Title: Cadmium accumulation in asparagus and potential driving factors

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1. Literature Review

1.1 General introduction to Cd

1.1.1 Cd in soil

In earth crust, including topsoil and subsurface soil system, the average of Cadmium (Cd) is 0.2 mg kg^{-1}. Cd in soil comes from both natural and anthropogenic sources. Natural sources include underlying bedrock or transported parent material, such as glacial till and alluvial deposits. Anthropogenic activities are responsible for Cd contamination in many soils, especially in agricultural soils. The major species of Cd in surface soil can be categorized as exchangeable Cd, Cd associated with carbonates, and Cd in organic compounds, Cd in inorganic compounds and residual (Xian 1989).

Metal-organic complex-bound Cd is Cd in chemical compounds contain Cd and organic ligands. (Krishnamurti et al. 1995), which is a kind of Cd speciation in organic compounds. It is the most common form among the particulate-bound Cd species of surface soils in temperate and tropical regions where the Peruvian soils are in. Cadmium is particularly enriched as metal-organic complex-bound species in the rhizosphere soils after applying phosphate fertilizer (Krishnamurti et al. 2005, Chanmugathas and Bollag 1988). As this study is based on the soil in which asparagus is grown, the following discussion of Cd contamination is focused on agricultural sites. In the subsurface soil, the sediment as solid-phase Cd is essentially immobile (Ge et al. 2009), while in the surface soil, especially soil solution, Cd is more reactive. Inorganic fertilizers are important sources of heavy metals released to soils. Some phosphorus fertilizers contain Cd that is released into the soil with every fertilizer application. The presence of cadmium in some fertilizers, such as lime and calcium superphosphate, is a concern because the metal is highly toxic to living things and can accumulate in the soil, subsequently entering plants and then the food chain (Eisazadeh et al. 2019).

Exceeding a certain limit of Cd not only seriously affects the yield and quality of crops and harms human health through the digestion of the edible part. Since cadmium cannot be degraded by microorganisms in the soil and its pollution is an irreversible accumulation process in soil (Zhang et al. 2009), detoxification is the only remediation strategy to help the heavy metal contained in soil be removed to clean the soil.

1.1.2 Cd bioavailability in soil and influencing factors

Availability refers to the rate and degree at which a chemical is released from the medium of concern. The biological availability of the chemical is released to a living receptor by direct contact or absorption (Kirkham 2006). In other words, the bioavailability of a chemical substance can be used to indicate its availability, and the two terms are often used interchangeably (Shahid et al. 2017). Apoplast and symplast pathways are the two main mechanisms of Cd uptake. The apoplast pathway refers to the transport of cadmium by going between intercellular spaces and cell walls. In contrast the symplast pathway describes the passing of Cd from cells to cells through plasmodesmata (Chen et al. 2018).

The bioavailability of Cd in soils is influenced by the binding strength of Cd by soil, which is subsequently regulated by environmental factors including soil pH, soil organic matter, cation exchange capacity (CEC), and fertility (Eggleton and Thomas 2004). It is well documented that Cd availability is negatively correlated with soil pH (Kim, Owens and Naidu 2009). Cadmium is prone to be absorbed by negatively charged organic and mineral surfaces under alkaline conditions. High levels of organic matter and clay content help to reduce Cd availability by providing binding sites (Hong, Tokunaga and Kajiuchi 2002). Since organic matter and fine particles are two primary sources of CEC in soils, soils with high CEC also tend to have low Cd availability. Cd uptake is also influenced by the absorption of other elements by plants such as potassium (K), zinc (Zn), manganese (Mn), copper (Cu), and iron (Fe). For example, Cd uptake significantly increased with additional application of K fertilizer (KCl and KNO_3) (Zhao et al. 2004), as K nutrient likely increased plants' tolerance to Cd.

Plants also play an active role in increasing Cd bioavailability. Plant roots secrete organic acids, such as oxalic acid, that can form organic complexes with Cd and contribute to Cd detoxification. Chelation of metal ions occurring in the vicinity of plant roots enhances supplying Cd as well as nutrients to plants (Wolterbeek, Vandermeer and Debruin 1988). Adding chelating agents to the soil to form the metal complex is a way to increase Cd's bioavailability. The addition of organic acids often increased plant Cd uptake. The addition of phenylglyoxal, which blocks the oxalic acid secretion of plant roots, has been reported to decrease Cd uptake by plant (Tao et al. 2016). Meanwhile, environmental factors such as instance temperature can indirectly affect Cd

bioavailability via regulating the biological activities in the soil-plant system(Jalil, Selles and Clarke 1994).

1.1.3 Impact of Cd on plant growth

Cadmium in the soil is absorbed by the roots and transported through plant tissue, eventually accumulating in roots, shoots, fruits and grains (Liu et al. 2014) . Cd has a strong inhibitory effect on plant root growth and affects root morphology as it results in the disorder of plant biochemical and physiological processes, and thus affecting plant growth and morphology (Ci et al. 2009). The roots are likely to be affected first by heavy metals because more metal ions accumulate in the roots than in the shoots. Thus, heavy metals are more toxic to roots than to shoots (Papoyan, Pineros and Kochian 2007). Furthermore, the presence of Cd in soil may influence the availability of other elements (Benavides, Gallego and Tomaro 2005). As some elements are essential nutrients for plant growth, the absence or shortage of them would cause serious problems such as stunted growth, chlorosis, interveinal chlorosis, purplish-red coloring and necrosis (Messing and Owen 1954). The maximum Cd concentrations in non-contaminated soil is 3 to 8 ppm in soil and 0.04 to 0.32 mM Cd in soil solution, also known as the critical limit of Cd concentration (Sukarjo, Zulaehah and Purbalisa 2019).

1.1.4 Impact of Cd on human health

Soil Cd pollution is a great threat to human health because soil cadmium can enter the human body through the food chain and cause serious harm. Cadmium is mainly stored in the kidney and liver, which account for about 50% of the total cadmium in the body (Papoyan et al. 2007) . The Cd poisoning mainly leads to damage of kidney function and lung injury, leading to renal cortical necrosis, renal tubular damage, emphysema, pulmonary edema. It can also cause heart expansion and high blood pressure (Satarug et al. 2005). Cd taken by the body for a long time can lead to osteoporosis, embrittlement, lumbar disease, spinal deformity (Papoyan et al. 2007). Besides, there is also the risk that damage of target organs and non- specific changes for the population such as weakness, ease of suffering from other illness, and rise of morbidity and mortality, etc (Du and Shang 2006). According to FAO/WHO, fruit vegetables have a maximum level of Cd at 0.05 mg kg^{-1} (Stan 2009).

1.2 General introduction to Asparagus

Asparagus (*Asparagus Officinalis L*) is suitable for growing in well-drained soil with a neutral pH. It particularly favors sandy loam rich in organic matters. It grows well in fertile soil with loose soil, deep soil layer, soil with adequate fertilizer and good water retention capacity, and good air permeability (Satarug et al. 2005). Asparagus can tolerate slight salinization, but when the soil salt content is more than 0.2%, the plant development can be adversely affected, leading to atrophied roots, thin and weak stems and leaves, and eventually death (Uno et al. 1996). Asparagus has strong adaptability to soil acidity and alkalinity and can be cultivated in soils with a pH between 5.5 and 7.8 (Casas and Sanchez 2008). Thanks to the low transpiration and highly developed root system, asparagus is drought tolerant. Asparagus crowns are often used to grow the edible part of asparagus.

Peru is the largest exporter of fresh asparagus. Asparagus is a vegetable of high economic value and it is produced in Peru is of good quality and moderate price, which makes it very popular in the international market. In recent years, Peruvian asparagus has been widely sold in the United States and Europe and began to enter the Asian market in large quantities. Low precipitation is a key challenge to asparagus plantation in Peru, so that trickle irrigation is widespread in Peru.

My major paper is motivated by the problem that Cd uptake by asparagus is unusually high in alkaline soils in Peru. In order to control the Cd level in the edible part(shoot) of the asparagus, one has to understand the controlling factors of Cd uptake by Asparagus.

2. Rationale, hypotheses and objectives

The soils in Peru contain a naturally high content of Cd, which is not the result of farming practices or due to any other man-made actions (Argota-Perez et al. 2014). Asparagus crops in Peru are often found to have high levels of Cd. For instance, my collaborators reported Cd concentrations of asparagus crops in the range from 0.35 to 6.2 mg Cd kg^{-1} (C. Quiros and J. de los Rios, personal communications). These values are all significantly higher than the FAO's maximum level of Cd set for stalk and stem vegetables (including asparagus) at 0.1 mg Cd kg^{-1}. This is surprising because these soils have slightly alkaline pH (7.8-8.0; C. Quiros, personal communications) that is known to limit Cd absorption by plants (Adamczyk-Szabela, Markiewicz and Wolf 2015).

To develop management practices for decreasing Cd uptake by asparagus, my study here focuses on understanding the interactions between the uptake of Cd and other elements by asparagus. Past studies have suggested that P, Ca, Mg, Fe, and Zn impact plant Cd uptake, but it is unclear which one is more important for Asparagus. If Cd uptake was strongly coupled to another element, regulating the concentration and availability of that element would be a feasible solution for reducing Cd uptake. In a hydroponic setting, I grew asparagus from crowns. I selected five elements (P, Ca, Mg, Zn, and Fe) and developed five treatments where I added Cd and reduced the concentration of one element to 10% of its full strength each time. I have also included two types of control treatments where all elements were supplied at their full strength with and without Cd addition. I determined the Cd concentration of asparagus shoot and examined the interactions between the uptake of Cd and other elements.

Ca is the essential element affecting the Cd adsorption because of most Cd and Ca ion share the same transport routes moving across the roots (He et al. 2017). Thus, I hypothesize that reducing Ca availability in the hydroponic solution would increase Cd adsorption via relieving competition for transport. Similarly, I also hypothesize that lowing Zn, Fe, and Mg would also increase Cd uptake, as both elements were found to compete with Cd for transport channels (Li et al. 2009, Kashem and Kawai 2007, Astolfi et al. 2014). Phosphate is a common amendment added to the soil for tying up heavy metals. Thus, I expect that reducing P concentration would increase Cd uptake.

3. Materials and methods

3.1 Experiment setup

The cultivar UC157 asparagus crowns were selected due to its popularity in Peru. In 1986, the first commercial green asparagus fields were planted in the southern part of Ica, Peru. "UC 157 F1" was introduced, and the first harvest occurred at the end of the year, mainly to provide fresh green spears for the United States. Since then, the area of green asparagus in the south has grown rapidly, and the northern hybrid, UC 157 F1, has begun to replace the open-pollinated white asparagus. Hoagland's nutrient solution (HNS) was used as a growth medium (Hothem, Marley and Larson 2003). The full-strength HNS was purchased from Fisher Scientific, containing 4.0 mM $Ca(NO_3)_2$,

2.0 mM $MgSO_4$, 5.0 mM KNO_3, 1.0 mM NH_4NO_3, 1.0 mM KH_2PO_4, 0.132 mM $MnSO_4$, 0.1 mM H_3BO_3, 0.03 mM $ZnSO_4$, 0.1 mM $CuSO_4$, 0.1 mM $CoCl_2$, 1.0 mM Na_2MoO_4, 5.0 mM KI, 0.1 mM $FeSO_4$, and 0.1 mM Na_4EDTA. Crowns of Asparagus (*Asparagus Officinalis* L.) were rinsed with deionized water and then acclimatized to a 10 L solution containing full-strength HNS for each. Efforts were made to ensure that the plants were uniform and similar in size. Following two weeks in full-strength HNS, asparagus crowns were removed and transferred to the solutions for a total of seven treatments. In the first five treatments, one of the five selected elements (i.e., Ca, Mg, K, Zn, and Fe) were kept at 10% of full-strength HNS for four weeks, which corresponds to decreasing their concentrations to 0.4 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.003 mM $ZnSO_4$ and 0.01 mM $FeSO_4$, once in each treatment. Two types of control were established using HNS with and without 0.2 mM $CdCl_2$. With four duplicates of each treatment, there was a total of 28 samples. The solutions were aerated continuously.

HS	Low Ca	low Mg	low P	low Zn	low Fe
full-strength	0.4 mM $Ca(NO_3)_2$	0.2 mM $MgSO_4$	0.1 mM KH_2PO_4	0.003 mM $ZnSO_4$	0.01 mM $FeSO_4$

Table. 1 Concentrations of treatments

After four weeks of treatments, the shoots of plants, which we call spears, were harvested after they reached the maturity that is between seven to nigh inches and oven-dried (65 °C on a Hotblock (Environmental Express, Ventura, CA).

For each digestion procedure, samples were weighed to the nearest 0.01 g and transferred a 0.5 to 1-gram aliquot to the digestion vessel coupled. For best results, each sample was weighed directly in the vessel on a balance. Each sample was added 5 mL (1:1) HNO_3 (in trace metal grade) and DI Water and shook well. Each sample covered with a ribbed watch glass was heated in the HotBlock at 90° to 100°C for 15 minutes without boiling. Allow the samples to cool, then 2.5mL concentrated HNO_3 was added and reflux at 95 °C for 30 minutes. This step was repeated until no brown fumes were given off by the sample. Each sample with a ribbed watch glass was heated to a volume of about 5 ml for 2 hours at 90 to 100 °C. Samples were removed from the HotBlock using transfer racks and allowed to cool completely.

The Cd concentration in digested solutions was analyzed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Perkin-Elmer Corp., Norwalk, CT). The operational conditions were quadrupole mass analyzer, Ni sampler, skimmer cones, 1600 W RF power, and plasma argon gas at 16 L min^{-1} (da Silva et al. 2019). Other elements were determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES; Perkin-Elmer Corp., Norwalk, CT).

In addition, standard reference materials from the National Institute of Science and Technology (NIST 1547 – peach leaves, Gaithersburg, MD) and appropriate reagent blanks were used as quality checks to ensure method accuracy and precision.

3.2 Statistical analysis

All data are presented as the mean of three replicates with standard deviation. Data analysis (one-way ANOVA) showed significant differences ($P < 0.05$) in the concentrations of elements in the roots and shoots across the different Cd concentrations in soil.

I simulated each treatment by running Visual MINTEQ to determine the main species of Ca and Cd, and Saturation Index (SI) of Ca speciation and Cd speciation.

4. Results

4.1 Concentrations of Cd and other nutrient elements

The total Cd concentration was highest in low Ca concentration compare to other treatments, meaning low P, Zn, Mg and Fe treatments reduced Cd uptake by asparagus (Fig.1).

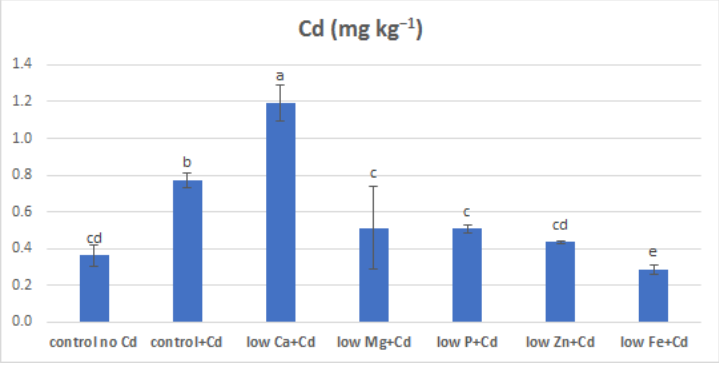


Fig. 1 Cd concentration in asparagus (mg kg^{-1}, treatments followed by the same letters are not significantly different at $p<0.05$. Bars represent the standard deviations (n=3).

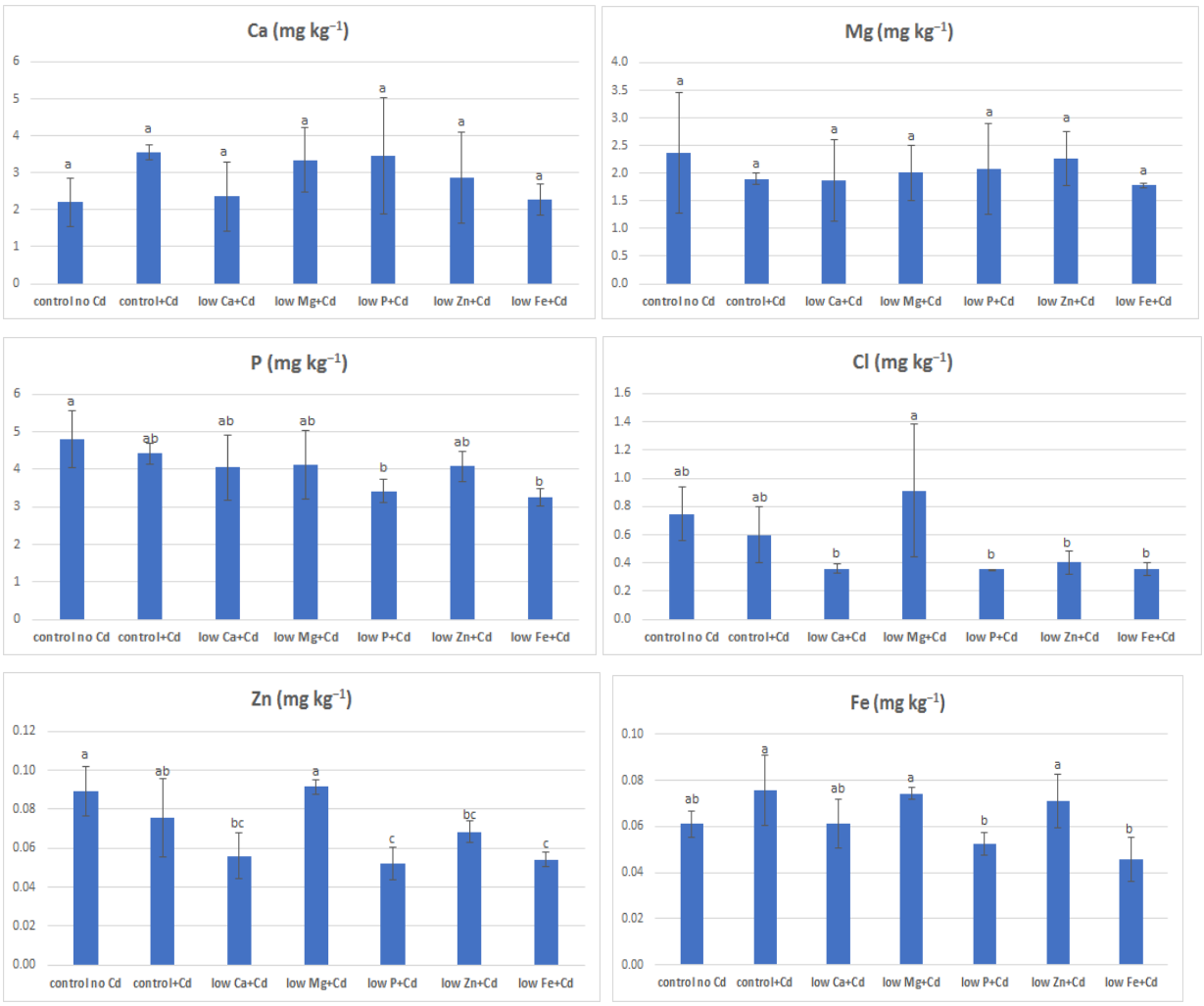


Fig 2. Nutrient concentrations in asparagus (mg kg^{-1}). Treatments followed by the same letters are not significantly different at $p<0.05$. Bars represent the standard deviations (n=3).

The concentrations of nutrients in shoots and roots of asparagus plants are presented in Fig. 2. There are complicated relations between the accumulation of Cd and nutrients that were determined, including Ca, Mg, P, Cl, Zn and Fe. These results implied that significant differences existed among those treatments in absorbing and translocating those nutrients.

4.2 Species and Saturation Indices (SIs) of Cd and Ca speciation

CaSO4 (aq) and $CaNO_3^+$ are mainly Ca species stay in solutions (Table 2). Each treatment had same Cd species, which are CdI_4^{2-}, CdI_2 (aq) and CdI_3^-. $CdI_4^{2-}$is the main species in all treatments while the proportions of CdI_2 (aq) and CdI_3^- were relatively lower (Table 3).

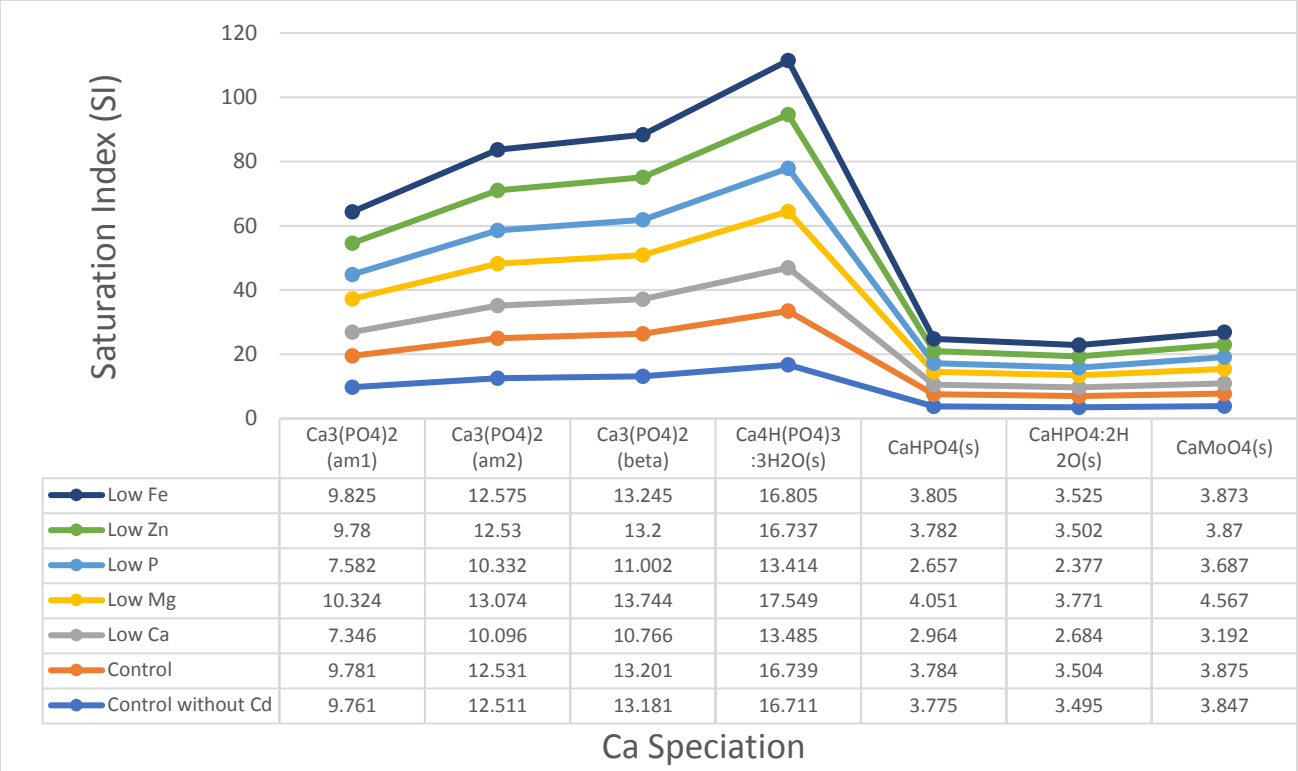
	low Ca	low Mg	low P	low Zn	low Fe
CaSO4 (aq)	30.442	6.663	10.609	10.402	14.714
CaNO3+	58.191	81.269	87.258	87.383	78.724

Table 2. Ca species and percentage of the total concentration (%)

	low Ca	low Mg	low P	low Zn	low Fe
CdI_2 (aq)	0.356	0.241	0.241	0.283	0.245
CdI_4^{2-}	82.044	85.02	85.013	83.848	84.9
CdI^{3-}	17.595	14.736	14.743	15.865	14.852

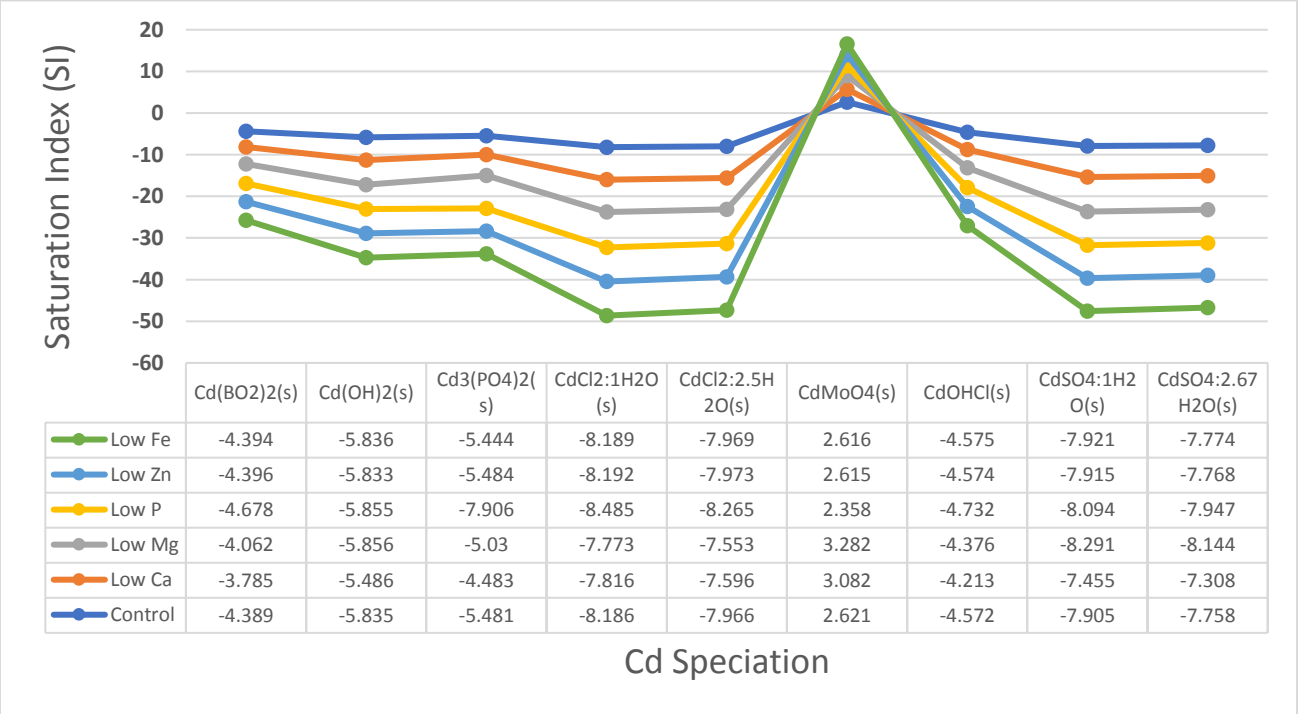
Table 2. Cd species and percentage of the total concentration (%)

I determined saturation indices (SIs) for mineral species in MINTEQ. Mineral species of Ca were $Ca_3(PO4)_2$(am1), $Ca_3(PO_4)_2$(am2), $Ca_3(PO_4)_2$(beta), $Ca_4H(PO_4)_3$:$3H_2O$(s), $CaHPO_4$(s), $CaHPO_4$:$2H_2O$(s) and $CaMoO_4$(s). Mineral species of Cd are $Cd(BO_2)_2$(s), $Cd(OH)_2$(s), $Cd_3(PO_4)_2$(s), $CdCl_2$:$1H_2O$(s), $CdCl_2$:$2.5H_2O$(s), $CdMoO_4$(s), CdOHCl(s), $CdSO_4$:$1H_2O$(s), $CdSO_4$:$2.67H_2O$(s). If SI > 0, it demonstrates that the solution is oversaturated; if SI < 0, the solution is under-saturated; and if SI = 0, the solution is reached at an apparent equilibrium(Sahariah et al. 2015). It indicates that Ca minerals were oversaturated with positive values of SIs. Treatments can increase Sis of Ca and low Fe treatment had the most significant influence (Fig.3). Conversely, Cd minerals are undersaturated with negative values of SIs. Treatments decreased SIs of Cd and low Fe treatment had the most significant influence as well (Fig.4).



	Ca3(PO4)2 (am1)	Ca3(PO4)2 (am2)	Ca3(PO4)2 (beta)	Ca4H(PO4)3 :3H2O(s)	CaHPO4(s)	CaHPO4:2H 2O(s)	CaMoO4(s)
Low Fe	9.825	12.575	13.245	16.805	3.805	3.525	3.873
Low Zn	9.78	12.53	13.2	16.737	3.782	3.502	3.87
Low P	7.582	10.332	11.002	13.414	2.657	2.377	3.687
Low Mg	10.324	13.074	13.744	17.549	4.051	3.771	4.567
Low Ca	7.346	10.096	10.766	13.485	2.964	2.684	3.192
Control	9.781	12.531	13.201	16.739	3.784	3.504	3.875
Control without Cd	9.761	12.511	13.181	16.711	3.775	3.495	3.847

Fig.3 Saturation Indices (SIs) of Ca speciation



	Cd(BO2)2(s)	Cd(OH)2(s)	Cd3(PO4)2(s)	CdCl2:1H2O (s)	CdCl2:2.5H 2O(s)	CdMoO4(s)	CdOHCl(s)	CdSO4:1H2 O(s)	CdSO4:2.67 H2O(s)
Low Fe	-4.394	-5.836	-5.444	-8.189	-7.969	2.616	-4.575	-7.921	-7.774
Low Zn	-4.396	-5.833	-5.484	-8.192	-7.973	2.615	-4.574	-7.915	-7.768
Low P	-4.678	-5.855	-7.906	-8.485	-8.265	2.358	-4.732	-8.094	-7.947
Low Mg	-4.062	-5.856	-5.03	-7.773	-7.553	3.282	-4.376	-8.291	-8.144
Low Ca	-3.785	-5.486	-4.483	-7.816	-7.596	3.082	-4.213	-7.455	-7.308
Control	-4.389	-5.835	-5.481	-8.186	-7.966	2.621	-4.572	-7.905	-7.758

Fig. 4 Saturation Indices (SIs) of Cd speciation

5. Discussion

This study assessed the interactions between uptake of Cd and other elements in a hydroponic setting. I found that lowering Ca concentration was the only treatment that significantly increased Cd accumulation in asparagus (Fig. 1). Past studies suggested that Cd could be absorbed via the Ca ion channels on the root membrane (Wolterbeek et al. 1988, McLaughlin et al. 1998, Chen et al. 2018). Thus, my results support the idea that the competition between Cd and Ca transport across the root membrane is a key factor regulating Cd availability in asparagus (McLaughlin et al. 1998). Meanwhile, I found Cd accumulations were reduced in asparagus with low P, Zn, Mg and Fe treatments, respectively, which is likely due to effects of Cd on transporting pathways of those mineral elements (P, Zn, Mg and Fe), such as *ZNT1*(Zn transporter 1), *IRT1*(Iron-regulated transporter 1) and OsNRAMP1 (Cohen et al. 1998, Barberon et al. 2014). I found a relatively high Cd concentration (~ 0.3 mg kg^{-1} Cd) of asparagus plants in the control treatment where no Cd was added to the hydroponic (Fig. 1). I can rule out analytical contamination for the following reasons: my analytical blanks did not return meaningful concentrations of Cd, and the Cd recovery was reasonable in my check standards (NIST tomato leaves). Even without soil cadmium pollution, asparagus contains a small amount of Cd, which is most likely responsible for the Cd detected in plants of the control treatment. The concentrations of nutrients we determined were also affected by those treatments. For example, Mg and P concentrations were reduced by different levels with treatments but no significance among those treatments. A decrease in Cd addition is correlated with an increase in the concentration of Fe because both Fe and Cd are transported by same transporters (*OsNRAMP1*) (Khaliq et al. 2019).

Soil pH is an important factor regulating Cd bioavailability in soil. The pH of the Hoagland solution was slightly acidic due to the use of acidic salts and boric acid. In my experiment, decreasing the concentration of elements (P, Cl, Ca, Mg, Zn, Fe) had limited impacts on the pH of solution. However, this acidic environment contrasts with the alkaline nature of Peruvian soils. In future studies, the pH of the hydroponic solution can be increased to better resemble the soil pH of Peruvian soils. A key issue is this scenario is to ensure the availabilities of major nutrients under alkaline conditions. For instance, the bioavailability of Fe is low in alkaline soils, which can influence Cd uptake according to my results (Fig. 1). Using MINTEQ or MINEQL+ software, simulations can be run to determine the speciation of major nutrients and to predict the formation

of metal precipitates. These simulations would also be valuable to verify the effectiveness of pH manipulation.

One thing to note in future experiments is that roots were often damaged or killed by microorganisms in my experiment. The root epidermis was damaged and could not absorb enough water to support plant growth, resulting in incomplete root formation. It is likely because the HNS is high in nutrients, leading to not only fast asparagus growth but also the proliferation of microorganisms. In the future study, asparagus roots should be washed regularly, e.g., whenever the hydroponic solution needs to be changed.

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