INSTITUTO POLITÉCNICO DE BRAGANÇA
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# Combinatorial effect of *Rhizophagus irregularis* and *Trichoderma harzianum* on the Silicon accumulation in wheat and maize, and the improvement of wheat resistance against *Zymoseptoria tritici*

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"Success is not final, failure is not fatal: It is the courage to continue that counts." Winston Churchill

Reflecting on this journey, this quote resonates deeply with me. The journey to finishing this thesis has been filled with challenges and obstacles including meticulous and often frustrating experiments to late-night data analysis. There were moments of doubt and tiredness when the outcomes did not match expectations and the road ahead appeared difficult. However, it was only through perseverance, resilience, and the unfailing support of my family and friends that I discovered the fortitude to keep going.

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# Abstract

Wheat and maize are two of the most important crops in the world, however, pathogen transmission threatens global cereal supply. *Zymoseptoria tritici* causes septoria tritici blotch (STB), which inflicts the most yield-reducing disease on wheat production. One of the most promising solutions to control this disease is the use of specialized plant-beneficial microbes like arbuscular mycorrhizal fungi (AMF) and *Trichoderma* spp. Also, silicon (Si) has been considered to induce protection against pest infection.

The present study aimed at studying the effect of the AMF *Rhizophagus irregularis* MUCL 43194, alone or combined with *Trichoderma harzianum* MUCL 29707, to improve plant growth and Si uptake in wheat and maize plants, and the impact of the three factors to control STB on durum wheat, thus working as biostimulants. For this, two experiments were developed. In the first, after 5 weeks of pre-colonization with *Rhizophagus irregularis* in trays, the wheat and maize plants were transferred to individual pots with the respective treatments (with addition of Si and presence of *T. harzianum*) and harvested 30 and 45 days after transfer. The second experiment was as previously described, but with the inoculation of the pathogen *Z. tritici* on the leaves 30 days after the moment of plant transfer. The parameters analyzed were fresh and dry weight of the aerial part and roots, the Si concentration in the leaves and the Si content in the plant, the percentage of AMF colonization and, for the second experiment only, the disease severity.

Results showed that, in the first experiment, for maize plants the interaction between AMF and *T. harzianum* was negative, since it reduced the growth and biomass of maize plants and did not impact the Si uptake and accumulation. AMF treatment alone was the best treatment for improving the growth and colonization of maize plants. For wheat plants, in root growth and biomass, AMF and *T. harzianum* had a positive interaction. However, the same was not observed for the Si accumulation and uptake. So, for the growth and biomass of wheat roots, the use of AMF or both fungi is recommended, whereas the use of Si is rejected. For improved Si accumulation and uptake, the use of this mineral alone is a better fit. Regarding the second experiment, the use of

microorganisms and Si did not prove to increase the tolerance against the disease efficiently.

Further studies are necessary to elucidate the interaction between the two fungi in these plants by applying Si.

**Keywords:** Plant-beneficial microbes, arbuscular mycorrhizal fungi, phytopathology

# Resumo

O trigo e o milho são duas das culturas mais importantes no mundo, no entanto, a transmissão de agentes patogénicos ameaça o fornecimento global de cereais. *Zymoseptoria tritici*, agente causal da septoriose do trigo (*septoria tritici blotch*) causa a maior redução de produtividade na produção de trigo. Uma das soluções mais promissoras para o controlo desta doença é a utilização de organismos com efeitos benéficos na planta, como fungos micorrízicos arbusculares e *Trichoderma* spp. Também o Silício (Si) foi considerado útil na indução da proteção contra ataque de pragas.

O presente trabalho teve como objetivos avaliar o efeito do fungo micorrízico *Rhizophagus irregularis* MUCL 43194, isolado ou combinado com *Trichoderma harzianum* MUCL 29707, no crescimento da planta e na acumulação de silício no trigo e no milho, e o efeito destes três fatores no controlo da septoriose no trigo. Para tal, foram desenvolvidos dois ensaios. No primeiro, após 5 semanas de pré-colonização com *R. irregularis* em tabuleiros, as plantas de trigo e milho foram transferidas para vasos individuais com os respetivos tratamentos (com adição de Si e presença de *T. harzianum*) e colhidas 30 e 45 dias após a transferência. No segundo ensaio, equivalente ao anterior, o agente patogénico *Z. tritici* foi aplicado nas folhas 30 dias após o momento de transferência das plantas. Os parâmetros analisados foram peso fresco e seco da parte aérea e das raízes das plantas, a concentração de Si nas folhas e teor de Si na planta, a percentagem de colonização do fungo micorrízico e, apenas para o segundo ensaio, a severidade da doença.

Os resultados mostraram que, para plantas de milho, a interação entre AMF e *T. harzianum* foi negativa, uma vez que reduziu o crescimento e a biomassa das plantas de milho e não teve impacto na captação e acumulação de Si. Assim, o tratamento apenas com AMF foi o melhor tratamento para melhorar o crescimento e a colonização de plantas de milho. Para as plantas de trigo, no crescimento radicular e na biomassa, AMF e *T. harzianum* tiveram interação positiva. No entanto, o mesmo não é observado para a acumulação e absorção de Si. Assim, para o crescimento e biomassa de raízes de trigo, recomenda-se a utilização de AMF ou ambos os fungos, enquanto a utilização de Si é rejeitada. A

acumulação e absorção de Si foi mais favorável quando este mineral foi aplicado de forma isolada. O uso de microrganismos e de Si não mostrou aumentar a tolerância das plantas de trigo contra a septoriose.

São necessários mais estudos para elucidar a interação entre os dois fungos e a aplicação de Si nestas plantas.

**Palavras-chave:** microrganismos benéficos, fungos micorrízicos arbusculares, fitopatologia

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# I. Introduction

## I.1. Framework

Wheat (*Triticum* spp.) and maize (*Zea mays*) are two of the most important crops in the world and, in 2022, annual productions in Europe achieved 282 million tons and 102 million tons, respectively (FAOSTAT, 2024).

However, a constant pressure to increase quality and yield, in the pursuit of elite high-performing cultivars, results in a reduction in genetic diversity and adaptability, which contributes to pathogen susceptibility to the point where diseases threaten global wheat supply (Figueroa et al., 2018).

*Zymoseptoria tritici*, an ascomycete fungus, causes septoria tritici blotch (STB), which inflicts the most yield-reducing disease on wheat production (McDonald et al., 2015; Kettles & Kanyuka, 2016; Barakat et al., 2021). Fungicides are frequently used to control this disease (Stocco et al., 2015). However, the regular use of fungicides results in fungicide resistant and/or less fungicide sensitive strains (Figueroa et al., 2018; Birr et al., 2021). Under this scope, innovative and environmentally friendly alternatives to STB control are mandatory and urgent.

One of the most promising solutions is the use of specialized and/or plant beneficial microbes that interfere with plant pathogens and pests (Saba et al., 2012). Biological control, or *biocontrol*, is the exploitation of living agents to combat pathogens, pests, and weeds for diverse purposes to provide human benefits (Stenberg et al., 2021). Among them, arbuscular mycorrhizal fungi (AMF) and *Trichoderma* spp. can protect plant against pathogens infection (Sarkar & Sadhukhan, 2022; Asad, 2022).

Arbuscular mycorrhizal fungi (AMF) can improve the resistance/tolerance of their host plant, via the production of antimicrobial compounds, as well as triggering disease resistance and defense systems in plants (Weng et al., 2022). Other fungi can directly attack pathogens and protect plants against pathogens infection. Among them, *Trichoderma harzianum*, a saprotrophic fungus, is an opportunistic, avirulent plant colonizer that can act as parasite and antagonist of numerous plant pathogens and has been employed to manage foliar diseases in many crops (De Jaeger et al., 2011; Stocco et al., 2015).

In addition, silicon (Si) has been considered to induce protection against pest infection. This may be related to its accumulation and polymerization in the cells, providing a mechanical barrier that makes pest infection harder (Gomes et al., 2005). Furthermore, it has been reported to enhance plant defense systems by eliciting defense genes (Gbongue et al., 2019).

To date, no studies focused on the potential improved accumulation of Si in leaves of mycorrhizal- and *Trichoderma*-associated wheat and maize plants and on the effect of the combination of the three biostimulants on the maize and wheat growth, as well as protection of wheat against foliar disease caused by *Z. tritici*.

## I.2. Objectives

The present study thus aimed at studying the effect of the AMF *Rhizophagus irregularis* MUCL 43194, alone or combined with *Trichoderma harzianum* MUCL 29707, to improve plant growth and Si uptake, and the effect of the three factors to control STB on durum wheat, thus working as biostimulants. According to this, the following tasks were addressed:

1. Evaluate the compatibility of the AMF and *T. harzianum* strains in the rhizosphere of wheat and maize, by evaluating the intraradical growth of the AMF.

2. Evaluate the effect of the combination of both fungi on plant Si uptake by wheat and maize plants.

3. Determine the impact of the combination of the AMF, *T. harzianum* and Si on the growth stimulation of wheat and maize, as well as on the biocontrol of STB on wheat plants.

It is expected that this research will shed light on the role of these organisms and mineral on plant growth and defense against pathogen.

# II. Literature review

## II.1. The importance and evolution of agriculture

The agricultural revolution was an important turning point in human history (Braidwood, 1960). This revolution enabled the domestication of plants through the human management and the use of soil and water resources. This allowed society to thrive in complex and urban civilizations, eventually leading to the development of modern life (Parikh & James, 2012). However, modern agriculture led to a dependence on a small number of genetically uniform crops. This fact, along with frequent and severe mechanical soil disruptions (that leads to erosion, pollution, and soil deterioration) and decrease of the diversity of natural pest enemies, results in pest outbreaks (Wilby & Thomas, 2002; Wilkinson et al., 2019).

Given these threats, along a with world's growing population, increasing agricultural productivity is critical to achieve food security, as a vital component of health and driver of economic growth (Duveiller et al., 2007; Lipper et al., 2014).

## II.2. The importance of wheat and maize crops worldwide

Cereal grains are the fruit of cultivated grasses. They give more nourishment to humans than any other food type, accounting for roughly half of overall caloric requirements. While there are over a dozen cereal crops used for food, only wheat, maize, and rice are essential human food sources, accounting for 94% of total cereal consumption (Ranum et al., 2014).

Wheat is one of the oldest and most important crops, providing a considerable supply of calories and protein in human diets. In 2022, annual wheat production in Europe achieved 282 million tons, and globally reached 808 million tons worldwide, an increasing production since 2018 on a constant cultivated area (Figure 1) (FAOSTAT, 2024). This crop can also be used for animal feed, alcohol distillation, and as a raw material for biofuels (Fones & Gurr, 2015).

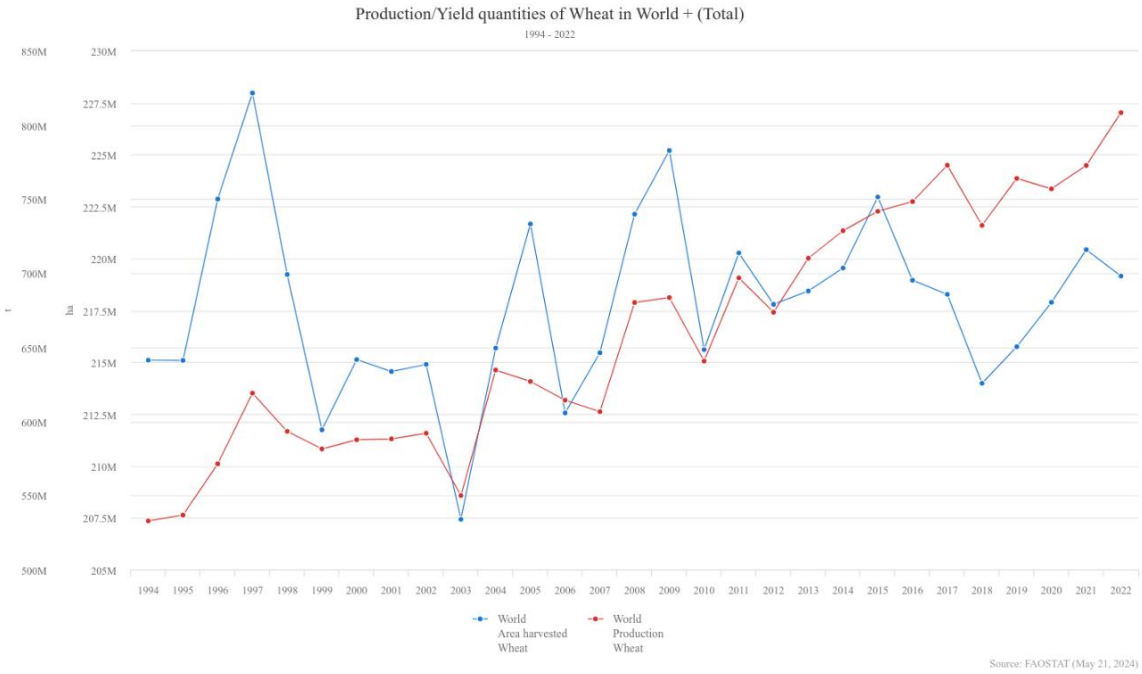


**Figure 1-** Wheat production worldwide in tonnes per hectare, from 1994 to 2022 (FAOSTAT, 2024).

Wheat is a member of the genus *Triticum*, which has many species, but only two are widely grown commercially: *Triticum aestivum* (common winter wheat) and *Triticum durum* (durum wheat) (Golovnina et al., 2007; Uthayakumaran & Wrigley, 2010). Because of its distinctive properties and end products, such as pasta, couscous, and numerous forms of bread, durum wheat is an economically important crop (Elias, 1995; Troccoli et al., 2000). It is commonly produced as a spring crop in Central Europe, with sowing in April and harvest in August due to its frost susceptibility (Longin et al., 2013). On the other hand, winter wheat is used for bread, cakes, pastries, biscuits, puddings and noodles (Uthayakumaran & Wrigley, 2010).

Maize (*Zea mays*) is one of the most important cereal crops worldwide. This cereal is used to produce food, fuel, and as a raw material for diverse products at industrial level (Ranilla, 2020). Maize annual production reached 102 million tons in Europe (Figure 2) in 2022 (FAOSTAT, 2024).

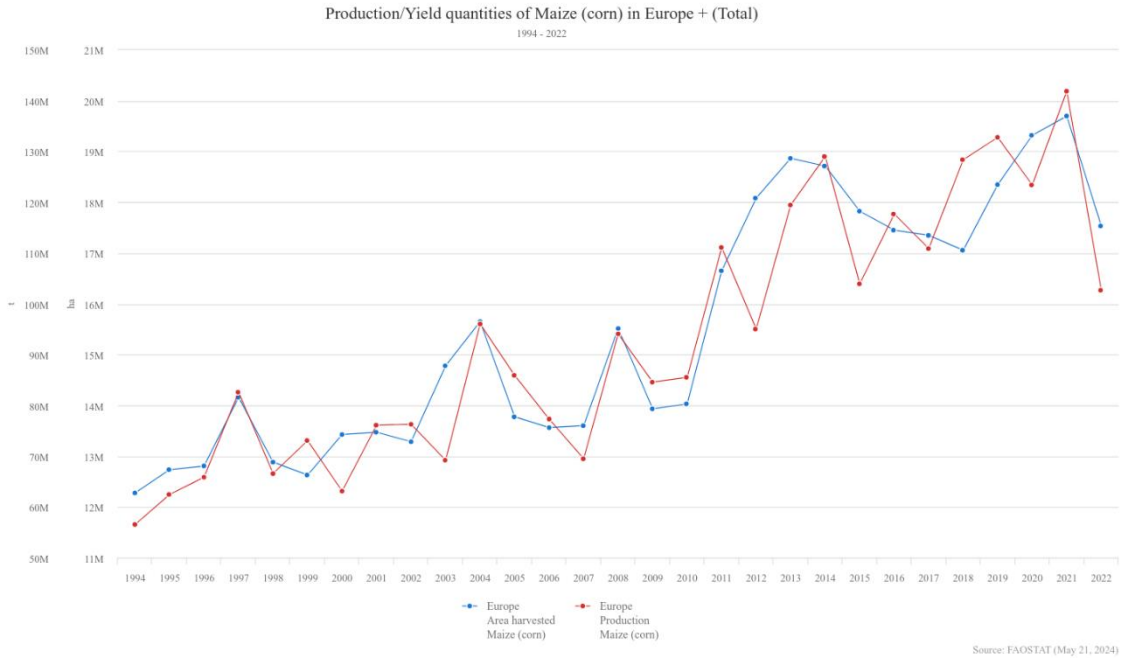


**Figure 2-** Maize production in Europe in tonnes per hectare, from 1994 to 2022 (FAOSTAT, 2024).

Maize is thought to have been one of the earliest plants cultivated by farmers, between 7,000 and 10,000 years ago, with evidence of its use as food found at some archeological sites in Mexico (Ranum et al., 2014). It originated in Central America and spread to Europe and other continents during Columbus' travels at the end of the 15th century. Because of maize's excellent adaptability, farmers have developed a wide range of genetic resources with diverse adaptations, traits, and applications (Revilla et al., 2022).

A constant pressure to increase quality and yield, in the pursuit of elite high-performing cultivars, results in a reduction in genetic diversity and adaptability, which contributes to pathogen susceptibility to the point where diseases threaten global wheat supply (Figueroa et al., 2018).

## II.3. Septoria tritici blotch (STB) as an alarming wheat disease

Pathogenic fungi are a major limitation to wheat productivity. Rust pathogens and Ascomycete fungus are two examples of dangerous fungi for wheat production (Figueroa et al., 2018). The ascomycete fungal pathogen of wheat, *Zymoseptoria tritici*, is the causal

agent of Septoria tritici blotch (STB), a worldwide significant disease that threatens food security (Chen et al., 2023).

### II.3.1 *Zymoseptoria tritici*: the causal agent of STB

*Zymoseptoria tritici*, an fungus causing septoria tritici blotch (STB), inflicts the most yield-reducing disease on wheat production, which makes it the most important foliar disease of wheat worldwide (Figure 3) (McDonald et al., 2015; Kettles & Kanyuka, 2016). This fungus has the ability to damage the leaves and disrupt the grain filling process, making it also the most economically damaging disease of wheat in Europe, with an estimated €1 billion per year in fungicide expenditure directed toward its control, as well as a 30-50% reduction in wheat yields (Stocco et al., 2015; Kettles & Kanyuka, 2016; Duba et al., 2018).

**Figure 3-** Leaves of wheat damaged by *Zymoseptoria tritici* (Source: Ponomarenko et al., 2011).

### II.3.2. *Zymoseptoria tritici* infection cycle

The infection cycle of *Z. tritici* is divided in two stages (Figure 4). It starts with its inoculation onto the leaf surface, where spores germinate and invade the plant through the stomata. After that, the fungus undergoes a prolonged asymptomatic phase of very

slow hyphal growth in the apoplastic space between mesophyll cells. This asymptomatic phase usually lasts 7-10 days (Kettles & Kanyuka, 2016). The next phase, frequently referred to as 'necrotrophic', is characterized by large-scale reprogramming of both host and pathogen transcriptomes. A strong activation of host defense responses culminating in apoptotic-like cell death and nutrient release into the leaf apoplast, results in a significant buildup of fungal biomass. Then, dead leaf areas expand to form irregularly shaped blotches (lesions) in which fungal asexual sporulation structures develop. These erupt and release pycnidiospores, which may cause additional rounds of infection if they are transmitted to healthy tissues via rain splash (Kettles & Kanyuka, 2016).

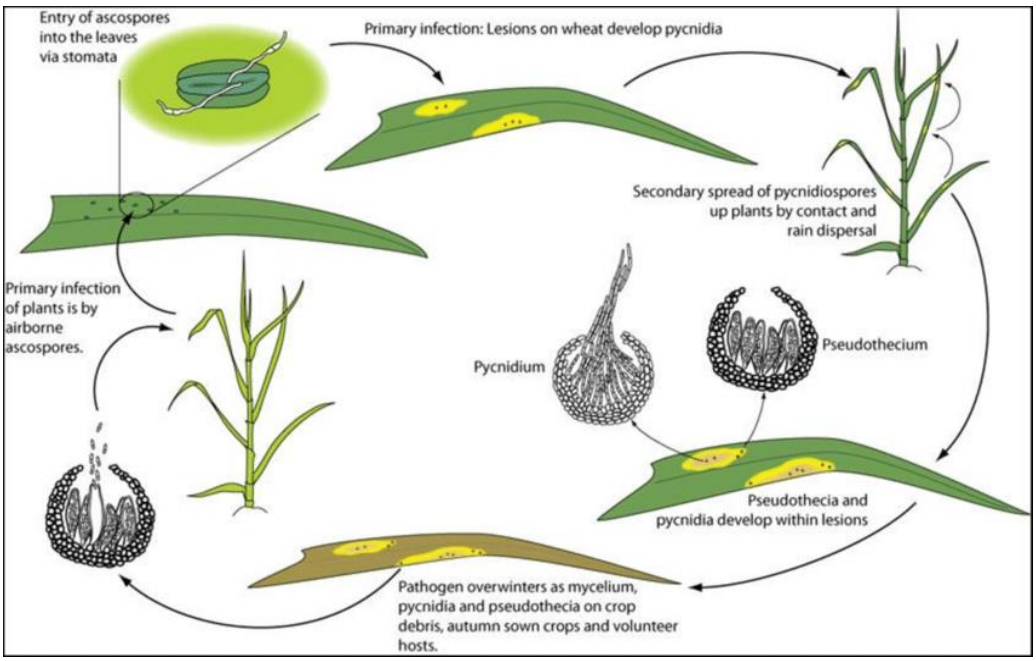


**Figure 4-** Life cycle of the fungal wheat pathogen *Zymoseptoria tritici* (Source: Ponomarenko et al., 2011).

The incidence, course, and severity of STB outbreaks are determined by climatic conditions such as constant precipitation, moderate temperatures, and high humidity (Prahl et al., 2023). Incidence is the proportion (or percentage) of plants within a population that show symptoms of a particular disease, indicating if a plant is either affected or not affected. On the other hand, severity is the number of disease-affected entities within a sampling unit, which signifies the area of plant tissue afflicted by disease (Seem et al., 1984).

## II.4. Approaches for controlling STB and its causal agent

Fungicides are frequently used to control wheat diseases (Stocco et al., 2015). In Europe, STB control accounts for over 70% of yearly fungicide consumption (Birr et al., 2021). These fungicides include quinone outside inhibitors, succinate dehydrogenase inhibitors, demethylation inhibitors, and multi-site fungicides (Torriani et al., 2015). However, the regular use of fungicides to reduce STB results in an increase in resistance of the pathogen and less fungicide sensitive strains within the *Z. tritici* population (Birr et al., 2021).

Other issues related to the use of fungicide are pollution of the atmosphere, water, and soil, as well as impact on non-targeted organisms, particularly key pollinators and humans via the food chain, harming human health (Rosa et al., 2022; Weng et al., 2022). Furthermore, the Regulation (EC) No. 1107/2009 of the European Commission (EC, 2009) has introduced strong limitations to the use of several fungicides and to new approvals due to environmental concerns, stressing even further the challenges with wheat disease control. As a result, new tools and methodologies are required to create an integrated disease management strategy (McDonald et al., 2015). There is currently a need for environmentally friendly biocontrol agents that can help to tackle some of these issues.

A promising solution more and more developed is the use of specialized microbes that interfere with plant pathogens and pests, in a natural, ecological way to overcoming the challenges produced by traditional chemical techniques of plant protection (Saba et al., 2012).

## II.5. Biocontrol as an eco-friendly alternative to chemicals

Biological control, or *biocontrol*, is the exploitation of living agents to combat pathogens, pests, and weeds for diverse purposes to provide human benefits (Stenberg et al., 2021).

One of the disease control strategies currently on the table is the increase of plant disease resistance by using microorganisms and molecules they produce. Indeed, the use of short peptides and double-stranded RNA molecules can be used as fungicide alternatives. These agents are highly selective, rapidly degradable, and efficient at low

dosages; yet these technologies still require the resolution of critical difficulties concerning their design, manufacture, and delivery (Rosa et al., 2022).

Plant growth promoting microorganisms (PGPM) are microbes that have beneficial effects on plants. They can be bacteria (such as *Bacillus spp.* and *Rhizobia spp.*) and fungi (particularly *Trichoderma spp.*). The usage of these inoculants has resulted in an average of 10-20% increase in crop productivity (Sellitto et al., 2021). Their biocontrol effects include direct competition for nutrients and space, for photosynthetic products, antibiosis, changes of rhizospheric microbial composition, as well as induction of host resistance. Furthermore, they may manage the disease indirectly by root damage compensation, plant health improvement, morphological alteration of the plant root, and/or induction of plant resistance (Al-Asbahi, 2012; El-Sharkawy et al., 2018). Among them, arbuscular mycorrhizal fungi (AMF), associated to plant roots, and *Trichoderma* spp., are able to protect plant against pathogens infection (Sarkar & Sadhukhan, 2022; Asad, 2022).

## II.6. Arbuscular mycorrhiza fungi and *Trichoderma* as plant beneficial microorganisms

Arbuscular mycorrhiza fungi are obligatory biotrophs with plants, with which they make a mutualistic association with plants (Al-Asbahi, 2012; Baum et al., 2015; El-Sharkawy et al., 2018). This symbiosis played a critical role in the early colonization of land by plants, as evidenced by molecular analyses and investigations of fossilized material (Rosendahl, 2008; Al-Asbahi, 2012).

The symbiosis between AMF and the host plant is thought to be initiated via reciprocal signal exchange by coordination. A complex sequence of biochemical and cytological activities, as well as intracellular alteration, are associated with the penetration and intercellular development of the AMF into cortical cells of the root plant, creating branched hyphae known as arbuscules (Figure 5) (Al-Asbahi, 2012). Upon the success of AMF penetration, this fungus will produce lipid storage vesicles, which are thick-walled, irregular lobes that are intraradically formed either within or between host cells (Figure 5) (Choi et al., 2018).

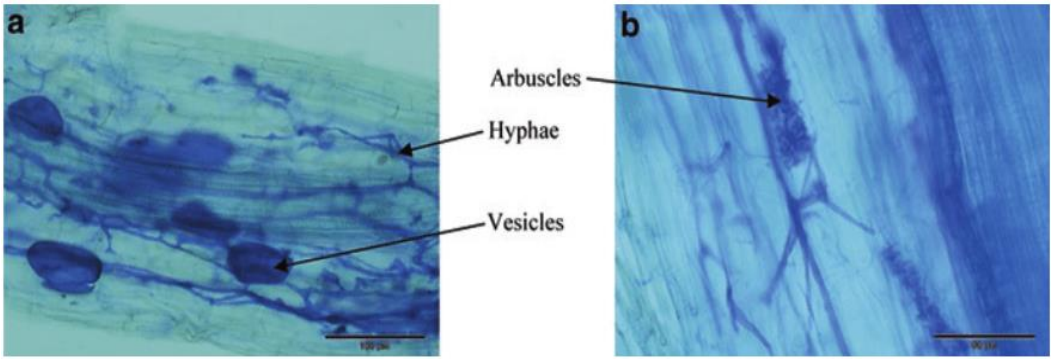


**Figure 5-** Microscopic visualization of arbuscular mycorrhizal fungi (a) showing vesicles and hyphae; (b) showing arbuscules (Source: Akhtar et al., 2011).

The *Trichoderma* genus includes a group of anamorphic filamentous fungi, characterized by a rapid growth and production of large amounts of conidia whose pigmentation can vary from dark to light green. They grow naturally in different habitats in a wide range of climatic zones from polar to equatorial latitudes; hence, they are the most isolated fungi from the soil (Poveda et al., 2020). *Trichoderma spp.* are ubiquitous colonizers of cellulosic materials and can thus often be found wherever decaying plant material is available as well as in the rhizosphere of plants (Schuster & Schmoll, 2010).

## II.7. Mechanisms of protection conferred by beneficial microorganisms

The AM fungus provides numerous benefits to the host plant, including interactions with other microorganisms in the soil around the fungal hyphae, whose synergistic effects increase plant growth. In the absence of symbionts, a rapid depletion of mineral nutrients is measured in the rhizosphere, whereas the high volumes of soil explored by the mycelium of AMF are able to take up nutrients such as nitrogen and phosphate and water to transfer them to the host plant (Figure 6). In return, up to 20% of plant-fixed carbon is transferred to the fungus (Baum et al., 2015; Etesami et al., 2021).

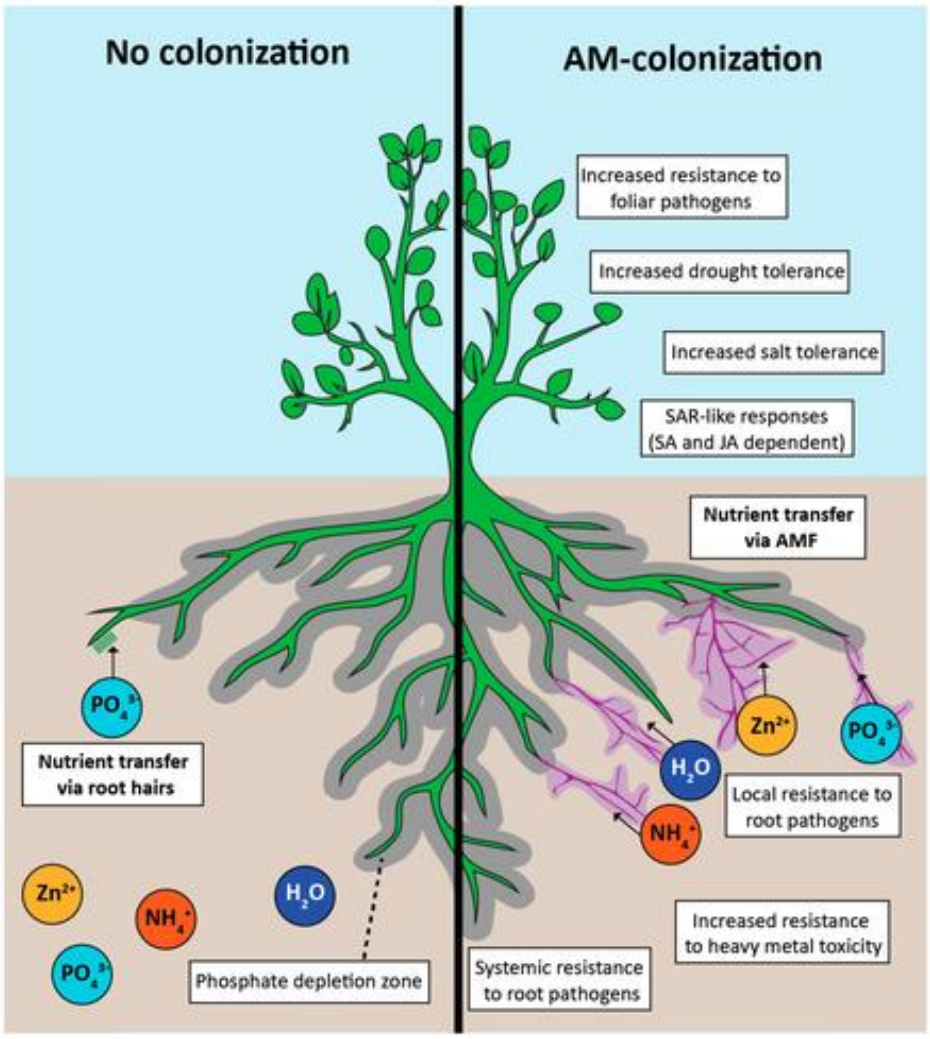


**Figure 6**- Comparison of the benefits between non-colonized plants and plants colonized with AMF (Source: Jacott et al., 2017).

Arbuscular mycorrhiza fungi have also been linked to a decrease in pest infection via increased plant response (Willis et al., 2013; Garg & Singh, 2018). In wheat, it has been proven that AMF inoculation substantially decreased the negative effects caused by the pathogen *Fusarium pseudograminearum*, suggesting that AMF inoculation significantly reduces pathogen population density and disease severity (Spagnoletti et al., 2021). Another study showed that AMF was able to control STB on bread wheat (Allario et al., 2022). In maize, a study showed improved plant growth and grain yield when combined with AMF inoculum (Cozzolino et al., 2013).

For the defense activation on the plant by AMF, recognition is the first step, where several volatile compounds are secreted by the plant roots that are sensitized by the AMF and induces its germination of spore. During this phase, a small molecule known as *myc* factor is produced. The host plant senses the presence of this molecule and triggers

downstream responses including $Ca^{2+}$ responses that finally lead to the establishment of a symbiotic relationship with the host plant. A Ca elevation induces an increment in the reactive oxygen species (ROS) generation which is an initial step of active defense, being superoxide anion and hydrogen peroxide the main ROS produced. At the site of pathogen infection, ROS are the major responsible factors for the membrane destruction, resulting in hypersensitive response or systemic resistance by inducing oxidative burst. Peroxide exhibits antimicrobial activity by inhibiting fungal spore germination as well as participates in phenoxyl-radicals synthesis during phenol-polymerization within the plant cell wall (Maharshi et al., 2019).

The saprotrophic fungi *Trichoderma* spp. are generally avirulent root endophytes that can act as parasites and antagonists of numerous plant pathogens and have been employed to manage foliar diseases in many crops (De Jaeger et al., 2011; Stocco et al., 2015; Abdullah et al., 2021; Guzmán-Guzmán et al., 2023). Commercially, *Trichoderma* spp. represent one-third of all fungal biocontrol agents (El-Sharkawy et al., 2018). Examples of these products are Anti-Fungus, Biofungus, Harzian 20 and Binap-T&W (Ghazanfar et al.,2018).

Its biocontrol activity has been attributed to several mechanisms of action (Barakat et al., 2021; Poveda, 2021; Guzmán-Guzmán et al., 2023):

(1) competition for nutrients and space in roots,
(2) antibiosis, via the production of antifungal metabolites, such as triol and octaketide,
(3) mycoparasitism via the production of specific cell wall degrading enzymes such as chitinases and proteases,
(4) induction of systemic resistance, and stimulation of plant growth via an increased nutrition. *Trichoderma* spp. colonizes the outer layers of the plant root and activate the systemic plant defenses against the infection of pests and/or pathogens.

*Trichoderma* also modulates the composition of and interactions with other microorganisms and brings numerous benefits to its host, such as plant growth promotion (Woo et al., 2022).

*Trichoderma harzianum* has been shown to have biocontrol actions on wheat against *Fusarium solani* through competition, antibiosis, and eliciting plant defense

responses (Guzmán-Guzmán et al., 2023). Two formulation types, spore suspension and the coated-seed technique, containing various *T. harzianum* strains, were found to decrease the incidence and severity of STB in wheat during the early stages of the disease (Perelló et al., 2009). In maize, *T. harzianum* caused increases in growth parameters, chlorophyll content, starch content, nucleic acids content, total protein content and phytohormone content of maize plants (Akladious & Abbas, 2013).

The use of these two plant beneficial microorganisms could thus help plant fight against pathogens, but currently, little is known about their interaction. Knowing that *Trichoderma* spp. are mycoparasites, some studies focused on their interaction with AMF.

## II.8. Interaction between AMF and *Trichoderma*

Melon crops, inoculated with *Glomus intraradices* and *T. harzianum* produced a more effective control of Fusarium wilt than with AMF inoculated alone (Martínez-Medina et al., 2011). Another study showed a growth increment in tomato plants when AMF and *Trichoderma* were combined (Singh, 2015). It has also been reported that the presence of *T. harzianum* significantly increased root colonisation by several AMF species, reaching values significantly higher than the most effective AMF (*Funneliformis mosseae*) inoculated alone (Pascual, 2016).

## II.9. Silicon as chemical biostimulants useful for plants resistance against pathogen infection

Besides the use of biological control agents, biostimulants are also considered useful in the management of biotic stresses. Elements, among them silicon (Si) were demonstrated to improve plant resistance against pathogen infection. Si dioxide ($SiO_2$) is the most prevalent form in soil and is the second most abundant element in the lithosphere (about 28%). The majority of Si is found in silicate minerals, and only a small part of the Si present in nature is available to plants. However, Si has not been identified as an important nutrient for plant growth and development. Despite that, due to its beneficial characteristics, particularly when subjected to various conditions such as drought, heavy metal toxicity, nutritional imbalance, plant infections, and salt, it has been identified as a quasi-essential (Etesami et al., 2021).

Plant roots absorb the Si present as silicic acid [$Si(OH)_4$] in the soil solution, where along with water, follows the transpiration stream to finally deposit within plant

tissue as $SiO_2$, commonly known as phytoliths or silica bodies (Guével et al., 2007; Frew et al., 2017; Etesami et al., 2021). Accumulation of Si was demonstrated to increase plant growth and development, defense against pathogens, and stress tolerance (Dalal et al., 2019).

The mineral Si is considered a biostimulant able to induce protection against pest infection and is used commercially (e.g., Codasil, Hubel Verde®) in cereals and other crops to enhance nutrient uptake and stress tolerance. It is known to enhance primary metabolism by increasing photosynthesis and nutrient uptake, and secondary metabolism by promoting the production of phenolic compounds, also favoring the antioxidant defense systems (Maghsoudi et al., 2016; Vega et al., 2019). In this sense, it has been reported to enhance plant defense systems by eliciting defense genes in banana (Gbongue et al., 2019). Resistance induction in plants is associated with increased activity of defensive enzymes such as peroxidase (POX), polyphenoloxidase (PPO), and phenylalanine ammonia lyase (PAL). The POX are engaged in the lignification and suberization processes, while the PPO enzyme catalyzes the oxidation of phenolic substances to quinones, lowering the nutritional quality and protein digestibility of meals (Gomes et al., 2005).

Finally, Si's defense of plants may be related to its accumulation and polymerization in the cells, providing a mechanical barrier that makes pest infection harder (Gomes et al., 2005). Rice, cucurbits, wheat, corn, sorghum, and banana are examples of these accumulations in plant tissues (Gbongue et al., 2019).

Aside from biocontrol abilities, this mineral has other benefits such as reduced water loss by transpiration caused by Si deposits under the cuticle, reduced absorption of harmful minerals due to Si deposits in the root, promoted orientation of leaves toward the sun in such a way as to maximize light interception and thus, photosynthesis, and influenced the absorption and transport of several mineral elements (Rains et al., 2006; Oye Anda et al., 2016).

Some studies have demonstrated that combining Si with AMF can reduce disease severity in *Cajanus cajan* and banana and even helps plants thrive under stress conditions (Garg & Singh, 2018; Gbongue et al., 2019). In addition, combining *Trichoderma* strains with Si improved resistance to a disease caused by *Pyricularia oryzae* in *Lollium multiforum* plants (Arellano et al., 2021).

So far, no research aimed at studying the potential improved accumulation of Si in leaves of mycorrhizal- and *Trichoderma*-associated wheat and maize plants and the effect of the combination of the three biostimulants on the maize and wheat growth, as well as protection of wheat against foliar disease caused by *Z. tritici*.

# III. Materials and Methods

## III.1. Biological material

### III.1.1. Plant material

The plant material used for the experiment was non-treated (non-coated with pesticides) seeds of durum wheat (*Triticum turgidum*), variety Casteldoux, provided by the Centre Wallon de Recherches Agronomiques (Walloon Agricultural Research Center, Gembloux, Belgium). *Triticum turgidum* was chosen for the experiments due to its sensitivity to the pathogenic agent *Z. tritici* and its ability to be associated with arbuscular mycorrhizal fungi (AMF).

The second plant material used was non-treated seeds of maize (*Zea mays* L.), variety Troizi, provided by "Caussade Semences" (www.caussade-semences.com, France). This plant was chosen due to its rapid growth and aptitude for mycorrhization.

Seeds were surface-disinfected by immersion in 30 mL of sodium hypochlorite at 7.5% in a 50-mL Falcon tube for 10 minutes with shaking at 100 rpm. Seeds were then rinsed four times in 30 mL of type II water, with shaking for 5 minutes at 100 rpm (Oye Anda et al., 2016). Seeds were placed in colonized and non-colonized pots and germination was observed between three to five days.

### III.1.2. Arbuscular mycorrhizal fungi

*Rhizophagus irregularis* MUCL 43194 (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as [‘irregulare'], formerly named *Glomus intraradices/irregulare* (Redecker et al., 2013; Walker et al., 2021) was isolated from Pont-Rouge, Québec, Canada. This strain was supplied by the Glomeromycota *in vitro* collection of the Belgian Coordinated Collection of Microorganisms (BCCM-MUCL).

*Rhizophagus irregularis* was maintained *in vitro* on carrot (*Daucus carota* L.) roots clone 3G in 90 mm diameter bi-compartmented Petri plates with a vertical wall separating the root compartment (RC), containing the root and AMF, from the hyphal compartment (HC), in which only the fungus was allowed to grow (St Arnaud et al. 1996). After two months, mycelium from the *in vitro* culture was then mass-produced in pot culture in lava stone with maize for several months. This species was chosen due to its characteristics of rapid growth and easy cultivation. It’s also a very well-studied model species in the scientific literature that has its genome sequenced.

### III.1.3. *Trichoderma harzianum*

*Trichoderma harzianum* MUCL 29707 was isolated from Heverlee, Belgium. This strain was also provided by the BCCM-MUCL collection and was subcultivated on potato dextrose agar (PDA) medium (see composition in Annex 1) every 15 days in 9-cm mono-compartmented Petri dishes by transferring a medium plug (~0.5*0.5 cm) containing mycelium on a fresh medium. It was maintained in a dark room at 25 °C (Anrici et al., 2010). This species was chosen for its acknowledged protecting plant properties against pathogenic agents (De Jaeger et al., 2011).

### III.1.4. Pathogenic agent

*Zymoseptoria tritici* strain T02596 was provided by Junia-Yncrea (Lille, France) and the strain MUCL 45404 (Figure 7) was isolated at Acoz, Belgium and was provided by the BCCM-MUCL. This strain was subcultivated every 15 days on PDA medium in 9-cm mono-compartmented Petri dishes by transferring a medium plug (~0.5*0.5 cm) containing mycelium on a fresh medium. It was maintained in a dark room at 25 °C (Stocco et al., 2015). This pathogenic agent was chosen for its well-known capacity to infect wheat plants.

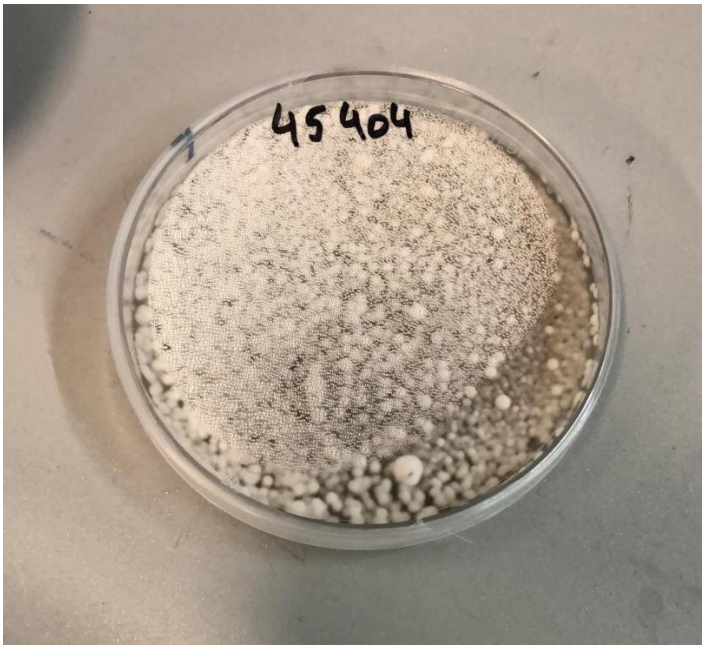


**Figure 7-** Pure culture of *Zymoseptoria tritici* strain MUCL 45404.

## III.2. Preparation of bioavailable silicon

To obtain the mineral Si in the bioavailable form for plant and fungal uptake ($H_4SiO_4$), 100 mL of amberlite were saturated with 100 mL HCl 2N and mixed in a stirrer for two hours. This allowed the adsorption of $H^+$ ions on amberlite beads. After saturation, the material was cleaned with type II water three consecutive times until conductivity reached the one of water. Next, 106,07 g of sodium metasilicates ($Na_2SiO_3.5H_2O$ – non-available for plant and fungi uptake) were weighed and dissolved in 1 L of type II water. This solution was divided in 5 beakers of 500 mL (200 mL in each). In each beaker, 200 mL of saturated amberlite was added and mixed for two hours with a stirrer. This allowed the replacement of $Na^+$ ions of $Na_2SiO_3.5H_2O$ by $H^+$ ions given by the amberlite to obtain a solution of $H_4SiO_4$, available for uptake by plants and fungi. This final solution was filtered in a 250 µm porosity sieve and finally on 25-µm porosity filter (Whatman) using a vacuum pump. Finally, the obtained solution was diluted until 10 L.

This solution was stored at 4 °C during one day for homogenization. Before use, the concentration of Si and Na was quantified via ICP-AES (Henriet et al., 2006; Walgraffe, 2018). After the quantification of Si, the value obtained was 1024.667 mg/L. From this solution, a 0.9 mM Si solution was prepared. The concentration of 0.9 mM was chosen to be applied in the substrate based on Henriet et al. (2006).

## III.3. Selection of the cultivation substrate

Three substrates usually used to cultivate mycorrhizal plants (Coelho et al., 2014; Garcés-Ruiz et al., 2017; Tigka & Ipsilantis, 2020) were tested to select the best substrate for the experiment, i.e. containing the minimal concentration of Si possibly released and thus available for plants:

- Sand,
- A mix of sand and vermiculite (50/50, v/v),
- Lava stone

For this, the concentration of Si in each substrate was determined using the following protocol: One hundred mg of each substrate were mixed with 100 mL of a 2 M HCl solution for 72 h in a chemical hood and mixed two times a day. After this, the HCl was removed, and the substrate was washed three times with type II water. The substrate was then transferred to new Erlenmeyers, and 100 mL of type II water was added. Six

mL of liquid were immediately sampled and then again after one and two weeks to quantify the Si release by the substrate over time Si was quantified in ICP-AES (Gbongue et al., 2019) (see protocol below in *III.5.2. Silicon concentration in the leaves*). The concentration of Si in each substrate is shown in Figure 8.

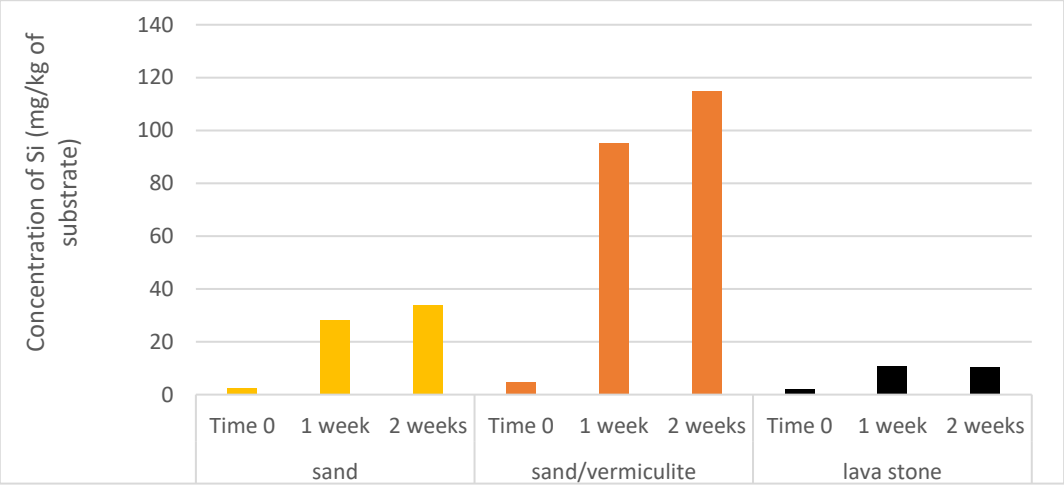


**Figure 8-** Concentration of Si in sand, a mix of sand/vermiculite (50/50, v/v) and lava stone (mg/kg of substrate), immediately (T0), and after one and two weeks in a water bath.

Because low concentrations of Si were released by lava stone (less than 11 mg/kg after two weeks) in comparison with sand mixed with vermiculite or only sand (up to 114,7 mg/kg after two weeks), lava stone was selected as substrate to conduct the experiments.

## III.4. Experimental design

Two consecutive experiments were designed to study the effect of the association of plants with AM fungi and/or *T. harzianum* on the Si uptake by plants and accumulation in leaves, and the impact on disease severity caused by *Z. tritici* on wheat leaves:

- Pre-test (see *III.3. Selection of the cultivation substrate*): effect of *R. irregularis* and/or *T. harzianum* on the growth and Si uptake and accumulation in leaves of wheat and maize.

=> Because growth of plants was hampered by the substrate preparation in the pre-test, this experiment was repeated after improvement of the substrate preparation protocol.

- The first experiment was done to evaluate the effect of *R. irregularis* and/or *T. harzianum* on the growth and Si uptake and accumulation in leaves of wheat and maize and in roots of maize.

- The second experiment was led to evaluate the effect of *R. irregularis* and/or *T. harzianum* on the growth and Si uptake and accumulation in leaves of wheat and the disease severity caused by *Z. tritici* on the leaves.

For both experiments, the donor plant culture systems of maize and wheat were previously prepared and colonized.

### III.4.1. Preparation of the donor plant culture system and pre-colonization of wheat and maize

Mycelium of *R. irregularis* was mass-produced in maize roots. The objective was the formation of a dense and active mycelial network, able to homogeneously colonize receiver plants (wheat and maize used for the experiments) in a short time.

For this, donor maize plants were grown in two trays (40*60*7 cm or 16.8 L) for each condition (mycorrhizal (AMF) and non-mycorrhizal (NM)). The trays were previously washed and disinfected with ethanol 70%. Dried trays were filled with 16 L of sterilized lava stone (formerly autoclaved twice at 120 °C for 15 min).

For AMF trays (Figure 9), already colonized pots with maize plants (see *III.1.2. Arbuscular mycorrhizal fungi*) associated to *R. irregularis* 43194 in the greenhouse were used. Four maize plants and roots were removed from this pot and added to the lava stone for each tray, with a small portion of soil from the colonized pot, to ensure the presence of spores, able to colonize the receiver plants.

**Figure 9-** Arbuscular mycorrhizal trays prepared for colonization of the wheat and maize plants.

For NM trays, the same substrate from pots cultivated with *R. irregularis,* used to inoculate mycorrhizal trays, was used to sample the microbiota (except the AM fungus). The microbiota from 100 g of lava stone was collected and mixed with 1L of type II water. With the help of a vacuum pump, the substrate solution was filtered with three types of filters (20, 11 and 8 µm, Whatman), to remove the AM fungal mycelium. The obtained solution was applied on the NM trays to ensure a similar micro-flora with the mycorrhizal trays. Surface-disinfected seeds of maize and wheat were then seeded at two cm deep.

These trays were stored in a greenhouse at 25/20 °C (day/night), photoperiod of 16 h $day^{-1}$, relative humidity (RH) of 38%, and photosynthetic photon flux (PPF) of 120 µmol $m^{-2}$ $s^{-1}$. The plants in trays were nourished once a week with 500 mL of Hoagland low-P solution, impoverished by 90% in P (Hoagland and Arnon, 1950; Garcés-Ruiz et al., 2017; see composition in Annex 2). The reduction of P of this solution ensures the root colonization by the AM fungus.

After 5 weeks in these trays, corresponding to the time 0 (T0) of the experiment (Figure 11), 5 AMF and 5 NM plants were harvested to assess their fresh weight, dry weight and Si accumulation in the leaves. The root colonization by the AM fungus was only assessed for the AMF plants (see protocol below in *III.5.1 Root colonization*). Roots from NM plants were stained with ink and observed under the microscope to confirm the

absence of AMF structures. Because AM fungal structures were observed in roots (Table 1), plants were ready to be transferred to individual pots to conduct the experiments.

**Table 1**- Percentage of total, arbuscular and vesicular root colonization of wheat and maize by *R. irregularis* MUCL 43194, after 5 weeks of pre-colonization in mycorrhizal trays, for the first experiment.

| wheat | | | maize | | |
|---|---|---|---|---|---|
| Total colonization | Arbuscular colonization | Vesicular colonization | Total colonization | Arbuscular colonization | Vesicular colonization |
| 68,33% | 50,42% | 5,14% | 71,33% | 56,33% | 7,67% |

### III.4.2. Pre-test

For this, after pre-colonization in trays, the plants were transferred into individual pots with the respective treatments (with addition of Si and presence of *T. harzianum*). Pots, bottoms, and tags were previously washed and disinfected with ethanol 70%. Then, an aluminum foil was added at the bottom of the pot to limit the growth of roots outside the pot.

To remove as much Si as possible, the substrate for the individual pots was washed with HCl, as described in the previous protocol (see *III.3. Selection of the cultivation substrate*). However, this led to the death of plants due to the acidity of the substrate, and the protocol was changed. So, the substrate was only autoclaved before being applied in the individual pots.

### III.4.3. Experiment 1: Effect of Si, AMF and *Trichoderma* on the growth, uptake of Si and colonization by wheat and maize

The aim of the first experiment was to assess the effect of *R. irregularis*, associated or not with *T. harzianum* on plant growth, AM fungal root colonization and Si accumulation in wheat and maize plants.

For this, after 5 weeks of pre-colonization in trays, the plants were transferred into individual pots with the respective treatments (Figure 10) (with addition of Si and presence of *T. harzianum*) and harvested 30 (T1) and 45 (T2) days after transfer (Figure 11).

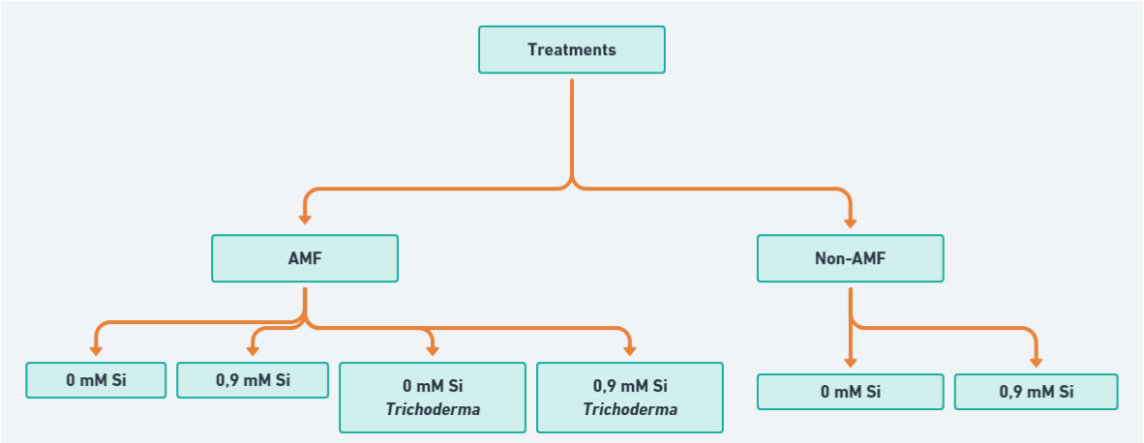


**Figure 10**- Treatments used in the first experiment in both wheat and maize plants.

The following timeline was followed for the first experiment (Figure 11):

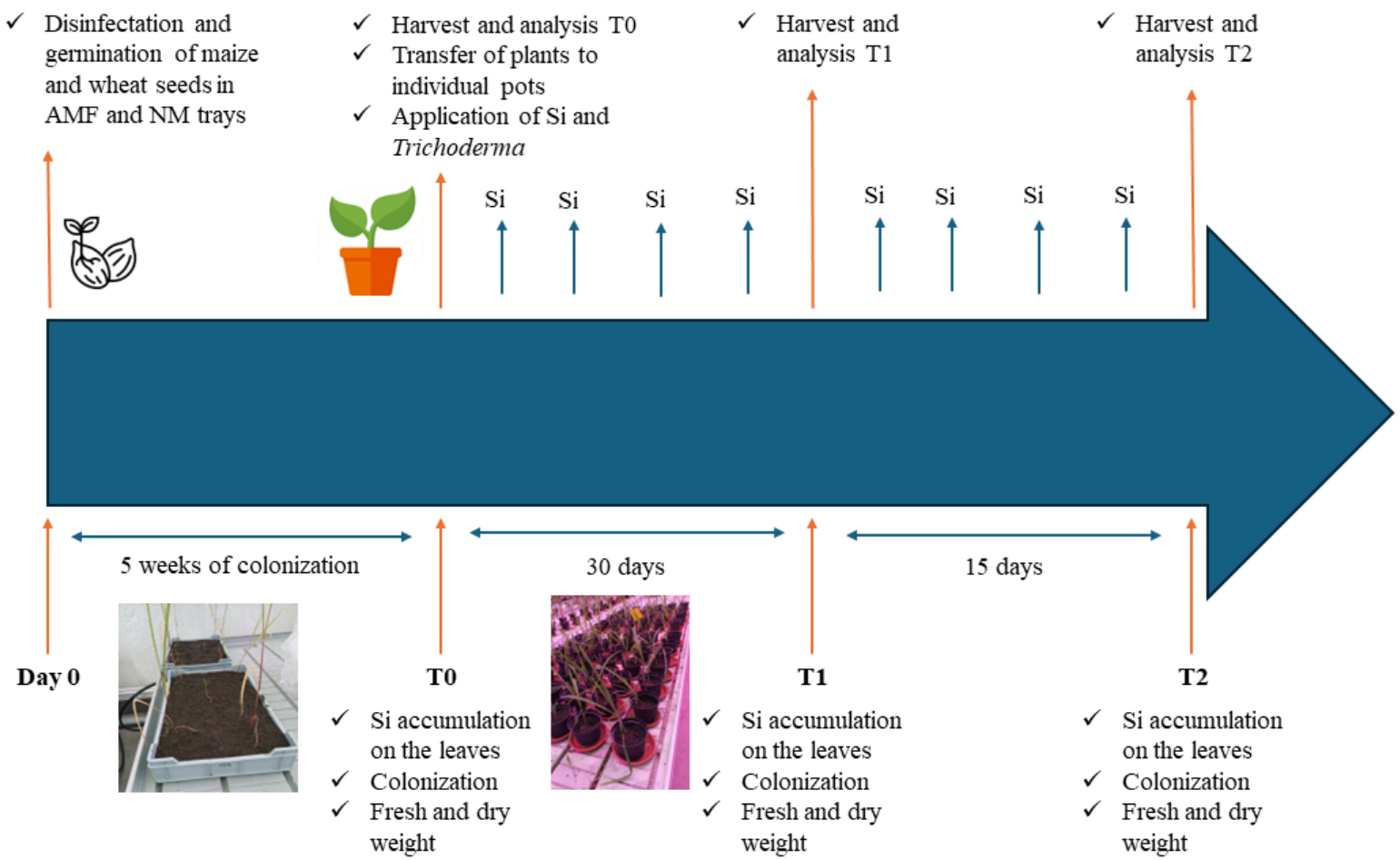


**Figure 11-** Timeline for the first experiment for wheat and maize plants with the required steps.

### III.4.3.1. Preparation of individual pots

The preparation of the pots, bottoms, and tags was done as previously explained (see *III.4.2. Pre-test*). Each pot was filled with approximately 1 L of autoclaved lava stone.

Maize and wheat plants were carefully removed from the AMF and NM trays and transferred to individual pots (Figure 12). For plants associated with *T. harzianum*, roots were submerged in a conidial suspension of the fungus ($10^7$ conidia/mL) for 5 minutes.

To obtain this conidial suspension, 10 mL of tap water were added to Petri plates and mycelium was scratched with a scalpel to collect the spores. The quantification of the spores was performed using a Thoma cell counting chamber to obtain a final concentration of $10^7$ conidia/mL (Mwangi et al., 2011). Dilutions were made with tap water. For control plants (non-treated with *T. harzianum* conidia), the roots were submerged in tap water for 5 minutes.

**Figure 12**- Individual pots with maize plants and their respective treatments.

The addition of Si (at 0.9 mM) was made after the transfer of plants to their individual pots. Control plants without Si were watered only with Hoagland low-P solution. Each pot was nourished twice a week with 100 mL of Hoagland low-P solution.

Fourteen maize plants and 10 wheat plants were considered for each treatment: NM, AMF and AMF/T (plants associated with both fungi *R. irregularis* and *T. harzianum*), with the addition or not of 0.9 mM of Si. Plants were harvested 30 and 45 days after their transfer to individual pots. The number of pots in each treatment for each plant is summarized in Table 2.

**Table 2-** Treatments applied to wheat and maize plants in the first experiment, the respective number of replicates and concentration of Si for each one. NM= non-mychorrized; AMF= *Rhizophagus irregularis*; AMF/T= *R. irregularis* with *Trichoderma harzianum*.

| | Treatments [Si] | NM | AMF | AMF/T |
|---|---|---|---|---|
| **Maize** | **0 mM** | 14 | 14 | 14 |
| | **0,9 mM** | 14 | 14 | 14 |
| **Wheat** | **0 mM** | 10 | 10 | 10 |
| | **0,9 mM** | 10 | 10 | 10 |

The pots were randomly distributed in the greenhouse. To determine each position, all the treatments and respective replicates were inserted in an excel file. A column was added with the following formula "=ALEA()", which gives a random number to each replicate. The pots were organized in the greenhouse according to the given number, from lower to higher.

### III.4.4. Experiment 2: Effect of Si, AMF and *Trichoderma* on growth, Si uptake and the biocontrol of *Zymoseptoria tritici* on wheat

The aim of the second experiment was to assess the effect of *R. irregularis*, associated or not with *T. harzianum* with the presence of Si on plant growth, Si accumulation in leaves and disease severity on wheat plants.

To do so, seeds of wheat were pre-colonized with AMF as previously described. After 5 weeks of pre-colonization in trays, the plants were transferred to their individual pots and the respective treatments were applied (with Si and *Trichoderma*), as explained for experiment 1. The preparation of individual pots was the same as for the first experiment (see *III.4.3.1. Preparation of individual pots*). The pathogens *Z. tritici* T02596

and MUCL 45404 were applied on the leaves 30 days after the transfer and disease severity was assessed after 21 days.

The timeline followed for the second experiment is shown in Figure 13. The addition of Si (at 0.9 mM) was made after the transfer of plants to their individual pots. Control plants without Si were watered only with Hoagland low-P solution. Each pot was nourished twice a week with 100 mL of Hoagland low-P solution.

After 21 days from the application of the pathogenic agent (51 days after their transfer into pots), 7 NM, 7 AMF and 7 AMF/T plants grown in the absence or presence of Si were harvested. The different combinations of treatments are summarized in Table 3. The pots were randomly distributed in the greenhouse as previously described (see *III.4.3.1. Preparation of individual pots*).

**Table 3-** Treatments applied to wheat plants in the second experiment, the respective number of replicates and concentration of Si for each one. NM= non-mychorrized; AMF= *Rhizophagus irregularis*; AMF/T= *R. irregularis* with *Trichoderma harzianum*.

| Treatments [Si] | NM | AMF | AMF/T |
|---|---|---|---|
| **0 mM** | 7 | 7 | 7 |
| **0,9 mM** | 7 | 7 | 7 |

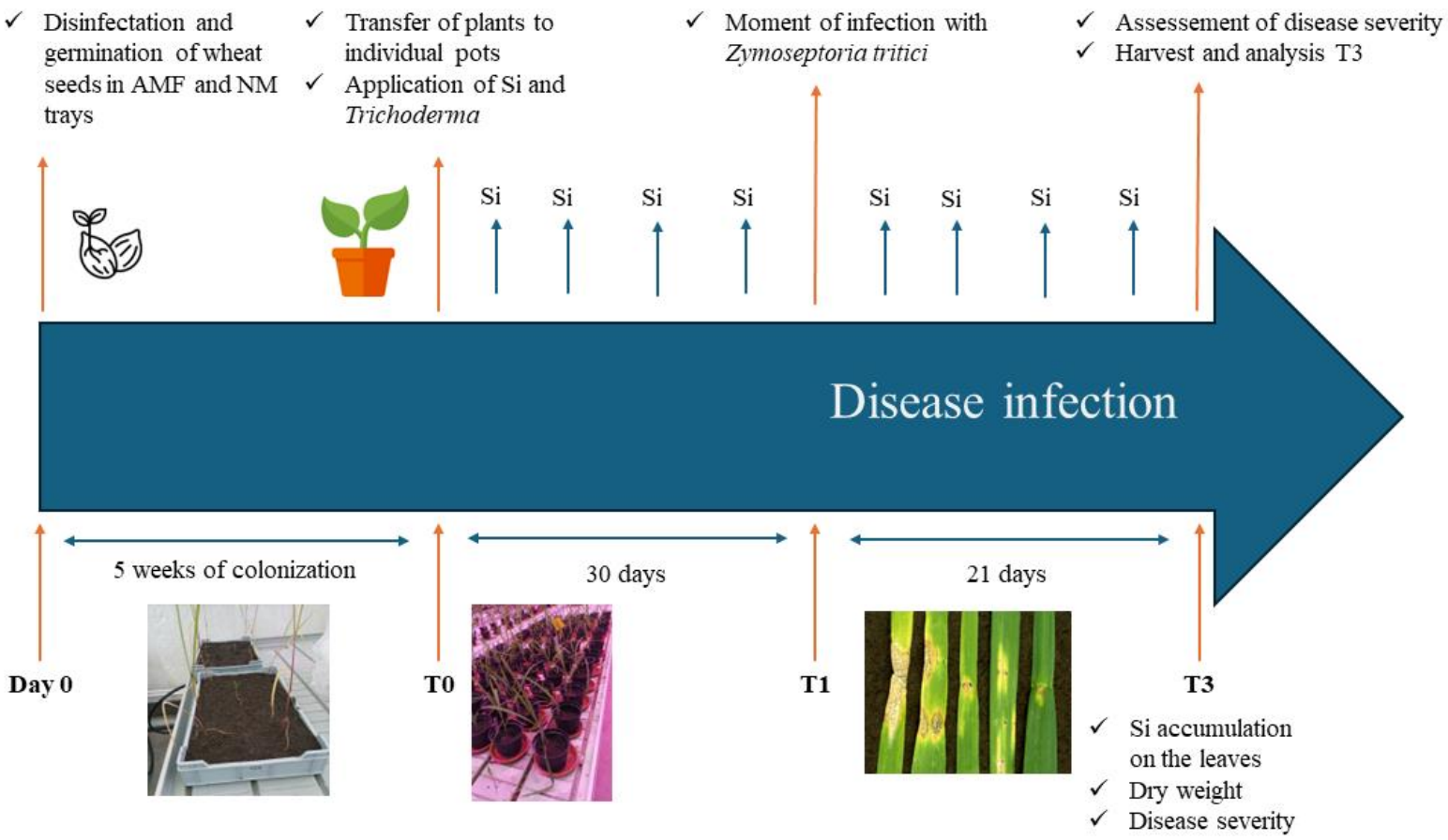


**Figure 13-** Timeline for the second experiment for wheat plants with the required steps.

### III.4.4.1. Preparation of the pathogenic agent

To prepare the *Z. tritici* T02596 and MUCL 45404 suspension, 10 mL of tap water were added to the Petri plates and mycelium was scratched with a scalpel to harvest the sporangia, in a laminar flux chamber. The quantification of the sporangia was performed with a Thoma cell counting chamber to obtain a final concentration of $10^6$ sporangia/mL. A Tween 20 solution (0.05% in deionized water) was prepared. This wetting agent allows a better pathogen adhesion on the leaves. The Tween 20 solution was first sprayed on the leaves, before application of the pathogen. Then the spore's suspension was applied throughout the leaves using a Pasteur pipette, on the 1st, 2nd and 3rd leaves. Plants were then covered with a plastic bag for 3 days to ensure around 100% humidity, condition to improve the sporangia germination and plant infestation (Le Mire et al., 2018; Mejri et al., 2018).

Twenty-one days after the infection, disease severity was quantified according to the protocol described below (see *III.5.3. Disease severity caused by* Z. tritici *on wheat leaves*).

## III.5. Plant harvest

The plants were harvested 30 days and 45 days after their transfer to individual pots (for experiment 1) and 51 days after their transfer to individual pots (for experiment 2). Plants that died during the experiment were excluded from all analyses.

Plants were harvested from the substrate with the help of a spoon. Shoots and roots were separated with a scissor, and roots were rinsed with water to remove substrate residues. The fresh weight (FW) for both parts was recorded and placed in labeled bags. The bags were introduced in an incubator at 55 °C for 7 days. After that, the dry weight (DW) was assessed for both parts.

Dry roots were used to quantify the percentage of root colonization whereas the dry aerial part was used to quantify Si concentration and Si content (see calculations in *III.5.2. Silicon concentration in the leaves*).

## III.5. Physiological and biochemical analyses

### III.5.1 Root colonization

The roots of wheat and maize were first re-hydrated with water for 24 h before staining. For this, a solution of KOH at 10% was added to the roots, and samples were placed in an incubator at 70 °C (45 min for maize and 1h30 for wheat roots) to clear the root cells from cell components. The KOH solution was then removed, and the roots were rinsed with tap water. A HCl at 1% solution was applied to the roots for 5 minutes at room temperature, to neutralize KOH and thus acidify roots, which is the optimal environment to stain roots. The solution was discarded, and a solution of ink 2% (Parker) in HCl 1% was added to the roots. The roots were incubated at 70 °C for 45 minutes. To ensure the conservation of roots before observation, roots were rinsed with water and stored in a solution of lactoglycerol (glycerol/lactic acid/deionized water, 33/33/33, v/v/v) at 4 °C.

The roots were placed on a Petri dish and were cut into 1-cm fragments with the help of a scalpel. Fifteen fragments were placed on a slide with glycerol, and a coverslip was placed on top (Figure 14). Two slides were considered for each sample. The observation of fungal structures in roots was performed using a bright field light microscope (x200). Six observations were randomly made for each fragment.

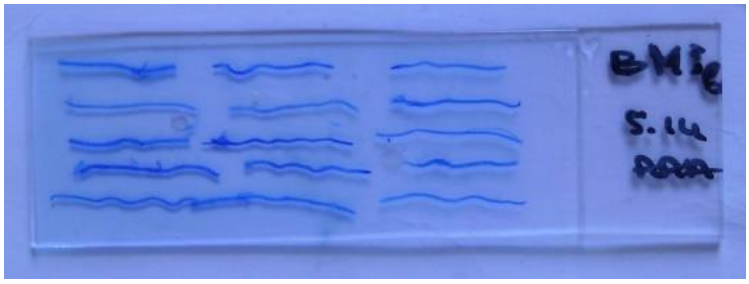

**Figure 14-** Mounted slide containing stained roots (copyright: Laboratory of Mycology, UCLouvain).

Root colonization was quantified by the observation of a minimum of 100 to 150 intersections between root and vertical eyepiece crosshair using the bright field light microscope (Figure 15) following the method of McGonigle et al. (1990).

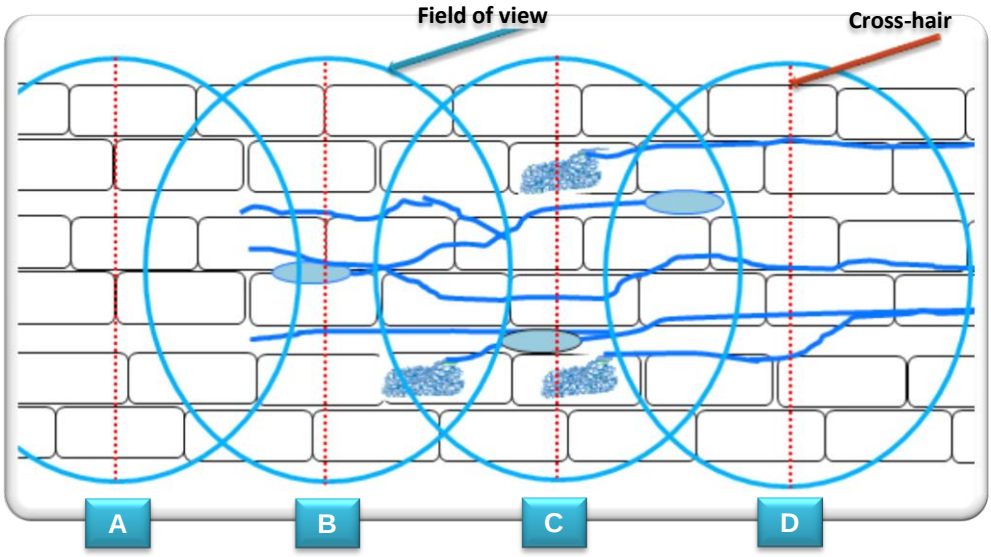


**Figure 15-** Evaluation of the AM fungal root colonization using the method of McGonigle et al. (1990).

**A** In absence of AMF at the intersection, mark one point for "negative-intersect".

**B** When spores/vesicles or arbuscules are observed inside the root, note one point for the "presence" of the observed structure (spores/vesicles or arbuscules). Note that here, even if hyphae are on the cross-hair, it is not counted.

**C** When at the same time spores/vesicles, arbuscules and hyphae are observed in the intersection, note one point for the "presence" of spores/vesicles and one point for the "presence" of arbuscules; but again, nothing for hyphae.

**D** When only hyphae are observed at the intersection (D), note one point for the "presence" of hyphae.

The percentages of arbuscule colonization (%AC) and spore/vesicle colonization (%VC) are calculated by dividing the number of intersections with the presence of arbuscules or spores/vesicles, respectively, by the total number of intersections examined. Total colonization (%TC) is calculated as the proportion of non-negative intersections.

The following formulae were applied to calculate the arbuscules, vesicles and total AMF colonization percentages, respectively (McGonigle et al., 1990):

$$\%AC = \frac{\text{Number of intersections with the presence of arbuscules}}{\text{Total number of intersections examined}} * 100$$

$$\%VC = \frac{\text{Number of intersections with the presence of vesicles}}{\text{Total number of intersections examined}} * 100$$

$$\%TC = \frac{(\text{Total number of intersections examined} - \text{Intersections without AMF})}{Total\ number\ of\ intersections\ examined} * 100$$

### III.5.2. Silicon concentration in the leaves

To quantify Si in the leaves, the leaves were first crushed in liquid nitrogen and 100-150 mg of three randomly selected samples were weighted and placed in a 15 mL Falcon tube. After, 4 mL of NaOH 0,2 M was added to each tube. Three negative controls containing only NaOH were also prepared. The tubes were placed in a water bath at 100 °C during 1h. Once they cooled down, one mL of HCl 1 M 30% suprapur (Merck, Germany) was added in each tube and left at 4 °C overnight. The next day, samples were centrifuged for 10 minutes at 2500 rpm. Then, they were left in the fridge for three days. Finally, the supernatant was transferred to a 15 mL Falcon tube and analyzed via ICP-AES (ICAP 6500, Thermo-Scientific, United States) (Reynolds et al., 2008).

The Si content was determined with axial viewing of the emitted radiation. A peristaltic pump was used to introduce the solutions into the ICP-AES at a flow rate of 1.5 mL/min. Operating parameters for the instrument included forward power 1150 W, coolant gas flow rate 12 L/min, auxiliary gas flow rate 1 L/min (or L $min^{-1}$, you can choose what you prefer) and nebulizer gas flow rate 0.6 L/min. Si quantification was analyzed under a wavelength of 251.611 and 288.158 γ/nm. The limit of detection was <100 ppb. Data obtained (in mg/L) were converted in µg/g of plant.

The following formulae were used to assess Si concentration (µg/mg of dry weight) in maize leaves and Si content (mg/plant) in the plant:

$$Si\ concentration = \frac{\text{Si concentration measured via ICP} - \text{AES} * \text{mass of solution}}{\text{weight of plant quantified in the Falcon tube}}$$

$$Si\ content = \frac{\text{Si concentration} * \text{total weight of the plant}}{1000}$$

### III.5.3. Disease severity caused by *Z. tritici* on wheat leaves

To assess disease severity in wheat plants, four leaves of each plant from each treatment were observed and disease percentages estimated according to the scale developed by Koyshibayev and Muminjanov (2016) (Figure 16).


**Figure 16-** Disease severity analysis scale used for the second experiment (Source: Koyshibayev and Muminjanov, 2016).

## III.5 Statistical analysis

For all the analyses, because of the relatively low numbers of replicates, all samples were subjected to a non-parametric Kruskall-Wallis test ($p \leq 0.05$). If the result was significant, a non-parametric multiple comparison between the treatments was used (Steel-Dwass and/or Wilcoxon each pair when Steel-Dwass results were not in accordance with the Kruskall-Wallis significance, $p \leq 0.05$)). All analyses were performed using the JMP software, version Pro 17.

# IV. Results and Discussion

## IV.1. Effect of Si, AMF and *Trichoderma* on the growth, uptake of Si and colonization by maize

### IV.1.1. Effect of AMF on the growth of maize at the moment of transfer to individual pots (T0)

The roots and aerial fresh (FW) and dry (DW) weights of maize at the moment of transfer to individual pots are shown in Figure 17.

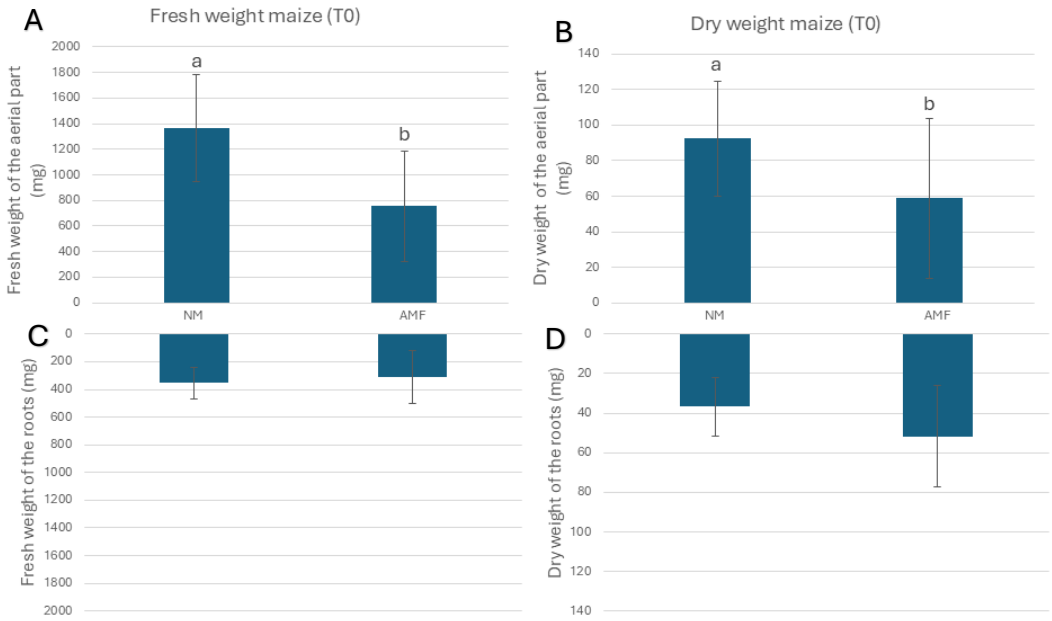


**Figure 17-** Aerial fresh (A) and dry (B) weight (mg); roots fresh (C) and dry (D) weight of maize inoculated (AMF) or not (NM) by *Rhizophagus irregularis* MUCL 43194 at the moment of transfer (T0), 5 weeks after sowing in donor trays. Different lower-case letters indicate significant differences between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$); n = 10 replicates. The absence of letters indicates the absence of difference between the treatments.

For the first experiment, the FW and DW biomass of the aerial part was significantly higher ($p = 0.0101$ for FW and $p = 0.0311$ for DW) for the NM plants (FW = 1367 ± 420 mg; DW = 92 ± 32 mg) compared to AMF plants (FW = 755 ± 432 mg; DW = 59 ± 45 mg), whereas the root fresh and dry biomasses remained unchanged between both mycorrhizal and non-mycorrhizal treatments.

### IV.1.2. Effect of Si, AMF and *Trichoderma* on the growth of maize after 30 and 45 days of transfer to individual pots (T1 and T2)

The roots and aerial fresh and dry weights of maize, 30 days after transfer to individual pots are shown in Figure 18.

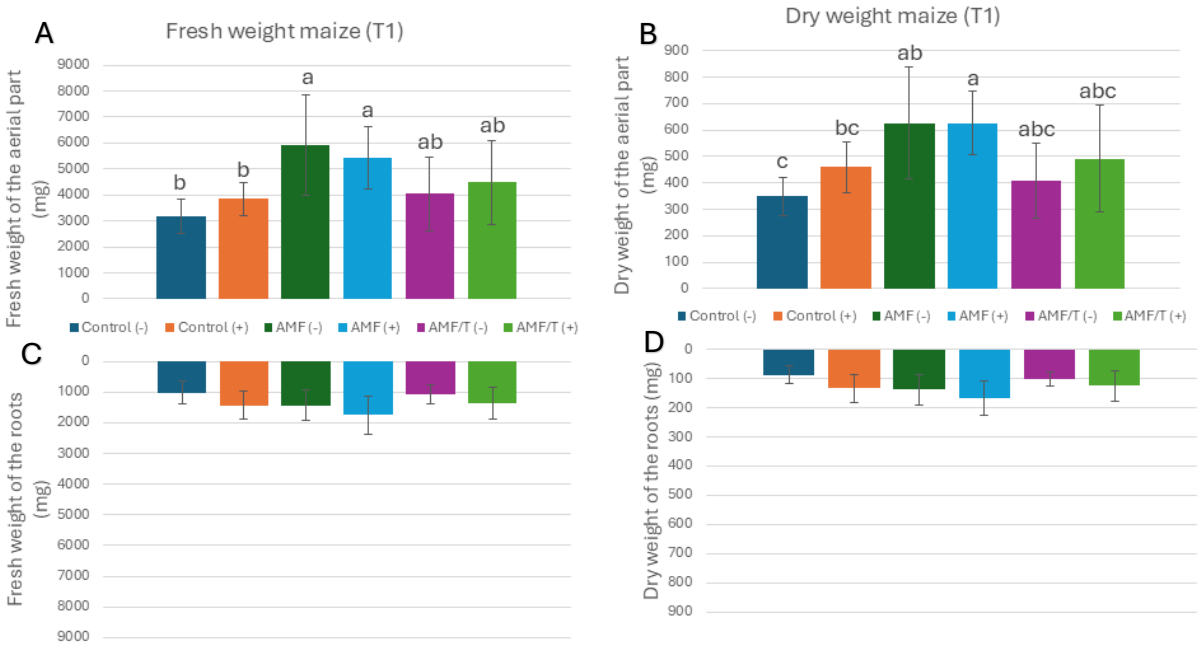


**Figure 18**- Aerial fresh (A) and dry (B) weight; roots fresh (C) and dry (D) weight of maize 30 days after the moment of transfer (T1). Different lower-case letters indicate significant differences between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$). The absence of letters indicates the absence of difference between the treatments.
Control (-) (non-inoculated control without addition of Si); n = 7; Control (+) (non-inoculated control with addition of Si); n = 7; AMF (-) (maize inoculated by *Rhizophagus irregularis* MUCL 43194 without the addition of Si); n = 7; AMF (+) (maize inoculated by *R. irregularis* MUCL 43194 with the addition of Si); n = 6; AMF/T (-) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and without the addition of Si); n = 6; AMF/T (+) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and addition of Si); n = 6.

Thirty days after transfer (T1), both aerial FW and DW presented significant differences ($p = 0.0177$ and $p = 0.0247$, respectively) between treatments. The FW of plants associated with *R. irregularis* alone were significantly higher than the control plants, both in presence (FW of AMF plants = 3849±643 mg) and absence of Si (FW of AMF plants = 3186±664 mg). Similar results were observed for the DW of mycorrhizal plants in the presence of Si, which were significantly higher than control plants. On the other hand, the aerial FW of plants associated with both *R. irregularis* and *T. harzianum* remained similar to the controls and plants associated with the AM fungus alone. On the

contrary, the statistical analysis of the treatments didn't show significant differences for the root FW and DW between treatments.

The roots and aerial FW and DW of maize 45 days after the moment of transfer to individual pots are shown in Figure 19.

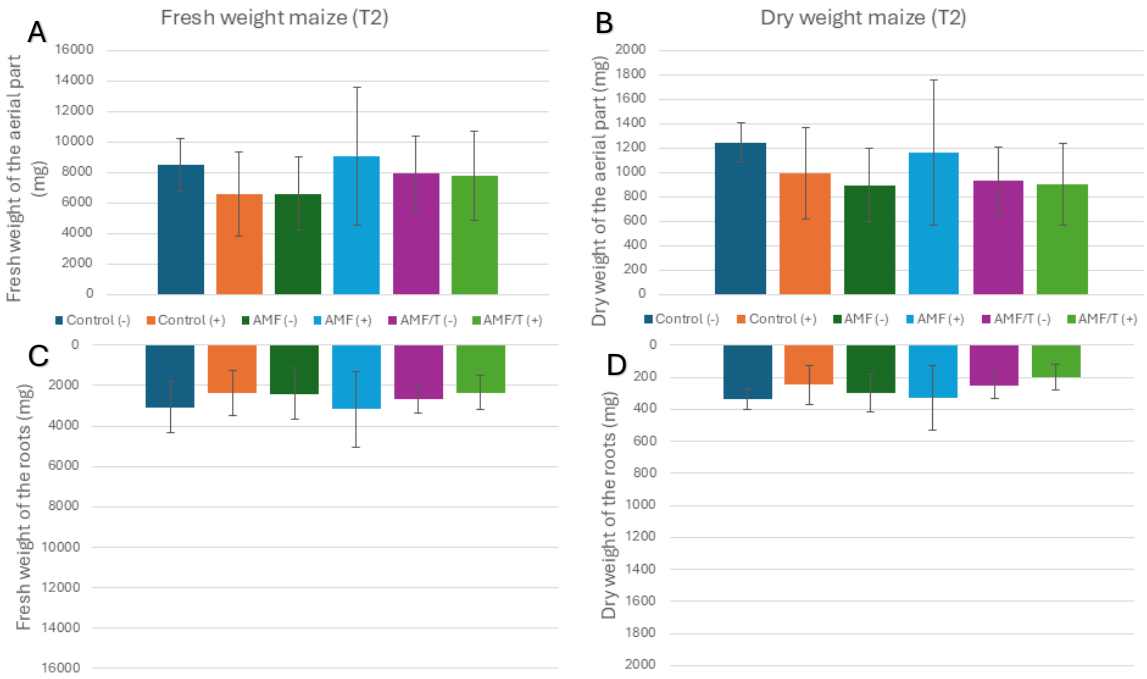


**Figure 19-** Aerial fresh (A) and dry (B) weight; roots fresh (C) and dry (D) weight of maize 45 days after the moment of transfer (T2). Different lower-case letters indicate significant differences between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$). The absence of letters indicates the absence of difference between the treatments.
Control (-) (non-inoculated control without addition of Si); n= 7; Control (+) (non-inoculated control with addition of Si); n = 6; AMF (-) (maize inoculated by *Rhizophagus irregularis* MUCL 43194 without addition of Si); n = 7; AMF (+) (maize inoculated by *R. irregularis* MUCL 43194 with addition of Si); n = 7; AMF/T (-) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and without addition of Si); n = 7; AMF/T (+) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and addition of Si); n = 7.

After 45 days (T2) from transfer to individual pots, no significant differences in the root and aerial FW and DW were measured between treatments. However, the control treatments increased in the weight in only 15 days; on the other hand, the AMF treatments had only a small increase. This may be due to the limiting space in the pot for both the plant and the AMF which might have led to a competition for nutrients and space.

### IV.1.3. Effect of AMF on the Si accumulation of maize leaves at the moment of transfer to individual pots (T0)

The Si concentration in maize leaves and Si content in maize plants at the moment of transfer to individual pots are shown in Table 4.

**Table 4-** Si concentration (µg/mg of DW) in maize leaves and Si content (mg/plant) in maize plants, inoculated (AMF) or not (NM) with *Rhizophagus irregularis* at the moment of transfer (T0). Different lower-case letters in a column indicate significant differences among treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$); n = 3; average ± SD. The absence of letters in a column indicates the absence of difference between the treatments.

| | Si concentration (µg/mg of dry weight) | Si content (mg/plant) |
|---|---|---|
| **NM** | 1.42 ± 0.32 | 0.17 ± 0.002a |
| **AMF** | 0.94 ± 0.30 | 0.08 ± 0.05b |

At the moment of transfer to individual pots (T0), the Si concentration remained similar between NM and AMF leaves. However, when reporting the concentration of the plant (Si content), a significant difference (p=0.0495) was observed. NM accumulated higher Si content than mycorrhizal plants.

### IV.1.4. Effect of Si, AMF and *Trichoderma* on the Si accumulation of maize leaves, 30 and 45 days after transfer to individual pots (T1 and T2)

The Si concentration in maize leaves and Si content in maize plants 30 days and 45 days after the moment of transfer to individual pots are shown in Table 5.

**Table 5-** Si concentration (µg/mg of DW) in maize leaves and Si content (mg/plant) in maize plants 30 days and 45 days after the moment of transfer (T1 and T2). Different lower-case letters in a column indicate significant differences between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$); n = 3; average ± SD. The absence of letters in a column indicates the absence of difference between the treatments. The treatments used are identical to those in the fresh and dry weight figures.

| | Si concentration (µg/mg of dry weight) | Si content (mg/plant) |
|---|---|---|
| T1 | | |
| **Control (-)** | 1.57 ± 0.07 | 0.56 ± 0.04 |
| **Control (+)** | 0.94 ± 0.19 | 0.52 ± 0.09 |
| **AMF (-)** | 0.91 ± 0.27 | 0.36 ± 0.06 |
| **AMF (+)** | 0.98 ± 0.30 | 0.61 ± 0.28 |
| **AMF/T (-)** | 1.56 ± 0.68 | 0.77 ± 0.27 |
| **AMF/T (+)** | 0.75 ± 0.09 | 0.42 ± 0.09 |
| T2 | | |
| **Control (-)** | 0.96 ± 0.29 | 1.14 ± 0.22 |
| **Control (+)** | 0.99 ± 0.46 | 1.18 ± 0.39 |
| **AMF (-)** | 1.17 ± 0.12 | 0.95 ± 0.33 |
| **AMF (+)** | 0.95 ± 0.33 | 1.48 ± 1.10 |
| **AMF/T (-)** | 1.13 ± 0.49 | 1.00 ± 0.43 |
| **AMF/T (+)** | 1.12 ± 0.45 | 1.01 ± 0.24 |

After 30 and 45 days of growth in individual pots inoculated or not with *R. irregularis* and/or *T. harzianum* and amended or not with Si, no significant differences were demonstrated on both Si concentration and Si content in maize. In this case, the application of Si as well as the inoculation of maize with *R. irregularis* and *T. harzianum* had no impact on the Si accumulation.

Although the concentration remained similar between T1 and T2, the Si content in plants increased concomitantly with the growth of the plants. This suggests a constant translocation or release of Si from the roots to the leaves of maize.

As the values for Si concentration in leaves were so low, it could be hypothesized that the Si was mainly stored in the roots of the maize plants with a limited transfer to the leaves. A second experiment was performed to assess the Si accumulation in the roots to verify this hypothesis.

### IV.1.5. Effect of Si, AMF and *Trichoderma* on the Si accumulation of maize leaves and roots, 30 and 45 days after transfer to individual pots (T1 and T2)

A second experiment was performed to confirm the previous results and to measure the Si accumulation in the roots of maize plants. The Si concentration and Si content in maize leaves and roots, 30 days and 45 days after the moment of transfer to individual pots are shown in Table 6.

Thirty and 45 days after transfer to individual pots, maize plants accumulated similar concentrations and contents of Si, meaning that the plants didn't accumulate more Si on the roots. However, significant differences were observed for Si content in the plants at T2, where the highest values were the treatments where AMF and *T. harzianum* were both present, especially without Si application. These treatments were significantly different than the treatment with AMF and Si present.

**Table 6-** Si concentration (µg/mg of DW) in maize leaves and roots and Si content (mg/plant) in maize plants and roots 30 days and 45 days after the moment of transfer (T1 and T2). Different lower-case letters in a column indicate significant differences between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$); n = 3; average ± SD. The absence of letters in a column indicates the absence of difference between the treatments. The treatments used are identical to those in the fresh and dry weight figures.

| | Si concentration leaves (µg/mg of dry weight) | Si content (mg/plant) | Si concentration roots (µg/mg of dry weight) | Si content roots (mg/plant) |
|---|---|---|---|---|
| **T1** | | | | |
| **Control (-)** | 1.88 ± 0.50 | 0.55 ± 0.32 | 0.67 ± 0.07 | 0.13 ± 0.03 |
| **Control (+)** | 1.17 ± 0.49 | 0.81 ± 0.47 | 0.74 ± 0.40 | 0.15 ± 0.06 |
| **AMF (-)** | 0.87 ± 0.35 | 0.55 ± 0.22 | 0.73 ± 0.33 | 0.16 ± 0.11 |
| **AMF (+)** | 0.52 ± 0.31 | 0.40 ± 0.15 | 0.92 ± 0.30 | 0.26 ± 0.16 |
| **AMF/T (-)** | 1.18 ± 0.93 | 0.60 ± 0.29 | 0.96 ± 0.37 | 0.14 ± 0.05 |
| **AMF/T (+)** | 0.72 ± 0.21 | 0.64 ± 0.12 | 0.87 ± 0.13 | 0.20 ± 0.11 |
| **T2** | | | | |
| **Control (-)** | 2.26 ± 0.60 | 1.19 ± 0.79bc | 1.90 ± 1.10 | 0.28 ± 0.20 |
| **Control (+)** | 3.14 ± 1.26 | 2.18 ± 1.72abc | 1.61 ± 0.22 | 0.20 ± 0.19 |
| **AMF (-)** | 3.35 ± 2.52 | 4.12 ± 3.46ab | 2.42 ± 0.94 | 0.72 ± 0.90 |
| **AMF (+)** | 0.95 ± 0.37 | 1.03 ± 0.53c | 2.22 ± 0.65 | 0.43 ± 0.12 |
| **AMF/T (-)** | 3.40 ± 1.42 | 6.38 ± 2.81a | 1.81 ± 0.04 | 0.83 ± 0.15 |
| **AMF/T (+)** | 3.00 ± 0.34 | 4.89 ± 1.04a | 2.13 ± 0.46 | 0.98 ± 0.22 |

### IV.1.6. Mycorrhizal colonization of maize roots at the moment of transfer (T0), 30 and 45 days of transfer in individual pots (T1 and T2)

The arbuscules percentages, vesicles percentages and total colonization percentages in roots of maize at the T0, T1 and T2 are shown in Figure 20. At transfer (Figure 20A), maize plants presented a high level of root colonization percentage, with a total colonization rate reaching more than 70%. This high colonization was mainly linked to the arbuscular colonization rate, which reached around 55%. The presence of a high number of arbuscules suggests an active transfer of nutrients between the plant and the fungus and, thus, an active association.

As visible in Figure 20B, the total colonization rate of maize increased after transfer to pots, reaching more than 90% after 30 days and 95% after 45 days, however, the percentage of arbuscules and vesicules remained relatively similar. The percentage of total colonization and arbuscular colonization was not different both in absence/presence of Si and *T. harzianum*. On the other hand, the percentage of vesicles showed a significant difference ($p = 0.0072$) between treatments. In the absence of Si and *T. harzianum*, the AMF plants presented a higher percentage of vesicles ($V = 11.2\% \pm 4.3$) as compared to the treatments where the *Trichoderma* and Si were present, which showed the lowest value ($V = 0.9\% \pm 1.4$).

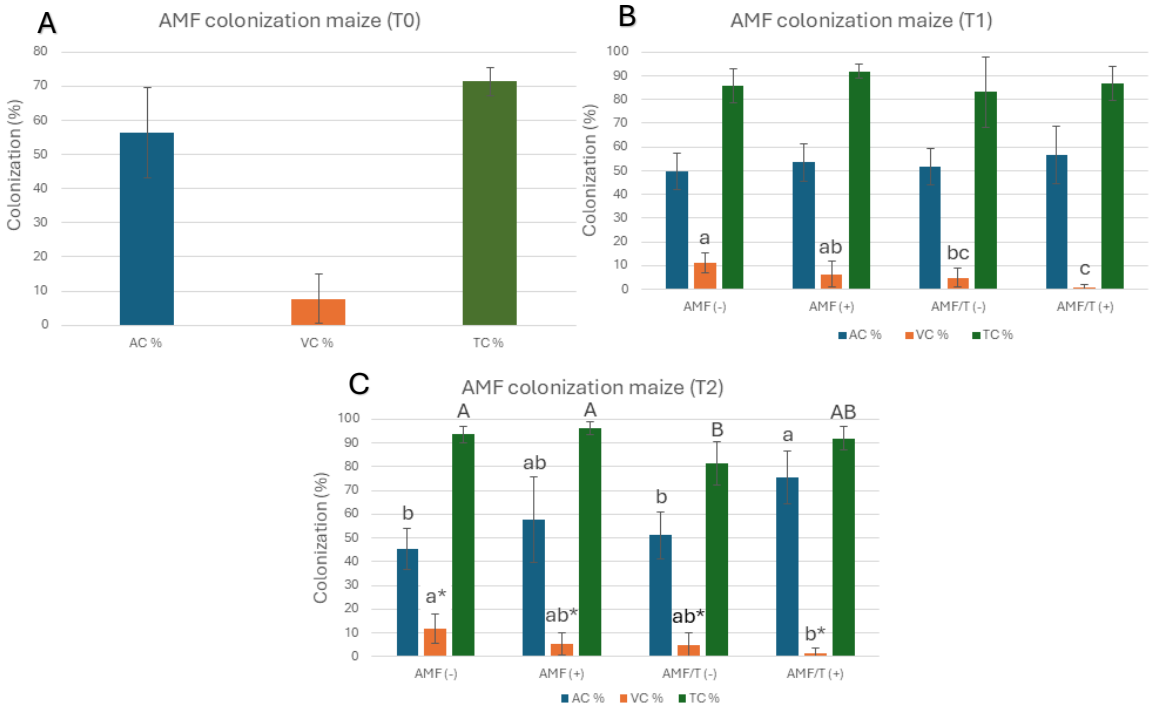


**Figure 20-** Arbuscular (AC%), Vesicular (VC%) and total colonization (TC%) percentages of maize inoculated with *Rhizophagus irregularis* MUCL 43194: (A) at the moment of transfer (T0), (B) 30 days after transfer (T1) and (C) 45 days after transfer (T2). Different lower-case letters indicate significant differences between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$); n = 5. The absence of letters indicates the absence of difference between the treatments. The treatments used are identical to those in the fresh and dry weight figures.

Fifteen days later (Figure 20C), the three parameters (arbuscules, vesicles and total percentage of colonization) presented significant differences. For the arbuscule percentage (p=0.0265), the treatment with higher values was when AMF was associated with Si and *Trichoderma* (A = 75.3% ± 11.2), which was significantly different from all the treatments where Si was absent (i.e. AMF(-) and AMF/T(-)). For the percentage of vesicle colonization (p = 0.0289), the addition of Si and *Trichoderma* significantly decreased the vesicular colonization (V = 1.4% ± 2). For the total colonization percentage (p=0.0094), the treatment with AMF and Si obtained the highest values, and the one where *Trichoderma* and Si were present had significantly lower values. These results on the vesicular colonization percentages are in accordance with T1.

## IV.1. General discussion of the effect of Si, AMF and *Trichoderma* on the growth, uptake of Si and colonization by maize

During the first experiment on maize, the absence of AMF was observed to be positive for the growth of the aerial part on T0. Several studies have shown that the

contribution of AMF is not always positive on maize growth, and it can depend on environmental conditions and the interaction between plant and fungus. A study has shown that *R. irregularis* by itself had no maize growth-promoting effect, even at a high mycorrhizal colonization level in roots under salt-stress conditions (Chen et al., 2022). Another study showed that *Funneliformis mosseae* (a type of AMF species) was more effective for maize growth than *R. irregularis* on maize (Hussain et al., 2021). It cannot be overlooked that the AMF trays had a higher number of plants, potentially limiting the space and growth of the plant.

Contrarily to the moment of transfer to individual pots, the AMF inoculation had a positive effect on the plant's growth after 30 days of growth in their individual pots. On the other hand, the addition of Si and *Trichoderma* with the AMF reduced the AMF beneficial effect on the growth of maize. One hypothesis can be that the use of *Trichoderma* negatively interacts or competes with the AMF, thus affecting the growth and biomass of the host plant. *Trichoderma* can affect AMF, reducing the positive effect of mycorrhizal association by competition for resources. One study showed that *T. harzianum* reduced root colonization by *Rhizophagus intraradices/irregularis* (Green et al., 1999). Another study also proved that *Trichoderma pseudokoningii* inhibited the germination of *Glomus mosseae* thus affecting the colonization of the roots (Martinez et al., 2003). Another hypothesis can be that the addition of *T. harzianum* does not promote maize growth due to the environmental conditions, or that the specific strain of *Trichoderma* used was not the most appropriate for this plant species under the test conditions. One study proved that *Trichoderma asperellum* LU1370 – used as a biocontrol product – could have a negative impact on *Arabidopsis thaliana* growth in sterile soil experiments (Nieto-Jacobo et al., 2017). Nonetheless, the results demonstrated an absence of influence of the microbial consortium on the growth of the maize roots. For T2, no significant differences were observed, which might be due to the limited space in the pot.

In conclusion, for maize growth and biomass, the application of AMF alone seems to be a good option, which increased the aerial part of maize plants at the early stage of growth. To the contrary, *T. harzianum* impaired this benefit. To increase the growth of plants, other *Trichoderma* strains or species could be tested for plant growth improvement as well as for compatibility with AMF. The use of bigger pots should be used to

determine, under less-limiting conditions, the influence of the fungi over a longer period. Nonetheless, a mix or other AMF species can lead to better outcomes.

The Si uptake and accumulation by maize plants was not influenced by the addition of Si in the substrate nor by the inoculation with beneficial microorganisms. Only at T0, NM plants accumulated higher Si content in maize plants, which may be due to higher aerial biomass, thus accumulating more Si than mycorrhizal plants. For T1 and T2, the application of Si as well as the inoculation of maize with *R. irregularis* and *T. harzianum* had no impact on the Si accumulation. However, a study by Silva et al. (2023) reported the opposite result, where the application of Si combined with *T. harzianum* promoted higher nutrient ion contents in *Sorghum sudanense*. Another study showed positive impacts of *R. irregularis* combined with Si on the Si uptake by *Cajanus cajan* (Garg & Singh, 2018). However, this outcome can be explained by the low concentration of Si (0,9 mM) applied to the maize plants. Malčovská et al. (2014) showed that the application of 5 mM of Si in maize plants enhanced growth and reduced the level of oxidative stress, while Moussa (2006) concluded that when Si is applied at 3 mM it counterbalanced the negative effects of salt-stressed conditions in maize plants.

In short, more studies are necessary, where higher Si concentrations need to be tested to determine the influence of the fungi on this mineral uptake and accumulation by maize plants. Also, other forms of Si application can be assessed, like foliar spray application with adjuvants and surfactants (for better absorption) or even Si nanoparticles. Again, testing other strains or species of *Trichoderma* and AMF that might have a greater impact on Si uptake and accumulation is also required.

Lastly, the application of AMF alone induced the formation of a significantly higher number of vesicles as compared to the application of Si and *T. harzianum*. However, for the formation of arbuscules, the interaction between AMF, *T. harzianum* and Si gave the best outcome. One hypothesis can be that Si stimulates the formation of arbuscules in AMF maize plants, which may be due to improved root growth, enhanced nutrient uptake and transfer of nutrients to the plant. One study confirmed this hypothesis in strawberry plants under drought stress (Moradtalab et al., 2019). Observing the results for the vesicle and total colonization percentage, *Trichoderma* and Si might have had an antagonistic effect on their formation. Martinez-Medina et al. (2009) also reported a decrease in colonization when *T. harzianum* was combined with *Glomus mosseae*.

Finally, for maize plants it is possible to affirm that the interaction between AMF and *T. harzianum* was negative, since it reduced the growth and biomass of maize plants and did not impact the Si uptake and accumulation. Nonetheless, the plant was not able to accumulate and uptake more Si, whether the Si was applied or due to its presence in the soil. According to all parameters observed, the AMF treatment alone was the best treatment for improving the growth and colonization of maize plants.

## IV.2. Effect of Si, AMF and *Trichoderma* on the growth, Si uptake and colonization of wheat

### IV.2.1. Effect of AMF on the growth of wheat at the moment of transfer to individual pots (T0)

The roots and aerial fresh and dry weights of wheat at the moment of transfer to individual pots are shown in Figure 21.

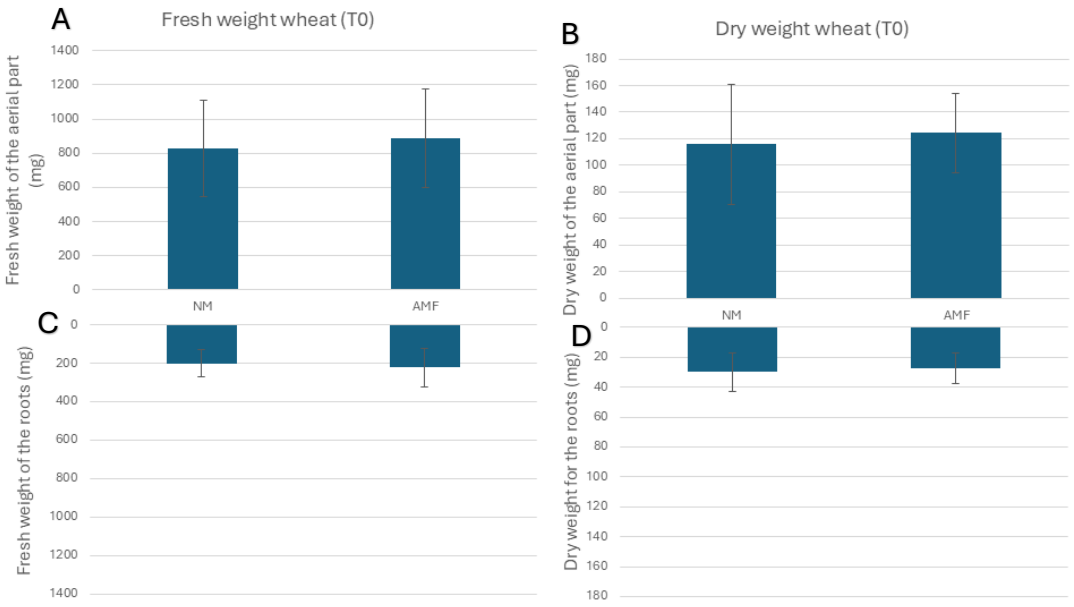


**Figure 21**- Aerial fresh (A) and dry (B) weight (mg); roots fresh (C) and dry (D) weight of wheat inoculated (AMF) or not (NM) with *Rhizophagus irregularis* MUCL 43194 at the moment of transfer (T0), 5 weeks after sowing in donor trays. Different lower-case letters indicate significant differences between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$); n = 7. The absence of letters indicates the absence of difference between the treatments.

For the first experiment with wheat, plants weighed around 1 g FW and 150 mg DW. No significant difference in aerial and root FW and DW was observed between NM and AMF plants at the time of transfer into individual pots (T0).

### IV.2.2. Effect of Si, AMF and *Trichoderma* on the growth of wheat after 30 and 45 days of transfer to individual pots (T1 and T2)

The roots and aerial fresh and dry weights of wheat 30 days (T1) and 45 days (T2) after transfer to individual pots are shown in Figure 22 and Figure 23.

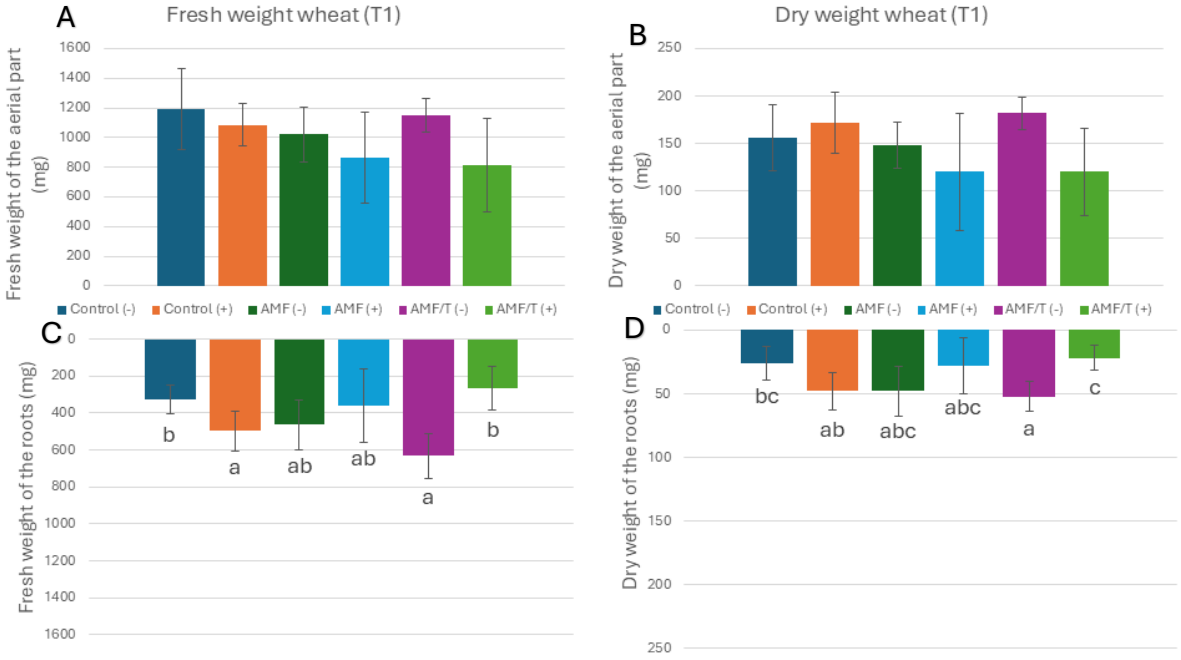


**Figure 22**- Aerial fresh (A) and dry (B) weight; roots fresh (C) and dry (D) weight of wheat 30 days after the moment of transfer (T1). Different lower-case letters indicate significant differences between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$). The absence of letters indicates the absence of difference between the treatments.
Control (-) (non-inoculated control without addition of Si); n = 5; Control (+) (non-inoculated control with addition of Si); n = 5; AMF (-) (maize inoculated by *Rhizophagus irregularis* MUCL 43194 without the addition of Si); n = 5; AMF (+) (maize inoculated by *R. irregularis* MUCL 43194 with the addition of Si); n = 5; AMF/T (-) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and without the addition of Si); n = 5; AMF/T (+) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and addition of Si); n = 5.

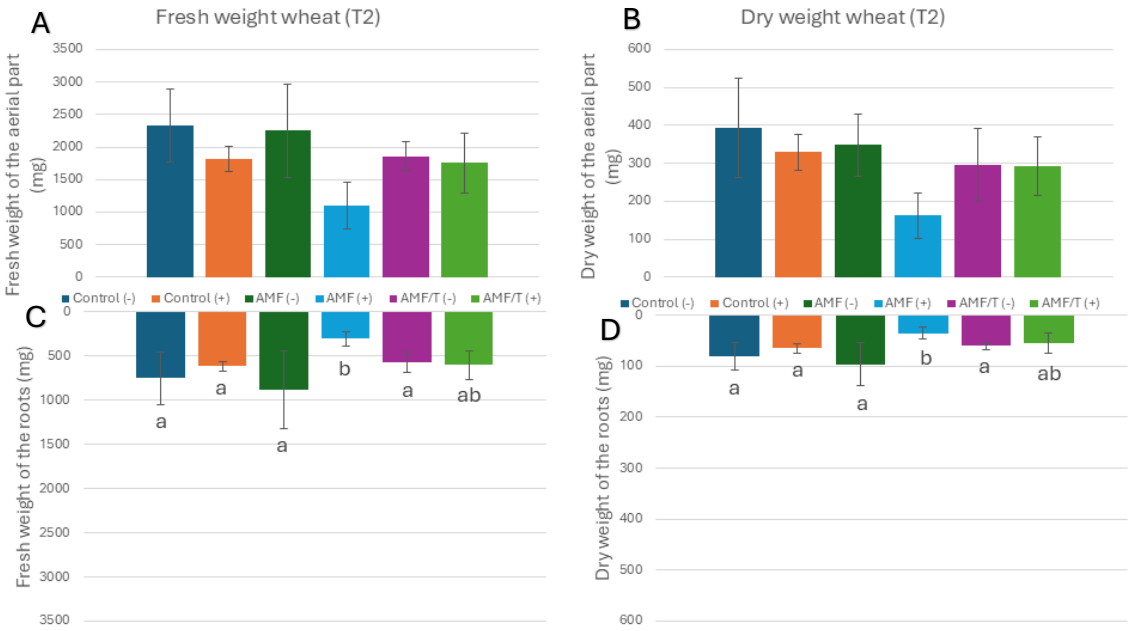


**Figure 23**- Aerial fresh (A) and dry (B) weight; roots fresh (C) and dry (D) weight of wheat 30 days after the moment of transfer (T1). Different lower-case letters indicate significant difference between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$). The absence of letters indicates the absence of difference between the treatments.
Control (-) (non-inoculated control without addition of Si); n = 5; Control (+) (non-inoculated control with addition of Si); n = 5; AMF (-) (maize inoculated by *Rhizophagus irregularis* MUCL 43194 without the addition of Si); n = 5; AMF (+) (maize inoculated by *R. irregularis* MUCL 43194 with the addition of Si); n = 5; AMF/T (-) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and without the addition of Si); n = 5; AMF/T (+) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and addition of Si); n = 5.

At T1, after 30 days of growth in individual pots, plant biomass reached around 1.5 g fresh (Figure 22A) and 200 mg dry (Figure 22B), with an increase of around 33% of their weight after transfer. The application of Si and *T. harzianum* did not significantly affect the FW and DW of the aerial part of the wheat plants.

Regarding the roots, a significant difference was measured for the roots FW ($p=0.0190$) and DW ($p = 0.0386$). The treatments with higher values were AMF/T in the absence of Si (FW = 632 mg ± 120.6; DW = 52 mg ± 11.7) and control in the presence of Si (FW=498 mg ± 110.3; DW= 48 ± 14.69), compared with the treatment AMF/T in the presence of Si (FW = 268 mg ± 118.45; DW = 22 mg ± 9.79) and control without Si (FW = 326 mg ± 78.63; DW = 26 mg ± 13.56).

Fifteen days after T1 (T2), there was no significant difference between the treatments for the aerial part, for both FW (Figure 23C) and DW (Figure 23D). On the other hand, a significant difference was observed in FW roots ($p = 0.0470$) and DW roots ($p = 0.0461$). The treatment with better results was AMF in the absence of Si (FW =

880 mg ± 445; DW = 96 mg ± 41), which was significantly different from AMF in the presence of Si (FW = 302 mg ± 78; DW = 35 mg ± 11). These results show that, by T2, the combination of Si and AMF had a negative impact on root growth and biomass.

### IV.2.3. Effect of AMF on the Si accumulation of wheat leaves at the moment of transfer to individual pots (T0)

The Si concentration in wheat leaves and Si content in wheat plants at the moment of transfer to individual pots are shown in Table 7. At T0, there was only a significant difference in the Si concentration in the leaves (p=0.0495), where NM plants had higher values than AMF plants.

**Table 7-** Si concentration (µg/mg of DW) in wheat leaves and Si content (mg/plant) in wheat plants, inoculated (AMF) or not (NM) with *Rhizophagus irregularis* at the moment of transfer (T0). Different lower-case letters in a column indicate significant differences between treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$); n = 3; average ± SD. The absence of letters in a column indicates the absence of difference between the treatments.

| | Si concentration (µg/mg of dry weight) | Si content (mg/plant) |
|---|---|---|
| **NM** | 8.84 ± 1.58a | 0.99 ± 0.72 |
| **AMF** | 5.73 ± 0.85b | 0.66 ± 0.21 |

### IV.2.4. Effect of Si, AMF and *Trichoderma* on the Si accumulation of wheat leaves 30 and 45 days after transfer in individual pots (T1 and T2)

The Si concentration in wheat leaves and Si content in wheat plants, 30 days (T1) after the moment of transfer to individual pots are shown in Table 8.

**Table 8**- Si concentration (µg/mg of DW) in wheat leaves and Si content (mg/plant) in wheat plants 30 days after the moment of transfer (T1). Different lower-case letters in a column indicate significant difference between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$); n = 3; average ± SD. The absence of letters in a column indicates the absence of difference between the treatments. The treatments used are identical to those in the fresh and dry weight figures.

| | Si concentration (µg/mg of dry weight) | Si content (mg/plant) |
|---|---|---|
| **Control (-)** | 6.23 ± 2.55 | 0.84 ± 0.32cd |
| **Control (+)** | 8.15 ± 0.85 | 1.57 ± 0.08a |
| **AMF (-)** | 7.79 ± 2.09 | 1.15 ± 0.07b |
| **AMF (+)** | 7.97 ± 1.37 | 0.67 ± 0.24d |
| **AMF/T (-)** | 6.18 ± 2.29 | 1.05 ± 0.23bc |
| **AMF/T (+)** | 6.39 ± 1.58 | 0.89 ± 0.20c |

Thirty days after transfer in their individual pots (T1), the Si concentration in wheat leaves did not present a statistical difference between all the treatments. On the other hand, the Si content differed between treatments (p=0.0269), where the higher Si content values belong to the control (+) from all the treatments where Si was present, i.e. AMF (+) and AMF/T (+). So, when Si is combined with AMF and AMF/T the values are significantly lower.

The Si concentration in wheat leaves and Si content in wheat plants 45 days (T2) after the moment of transfer to individual pots are shown in the Table 9. Fifteen days later, no significant difference of Si concentration and Si content was measured in wheat plants, whatever the addition of Si or the inoculation with beneficial microorganisms.

**Table 9**- Si concentration (µg/mg of DW) in wheat leaves and Si content (mg/plant) in wheat plants 45 days after the moment of transfer (T2). Different lower-case letters in a column indicate significant difference between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$); n = 3; average ± SD. The absence of letters in a column indicates the absence of difference between the treatments. The treatments used are identical to those in the fresh and dry weight figures.

| | Si concentration (µg/mg of dry weight) | Si content (mg/plant) |
|---|---|---|
| **Control (-)** | 9.33 ± 5.04 | 4.49 ± 3.66 |
| **Control (+)** | 14.97 ± 2.13 | 4.52 ± 0.45 |
| **AMF (-)** | 7.92 ± 3.32 | 3.02 ± 1.23 |
| **AMF (+)** | 10.58 ± 2.98 | 2.07 ± 1.02 |
| **AMF/T (-)** | 12.32 ± 1.23 | 4.18 ± 0.38 |
| **AMF/T (+)** | 12.56 ± 1.38 | 3.16 ± 0.65 |

### IV.2.5. Mycorrhizal colonization of wheat roots at the moment of transfer (T0), 30 and 45 days of transfer to individual pots (T1 and T2)

The arbuscule percentage, vesicles percentage and total colonization percentage in wheat at T0, T1 and T2 are shown in Figure 24. When analyzing wheat plants for the first experiment (T1 and T2), the percentages of colonization (arbuscules, vesicles and total percentage) remained similar between the treatments, independently of the application of Si and inoculation with *T. harzianum*. Nonethess, it is possible to declare that as time increases, so does the percentage of colonization.

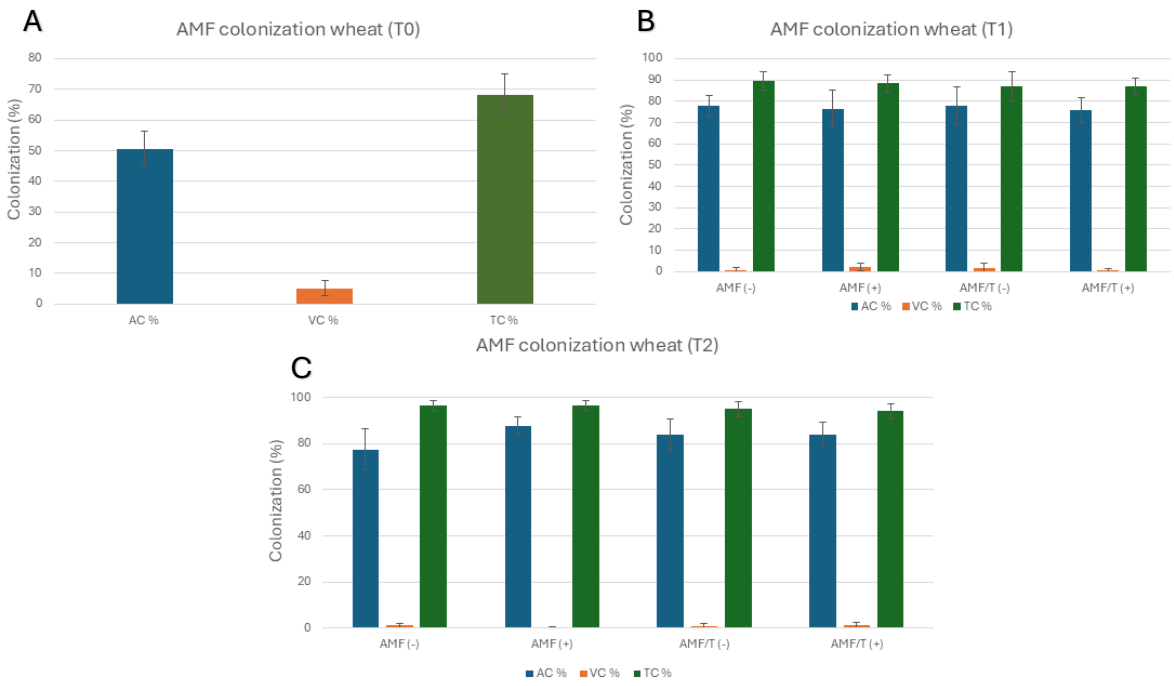


**Figure 24-** Arbuscular (AC%), Vesicular (VC%) and total colonization percentages (TC%) of wheat inoculated with *Rhizophagus irregularis* MUCL 43194: (A) at the moment of transfer (T0), (B) 30 days after transfer (T1) and (C) 45 days after the moment of transfer (T2). Different lower-case letters indicate significant differences between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$); $n = 5$. The absence of letters indicates the absence of differences between the treatments. The treatments used are identical to those in the fresh and dry weight figures.

## IV.2. General discussion of the effect of Si, AMF and *Trichoderma* on the growth, Si uptake and colonization of wheat

Analyzing the results for the first experiment on wheat plants, AMF alone and in combination with *T. harzianum*, showed the best results for root growth and biomass. However, the addition of Si had a negative impact on the wheat growth in both T1 and T2. This can be due to a potential negative interaction between Si and *Trichoderma*, as demonstrated by Rachniyom & Jaenaksorn (2008), who showed that soluble Si could significantly retard the vegetative and reproductive growth of *T. harzianum* and thus might alter the biomass of the roots. Similar trends were observed for T2, in which Si was also negative for plant growth when combined with AMF. While some studies have proven the beneficial combinatorial effect of Si and AMF on plant growth (Hajiboland et al., 2018; Islam et al., 2023), this effect can depend on the plant species or the biochemical interactions between Si and AMF. In the present study, the application of Si was not beneficial for the wheat aerial and roots growth and biomass, and the use of AMF alone or in combination with *Trichoderma* can be more suitable to increase the growth of wheat

plants. As for the maize plants, different species, or even different fungi can be applied to obtain higher biomass for wheat plants. As for the Si application, other concentrations and different times of application can be tested.

At the moment of transfer to individual pots, AMF had a negative effect on the absorption and accumulation of Si in the leaves at early plant stages. One hypothesis can be that as Si and AMF are a facilitator for phosphorus uptake, or other nutrients, this might limit the Si uptake and transport within the plant (Kostic et al., 2017; Zhang et al., 2019). As for T1 and T2, the application of Si was more positive when applied alone and lower when AMF and *Trichoderma* were inoculated. This can mean that for wheat plants, to obtain more Si accumulation, it is recommended to apply Si alone.

In conclusion, the use of one or both fungi had a negative impact on Si accumulation, whereas the use of Si alone was able to accumulate more of this mineral in the leaves, which makes it more recommendable. Again, other concentrations and fungi can be studied for a better understanding of the mechanisms.

Observing the results of colonization, the treatments did not significantly change the outcome. It means that, contrarily to maize (i.e., reduction of vesicular colonization and increase of arbuscular colonization), the organisms in the rhizosphere of wheat seem not to interact.

Finally, for wheat root growth and biomass, AMF and *T. harzianum* had a positive interaction. However, the same is not observed for the Si accumulation and uptake. So, for the growth and biomass of wheat roots, the use of AMF or both fungi is recommended, whereas the use of Si is rejected. If the desired result is more Si accumulation and uptake, the use of this mineral alone is a better fit.

## IV.3. Effect of Si, AMF and *Trichoderma* on growth, Si uptake and biocontrol of *Zymoseptoria tritici* on wheat

### IV.3.1. Effect of Si, AMF and *Trichoderma* on the growth of wheat after 21 days of infection with *Z. tritici*

The roots and aerial dry weight of wheat 21 days after the infection with *Z. tritici* is presented in Figure 25. Twenty-one days after the infection with the pathogen, a significant difference in the aerial DW ($p = 0.0175$) was calculated. The control treatment

with Si had the highest values (DW = 531 mg ± 148) and was significantly different from AMF with Si and AMF/T without Si. For the roots DW, no significant difference was measured.

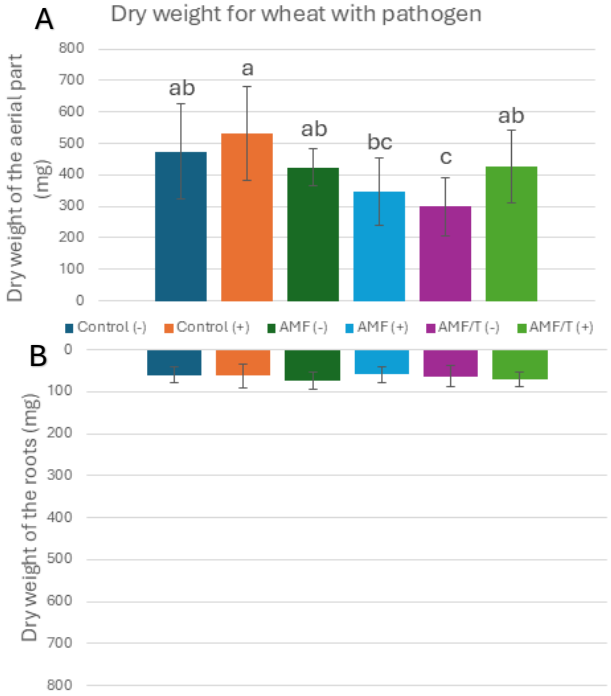


**Figure 25**- Aerial (A) and roots (B) dry weight of wheat 21 days after infection with *Zymoseptoria tritici*. Different lower-case letters indicate significant differences between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$). The absence of letters indicates the absence of difference between the treatments.
Control (-) (non-inoculated control without addition of Si); n = 7; Control (+) (non-inoculated control with addition of Si); n = 7; AMF (-) (maize inoculated by *Rhizophagus irregularis* MUCL 43194 without addition of Si); n = 7; AMF (+) (maize inoculated by *R. irregularis* MUCL 43194 with addition of Si); n = 7; AMF/T (-) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and without addition of Si); n = 7; AMF/T (+) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and addition of Si); n = 6.

### IV.3.2. Effect of Si, AMF and *Trichoderma* on the Si accumulation of wheat leaves after 21 days of infection with *Z. tritici*

The Si concentration in infected wheat leaves and Si content in infected wheat plants 21 days after the infection with *Z. tritici* is presented in Table 10. The treatments had no significant difference in both Si concentration and Si content. The control (-) had the highest Si concentration and Si content, in opposition to AMF (-) with the lowest values. When wheat plants were submitted to the pathogenic agent, the use of no treatment was able to accumulate more Si in the plant.

**Table 10**- Si concentration (µg/mg of DW) in infected wheat leaves and Si content (mg/plant) in infected wheat plants 21 days after infection with *Zymoseptoria tritici*. Different lower-case letters in a column indicate significant difference between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$); n = 3; average ± SD. The absence of letters in a column indicates the absence of difference between the treatments. The treatments used are identical to those in the fresh and dry weight figures.

| | Si concentration (µg/mg of dry weight) | Si content (mg/plant) |
|---|---|---|
| **Control (-)** | 15.32 ± 0.91 | 7.26 ± 0.73 |
| **Control (+)** | 10.10 ± 1.52 | 5.22 ± 1.54 |
| **AMF (-)** | 7.84 ± 5.43 | 3.52 ± 2.67 |
| **AMF (+)** | 11.90 ± 3.34 | 4.66 ± 0.73 |
| **AMF/T (-)** | 12.26 ± 7.81 | 3.28 ± 2.00 |
| **AMF/T (+)** | 11.75 ± 0.72 | 5.87 ± 1.31 |

### IV.3.3. Effect of Si, AMF and *Trichoderma* on disease severity of wheat plants after 21 days of infection with *Z.tritici*

The disease severity in wheat leaves 21 days after the infection with *Z. tritici* is presented in Figure 26. All the wheat leaves sprayed with *Z. tritici* showed necroses and pycnidia, 3 weeks after the pathogen inoculation.

The disease severity was quantified on the first four leaves (Figure 26). As visible in Figure 26, the 1$^{st}$ leave presented less than 20% of severity. Indeed, this first leave was not fully expanded when the pathogen was sprayed, thus limiting its infection. On the other hand, the 3$^{rd}$ and 4$^{th}$ leaves presented high levels of severity, comprising between 40 and 60%. The level of infection was similar whether the addition of Si or the inoculation of beneficial microorganisms.

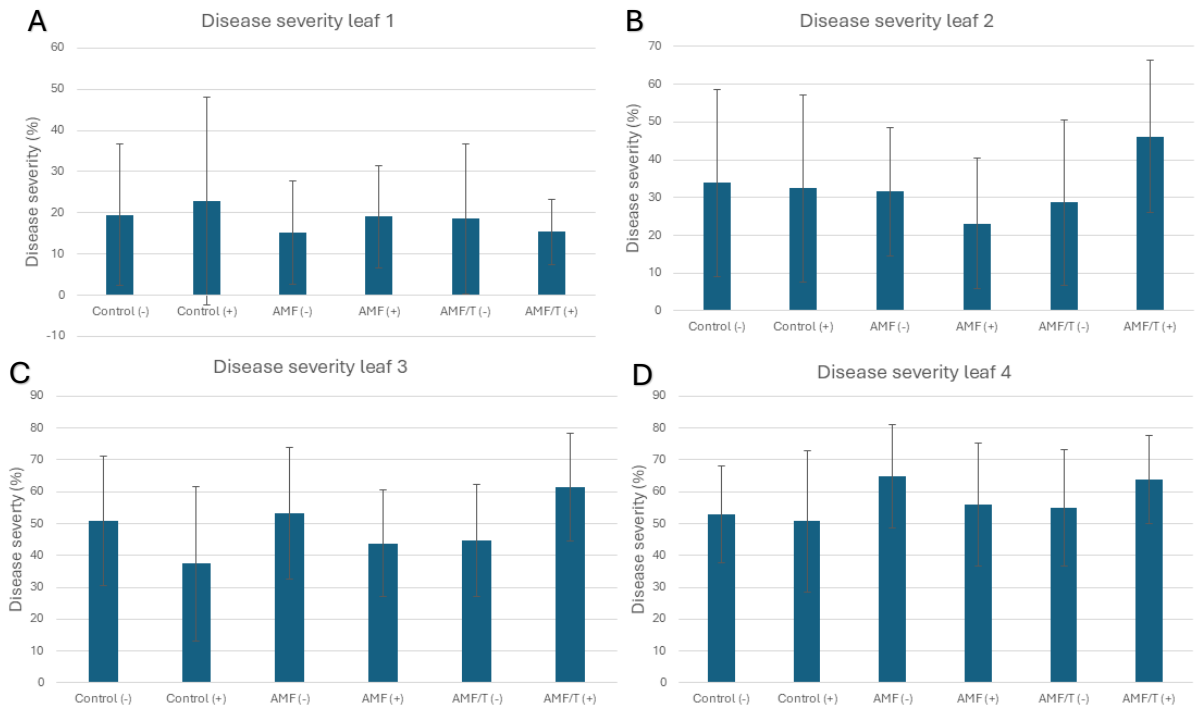


**Figure 26-** Disease severity in leaf 1 (A), leaf 2 (B), leaf 3 (C) and leaf 4 (D) in wheat 21 days after infection with *Zymoseptoria tritici*. Different lower-case letters indicate significant difference between the treatments according to a non-parametric Kruskall-Wallis test ($P \leq 0.05$). The absence of letters indicates the absence of difference between the treatments.
Control (-) (non-inoculated control without addition of Si); n = 7; Control (+) (non-inoculated control with addition of Si); n = 7; AMF (-) (maize inoculated by *Rhizophagus irregularis* MUCL 43194 without addition of Si); n = 7; AMF (+) (maize inoculated by *R. irregularis* MUCL 43194 with addition of Si); n = 7; AMF/T (-) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and without addition of Si); n = 7; AMF/T (+) (maize inoculated with *R. irregularis* MUCL 43194 and *T. harzianum* MUCL 29707 and addition of Si); n = 6.

## IV.3. General discussion of the effect of Si, AMF and *Trichoderma* on growth, Si uptake and the biocontrol of *Zymoseptoria tritici* on wheat

Analyzing the results for the second experiment, for the higher growth and biomass of the aerial part of infected wheat plants, the use of Si alone was the best option, whereas the use of both fungi gave significantly lower results. One hypothesis can be that, under certain conditions, *Trichoderma* might compete for space and nutrients with AMF, potentially leading to a negative impact on plant growth. One study proved that *T. harzianum* and *Glomus* spp. had a competitive interaction, where their combinatorial application did not provide a synergistic effect against Fusarium wilt on banana plants (Castillo et al., 2019).

For a higher aerial growth and biomass of infected wheat plants, the use of Si alone was shown to be a more viable option than the use of the microorganisms. Other

concentrations or applications could lead to a better outcome and, on the other hand, other fungi can be used and studied. Nonetheless, other varieties of wheat can be studied to determine which one has a higher disease resistance against this pathogen.

Observing the results for Si accumulation and uptake, the control treatments were able to accumulate more Si than other treatments where AMF and AMF/T were present. In conclusion, for better Si accumulation, the lack of fungi can be more desirable. Again, testing other concentrations and applications of Si can lead to more viable outcomes.

The results for the disease severity were very scattered, and whatever the considered leave, the area covered by the necroses induced by *Z. tritici* remained similar between the treatments, suggesting that both the inoculation with beneficial organisms and the amendment of Si did not induce a protection on the wheat leaves. One hypothesis that can explain the absence of biocontrol, is the absence of a higher accumulation of Si, which accumulates in the cell walls of the plants and thus blocks the entry of the pathogen and reduces the disease severity. Another hypothesis can be that, while both organisms are known for their biocontrol properties, their combination doesn't reduce the impact of *Z. tritici* beyond their individual effects. Our study relates to that of Bellameche et al. (2020), who reported that the combination of *Pseudomonas protegens* and *Pseudomonas chlororaphis* did not influence the resistance of wheat against STB disease. Another study showed that Si amendment did not increase wheat plant growth under powdery mildew infection (Guével et al., 2007).

Overall, the use of microorganisms and Si didn't prove to efficiently increase the tolerance against the disease. So, for a better understanding of the disease resistance against *Z. tritici*, more studies are required, such as different concentrations and applications of Si, a more resistant variety of wheat and a different mix of fungi.

# V. Conclusions and work perspectives

The experiments described in this thesis aimed at assessing the effect of the arbuscular mycorrhizal fungus *Rhizophagus irregularis*, alone or in combination with *Trichoderma harzianum*, on the improvement of maize and wheat plant growth and Si uptake, and also the effect of the three factors to control STB on wheat plants.

For maize plants the interaction between AMF and *T. harzianum* was negative, where it reduced the growth and biomass of maize plants and didn't impact the Si uptake and accumulation. Nonetheless, the plant was not able to accumulate and uptake more Si, whether the Si was applied or not. According to all parameters observed, the AMF treatment alone was the best for improving the growth and colonization of maize plants.

For wheat root growth and biomass, AMF and *T. harzianum* had a positive interaction. However, the same was not observed for the Si accumulation and uptake. So, for growth and biomass of wheat roots the use of AMF or both fungi is better, whereas the use of Si should be rejected, unless the desired result is more Si accumulation and uptake. Finally, the use of microorganisms and Si didn't prove to efficiently increase the tolerance against STB.

Overall, more studies are mandatory, where other strains or species of fungi, as well as their interaction, can be studied. Other forms of fungi application can be a good option, like seed coating or even other biostimulants. Different Si concentrations or application protocols (like foliar application), different plant varieties with more desirable characteristics (more resistant or more responsive to the microbial inoculation) and other environmental conditions can be more suited (different temperatures, humidity and a higher percentage of organic matter). Also, longer cultivation periods (covering different seasons) and bigger pots could lead to better results and a better understanding of the overall experiment.

Finally, for a more profound comprehension of the overall interactions, other parameters can lead to better conclusions, for example, testing the soil enzyme activity (enzymes such as phosphatase, dehydrogenase, and urease, which are indicators of soil biological activity), soil nutrient levels (to determine soil fertility), soil microbial biomass and quantification of the microbial community in the soil by PCR methods.

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# Annex 1

**Table A1**- PDA medium components necessary composition.

| Components | Composition |
|---|---|
| **Starch** | 19,5 g |
| **Agar** | 1 g |
| **Type II water** | 500 mL |

# Annex 2

**Table A2-** Hoagland's solution composition.

| Solution | Salts | Solution stock (g/L) |
|---|---|---|
| **A** | KH2PO4 | 2,74 |
| **B** | Ca(NO3)2·4H2O | 82,6 |
| **C** | KNO3 | 40,7 |
| | NH4NO3 | 8 |
| | MgSO4·7H2O | 12,04 |
| | KCl | 4,51 |
| | K2SO4 | 10,54 |
| **D** | MnSO4·H20 | 0,053 |
| | H3BO3 | 0,14 |
| | CuSO4·5H2O | 0,015 |
| | (NH4)6Mo7O2·4H2O | 0,008 |
| | ZnSO4·7H2O | 0,06 |
| **E** | C10H12FeN2NaO8 | 1,9 |