower translocation of the toxicant from the roots to the leaves, respect to the control treatment. In this sense, Khan et al. (2009) reported that a TF value ≤ 0.1 would indicate that the plant reduces the amount of accumulated toxicant by expelling from the plant tissue, as a detoxification mechanism. The same phenomenon was reported by Caporale et al. (2014), who demonstrated that lettuce plants inoculated with two *Trichoderma* strains, and irrigated with As-contaminated water (5 or 10 mg L^{-1}), showed a significantly lower concentration of As in leaves, respect to the non-treated control. Also, Tripathi et al. (2017) published that As concentration decreased in chickpea plants (root, stem, seeds) inoculated with *Trichoderma* sp. Thus, a low As concentration in the leaves could be due to limited translocation at a systemic level (Smith et al. 2009). In addition, the higher retention of As in roots could be caused by a process saturation, where plants exceed their capacity of translocating the toxicant to aerial parts (Gusman et al. 2013).

The TF of 0.33 determined for the broad bean crop treated with the llama manure/ *Trichoderma* T1R3 combination showed a higher As translocation from root to leaves, respect to the control (TF of 0.15). Considering that the As is translocated from the root to the leaves through phosphate channels (Niazi et al. 2016), this may be attributable to better solubilization of phosphorus caused by the T1R3 strain and to the bioavailable phosphorous present in llama manure. Yao et al. (2009), also observed that the application of 4% chicken manure and 4% pig manure to water spinach enhanced As translocation. Also, Niazi et al. (2017) obtained higher TF values in *B. napus*, which suggests that this plant species is efficient in transferring As from roots to shoots in presence of phosphate.

In our study, broad bean plants showed a greater capacity of translocate As than chard plants, except in the *Trichoderma* T1R3 treatment. In the llama manure treatments and in those with the llama manure/ *Trichoderma* T1R3 combination, the bean crops had As TF values which were two-fold and three-fold higher than those of chard crops, respectively.

3.5 Potential health risks associated with the consumption of Swiss chard leaves from crops exposed to arsenic, and supplemented with llama manure and *Trichoderma* T1R3

The food chain is an important pathway for human exposure to As. The risk to human health by the intake of As through consuming chard crops was assessed using the hazard quotient (HQ). As shown in Table 6, the applications with llama manure and the llama manure/ *Trichoderma* T1R3 combination to chard crops grown in As-contaminated soil and irrigated with As-contaminated water brought HQ indices below 1, compared to the control treatment. According to USEPA (2000) guidelines, potential adverse impact on human health would occur when $HQ \geq 1$, whereas $HQ < 1$ values mean that the exposed population is unlikely to experience adverse health effects. Recently, Mandal et al. (2019) showed that adding farmyard manure to a soil contaminated by As (10, 20, 30, and 40 mgAs kg⁻¹) reduces hazard quotient values for intake of As through the consumption of wheat grown in contaminated soil.

The carcinogenic risk (CR) posed by consuming As-contaminated chard leaves is shown in Table 6. Applications of llama manure and the llama manure/ *Trichoderma* T1R3 combination to chard crops significantly decreased the CR, compared to the control. However, CR values obtained for As were not within the acceptable range, and exceeded the threshold value (1×10^{-4}), which suggests that consuming leafy vegetables involves a considerably high risk of developing cancer (Muñoz et al. 2017). This CR values were consistent with those reported by Ma et al. (2017), who evaluated the consumption of leafy vegetables from thirteen different crops, reporting CR values between 1.28×10^{-4} and 4.57×10^{-4} . Also, risk assessment studies conducted by Nawab et al. (2018) showed that applying 1, 2 and 5% farm manure and peat to agricultural soils contaminated with Ni, Cr, As, Zn, Cd and Pb decreased their daily intake of these metals, as well as the cancer risks associated with rice consumption. The major source of human exposure to As is through consumption of As-accumulating crops and vegetables. However, the inhalation of soil particles, drinking water and dermal contact are important pathways for human exposure to As (Li et al. 2017).

4. Conclusion

In the present study evaluated the effect of llama manure amendment and *Trichoderma* strain T1R3 inoculation on As uptake and toxicity in chard and broad bean crops, together with human health risks associated with the consumption from these crops. In both crops, the combined addition of llama manure and *Trichoderma* T1R3 was the treatment that most efficiently reduced As accumulation in chard leaves and in the roots of both crops, showing a great potential as an As complexing agent, and a capacity to reduce plant As uptake and its availability in the soil. The TFs of broad beans were also higher than those of chard; however, specifically in broad bean, treatments with *Trichoderma* T1R3 strain

led to a considerably lower translocation of the toxicant from the roots to the leaves. In addition, the llama manure/ *Trichoderma* T1R3 combination was more effective in reducing Hazard quotients (HQ) and Carcinogenic risk (CR) represented by consuming chard leaves. However, CR values obtained for As were higher than those acceptable, which means that there is a considerable carcinogenic risk in consuming leafy vegetables.

This work indicates that it is possible combine bio-fertilization and mitigation of As toxicity in important food crops by using selected *Trichoderma* strains. Further research is needed in relation to the role llama manure and *Trichoderma* T1R3 play in As immobilization/mobilization and its uptake by different plant species that are grown in a range of As-contaminated soils. Therefore, it becomes of primary importance to perform detailed studies and development of strategies that minimize the water-soil-plant transfer of arsenic or restrict As contamination of edible plant parts, preventing root-to-shoot translocation. These strategies would be sufficient to become another route for increasing food safety.

Declarations

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Authors Contributions

LMY: designed the study, and analyzed the data, discussion of the results, and writing of the manuscript.

JAA: designed the study, conducted the experiments and data compilation, discussion of the results.

NMEAC: analyzed the data, acquisition and administration of funds

GBM: methodology planning, acquisition and administration of funds.

All authors reviewed and approved the final manuscript.

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Tables

Table 1: Parameters and values used in risk assessment equations

Abbreviation	Parameter	Value
EF (days/year)	Exposure frequency	48
ED (years)	Exposure duration	70
FIR (g/day)	Food ingestion rate	200
C (mg/kg)	As concentration in Swiss chard	Table 2
RfD (mg/kg day)	Arsenic reference dose	0.0003
BW (kg)	Body weight	70
AT (days)	Average time for non-carcinogens	365
CSF (kg day/mg)	Arsenic oral cancer slope factor	1.5

Source: USEPA (2015); Shahid et al. (2017)

Table 2: Physicochemical properties of the soil, llama manure and irrigation water sampled from Pastos Chicos.

Soil	Value	Manure	Value Value	Water
pH (1:2.5, soil:water)	8.3	pH (1:2.5, soil:water)	7.6 8.25	pH
pH of extract	8.12	pH of extract	7.36 2.6	EC (dS m ⁻¹)
Organic carbon (%)	5.12	Organic carbon (%)	23 4.0	Ca (meq L ⁻¹)
Total nitrogen (%)	0.25	Total nitrogen (%)	1.6 2.6	Mg (meq L ⁻¹)
Ratio C/N	9.14	Ratio C/N	14.0 18.0	Na (meq L ⁻¹)
EC (dS m ⁻¹)	1.98	EC (dS m ⁻¹)	12.76 1.4	K (meq L ⁻¹)
Extractable P (mg kg ⁻¹)	72.8	Extractable P (mg kg ⁻¹)	418 1.02	Carbonate (meq L ⁻¹)
Ca (meq L ⁻¹)	8.8	Extractable K (mg kg ⁻¹)	9048 4.08	Bicarbonate (meq L ⁻¹)
Mg (meq L ⁻¹)	3.4	Na (cmol _c kg ⁻¹)	15.10 6.28	Chlorides (meq L ⁻¹)
Clay (%)	15.2	K (cmol _c kg ⁻¹)	23.20 14.61	Sulfates (meq L ⁻¹)
Silt (%)	7.5	Total As (mg kg ⁻¹)	13.3 9.92	SAR
Sand (%)	77.3		C4 S3	Riverside classification
Total As (mg kg ⁻¹)	49		1.44	Total As (mg L ⁻¹)

EC: electrical conductivity; P: phosphorus; Ca: calcium; Mg: magnesium; C: carbon; N: nitrogen; Na: sodium; K: potassium; SAR: sodium absorption ratio

Table 3: Arsenic concentration (mgAs kg⁻¹) in Swiss chard crops

Treatments	Roots		Leaves	
	dry weight	% Reduction	dry weight	% Reduction
Control	186.40 ± 5.12 ^a	.....	12.72 ± 0.88 ^a	.....
Llama manure	125.9 ± 3.55 ^b	32.46	8.25 ± 0.60 ^b	35.2
<i>Trich.</i> T1R3	171.00 ± 2.97 ^c	8.26	11.89 ± 0.37 ^a	6.6
Manure/ <i>Trich.</i> T1R3	67.06 ± 3.95 ^d	64.02	7.06 ± 0.33 ^b	44.5

Data are expressed as mean values ± SD ($n = 3$). The different letters within a column indicate a significant difference at $p \leq 0.05$ according to Duncan's multiple range tests. The % reduction was determined considering the As concentration of the control treatment as 100% As content.

Table 4: Arsenic concentration (mgAs kg⁻¹ dry weight) in broad bean crops

Treatments	Roots	% Reduction	Leaves	% Reduction
Control	160.62 ± 11.27 ^a	.....	24.73 ± 1.63 ^a	.....
Llama manure	100.81 ± 7.69 ^b	37.24	11.35 ± 1.22 ^b	54.10
<i>Trich.</i> T1R3	590.83 ± 14.14 ^c	.....	8.06 ± 0.25 ^c	67.42
Manure/ <i>Trich.</i> T1R3	68.33 ± 5.83 ^d	57.46	22.42 ± 1.05 ^a	9.32

Data are expressed as mean values ± SD ($n = 3$). The different letters within a column indicate a significant difference at $p \leq 0.05$ according to Duncan's multiple range tests. The % reduction was determined considering the As concentration of the control treatment as 100% As content.

Table 5: Arsenic translocation in crops

Treatments	Translocation factor	
	Chard	Broad beans
Control	0.07	0.15
Llama manure	0.07	0.11
<i>Trich.</i> T1R3	0.07	0.01
Llama manure/ <i>Trich.</i> T1R3	0.11	0.33

Table 6: Hazard quotients (HQ) and carcinogenic risk (CR) of arsenic exposure based on chard leave consumption

Treatments	HQ	CR
Control	1.50 ± 0.10^a	$6.7 \times 10^{-4} \pm 4.4 \times 10^{-5}^a$
Llama manure	0.97 ± 0.07^b	$4.4 \times 10^{-4} \pm 3.3 \times 10^{-5}^b$
<i>Trich.</i> T1R3	1.40 ± 0.04^a	$6.3 \times 10^{-4} \pm 1.6 \times 10^{-5}^a$
Llama manure/ <i>Trich.</i> T1R3	0.83 ± 0.04^b	$3.8 \times 10^{-4} \pm 2.0 \times 10^{-5}^b$

Data are expressed as mean values $\pm$ SD ($n = 3$). The different letters within a column indicate a significant difference at $p \leq 0.05$ according to Duncan's multiple range tests.

Figures

Figure 1

Growth of Swiss chard and broad bean crops exposed to arsenic, llama manure and *Trichoderma* strain T1R3. Data are expressed as mean values $\pm$ SD ($n = 15$). Bars with different letters significantly differ at the level of $p \leq 0.05$ according to Duncan's multiple range tests. Statistical comparisons among crop treatments, not among different crops.