UNIVERSIDADE FEDERAL DE VIÇOSA

ENDY LOPES KAILER

THE USE OF MYCORRHIZAL FUNGI FOR GROWING ZN-ENRICHED LETTUCE

VIÇOSA - MINAS GERAIS
2021

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Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitotecnia, para obtenção do título de *Magister Scientiae*.

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Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
Kailer, Endy Lopes, 1995-
K13u The use of mycorrhizal fungi for growing Zn-enriched
2021 lettuce / Endy Lopes Kailer. – Viçosa, MG, 2021.
49 f. : il. (algumas color.) ; 29 cm.

Inclui apêndice.
Orientador: Carlos Nick Gomes.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Referências bibliográficas: f. 37-47.

1. Melhoramento de cultivos agrícolas. 2. Minerais na nutrição humana. 3. Micro-organismos do solo. 4. Alimentos - Biotecnologia. I. Universidade Federal de Viçosa. Departamento de Fitotecnia. Programa de Pós-Graduação em Fitotecnia. II. Título.

CDD 22. ed. 635.52

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APROVADA: 16 de junho de 2021.

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Aos meus pais Gilmara, Edinilson e Luis Cláudio.

AGRADECIMENTOS

Agradeço primeiramente a Deus, pelo dom da vida, por todas as bênçãos e pela saúde que me permitiu vencer mais essa etapa, em tempos tão difíceis como esse.

Ao meu noivo Pedro, por todo o suporte em cada etapa desse projeto, por todos os dias e noites no laboratório e na casa de vegetação fazendo o possível e o impossível para que eu chegasse aqui hoje. Muito obrigada por tanto amor, paciência e compreensão. Que sorte a minha ter você em minha vida.

Aos meus pais Gilmara, Edinilson e Luís Claudio pelo apoio incondicional. Tudo o que eu sou, e ainda serei, devo inteiramente a todos vocês, que nunca mediram esforços para que esse dia chegasse. Vocês são a minha base, meus exemplos e donos da minha imensa admiração e gratidão.

A todos os professores que contribuíram para essa conquista, em especial os membros da minha comissão orientadora, professor Carlos Nick, professor Samuel Valadares, professora Maria Catarina e Doutora Marliane da Silva. Nunca esquecerei o quanto me ajudaram em cada etapa deste projeto, mesmo com todas as dificuldades impostas pela pandemia. Muito obrigada por todo o suporte, paciência e parceria, levarei comigo uma grande admiração por todos vocês.

Aos meus estagiários João, Mirelle e Aíssa. Ao Júlio Nunes, Françoise e Rafael Valadares. À família Lamic, em especial ao Paulo Prates e Leandro Lopes, por toda a ajuda e suporte. Vocês foram essenciais para este projeto, serei eternamente grata por tudo o que fizeram por mim nos últimos meses.

Aos meus amigos e familiares, em especial Sara Nacarate, Ryan Anderson, Rafaela Rodrigues, Rosângela Gonçalves e Pedro Abel pelo incentivo incondicional. Ao Milk Antônio por todo o carinho, suporte emocional e amor.

À UFV, aos departamentos de fitotecnia, microbiologia e solos, e ao CNPQ, á CAPES e à Fapemig pelo apoio financeiro e por toda a infraestrutura cedida.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

A todos vocês, minha sincera gratidão.

RESUMO

KAILER, Endy Lopes, M.Sc., Universidade Federal de Viçosa, junho de 2021. **O uso de Micorrizas Arbusculares para a Produção de Alface Enriquecida em Zinco.** Orientador: Carlos Nick Gomes. Coorientadores: Samuel Vasconcelos Valadares, Maria Catarina Megumi Kasuya e Marliane de Cássia Soares da Silva.

Hoje, mais de dois bilhões de pessoas enfrentam os resultados negativos das deficiências de micronutrientes em todo o mundo, especialmente quando se trata de zinco (Zn). A biofortificação agronômica é considerada a forma mais econômica de aumentar o teor de Zn em produtos alimentícios. Conhecidos como os "engenheiros invisíveis do ecossistema", os fungos micorrízicos arbusculares (FMA) estão associados a mais de 80% de todas as plantas vivas e são responsáveis por aumentar a absorção de nutrientes. A alface (*Lactuca sativa* L.) é uma das hortaliças mais consumidas em todo o mundo e seu valor nutricional pode impactar diretamente na ingestão diária de nutrientes de milhões de pessoas. Nesse sentido, esta pesquisa teve como objetivo avaliar o efeito da inoculação micorrízica na biofortificação de Zn em folhas de alface. Assim, vasos de alface foram cultivados em casa de vegetação em delineamento de blocos inteiramente casualizados em esquema fatorial duplo (5x2), composto por cinco doses de Zn (0, 8, 32, 64 e 96 mg/dm^3) como Zn sulfato ($ZnSO_4$) e dois níveis de inoculação micorrízica (presença e ausência) em sete repetições. Os dados experimentais foram submetidos à análise de variância (ANOVA) e regressão. Independentemente da dose aplicada, as plantas inoculadas com FMA apresentaram aumento na massa fresca da parte aérea, altura da parte aérea, matéria seca, volume radicular e número de folhas. Além disso, a inoculação com FMA não afetou a concentração de zinco nas folhas, mostrando que a biofortificação pode ocorrer independentemente da inoculação de FMA nas folhas de alface. As plantas inoculadas com FMA apresentaram um maior conteúdo de zinco total por planta, apresentando resposta linear à medida que aumentava a fertilização com Zn. Uma vez que as plantas inoculadas cresceram mais, o nutriente sofreu efeito de diluição do tecido, uma vez que as folhas foram mais largas. A matéria seca diminuiu com doses superiores a 32 mg/dm^3, apresentando alguns sinais de fitotoxicidade. As plantas foram biofortificadas, acumulando 25 vezes mais Zn na maior dose quando comparadas às testemunhas. Assim, a associação com FMA pode estimular o crescimento da planta e levar a um maior conteúdo de zinco nas folhas da alface.

Palavras-chave: Biofortificação agronômica. Absorção de nutrientes. Fome oculta. Fertilização com zinco. Microrganismos do solo.

ABSTRACT

KAILER, Endy Lopes, M.Sc., Universidade Federal de Viçosa, June, 2021. **The use of Mycorrhizal Fungi for Growing Zn-enriched Lettuce.** Advisor: Carlos Nick Gomes. Co-advisors: Samuel Vasconcelos Valadares, Maria Catarina Megumi Kasuya and Marliane de Cássia Soares da Silva.

Today, over two billion people face the negative outcomes of micronutrient deficiencies all over the world, especially when it comes to zinc. Agronomic biofortification is considered the most cost-effective way to increase zinc content in food products. Known as the “ecosystem’s invisible engineers”, arbuscular mycorrhizal fungi (AMF) are associated with more than 80% of all the living plants and are responsible for enhancing nutrient uptake. Lettuce (*Lactuca sativa L.*) is one of the most widely consumed vegetables worldwide and its nutritional value can directly impact the daily nutrient intake of millions of people. In this sense, this research aimed to assess the effect of mycorrhizal inoculation in the biofortification of Zn in lettuce leaves. Thus, lettuce pots were cultivated in a greenhouse in a completely randomized block design experiment in a double factorial design (5x2), composed by five doses of Zn (0, 8, 32, 64, and 96 mg/dm^3) as Zn sulphate ($ZnSO_4$) and two levels of mycorrhizal inoculation (presence and absence) in seven replications. Experimental data were subjected to variance (ANOVA) and regression analysis. Regardless of the applied dose, plants that were inoculated with AMF presented an increase in fresh weight of shoots, shoot height, dry matter, root volume and leaf number. Also, the inoculation with AMF did not show any effect on the zinc concentration of the leaves, showing that biofortification may happen regardless of AMF inoculation in lettuce leaves. Plants that were inoculated with AMF showed higher levels of total zinc content per plant, presenting a linear response as Zn fertilization was increased. As inoculated plants grew more, the nutrient suffered tissue dilution effect, as leaves were wider. Dry matter showed a decrease with doses higher than 32 mg/dm^3, showing some signs of phytotoxicity. Plants were biofortified, accumulating 25 times more Zn in the highest dose when compared to the control ones. Thus, association with AMF can stimulate plant growth and lead to higher zinc content in lettuce leaves.

Keywords: Agronomic biofortification. Nutrient uptake. Hidden hunger. Zinc fertilization. Soil microorganisms.

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1. INTRODUCTION

Feeding a growing population with very limited resources is one of the greatest challenges of modern agriculture (Godfray et al., 2010). The current population of 7.8 billion people is projected to achieve the mark of 10 billion people by 2057, setting a major pressure on the global food production system (Godfray et al., 2010; Worldmeters, 2021). It is estimated that over two billion people face micronutrient deficiencies all over the world (Allen et al., 2006). The lack of essential minerals and vitamins in human diet is known as “hidden hunger”, a current global public-health challenge that has greater impacts in developing countries where diet diversification might be scarce due to a frequent dependence on staple foods (Thompson & Amoroso, 2014).

A well-balanced diet assures the supply of over twenty mineral elements that are daily required by the human body (Thompson & Amoroso, 2014). Zinc, iron, iodine and vitamin A deficiencies are responsible for a wide-range of healthy related issues that mainly affect pregnant women and children (Bailey et al., 2015; Thompson & Amoroso, 2014). It is estimated that in the past few decades around 30% of the world’s population could be facing zinc (Zn) deficiency (Hotz & Brown, 2004). In this scenario, biofortification processes have been showing excellent results in enhancing nutrient concentrations in many crops around the world, directly increasing the dietary intake of micronutrients (Bouis et al., 2011).

Agronomic biofortification is considered the most cost-effective way to increase micronutrient concentration in food products (Bouis & Saltzman, 2017). This method aims to increase nutrient concentration in edible parts of the plant by applying mineral fertilizers and/or by improving the bioavailability of the nutrients in the soil, leading to a higher intake of nutrients by the final consumers (de Valença et al., 2017).

Even though some soils present a high content of nutrients, these elements are not always available for plants due its frequent low phytoavailability (Marschner, 1995). For instance, minerals like zinc (Zn^{2+}) and cupper (Cu^{2+}), which have small diffusion coefficients, are usually present in low concentrations in the soil solution, resulting in low mobility in soil (Barber, 1995), depending on root expansion to reach the demanding nutrient’s area (Rengel, 2001).

A holistic management is indispensable to produce high quality food in a cost-effective and environmentally safe way, where agricultural systems rely on ecosystem services to improve crop yields and enhance nutrients bioavailability in the soil (Moonen & Bàrberi, 2008; Smith

& Read, 2008a). Soil inoculation using beneficial microorganisms is one of the most effective ways to increase nutrient phytoavailability in the rhizosphere by turning complex structures of these nutrients into simpler forms that can be successfully absorbed by the plants (Gianinazzi et al., 1990; Smith & Read, 2008).

Mycorrhizal fungi are microorganisms that associate with plant species in a symbiotic relationship where the fungi facilitate the nutrient uptake by the plant, whereas the plant provides the carbon that the fungi depend on (Bago et al., 2000; Hodge et al., 2010; Smith & Read, 2008). Considered the most important type of mycorrhizal association, Arbuscular Mycorrhizal Fungi (AMF), phylum *Glomeromycota*, is broadly associated with the great majority of all the cultivated crops, being the most agronomically relevant type of mycorrhiza (He & Nara, 2007). Once the roots are colonized, the fungi allow the roots to explore greater volumes of soil, achieving areas further from the depletion zone, being able to absorb even the nutrients that are extremely poorly mobile in soil, as phosphorus and zinc (Bago et al., 2002; Marschner & Dell, 1994; Smith & Read, 2008).

Lettuce (*Lactuca sativa* L.) is one of the most widely consumed vegetables worldwide and the number one most consumed leafy vegetable in Brazil, being a great source of vitamins, minerals, carotenoids, flavonoids, phenolic acids, among other important antioxidant compounds (Kim et al., 2016; Sala & da Costa, 2012). Also, considering that lettuce leaves are broadly consumed in salads, uncooked, it is easier to preserve the nutrient content of the leaves, being an accessible great source of nutrients with a low-calorie content (Mota et al., 2012). Considering how popular lettuce is, a careful look on its nutritional value has to be taken, once it can directly impact in the daily nutrient intake of millions of people.

Recent studies have shown the beneficial effects of lettuce biofortification, with or without inoculation with beneficial microorganisms (Atsushi et al., 2021; H. de Almeida et al., 2020; Di Gioia et al., 2019; Giordano et al., 2019; Graciano et al., 2020; Machado de Lima, 2021; Moraes, 2020).

In this sense, this study was conducted to test the hypothesis that plants inoculated with mycorrhizal fungi would present higher concentrations of zinc when compared to the non-inoculated ones, expecting to find greater nutrient content in plants that received higher doses of zinc. Thus, this study aimed to assess the effect of mycorrhizal inoculation in the biofortification of Zn in lettuce leaves.

2. LITERATURE REVIEW

One of the major challenges to be faced in a near-future scenario is feeding a constantly increasing population with the same limited (or even fewer) resources (Godfray et al., 2010). It is estimated that the current population of 7.8 billion people is projected to achieve the mark of 10 billion people by 2057, setting a major pressure on the global food production system (Godfray et al., 2010; Worldmeters, 2021).

The global undernourishment index got reduced in around 6% mainly due to more efficient agricultural practices that resulted in greater crop yields (FAO et al., 2012). Yet, over two billion people still face the negative outcomes of micronutrient deficiencies all over the world (Allen et al., 2006). The lack of essential minerals and vitamins in human diet is broadly known as “hidden hunger”, a current global public-health challenge that has a greater impact in developing countries where the diet diversification is usually scarce (Thompson & Amoroso, 2014). However, micronutrient deficiency is commonly mistaken as an issue that only affects low-income developing countries, which was proven wrong by the data released from the National Health and Nutrition Examination Survey in the United States, showing that it is also an issue in well-developed countries. In the U.S. alone, around 35–45% of elderly adults show a zinc intake below the recommended daily requirement for their age. Additionally, elderly people from low-income families in the U.S. are getting less than 50% of the recommended zinc (and other essential vitamins and minerals) daily intake as a consequence of a not sufficiently diverse diet based on fruits, vegetables, grains and protein sources (Dixon et al., 2001; Ervin & Kennedy-Stephenson, 2002; National Institutes of Health, 2021).

Zinc, iron, iodine and vitamin A deficiencies play a major role in causing public-health issues worldwide, being responsible for a wide-range of healthy related life-long consequences that mainly affect pregnant women and children (Bailey et al., 2015; Thompson & Amoroso, 2014). It is worth to point out that micronutrient deficiencies are among many other more “visible” forms of hunger and malnutrition that bring a pressing challenge for agriculture and food production (Bailey et al., 2015). It is estimated that in the past few decades around 30% of the world’s population could be facing zinc (Zn) deficiency. In this sense, a suitable and well-balanced diet assures the supply of over twenty mineral elements that are daily required by the human body (Thompson & Amoroso, 2014).

Zinc is an essential micronutrient involved in many biological functions of the human body. Catalyst of over 100 enzymes (Sandstead, 1994), zinc is also involved in many protein and DNA synthesis reactions (Prasad, 1995), cellular division, wound healing processes and some immune functions (Prasad, 1995; Solomons, 1998). Additionally, zinc is extremely important to assure the proper gestational development of pregnant women and the growth process in children and teenagers (Maret & Sandstead, 2006; Simmer & Thompson, 1985a). Along with humans and other animals, plants also rely on zinc for many vital physiological processes, considering that it is responsible for controlling an expressive number of enzymes, protein structure's stability, and important reactions as transcription and translation (Taiz & Zeiger, 2017).

Considering that the human body is incapable of storing the required levels of zinc, its daily ingestion is highly important (Rink & Gabriel, 2000). The Food and Nutrition Board (FNB) states that the adequate zinc intake varies between 8-11 mg/day for adults and between 2-8 mg/day for children and teenagers (National Institutes of Health, 2021). Therefore, zinc can be found in a broad variety of food products, such as seafood, red meat, poultry, dairy products, whole grains, beans, nuts and vegetables. However, high levels of zinc do not ensure that this nutrient will be well-absorbed by the organism, considering that it can interact with some chemicals, as phytates, that can inhibit zinc's absorption (Sandström, 1997; Wise, 1995). Thus, if the daily ingestion of zinc is not met, many physiological issues arise. The most common symptoms of zinc deficiency includes loss of appetite, weakened immunity system, hair loss, diarrhea, late sexual maturation, taste abnormalities, among others (Hambidge, 1989; Heyneman, 1996; Nishi, 1996; Ploysangam et al., 1997; Prasad, 2004).

Especially dangerous for pregnant women, zinc deficiency can lead to preterm delivery, fetal growth restrictions, hemorrhage and maternal morbidity (King, 2000; Simmer & Thompson, 1985b). In addition, zinc deficiency is also hazardous for children, leading to growth and brain development impairment. Also, zinc deficient children may also face higher chances of getting infectious diseases like pneumonia, increasing the chances of mortality. Around half a million children die every year as a consequence of zinc deficiency, consolidating it as a public health issue (Black et al., 2008; Krebs et al., 2014; Terrin et al., 2015).

If the nutrient concentration was the only pattern to consider, most soils would have enough micronutrients to support even the most mineral-demanding crops (Frossard et al., 2000). However, over 90% of all Brazilian soils show low availability of micronutrients, naturally presenting low zinc content (0.6 to 2.0 mg/kg). Also, many soil characteristics are known for influencing the availability of this micronutrient, as the pH, redox conditions, organic matter content, phosphorus adsorption capacity, activity of microbes, cation exchange capacity, among others (Marschner, 1995; Shuman, 1998).

Micronutrients are not always available for the plants due to its frequently low phytoavailability. A great example of this is the frequent presence of zinc deficiency in crops cultivated in alkaline soils (Frossard et al., 2000; Shuman, 1998). For instance, minerals like zinc (Zn^{2+}) and cupper (Cu^{2+}), which have small diffusion coefficients, are usually present in low concentrations in the soil solution, which results in a low mobility in soil (Barber, 1995; Broadley et al., 2007; Whiting et al., 2003), depending on root expansion thorough the soil to reach the demanding nutrient's area (Rengel, 2001).

Zinc deficiency is a public health issue that can bring negative outcomes to billions of people all over the world, being aggravated by the poor availability of micronutrients in tropical soils (Hefferon, 2019; Wessells & Brown, 2012). Therefore, biofortification processes have been showing excellent results in enhancing nutrient concentrations in many crops through genetic engineering, classic plant breeding and agronomic approaches (Bouis et al., 2011).

Agronomic biofortification is considered the most cost-effective way to increase zinc and iron content in food products (Bouis & Saltzman, 2017). This method aims to increase the nutrient concentration in edible parts of the plant by applying mineral fertilizers and/or by improving the bioavailability of the nutrients in the soil, which leads to a higher intake of nutrients by the final consumers (de Valença et al., 2017). Mineral fertilizers tend to be applied straight to the leaves or in the soil, depending on the plant and soil properties (Graham et al., 2007; White & Broadley, 2009). Additionally, leafy vegetables tend to store higher concentrations of Zn when compared to other organs as seeds, fruits and tubers due to its main transportation via xylem (White et al., 2018; White & Broadley, 2011). Considering that the phloem-fed organs above cited might face issues regarding low zinc mobility, it can directly decrease its bioaccumulation in the edible parts of the plant (Rengel et al., 1999; White & Broadley, 2005).

Many agricultural systems rely on ecosystem services to improve crop yields and enhance nutrients bioavailability in the soil, considering that a holistic management is indispensable to produce high quality food products in a cost-effective and environmentally safe way (Moonen & Bàrberi, 2008). Soil inoculation using beneficial microorganisms is one of the most effective ways to increase nutrient phytoavailability in rhizosphere by turning complex structures of these nutrients into simpler forms that can be successfully absorbed by the plants (Gianinazzi et al., 1990; Marschner & Dell, 1994).

In the rhizosphere, many beneficial and deleterious organisms engage in complex interactions with each other, as well as with the plants from that area, developing a broad range of reactions that influence both the soil and the plants (Fitter & Garbaye, 1994). In this sense, it is extremely difficult to grow crops without acknowledging how important the biotic activities are for plant metabolism and soil health. Even though the details of these complex interactions are not entirely understood, it is proven that fungi, protozoa, bacteria, nematodes, arthropods and other living organisms are physically linked in many vital reactions that might lead to the success or the failure of the crops cultivated in that area (Fitter & Garbaye, 1994). Broadly known as the "ecosystem's invisible engineers", mycorrhizal fungi can be found associated with more than 80% of all living plants, playing a major role in the nutrition of almost all agricultural crops (Smith & Read, 2008b).

Mycorrhizal fungi are microorganisms that associate with a broad number of plant species in a symbiotic relationship where the fungi facilitate nutrient uptake by the plant, whereas the plant provides the carbon that the fungi depend on (Bago et al., 2000; Hodge et al., 2010). There are a wide number of positive outcomes that results from this symbiotic relationship, including positive effects in nutrient enrichment (Huang et al., 2020; Shao et al., 2021; Xie et al., 2020), plant growth and height (Cofré et al., 2020; Huang et al., 2020; Xie et al., 2020), biomass accumulation (Tran et al., 2020), resistance to several pathogens (Linderman, 2000; Wu et al., 2021), shoot : root ratio (Veresoglou et al., 2012), flower production (de Almeida et al., 2020), drought resistance (Augé, 2001; Malhi et al., 2020), salinity resistance (Romero-Munar et al., 2019; Santander et al., 2020), higher yields (Ghobadi et al., 2020), among others. Additionally, the presence of mycorrhizal fungi also improves soil aggregation and organic carbon storage (Al-Maliki & Breesam, 2020; Heydari et al., 2021). As an exchange, the plant provides the fungi with 4-20% of the carbon fixed in the photosynthesis process (Douds et al., 1988; Smith & Read, 2008b).

Arbuscular Mycorrhizal Fungi (AMF), phylum *Glomeromycota*, is largely associated with the great majority of all cultivated crops, being the most agronomically relevant type of mycorrhizal association (He & Nara, 2007). AMF aseptate hyphae develops within cells of the root, while ectomycorrhiza (phylum *Ascomycota* and *Basidiomycota*) fungi colonize the roots without penetrating into the cells (Smith & Read, 2008a). Once the roots are colonized, the fungi allow the roots to explore broader volumes of soil, achieving areas further from the depletion zone, being able to absorb even the nutrients that are poorly mobile in soil, such as phosphorus and zinc (Bago et al., 2002; Marschner & Dell, 1994). The root absorption zone in non-colonized plants is usually around 10 mm, while in the AMF colonized plants it can reach up to 110 mm, what can easily illustrate how powerful AMF can be in increasing the absorption of immobile nutrients (Li et al., 1991). In addition, not only do AMF increase phosphorus and zinc absorption, but also other nutrients such as N, K, Ca, Mg and micronutrients. Thus, it is clear that the root ability to explore a greater volume of soil ensures that the plant will have access to nutrients that it would not be able to reach without the presence of AMF, which gives a better chance to plants that struggle in depleted soils (Marschner, 1996).

AMF need the symbiotic association in order to sporulate and complete their lifecycles, once the fungi is unable to assimilate external carbon structures without being associated (Douds et al., 2006). For this reason, the spores of these organisms are unable to be multiplied on its own (axenic culture), requiring the presence of a host plant for inoculum production, which turns it into a challenging practice, especially for large-scale crop production (Ijdo et al., 2011).

The costs involved in inoculum acquisition could make inoculation economically nonviable, especially in large areas where an expressive amount of inoculant is needed. A sustainable alternative to overcome these costs is the production of the inoculum on site, known as the on-farm method, a practice that has become very popular in developing countries (Douds et al., 2005). This method relies on the multiplication of spores from a microbial community that are already present in that area, utilizing agro-industrial by-products, in order to produce the right strains for the specific conditions of that area, considering the weather, soil characteristics, crops produced, among others. Additionally, on-farm inoculum is known for producing a great number of spores that are well-adapted to the specific conditions of each place. Also, indigenous fungi community that are largely diverse might show a better performance when used to start the inoculum production than the introduction of commercial AMF products (Smith et al., 2000). Additionally, several cultural practices are known for stimulating the activities of native AMF communities, as the use of cover crops, crop rotation, and the reduced tillage/no-till approach, in order to ensure an adequate environment for the colonization of the new crops (Douds et al., 2005).

The tendency of adopting a healthier lifestyle has been increasing worldwide, especially when it comes to food habits. People began to seek food that have better nutrient content in order to decrease the risks of diseases and promote a healthier longer life. Thus, people who consume more vegetables, tend to show reduced risks of developing cardiovascular diseases, cancer and other chronic diseases (Hung et al., 2004; Kris-Etherton et al., 2002).

Lettuce (*Lactuca sativa* L.) is one of the most widely consumed vegetables worldwide and the number one most consumed leafy vegetable in Brazil, being a great source of vitamins, minerals, carotenoids, flavonoids, phenolic acids, among other important antioxidant compounds (Kim et al., 2016; Sala & da Costa, 2012). Also, considering that lettuce leaves are broadly consumed in salads, uncooked, it is easier to preserve the nutrient content of the leaves, being an accessible great source of nutrients with a low calorie content (Mota et al., 2012). Considering how popular lettuce is, a careful look on its nutritional value has to be taken, once it can directly impact in the daily nutrient intake of millions of people.

The curly group has become the most popular type of lettuce in Brazil, leading almost 70% of the lettuce internal market, due to its better resistance to pathogens, longer shelf-life, and easiness to handle and transport (Sala & da Costa, 2012). Additionally, considering that lettuce is a short-cycle crop (Spring/Summer- 6-8 weeks; Winter- 10-12 weeks), the nutrients should be available promptly in order to avoid deficiency symptoms, that might lead to expressive yield losses. In this sense, a great alternative to enhance the levels of nutrients in lettuce leaves and to ensure a better use of nutrients is the inoculation using beneficial microorganisms that increase nutrient phytoavailability and mineral accumulation in plants (Marschner, 1996).

For instance, recent studies have shown the beneficial effects of lettuce biofortification, with or without inoculation with microorganisms (Atsushi et al., 2021; H. de Almeida et al., 2020; Di Gioia et al., 2019; Graciano et al., 2020; Machado de Lima, 2021), emphasizing its importance in today’s scenario.

3. MATERIAL AND METHODS

3.1 Location and Experimental Design

A greenhouse experiment was carried out at Universidade Federal de Viçosa, Brazil (20°45′14” S, 42°52′55” W, altitude of 648 m) between February and April. According to the Köppen’s system, Viçosa is classified as mesothermic tropical highland (Cwb) with rainy summers and dry winters (Peel et al., 2007).

The completely randomized block design experiment was distributed in a double factorial design (5x2), composed by five doses of Zn (0, 8, 32, 64, and 96 mg/dm^3) as Zn sulphate ($ZnSO_4$) and two levels of mycorrhizal inoculation (presence and absence) in seven replications. The experimental unit was one plant per 3.0 dm^3 polyethylene pot.

3.2 Soil

A mixture (1:1) of sand and a loam Red-Yellow Latossol (Oxisol) from the city of Viçosa, located in the state of Minas Gerais, Brazil was used as the substrate for plant growth. The material was passed through a 2 mm sieve and submitted to chemical analysis (Table 1). After that, 2.5 dm^3 of the soil mixture was transferred to polyethylene pots (3.0 dm^3) inside plastic bags. Liming was executed based on the results of the chemical analyses, applying and homogenizing 2.03 g of $CaCO_3$ and $MgCO_3$ (ratio 3:1 mol) per pot. Then, the mixture was incubated for 30 days being irrigated up to 70% of the field capacity. During the course of the experiment, nutrients were added to the pots in six applications, starting one day before the seedlings were transferred to the pots. Phosphorus was the only nutrient that was applied at once, one day before the transplantation of the seedlings, using 70 mg/dm^3 as calcium phosphate ($Ca(H_2PO_4)_2.2H_2O$). The following nutrients were also applied during the course of the experiment, in a nutrient solution directly to each pot: 125 mg/dm^3 of N (calcium nitrate ($CaNO_3)_2$), 125 mg/dm^3 of K (potassium chloride (KCl)), 67 mg/dm^3 of sulfur (S) (50 mg/dm^3 of calcium sulfate ($CaSO_4.2H_2O$) and 17 mg/dm^3 of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)), 0.68 mg/dm^3 of boron (B) (boric acid (H_3BO_3)), 1.08 mg/dm^3 of copper (Cu) (copper(ii) chloride dihydrate ($CuCl_2.2H_2O$)), 3.08 mg/dm^3 of manganese (Mn) (manganese (ii) chloride tetrahydrate ($MnCl_2.4H_2O$)), 1.29 mg/dm^3 of iron (Fe) (Fe sulphate ($FeSO_4.7H_2O$))

and 0.13 mg/dm^3 of molybdenum (Mo) (ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)) (modified from Novais et al. 1991).

Table 1. Chemical and physical properties of the soil mixture (Red-Yellow Latossol + Sand).

Soil Attribute	Value
pH in water (1:2.5)	5,5
P (mg/dm^3)^1	2,1
K (mg/dm^3) ^{1}	26
Na (mg/dm^3) ^{1}	-
Ca^{+2} (cmol$_c$/dm^3) ^{2}	0,28
Mg^{+2} (cmol$_c$/dm^3) ^{2}	0,08
Al^{+3} (cmol$_c$/dm^3) ^{2}	0,0
H + Al (cmol$_c$/dm^3)	1,65
Sum of bases (cmol$_c$/dm^3)	0,43
Effective CEC (cmol$_c$/dm^3)	0,43
CEC at pH 7,0 (cmol$_c$/dm^3)	2,08
Base saturation index (%)	20,7
Al saturation index (%)	0,0
ISNa (%)	-
Organic carbon (dag/kg)^3	0,54
Remaining phosphorus (mg/L)	10,5
Zn (mg/dm^3) ^{1}	1,1
Fe (mg/dm^3) ^{1}	56,3
Mn (mg/dm^3) ^{1}	17,1
Cu (mg/dm^3) ^{1}	0,4
B (mg/dm^3)	0,14
S (mg/dm^3)	-

$^{1/}$Mehlich-1 extraction (Mehlich, 1953); $^{2/}$KCl 1 mol.L^{-1} extraction (Defelipo & Ribeiro, 1981); $^{3/}$ Walkley-Black Method (Gaudette & Wilson, 1974).

3.3 Plant

For being widely consumed in Brazil, the curly cultivar Amanda was the type of lettuce chosen for this study (*Lactuca sativa* L cv. Amanda). This tropical cultivar can be grown all year long and it also presents great tolerance to early bolting (Seminis, 2021).

The seedlings were cultivated in 64-cell plastic trays filled with commercial pine bark vermiculite-based substrate. After sowing, the seedlings were watered with deionized water for 30 days. When the seedlings presented three to four fully developed leaves, they were transferred to the 3.0 dm^3 pots filled with the soil-sand mixture (1:1, v:v).

3.4 Mycorrhizal Fungi Inoculum

The inoculum source consisted of a mixture of spores of *Paraglomus albidum* cf, *Paraglomus brasilianum*, *Claroideoglomus etunicatum, Paraglomus sp.,*and *Acaulospora scrobiculata* containing a rate of 150 spores/100g, of the inoculum obtained through the On-farm method, in which the indigenous spores present in the soil of a native forest region are multiplied in pots using sorghum as a host plant (Douds et al., 2005). The substrate is composed by native forest soil, sugarcane bagasse and vermiculite (1:1:1, v:v:v). Additionally, 1% of organic compost was added to the mixture. After 3 months of cultivation, the sorghum plants underwent a period of intentional hydric stress (1 month in the absence of water), which stimulated the multiplication of the spores. After that, the substrate was sieved and became ready to be used as an inoculum source. In this sense, right before transplanting, the inoculated experimental units (50 pots) received 35 grams of on-farm mycorrhiza fungi inoculum (around 53 spores). The remaining pots received the same amount of sterilized inoculum, to guarantee that all plants would have access to the same sources of nutrients (with the exception of each treatment's dose). After that, the inoculum was homogenized into the top 10 cm depth of the soil-sand mixture in each pot to ensure that the roots of the seedlings will be immediately in contact with the inoculum.

3.5 Conduction of the Experiment

The plants were manually irrigated utilizing deionized water. According to the growth stage, the frequency of irrigations was adjusted, starting with two daily watering shifts right after transplanting the seedlings, in order to keep the soil moisture index proper to the development of the plants. Additionally, manual weeding was executed as needed, with daily supervision of all pots in order to also monitor the presence of pests and diseases. All the plants were harvested 34 days after transplanting the seedlings.

3.6 Analytical Methods

All the plants were harvested by cutting the shoots at the soil level and the roots were carefully removed from the soil mixture and washed on running deionized water. After rinsing, the material was placed over a tissue paper to absorb the moist. Then, the shoots were weighted and the volume of the roots was measured using a graduated cylinder (1 dm^3). Also, shoot height and the number of leaves per plant were measured and recorded. The shoots were oven dried at 70 °C until constant weight was achieved in order to quantify the dry matter and proceed with the analyses to determine the concentration of the micronutrients. Additionally, some root samples were stored in 70% ethanol to analyze the colonization index.

To analyze Zn concentrations, the dried samples were finely ground in a Wiley mill (< 0.5 mm) and digested in a solution of HNO_3 and $HClO_4$ (4:1, v:v), having the nutrient content determined by an atomic absorption spectrophotometer (Silva, 2009; Zasoski & Burau, 1977; Zhao et al., 1994).

To evaluate the colonization of the roots, a modified root staining procedure was executed (Phillips & Hayman, 1970). In this approach, the roots are heated for five minutes in a 10% KOH solution. After that, the roots are washed three times in running water and acidified in 2% HCl for ten minutes. Then, the roots are submerged in a 0.05% trypan blue staining solution, heated for ten minutes and left overnight. After that, thirty root segments (1 cm) are randomly picked from each plant to be observed with a light microscope. To estimate the values of root colonization in each plant, the total number of colonized fragments are divided by the total number of root fragments analyzed and the result is multiplied by one hundred (Giovannetti & Mosse, 1980).

3.7 Statistical Analysis

Experimental data were subjected to a two-way analysis of variance (ANOVA) at a level of 5% ($p < 0.05$) by the F test. The variance considered the main factors (Mycorrhizal fungi inoculation and Zn fertilization) and the possible interaction between them. For the nutrient dose factor, regression analysis was performed by using the statistical software R (R Core Team, 2019).

4. RESULTS AND DISCUSSION

The results obtained in the present study indicate that a significant interaction was only observed ($p <0.01$) between the factors mycorrhizal fungi inoculation and zinc (Zn) fertilization for the variable total zinc content (Table 6-Appendix). For other variables, as there was no interaction between the factors, the results were individually presented and discussed. Additionally, Zn doses only lead to significant responses on the variables final zinc concentration and total zinc content. However, mycorrhizal inoculation increased fresh weight of shoots, shoot height, shoot dry matter, root volume, leaf number and total zinc content, regardless of the applied dose ($p < 0,05$). Also, the variable dry matter was submitted to a regression analysis, as the dose effect was presented significant at 15% by the F test.

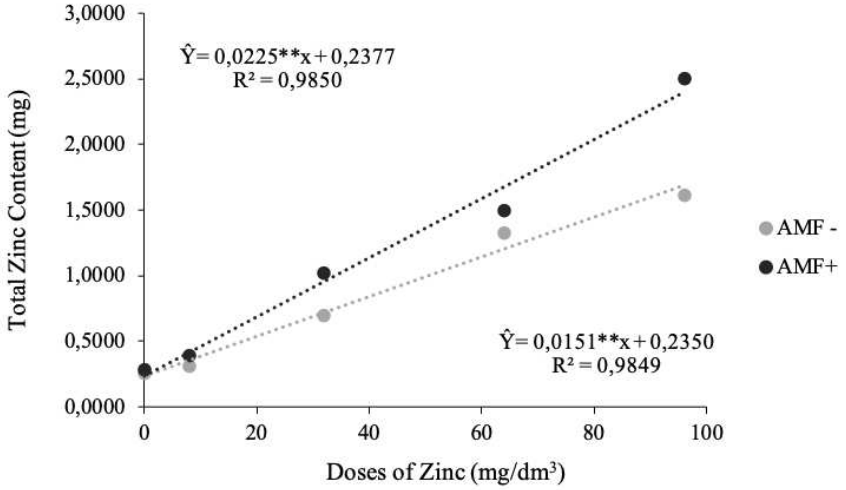

Figure 1. Total zinc content per plant (mg) in different doses of zinc (0, 8, 32, 64, 96 mg/dm^3) in inoculated and non-inoculated plants at 35 days after transplantation. 2021. **1%, * 5%, *** 10%, ° > 10% of significance.

For the variable total zinc content (TZC), there was a significant interaction between the factors Zn fertilization and AMF inoculation (Figure 1), where inoculated plants obtained the highest zinc content values. TZC shows how much zinc was accumulated in each plant, regardless of its concentration in leaves. However, when it comes to food biofortification, it is interesting that people find a great amount of nutrient in a small portion of the product, meaning that people will not have to eat large portions to fulfill their nutritional needs. In this sense, it is more important for this study that plants hold a high Zn concentration than a high plant Zn content. Therefore, even though AMF+ plants accumulated more zinc than AMF- ones, AMF+ inoculation also increased plant growth, reducing Zn concentration in the tissue, leading to a nutrient dilution effect (Burleigh et al., 2003; Clark & Zeto, 2000).

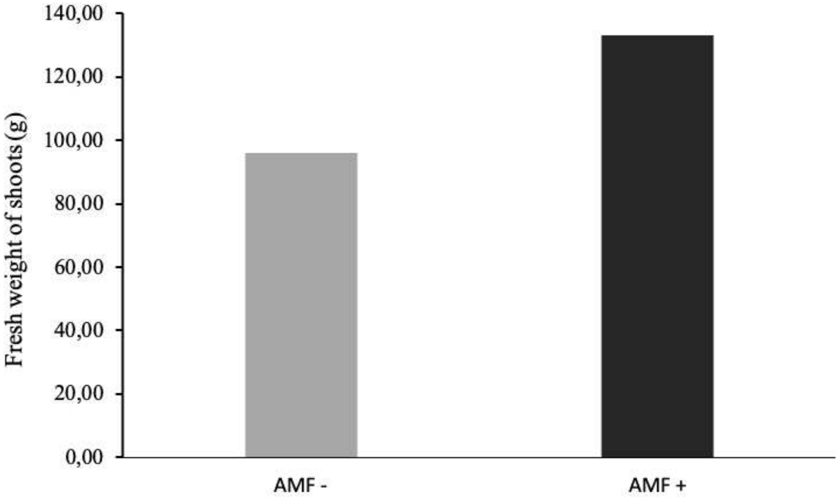

Figure 2. Fresh weight of shoots of inoculated and non-inoculated plants at 35 days after transplantation. 2021. There were significant differences ($p < 0,01$) between the means of the treatments by the F test.

Inoculated plants (AMF+) reached higher values of fresh weight of shoots (FWS) when compared to non-inoculated ones (AMF-), leading to an increase of around 30% in fresh matter, with their means varying between 132.98 and 95.88 g/plant for AMF+ and AMF- respectively (Table 3), results that are very similar to the ones obtained by Vargas et al. (2017). Similar results were also found by Sanmartín et al. (2014), where the fresh weight of AMF+ plants reached 196.4 g/plant, contrasting with 160.1 g/plant from AMF- plants. Sahin (2020) also found similar values for FWS on his experiment with iodine biofortification, where the control plants weighed around 175 g/plant. Interestingly, there were not any effects of the applied doses of Zn in FWS, contrasting with the research conducted by Graciano et al. (2020) where a 32% decrease in FWS was observed when plants received the highest dose of zinc (1600 g/ha), inhibiting plant growth. Sago et al. (2018) also found a drastic reduction on FWS of lettuce plants as higher doses of zinc were applied in the nutrient solution.

Also, considering that all plants were grown under an optimal irrigation regime, AMF inoculation played a major role in the uptake and translocation of water in the tissues of the shoots, optimizing the water uptake capacity of the plants, as described by Ruiz-Lozano & Azcón (1995), contributing to plants with greater values of FWS. In contrast, results obtained by Kohler et al. (2008) show that lettuce plants inoculated with *G. intraradices* and *G. mosseae* did not show higher content of water in shoots when compared to AMF- plants under different irrigation regimes.

Moraes (2020) found even higher numbers for FWS variable when Zn biofortifying two lettuce cultivars in different growing seasons, ranging between 234 and 210 g/plant for the cultivars ‘Saladela’ and ‘Vanda’ respectively. However, as more zinc was added to the soil, a linear decrease was observed for this variable, reaching an average of 144 g/plant under the highest applied dose (40 mg/dm^3). Additionally, it is important to mention that in the present study plants were harvested 14 days before the standard harvest time for its cultivar (49 DAT), in order to avoid having problems with *Bremia lactucae,* very common in that area. Presumably, if the plants had kept their growing rate, they would have achieved FWS numbers as high as found by Sousa et al. (2018), where plants ranged between 350.3 and 370.3 g/plant.

Despite the fact that lettuce can be cultivated during all growing seasons, these plants are more adapted to mild temperatures (around 15 and 24 °C), showing higher yields during the cool seasons in tropical countries as Brazil. Also, under higher temperatures, as faced during this study, lettuce plants tend to accelerate its growing cycle, producing smaller plants (Filgueira, 2013).

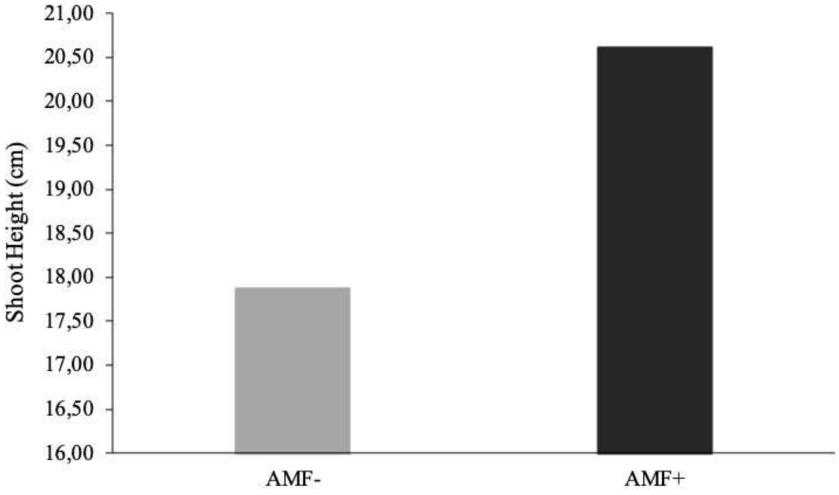

Figure 3. Shoot height of inoculated and non-inoculated plants at 35 days after transplantation. 2021. There were significant differences ($p < 0,01$) between the means of the treatments by the F test.

The application of ascending doses of zinc did not lead to effects on shoot height (SH) data. However, the inoculation with AMF increased shoot height regardless of the applied dose, with means varying between 17.87 and 20.61 cm for AMF- and AMF+ respectively, very similar to values (9.0-16.0 cm) found by Vargas et al., (2017) in their experiment regarding the performance of crispy lettuce cultivars in several soil covers. According to Blind & Silva Filho (2015), lettuce plants that contain larger leaves are usually most desirable for sale, which makes SH one of the most important variables on the consumer’s view. When evaluating Zn biofortification of four curly lettuce cultivars, Graciano et al. (2020) found similar values for SH, ranging between 15.76 and 19.58 cm, at 43 DAT. In this sense, as the increase of Zn fertilization does not decrease SH, it might be a great way to increase Zn daily intake by the population, once zinc is directly involved in many important reactions of the human body, including DNA/RNA and protein synthesis, cell growth and division, among others (Fukada et al., 2011).

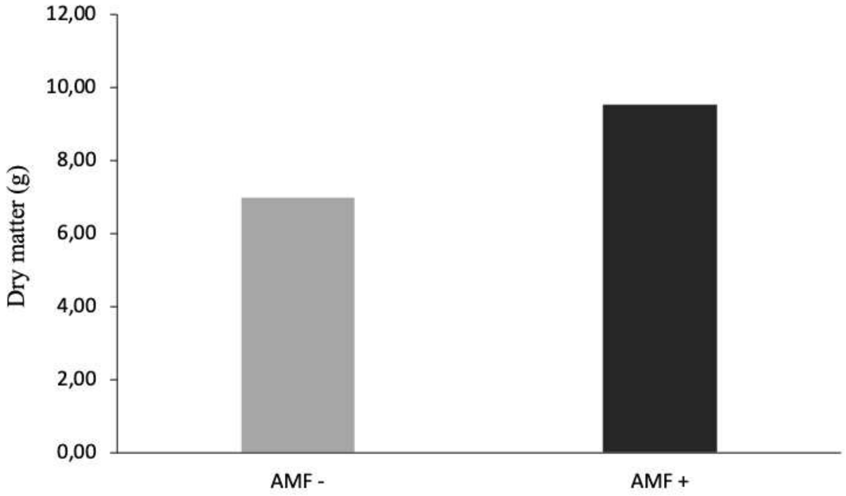

Figure 4. Dry matter of inoculated and non-inoculated plants at 35 days after transplantation. 2021. There were significant differences ($p < 0,01$) between the means of the treatments by the F test.

When it comes to dry matter (DM), inoculation with AMF increased shoot dry matter, being also influenced by Zn fertilization, as shown by figure 4. The dose that lead to the best levels of DM is around 36.75 mg/dm^3, presenting a decline on the following increasing doses.

Leafy vegetables are strongly influenced by micronutrients as Zn, once it has a significant ability to absorb them in excess, which may lead to symptoms of phytotoxicity, as shown by some of the plants from this study (Figure 5)(Alloway, 2008; Broadley et al., 2007; Chaney, 1993). Also, the soil used in the present study already contained a medium zinc content, around 175% more than the critical level (> 0.4 mg/dm^3), a condition that might have positively influenced the growth rates of the plants that did not receive any Zn fertilization, once Zn is closely related to growth hormones, nitrogen metabolism, among other processes that directly influence plant growth (Ribeiro et al., 1999).

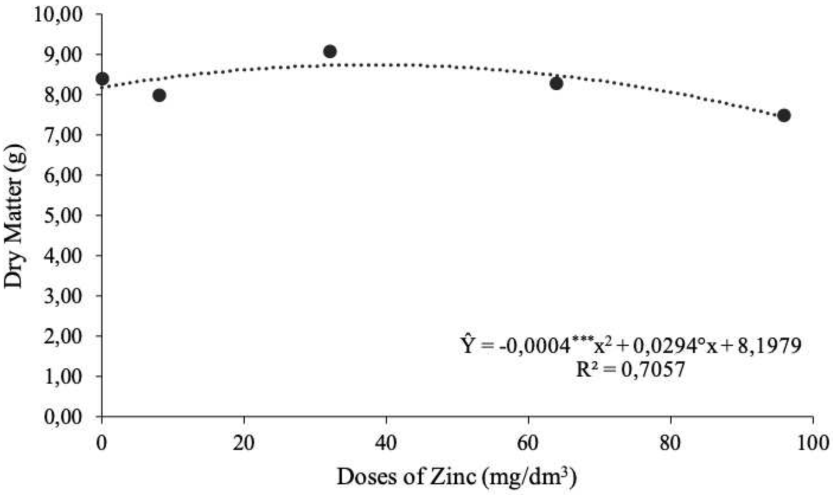

Figure 5. Dry matter (g) in different doses of zinc (0, 8, 32, 64, 96 mg/dm^3) in inoculated and non-inoculated plants at 35 days after transplantation. 2021. **1%, * 5%, *** 10%, ° > 10% of significance.

Likewise, Moraes (2020) observed a significant linear reduction in the DM of lettuce plants as zinc doses were increased, reaching 10.2 g/plant under the highest dose (40 mg/dm^3). Similarly, Barrameda-Medina et al. (2014) found that DM were significantly decreased when foliar zinc concentration achieved 218 mg/kg. In the present study, DM of lettuce plants reached an average of 9.53 g/plant in AMF+ and 6.98 g/plant in AMF-, similar to values found by other authors (H. de Almeida et al., 2020; Ferreira, 2016; Laurett et al., 2017; Sanmartín et al., 2014; Vargas et al., 2017), showing that despite the fact that the plants were harvested before achieving their maximum development, they were still suitable for commercialization. Also, the early harvesting ensured the absence of early bolting, a physiological process that induces undesirable traits for consumption, as latex production, that brings a bitter taste to lettuce leaves (Filgueira, 2013).

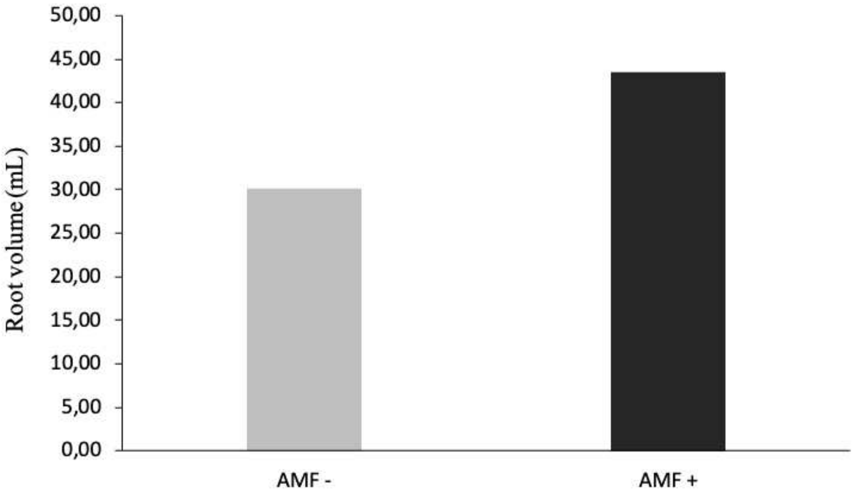

Figure 6. Root volume of inoculated and non-inoculated plants at 35 days after transplantation. 2021. There were significant differences ($p < 0,01$) between the means of the treatments by the F test.

Plants that were colonized by AMF showed a significant increase in root volume (RV) when compared to the non-inoculated ones (Figure 2), without a significant influence from zinc application, contrasting with the results obtained by de Almeida et al. (2020) and Watts-Williams et al. (2013), where plants showed a significant decrease in root volume as the levels of Zn were increased. Also, for having a significant degree of phenotypic plasticity, AMF+ root systems tend to occupy higher volumes due external hyphae expansion, exploring greater areas of soil and having access to nutrients that the plant itself would not be able to intercept (Heinonsalo et al., 2017; Smith & Read, 2008). As mycorrhizal fungi directly influence root morphology, root branching is an expected effect, favoring nutrient uptake when compared to the non-inoculated ones (Berta et al., 1993; Wang et al., 2017). Also, as root nutrition is improved, its architecture tend to be upgraded, as a response to different stimuli from growth regulator hormones and polyamines (Liu et al., 2018; Zhang et al., 2019, 2020). Therefore, AMF+ plants have a facilitate access to low mobility metal nutrients as P, Zn and Fe, as the extraradical hyphae tend to explore beyond the natural root absorbing area (Liu et al., 2000).

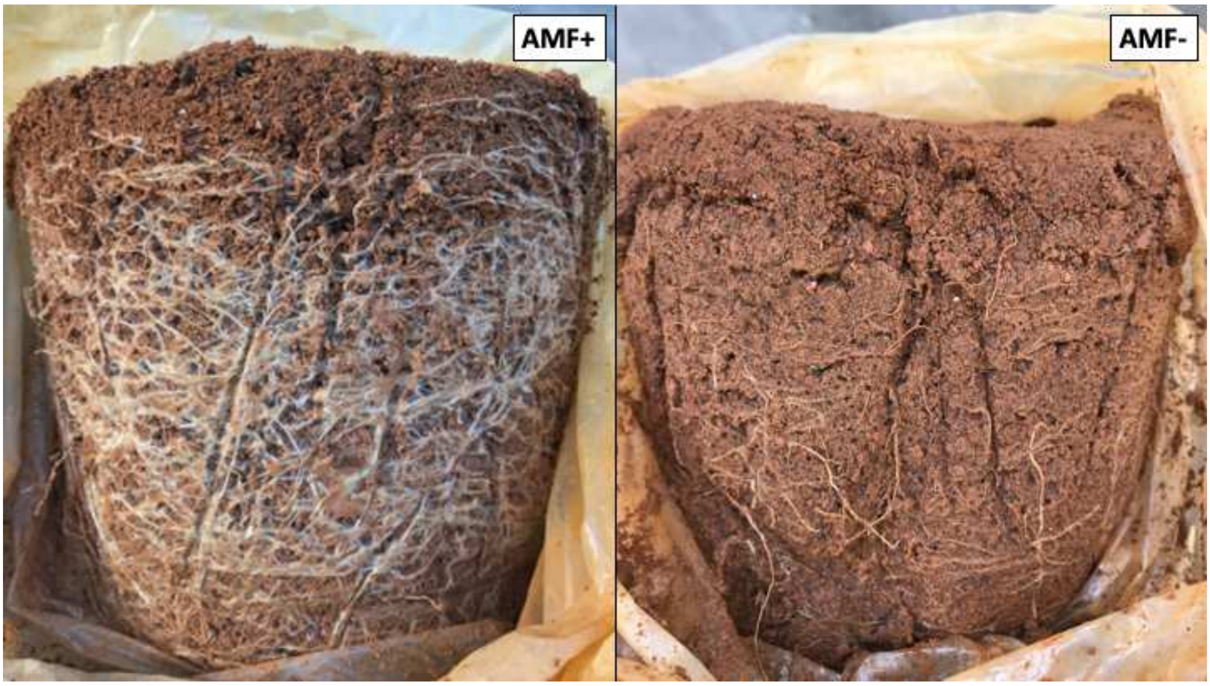

Figure 7. Differences in root volume between AMF+ and AMF– plants at 35 days after transplantation. Photo: Endy Kailer.

Figure 8. Differences in shoot height and leaf number between AMF+ and AMF– plants at 35 days after transplantation. Photo: Endy Kailer.

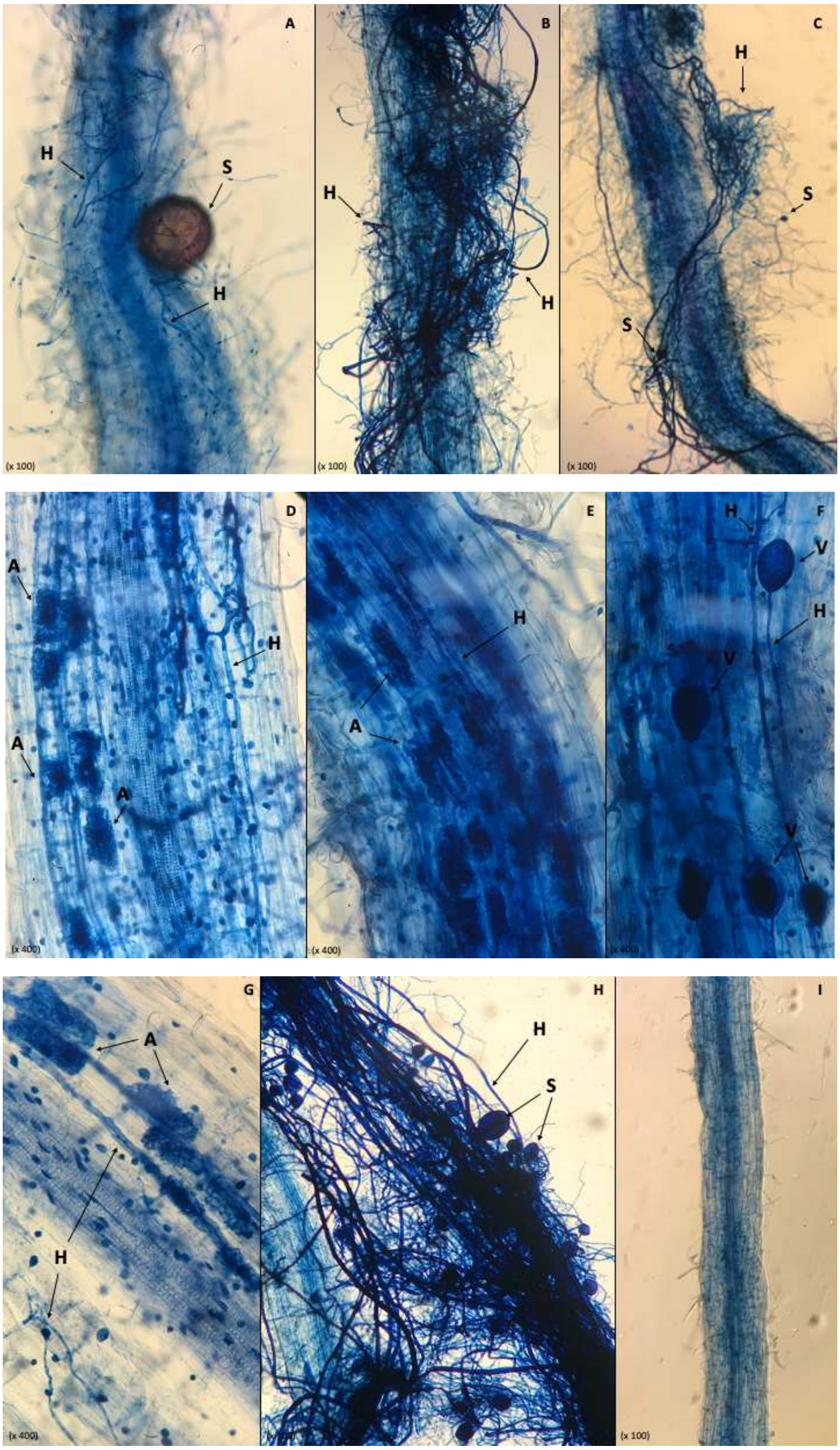

Figure 9. A-H = Microscopic images of roots from inoculated plants (AMF+) at 35 days after transplantation. Fungal structures: a = arbuscule; h = hyphae; s = spore; v = vesicle. I = Control plants (AMF-). Photo: Endy Kailer.

Figure 10. Symptoms of phytotoxicity in a D4 (96 mg/dm^3) plant at 35 days after transplantation. Photo: Endy Kailer.

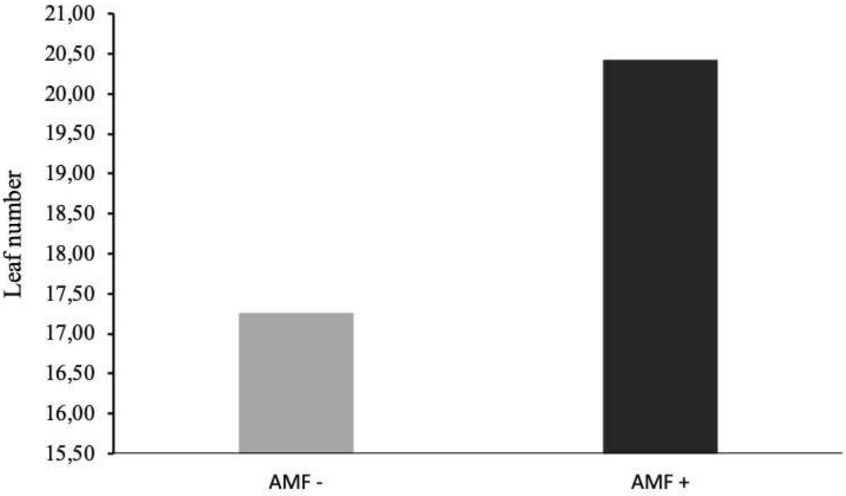

Figure 11. Leaf number of inoculated and non-inoculated plants at 35 days after transplantation. 2021. There were significant differences ($p < 0,01$) between the means of the treatments by the F test.

Considering that leaves are the edible parts of all lettuce plants, leaf number (LN) is one of the most important variables to determine whether the plant is suitable for commercialization (Filgueira, 2008). The number of leaves is also intimately related to how well-adapted that plant is to the environment that it has been cultivated, as well as to the temperature, luminosity, and characteristics of its surrounds (Diamante et al., 2013; Oliveira et al., 2004). Plants that present greater number of leaves also have a competitive advantage when it comes to sunlight interception, converting luminous into chemical energy, leading to well-developed and precocious plants (Taiz & Zeiger, 2017). The variable leaf number showed a significant positive effect of AMF inoculation, regardless of the zinc application, showing an average of 20.42 leaves/plant in AMF+ plants and 17.25 leaves/plant in AMF- (Figure 3), similar to the results found by Queiroz et al. (2014), Nespoli et al. (2017) and Vargas et al. (2017), where lettuce plants presented an average of 18 leaves/plant when cultivated with black mulching. Graciano et al. (2020) found a smaller number of leaves in Zn biofortified lettuce plants, ranging from 12.31 to 16.32 leaves/plant. On the other hand, De Lima, (2021) found greater number of leaves in the hydroponic Zn biofortification experiment, ranging from 20 to 28 leaves/plant, corroborating with data that showed that lettuce plants cultivated in hydroponic systems tend to grow 64.90% more than the same plants growing in soil (Steiner et al., 2009).

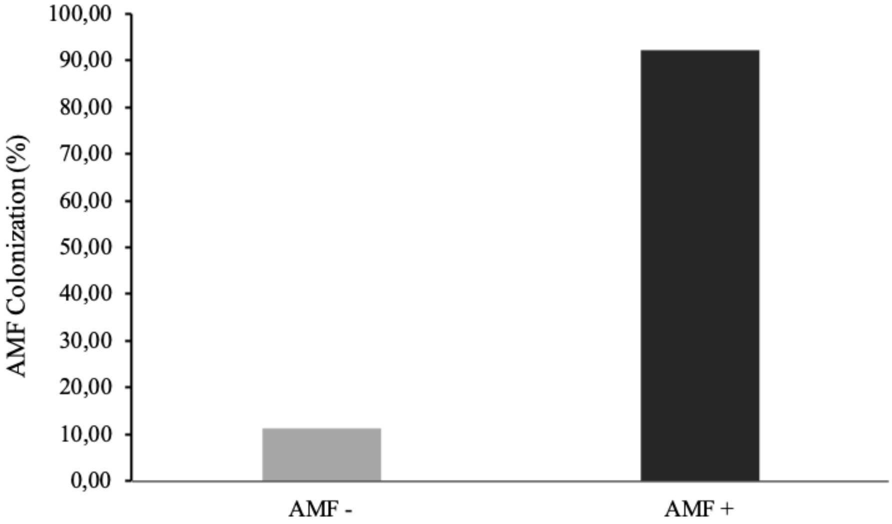

Figure 12. Mycorrhizal colonization of inoculated and non-inoculated plants at 35 days after transplantation. 2021. There were significant differences ($p < 0,01$) between the means of the treatments by the F test.

Plants that received the inoculum source presented an excellent colonization percentage (average of 92%), regardless the zinc fertilization, contrasting with the results obtained by Watts-Williams et al. (2013), where mycorrhizal colonization significantly decreased as zinc supply was increased in tomato plants. As a direct result of a great colonization, classic AMF structures as hyphae, arbuscules, vesicles, and spores were easily observed in analyzed root fragments (Figure 9). Also, a small amount of plants that did not receive the source of inoculum also presented a small number of AMF structures (average 11% of colonization) probably as a result of contamination. Interestingly, the prominent presence of arbuscules and hyphae in roots of inoculate plants indicates that the addition of crescent doses of zinc did not negatively influence the colonization, allowing the symbiosis relationship to be active up to the moment of the harvest (Sanmartín et al., 2014). When it comes to nutrient uptake and accumulation, some factors may influence on how helpful AMF would be for the lettuce plants, including the species that are present in the inoculant and the lettuce cultivar or variety to be produced (Baslam, et al., 2011).

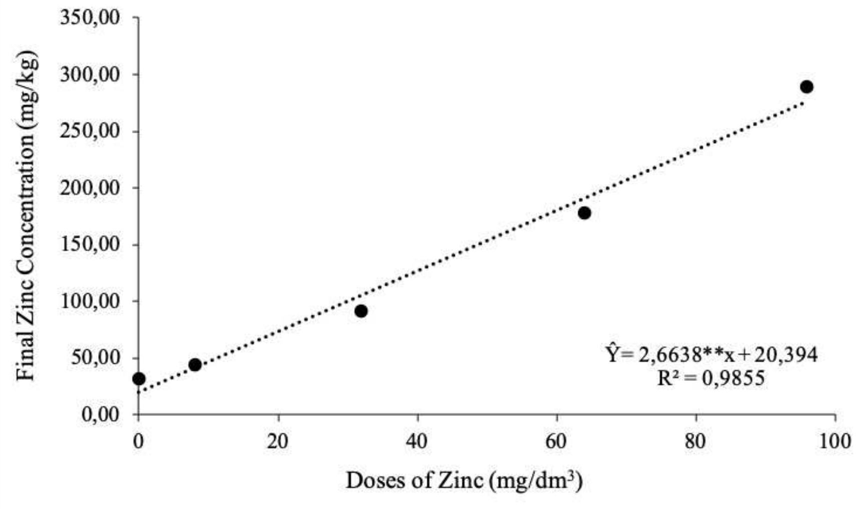

Figure 13. Final zinc concentration in leaves (mg/kg) in different doses of zinc (0, 8, 32, 64, 96 mg/dm^3) in inoculated and non-inoculated plants at at 35 days after transplantation. 2021. **1%, * 5%, *** 10%, ° > 10% of significance.

Consistent with what was expected, crescent Zn fertilization promoted a significant linear increase ($P < 0.01$) on zinc concentration in leaves (Figure 6), reaching an accumulation 25 times higher (517.93 mg/kg) with the highest dose when compared to the control one (20.30 mg/kg), regardless of the inoculation with AMF. As already discussed by Clemens (2017), *Asteraceae* plants might have a genetic susceptibility to hyperaccumulate minerals as Zn by vacuolar compartmentalization (Cappa & Pilon-Smits, 2014; Hall, 2002; Marschner, 1995; White & Broadley, 2011).

Among several mechanisms for Zn detoxification, its connection with phytic acid molecules is extremely important when it comes to biofortification, once phytic acid is a relevant antinutritional molecule that directly affects the nutrient absorption by the human body (Hunt, 2003). Insoluble complexes generally formed in the human digestive system, as polyphenols and phytates, are usually produced in more significant amounts in the digestion of grain crops, as well as in some staple foods (Luo & Xie, 2012; Wakeel et al., 2018). Leafy vegetables, on the other hand, do not show notable amounts of antinutritional factors, reinforcing its importance in biofortification programs, as in leaves nutrients are readily bioavailable for the human body (Broadley et al., 2012; Hunt, 2003; Sahin, 2020; White et al., 2018). As showed by Atsushi et al. (2020), Zn biofortified lettuce significantly increased Zn content in several parts of the human body, as the tibia, gastrocnemius, liver and kidneys.

Graciano et al. (2020) among other authors (H. de Almeida et al., 2020; Fontes et al., 2014; Meena et al., 2017; Moraes, 2020; Sago et al., 2018) also found a linear response on zinc concentration in the leaves as Zn fertilization was increased, finding 220 mg/kg of Zn when adding 1600g/ha of zinc sulphate. De Lima (2021), reported even higher values in the linear increase of Zn concentration, finding 733,3 mg/kg when adding 10,5 g/1000L in a nutrient solution for hydroponics. It is well-known that hydroponic systems are extremely efficient in providing nutrients, once the nutrients are readily available in a controlled solution. De Souza et al., (2019) found that lettuce plants cultivated in a hydroponic system accumulated more biomass than the ones cultivated in soil, probably due better nutrient availability. It is worth to point out that Zn uptake highly depend on many factors other than nutrient availability, being strongly influenced by the processes involved in root interception and penetration through the cuticle, intraradical transport, among others (Pearson & Rengel, 1995).

Table 2. Zinc content in a 100 g serving of biofortified lettuce leaves in different doses of zinc (0, 8, 32, 64, 96 mg/dm^3) and its contribution to achieve the recommended dietary allowances for Zinc, considering lettuce as the only source of zinc.

Nutrient		Lettuce Contribution to Zn Daily Intake (%) (100g serving)				
Gender	[1]Recommended Dietary Allowances for Zinc (mg/day)	D0	D1	D2	D3	D4
Male (19+)	11	28,73	40,28	83,44	161,63	262,94
Female (19+)	8	39,50	55,39	114,72	222,24	361,55
Pregnant (19+)	11	28,73	40,28	83,44	161,63	262,94
Lactating (19+)	12	26,33	36,92	76,48	148,16	241,03
Children (1-8)	4	79	110,77	229,45	444,47	723,10

[1]-National Institutes of Health, 2021.

Data obtained in this study showed that all plants, inoculated or not, got Zn biofortified. As lettuce is already daily present on the table of a great part of the population, its consumption would directly contribute to increase Zn intake, avoiding the negatives outcomes of hidden hunger without major changes in people's eating habits (White et al., 2018). Biofortified lettuce could also add value to the produce of many small-scale farmers, while it is still an affordable option for a great number of people, once lettuce is significantly cheaper than other standard Zn sources as red meat, seafood, and nuts (National Institutes of Health, 2021). Hypothetically, if lettuce was considered the only Zn source (Table 5), in order to meet Zn daily requirements of a male adult or a pregnant woman (11 mg/day), it would only be necessary to ingest 38,04 g of lettuce, contrasting with 348,10 g of the non-biofortified one (National Institutes of Health, 2021).

As shown on table 3, biofortified lettuce under 8 mg/dm^3 (D1) would already be enough for a child to achieve more than the recommended Zn daily intake, showing its potential as a powerful weapon in a war against hidden hunger, where around half a million children die every year as consequence of Zn deficiency (Black et al., 2008; Gibson, 2012; Krebs et al., 2014; Terrin et al., 2015). Likewise, at D3 (64 mg/dm^3), pregnant women would also dramatically reduce the chances of preterm delivery, fetal malformation, hemorrhage and maternal morbidity as it would provide more Zn than it is required for daily ingestion (King, 2000; Simmer & Thompson, 1985b).

Table 3. Zinc content in a 100 g serving of biofortified lettuce leaves in different doses of zinc (0, 8, 32, 64, 96 mg/dm^3) and its contribution to tolerable upper intake levels for Zinc, considering lettuce as the only source of zinc.

	Nutrient	Lettuce Contribution to Zn Daily Intake (%) (100g serving)				
Gender	Tolerable Upper Intake Levels for Zinc (mg/day)	D0	D1	D2	D3	D4
Male (19+)	40	7,90	11,08	22,94	44,45	72,31
Female (19+)	40	7,90	11,08	22,94	44,45	72,31
Pregnant (19+)	40	7,90	11,08	22,94	44,45	72,31
Lactating (19+)	40	7,90	11,08	22,94	44,45	72,31
Children (1-8)	9,5	33,26	46,64	96,61	187,15	304,46

[1]-National Institutes of Health, 2021.

When it comes to zinc toxicity, the present biofortified lettuce would only be unsuitable for children, on increasing doses after 32 mg/dm^3 (D2), for exceeding the recommended tolerable upper intake levels of zinc. Symptoms of Zn toxicity include diarrhea, abdominal pain, loss of appetite, nausea, vomiting and headaches (National Institutes of Health, 2021).

Table 4. Amount of biofortified lettuce in different doses of zinc (0, 8, 32, 64, 96 mg/dm^3) needed (g/day) to reach the recommended Zn daily intake, considering lettuce as the only source of zinc.

	Amount of Lettuce to Reach Zn Daily Intake (g/day)				
Gender	D0	D1	D2	D3	D4
Male (19+)	348,10	248,31	119,83	61,90	38,04
Female (19+)	253,16	180,59	87,15	45,02	27.66
Pregnant (19+)	348,10	248,31	119,83	61,90	38,04
Lactating (19+)	379,75	270,88	130,72	67,53	41,49
Children (1-8)	126,58	90,29	43,57	22,51	13,83

[1]-National Institutes of Health, 2021.

5. CONCLUSION

Mycorrhizal inoculation increased zinc uptake by plants. Inoculated plants accumulated more zinc, but the nutrient got diluted as plants were larger, leading to a decrease in Zn concentration when compared to non-inoculated treatments. Plants from this study were successfully biofortified, reaching Zn concentrations up to 517.93 mg/kg with the highest applied dose. Thus, more studies should be conducted regarding the use of soil microorganisms in agronomic biofortification in order to assist in decreasing worldwide consequences of hidden hunger.

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APPENDIX

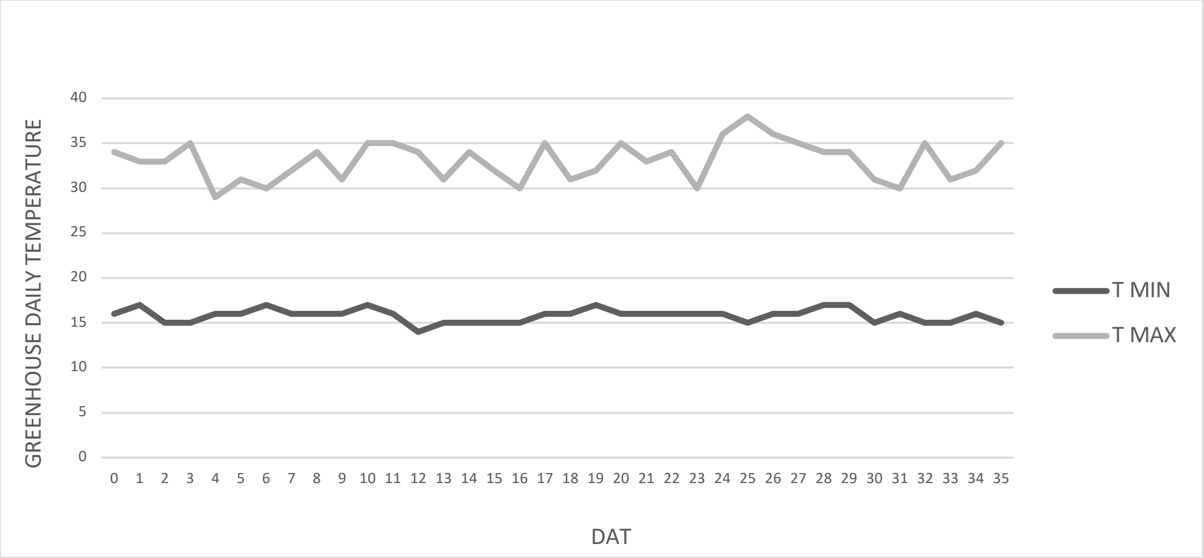

Figure 14. Minimal and maximum daily temperature inside de greenhouse during the course of the study.

Table 5. Analysis of variance for the characteristics: fresh weight of shoots (FWS), shoot dry matter (DM), root volume (RV), leaf number (LN), final zinc concentration (ZC), total zinc content (TZ) and colonization (COL).

	Mean Squares (MS)						
FV	FWS	DM	RV	LN	ZC	TZ	COL
Block	1429,1	6,479	131,80	6,995	2773	0,1941	137
Inoculum	24087,2**	112,548**	3115,56**	176,014**	5556^{NS}	1,5633**	114503**
Dose	601,0^{NS}	4,804^{NS}	27,34^{NS}	7,800^{NS}	161279**	7,9350**	42^{NS}
I x D	796,4^{NS}	2,793^{NS}	77,09^{NS}	8,943^{NS}	2328^{NS}	0,4183*	55^{NS}
Residual	475,9	2,581	91,21	3,821	3314	0,1463	116
CV (%)	19,06	19,46	25,92	10,37	45,35	38,69	20.83

*, ** = significant at 5 and 1 % probability by F test, respectively. NS- non-significant at 5% probability by F test.

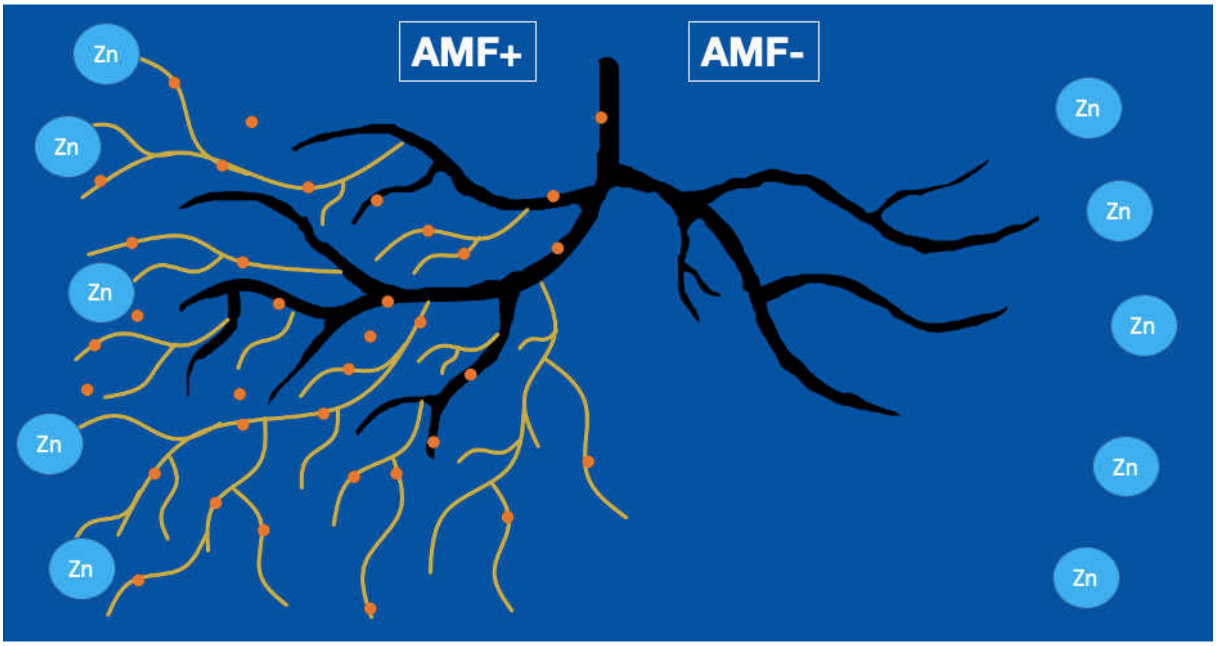

Figure 15. Root morphology with and without mycorrhizal inoculation. Source: Kailer, 2021.