EFFECTS OF ROOT MYCORRHIZAL COLONIZATION AND VARIED PHOSPHOROUS SUPPLY ON CADMIUM ACCUMULATION IN RICE PLANTS

by

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ABSTRACT

EFFECTS OF ROOT MYCORRHIZAL COLONIZATION AND VARIED PHOSPHOROUS SUPPLY ON CADMIUM ACCUMULATION IN RICE PLANTS

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Keywords: Arbuscular mycorrhizal fungi, cadmium, cadmium contamination, human health, phosphorus, rice.

Cadmium (Cd) is a heavy metal and exerts harmful effects on the environment and humans depending on its concentrations. Rice-based foods are known to be a major dietary source of Cd exposure to human populations. It is therefore important to study the factors affecting Cd accumulation in rice plants. Use of phosphorous (P) fertilizers is often associated with increased Cd accumulation in food crops and suggested that Cd-containing P fertilizers represent an important source of Cd in food crops. However, increases in plant Cd by P fertilizers are not necessarily related to the Cd content of the P fertilizers applied. This counterintuitive finding led us to investigate the factors affecting Cd accumulation in rice plants by paying particular attention to various P fertilizers differing in Cd content and root mycorrhizal activity. It is known that arbuscular mycorrhizal fungi (AMF) are an important barrier against the transfer of Cd from the soil to the shoots of plants. Rice plants (*Oryza sativa* L. cv. Rekor CL) were cultivated aerobically under greenhouse conditions and treated with increased P fertilization rates

with and without mycorrhizal inoculation and autoclaving the experimental soil. The results obtained showed that increasing the P application caused substantial increases in the shoot Cd concentrations in all of the experimental treatments, irrespective of the Cd content of the P fertilizers. These increases were pronounced when the experimental soil was sterilized. Soil inoculation with AMF significantly decreased the Cd accumulation in rice plants, especially when the experimental soil was sterilized. The results suggested that increase in plant Cd accumulation by P fertilization is related to diminished root mycorrhizal colonization, and root mycorrhizal activity plays a key role in limiting Cd transfer from soils in the above-ground parts of the rice plants.

ÖZET

ÇELTİK BİTKİSİNDE KÖK MİKORİZAL KOLONİZASYONU VE FARKLI FOSFOR UYGULAMALARININ KADMİYUM BİRİKİMİ ÜZERİNE ETKİLERİ

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Anahtar kelimeler: Arbusküler mikorizal funguslar, kadmiyum, kadmiyum kontaminasyonu, insan sağlığı, fosfor, çeltik.

Kadmiyum (Cd) ağır bir metal olup, konsantrasyonuna bağlı olarak çevre ve insanlar üzerinde zararlı etkiler göstermektedir. Pirinç temelli gıdaların, insanların Cd alımında çok önemli bir diyet kaynağı olduğu bilinmektedir. Bu nedenle, çeltik bitkilerinde Cd birikimini etkileyen faktörlerin incelenmesi önem taşımaktadır. Fosforlu (P) gübrelerin kullanımı genellikle gıda ürünlerinde artan Cd birikimi ile yakından ilişkilidir ve Cd içeren P gübrelerinin gıda ürünlerinde önemli bir Cd kaynağını temsil ettiği öne sürülmüştür. Bununla birlikte, P gübreleri tarafından neden olunan bitki Cd seviyesindeki artışların, uygulanan P gübrelerinin Cd içeriği ile mutlaka ilgili olmadığı görülmektedir. Bu pek beklenmedik bulgu bizi, başta Cd içeriği bakımından farklılık gösteren çeşitli P gübreleri ve kök mikorizal aktivitesini dikkate alarak, çeltik bitkilerinde Cd birikimini etkileyen faktörleri araştırmaya yönlendirdi. Arbusküler mikorizal mantarların (AMF), topraktan bitki yeşil aksamına gerçekleşen Cd transferine karşı önemli bir engel olduğu bilinmektedir. Bu çalışmada, çeltik bitkileri (*Oryza sativa* L. cv. Rekor CL), sera koşulları

altında aerobik toprak koşulları dikkate alınarak yetiştirildi. Denemelerde bitkilere steril ve doğal toprak koşullarında farklı oranda Cd içeren P gübreleri artan oranda uygulandı ve bitkiler farklı mikoriza inokulasyonuna tabii tutuldu. Elde edilen sonuçlara göre, fosfor uygulamasının arttırılması, P gübrelerinin Cd içeriğinden bağımsız olarak, denenen tüm uygulamalarda yeşil aksam Cd konsantrasyonunda önemli artışlara neden olmuştur. Bu artışlar, toprağın sterilize edildiği koşullarda daha çarpıcı biçimde ortaya çıktı. Denemelerde arbusküler mikorizal fungusun toprak aşılaması ile çalışılan tüm deneysel uygulamalarda, özellikle de toprağın sterilize edildiği koşullarda, bitkilerin Cd alımını önemli ölçüde azaltmıştır. Sonuçlar, P gübrelemesi ile bitkilerde ortaya çıkan Cd birikiminin, azalan kök mikorizal kolonizasyonu ile ilişkili olduğunu ve kök mikorizal aktivitesinin, çeltik bitkilerinin toprak üstü kısımlarında topraktan Cd transferini sınırlamada kilit bir rol oynadığını göstermektedir.

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This work is dedicated

To the memory of my father
who had always faith in me to be successful in my academic career.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C ...……degrees celcius

ABC ...……ATP binding cassette

Al .....……aluminum

AMF ...……..arbuscular mycorrhizal fungi

ANOVA ...…….……..analysis of variance

Ar ...……argon

ATP ...……adenosine triphosphate

B ...…….boron

C ...……...……..…carbon

Ca ...……calcium

$Ca(H_2PO_4)_2$ ……..……..calcium phosphate

$Ca(NO_3)_2$ .....……..……..……..…..calcium nitrate

$CaCl_2$ …..…..…….……calcium chloride

$CaCO_3$ ...……..…calcium carbonate

CaP ...……..…….……..calcium phosphate

$CaSO_4.2H_2O$ ...……....calcium sulfate dihydrate

Cd ...……..cadmium

$Cd(NO_3)^2$ ...…….……..cadmium nitrate

CDF ...……cation diffusion facilitator

cm …….....centimeter

CO_2 ……..carbon dioxide

Cu …….copper

cv .....cultivar

DAP ……..diammonium phosphate

DTPA ……..diethylenetriaminepentaacetic acid

DW ……dry weight

EFSA ……..European Food Safety Authority

EU ……European Union

Fe ……..iron

Fe^{3+} ……...ferric ion

g ……gram

GSH ……..glutathione

ha ……hectare

H_2O_2 ……..hydrogen peroxide

HC1 ……...hemicellulose 1

HCl ……hydrochloric acid

HMA ……..heavy metal ATPase

HNO_3 ……..nitric acid

HSD ……honestly significant difference test

ICP-MS ……inductively coupled plasma mass spectrometry

ICP-OES ……inductively coupled plasma optical emission spectroscopy

IMI ……...imidazolinone

K ……potassium

K_2SO_4 ……..potassium sulfate

kg ……..kilogram

KOH ……..potassium hydroxide

L ……...liter

La ……...lanthanum

LCT1 ……..low-affinity cation transporter

mg ……...…milligram

Mg ……magnesium

ml ……milliliter

Mn ……manganese

Mo ……..molybdenum

MS ……..Member States

N ……nitrogen

NAS ……...nicotinamide synthases

ng ……nanogram

NH_4NO_3 …….ammonium nitrate

NO_3^- ……..nitrate

NRAMP ……...natural resistance-associated macrophage protein

O_2^- ……..superoxide radical

OH^- …….....hydroxide ion

P ……phosphorus

P_2O_5 …….phosphorus pentoxide

PAL …….L-phenylalanine ammonia-lyase

PC ……....phytochelatins

Pi ……inorganic phosphate

PME …….pectin methylesterase

PO_4^{3-} ……...phosphate

ppm ……...parts per million

ROS ……..reactive oxygen species

S …….sulfur

Se ……..selenium

SSP …….superphosphate

v ……..volume

w ……..weight

ZIP …….ZRT-IRT-like proteins

Zn …….zinc

$ZnSO_4.7H_2O$ ……...zinc sulfate heptahydrate

µg ……...microgram

µM ……micromolar

1. INTRODUCTION

Heavy metal accumulation in the natural ecosystem is a worldwide issue. Soil quality is declining as excessive amounts of metals are released into the environment, followed by a loss in soil efficiency and food security (Xiao et al. 2017). Cadmium (Cd) is a heavy metal and may exert harmful impacts on the environment and humans depending on its concentrations (Genchi et al. 2020). The release of Cd to environment occurs because of both natural and anthropogenic practices such as industrial and agricultural activities (McLaughlin et al. 2021).

Aside from agricultural and industrial activities, soil and cultivation practice factors such as pH and salinity of the soil, patterns of cultivation, zinc (Zn) phyto-availability, liming, plant genotype, and nitrogen (N) and phosphorous (P) fertilization levels all have a marked effect on the Cd root uptake and soil solubility (Mench, 1998; Sheppard et al. 2009; McLaughlin et al. 2021). However, several previous and recent studies showed that Cd accumulation in plants treated with P fertilizers is independent of the Cd level of the applied P fertilizers (McLaughlin et al. 1995; Grant et al. 2002; Yazici et al. 2021). It was shown that diminished mycorrhizal root colonization by increasing P fertilization has a significant role in P-fertilization related Cd accumulation in plants as discussed recently by Yazici et al. (2021). It is known that mycorrhizal fungi are an important barrier against the transfer of Cd from the soil to the shoot parts of plants (Joner et al. 2000; Shi et al. 2020).

Cadmium can enter plants easily due to its extreme mobility in the soil-plant system and damages several crucial processes that lead to substandard plant development. Thus, the presence of Cd in soils in extreme amounts leads to Cd accumulation through the food chain (He et al. 2014; Xu et al. 2017). Consumption of food that has high Cd content causes negative effects on human health, such as renal dysfunction, cancer, major lung

damage, cardiac failure, cerebrovascular infarction, anemia, hypertension, cataract formation in the eyes, emphysema, proteinuria, and osteoporosis (Sebastian and Prasad 2013). Moreover, Cd is particularly damaging to the kidneys, specifically to the proximal tubular cells. The accumulation occurs in these cells over time and leads to a reduction in the glomerular filtration rate, which causes renal failure. Some recent studies have found that long-term dietary Cd exposure can harm the kidneys and bones (Song et al. 2017).

Food crops such as rice, wheat, corn, soybeans, and potatoes represent important food and feed crops, and can be an important cause of Cd contamination in human and animal diets (Clemens et al. 2013; Corguinha et al. 2015). Among these food crops, rice is the most studied crop in respect to Cd accumulation and contribution to dietary Cd intake, especially in Asia. Rice is a major staple crop, and together with wheat and maize contributes to the daily caloric intake worldwide population of the world (Awika, 2011). According to Shi et al. (2020), the highest Cd concentration in the polished market rice belongs to China with a 69.3 μg/kg median, while East Africa has the lowest median of 4.9 μg/kg. Nevertheless, rice genetics, bedrock geology, paddy soil management, soil formation, weathering, and the Cd level in fertilizers need to be taken into account and required to be further studied as these factors have a key role in grain Cd contents.

Human exposure to Cd-contaminated rice is a public health problem, emphasizing the importance of assessing food safety for the Cd concentrations in edible parts of food crops. Rice is known to be a principal source of the dietary intake of Cd for humans, especially in Asian countries. Consequently, China as well as European Union (EU) determined a strict standard of 200 μg Cd kg^{-1} allowable for rice and wheat (European Commission, 2006; Zhou et al. 2018; Shi, 2020).

In 2009, the European Food Safety Authority (EFSA) concluded that Cd in food is particularly toxic to the kidneys and can lead to kidney failure. Therefore, EFSA determined a weekly tolerable Cd intake limit of 2.5 μg/kg of body weight. Also, they indicated that groups of people such as smokers, vegetarians, and people living in severely contaminated locations can consume more than twice as much as the weekly limit. Furthermore, in 2021, the EU further decreased the maximum Cd concentration limit for a range of food products and established a new regulation for public health.

According to the regulation, the new maximum level of Cd in rice, quinoa, wheat bran, and wheat gluten has become 150 μg Cd kg^{-1} wet weight (European Food Safety Authority, 2009; European Commission, 2021).

1.1. Soil Contamination with Cadmium

Since the amount of numerous heavy metals such as Cd in certain food crops has reached health thresholds in recent decades, soil pollution by trace elements has now become a global problem (Rafiq et al. 2014). The release of Cd to environment occurs in various quantities due to activities that are natural and anthropogenic. Natural Cd sources in the atmosphere include mostly geological weathering (Liu et al. 2013). As ultramafic and mafic rocks have a large amount of Cd, a noteworthy amount of Cd to soil is released due to weathering (Khan et al. 2010). Cadmium concentrations in black shales can reach 100 mg kg^{-1}, and the soil generated through their deposits is enriched by Cd (He et al. 2005).

Anthropogenic sources such as metallurgical works, sewage sludge, combustion of fossil fuel, mining, phosphate fertilizers, smelting, and industrial and municipal wastes cause the release of over 90 percent of Cd to the environment. Production of polyvinyl chloride plastics, fungicides, motor oil, alloys, solders, and production of textile and rubber also cause Cd release (Khan et al. 2017). Total global Cd generation in 2017 was nearly 23,000 tons, according to the China Industrial Information Network (Shi et al. 2022). Mining activities in the mining regions release high amounts of Cd and are responsible for high Cd contamination of adjacent farms. According to geochemical similarities, Cd appears as a companion element in all forms of Zn ores. Hence, Cd is delivered to the atmosphere in significant quantities through Zn smelting. Also, dust containing high Cd, which can be disposed of locally with a brief residence time, is discharged into the environment during the Zn smelting processes (Roy and McDonald, 2013).

Cadmium release due to industrial practices and automobile emissions has a high potential to damage urban soil (Singh et al. 2018). The global overall estimated Cd entry

to the soil from various anthropogenic sources ranges from 5 to 38,000,000 kg $year^{-1}$, through wastes that are agricultural and animal, including fertilizers, accounting for 12 percent (Khan et al. 2017). Furthermore, the use of wastewater in irrigation also contaminates agricultural soils with Cd to an even greater extent (Farahat and Linderholm, 2015).

Among other major causes, the application of P fertilizers is often regarded as a significant source of Cd exposure in agricultural soil. On average, phosphate fertilizers applied in Europe contain 82.7 mg Cd kg^{-1}, expressed on the P basis, as reported by Nziguheba and Smolders (2008). Additionally, utilization of contaminated manure increases the amount of Cd in agricultural soil. Nicholson et al. (2003) demonstrated that when Cd was treated at an N rate that was 250 kg N ha^{-1} $year^{-1}$, the exposure of Cd into agricultural soil due to manure was in the range of 1.4 to 6.1 g Cd ha^{-1} $year^{-1}$, while sludge that contained 3.4 mg Cd kg^{-1} at the same N rate increased the Cd content by 19 g Cd ha^{-1} $year^{-1}$. Additionally, according to Khan et al. (2017), among many countries France has the maximum Cd concentration in soil, followed by Belgium and China.

1.2. Roles of Phosphorus Fertilizers in Cadmium Concentration of Plants

Phosphorus nutrient promotes plant growth, and if soils are deficient in P, the nutrient must be provided in whether organic or inorganic form to increase crop yield. However, P fertilizers contain Cd in varying levels as a contaminant due to its presence in phosphate rock (Grant et al. 2013; Jiao et al. 2004). The International Fertilizer Development Center (IFDC) made a list of the Cd level of phosphate rocks that were acquired from 20 countries. According to the list, the Cd content in sedimentary deposits was 21 mg/kg on average. The deposits of sedimentary phosphate rocks are approximately 69 times more concentrated in Cd than non-phosphate-containing rock. During the fertilizer manufacturing process, varying Cd levels in the phosphate rock pass the acidulation and beneficiation processes. The amount transferred to fertilizers is determined by the production procedures (Roberts, 2014).

Studies on the relationship between Cd accumulation and P fertilizers indicated an

inconsistent interaction between Cd and P in soil systems. Some researchers found that increasing P can inhibit Cd, while other studies concluded that Cd uptake can be increased by enhancing the P levels. Despite several controversial results, repeated applications of P fertilizers may enhance soil levels of Cd, which could ultimately enter the food chain by crop uptake (Wang et al. 2017; Grant et al. 2013). According to OPERA Research (OPERA Research, 2021), P fertilizers account for 45 percent of total Cd contamination of farmland in Europe. Concurrently, soil Cd accumulation accounts for 55 percent of the average European consumer's total dietary Cd consumption.

Although the EU has been worried about Cd since the 1970s, a regulation on the limits of the Cd content in P fertilizers was first adopted in June 2019. In Regulation 2019/1009 (EUR-Lex., 2019), the limits were adjusted to 60 mg/kg P_2O_5. Even though this maximum limit of Cd in P_2O_5 will become valid in July 2022 in the EU, it is difficult to consider the current situation as a progress. As 12 of the Member States (MS) have already adopted lower maximum limits, 8 of the MS have similar maximum limits to 60 mg/kg, and 2 of the MS have adopted higher maximum limits, thus EU could not maintain an internal market harmonization (OPERA Research, 2021).

It has also been observed that there is no consistent evident relationship between P and Cd. The increase in the Cd concentration of plants via P fertilization is not proportional to the Cd content of the P fertilizer (Grant et al. 2013; Yazici et al. 2021; Jiao et al. 2004; McLaughlin et al. 1995). Furthermore, in another study by Grant et al. (2002), even though the range of Cd content in P fertilizer differed between 0.2 to 186.0 mg per g P, the Cd level in wheat plants enhanced independently of the Cd level in the fertilizers applied.

Phosphorous fertilization can influence the accumulation of Cd in plants by various factors, which influence the availability of Cd, such as availability and supply of Zn, soil pH, root growth and distribution, ionic strength of the soil solution, mycorrhizal activity, and addition of Cd (Grant et al. 2013). Phosphorus-induced Zn deficiency is a well-documented phenomenon (Loneragan et al., 1982; Cakmak and Marschner, 1987). As Cd and Zn have similar atomic properties and similar uptake and transportation patterns in plants, high P applications may also induce high Cd uptake and accumulation in plants by inhibiting Zn absorption in plants (Grant et al. 2013; Sebastian and Prasad

2013). Zinc deficiency in plants may also enhance Cd uptake via upregulating the Zn acquisition mechanisms (Baxter et al. 2008; Küpper and Kochian 2010; Clemens et al. 2013; Chaney 2015).

1.3. Cadmium Uptake and Translocation

The phytoaccumulation factor index of Cd, which is the ratio of Cd content in the plant to the soil, is high and can exceed the index of several essential nutrients even though Cd is an unessential element. As Cd lacks a particular cell entrance pathway, plants take up Cd from soils generally by root uptake via essential element transporters that are specific and non-specific such as for Zn^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, and Cu^{2+} (Shahid et al. 2016). Mendoza-Co´zatl et al. (2011) reported that root uptake of Cd in plants is mediated by the ZIP family of transporters for Zn^{2+}, Mn^{2+}, and Fe^{2+}, or cation channels. Another mediator for Cd uptake is OsIRTs, although their part in Cd uptake may vary according to the plant type (Lee and An, 2009). Additionally, the entrance of Cd to the root cells of plants can occur via natural resistance-associated macrophage protein (NRAMP) family transporters, yellow-Stripe 1-Like proteins (YSL), heavy metal ATPase (HMA), the ATP binding cassette (ABC), cation diffusion facilitator (CDF), and nicotinamide synthases (NAS) as Cd-chelates (Maestri et al. 2010; Cui et al. 2019), as illustrated in Fig. 1.1. Furthermore, Clemens et al. (1998) discussed that low-affinity cation transporter (LCT1) performs as a calcium (Ca) transport system in plants and can have a role in the transport of Cd.

Root Cd uptake can be inhibited by several cationic nutrients and metals including Fe^{2+}, Ca^{2+}, La^{3+}, Mn^{2+}, Zn^{2+}, or Cu^{2+} existing in the rhizosphere solution. The competition between the cations for ion transport-specific channels affects the uptake of Cd (Lux et al. 2011). Furthermore, it has been demonstrated that when selenium (Se) was present in the soil at high levels, root Cd uptake was reduced by 44 percent in rice plants. Accordingly, Se also competes with Cd for uptake just as it does with cationic metals (Hu et al. 2014).

Generally, Cd accumulation is prone to be in the roots, and just a small percentage moves to the aerial parts. When the parts of the plants are compared in respect to their Cd content, roots have the highest content followed by stems, leaves, fruits, and seeds being the lowest (Benavides et al. 2005). Some species, which are known as low Cd accumulating plants, may sequester large quantities of Cd in their roots with minimal transfer to shoots, or mediate remobilization of Cd from leaves to roots via the phloem (Mendoza-Co´zatl et al. 2011). In a study, Cd was accumulated by 60-90 percent in the roots of the rice plants compared to the rest parts of the plants (Zhou et al. 2014). As Cd concentrations in the roots can be ten times more than in the shoots of the plants, roots function as an important barrier to the shoot translocation of Cd (Wang et al. 2009).

Cadmium is transported from the roots to the aerial tissues through transpiration-driven xylem loading, with apoplastic or symplastic transport of free Cd^{2+} or Cd complexed with different chelates. Transpiration has also an important effect on shoot Cd transport as shown by Zhao et al. (2006). Differences in transpiration rate among the rice plants were found to be responsible for the differences in shoot Cd transport (Shahid et al. 2016).

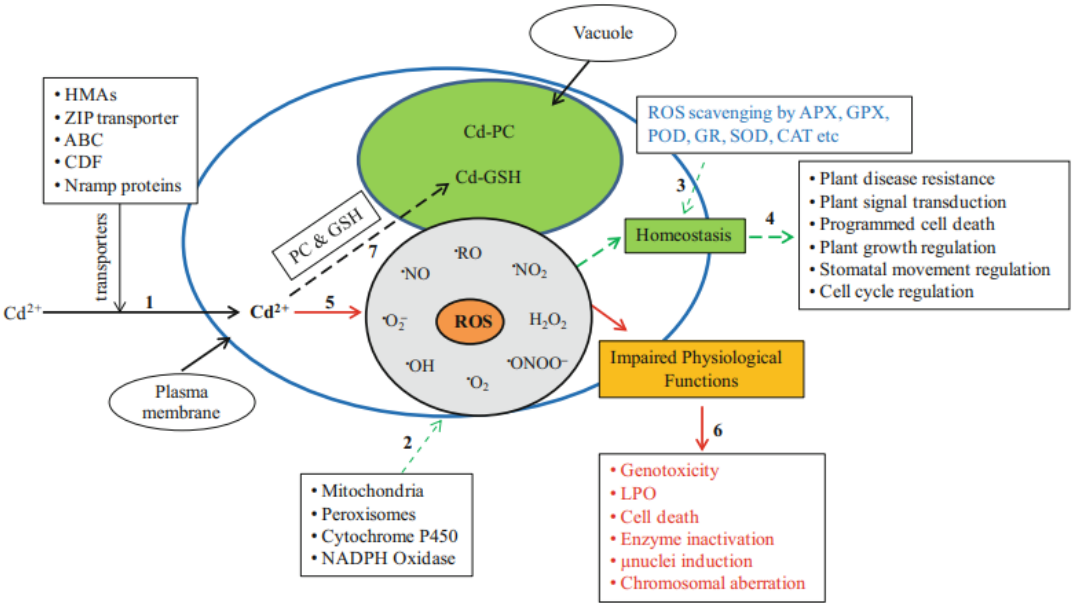

Figure 1.1: Schematic illustration representing potential Cd entrance, accumulation toxicity, and detoxification in a plant cell. 1) Membrane transporter gene families mediate Cd entry through the plasma membrane. 2) Under normal conditions, reactive oxygen species (ROS) are generated in several cell organelles 3) and an ideal ROS level (homeostasis) is preserved via antioxidant enzymes. 4) These ROS have a variety of critical functions in living organisms. 5) The Cd occurrence can enhance the generation of ROS, 6) which may result in serious physiological problems for the plant, known as "oxidative stress." 7) Alternatively, glutathione (GSH) and phytochelatins (PC) may sequester Cd into the vacuole (Shahid et al. 2016).

1.4. Mycorrhiza in Nutrient Uptake of Plants

Arbuscular mycorrhizal fungi (AMF) have a symbiotic relationship with more than 80 percent of terrestrial plant species which are grown in mineral soils with abundant inorganic P and N sources. Members of the fungal phylum Glomeromycota constitute the AM fungi. The studies on AM fungi showed that most of the plant species have the potential to form arbuscular mycorrhizas, even though studies have been done on only a small percentage of plant species. AM fungi can live in two environments, which are roots in which they get organic C and give back nutrients, and soil in which they can absorb those nutrients (Smith and Smith, 2011).

The symbiosis between AM fungi and plants represents the most common root symbiosis. AM fungi are obligate symbionts that get all their organic carbon (C) from their host plants. Although the activities of various free-living microorganisms such as microbes included in nutrient cycling are constrained via organic C substrates in soil, it is not the case for AM fungi (Smith and Smith, 2011). The symbiosis that depends mostly on the transfer of C from the plant and P from the fungi is frequently mutualistic. AM fungi have multiple benefits, such as altering the pollutant mobility by altering the soil properties (water retention, pH, and aggregate as a result of glomalin creation) due to absorbing anions such as NO_3^- and PO_4^{3-} and enhancing OH^- ions and soil pH. Furthermore, AM fungi can enhance nutrition such as P, Zn, and N and affect the resilience of the ecosystem to abiotic stresses such as temperature stress, water stress, nutrient deprivation, and biotic stresses, and may play a key role as ecosystem drivers (Marschner, 2012; Veresoglou et al. 2012; Li et al. 2021).

1.4.1. Role of Mycorrhizae in Interaction between Zinc and Phosphorous

Zinc is an essential micronutrient needed for plant growth and development. Even though Zn is not required in large concentrations, the critical Zn levels enable various crucial plant physiological mechanisms to function properly. Zinc is a significant structural constituent as well as a regulatory cofactor of a variety of different enzymes and proteins in several significant biochemical pathways, including protein, auxin biosynthesis, carbohydrate metabolism, pollen development, and resistance to biotic and abiotic stress. (Cakmak, 2000; Marschner, 2012). Zinc is also a critical element in human nutrition. Around 3 billion people, living mainly in developing countries, struggle with dietary deficits caused by micronutrient malnutrition, particularly Zn (Cakmak and Kutman, 2017; Suganya et al. 2020).

Another important nutrient is phosphorus, which is a significant macronutrient that performs a variety of critical roles in plant cells. Phosphorus is an integral part of ATP, phospholipids, DNA, RNA, and nucleic acids. Furthermore, P has a favorable impact on photosynthesis and the utilization of photoassimilates. Thus, it has an impact on plant

growth, development, energy transfer, and storage (Marschner, 2012). During the initial stages of crop development, plants require an adequate supply of P, which is provided by the utilization of P fertilizers. Moreover, P uptake is higher in mycorrhizal plants than in nonmycorrhizal plants. According to Li et al. (2006), arbuscular mycorrhizal fungi (AMF) increased the plant P uptake by fifty percent. However, high soluble P ions in agricultural soils, combined with soil alkalinity, drastically reduce the Zn availability (Akhtar et al. 2019). High application of P fertilizers is also involved in the reduction of root Zn uptake by diminishing AMF activity since AMF affect up to 50% of root Zn uptake in many plant species (Marschner, 2012; Ova et al., 2015; Watts-Williams et al. 2015; Coccina et al. 2019.)

1.4.2. Role of Mycorrhiza in Cadmium Uptake of Plants

AM fungi have been shown to benefit host plants through different ways. The heavily branched outer mycelium acts as a mycorrhizal pathway for the uptake and transfer of vital nutrients, particularly P and Zn (Marschner, 2012). AMF are also effective in reducing shoot Cd accumulation in plants. AM fungi can prevent the uptake of Cd by the roots and subsequent transfer into the shoots by immobilizing Cd in soils and cell walls (Joner et al., 2000; Ferrol et al., 2016). Immobilization of Cd in root tissues by AMF has also an important role in preventing Cd toxicity in shoot parts of plants.

It is well known that AMF are highly sensitive to high applications of P fertilizers. The increasing number of reports is available showing very significant decreases in root mycorrhizal activity by increasing P fertilization (Smith et al. 2011; Ova et al. 2015; Zhang et al., 2020). A decrease in mycorrhizal activity in the roots due to high P fertilization may increase the uptake of Cd through a lack of competition between Cd and Zn for root uptake, as discussed above (Ova et al., 2015; Yazici et al. 2021). Furthermore, AM fungi directly limit the uptake and accumulation of Cd from the root to the shoot (Yazici et al. 2021). Plants benefit from AM fungi as harmful elements are immobilized inside the mycelia or in the root system. These results indicate that AMF play an important role in reducing Cd transfer in the shoots of plants by immobilizing (phytostabilizing) Cd as well as by improving root Zn uptake.

AM fungi have the capability to modify root cell walls which leads to an increase in the properties of Cd fixation. In a hydroponic experiment, Gao et al. (2021) discussed that components of the cell wall may be Cd fixation sites, reducing Cd transfer from root to shoot in rice plants. Accordingly, inoculation with AM fungi enhanced the levels of pectin, lignin, and hemicellulose which have high Cd binding capacity.

1.5. Effects of Cadmium Toxicity on Plants

As a heavy metal, Cd is very toxic to plants, even at low tissue concentrations. Several plant physiological, morphological, and biochemical processes are detrimentally affected by Cd (Clemens, 2006). Conditions in which Cd concentrations exceed 5-10 mg Cd g^{-1} leaf dry weight can severely inhibit plant growth and lead to plant death. The main morphological and biochemical defects in plants can be observed as retarded growth, chlorosis, inhibition in root growth, repression of photosynthesis, impairments in CO_2 fixation, lipid peroxidation, disruption of N and sulfur (S) metabolism (Shahid et al. 2015; 2016).

Cadmium toxicity impairs the structural stability of cell membranes and increases the permeability of the plasma membranes via promoting lipid peroxidation. Cadmium is known to be responsible for the increases in the production of reactive oxygen species (ROS) and consequently induces oxidative damage to cellular systems and vital cell constituents (Rodríguez-Serrano et al. 2009). As discussed by Shah et al. (2001), when rice plants are exposed to 100 and 500 μM $Cd(NO_3)_2$, an increased ROS production occurs, especially superoxide anion (O_2^-). In addition, alterations in bio-membranes caused by Cd modify the functioning of membrane-bound enzymes, as well as their permeability to nutrients, water, and protons (Shahid et al. 2016).

Cadmium exposure can also cause impairments in seed germination and results in adverse effects on root anatomy and elongation as Cd preferentially accumulates in the roots (Meng et al. 2008; Lux et al. 2011), as already discussed above.

Cadmium has been demonstrated to have an impact at the biochemical stage on premature xylogenesis, and lignification of the root cell wall decreased root length and root browning. Additionally, Cd exposure can also lead to an enhancement in root diameter, which may be related to an elevation in cell size of parenchyma, which increases the radial flux resistance of solutes and water (Rady 2011; Mohamed et al. 2012).

1.6. Rice Production

Rice, which is a semi-aquatic stable crop, is adapted to a variety of growth conditions including aerobic soils in uplands, anaerobic and flooded lowlands, and even deeply submerged soils in places that are prone to flood (Miro and Ismail, 2013). The anaerobic rice cultivation system, which is also known as the flooded rice system, is a traditional rice production method in which paddy rice varieties are grown without oxygen during the rice-growing season in flooded fields (Iqbal et al. 2020).

Specially created varieties of rice are cultivated in well-drained, non-puddled, and non-saturated soils in the aerobic rice production system. Therefore, during the rice-growing season, the soil is with oxygen, also referred to as aerobic (Iqbal et al. 2020). The rice crop is cultivated by direct seeding via utilizing a dry or water-soaked seed in a non-puddle field and under unflooded field circumstances. The aerobic rice production system is the most effective method for reducing labor and water use. Aerobic rice utilizes thirty to fifty percent less water than lowland rice that is submerged in water (Jana 2018). Similar to other crops such as maize, wheat, or sorghum, aerobic rice is cultivated on dry soils and supplied with extensive agronomic procedures and surface irrigations when required (Bouman et al. 2005).

1.7. Scope

One of the important sources of Cd in plants has been attributed to the use of Cd-containing P fertilizers. However, the increase in the Cd concentration of plants is not

always proportional to the Cd levels of the P fertilizers. Published results show that P fertilization increases plant Cd accumulation regardless of the Cd concentration of the P fertilizers applied (Grant et al. 2013; Yazici et al. 2021; Jiao et al. 2004; McLaughlin et al. 1995). It is obvious that whenever P fertilizers are applied, there is a risk for high Cd accumulation in plants. Recently, Yazici et al (2021) showed in studies with wheat plants that the reductions in root mycorrhizal activity by P fertilization play a key role in P-induced Cd accumulation in plants. Increases in the application of P fertilizers were found to be associated with decreases in AMF activity and increases in shoot Cd accumulation in wheat regardless of the Cd content of the P fertilizers applied.

To our knowledge, there is no published result about the role of P fertilizers differing in Cd content in Cd accumulation in rice and its relation to the root mycorrhizal activity. Considering the fact that rice represents the main dietary source of Cd in human populations, especially in Asia, it is of great importance to study the role of P fertilizers and root AMF activity in Cd accumulation in rice.

In this thesis work, the main aim was to study the role of P fertilizers differing in Cd concentration and mycorrhizal inoculation on shoot Cd concentrations of rice plants grown aerobically.

2. MATERIALS AND METHODS

2.1. Plant Material

A paddy cultivar, which is developed by Trakya Agricultural Research Institue from HALİLBEYXIMI ÇEŞİT backcross and registered in 2018 (*Oryza sativa* L. cv. Rekor CL), was used in all experiments conducted throughout this thesis. As reported by the Trakya Agricultural Research Institue (Trakya Tarımsal Araştırma Enstitüsü Müdürlüğü, 2018), the leaves of the cultivar Rekor CL are horizontal, and its clusters are semi-tilted, and the appearance of the rice grains is long, yellow, glassy, and matte. Rekor CL, a Clearfield variety resistant to IMI group weed pesticides, has a yield potential of 8000-10000 kg per hectare.

2.2. Soil Culture

The soil utilized in this study was supplied from the Eskisehir region in Central Anatolia. All studies were carried out on this calcareous soil, which had an 18 percent $CaCO_3$ content and a pH of 7.8, contained 1.5 percent low organic matter and had a clay loam texture. The soil contained 0.44 mg kg^{-1} Cd and 43 mg kg^{-1} Zn, while 0.05 M $CaCl_2$-extractable levels were 2.6 g Cd kg^{-1} soil and 77 µg Zn kg^{-1} according to the DTPA analysis technique which were reported recently by Yazici et al (2021). Furthermore, the extractable P concentration in the soil was 2.28 mg kg^{-1} measured by Olsen et al. (1954).

2.3. Fertilizers

In the experiments, two types of P fertilizers were used including diammonium phosphate (DAP) containing 28 mg Cd per kg fertilizer and analytical grade calcium phosphate $Ca(H_2PO_4)_2$ (CaP) received by Sigma Aldrich (Steinheim, Germany) and containing virtually no Cd (i.e., 0.09 mg Cd kg^{-}). Phosphorus fertilizers have been applied at 3 rates as following: 20 mg P kg^{-1} (low), 60 mg P kg^{-1} (medium), and 180 mg P kg^{-1} (high). Plants were treated with N in the form of ammonium nitrate (NH_4NO_3) in addition to DAP to attain a level of 250 mg N per kg of soil. All experimental pots were supplied with 25 mg S per kg of soil in the form of K_2SO_4, and 11 mg S per kg of soil in the form of calcium sulphate dihydrate ($CaSO_4.2H_2O$). Zinc was supplied in the form of $ZnSO_4.7H_2O$ at different rates as indicated for each experiment.

2.4. Mycorrhizal Fungi

Mycorrhizal inoculum, *Claroideoglomus etunicatum*, BEG 24, which was propagated on sudangrass, *Sorghum bicolor* (L.) Moench was utilized in the experiments.

2.5. Plant Growth and Experimental Design

The experiments were set up as a full factorial design completely randomized with 5 independent replicates per treatment. Experimental plants were grown aerobically in plastic pots with 3.2 kg of a calcareous soil with high pH (7.8), as described above, under greenhouse conditions at Sabanci University. Deionized water was utilized to water the plants on a daily basis.

2.5.1. First Experiment

The first experiment was conducted to study the role of increasing P fertilization in the form of DAP (containing 28 mg Cd kg^{-1}) and CaP (Ca-phosphate, containing 0.09 mg Cd kg-^{1}) as well as inoculation of a mycorrhizal fungi on growth of plants and shoot Cd concentrations of rice plants. All experimental plants have received adequate amounts of N, K and S as mentioned above under 2.3 Fertilizers. Plants were also treated with 0.25 mg Zn kg^{-1} in the form of $ZnSO_4$ and 10 mg Fe kg^{-1} in the form of sequestrene. Then the soil was homogenously mixed. Inoculation of the soil with mycorrhizal inoculum (i.e., *Claroideoglomus etunicatum*, BEG 24) was realized by applying 500 spores in each pot as described by Yazici et al (2021). The first half of the 500 spores were thoroughly mixed in the soil, and the second half was put 3 cm below the seeds in a layer.

Each pot contained fourteen seeds, which were thinned to ten seedlings upon emergence. After 31 days of sowing, plants were harvested to determine the shoot dry matter production and nutrient accumulation. The dried plants were weighed, ground and analyzed for elemental compositions as shown below. The total uptake of Cd by shoots was also calculated by multiplying shoot dry weight with the shoot Cd concentrations.

2.5.2. Second Experiment

Since the results of the first experiment were highly interesting in terms of the impact of mycorrhizae on shoot Cd accumulation and the plants had lower Zn concentrations, a new experiment has been established to analyze same treatments mentioned in the 1st experiment at a higher Zn supply (i.e., 0.5 mg Zn kg^{-1} in form of $ZnSO_4$). In this experiment, plants were grown for 24 days and then analyzed for the shoot growth, shoot concentrations of Cd and other related elements and shoot Cd uptake.

2.5.3. Third Experiment

The aim with the 3rd experiment was to investigate the role of soil sterilization with and without a mycorrhizal inoculum on shoot Cd accumulation and root mycorrhizal activity of plants. Also in this experiment, plants were grown aerobically. Each plastic pot was supplied with 3.2 kg of unsterilized or sterilized soil. Soil sterilization was done by autoclaving the soil at 121°C for 2 hours to eradicate the native mycorrhizal components as well as other microorganisms. Iron treatment was made again in the form of sequestrene, but the rate was increased to 20 mg Fe kg^{-1} soil due to development Fe deficiency symptoms in the plants. Inoculation of sterilized and native soil with mycorrhizal inoculum was realized as mentioned above by using 500 spores (i.e., *Claroideoglomus etunicatum*, BEG 24) per pot.

Each pot contained fourteen seeds of rice, which were thinned to seven seedlings upon emergence. Plants were irrigated with deionized water when required during the whole growth period. The shoot harvest has been made when the plants were 32 days old. The harvested plants were dried to determine the shoot dry matter production and ground to analyze shoot concentrations of Cd and other related elements as indicated below.

2.6. Determination of Shoot Dry Matter Production

The details of the determination of shoot dry matter production and preparation of the samples for the element analysis were as following. The shoot samples of rice plants collected at the harvests were first washed in deionized water and dried in a forced oven at 65°C until a constant weight was achieved. Then, the measurement of shoot dry matter was made by weighing the dried samples. The dried samples were then subjected to grinding process in a very fine powder for the elemental analysis as described below.

2.7. Digestion and Element Analysis

Using an agate vibrating cup mill (Pulverisette 9; Fritsch GmbH; Germany), oven-dried shoot samples were first ground into fine powder. Then, the powdered samples were weighed at around 0.4 g and digested in a closed-vessel microwave system (MarsExpress, CEM Corp., Matthews, NC, USA) using 2 ml of 30% (v/v) H_2O_2 (Merck, EMSURE®, Darmstadt, Germany) and 5 ml of 65% (v/v) HNO_3 (Merck, EMSURE®, Darmstadt, Germany). After the digestion, the volume of the samples was increased to 20 mL using ultrapure water (18.2 MΩ).

After filtration, mineral element concentrations except Cd in the digested plant samples were measured using an inductively coupled plasma optical emission spectrometer (ICP-OES) (Vista-Pro Axial, Varian Pty Ltd., Mulgrave, Australia). As the Cd level of the samples were less than 30 µg Cd kg^{-1} dry weight, determination of Cd concentrations was done with utilizing ICP-MS (inductively coupled plasma mass spectrometry; Agilent 7700x).

2.8. Statistical Analysis

The statistical analyses were carried out by utilizing the Statistix 10 program. ANOVA (Analysis of variance) was used to determine the interactions of treatments and their effectiveness. The treatments with significant F test ($P<0.05$) results were subjected to Tukey's honestly significant difference (HSD) test to determine the significance of differences between treatment means.

3. RESULTS

3.1. First Experiment

3.1.1. Growth of Rice Plants

In the first experiment, the impacts of P fertilization and mycorrhizal inoculation were studied in rice plants that were grown aerobically on the native soil (Fig. 1). When the plants 31-days-old following the sowing time, plants were harvested for the determination of shoot dry matter production, shoot mineral nutrient and Cd concentrations. The plants without mycorrhizal inoculation and with medium P supply produced higher dry matter at both P application forms. Plants were slightly chlorotic with mycorrhizal inoculum which might be an indicator of iron (Fe) deficiency. The lower shoot Fe concentrations in mycorrhizae-treated plants (Table 3.8) support the idea that the plants with mycorrhizal treatments are probably under Fe deficiency stress. This is an interesting observation that is discussed below.

Figure 3.1. General photograph of the rice plants from the 1st experiment.

3.1.2. Shoot Dry Matter Production

Analysis of variance was carried out for the shoot dry weight of rice plants (Table 3.1). Mycorrhiza and P supply as well as mycorrhiza x P supply had significant effects on the shoot dry weight.

Table 3.1: Analysis of variance (ANOVA) of the effects of mycorrhiza, P source and P supply on the shoot dry weight of 31-days-old rice plants.

		Shoot Dry Weight	
Source of Variation	DF	P	$HSD_{0.05}$
Mycorrhiza (A)	1	<0.001	12
P Source (B)	1	0.673	*n.s*
P Supply (C)	2	<0.001	18
A x B	1	0.013	23
A x C	2	<0.001	32
B x C	2	0.143	*n.s*
A x B x C	2	0.744	*n.s*

The shoot dry matter production of the 31 days old rice plants was affected by the different rates of P treatments in the forms of DAP and CaP, as well as by the AMF application (Table 3.2). Adding the mycorrhizal inoculum tended to reduce the shoot dry matter production, especially at the lower P rates. There was a clear increase in shoot dry matter production of plants treated with AMF by increasing P supply in both forms.

Table 3.2: Effect of increasing P supply in the form of calcium phosphate (CaP) with virtually no Cd and DAP (containing 28 mg Cd kg^{-1}) on the shoot dry weight of 31-days-old rice plants grown in native soil with and without mycorrhizal inoculation and with a marginal Zn supply (0.25 mg Zn kg^{-1}) in aerobic conditions.

Mycorrhizae	P Source	P Supply (mg kg^{-1})	DW (mg $plant^{-1}$)
Uninoculated	CaP	20	128 ± 17
		60	175 ± 18
		180	130 ± 38
	DAP	20	158 ± 20
		60	179 ± 17
		180	136 ± 40
Inoculated	CaP	20	71 ± 13
		60	117 ± 5
		180	158 ± 34
	DAP	20	69 ± 4
		60	101 ± 13
		180	120 ± 25

3.1.3. Shoot Concentrations of Cd, Zn, P, and Shoot Cd Accumulation

Analysis of variance was carried out for 4 parameters including shoot Cd concentration and Cd content as well as shoot Zn and P concentrations of rice plants (Table 3.3). All treatments had significant effects on shoot Cd concentration and Cd accumulation (i.e., total Cd uptake). Shoot Zn concentration was markedly affected by mycorrhiza and P source. In the case of shoot P concentration, mycorrhiza, P supply and mycorrhiza x P source had significant effects.

Table 3.3: Analysis of variance (ANOVA) of the effects of mycorrhiza, P source and P supply on the shoot Cd concentration, total Cd uptake and shoot Zn and P concentrations of 31-days-old rice plants.

Source of Variation	DF	Cd		Cd Uptake		Zn		P	
		P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$
Mycorrhiza (A)	1	<0.001	0.85	<0.001	0.24	<0.001	0.65	<0.001	0.01
P Source (B)	1	<0.001	0.85	<0.001	0.24	<0.001	0.65	0.630	*n.s*
P Supply (C)	2	<0.001	1.26	<0.001	0.35	0.008	0.95	<0.001	0.02
A x B	1	<0.001	1.60	<0.001	0.44	0.714	*n.s*	<0.001	0.02
A x C	2	<0.001	2.19	0.017	0.61	0.018	1.66	0.071	*n.s*
B x C	2	<0.001	2.19	<0.001	0.61	0.078	*n.s*	0.287	*n.s*
A x B x C	2	<0.001	3.59	0.029	1.00	0.190	*n.s*	0.356	*n.s*

Under growth conditions without mycorrhizal inoculation, enhancing the P treatment caused a significant increase in the shoot Cd concentrations in all of the treatments, in particular in the plants treated with the DAP fertilizer (Table 3.4), which can be attributed to the existence of higher Cd in the DAP fertilizer. For example, increase in shoot Cd with the increasing DAP application was almost 6-fold. Similarly, also calculated total Cd uptake (i.e., Cd accumulation) showed a marked increase with the P application. When the soil was inoculated with mycorrhiza, both shoot Cd concentrations and shoot Cd accumulation showed particular decreases, especially in the case of lower P rates. The Cd uptake values for the low P conditions were even not delectable as presented in Table 3.4.

The shoot Zn concentration of the plants was slightly affected by the treatments (Table 3.4). However, a general decreasing trend in shoot Zn was found in the case of the CaP treatments. It was important to notice that the plants inoculated with mycorrhizae exhibited significant increases in shoot Zn. As expected, the increases in P supply had a substantial effect on the shoot P concentration of plants regardless of the other treatments (Table 3.4). In contrast, to shoot Zn, there was a decline in shoot P concentrations after mycorrhizal inoculation of plants at each P fertilizer form.

Table 3.4: Effect of increasing P supply in the form of calcium phosphate (CaP) with virtually no Cd and DAP (containing 28 mg Cd kg^{-1}) on the shoot Cd concentration, total Cd uptake and shoot Zn and P concentrations of 31-days-old rice plants grown in native soil with and without mycorrhizal inoculation and with a marginal Zn supply (0.25 mg Zn kg^{-1}) in aerobic conditions.

Mycorrhizae	P Source	P Supply (mg kg^{-1})	Cd (µg kg^{-1})	Cd Uptake (ng $plant^{-1}$)	Zn (mg kg^{-1})	P (%)
Uninoculated	CaP	20	2.03 ± 0.27	0.26 ± 0.06	7.31 ± 0.68	0.10 ± 0.03
		60	3.41 ± 0.51	0.60 ± 0.12	6.82 ± 0.39	0.19 ± 0.02
		180	5.05 ± 1.31	0.69 ± 0.35	5.72 ± 0.58	0.33 ± 0.03
	DAP	20	4.01 ± 0.93	0.64 ± 0.21	7.36 ± 0.63	0.12 ± 0.01
		60	11.83 ± 2.51	2.12 ± 0.53	7.81 ± 0.66	0.21 ± 0.02
		180	23.58 ± 4.41	3.27 ± 1.32	8.67 ± 0.73	0.35 ± 0.02
Inoculated	CaP	20	<d.l*	-	12.07 ± 2.44	0.06 ± 0.01
		60	1.56 ± 0.39	0.18 ± 0.04	12.57 ± 0.72	0.15 ± 0.03
		180	2.79 ± 0.32	0.44 ± 0.09	10.51 ± 0.95	0.28 ± 0.02
	DAP	20	<d.l*	-	12.43 ± 1.28	0.06 ± 0.00
		60	1.61 ± 0.47	0.17 ± 0.06	14.44 ± 1.99	0.13 ± 0.03
		180	9.03 ± 1.54	1.11 ± 0.37	11.57 ± 1.62	0.23 ± 0.03

* Lower than the detection limit of ICP-MS (<0.0000)

3.1.4. Concentrations of Other Elements

The plant shoot samples were also analyzed for potassium (K), calcium (Ca), magnesium (Mg), sulphur (S) and for the micronutrients copper (Cu), iron (Fe) and manganese (Mn) to understand to what extend the changes found for shoot Cd by the treatments are specific. Analysis of variance was carried out for the mentioned mineral nutrients and the results are presented in Table 3.5 and Table 3.6. Shoot K concentration was significantly

affected by mycorrhiza and mycorrhiza x P supply. The effect of the treatments except for mycorrhiza x P supply on shoot Mg concentration was the same as the shoot K concentration. Shoot Ca concentration was significantly affected only by P supply, while shoot S concentration was significantly affected by mycorrhiza, P supply, and mycorrhiza x P supply. Shoot concentration of Cu and Mn were found to be significantly affected by mycorrhiza, P source, P supply, P source x P supply, and the triple interaction. Finally, shoot Fe concentration was significantly affected only by mycorrhiza.

Table 3.5: Analysis of variance (ANOVA) of the effects of mycorrhiza, P source and P supply on the shoot concentrations of K, Ca, Mg, and S in 31-days-old rice plants.

		K		Ca		Mg		S	
Source of Variation	DF	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$
Mycorrhiza (A)	1	<0.001	0.06	0.595	*n.s*	<0.001	0.01	<0.001	0.01
P Source (B)	1	0.002	0.06	0.539	*n.s*	0.041	0.01	0.118	*n.s*
P Supply (C)	2	<0.001	0.10	<0.001	0.05	<0.001	0.02	<0.001	0.02
A x B	1	0.001	0.12	0.208	*n.s*	0.592	*n.s*	0.959	*n.s*
A x C	2	<0.001	0.17	0.663	*n.s*	0.002	0.04	<0.001	0.03
B x C	2	0.620	*n.s*	0.330	*n.s*	0.108	*n.s*	0.137	*n.s*
A x B x C	2	0.309	*n.s*	0.715	*n.s*	0.381	*n.s*	0.120	*n.s*

Table 3.6: Analysis of variance (ANOVA) of the effects of mycorrhiza, P source and P supply on the shoot concentrations of Cu, Fe, and Mn in 31-days-old rice plants.

		Cu		Fe		Mn	
Source of Variation	DF	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$
Mycorrhiza (A)	1	<0.001	0.79	<0.001	2.64	<0.001	2.66
P Source (B)	1	<0.001	0.79	0.026	2.64	<0.001	2.66
P Supply (C)	2	<0.001	1.16	0.044	3.9	<0.001	3.92
A x B	1	0.041	1.47	0.594	*n.s*	<0.001	4.98
A x C	2	0.196	*n.s*	0.050	6.76	0.187	*n.s*
B x C	2	<0.001	2.01	0.146	*n.s*	<0.001	6.8
A x B x C	2	<0.001	3.29	0.204	*n.s*	0.003	11.14

The shoot concentrations of Ca, K, Mg, and S were found to be similarly affected by the changes in the P treatment (Table 3.7). For the condition that AM fungi was applied to soil, there was a linear rise in the concentrations of the macro elements when the P supply was also increased. Same observations were also made for Mg and S concentrations of the plants that were not treated with mycorrhiza. However, although the lowest concentration of K was found at the low P supply, it was decreased by increasing P supply from medium to high P supply when no mycorrhiza was applied.

In case of the addition of the mycorrhizal inoculum, shoot Mg and K concentrations were generally decreased, while shoot Ca concentrations were not clearly affected. Similarly, AMF treatment did not affect also shoot S concentration except the highest P treatment (Table 3.7). The form of the P fertilization, whether DAP or CaP, did not result in different effects on shoot concentrations of Ca, and S. Also, K concentration was not affected by the P form when the soil was inoculated with mycorrhiza. However, K concentration was higher when plants were supplied with DAP fertilizer and soil was not treated with AMF.

Table 3.7: Effect of increasing P supply in the form of calcium phosphate (CaP) with virtually no Cd and DAP (containing 28 mg Cd kg^{-1}) on the shoot K, Ca, Mg and S concentrations of 31-days-old rice plants grown in native soil with and without mycorrhizal inoculation and with a marginal Zn supply (0.25 mg Zn kg^{-1}) in aerobic conditions.

Mycorrhizae	P Source	P Supply (mg kg^{-1})	K	Ca	Mg	S
			(%)			
Uninoculated	CaP	20	2.21 ± 0.07	0.59 ± 0.05	0.35 ± 0.03	0.26 ± 0.02
		60	2.57 ± 0.09	0.61 ± 0.04	0.43 ± 0.02	0.32 ± 0.03
		180	2.30 ± 0.22	0.74 ± 0.09	0.49 ± 0.03	0.36 ± 0.02
	DAP	20	2.49 ± 0.18	0.55 ± 0.04	0.34 ± 0.02	0.26 ± 0.01
		60	2.68 ± 0.14	0.61 ± 0.07	0.42 ± 0.03	0.34 ± 0.02
		180	2.59 ± 0.13	0.75 ± 0.11	0.45 ± 0.04	0.37 ± 0.01
Inoculated	CaP	20	1.98 ± 0.13	0.59 ± 0.04	0.26 ± 0.01	0.26 ± 0.02
		60	2.23 ± 0.08	0.62 ± 0.02	0.32 ± 0.02	0.34 ± 0.05
		180	2.53 ± 0.09	0.70 ± 0.07	0.43 ± 0.03	0.47 ± 0.02
	DAP	20	1.97 ± 0.09	0.60 ± 0.03	0.27 ± 0.02	0.25 ± 0.01
		60	2.25 ± 0.09	0.62 ± 0.02	0.30 ± 0.02	0.33 ± 0.03
		180	2.51 ± 0.11	0.77 ± 0.08	0.41 ± 0.03	0.52 ± 0.03

The shoot concentrations of Cu, Mn and Fe were also affected by the treatments. Increasing application of P, regardless of the mycorrhizal inoculation, linearly increased the concentrations of Cu, and Mn, while no effect was found on Fe concentrations (Table 3.8). The concentration of Cu, Mn and Fe showed very clear decreases when the soil was treated with AMF in both forms of the P fertilization. The decreases in shoot Fe concentrations by adding AMF were interesting and resulted in expression of leaf chlorosis.

Table 3.8: Effect of increasing P supply in the form of calcium phosphate (CaP) with virtually no Cd and DAP (containing 28 mg Cd kg^{-1}) on the shoot Cu, Fe, and Mn concentrations of 31-days-old rice plants grown in native soil with and without mycorrhizal inoculation and with a marginal Zn supply (0.25 mg Zn kg^{-1}) in aerobic conditions.

Mycorrhizae	P Source	P Supply (mg kg^{-1})	Cu	Fe (mg kg^{-1})	Mn
Uninoculated	CaP	20	15 ± 1.4	45 ± 1	65 ± 4
		60	20 ± 1.4	51 ± 6	73 ± 5
		180	27 ± 3.5	59 ± 8	66 ± 6
	DAP	20	15 ± 0.5	49 ± 8	68 ± 3
		60	19 ± 0.9	48 ± 6	81 ± 6
		180	20 ± 0.5	51 ± 4	96 ± 7
Inoculated	CaP	20	9 ± 0.6	38 ± 2	34 ± 3
		60	12 ± 0.9	38 ± 4	41 ± 5
		180	17 ± 1.3	38 ± 2	47 ± 4
	DAP	20	8 ± 0.4	35 ± 3	34 ± 4
		60	11 ± 0.8	33 ± 1	39 ± 5
		180	16 ± 2.1	35 ± 5	52 ± 6

The changes in shoot concentrations of Cu, Mn and Fe reported were also very similar to the changes found with the total uptake of Cu, Mn, and Fe (Figures 3.2; 3.3; 3.4). The total uptake of Fe and Mn was higher in the plants supplied no mycorrhiza and largely reduced by adding mycorrhizae. (Figures 3.2 and 3.3).

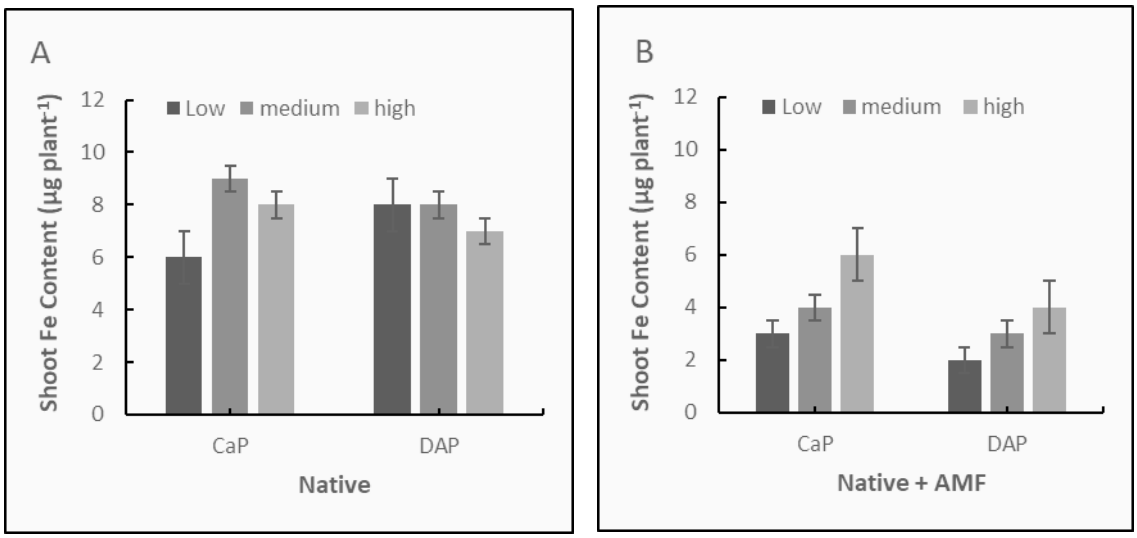

Figure 3.2: Fe uptake of the 31-days-old rice plants grown with low, medium, and high P fertilization in the form of CaP and DAP in native soil (A), and in native soil treated with mycorrhizal inoculum (B).

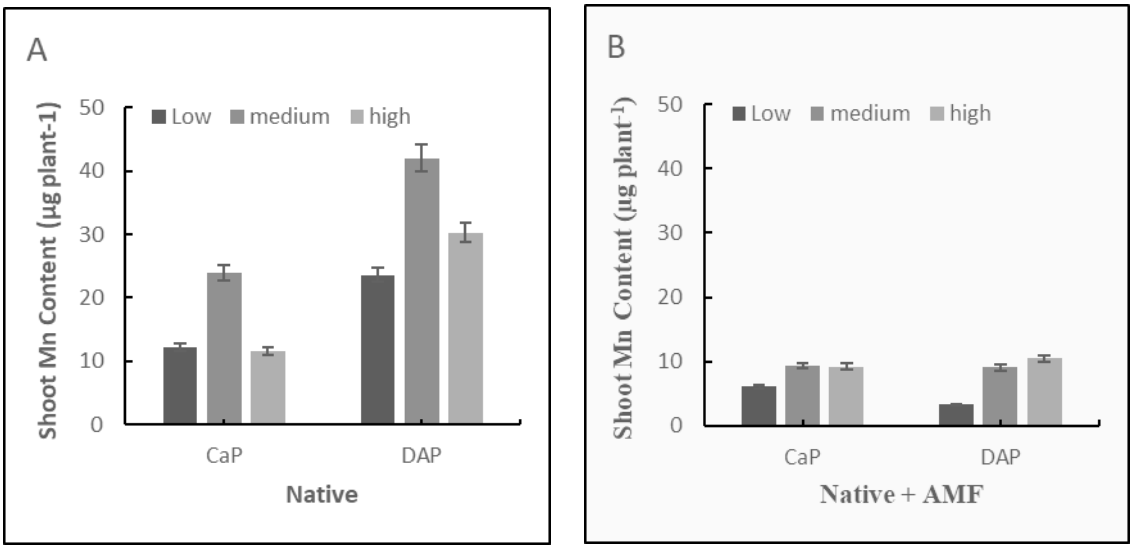

Figure 3.3: Mn uptake of the 31-days-old rice plants grown with low, medium, and high P fertilization in the form of CaP and DAP in native soil (A), and in native soil treated with mycorrhizal inoculum (B).

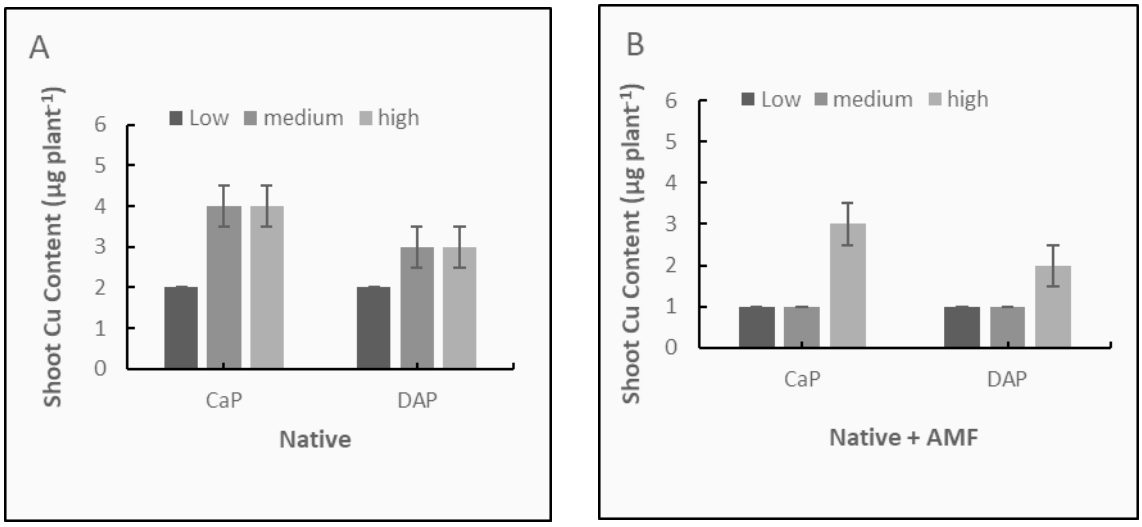

Figure 3.4: Cu uptake of the 31-days-old rice plants grown with low, medium, and high P fertilization in the form of CaP and DAP in native soil (A), and in native soil treated with mycorrhizal inoculum (B).

3.2. Second Experiment

3.2.1. Growth of Rice Plants

In the first experiment, plants contained low amounts of Zn in shoot (around 6 mg kg^{-1}) which might be associated with, at least, a marginal Zn deficiency stress, especially in case of the no mycorrhizae addition (Table 3.4). Since the effects of mycorrhizae addition on Cd levels of the plants were impressive, the experiment has been repeated by using higher Zn application rate (i.e., 0.5 mg kg Zn).

The plants were grown aerobically under same treatments with the P applications and mycorrhizal inoculum for 24 days and then harvested for the determination of shoot dry matter production, shoot nutrient concentrations and Cd. When the rice plants were supplied with Zn at the rate of 0.5 mg kg^{-1}, plants had no sign of Zn deficiency symptoms and looked healthier. However, when plants were inoculated with mycorrhiza, their leaves were slightly yellowish which was also in good agreement with reduced Fe concentration of plants with mycorrhizal inoculation (Table 3.6).

3.2.2. Shoot Dry Matter Production

Analysis of variance was carried out for the shoot dry weight of rice plants (Table 3.9). The results showed that shoot dry weight was significantly affected by mycorrhiza and P source.

Table 3.9: Analysis of variance (ANOVA) of the effects of mycorrhiza, P source and P supply on the shoot dry weight of 24-days-old rice plants.

		Shoot Dry Weight	
Source of Variation	DF	P	$HSD_{0.05}$
Mycorrhiza (A)	1	<0.001	3.7
P Source (B)	1	<0.001	3.7
P Supply (C)	2	0.033	5.5
A x B	1	0.566	*n.s*
A x C	2	0.009	9.5
B x C	2	0.085	*n.s*
A x B x C	2	0.025	15.6

The shoot dry matter production of the 24 days old rice plants was slightly affected by the different P treatments in both forms. (Table 3.10). However, mycorrhizal inoculation treatment tended to decrease shoot dry weight. The differences in shoot dry matter production among the treatments were rather minimal.

Table 3.10: Effect of increasing P supply in the form of calcium phosphate (CaP) with virtually no Cd and DAP (containing 28 mg Cd kg^{-1}) on the shoot dry weight of 24-days-old rice plants grown in native soil with and without mycorrhizal inoculation and with a marginal Zn supply (0.5 mg Zn kg^{-1}) in aerobic conditions.

Mycorrhizae	P Source	P Supply (mg kg^{-1})	DW (mg $plant^{-1}$)
Uninoculated	CaP	20	53 ± 10
		60	62 ± 7
		180	53 ± 12
	DAP	20	53 ± 10
		60	50 ± 5
		180	47 ± 7
Inoculated	CaP	20	41 ± 4
		60	47 ± 2
		180	60 ± 8
	DAP	20	36 ± 7
		60	47 ± 4
		180	41 ± 4

3.2.3. Shoot Concentrations of Cd, Zn, P, and Shoot Cd Accumulation

The shoot samples were analyzed for shoot concentrations of Cd, Zn, P, and shoot Cd accumulation. Mycorrhiza, P source, P supply, and the interaction between P source x P supply markedly affected the shoot Cd concentration and Cd accumulation. In addition, Cd uptake was also significantly affected by the triple interaction of mycorrhiza x P source x P supply. Lastly, while shoot Zn concentration was significantly affected by mycorrhiza and P supply, shoot P concentration was only significantly affected by P supply.

Table 3.11: Analysis of variance (ANOVA) of the effects of mycorrhiza, P source and P supply on the shoot Cd concentration, total Cd uptake and shoot Zn and P concentrations of 24-days-old rice plants.

		Cd		Cd Uptake		Zn		P	
Source of Variation	DF	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$
Mycorrhiza (A)	1	<0.001	1.09	<0.001	0.05	<0.001	1.81	0.018	0.03
P Source (B)	1	<0.001	1.09	<0.001	0.05	0.758	*n.s*	0.051	*n.s*
P Supply (C)	2	<0.001	1.61	<0.001	0.07	<0.001	2.67	<0.001	0.04
A x B	1	0.011	2.05	0.004	0.09	0.113	*n.s*	0.033	0.05
A x C	2	0.372	*n.s*	0.175	*n.s*	0.350	*n.s*	0.468	*n.s*
B x C	2	<0.001	2.80	<0.001	0.13	0.225	*n.s*	0.075	*n.s*
A x B x C	2	0.052	*n.s*	<0.001	0.21	0.862	*n.s*	0.305	*n.s*

Similar to the results obtained in the 1st experiment, increasing P supply resulted in significant increases in the shoot Cd concentrations which was more apparent in the plants treated with DAP (Table 3.12), probably because of higher Cd content of the DAP fertilizer. When the soil was inoculated with mycorrhiza, Cd uptake of plants was drastically decreased in all conditions, indicating a key role of AMF in reducing Cd transport from roots or soils in the shoots. Since the shoot dry weight of the plants was not clearly affected from the treatments, the results of the shoot Cd accumulation (i.e., total Cd uptake) were similar to the shoot Cd concentration results (Table 3.12).

Increasing Zn supply to the plants in this 2nd experiment resulted in higher shoot Zn concentrations (almost up to 30 mg Zn kg^{-1}) compared to the shoot Zn levels in the 1st experiment (Table 3.12). There was a decreasing trend in shoot Zn with the P application rate regardless of the mycorrhizal inoculation in both P forms. Also in this experiment, mycorrhizal inoculation resulted in clear increases in shoot Zn concentrations in both P forms. As expected, enhancement in P supply increased shoot P concentrations in all treatments, while the treatment of the soil with mycorrhizal inoculum had a reducing effect on shoot P, mainly at lower P rates (Table 3.12).

Table 3.12: Effect of increasing P supply in the form of calcium phosphate (CaP) with virtually no Cd and DAP (containing 28 mg Cd kg^{-1}) on the shoot Cd concentration, total Cd uptake and shoot Zn and P concentrations of 24-days-old rice plants grown in native soil with and without mycorrhizal inoculation and with a marginal Zn supply (0.5 mg Zn kg^{-1}) in aerobic conditions.

Mycorrhizae	P Source	P Supply (mg kg^{-1})	Cd (µg kg^{-1})	Cd uptake (ng $plant^{-1}$)	Zn (mg kg^{-1})	P (%)
Uninoculated	CaP	20	4.47 ± 0.91	0.27 ± 0.04	21.80 ± 2.84	0.24 ± 0.05
		60	6.59 ± 0.98	0.41 ± 0.08	18.24 ± 1.84	0.33 ± 0.05
		180	6.35 ± 1.59	0.27 ± 0.02	16.72 ± 2.02	0.44 ± 0.11
	DAP	20	8.35 ± 1.72	0.44 ± 0.10	22.24 ± 3.40	0.20 ± 0.04
		60	8.30 ± 2.52	0.35 ± 0.03	18.60 ± 5.14	0.24 ± 0.05
		180	19.46 ± 4.93	0.91 ± 0.23	19.44 ± 5.04	0.40 ± 0.04
Inoculated	CaP	20	1.42 ± 0.40	0.07 ± 0.01	29.75 ± 2.14	0.19 ± 0.04
		60	3.42 ± 1.01	0.16 ± 0.05	26.04 ± 2.10	0.27 ± 0.05
		180	4.94 ± 0.68	0.30 ± 0.06	20.77 ± 3.73	0.37 ± 0.04
	DAP	20	2.25 ± 0.17	0.08 ± 0.01	26.41 ± 3.04	0.14 ± 0.03
		60	5.70 ± 1.63	0.26 ± 0.06	23.00 ± 2.55	0.24 ± 0.04
		180	11.94 ± 2.76	0.49 ± 0.14	21.95 ± 4.12	0.44 ± 0.06

3.2.4. Concentrations of Other Elements

Also in this experiment, shoot concentrations of Ca, Mg, S, Cu, Fe, and Mn were measured. Analysis of variance was carried out for these 7 elements and the results are presented in Table 3.13 and 3.14. Mycorrhiza and P supply significantly affected the shoot concentrations of K, Mg, S and Cu. Shoot Mg concentration was also significantly affected by P source x P supply. In the case of shoot Ca concentration, P source and mycorrhiza x P source had significant effects. Shoot Mn concentration was markedly affected by mycorrhiza and P source x P supply. Finally, shoot Fe concentration was only affected by mycorrhiza.

Table 3.13: Analysis of variance (ANOVA) of the effects of mycorrhiza, P source and P supply on the shoot K, Ca, Mg, and S concentrations of 24-days-old rice plants.

		K		Ca		Mg		S	
Source of Variation	DF	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$
Mycorrhiza (A)	1	<0.001	0.1	0.562	*n.s*	<0.001	0.01	<0.001	0.02
P Source (B)	1	0.762	*n.s*	<0.001	0.03	0.027	0.01	0.027	0.02
P Supply (C)	2	<0.001	0.15	0.007	0.04	<0.001	0.02	<0.001	0.03
A x B	1	0.301	*n.s*	<0.001	0.06	0.004	0.02	0.084	*n.s*
A x C	2	0.004	0.26	0.610	*n.s*	0.518	*n.s*	0.173	*n.s*
B x C	2	0.003	0.26	0.002	0.08	<0.001	0.03	0.084	*n.s*
A x B x C	2	0.279	*n.s*	0.604	*n.s*	0.023	0.05	0.230	*n.s*

Table 3.14: Analysis of variance (ANOVA) of the effects of mycorrhiza, P source and P supply on the shoot Cu, Fe, and Mn concentrations of 24-days-old rice plants.

Source of Variation	DF	Cu		Fe		Mn	
		P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$
Mycorrhiza (A)	1	<0.001	0.70	<0.001	2.99	<0.001	5.7
P Source (B)	1	0.370	*n.s*	0.022	2.99	0.004	5.7
P Supply (C)	2	<0.001	1.04	0.121	*n.s*	0.052	*n.s*
A x B	1	0.971	*n.s*	0.588	*n.s*	0.696	*n.s*
A x C	2	0.096	*n.s*	0.070	*n.s*	0.048	14.6
B x C	2	0.011	1.80	0.123	*n.s*	<0.001	14.6
A x B x C	2	0.018	2.95	0.002	12.55	0.635	*n.s*

Shoot concentrations of Ca, K, Mg, and S were similarly affected by the P treatments (Table 3.15). The differences found among the treatments were generally minimal. However, increasing P application generally resulted in a slight increasing effect on the shoot concentrations of these elements.

At each mycorrhizal inoculum, increasing P application did not result in a consistent effect on shoot concentrations of Cu, Fe and Mn (Table 3.16). However, treatment of the soils with mycorrhizal inoculum caused decreases in shoot concentrations of Cu, Fe and Mn, especially in case of Mn and Fe.

Table 3.15: Effect of increasing P supply in the form of calcium phosphate (CaP) with virtually no Cd and DAP (containing 28 mg Cd kg^{-1}) on the shoot K, Ca, Mg, and S concentrations of 24-days-old rice plants grown in native soil with and without mycorrhizal inoculation and with a marginal Zn supply (0.5 mg Zn kg^{-1}) in aerobic conditions.

Mycorrhizae	P Source	P Supply (mg kg^{-1})	K	Ca	Mg	S
			(%)			
Uninoculated	CaP	20	2.71 ± 0.21	0.66 ± 0.04	0.35 ± 0.01	0.37 ± 0.03
		60	2.85 ± 0.14	0.71 ± 0.04	0.41 ± 0.02	0.40 ± 0.03
		180	2.69 ± 0.35	0.66 ± 0.05	0.41 ± 0.03	0.40 ± 0.04
	DAP	20	2.63 ± 0.18	0.72 ± 0.04	0.35 ± 0.02	0.35 ± 0.03
		60	2.57 ± 0.24	0.78 ± 0.07	0.41 ± 0.03	0.36 ± 0.03
		180	2.85 ± 0.13	0.87 ± 0.09	0.49 ± 0.03	0.44 ± 0.02
Inoculated	CaP	20	2.74 ± 0.16	0.72 ± 0.07	0.33 ± 0.02	0.45 ± 0.03
		60	2.95 ± 0.24	0.77 ± 0.05	0.38 ± 0.02	0.54 ± 0.05
		180	3.01 ± 0.06	0.72 ± 0.05	0.41 ± 0.01	0.53 ± 0.05
	DAP	20	2.54 ± 0.22	0.70 ± 0.03	0.32 ± 0.01	0.44 ± 0.04
		60	2.93 ± 0.24	0.74 ± 0.06	0.37 ± 0.02	0.48 ± 0.04
		180	3.35 ± 0.07	0.78 ± 0.06	0.42 ± 0.02	0.49 ± 0.02

Table 3.16: Effect of increasing P supply in the form of calcium phosphate (CaP) with virtually no Cd and DAP (containing 28 mg Cd kg^{-1}) on the shoot Cu, Fe, and Mn concentrations of 24-days-old rice plants grown in native soil with and without mycorrhizal inoculation and with a marginal Zn supply (0.5 mg Zn kg^{-1}) in aerobic conditions.

Mycorrhizae	P Source	P Supply (mg kg^{-1})	Cu	Fe (mg kg^{-1})	Mn
Uninoculated	CaP	20	18 ± 1.0	72 ± 6	105 ± 4
		60	22 ± 0.8	62 ± 8	107 ± 4
		180	21 ± 1.7	58 ± 7	95 ± 10
	DAP	20	17 ± 0.8	59 ± 4	95 ± 8
		60	21 ± 2.9	58 ± 7	106 ± 13
		180	24 ± 0.5	66 ± 8	129 ± 16
Inoculated	CaP	20	17 ± 1.0	45 ± 3	84 ± 11
		60	18 ± 0.4	49 ± 6	64 ± 10
		180	19 ± 0.4	44 ± 6	57 ± 8
	DAP	20	16 ± 1.2	45 ± 2	69 ± 11
		60	19 ± 2.0	44 ± 3	68 ± 9
		180	20 ± 1.2	37 ± 2	98 ± 16

3.3. Third Experiment

3.3.1. Growth of Rice Plants

In order to further analyse the effects of AMF on Cd accumulation in plants, the 3rd experiment has been conducted in soils with and without soil sterilization to eliminate mycorrhizal activity and structures in the soil (Figure 3.5). Soil sterilization was realized by autoclaving the soil at 121°C for 2 h. Additionally, the experiment was realized with and without mycorrhizal inoculation and by increasing P fertilization from 20 mg P kg^{-1} to 180 mg P kg^{-1}.

Plants were grown until 32 days after sowing, and then harvested for the determination of shoot dry matter production and shoot nutrient concentrations. At the highest P rate plant growth showed a decreasing trend, especially in case of soil sterilization. When plants were around 25 days old, the color of leaves turned yellow, possibly due to Fe deficiency, and an additional 10 ppm Fe was applied to all in the form of sequestrene. Generally, even under natural conditions, rice plants initially show chlorosis problem, but the reason is not well understood and possibly related to reduced Fe acquisition capacity of plants.

In case of the highest P fertilization, plants develop also slight necrotic patches on the leaves which have been ascribed to expression of Zn deficiency due to low amounts of Zn in leaf tissues (see below). These Zn deficiency symptoms were more apparent when soil was sterilized; but less when the plants were inoculated with mycorrhizae.

Figure 3.5: General photograph of the rice plants from the 3rd experiment.

3.3.2. Shoot Dry Matter Production

Analysis of variance was carried out for the shoot dry weight of rice plants and the results presented in Table 3.17. The shoot dry matter production of the 32-day-old rice plants was significantly affected by increasing rates of P treatment, especially when the soil was sterilized and not inoculated with mycorrhizae (Table 3.18). Plants with soil sterilization and without mycorrhizal inoculation had lowest Zn in shoot tissues (Table 3.20) and therefore the reduced growth is probably related to Zn deficiency stress. Plants had better growth under low P rates and with mycorrhiza inoculation.

Table 3.17: Analysis of variance (ANOVA) of the effects of soil sterilization, mycorrhiza, and P supply on the shoot dry weight of the 32-days-old rice plants.

		Shoot Dry Weight	
Source of Variation	DF	P	$HSD_{0.05}$
Soil (A)	1	0.994	*n.s*
Mycorrhiza (B)	1	0.005	17
P Supply (C)	2	0.002	25
A x B	1	0.003	32
A x C	2	0.006	44
B x C	2	0.348	*n.s*
A x B x C	2	0.233	*n.s*

The shoot biomass of the rice plants grown on sterilized soil with mycorrhizal inoculum was less than the plants grown on native soil with AMF application (Table 3.18). This difference could be attributed to the removal of beneficial microbes (i.e., AMF) from the soil with sterilization.

Table 3.18: Effect of increasing P supply in the form of DAP (containing 28 mg Cd kg^{-1}) on the shoot dry weight of 32-days-old rice plants grown in native (unsterilized) and sterilized soil, with and without mycorrhizal inoculation and with a marginal Zn supply (0.1 mg Zn kg^{-1}) in aerobic conditions.

Soil	P Supply (mg kg^{-1})	DW (mg $plant^{-1}$)
Native	20	209 ± 46
	60	132 ± 15
	180	160 ± 19
Native + Mycorrhizae	20	199 ± 46
	60	216 ± 43
	180	167 ± 38
Sterilized	20	197 ± 37
	60	171 ± 34
	180	138 ± 26
Sterilized + Mycorrhizae	20	142 ± 19
	60	162 ± 26
	180	123 ± 11

3.3.3. Shoot Concentrations of Cd, Zn, P, and Shoot Cd Accumulation

Analysis of variance was carried out for shoot Cd concentration and Cd content as well as shoot Zn and P concentrations of rice plants (Table 3.19). All sources of variation except for interaction of mycorrhiza x P supply, soil x P supply, and the triple interaction had significant effects on shoot Cd concentration and Cd content. Shoot Zn concentration was markedly affected by soil, mycorrhiza, P supply, and soil x P supply. In the case of shoot P concentration, P supply and soil x P supply had significant effects.

Table 3.19: Analysis of variance (ANOVA) of the effects of soil sterilization, mycorrhiza, and P supply on the shoot Cd concentration, Cd content and shoot Zn and P concentrations of 32-days-old rice plants.

Source of Variation	DF	Cd		Cd Uptake		Zn		P	
		P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$
Soil (A)	1	<0.001	1.5	<0.001	0.36	<0.001	0.38	0.180	*n.s*
Mycorrhiza (B)	1	<0.001	1.5	<0.001	0.36	<0.001	0.38	0.483	*n.s*
P Supply (C)	2	<0.001	2.2	<0.001	0.53	<0.001	0.56	<0.001	0.02
A x B	1	<0.001	2.8	<0.001	0.67	0.768	*n.s*	0.031	0.02
A x C	2	<0.001	3.8	0.161	*n.s*	<0.001	0.96	<0.001	0.03
B x C	2	0.001	3.8	0.552	*n.s*	0.632	*n.s*	0.093	*n.s*
A x B x C	2	0.244	*n.s*	0.998	*n.s*	0.946	*n.s*	0.097	*n.s*

When P supply to plants was increased, shoot Cd concentrations in all treatments were dramatically increased (Table 3.20). Similarly, also shoot Cd accumulation (total Cd uptake) showed distinct increases with P fertilization. Increases in shoot Cd were more noticeable in the plants that were not treated with mycorrhizal inoculum. Additionally, plants grown in the sterilized soil had also much higher Cd concentration in shoots than the plants grown in native soil. Generally, soil sterilization almost doubled shoot Cd concentrations which showed up to 5-fold decreases (mainly at lower P rates) when plants were treated with AMF. These results indicate an impressive role of AMF in reducing Cd accumulation in plants.

As shown in Table 3.20, plant shoot Zn concentrations showed a decrease when the P rate was highest; but in the native soil without mycorrhizal treatment, P treatments had no effect on shoot Zn. Soil sterilization had a clear reducing effect on shoot Zn and this effect was more pronounced when the soil was not inoculated with mycorrhizae. Indeed, adding the mycorrhizal inoculum in the soil resulted in very clear increases in shoot Zn concentrations both in native soil and sterilized soil, more evidently at lower P rates (Table 3.20). The P supply rates significantly increased the shoot P concentration of plants regardless of the other applications (Table 3.20). Mycorrhizal treatment enhanced shoot P concentrations at the lowest P treatment but remained less effective at higher P rates. Generally, soil sterilization caused minimal changes in shoot P concentrations.

Table 3.20: Effect of increasing P supply in the form of DAP (containing 28 mg Cd kg^{-1}) on the shoot Cd concentration, total Cd uptake and shoot Zn and P concentrations of 32-days-old rice plants grown in native (unsterilized) and sterilized soil, with and without mycorrhizal inoculation and with a marginal Zn supply (0.1 mg Zn kg^{-1}) in aerobic conditions.

Soil	P Supply ($mg\ kg^{-1}$)	Cd ($\mu g\ kg^{-1}$)	Cd uptake ($ng\ plant^{-1}$)	Zn ($mg\ kg^{-1}$)	P (%)
Native	20	6.32 ± 1.35	1.29 ± 0.23	5.00 ± 0.34	0.08 ± 0.01
	60	13.42 ± 3.94	1.75 ± 0.46	4.73 ± 0.50	0.17 ± 0.02
	180	20.61 ± 2.60	3.33 ± 0.74	5.08 ± 0.59	0.27 ± 0.02
Native + mycorrhizae	20	3.78 ± 0.27	0.75 ± 0.18	8.50 ± 1.35	0.15 ± 0.03
	60	3.33 ± 0.46	0.71 ± 0.11	7.15 ± 1.12	0.16 ± 0.02
	180	11.79 ± 2.82	1.95 ± 0.53	5.73 ± 0.41	0.19 ± 0.02
Sterilized	20	15.45 ± 1.33	3.04 ± 0.60	4.33 ± 0.54	0.08 ± 0.02
	60	23.13 ± 3.60	3.98 ± 1.12	3.90 ± 0.29	0.16 ± 0.02
	180	38.54 ± 5.90	5.40 ± 1.54	3.84 ± 0.16	0.26 ± 0.03
Sterilized + mycorrhizae	20	3.30 ± 0.83	0.46 ± 0.08	7.82 ± 1.12	0.13 ± 0.03
	60	5.35 ± 0.79	0.86 ± 0.18	6.39 ± 0.69	0.18 ± 0.02
	180	16.03 ± 3.30	1.99 ± 0.55	4.77 ± 0.36	0.24 ± 0.04

3.3.4. Concentrations of Other Elements

Analysis of variance was carried out for the shoot concentrations of K, Ca, Mg, S, Cu, Fe and Mn of rice plants (Table 3.21 and 3.22). Shoot K concentration was significantly affected by soil, mycorrhiza, soil x mycorrhiza, and mycorrhiza x P supply (Table 3.21). Soil, P supply, and soil x P supply significantly affected the shoot Ca concentration. In the case of shoot Mg concentration, soil, mycorrhiza, P supply, and soil x mycorrhiza had significant effects. Finally, shoot S concentration was significantly affected by P supply, mycorrhiza x P supply, and the triple interaction of soil, mycorrhiza, and P supply.

Soil and mycorrhiza significantly affected the shoot concentrations of Cu and Mn (Table 3.22) while P supply significantly affected the shoot concentrations of Cu and Mn. Soil x mycorrhiza interaction resulted in significant effects on the Mn concentrations, while soil x P supply interaction significantly affected Cu concentrations. In the case of mycorrhiza x P supply interaction, the effect turned out to be significant for Mn concentrations.

Table 3.21: Analysis of variance (ANOVA) of the effects of soil sterilization, mycorrhiza, and P supply on the shoot concentrations of K, Ca, Mg, and S in 32-days-old rice plants.

		K		Ca		Mg		S	
Source of Variation	DF	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$
Soil (A)	1	<0.001	0.07	<0.001	0.03	<0.001	0.02	0.002	0.007
Mycorrhiza (B)	1	<0.001	0.07	0.014	0.03	<0.001	0.02	0.298	*n.s*
P Supply (C)	2	0.260	*n.s*	<0.001	0.05	<0.001	0.03	<0.001	0.01
A x B	1	<0.001	0.12	0.202	*n.s*	<0.001	0.04	0.003	0.01
A x C	2	0.001	0.17	<0.001	0.09	0.004	0.05	<0.001	0.02
B x C	2	<0.001	0.17	0.017	0.09	0.000	0.05	0.094	*n.s*
A x B x C	2	0.052	*n.s*	0.440	*n.s*	0.072	*n.s*	<0.001	0.03

Table 3.22: Analysis of variance (ANOVA) of the effects of soil sterilization, mycorrhiza, and P supply on the shoot concentrations of Cu, Fe, and Mn in 32-days-old rice plants.

		Cu		Fe		Mn	
Source of Variation	DF	P	$HSD_{0.05}$	P	$HSD_{0.05}$	P	$HSD_{0.05}$
Soil (A)	1	<0.001	1.29	0.002	3.55	<0.001	22
Mycorrhiza (B)	1	<0.001	1.29	0.021	3.55	<0.001	22
P Supply (C)	2	<0.001	1.90	0.043	5.23	<0.001	32
A x B	1	0.138	*n.s*	0.003	6.65	<0.001	40
A x C	2	<0.001	3.30	0.067	*n.s*	0.058	*n.s*
B x C	2	0.005	3.30	0.197	*n.s*	<0.001	55
A x B x C	2	0.220	*n.s*	0.380	*n.s*	0.222	*n.s*

The concentrations of Ca, Mg, and S were found to be similarly modified by varied P supply unlike K concentrations (Table 3.23). Under conditions where there was an application of AM fungi to native or sterilized soil, there was a linear increase in the concentrations of these macro elements when the P supply was also increased. However, while the lowest concentrations were observed on low P supply, concentrations of these nutrients were not affected by the change from adequate P to high P for the condition where no mycorrhiza was added to native soil.

Also, the addition of mycorrhizal inoculum caused some alterations in the concentrations of macronutrients (Table 3.23). When plants were grown in sterilized soil, Mg concentrations were lower when compared to plants that were not treated with AMF. Same results were obtained at almost all treatments for Ca concentrations of the plants grown in both soils. However, these effects were the opposite for K concentrations. The addition of AMF increased K concentrations of plants grown in sterilized soil. Nevertheless, sterilization decreased K concentrations when compared to plants grown in native soil regardless of the AMF. Although Mg concentrations of plants with no AMF treatment increased when the soil was sterilized, there was no significant difference when the soil was treated with AMF. Furthermore, Ca concentration was enhanced by soil

sterilization only when treated with high P. However, S concentration wasn't affected by soil conditions whether mycorrhiza was added or not.

Table 3.23: Effect of increasing P supply in the form of DAP (containing 28 mg Cd kg^{-1}) on the shoot K, Ca, Mg, and S concentrations of 32-days-old rice plants grown in native (unsterilized) and sterilized soil, with and without mycorrhizal inoculation and with a marginal Zn supply (0.1 mg Zn kg^{-1}) in aerobic conditions.

Soil	P Supply (mg kg^{-1})	K	Ca	Mg	S
		(%)			
Native	20	2.14 ± 0.11	0.57 ± 0.06	0.37 ± 0.04	0.21 ± 0.01
	60	2.09 ± 0.08	0.80 ± 0.04	0.50 ± 0.02	0.30 ± 0.01
	180	2.42 ± 0.19	0.66 ± 0.07	0.46 ± 0.04	0.29 ± 0.01
Native + mycorrhizae	20	2.26 ± 0.08	0.45 ± 0.05	0.35 ± 0.03	0.25 ± 0.02
	60	2.27 ± 0.10	0.53 ± 0.08	0.41 ± 0.03	0.26 ± 0.01
	180	2.18 ± 0.17	0.64 ± 0.10	0.51 ± 0.05	0.30 ± 0.02
Sterilized	20	1.87 ± 0.17	0.59 ± 0.06	0.43 ± 0.05	0.21 ± 0.02
	60	1.70 ± 0.12	0.76 ± 0.06	0.61 ± 0.05	0.27 ± 0.01
	180	1.62 ± 0.14	0.75 ± 0.09	0.69 ± 0.06	0.27 ± 0.02
Sterilized + mycorrhizae	20	2.11 ± 0.05	0.45 ± 0.05	0.32 ± 0.03	0.23 ± 0.01
	60	2.20 ± 0.16	0.58 ± 0.07	0.43 ± 0.03	0.27 ± 0.01
	180	1.90 ± 0.08	0.79 ± 0.04	0.54 ± 0.04	0.33 ± 0.02

The changes in shoot Cu, Fe and Mn are presented in Table 3.24. While increasing P supply, whether with the application of AMF or not, linearly increased the Mn concentration, but it had no significant effect on shoot Fe concentration of plants (Table 3.24). Effects of increasing P supply on Cu concentrations were not homogenous. Autoclaving the soil generally increased the concentrations of Cu and Mn. Shoot Mn concentrations were remarkably changed by the soil sterilization. As average, shoot Mn concentrations of plants grown in native soil was about 74 mg kg^{-1} and sterilization of the soil increased shoot Mn concentration to 487 mg kg^{-1}. These increases were markedly alleviated by inoculation of the soil with mycorrhizae (Table 3.24). Interestingly, soil sterilization did not clearly affect shoot Fe concentrations. However, the addition of mycorrhiza decreased the shoot Fe concentration in native soil, and Cu concentrations in both soils.

Table 3.24: Effect of increasing P supply in the form of DAP (containing 28 mg Cd kg^{-1}) on the shoot Cu, Fe, and Mn concentrations of 32-days-old rice plants grown in native (unsterilized) and sterilized soil, with and without mycorrhizal inoculation and with a marginal Zn supply (0.1 mg Zn kg^{-1}) in aerobic conditions.

Soil	P Supply ($mg\ kg^{-1}$)	Cu	Fe ($mg\ kg^{-1}$)	Mn
Native	20	18 ± 2.5	59 ± 9	59 ± 8
	60	30 ± 1.9	66 ± 8	81 ± 8
	180	26 ± 1.3	58 ± 5	82 ± 6
Native + mycorrhizae	20	15 ± 1.2	46 ± 2	30 ± 6
	60	18 ± 3.6	54 ± 12	34 ± 3
	180	23 ± 2.8	49 ± 3	41 ± 8
Sterilized	20	26 ± 2.4	53 ± 8	383 ± 33
	60	40 ± 3.5	51 ± 1	512 ± 78
	180	36 ± 3.6	51 ± 8	567 ± 103
Sterilized + mycorrhizae	20	17 ± 1.2	43 ± 3	79 ± 14
	60	27 ± 2.4	54 ± 7	107 ± 14
	180	34 ± 3.3	56 ± 4	173 ± 38

4. DISCUSSION

In this thesis project, P fertilizers differing in Cd concentrations and the addition of mycorrhizal inoculum were studied for their impacts on shoot Cd concentration of rice plants grown in soils with and without sterilization. The results obtained showed clearly, that increasing P fertilization increases shoot accumulation of Cd and this effect was more pronounced with the P fertilizer containing higher Cd (Table 3.4; 3.12). It was interesting to notice that even the P fertilizer with almost no Cd (i.e., CaP) enhanced the Cd concentrations of plants. These results are similar to the results in wheat published recently by Yazici et al (2021). However, in the studies with wheat, P fertilizers varying in Cd concentrations resulted in more or less similar Cd concentrations in the plants. The reason for this differential response of rice and wheat plants to P fertilization in respect to shoot Cd accumulation could not be understood. In the previous studies, it has been discussed that rice plants are generally less mycorrhizal plants depending on the growth conditions (Ruíz-Sánchez et al. 2011; Campo et al. 2020; Ilag et al. 1987; Vallino et al. 2009). Since Cd accumulation in plants is strongly reduced by AMF as discussed below, the low colonization of roots with AMF in rice might be one reason why Cd uptake could not be better controlled in case of the P fertilizers containing higher Cd such as DAP. The plant species with a high root mycorrhizal colonization rate, such as wheat, have a better capacity to control and reduce Cd transport in the shoots (Yazici et al. 2021).

In good agreement with these observations, rice plants inoculated with arbuscular mycorrhiza showed drastic decreases in shoot accumulation of Cd, and elimination of mycorrhizae in the soil by sterilization resulted in marked increases in shoot Cd. These results highlight the particular role of AMF in reducing Cd transfer from soils and roots in the shoot parts of the plants. AMF are known to be highly Cd biosorbent and act as an important physical barrier against Cd transfer in the shoots by immobilizing Cd in their structures (Joner et al., 2000; Ferrol et al., 2016). AMF have very high Cd adsorption

capacity through their cell walls. There are published reports showing that mycorrhizal tissues contain much higher Cd binding sites than the root tissues (Chen et al., 2018).

These findings indicate that any factor reducing root mycorrhizal colonization may promote Cd uptake and accumulation in plants which is the case with P fertilization. The higher sensitivity of AMF to P fertilization is a very well-documented phenomenon and shown in several studies (Smith et al. 2011; Ova et al. 2015; Ryan and Graham 2018). Therefore, the increases in Cd accumulation in rice plants by P fertilizers regardless of their Cd content can be ascribed to diminished activity in AMF through P fertilization.

As shown in the present study, suppression of root mycorrhizal colonization due to increasing P fertilization is mostly responsible for the marked increases in shoot Cd concentrations of rice in unsterilized soil (Table 3.4, 3.12). The removal of arbuscular mycorrhizal fungi by autoclaving the soil or deactivating AMF by high P supply led to a marked increase in the shoot Cd concentrations (Table 3.20). For example, the shoot Cd concentration increased from 6.32 µg kg^{-1} to 15.45 µg kg^{-1} with autoclaving the soil under the low P treatment without the application of mycorrhizal inoculum (Table 3.20). When P was applied at a higher rate to the plants, autoclaving the soil had a lower impact on shoot Cd concentrations, due to reduced root mycorrhizal colonization. These findings show that rice plants accumulate more Cd in shoots when root mycorrhizal colonization is eliminated through a high P treatment or soil autoclaving. The marked decrease in shoot Cd concentration upon mycorrhizal inoculation to sterilized soil further supports this negative interaction between the root AMF colonization and plant Cd uptake (Table 3.20). Mycorrhizal inoculation was similarly shown to be highly effective in decreasing the Cd accumulation in maize (Liu et al. 2014), pigeon pea, and pea (Garg et al. 2015), which is consistent with the findings for rice in the present study.

The augmenting effect of P fertilizers on plant Cd concentration has also been reported in potato and durum wheat cultivated under field conditions (Grant et al. 2002, 2013). Similar to the present findings, Bošković-Rakočević et al. (2017) demonstrated that the Cd concentration in potato tubers increased with the increasing rate of various P fertilizers containing 56.51, 0.84, 0.65, 23.69, and 14.02 mg Cd kg^{-1}. Maqbool et al. (2022) also demonstrated that the shoot Cd concentration of *Solanum nigrum* L. increased with an increasing rate of P treatment in the form of single superphosphate (SSP) and DAP in a

greenhouse study. However, the results of these studies may also be affected by other factors than the Cd contents of P fertilizers that are involved.

Phosphorus fertilization can also increase shoot Cd accumulation by inducing Zn deficiency stress in plants. It is known that Cd and Zn have very similar atomic properties and are transported through the same channels within plants (Cakmak et al. 2000; Jiao et al. 2004; Hart et al. 2005; Qin et al. 2013; Gao et al. 2016). Consequently, there is a competition between these 2 divalent cationic metals during their root uptake and shoot transport. Phosphorus is antagonistic to root Zn uptake and utilization in plants and depending on the application rate, p fertilizers can easily increase Zn deficiency stress in plants (Loneragan et al., 1982; Cakmak and Marschner, 1986; Ova et al. 2015). Also in the present study, we have seen that rice plants showed signs of Zn deficiency in shoots (i.e., reduced shoot elongation and expression of some necrotic spots on leaves) when P fertilizers have been applied at high rates. Consequently, P fertilization may stimulate Cd accumulation in plants by reducing Zn completion against Cd. Most probably, this is another critical factor involved in P-fertilization-related Cd accumulation in plants.

Baghaie et al. (2021) observed on wheat plants that arbuscular mycorrhiza enhanced the Zn uptake from mineral fertilizer and decreased the Cd concentration in the wheat grains in a pot experiment. In several crop species such as wheat and tomato, AMF is accountable for 24 to 50 percent of the overall Zn uptake (Bhantana et al. 2021; Watts-Williams et al. 2015; Yazici et al. 2021). Hui et al. (2022) also demonstrated a positive impact of mycorrhizal root colonization on the Zn concentrations in wheat plants. Consequently, in the present study, shoot Zn concentration was increased in plants with AMF inoculation compared to plants grown without AMF inoculation (Table 3.4, 3.12). In a greenhouse study by Krishna et al. (1984), a substantial reduction of Zn uptake was reported in peanut plants cultured in sterilized soil to prevent root mycorrhizal colonization. Similar findings were also obtained in the present study. Soil sterilization and application of high P reduced the Zn concentration in rice shoots, as shown in Table 3.20. These results indicate that autoclaving and/or fertilizing the soils with high P rates may hamper the AMF-mediated Zn uptake, and subsequently lead to an induced/selective uptake and accumulation of Cd in crop plants.

In the present study, there was a significant reduction in the shoot Fe accumulation following the mycorrhizal inoculum which could be, in part, related to the improved Zn uptake capacity of plants. Like Zn and Cd, also Zn and Fe are antagonistic and compete for the same binding sites and transporters in the plants (Cakmak, 2000; Grotz et al., 2006; Marschner, 2012). Improving the Zn nutritional status of plants by mycorrhizal inoculation would reduce root Fe uptake and cause Fe deficiency in plants if the soils are marginally supplied with Fe as was the case in the present study. Therefore, in the current experiments, we applied Fe to the plants twice to minimize the risk of Fe deficiency in plants.

Similarly, also shoot Mn concentration showed decreases with the addition of mycorrhizal inoculum, and these decreases were much more pronounced with Mn than Fe (Table 3.8; 3.16). Similar results where arbuscular mycorrhiza decreased the Fe and Mn concentration of plants have also been reported for maize (Kothari et al. 1990; 1991). According to Kothari et al. (1990), a decrease in shoot Fe concentration is related to a reduction in the lateral root formation in mycorrhizal plants. Additionally, Kothari et al. (1991) observed that the decrease in the density of root length in the AMF treatment led to a reduction of the Fe- and Mn- reducing microorganisms in the rhizosphere soil, which eventually decreased the Fe and Mn concentrations in maize plants. Most probably, reductions in Mn reducing bacteria with mycorrhiza inoculum plays a key role in why plant showed decreases in Mn concentration after AMF inoculation. The reason is not well understood, but it is known that soil sterilization markedly increases soluble levels of Mn in soils which maybe even too much resulting in toxicity in plants (Boyd, 1971; Berns et al. 2008).

5. CONCLUSION

Rice-based foods are known to be a major dietary source of Cd exposure to human populations. Therefore, understanding the mechanisms affecting Cd accumulation in rice plants is of great importance. Today, high Cd levels in food crops represent both an important scientific issue and political topic.

There are controversial discussions about the sources of Cd in food crops (Grant et al. 2013; McLaughlin et al. 2021; Yazici et al. 2021). Very recently, the EU has reduced the allowable Cd limits further for a number of food sources including cereals (https://eur-lex.europa.eu/eli/reg/2021/1323/oj). Phosphorus fertilizers have been often discussed as the most important source of Cd in plants and therefore the levels of Cd in plants have been suggested to be reduced significantly. For example, Carne et al. (2021) suggested that Cd concentrations of P fertilizers should be reduced to minimize Cd contamination in food crops and dietary Cd exposure of humans.

However, the present results obtained from the rice experiments as well as the results published by Yazici et al. (2021) on wheat show that the Cd accumulation in plants enhanced by P fertilizers is not strictly related to Cd concentrations of the P fertilizers applied. It is obvious that as long as P is applied, there is a risk for enhanced Cd accumulation in plants because P fertilization has a diminishing impact on root mycorrhizal activity of plants. As shown in the present study, reducing the mycorrhizal activity of plants by high P fertilization or soil sterilization promoted shoot Cd accumulation. The addition of a mycorrhizal inoculum in the sterilized soil or native soil significantly reduced shoot Cd concentrations of plants. These results strongly suggest that AMF represent a very critical factor in crop production systems in controlling and reducing Cd accumulation in plants. Therefore, besides the discussions on reducing Cd in P fertilizers, a particular attention should be also given to the maintenance of a

functional AMF in cultivated soils. It is known that there are several crop and soil management practices that also reduce mycorrhizal activity in soils, like high P fertilization, such as intensive soil use and tillage, monoculture cropping systems, and high nitrogen fertilization (Bowles et al. 2017; Dietrich et al. 2020; Ma et al. 2021). Therefore, for an effective reduction of Cd accumulation in food crops, besides P fertilization, an equally high priority should be also given to the mentioned soil and crop management practices which also detrimentally influence AMF activity in soils and potentiate Cd accumulation in plants.

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