Szent István University

Effect of mycorrhizal fungi on stress responses to environmental factors in sunflower and maize

PhD thesis

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BACKGROUND AND OBJECTIVES

One of the most important challenges of agricultur these days is to minimize the economic losses caused by the extreme weather conditions induced by climate change. The inducer of this global problem is the global warming, which exceeds the global trend in Hungary. Thus, uneven rainfall distribution, mechanical damage caused by hail, and increased salinisation due to inappropriate irrigation and specific field characteristics are problems with utmost importance.

Various biotic and abiotic stresses are the most limiting factors on plant growth and crop yield. Under stress conditions highly reactive oxygen species (ROS) are generated in plant cells and ROS-scavenging enzymes such as catalase, peroxidase, glutathione S-transferase (Ergun et al. 2014), together with antioxidants of the non-enzymatic way (ascorbic acid, glutathione etc.) are able to detoxify these toxic molecules (Ordoñez et al. 2014).

Agricultures most burning question is how to mitigate stress effects and how to produce plants that are more resistant to these stresses. In Hungary, sunflower (sown area in Hungary increased significantly between 2004 and 2013, 628 thousand ha in 2018, the total harvested crop is 2 960 thousand tons (KSH 2019)) and maize (sown area in Hungary is 956 thousand ha in 2018, the total harvested yield is 8 440 thousand tons (KSH 2019)), besides wheat, are the most important field crops. Their sown period is in April, when the daily average temperature ranges between the extremes and unpredictable rainfalls are common, both of these factors can have a severe effect on crops. May is a critical period for young plants, as they are more sensitive to mechanical damage caused by hail and soil particles, these environmental factors are responsible for significant losses. In addition to the effects of temperature, drought and wound stress, the negative effects of inadequate irrigation or salinisation due to specific field characteristics, and salinity stress also make it difficult to ensure the desired yield. In order to avoid losses, it is essential to grow plants that are more resistant to these stress factors, this can be achieved by the inoculation of plants with arbuscular mycorrhizal (AM) fungi. Therefore, we investigated how the defense system of sunflower and maize is affected by the arbuscular mycorrhizal fungi inoculation during the early development stages when the plants are the most vulnerable.

In addition to abiotic stress effects, crop production alos suffers severe losses in yield and quality due to *Fusarium* infections. The main source of danger they pose is through their secondary metabolites called mycotoxins, which present health risks towards animals and humans alike.

Besides soil and postharvest management, there is an increased demand to use environmentally-friendly methods to control mycotoxin producing organisms. Among these strategies, there has been an increasing interest in beneficial microbes, such as arbuscular mycorrhiza (AM).

The symbiotic relationship between plants and arbuscular mycorrhizal fungi has been established more than 400 million years ago. The benefits to the target plant, include enhanced nutrient uptake, mostly phosphorus and water. Moreover, AMF may induce a systemic defense mechanism called mycorrhiza-induced resistance (MIR) in host plants. It activates the plant's defense system against biotic pathogens by changing the concentration of jasmonic acid in a priming state (Minton et al. 2016). Mycorrhizal colonization is also beneficial for the plant, by changing its energy management and response speed to an ideal state. The mycorrhiza inoculation induced protection against stressors is often accompanied by higher levels and activity of antioxidant enzymes, however the regulatory mechanisms of these changes are not well known, at early plant development stages, when AM colonization is still at low levels. Moreover AMF dramatically alters plant primary and secondary metabolism in affected roots, thereby influencing microorganisms living in the rhizosphere.

The decreased growth of filamentous fungi in the presence of root exudates has already been investigated but how it is altered in the presence of arbuscular mycorrhizal fungi is not well known. Hence, the aims of this work was to study the effects of root exudates derived from mycorrhizal plants grown under different nutrient levels on the growth and fumonisin production of *F. proliferatum*.

Our investigations were targeted to:

1. How arbuscular mycorrhizal fungal colonization affect the activity of some defense enzymes (polyphenol oxidase, peroxidase, glutathione S-transferase) involved in reducing abiotic stress effects (low temperature, high temperature, mechanical stress) at an early development stage (9, 15 and 42 days) of sunflower?

2. Investigation of the effect of arbuscular mycorrhizal fungi colonization by monitoring the activity and gene expression of plant defense enzymes (polyphenol oxidase, peroxidase, catalase, glutathione S-transferase) in 21 and 42-day-old maize plants exposed to abiotic stresses (high temperature, salt and drought stress).

3. How the presence of mycorrhizal fungi influences through root exudates the in vitro growth of *Fusarium proliferatum* fungi and the expression of the fumonisin production gene (*FUM1*) and the stress response involved HOG-type MAP kinase gene (*HOG1*)?

MATERIALS AND METHODS

Plant material and growth conditions

In the sunflower (*Helianthus annuus* L. var. Iregi, untreated) pot experiment two inoculation treatments were performed. Plants were inoculated with a commercial mycorrhizal product (mixture of *Rhizophagus irregularis* BEG140, *Funneliformis mosseae* BEG95, *Claroideoglomus etunicatum* BEG92, *Claroideoglomus claroideum* BEG96, *Rhizoglomus microaggregatum* BEG56, *Funneliformis geosporum* BEG199). For non-mycorrhizal treatments sterilized mycorrhizal inoculant was prepared. Plants were exposed to low temperature (AH, 4°C), high temperaure (MH, 38°C) and mechanical wounding (M) stress. Non-stressed plants were used as control (K). PPO, PER, GST enzyme activity and *GST* gene expression was determined.

In the maize (*Zea mays* L., Golda F1, untreated) pot experiment two inoculation treatments were performed. Plants were inoculated with a commercial mycorrhizal product (mixture of *Rhizophagus irregularis* BEG140, *Funneliformis mosseae* BEG95, *Claroideoglomus etunicatum* BEG92, *Claroideoglomus claroideum* BEG96, *Rhizoglomus microaggregatum* BEG56, *Funneliformis geosporum* BEG199). For non-mycorrhizal treatments sterilized mycorrhizal inoculant was prepared. Plants were exposed to salt (SÓ), high temperaure (MH, 42°C) and drought (D) stress. Non-stressed plants were used as control (K). PPO, PER, CAT, GST enzyme activity and *PPO*, *PER*, *CAT*, *GST* gene expression was determined.

For root exudate collection, two inoculation treatments with maize (*Zea mays* L., Golda F1) were performed. Half of the pots were inoculated with the mixture of mycorrhizal fungi (*Funneliformis mosseae* BEG12 (Glomerales: Glomeraceae); *Rhizophagus irregularis* BEG53 (Glomerales: Glomeraceae)). Inoculants were propagated on maize (*Zea mays* L. 'Golda F1′). For control treatments, sterilized mycorrhizal inoculum was prepared. Every second day, the plants were irrigated with 50 mL tap water as low nutrient supply (LN) or 5× Long Ashton representing high nutrient supply (HN). Root exudates were collected according to Da Silva Lima et al. (2014) and Lioussanne et al. (2009).

Plant biomass and root colonization were determined (Giovannetti and Mosse 1980, Vierheilig et al. 1998).

Growth assessment of *Fusarium proliferatum* under different root exudates

Fusarium proliferatum ITEM 2287 was used in the study. Mycelium for fumonisin production was prepared in 50 mL DM, supplemented with 30 mM ammonium dihydrogen phosphate and inoculated with 10^6 mL^{-1} conidia. Cultures were incubated at 26 °C with shaking (150 rpm) for 48 h then filtrated and washed. The mycelium was transferred directly to 50 mL of new DM medium. The co-cultures were inoculated separately with 5 mL of each of root exudate. The control treatment was prepared by adding 5 mL of sterilized distilled water. All treatments, were incubated at 26 °C using a shaker (150 rpm) for 24 h and 5 days.

The growth of *Fusarium proliferatum* ITEM 2287 due to various root exudates was followed on PDA. 100 µL of different types of concentrated root exudates were spread on the surface of PDA plates, and 10^7 mL^{-1} conidia of stock culture was placed in the center of the plate. Plates without root exudates had 100 µL sterilized distilled water spread over their surface and were prepared as described above, thes served as control.

Gene expression studies

qRT-PCR was performed on a Stratagene Mx3000P QPCR System. *GST* gene-specific and *actin* primer in the sunflower experiment, catalase (*CAT*), polyphenol oxidase (*PPO*), guaiacol peroxidase (*PER*), glutathione S-transferase (*GST*) gene specific and *actin* as a constitutively expressed control primer were used in maize expreiment. Fumonisin (*FUM1*) and HOG1-type MAP kinase (*HOG1*) gene specific and *histone H3* gene-specific primer were used as a constitutively expressed control. The gene expressions were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Enzyme assays

Measurement of polyphenol peroxidase (EC 1.10.3.1) enzyme activity was determined by a modified method by Fehrmann and Dimond (1967), the activity of guaiacol peroxidase (EC 1.11.1.7) was determined by a method developed by Rathmell and Sequeira (1974). The enzyme activity of catalase (EC 1.11.1.6) was determined by the method of Aebi (1984). Measurement of the activity of the glutathione S-transferase (EC 2.5.1.18) was determined according to Habig et al. (1974). Total protein was determined by the method of Bradford (1976).

All data were statistically analyzed with the R Statistical Software (R Foundation for Statistical Computing, Wien, Austria).

RESULTS

AM fungi colonisation and plant growth

No colonization was recognized when sterilized inoculant were used for inoculation, while high percentage of AM colonization was observed in inoculated plants, the rate of colonization also increased significantly over time. No growth responses could be observed in the sunflower pot experiment between mycorrhizal and non-mycorrhizal treatments. In maize pot expreiment the highest level of mycorrhizal colonization was measured after 21 days of growth under drought stress. Higher biomass was measured in case of the 21-day mycorrhizal, control plants. Under the influence of high temperature stress treatments, only the wet shoot and dry root weights were different in the mycorrhizal plants. After 42 days of incubation, the effect of mycorrhizal colonization on plant biomass was more observable. Higher values were found for wet, dry shoot and wet dry root weights of mycorrhizal plants, except for the wet shoots weight of plants treated with drought stress. After 6 weeks of incubation in the root exudate experiment maize plants grown under low nutrient treatment showed much higher colonization levels than plants in the high nutrient treatment.

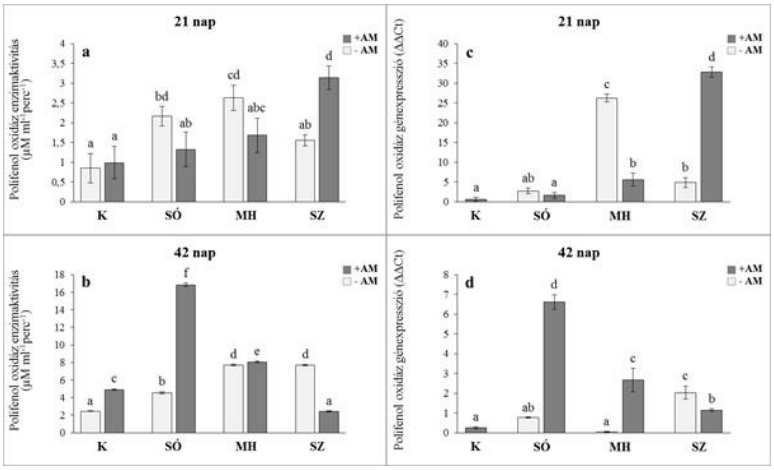
The percentage of mycorrhizal root colonization showed significant differences between samples collected from +AM LN and +AM HN. Plants colonized by AM fungi in low nutrient solution had significantly higher shoots and higher root growth than non-mycorrhizal plants. The main effects in fresh weight of plant shoots were all significant, except between mycorrhizal and non-mycorrhizal treatments at high nutrient supply. Plants colonized by mycorrhiza in low nutrient solution had significantly higher wet root weight than plants at high nutrient supply.

Polyphenol oxidase enzyme activity and gene expression

Only the mechanical wounding caused a significant increase in the activity of the PPO enzyme in the youngest 9-day old sunflower plants. In the two-week-old plants, mycorrhiza inoculation did not result in significant differences in the activity of the PPO enzyme, however, different stress effects increased the enzyme activity of the non-mycorrhizal plants. After 6 weeks, only high-temperature stress caused a significant difference in enzyme activity between mycorrhizal and non-mycorrhizal plants.

After three weeks of incubation drought stress induced significantly increased PPO enzyme activity and gene expression in mycorrhizal maize plants. Different stresses caused various changes in enzyme activities but only salt and high temperature stress increased enzyme accumulation of this enzyme in a high rate in mycorrhizal plants after 42 days of growth. In

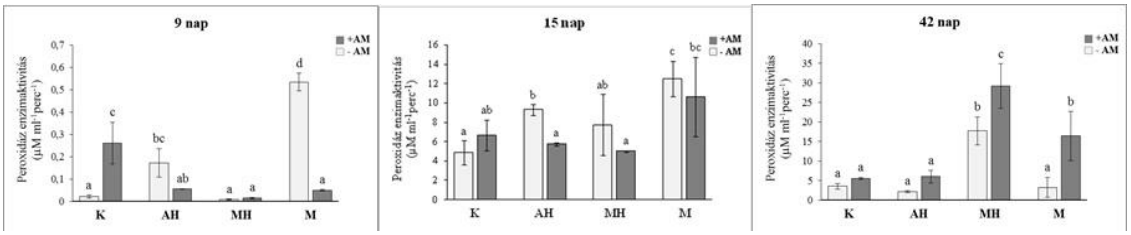
contrast to 21-day old plants, drought caused increased enzyme activity and *PPO* gene expression in the -AM plants after 42-days.



Polyphenol oxidase gene expression and enzyme activity in 21 and 42 days old maize plants
-AM – non-mycorrhizal, +AM – mycorrhizal; K – controll, SÓ – salt stress, MH – high temperature stress, SZ – drought stress. Different letters represent significantly different ($p < 0.05$) values.

Peroxidase enzyme activity and gene expression

Peroxidase enzyme activity increased in the 9-day old +AM control plants, this change was no longer observable at 15 and 42 days. At 9 day of incubation, only mechanical wounding caused higher enzyme activity in the -AM plants. Mycorrhizal inoculation did not cause any difference in PER enzyme activity in the 15-day old plants. After 42 days, high temperature and mechanical stress induced increased peroxidase activity in the mycorrhizal plants.



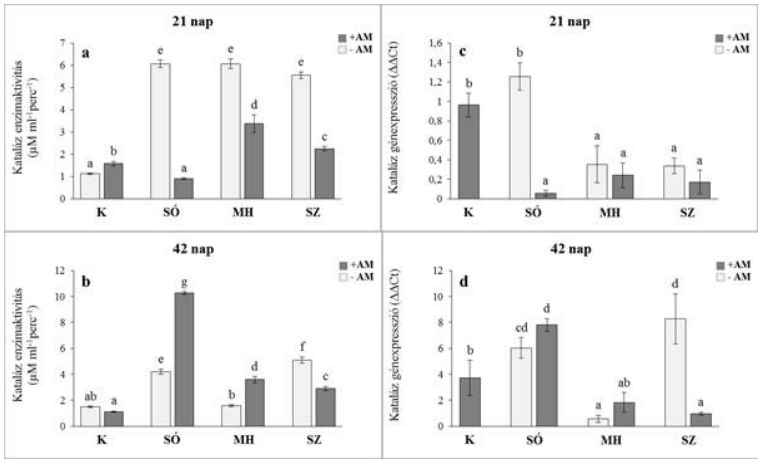
Peroxidase enzyme activity in 9, 15 and 42 days old sunflower plants
-AM – non-mycorrhizal, +AM – mycorrhizal; K – controll, AH – low temperature stress, MH – high temperature stress, M – mechanical wounding stress. Different letters represent significantly different ($p < 0.05$) values.

After 21 days of growth inoculated plants demonstrated higher PER enzyme activity and gene expression under salt and high temperature stresses meanwhile drought caused higher enzyme activity in –AM plants. In the 42-day old plants, the tendency of enzyme activity also changed in case of high temperature and drought treatments. Salt stress induced a higher level of enzyme activity in the + AM plants, but this was not observable in gene expression.

Catalase enzyme activity and gene expression

In 21-day old maize plants, treatments induced significantly higher catalase enzyme activity in the -AM plants compared to + AM and control plants. In the 42-day old maize plants,

the tendency was changed during salt and high temperature stress, + AM plants had the higher enzyme activity. Drought resulted in an increased enzyme activity and *CAT* gene expression in the –AM plants, this is contrary to the peroxidase enzyme activity and gene expression in the 42 day old plants.

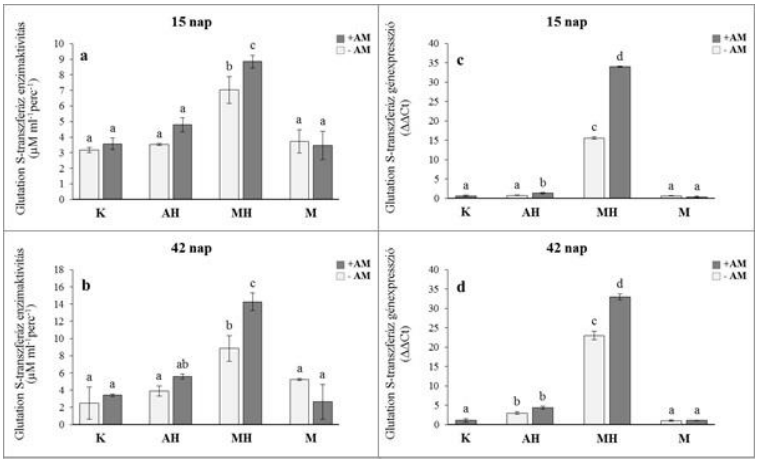


Catalase gene expression and enzyme activity in 21 and 42 days old maize plants

-AM – non-mycorrhizal, +AM – mycorrhizal; K – controll, SÓ – salt stress, MH – high temperature stress, SZ – drought stress. Different letters represent significantly different ($p < 0.05$) values.

Glutathione S-transferase enzyme activity and gene expression

High temperature stress induced an increase in glutathione S-transferase enzyme activity and gene expression in the 15 and 42-day old +AM sunflower plants compared to the control and –AM plants.



Glutathione S-transferase gene expression and enzyme activity in 15 and 42 days old sunflower plants

-AM – non-mycorrhizal, +AM – mycorrhizal; K – controll, AH – low temperature stress, MH – high temperature stress, M – mechanical wounding stress. Different letters represent significantly different ($p < 0.05$) values.

Salt stress induced an increase in GST enzyme activity and gene expression in the 21-day old –AM maize plants, whereas the difference caused by salt stress was abolished in the 42-day old plants, but the enzyme activity increased compared to the control. In the 42-day old +AM

maize plants, high temperature treatment induced significantly higher enzyme activity and *GST* gene expression compared to the non-mycorrhizal and control plants.

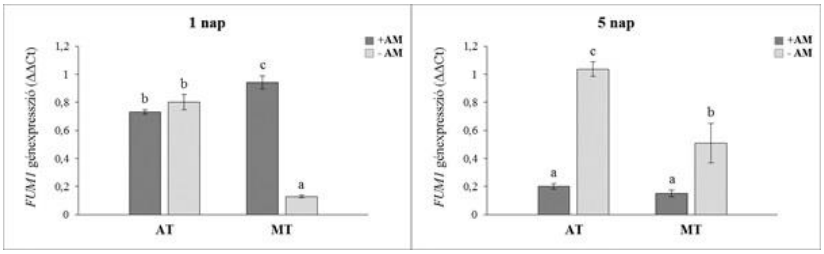
Growth of *Fusarium proliferatum*

After 1 day of incubation, there was a significant difference in the colony diameter of *Fusarium proliferatum* treated with the root exudates of non-mycorrhizal, LN plants only. After 5 days of incubation, the colony diameters were larger than in the previous measurement time, but there was no difference regardless of nutrient levels (low and high) and mycorrhizal inoculation.

***FUM1* gene expression**

After 1 day of incubation, the root secretion of the AT plant showed no difference regardless of the presence or absence of inoculation. Root exudates from low colonized roots (+AM HN) increased the expression of the *FUM1* gene compared to root-exudates from roots with a higher mycorrhizal colonization level (+AM LN).

After 5 days, mycorrhizal root exudates significantly reduced the relative expression of the *FUM1* gene, irrespective of the extent of the nutrient supplement of the target plants. Parallel with this decrease, increased *FUM1* gene expression was measured in the presence of −AM root exudates.



***FUM1* gene expression after 1 and 5 days of incubation**

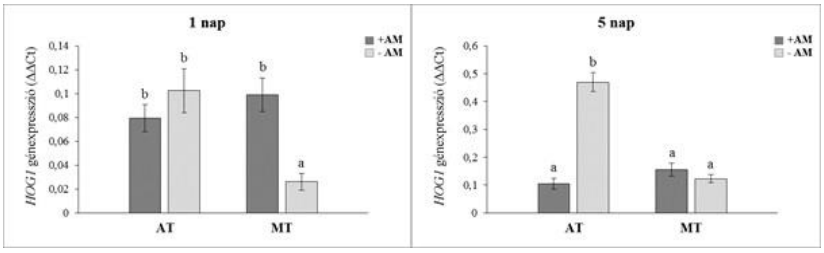
−AM: non-mycorrhizal plants, +AM – mycorrhizal plants; AT – low nutrient, MT – high nutrient. Different letters represent significantly different ($p < 0.05$) values.

***HOG1* gene expression**

After 1 day of incubation no significant differences were recognized among the treatments at low nutrient supply regardles of the presence or lack of mycorrhizal inoculation. Under –AM HN treatment, the gene expression of *HOG1* was significantly lower than in other treatment groups.

HOG1 gene expression was significantly lower after 5 days of incubation with +AM LN plant root exudates compared to the effect of −AM plant root exudates. There was no significant difference in *HOG1* gene expression between the root exudates of plants at a high nutrient

supply. Gene expression of *HOG1* was only significantly higher under −AM LN treatment compared to the other treatments.



***HOG1* gene expression after 1 and 5 days of incubation**
−AM: non-mycorrhizal plants, +AM – mycorrhizal plants; AT – low nutrient, MT – high nutrient.
Different letters represent significantly different ($p < 0.05$) values.

New scientific results

1. The measured enzyme activities (PPO, PER, GST) and GST gene expression showed significant differences depending on the age of the plant (9, 15 and 42 days) and the type of stress effect.

2. The presence of mycorrhizal fungal strains increased the peroxidase activity of the 9-day old sunflowers compared to the non-mycorrhizal plants, this change was no longer present after 15 and 42 days. The cause of this change is the initial detection of the mycorrhizal fungus as a pathogen and its transition to a symbiotic state over time.

3. The highest differences in the defense enzymes (PPO, CAT, GST) and gene expression (*PPO*, *CAT*, *GST*) of the colonized and non-mycorrhizal plants (maize, sunflower) were found under the influence of high temperature stress. Increased enzyme activity and gene expression of mycorrhizal plants under heat stress contribute to the elimination of reactive oxygen species, which thereby becomes more efficient in the colonized plant after a well established symbiosis.

4. We were the first to publish data on the alteration of glutathione S-transferase gene expression and enzyme activity induced by the combination of mycorrhizal inoculation, temperature and mechanical wounding stresses. The changes in glutathione S-transferase (GST) enzyme activity and expression followed the same tendency, peaking in the presence of mycorrhiza and high temperature stress.

5. The root exudates of the plants co-colonized with the two mycorrhizal fungal strains (*Funneliformis mosseae*, *Rhizophagus irregularis*) caused a significant decrease in the growth of *Fusarium proliferatum* after 24 hours *in vitro*, compared to the root exudates of non-mycorrhizal, low nutrient-grown plants.

6. *FUM1* gene expression was reduced after 5 days of incubation in the presence of root exudates originating from low- and high-nutrient supply plants colonized with two mycorrhizal fungal strains (*Funneliformis mosseae*, *Rhizophagus irregularis*) compared to the root exudates of non-mycorrhizal plants.
7. Root exudates of mycorrhizal maize plants grown under low nutrient-level and colonized with two mycorrhizal fungi strains (*Funneliformis mosseae*, *Rhizophagus irregularis*) at a higher rate resulted in a more notable decrease in *FUM1* gene expression after 5 days of incubation than root exudates of mycorrhizal plants grown under higher nutrient-level.
8. After 5 days of incubation root exudates of mycorrhizal plants grown under low nutrient-level colonized with two mycorrhizal fungi strains (*Funneliformis mosseae*, *Rhizophagus irregularis*) at a higher rate resulted in lower MAP kinase (*HOG1*) gene expression compared to the root exudates of non-mycorrhizal plants.

CONCLUSIONS AND PROPOSITIONS

The beneficial effects that AM fungi provides to host plants, including increased uptake of water and nutrients, especially phosphorus, and the protection of older plants against biotic and abiotic stress effects have been studied. The results support the stress tolerance-enhancing effect of mycorrhizal fungi in plants (Lenoir et al. 2016) and the role of elevated antioxidant levels in host plants, however, studies on young plants at their most sensitive age are insufficient.

Because of this we chose to monitor the changes in the enzyme activities and gene expressions induced by the most common stresses (drought, mechanical, salt and heat) in paralell with AM fungi inoculation during the emergence and the early stages of development of sunflower and maize plants.

In our experiment with sunflower, mycorrhizal inoculation did not cause significant difference in the dry and wet weights of the plants, not even after six weeks. This is probably due to the near-optimal phosphorus content of the medium, which was intended so we could study the effects of mycorrhizal colonisation while ignoring the effects of the enhanced nutrient uptake. However, prolonged drought and salt stress are known to reduce the number and size of leaves by decreasing the water potential of the soil and to reduce the biomass of the plants (Zhao et al. 2015), which we also observed while comparing treated to control plants at 21 and 42 days in case of maize. During the study period (21 and 42 days), the role of mycorrhizal inoculation in promoting plant growth, and its effect in the mitigation of stresses, was moderately observed under constant stress. Only plants under control or non-continuous stress (high temperature

stress) showed higher mycorrhizal dependence, which is also consistent with the results of the root colonization assay. In addition, continuous stress on the plants could be traced not only to the loss of biomass but also in the change of selected enzymes activities and gene expression involved in abiotic stress effects. Our results confirm the literature references (Lone et al. 2018), which show the sensitivity of early developmental stage plants based on studies related to changes in stress enzymes.

As an obligate biotrophic fungi, AM is known to utilize close to 5-22% of the plant's assimilates (Wright et al. 1998), which results in a significant loss of energy, especially in the early stages of plant development. Therefore, it is not surprising that constant stress induces changes in the mycorrhizal plant to such an extent that cannot been compensated by the AM fungi through excessive plant growth. The stress-relieving effects of mycorrhiza in salt and drought stressed plants is more likely to emerge at an advanced age, although their role is not negligible at the early developement stages neither.

Nine days after mycorrhizal inoculation, at early stages of symbioses, only appressoriums and external hyphae together with germinated spores of AMF were detected, however, internal hyphae and arbusculum were recorded after 42 days of growth. In addition, the highest percentage of colonization was observed under drought stress, as the plant attempts to compensate for water shortage (Pagano 2014) and to increase plant assimilates through nutrient uptake in this manner. The negative effects of constant salt stress on colonization have also been demonstrated (Hashem et al. 2018), which we have confirmed. However, only two (*Rhizophagus irregularis* and *Funneliformis mosseae*) and three (*Claroideoglomus etunicatum*, *Rhizophagus intraradices* and *Funneliformis mosseae*) AM fungi have been tested in these studies, compared to the six AM fungi strains what we used.

One of the earliest responses of plants to different stresses, is the rapid accumulation of reactive oxygen species (ROS), which molecules can be eliminated by the plant enzymes and the non-enzymatic pathway (Zenda et al. 2018). ROS is not only harmful to plant cells, but also to the mycorrhizal fungi, so elimintaion is essential for both parties.

In addition to the abiotic stress effects within two weeks of germination, the presence of AM fungi is also regarded as a stress factor for the young plant. The higher peroxidase activity in the 9-day old, mycorrhizal control plants indicate that the presence of mycorrhiza in the host plant appears as a stress effect, which is also indicated by the increased stress enzyme activity. Similar results were obtained by Spanu and Bonfante-Fasolo (1988) in 16-day old onion plants. This is not surprising since mycorrhizal fungi are often perceived by the plant as a biotrophic pathogen in the early stages of colonization (Pozo and Azcón-Aguilar 2007). Although not

measured by us, there are several references in the literature on how the relationship between the fungi and the plant changes from perceiving as a pathogen to a stable symbiotic relationship, this is achieved by altering the quantity and quality of plant hormones (Pozo et al. 2015). This is proved by the disappearance of differences in peroxidase enzyme activity, which is responsible for the production of monomers, dimers and phenoxy-free radicals and the regulation of the harmful accumulation of hydrogen peroxide in the 15 and 42 day old sunflower plants. The 58% high colonization level by these dates also further proves this.

Suprisingly, we only observed an increase in the peroxidase enzyme activity that normally occurs as a result of stress, (compared to the control) in non-mycorrhizal plants, and in mechanical wounding and high temperature stress treatments with a colonization rate over 50%. The mycorrhizal plants, with their increased enzyme activity, are ready to eliminate the stress effects since besides peroxidase the levels of polyphenol oxidase and glutathione S-transferase are also increased. Heat tolerance of mycorrhizal plants has already been described (Maya and Matsubara 2013), but there is no data available on the age from which the plant is capable of developing it.

In the 9-day old plants with a colonization level of 24%, due to the stress effects they suffered the enzyme activity of the peroxidase was reduced compared to the non-mycorrhizal plants, which is likely due to the energetic conditions of the plant. At this early stage of development, the photosynthetic surface is small and amount of assimilates is little, which has to cover the energy needs of both the fungi and plant development and also the synthesis of enzymes involved in stress reduction. Therefore, the plant has to reconsider its energy management at this early stage and wont necessarily raise the level of antioxidants, in this case the level of peroxidase. At later developement when it has more assimilates, it can increase the amount of antioxidants without the inhibition of plant growth. After 6 weeks, an increase in enzyme activity was observed in mycorrhizal plants, indicating a more balanced energy management from the plant. Increased levels of peroxidase in mycorrhizal plants for abiotic stress effects have been reported, using either 7-week old maize plants (Zhu et al. 2010) or 28-day old ryegrass (Lee et al. 2012) as model, for sunflower and at such an early stage of development (9 days) the experiments are missing.

The coordinated action of antioxidant enzymes ensures ROS eilimination. Thus, in addition to the peroxidase enzyme activity, changes in the catalase enzyme activity involved in controlling the harmful accumulation of hydrogen peroxide that damage the cell membrane, DNA, RNA, and proteins must be monitored (Estrada et al. 2013). The catalase and the peroxidase enzyme showed significant increases in both time points (21 and 42 days) compared

to non-mycorrhizal control plants. The effect of salt stress did not result in an increase in the catalase enzyme activity and gene expression but peroxidase activity did increase in the 21-day old inoculated plants, this might explained with the energy management of the young plant and the similar role of the enzymes in the plant defense systems. These results are in line with those of Estrada et al. (2013) in maize. In the presence of arbuscular mycorrhizal inoculation and stress, an increse in the activity of enzymes (superoxide dismutase, catalase, peroxidase) was observed in 10-week old bean (Lambais et al. 2003) and 16-day old sunflower plants (Vangelisti et al. 2018).

The glutathione S-transferase enzyme plays a key role in the inactivation of oxidative stress metabolites, however, no data are available on its changes in mycorrhizal plants under stress conditions. Vangelisti et al. (2018) studied the interaction of 16-day old sunflower plants and AM fungi under controlled, stress-free conditions. Surprisingly, GST showed a lower level of enzyme activity and gene expression in the early stages of plant development under stress conditions compared to the control mycorrhizal plants, with the exception of drought in maize and high temperature in sunflower.

We found increased levels of PPO enzyme as a result of salt stress, thishas already been reported previously (Kaya et al. 2013), but unlike us Kaya et al., also found increased PPO levels under temperature stress. All of this is explained by stimulated efficiency of PS II (Tang et al. 2009), as well as increased water uptake, osmotic potential change and elevated N and Mg uptake (Talaat and Shawky 2014). Not only elevated antioxidant enzyme activity but also changes in osmolite accumulation, photosynthesis and secondary metabolism play a role in the elimination of low temperature-induced ROS.

Our findings confirm that abiotic stress induced changes in antioxidant enzyme activities are influenced by the age of the plant, and these changes are not solely the individual effect of AM inoculation, although it plays a crucial role in them.

The beneficial effects of arbuscular mycorrhizal fungi include its protection against pathogen infections. Arbuscular mycorrhizal fungi during colonization stimulate the plant's defense system through mycorrhizal induced resistance (Minton et al. 2016). AM colonization induces physiological changes in the root of the plant during colonization, which may affect the composition of root exudates (Lioussanne et al. 2009), and through them the microflora of the rhizosphere and mycorrhizosphere. The inhibitory effect of AM fungi on the growth of filamentous fungi has been observed several times (Wang et al. 2018).

The effect of root exudates, and through them the effect of arbuscular mycorrhizal fungi, on the growth of *Fusarium proliferatum* and its fumonisin production has not been studied.

Therefore, we investigated the effect of the root exudates of maize plants grown under two nutrient levels, coupled with AM fungi inoculation for each treatment.

AM fungi colonization had no indirekt effect through root exudates on colony growth. Previously, Ismail et al. (2011) and Filion et al. (1999) also observed the opposite of this. They described the inhibitory effect of *Rhizophagus irregularis* on the growth of *Fusarium sambucinum* and the inhibitory effect of *R. intraradices* on the germination of *F. oxysporum* conidials. However, unlike our experiment, they performed their tests on a "sterile" AM fungal strain maintained on the root of *Daucus carota*. Lioussanne et al. (2008) showed that mycorrhizal colonization in its early stages have a positive effect on the zoospore germination of the pathogene *Phytopthora nicotianae* (Peronosporales: Peronosporaceae) while root exudates from plant roots extensively colonized by AM fungi show inhibitory effects.

Our study was carried out with root exudates of plants from pot cultures, which contained other microorganisms besides AM fungi. Colony growth may have been influenced by PDA medium, which provides the adequate amount of nutrients needed for growth, and also the effect of AM fungal colonization was not significant, despite the biomass of the plant (Frater et al. 2018) and the percentage of colonization in our experiments with low- and high-nutrient grown plants confirmed earlier results that plants depend more on the beneficial effects of AM fungal colonization under low nutrient levels than in more favorable nutrient conditions.

Only root exudates of non-mycorrhizal plants grown at low nutrient levels after 24h of incubation showed a significantly greater colony growth of *Fusarium proliferatum*, this difference did disappear after 5 days of incubation. Our result are opposing previous studies, this may be due to the different set-up of our experiment, the difference in tested strains, and due to our experiment only studying the effect of root exudates in short-term. Our study focused not only on the root extracts effect on colony growth but also on the fumonisin production ability of *Fusarium proliferatum*.

Our results were the first to show that root exudates of mycorrhizal plants after 5 days of incubation significantly reduced the fumonisin producing ability, this effect was more prominent when the root exudates of low-nutrient plants was applied. The root exudates of the mycorrhizal and non-mycorrhizal maize plants had the same effect on the expression of the *HOG1* gene as the expression of the *FUM1* gene, and it was observed that the low nutrient supply was a stress factor at both time points. Increased *HOG1* and *FUM1* gene expression is documented under nitrogen starvation (Kohut et al. 2009), but their changes under mycorrhizae influence have not been tested until now. Various biotic and abiotic factors, such as water capacity (Ferrochio et al. 2014), different plant extracts (Górna et al. 2016), temperature (Cendoya et al. 2018) and carbon

sources (Jian et al. 2019) influence the mycotoxin production of *F. proliferatum*. It is known that *FUM1* gene catalyzes the biosynthesis of the polyketide from acetyl-CoA, malonyl-CoA and methionine molecules (Proctor et al. 1999) from which the fumonisin is formed.

Mitogen-activated protein kinase (MAPK) cascades are important in stress-responsive signaling pathways for both plants and fungi. The cascade is activated by and reduces the accumulation of induced reactive oxygen species (ROS) in fungal cells (Jiang et al. 2018).

The initial increase (after 24h) in gene expression of *HOG1* can be attributed to the fungi percieving HN root exudates as a stress factor also described by Zheng et al. (2012) measuring the HOG-type mitogen-activated protein (MAP) kinase gene (*HOG1*) expression.

Studying the effects of AM fungi colonized plants root exudates on pathogens could yield significant results for the development of new technologies in agriculture, for this the next step could be the inclusion of additional genes involved in mycotoxin production and the further involvement of *Fusarium* and other species that cause crop failure in agriculture.

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