Investigating the Effects of Future Climate on Arbuscular Mycorrhizal Fungal Spore Dynamics in a Belgian Pear Orchard Ecosystem

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*Running title: *Future Climate Effects on Mycorrhizal Fungi*

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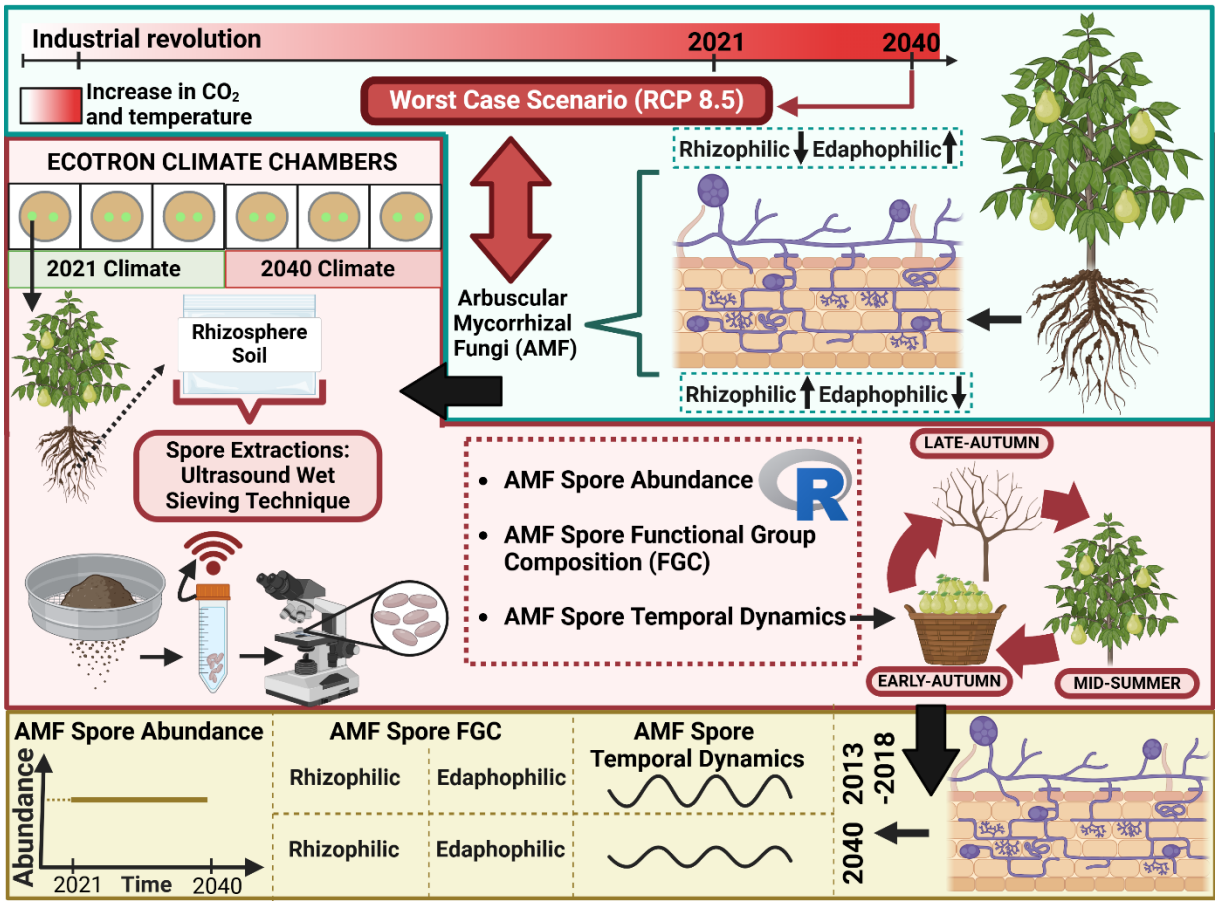
Graphical Abstract



ABSTRACT (236 words)

Climate change affects soil microbial communities, including arbuscular mycorrhizal fungi (AMF), which play a crucial role in plant resilience and nutrient uptake. This study examines the impact of projected climate change (2040) on the functional group composition and temporal dynamics of AMF spores in Belgian pear orchards using an advanced Ecotron facility. By simulating present (2013-2018) and future (2040) climate conditions under the RCP 8.5 scenario, AMF functional group (i.e. rhizophilic and edaphophilic) responses were assessed in response to climate change. The results indicate that overall AMF spore abundance remained stable between climate periods, suggesting resilience to climate change. *Glomeraceae* consistently exhibited higher spore abundance than *Gigasporaceae* across all seasons, but functional group composition remained unaffected by climate change. However, seasonal shifts in spore production were observed, with a pronounced mid-summer peak in *Glomeraceae* spore counts in 2013-2018, which diminished by 2040, leading to a more stable distribution across seasons. This shift suggests that climate change may influence AMF phenology, altering phenological patterns rather than overall species abundance and functional group composition. These findings contribute to understanding how AMF respond to climate change, revealing phenological shifts. While this study focuses on a specific Belgian context, broader and long-term studies are needed to fully assess AMF adaptability under future climatic conditions. Our research advances the understanding of climate-driven dynamics of AMF in agricultural systems, providing insights into sustainable crop production and soil fertility under future climate conditions.

INTRODUCTION

Since the onset of the Industrial Revolution, atmospheric CO_2 concentrations have risen from 278 ppm in 1750 to 420 ppm in 2023, a 51% increase, significantly amplifying the greenhouse effect [1]. As a result, global temperatures from 2014 to 2023 averaged 1.20°C (±0.12°C) above pre-industrial (1850–1900) levels, with the annual average in 2023 reaching 1.45°C (±0.12°C) above pre-industrial conditions [2]. This global climate change is predicted to lead to major shifts in weather patterns, intensifying heatwaves, prolonging droughts, and increasing both the frequency and severity of heavy rainfall [3]. Additionally, it has driven compound events, such as simultaneous drought and extreme heat that heighten wildfire risks. This trend is expected to persist and intensify as global temperatures continue to rise [3]. These climate-driven shifts pose serious challenges to both natural and agricultural ecosystems. As terrestrial plants are sessile organisms, unable to migrate to evade environmental stressors, it is crucial to understand their adaptive mechanisms to climate change, such as adaptations to water- and nutrient-limitations and plant pathogens. A critical plant adaptation strategy to environmental stresses, including abiotic stressors associated with climate change (e.g., heat and drought) and nutrient limitation, is a symbiotic relationship with arbuscular mycorrhizal fungi (AMF), which can ameliorate these stressors [4].

These fungi form symbiotic associations with up to 80% of terrestrial plant species, including economically valuable crops such as fruit trees, which hold significant value for the European agricultural industry [5]. AMF play a crucial role in various plant functions, such as enhancing phosphorus (P) uptake and improving plant resistance to soil-borne pathogens, heavy metals, and water stress [5]. To facilitate this symbiosis, AMF develop both extraradical and intraradical structures (Figure 1). The extraradical structures include the mycelium, which extends beyond the root system, expanding soil exploration and thereby increasing P availability and translocation, ultimately enhancing plant nutrition and growth [6]. Research shows that AMF can contribute up to 90% of the plant's P supply [7]. Additionally, extraradical structures that extend beyond the plant roots into the soil, consist of spores that are reproductive units dispersed in soil, and hyphae that extend from the mycelium to aid in nutrient uptake. In contrast, intraradical structures are formed inside the plant roots and include hyphae, which are involved in root colonization, arbuscules, which serve as the primary sites for nutrient and carbon exchange, and vesicles, specialized for lipid storage, and spores [8].

Besides nutrient uptake, AM fungi enhance litter decomposition and soil nutrient availability by facilitating interactions with soil microbial communities, particularly saprotrophic microorganisms that help mobilize nitrogen from soil organic matter (SOM). While AM fungi don't directly mineralize SOM, their hyphal exudates stimulate microbial activity— a process called 'priming'— thereby improving soil structure, texture, and overall plant health [9].

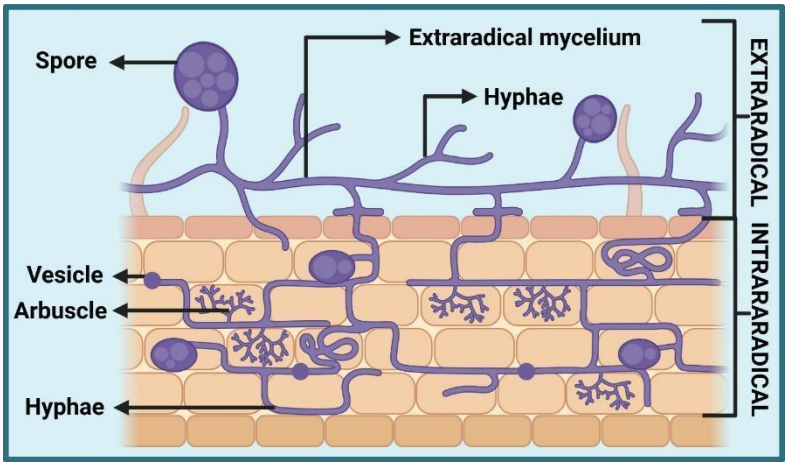


Figure 1: Plant Root Colonization by Arbuscular Mycorrhizal Fungi (AMF): Extraradical and Intraradical Structures. *Figure created with BioRender.com.*

In particular, spores, being reproductive units, are essential for the long-term survival of AM fungi and the re-establishment of mycorrhizal networks following disturbances [10]. *Gigasporaceae* and *Gigasporaceae*, the two largest AM fungal functional groups, rely on distinct life-history strategies to fulfill their specific roles in relation to host plants [10]. *Gigasporaceae* follow an edaphophilic (i.e. soil-loving) strategy, prioritizing biomass allocation to extraradical hyphae for long-term persistence in stable environments by acquiring growth-limiting resources to recolonize when conditions become favorable. These fungi play a key role in ensuring survival in low-stress and low-disturbance environments by delaying sporulation to support dormancy and by maintaining the slow-growing competitor strategy that thrives in mature ecosystems [11-15]. In contrast, *Glomeraceae* employ a rhizophilic (i.e. root-loving) and ruderal strategy, prioritizing fast intraradical colonization. These fungi are adapted for rapid colonization in highly disturbed and low stress environments by rapid re-establishment of mycorrhizal networks using high hyphal turnover and frequent reproduction at the expense of low biomass allocation to extraradical hyphae. This strategy enables faster plant colonization and nutrient acquisition, particularly in high-disturbance environments [16-18].

Earlier research demonstrated that AM fungi promote plant growth under abiotic stress conditions by mediating complex signaling pathways between plant and fungus. Plants inoculated with AM fungi exhibit enhanced resilience to various environmental stressors, including salinity, drought, nutrient stress, alkali stress, cold stress, and extreme temperatures. This increased stress tolerance translates into higher crop yields per hectare across a wide range of agricultural species [6]. In addition to improving plant health, AM fungi may also indirectly influence atmospheric CO_2 fixation through biogeochemical cycling, contributing to soil carbon storage—a process known as the 'sink effect'. Estimates suggest that mycorrhizal mycelium sequesters 13.12 Gt CO_2e each year or about 36% of annual CO_2 emissions from fossil fuels, highlighting the vital role of these fungi in global carbon dynamics and ecosystem stability [8,19,20]. Given their substantial impact on both plant health and ecosystem functioning, it is crucial to examine how AMF communities are affected by climate change. With climate change becoming an increasing concern, research on how shifting climatic variables impact AMF communities has become increasingly critical.

Studies show that AM fungi exhibit significant sensitivity to changing climatic parameters, which can both directly and indirectly affect their diversity, distribution, and functions within ecosystems. Indirect effects arise from climate-driven changes in host plants, soil properties (e.g. pH), and nutrient availability [5,21]. Although research attempts to understand the effects of altered water availability on AMF communities are more abundant compared to studies of the effects of drought, the findings remain controversial, often inconsistent, and context-dependent [5]. Furthermore, the effects of increased rainfall on AMF diversity vary, with increases, decreases, and no change being reported [5]. Nevertheless, both drought and increased rainfall show to influence AMF community composition in certain ecosystems [5]. In contrast to increased rainfall, drought has been reported to negatively affect AMF [21]. Further, most studies indicate that increased CO_2 does not significantly affect AMF richness or diversity; however, it alters community composition, thereby changing community structure. Nevertheless, this shift is primarily driven by changes in carbon allocation to AMF, favoring *Glomeraceae* species while disadvantaging *Gigasporaceae*, rather than broader climatic factors [5].

A critical aspect of AMF dynamics under climate change that has gained increasing attention is the role of AMF spores [22-24]. Research suggests that in cold and temperate climates, higher temperatures promote AMF colonization. As reproductive units, spores enable AM fungi to establish contact with plant roots, which is essential for their survival, particularly in harsh environmental conditions, such as drought or extreme temperatures [24,25]. AMF spores can remain dormant in the soil until favorable conditions trigger their germination, allowing them to colonize plant roots when conditions are optimal [26]. Consequently, seasonal climatic variations that influence host plant phenology can affect the timing of root colonization and thereby nutrient exchange, ultimately impacting plant health and ecosystem productivity [27]. With projected temperature increases by 2040, enhanced CO_2 assimilation and transport to roots [28] may delay AMF dormancy onset. Yet these effects are taxon-dependent, as research has shown that spores of *Glomeraceae* species are often more resilient to environmental stressors, such as elevated CO_2, which suggests their potential to become more dominant in future climate [22]. Thus, climate change could alter the temporal dynamics of AMF, making it crucial to understand these phenological shifts in order to predict their future ecological roles in soil ecosystems and their potential implications for agricultural practices in a changing climate.

Previous research has predominantly focused on the effects of individual climatic parameters, creating a gap in understanding the joint impacts of full climate exposure. This study aims to address this gap by exploring a comprehensive climate scenario for 2040 in Belgium. The scenario is based on a Representative Concentration Pathway (RCP), as defined by the Intergovernmental Panel on Climate Change (IPCC) in their Fifth Assessment Report of 2014 [29,30]. These RCPs represent standardized greenhouse gas concentration pathways that project future climate outcomes, ranging from low-emission scenarios with active mitigation (RCP 2.6), through two intermediate pathways (RCP 4.5 and RCP 6.0), to a high-emission scenario (RCP 8.5). The RCP 8.5 scenario (i.e., worst-case emission pathway), characterized by a continuous rise of greenhouse gas emissions throughout the 21st century, projects a mean global temperature increase of +2.0°C by 2046-2056 and of +3.7°C by 2081-2100, leading to more rapid warming and more significant climate change [29,30].

In this study, the worst-case RCP8.5 scenario for Belgium in 2040 is used, as it is expected to result in the most severe ecological effects. To simulate this comprehensive climate scenario and assess its impact on AMF spore abundance, functional group composition and temporal dynamics, we used the state-of-the-art Ecotron facility at Hasselt University (Figure 2). The Ecotron offers precise control over key climatic parameters, such as soil temperature, air humidity, and CO_2 levels, enabling the replication of both ambient (2013–2018) and future (2040) climate conditions. By simulating the full climate scenario rather than individual climatic parameters, the Ecotron bridges the gap between controlled laboratory experiments and field studies. This innovative approach provides valuable insights into the ecological impacts of climate change, particularly in understanding the complex interactions among environmental drivers and their effects on AMF spore communities [31].

Figure 2: Ecotron Facility Maasmechelen, National Park Hoge Kempen (NPHK), Belgium

Addressing the gaps in current research on AMF responses to climate change, this study assesses how AMF spores, specifically associated with pears (*Pyrus communis L.*), respond to the projected 2040 climate conditions in Belgium, based on the worst-case RCP 8.5 scenario. This research focuses on the abundance, functional group composition and temporal dynamics of AMF spores under these climate conditions. The study hypothesizes that the AMF spore community in a pear orchard will exhibit more rapid and pronounced temporal changes under future climate conditions (RCP 8.5, 2040), with a significant shift in AMF spore functional group composition, including an increased dominance of *Glomeraceae*, but no change in overall AMF spore abundance.

MATERIAL AND METHODS

We examined pear tree rhizosphere soil samples under both ambient (2013-2018) and future (2040, RCP8.5) climate conditions, addressing climate change impacts on the AMF spore abundance, functional group composition and temporal dynamics across different pear tree phenophases (i.e., temporal dynamics) of AMF spores, such as fruit growth, harvest, and dormancy. Therefore, samples were obtained of rhizosphere soil at three different timepoints during summer and autumn to capture phenological shifts in AMF functional group composition.

Climate manipulations.

This study is part of the broader QPear experiment, which aims to evaluate the impact of the projected 2040 Belgian climate under the RCP 8.5 scenario on pear tree growth, fruit quality, and orchard ecosystem functioning in Europe, compared to ambient conditions from 2013-2018. To replicate these conditions, the innovative Ecotron infrastructure in Maasmechelen at the National Park Hoge Kempen, operated by Hasselt University, was utilized. This facility entails 12 advanced, enclosed macro-scale sun-lit climate chambers (167 m^3) designed to precisely regulate and monitor key climatic parameters, including air and soil temperature, air humidity, CO_2 levels, precipitation, groundwater content, and windspeed upon a lysimeter containing a soil-canopy column to monitor real-time ecosystem processes (Figures 2 and 3) [31]. Detailed description of the macro-scale Ecotron facility is provided by Rineau et al (2019) [32]. In late autumn 2021, twelve adult pear trees, each measuring three meters in height, were excavated along with their corresponding intact soil cylinders from an experimental orchard at PCFruit (ProefCentrum Fruitteelt) in Limburg province, Belgium. The trees were then placed into six macro-scale lysimeters (two meters in diameter and 1.5 meters in depth), with two trees positioned per lysimeter. Subsequently the trees were grown for one year in the lysimeters in open air, exposing them to ambient climatic conditions. This pre-treatment facilitated the acclimatization of both the trees and the soil to the lysimeter environment.

In January 2022, the lysimeters containing the trees were transported to the Ecotron, where they were exposed to one of two climatic conditions until December 2024: a typical climate of 2013-2018 period, or a typical year of 2040 climate, with three biological replicates per climate condition. The climate scenarios were generated by selecting a simulation from an ensemble of dynamically downscaled regional climate model (RCM) outputs, ensuring it accurately represented present-day climate conditions for the key variables in the region of interest, while also aligning with future multi-model mean projections. More info on the climate projections can be found in Supplementary Methods 1. This methodology incorporated the co-variance of climate variables, natural climate variability, and extreme events, providing a robust framework for generating realistic climate forcing for ecosystem manipulation experiments [33,34]. The experiment included two exposure periods: from 2022 to 2023 and from 2023 to 2024.

Sampling and Sample Processing.
Rhizosphere soil sampling was conducted in 2024 at three key phenological stages (summer and autumn) of the pear tree: fruit growth (16/07), harvest (17/09), and dormancy (26/11) (Figure 3). To account for technical replicates in each climate chamber, three rhizosphere soil samples were taken from random locations within each lysimeter at a depth of 0-30 cm. Sampling occurred within a 60 cm radius of both three trunks, with one sample taken near each trunk and a third positioned between trunks, yielding a total of 54 soil samples. Rhizosphere soil was collected from both the soil directly surrounding the roots and the soil attached to the roots. Sampling depths and three phenological states (i.e., fruits growing, pears an or leaves present on tree, leaves fallen, or pears harvested) were documented, and sampled regions were labelled to prevent re-sampling at the same location. After collection, soil samples were thoroughly mixed, cleared of debris and roots, and stored in labeled plastic bags for further analysis.

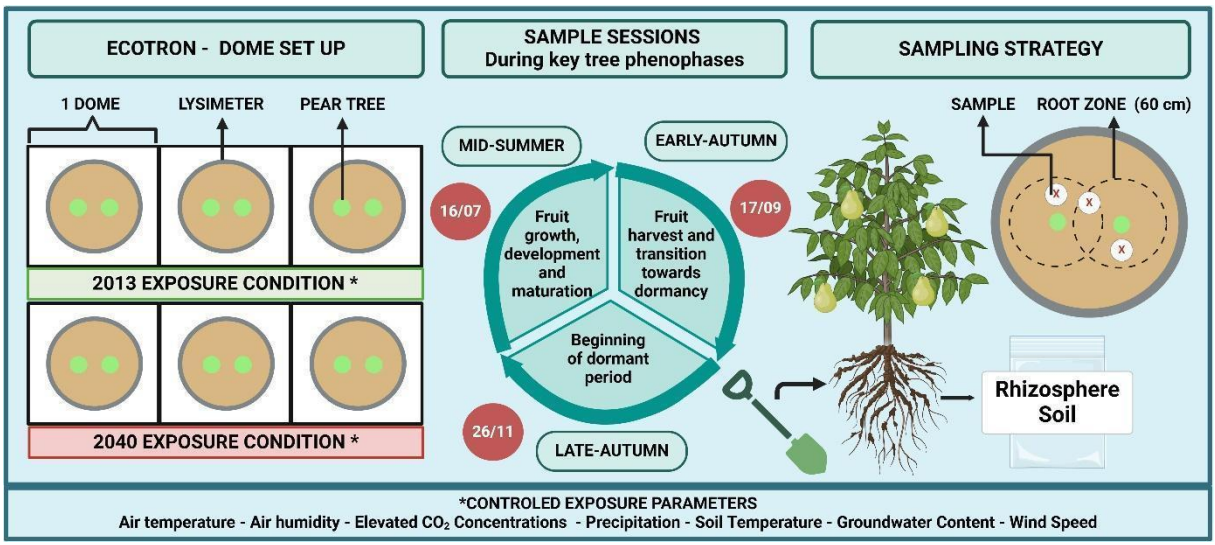


Figure 3: ECOTRON Dome Setup, Sample Sessions, and Sampling *Strategy – White circles with a red cross indicate samples randomly collected at each sampling point to ensure unbiased data collection. Figure created with BioRender.com.*

Pilot Experiments – To optimize spore extraction techniques, two pilot experiments were conducted comparing the Ultrasound Wet-Sieving Technique (UWST) and Ultrasound Centrifuge Technique (UCT) [35]. Both methods were tested using a combination of Mycorrhiza Mix ("Mycorrhiza Mix" Snelkiemende Endomycorrhiza 50Gr—by Dutch Garden Seeds)[36] and bulk soil samples, collected near a pear orchard with a low spore density, to assess their efficiency in spore recovery. The first pilot experiment aimed to identify the most effective technique, while the second focused on the impact of ultrasound exposure on spore integrity and recovery. These experiments guided the development of the final protocol, which employed UWST with ultrasound exposure to enhance spore extraction and

recovery. The ultrasound effectively dislodged AM fungal spores from soil particles, optimizing reliability and the subsequent visualization and analysis of the AMF spores. A full description of the pilot experiments can be found in Supplementary Methods 2.

Spore Extractions and Spore Quantification—AM fungal spores were extracted from the 54 processed rhizosphere soil samples using the UWST method, selected based on the results of the pilot experiment. Subsequently, spore visualization was performed by administering 200 µL of spore suspension from the primary AM fungal spore extractions on a microscope slide, with digital images being captured using the Nikon SMZ800N stereomicroscope at 30× or 40× magnification and processed with NIS Elements software. The procedure was performed in triplicate, performing three visualizations per spore suspension sample, to ensure accuracy. Thereafter, the numbers of spores per 200 µL of spore solution were determined using ImageJ software, allowing for the quantification of the total AMF spore number (TSN) per gram of soil. TSN was determined using the following formula:

$$TSN = \frac{SN \times W}{S},$$

where SN indicates the AMF spore numbers in 1 mL of spore suspension, W denotes the total volume of water used (mL), and S corresponds to the amount of soil processed (g). SN was initially determined for 200 µL of suspension and subsequently scaled to 1 mL (i.e. multiplication by 5), ensuring *W* is also standardized to 1 mL. In addition to these measurements, the lengths of all visualized spores were measured using ImageJ and systematically documented to help in spore species identification.

Spore Species Identification— The images were analyzed to identify the AMF functional groups present (i.e. *Gigasporaceae* and *Glomeraceae*). *Gigasporaceae* spores were distinguished from *Glomeraceae* based on size, with *Gigasporaceae* spores typically measuring > 150 µm and *Glomeraceae* spores < 150 µm [37-39].

Statistics –To assess differences in AMF spore counts between ambient (2013-2018) and future (2040, RCP 8.5) climate conditions and between seasons, data analysis was conducted using R version 4.4.2. The statistical approach employed a Negative Binomial Generalized Linear Mixed Model (GLMM), implemented using the package glmmTMB (version 1.1.5), with additional diagnostics performed using performance (version 0.10.3) and DHARMa (version 0.4.6). The previously calculated Total AMF Spore Numbers (TSN) were used as the response variable to assess spore counts.

Before model selection, data were checked for overdispersion by comparing the mean and variance of TSN, along with evaluating residual patterns using the DHARMa package. Given that variance exceeded the mean, a Negative Binomial GLMM (nbinom2) was chosen to account for overdispersion. The model included climate condition (ambient vs. future), season (early autumn, late autumn and middle summer), and spore type (*Gigasporaceae* and *Glomeraceae*) as fixed effects, while Unit was included as a random effect to account for within-experiment variability. To assess statistical differences in spore counts between climate conditions within each season, post-hoc pairwise comparisons were conducted using emmeans(), applying Tukey's adjustment for multiple comparisons. Additionally, differences among seasons within each climate condition were analyzed using the same approach. For data visualization, predicted spore counts were extracted using emmeans() and visualized with bar plots to show the adjusted estimates for each condition and season using ggplot2 (version 3.3.5), while observed spore counts represent the raw data, shown with boxplots for each climate condition and season. A scatter plot was specifically used to compare the predicted versus observed spore counts. Additionally, AMF functional group composition (*Gigasporaceae* and *Glomeraceae*) was analyzed separately using stacked bar plots, boxplots, and line plots.

PRELIMINARY RESULTS

The pilot experiments aimed to optimize spore extraction techniques and assess the impact of ultrasound on spore recovery and integrity. The Ultrasound Wet-Sieving Technique (UWST) proved to be the most effective method for spore extraction, ensuring maximum recovery efficiency, while the Ultrasound Centrifuge Technique (UCT) showed satisfactory results but was not as effective as UWST. The second pilot experiment demonstrated that ultrasound exposure enhanced spore recovery without compromising spore integrity.

The reported p-values were derived from a negative binomial generalized linear mixed model (GLMM), which assessed the relationship between spore counts (i.e., Total Spore Number) and the interaction between climate, season, and spore type (*Gigasporaceae* and *Glomeraceae*) while accounting for the random effects of Units. Figures 4 and 5 illustrate observed versus GLMM predicted spore counts across both climate conditions (2013 and 2040) at key phenological time points (Early-autumn, Late-autumn, and Mid-summer) (Figure 4), as well as between time points within each climate condition (Figure 5). Even though figures four and five show an increasing trend in spore counts, results from the estimated marginal means (emmeans) test indicate that spore counts did not differ significantly ($p > 0.05$) between the ambient (2013–2018) and future (2040) climate conditions within each season.

However, seasonal variations were significant ($p < 0.05$) within both climates, with mid-summer exhibiting significantly higher median spore counts than late-autumn ($p = 0.0175$ for 2013–2018; $p = 0.0158$ for 2040). Additionally, mid-summer spore counts were higher than those in early-autumn ($p = 0.1289$ for 2013–2018; $p = 0.0614$ for 2040), although these differences were not statistically significant ($p > 0.05$). Moreover, no significant ($p > 0.05$) difference was found between early- and late-autumn spore counts ($p = 0.7118$ for 2013–2018; $p = 0.8714$ for 2040) (Figure 4 and 5). Additionally, results from the GLMM indicate that predicted spore counts did not differ significantly between climate conditions or in seasonal patterns between the climate conditions (Suppl. Figure 2A and B). This was further evidenced by the observed versus predicted spore counts (Suppl. Figure 3A and B).

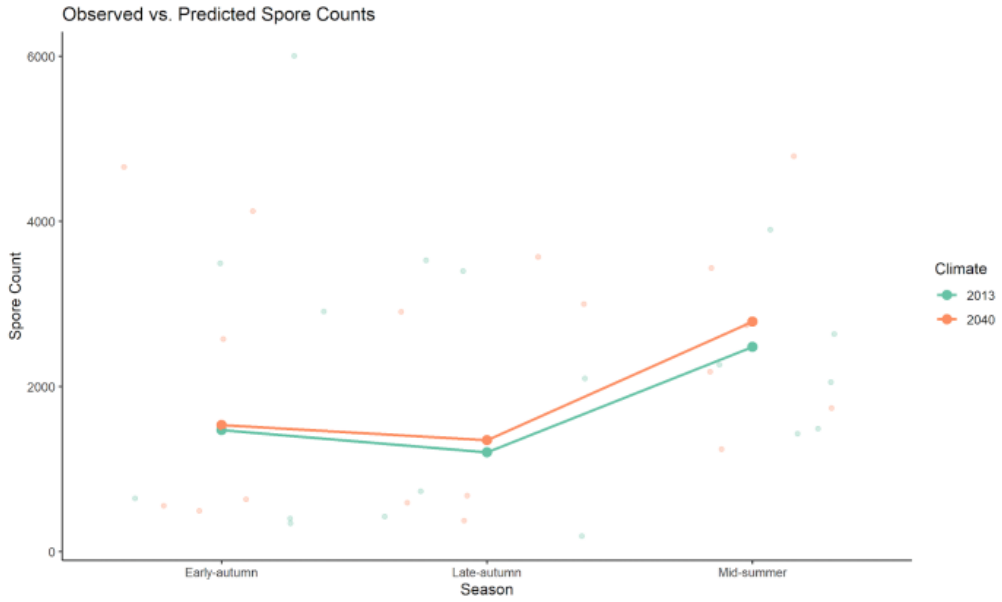


Figure 4: Comparison of observed to predicted spore counts between key phenological time points (Early-autumn, Late-autumn, and Mid-summer) for each of the climate conditions (2013 and 2040) — Dots represent observed spore counts, while lines represent predicted spore counts from the Generalized Linear Mixed Model, showing estimated trends across seasons and climate conditions.

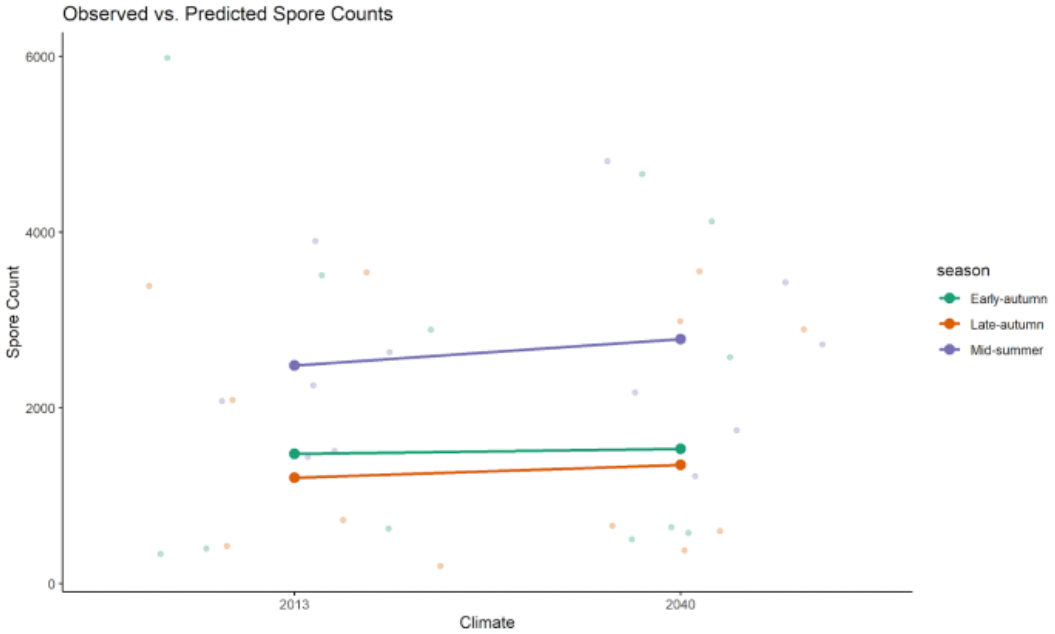


Figure 5: Comparison of observed to predicted spore counts between both climates (2013 and 2040) during each of the key phenological time points (Early-autumn, Late-autumn, and Mid-summer)— Dots represent observed spore counts, while lines represent predicted spore counts from the Generalized Linear Mixed Model, showing estimated trends across seasons and climate conditions.

The emmeans analysis that compared spore types across different climates and seasons showed consistent significant ($p < 0.0001$) higher spore counts (i.e. spore abundance) of *Glomeraceae* produces compared to *Gigasporaceae* across all seasons and climate conditions (Figure 6, Supll. Figure 3 and 4). The largest gap is observed in mid-summer. The contrast between *Gigasporaceae* and *Glomeraceae* was especially pronounced in mid-summer, where the difference in spore counts was the largest, with estimates of -3.57 in 2013 and -3.26 in 2040. Notably, climate change did not lead to significant differences in spore production for either fungal family, as the pattern of higher spore counts in *Glomeraceae* compared to *Gigasporaceae* remained consistent across both 2013 and 2040 climates.

The results from the emmeans test indicated seasonal variations within climate, resulting in distinct effects on spore counts (i.e. spore abundance) depending on spore type (Figure 6, Supll. Figure 3 and 4). For *Gigasporaceae*, spore counts remained relatively stable across seasons, with no significant differences detected in either 2013 or 2040. In contrast, for *Glomeraceae* mid-summer spore counts were significantly higher than in early- and late-autumn in 2013. However, this seasonal effect weakened by 2040, becoming non-significant. Specifically, in 2013, *Glomeraceae* spore counts were significantly higher in mid-summer compared to early-autumn ($p = 0.0218$) and late-autumn ($p = 0.0013$). By 2040, these differences were no longer statistically significant ($p = 0.2924$ and $p = 0.1915$, respectively). For *Gigasporaceae*, no significant seasonal differences were found in either climate condition ($p > 0.05$).

Further, the results from the emmeans test examining the impact of climate on overall spore counts for *Gigasporaceae* and *Glomeraceae* across seasons revealed that climate change had minimal effect on spore production for both fungal families (Figure 6, Supll. Figure 3 and 4). The effect of seasons is not visible in this test as seasons are not evaluated individually. For *Gigasporaceae*, no significant differences ($p > 0.05$) in overall spore counts between 2013 and 2040 were observed, indicating no significant climate effect. Similarly, for *Glomeraceae*, no significant changes ($p > 0.05$) in spore counts between the two climate conditions across the seasons were observed.

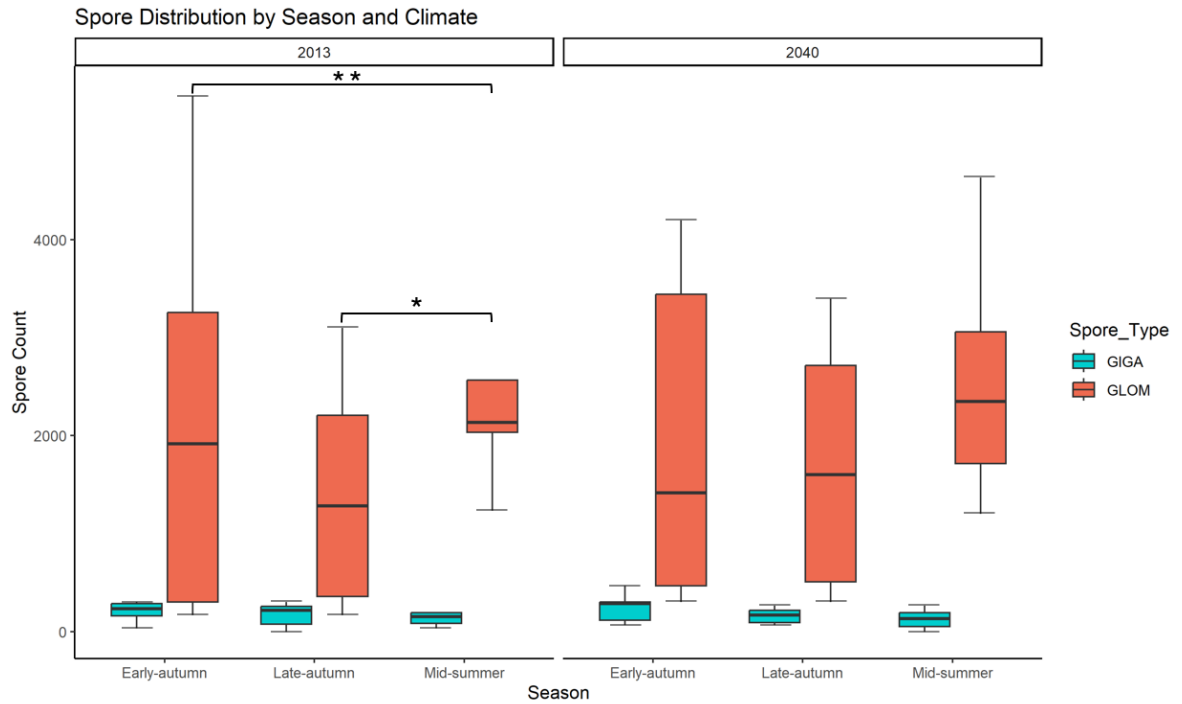


Figure 6: Comparison of spore distribution between both climates (2013 and 2040) during each of the key phenological time points (Early-autumn, Late-autumn, and Mid-summer) for both *Gigasporaceae* (GIGA) and *Glomeraceae* (GLOM) spore types— * represents significance $p < 0.05$ and ** $p < 0.005$ across seasons within climate

DISCUSSION

Climate change is a global challenge with profound effects on ecosystems and agriculture. The consequences of climate change become increasingly evident, leading to shifts in weather patterns, ecosystem dynamics, and agricultural practices [1-3], intensifying the need of plants—being sessile—for adaptation strategies. Arbuscular Mycorrhizal Fungi (AMF) play a vital role in plant resilience by enhancing nutrient uptake and protecting against soil pathogens [5]. Understanding how climate change affects these key microbial communities is essential for sustainable agriculture. This study examines the impact of projected climate change in Belgium on AMF in Belgian pear orchards, using an advanced Ecotron facility to simulate present (2013-2018) and future (2040) climate conditions under the RCP 8.5 scenario. The effects of climate change on AMF spore abundance, functional group composition and temporal dynamics were investigated by analyzing spores using spore extractions.

The results indicate that while spore counts show an increasing trend under future (2040) climatic conditions (Figure 4), these differences are not statistically significant ($p > 0.05$). However, seasonal patterns exhibit significant ($p < 0.05$) changes between each other within the same climate, particularly between mid-summer and late autumn ($p = 0.0175$ for 2013–2018; $p = 0.0158$ for 2040), as well as a notable, though non-significant ($p > 0.05$) increasing trend between mid-summer and early autumn ($p = 0.1289$ for 2013–2018; $p = 0.0614$ for 2040). These findings suggest that neither climate nor climate-induced temporal dynamics have a significant effect on overall spore abundance (i.e. spore counts). Instead, significant differences were observed only within seasons for each climate condition, but not between climate conditions. The rise in mid-summer spore counts aligns with previous research indicating that higher temperatures enhance AMF sporulation and colonization in temperate regions [40,41]. However, the absence of significant differences between current and future climate conditions suggests that AMF communities may exhibit a degree of resilience to climate change, at least within

the temporal and regional scale of our study. Longer-duration studies could help capture potential long-term shifts in AMF communities under changing climate conditions.

This observation contrasts with the limited existing research. Although, the findings of prior studies are performed on individual climatic parameters, showing varying results. A prior study that investigated the effects of elevated temperature and nitrogen addition on AMF and their role in plant community structure and productivity in a temperate meadow in northeast China suggests that individual climate parameters (i.e., warming and elevated CO_2) can influence AM fungal abundance, including spore production, as warming and elevated CO_2 both significantly increased AM fungal abundance. Warming showed to positively impact AMF abundance, including AMF spore abundance, but when temperatures exceed 4°C, this effect turned negative. The responses of AMF to these factors varied with the degree of warming and CO_2, with warming's effects decreasing as temperatures rise, while CO_2's effect strengthens with increased concentration [42]. On the contrary, a global-scale meta-analysis examining the impact of warming, elevated CO_2, and nitrogen addition on AMF abundance found that elevated temperatures reduced AMF spore density and diameter but increased hyphal length density. Fewer, smaller spores were produced, but the fungal hyphae grew more, which could help plants better tolerate heat by improving nutrient and water absorption [23]. However, a mini-review on the effects of individual global climate change parameters (e.g., altered precipitation and elevated CO_2) on AMF abundance also showed varying results, with only elevated CO_2 having no significant effect on AMF abundance [5]. The results of our study, which consider a full climate change scenario rather than isolated climatic factors, align with the hypothesis that overall AMF spore abundance remains stable despite climatic shifts.

This study also examined the effects of climate change upon AMF spore functional group composition, analyzing both rhizophilic (i.e. dominated by *Glomeraceae*) and endaphophlic (i.e. dominated by *Gigasporaceae*) AMF groups. A previous study in southern California highlighs how global change drivers like aridity, nitrogen deposition, and plant invasions affected AMF abundance. Drought reduced root colonization and extraradical hyphal density, while invasive grasses showed increased intraradical AM fungi and parasitic fungi. The study emphasized that water availability plays a key role in shaping AMF communities, with environmental changes influencing spore diversity and composition in complex ways [21]. Therefore it is important to not look only at overall AMF abundance (i.e., spore counts in this study), but also to examine AMF spore functional group composition.

While evaluating AMF functional groups (*Glomeraceae* and *Gigasporaceae) Glomeraceae* exhibited significantly higher ($p < 0.0001$) spore counts compared to *Gigasporaceae* across all climate and seasonal conditions, with the largest differences observed in mid-summer (Figure 5, Suppl. Figure 4 and 5). These findings suggest that *Glomeraceae* is more abundant in terms of spore production than *Gigasporaceae*, consistently in all seasons and across both climate periods. While analyzing the impact of climate on overall AMF spore abundance for *Glomeraceae* and *Gigasporaceae* across climates within each season, climate change showed minimal effect on overall spore production for both fungal types (Figure 5, Suppl. Figure 4 and 5). A key finding are the seasonal variations that have a pronounced effect on spore production, particularly of *Glomeraceae*. In 2013-2018, *Glomeraceae* exhibited significantly ($p < 0.05$) higher spore counts in mid-summer compared to early- and late-autumn, indicating a seasonal peak in spore production. However, by 2040, this effect diminished, with no significant ($p > 0.05$) seasonal differences observed. This shift reflects a response of AMF spores to climate change, leading to a reduction in the pronounced mid-summer peak observed in 2013-2018, pointing towards a change in the seasonal dynamics of *Glomeraceae*— that is an even distribution across the growing season by 2040. On the other hand, *Gigasporaceae* demonstrated already a stable spore distribution across seasons in both climate years, suggesting that this family's spore production is less influenced by seasonal changes.

The study results showing an increased dominance of *Glomeraceae* over *Gigasporaceae* aligns with the hypothesis of the study, as well as with prior research evaluating the effects of individual climatic parameters on AMF species composition. For example, previous findings evaluating the impact of global environmental changes (e.g., rising atmospheric CO_2) on AMF found that elevated CO_2 significantly altered AMF community composition, increasing the ratio of *Glomeraceae* to *Gigasporaceae*. This has been attributed to increased carbon allocation belowground, favoring rhizophilic AM fungi (i.e., *Glomeraceae*) that absorbs carbon more efficiently due to quicker absorption [5]. These changes are attributed to a decrease in carbon allocation to AMF caused by climate change induced elevated nitrogen, with *Glomeraceae* being less affected than *Gigasporaceae* (i.e. edaphophilic AM fungi) due to their lower carbon requirements that stems from their smaller extraradical hyphal networks [41]. Additionally, it has been shown that spores of *Glomeraceae* species are often more resilient to environmental stressors, such as elevated CO_2, which suggests their potential to become more dominant in future climate [22]. Other factors explaining the increased dominance of *Glomeraceae* are linked to their life history traits, including their ruderal strategy, which enables rapid colonization, high hyphal growth, and the ability to thrive in frequently disturbed (e.g., agroecosystems) or rapidly changing environments [16-18]. In contrast, *Gigasporaceae*, which grow more slowly and rely on competitive strategies, are better suited to stable environments and may require longer periods to establish dominance [43,44]. This over-selection of *Glomeraceae* could lead to a shift towards greater representation of these species in mixed communities, potentially reducing the abundance of other fungal species like *Gigasporaceae* [10].

This dominance may be further reinforced by *Glomeraceae's* potentially greater investment in absorptive hyphae, which enhances nutrient uptake efficiency [40,43,45]. Additionally, *Glomeraceae's* rapid hyphal growth and efficient phosphorus (P) uptake make them more effective at increasing plant biomass compared to *Gigasporaceae* [46,47]. In contrast, Gigasporaceae may allocate more resources to transport hyphae rather than absorptive hyphae, reflecting a different nutrient acquisition strategy [48,49]. *Gigasporaceae's* investment in extraradical hyphae suggests potential advantages in stable environments [11-15]. Furthermore, structural differences in hyphal networks offer additional insights into the contrasting ecological strategies of these two families. Moreover, *Glomeraceae* demonstrate greater flexibility in forming anastomoses (i.e., connections between different fungal hyphae), allowing them to integrate more effectively into large-scale mycorrhizal networks and facilitate improved nutrient exchange between plants. In contrast, *Gigasporaceae* tend to form anastomoses within their own hyphae, which limits their capacity to interact with other mycorrhizal networks, potentially constraining their ecological functionality [50]. Additionally, niche partitioning between the two families suggests that *Glomeraceae* preferentially colonize younger roots, while *Gigasporaceae* may establish in older roots over time, particularly in perennial plants [51,52]. This ecological differentiation could support the long-term coexistence of both groups, but in dynamic or frequently disturbed conditions, *Glomeraceae* are more likely to stabilize as the dominant group.

It has been suggested that when both fungal groups were combined, a synergistic effect is observed in root colonization, but not in plant growth or nutrient uptake, likely due to the over-selection of *Glomeraceae* [10]. This may indicate that the benefits of these fungal communities may be context-dependent, with *Glomeraceae* dominating in early-stage colonization and short-term systems while *Gigasporaceae* may play a larger role in long-term stability. While the study results align with this general trend, it was hypothesized that climate change would further increase the dominance of *Glomeraceae* over *Gigasporaceae*, altering AMF functional group composition. However, the findings do not provide strong evidence for this increased trend under climate change, suggesting that the expected shift may not be as pronounced as initially anticipated. This may indicate that the effects of climate change on AMF community dynamics may be more complex than initially predicted, potentially requiring longer experimental durations to detect significant changes in AMF spore abundance under climate change.

Climate change did not show an effect on overall spore abundance nor on functional group composition. However, when examining seasonality, significant differences were found in *Glomeraceae* community dynamics between mid-summer and autumn conditions (both early- and late-autumn) during 2013-2018. By 2040, this seasonal effect disappeared, indicating that climate change impacted AMF spore temporal dynamics. This suggests a shift in the phenology of *Glomeraceae*, leading to a more continuous spore production throughout the examined experimental period. This shift aligns with previous studies, which show that climate change influences soil organism phenology [28]. Similarly, seasonal climatic variations have been shown to affect host plant phenology, altering AMF root colonization timing and nutrient exchange, which in turn impacts plant health and ecosystem productivity [53]. Rising temperatures in 2040 may enhance CO_2 assimilation and transport to roots, potentially delaying AMF dormancy [54,55] and sustaining spore production across seasons, thereby driving phenological shifts.

These findings suggest that climate change will likely alter AMF temporal dynamics. While the study hypothesized that climate change would lead to more rapid and pronounced seasonal shifts, the results instead reveal a trend toward stabilization across seasons by 2040. This indicates a reduction in seasonal fluctuations, highlighting the need to understand these changes in order to predict AMF's future ecological roles and their implications for agriculture in a changing climate. Several factors may explain this trend toward stabilization. First, warmer winters could reduce the environmental cues that trigger dormancy, such as the drop in temperature or reduced resource availability that typically signals the need for a dormant phase, this could change AMF dormancy patterns and result in more consistent fungal activity across seasons or more evenly spread spore production throughout the year [24,25,27,55,56]. In addition, increased resilience of *Glomeraceae* in response to environmental stressors, such as elevated CO_2, may be contributing to the stabilization [22]. *Glomeraceae* species, which are more efficient in carbon uptake and better suited to changes in carbon allocation belowground [5,57], may dominate the community, leading to more consistent spore production across seasons. Finally, shifts in host plant phenology may also be influencing AMF dynamics [5,21,56]. As plants adapt to longer growing seasons and altered growth patterns due to climate change [4,5,58], the timing of AMF root colonization and nutrient exchange could become more consistent, contributing to the observed trend of stabilized spore production.

Since this study was designed to investigate the effects of climate change on AM fungi associated with pear trees in Belgium, specifically to assess potential impacts on Belgian agriculture, one key limitation is that the research was conducted within the specific Belgian regional climate. As a result, the findings may not be fully applicable to other regions or to the broader global climate. Broader studies that consider multiple regions with diverse climatic conditions would provide a more comprehensive understanding of AMF responses to global climate change. Additionally, the temporal resolution of the study (i.e., spore extractions over one year period) may not fully capture the long-term, cumulative effects of climate change on AMF family composition and activity. Longer-term studies examining AMF communities at different stages of climate adaptation would help assess the durability and resilience of these microbial communities over extended periods.

This research represents a significant step toward understanding climate-driven AMF dynamics in agricultural systems using full climate simulation and investigating both AMF functional groups (i.e. *Glomeraceae* and *Gigasporaceae*). It provides valuable insights that will contribute to refining global climate change models and inform sustainable agricultural strategies for maintaining crop productivity in the face of an increasingly unpredictable climate.

CONCLUSION

This study provides valuable insights into the effects of projected climate change on Arbuscular Mycorrhizal Fungi (AMF) in pear orchards in Belgium, specifically comparing the climate conditions of 2013-2018 with the projected 2040 climate under the worst-case RCP 8.5 scenario using the advanced Ecotron facility. Using spore extractions AMF spore abundance, functional group composition and temporal dynamics were evaluated. Over both climate conditions, *Glomeraceae* consistently exhibited higher spore abundance compared to *Gigasporaceae* across all seasons. However, no significant differences were observed in spore abundance or functional group composition between the two climate periods, suggesting that climate change did not have a notable impact on overall AMF abundance or functional group composition. In contrast, seasonal shifts in spore production were evident. The 2013-2018 climate condition showed a significant difference in *Glomeraceae* community dynamics between mid-summer and autumn conditions. However, climate change contributed to a more stable temporal pattern in AMF activity by 2040, leading to a more continuous spore production. This shift indicates that climate change could influence AM fungal phenology. Although this study is limited by its regional focus and relatively short time frame, it underscores the need for broader, long-term studies across diverse climatic regions to fully assess the resilience and adaptability of AMF communities to a changing climate. Such research is essential for informing sustainable agricultural practices and ensuring crop productivity in the face of climate change.

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Supplementary Methods 1.

Climate projections were generated by integrating both large-scale and regional models to estimate local conditions at a 15 km spatial resolution. The model output, consisting of 3-hourly data for air temperature, relative humidity, precipitation, and wind speed, was downscaled to half-hour intervals (in accordance with the Ecotron's operational scale) through linear interpolation. To account for atmospheric CO_2 levels, real-time measurements from the nearby Integrated Carbon Observation System (ICOS) were incorporated, with projections for 2040-2045 suggesting an increase of +133 µmol.mol−1. Soil water potential and air temperature followed field data from the ICOS station, encompassing both ambient and an elevated +2.0°C scenario. Moreover, plant drought stress was quantified using the Ri ratio, with values below 0.6 indicating the onset of water stress conditions [32-34].

Supplementary Methods 2.

Pilot Experiments – Prior to performing spore extractions on the rhizosphere soil samples, two pilot experiments were conducted to optimize spore extraction technique: Ultrasound Wet-Sieving Technique (UWST) or Ultrasound Centrifuge Technique (UCT). While conducting these techniques two substrate sources were utilized: a bulk soil sample collected near a pear orchard with a low spore density and a commercial Mycorrhiza Mix ("Mycorrhiza Mix" Snelkiemende Endomycorrhiza 50Gr—by Dutch Garden Seeds) containing AMF spores [36]. The protocols for UWST and UCT were based on previous research [35], with modifications to the sieve sizes used (1 mm, 100 µm, and 40 µm). These pilot experiments allowed for the optimization of spore extractions, ensuring maximum recovery efficiency for subsequent analyses.

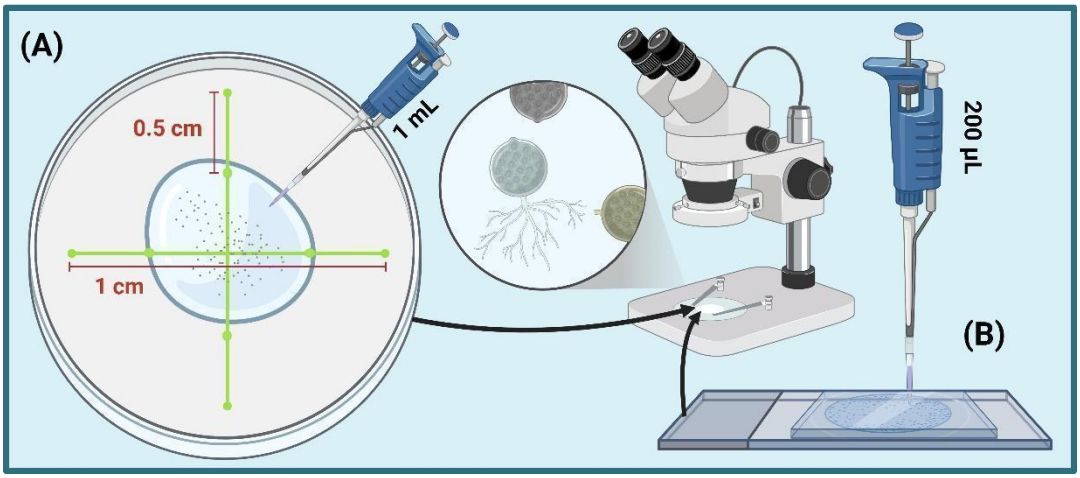
When using the UWST technique, a spore suspension was prepared by suspending one gram of Mycorrhiza Mix or a 1:1 mixture of Mycorrhiza Mix and bulk soil in dH_2O, using a magnetic stirrer for 1 min. The suspension was then subjected to an ultrasound bath (30 sec at 28 kHz) and subsequentially filtered through 1 mm, 100 µm, and 40 µm sieves. The 100 µm and 40 µm sieve contents were collected, washed with dH_2O, and exposed to ultrasound (30 sec at 28 kHz) three times. The final sieve content was washed with a 55% sucrose solution and centrifuged (7 min at 1500 rpm) to create gradient separation. The supernatant was then passed through a 40 µm sieve, and the retained sieve content was thoroughly washed to remove sucrose before being resuspended in 20 mL of MiliQ H_2O. In contrast, while using the UCT method, a spore suspension was created by mixing one gram of bulk

soil and one gram of Mycorrhiza Mix (1:1) in 40 mL of dH_2O, using a magnetic stirrer (5 min at 4-5 rpm), and subjected to an ultrasound bath (30 sec at 28 kHz). The suspension was then centrifuged (3 min at 3000 rpm), and the supernatant was passed through a 40 µm sieve. The final sieve content was collected using 20 mL of MiliQ H_2O. Spore counts were determined, for both UWST and UCT techniques, by placing one milliliter of spore suspension at the center of a Petri dish, which was prepared with a measuring grid (Figure 4.A). Visualization was conducted using a stereomicroscope (Nikon bino SMZ 800), and images were captured for subsequent analysis in ImageJ (win64). The number of spores per milliliter of spore solution was quantified through ImageJ analaysis.

Pilot experiment 1— The first pilot experiment aimed to compare UWST and UCT to determine the most effective spore extraction technique. One gram of Mycorrhiza Mix in powdered form, obtained by sieving to isolate fine particles, was used. This pilot experiment was crucial, as subsequent analyses will utilize homogenized soil rather than the aggregate clusters present in the Mycorrhiza Mix, ensuring a more standardized basis for evaluating both methods.

Pilot experiment 2—The second pilot experiment aimed at assessing the impact of ultrasound on spore integrity and recovery. One gram of powdered Mycorrhiza Mix underwent UWST without ultrasound exposure, serving as a control. Additionally, a 1:1 mixture of bulk soil (0.5 g) and powdered Mycorrhiza Mix (0.5 g) was subjected to UWST with ultrasound exposure. This comparison evaluated whether ultrasound exposure enhanced spore recovery while maintaining their spore integrity.

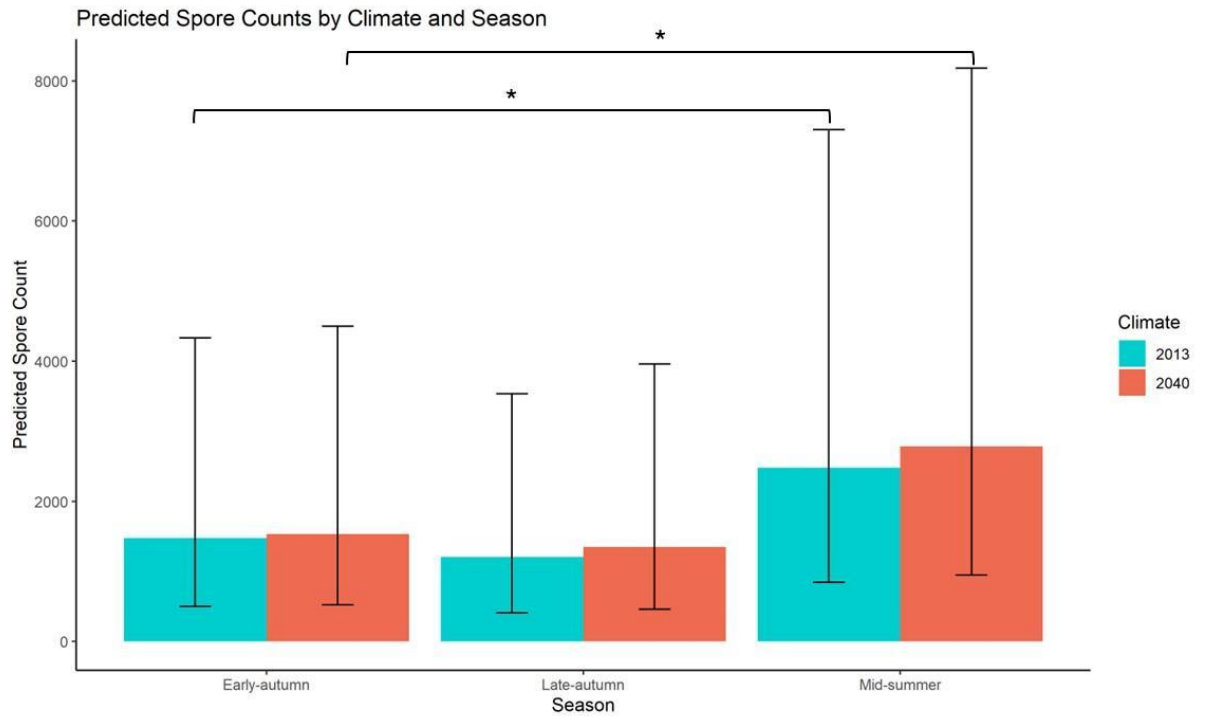
Enhancement of Spore visualization— To optimize spore analysis, the visualization technique was refined using primary spore extractions from the rhizosphere soil samples. 200 µL of spore suspension was placed on a microscope slide and covered with a cover slip (Figure 4.B). Digital images of all observed spores on the slide were captured using a stereomicroscope (Nikon SMZ800N) at 30× or 40× magnification and processed with NIS Elements software. The procedure was conducted in triplicate, ensuring three technical replicates for accuracy.



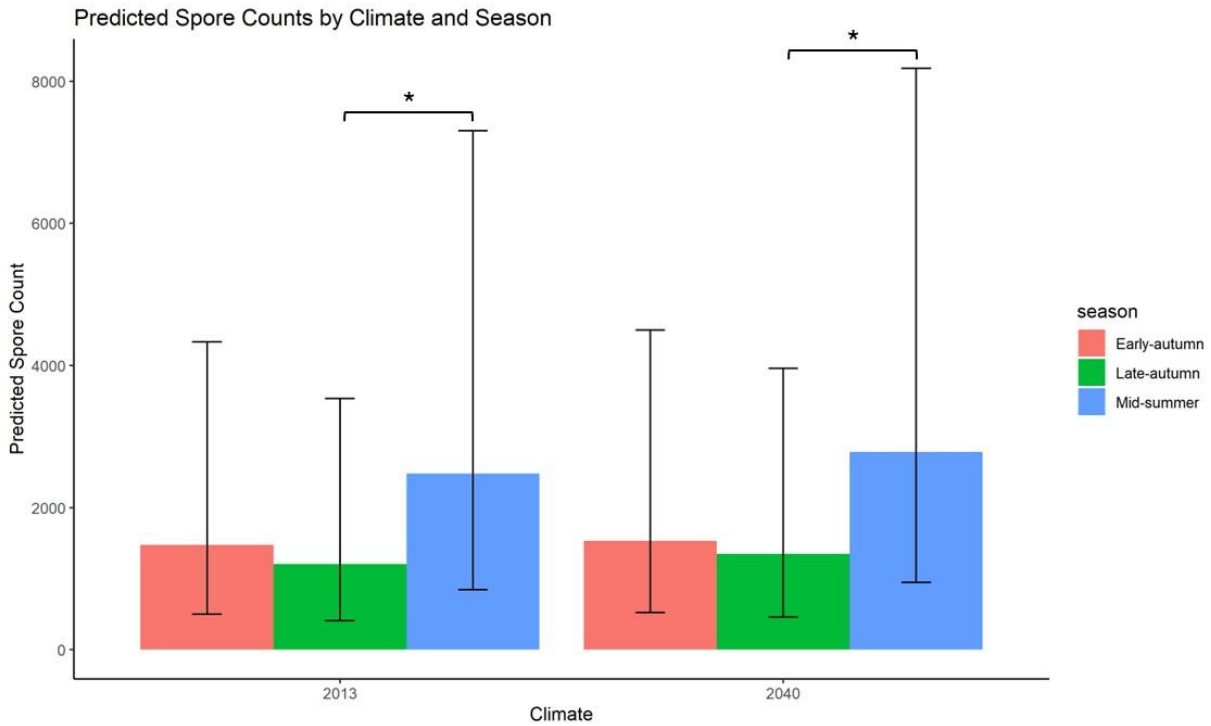
Supplementary Figure 1: Spore visualization methods (A) Measuring grid on Petri dish with 1 mL of spore solution. (B) Microscopic slide with 200 µL of spore solution covered with a slide cover. (A-B) Both are visualized for arbuscular mycorrhizal fungal (AMF) spores using a stereomicroscope. *Figure created with BioRender.com.*

Supplementary Figure 2: (A) Comparison of predicted spore counts between both climates (2013-2018 and 2040) during each of the key phenological time points (Early-autumn, Late-autumn, and Mid-summer) (B) Comparison of predicted spore counts between key phenological time points (Early-autumn, Late-autumn, and Mid-summer) for each of the climate conditions (2013 and 2040).
* represents significance ($p < 0.05$) across climates within each climate condition.

A

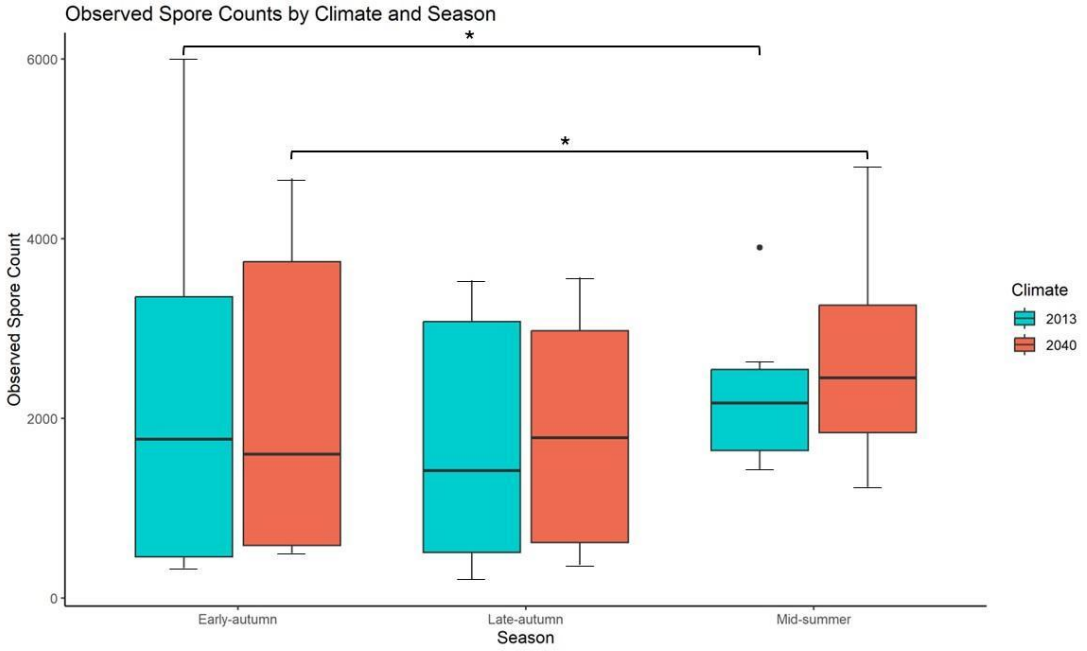


B

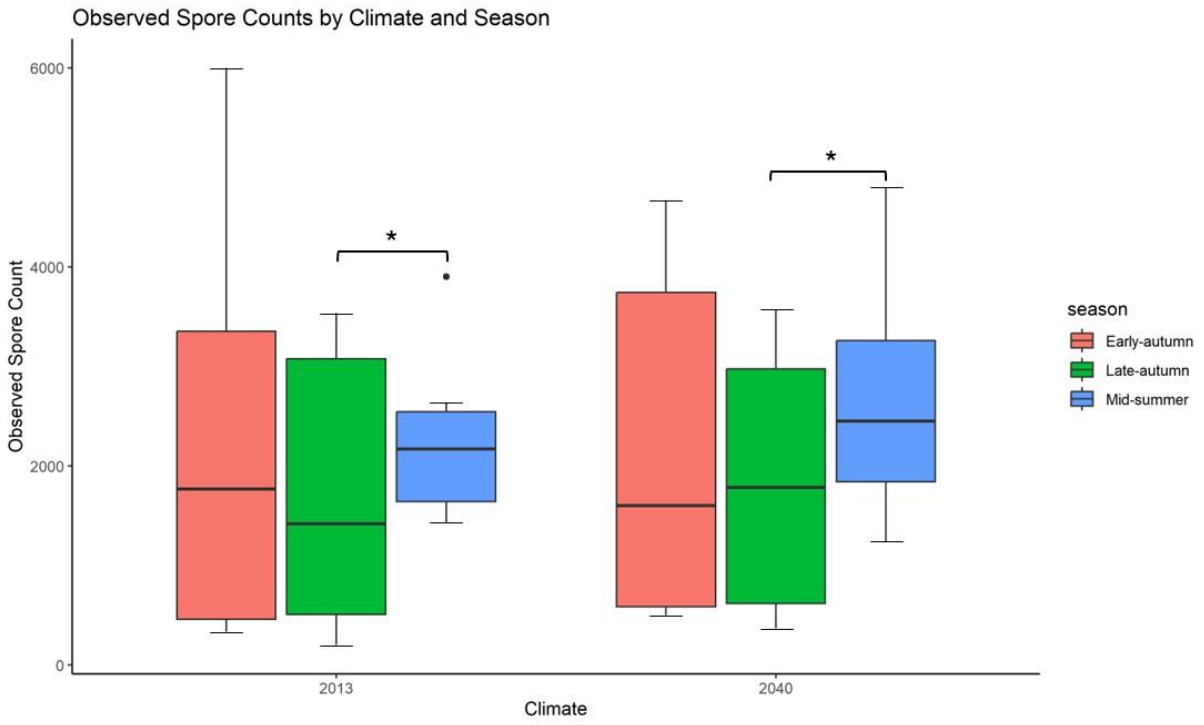


Supplementary Figure 3. (A) Comparison of observed spore counts between both climates (2013-2018 and 2040) during each of the key phenological time points (Early-autumn, Late-autumn, and Mid-summer). (B) Comparison of observed spore counts between key phenological time points (Early-autumn, Late-autumn, and Mid-summer) for each of the climate conditions (2013 and 2040). * represents significance ($p < 0.05$) across climates within each climate condition.

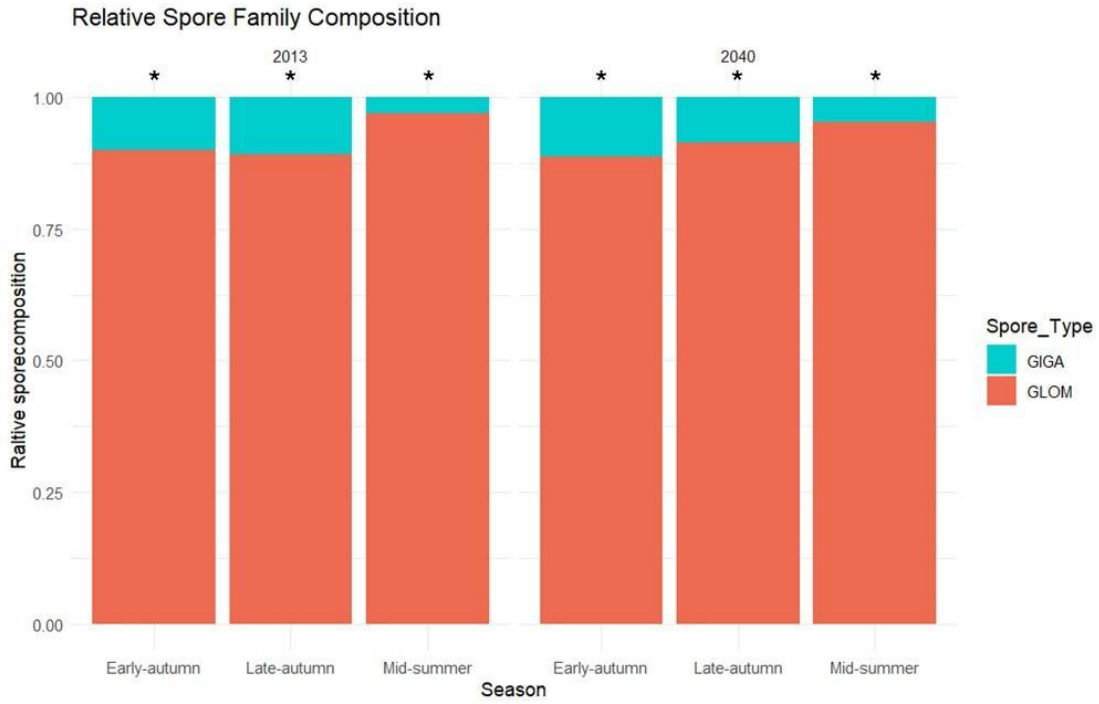
A.



B.



Supplementary Figure 4. Relative AMF family composition between both climates (2013-2018 and 2040) during each of the key phenological time points (Early-autumn, Late-autumn, and Mid-summer) for both *Gigasporaceae* (GIGA) and *Glomeraceae* (GLOM) spore types— * represents significance ($p < 0.05$) between *Gigasporaceae* and *Glomeraceae* functional groups within each season and within each climate condition.



Supplementary Figure 5. Ration of spore types over the key phenological time points (i.e. seasons) (Early-autumn, Late-autumn, and Mid-summer) for both *Gigasporaceae* (GIGA) and *Glomeraceae* (GLOM) spore types across both climates (2013-2018 and 2040).