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

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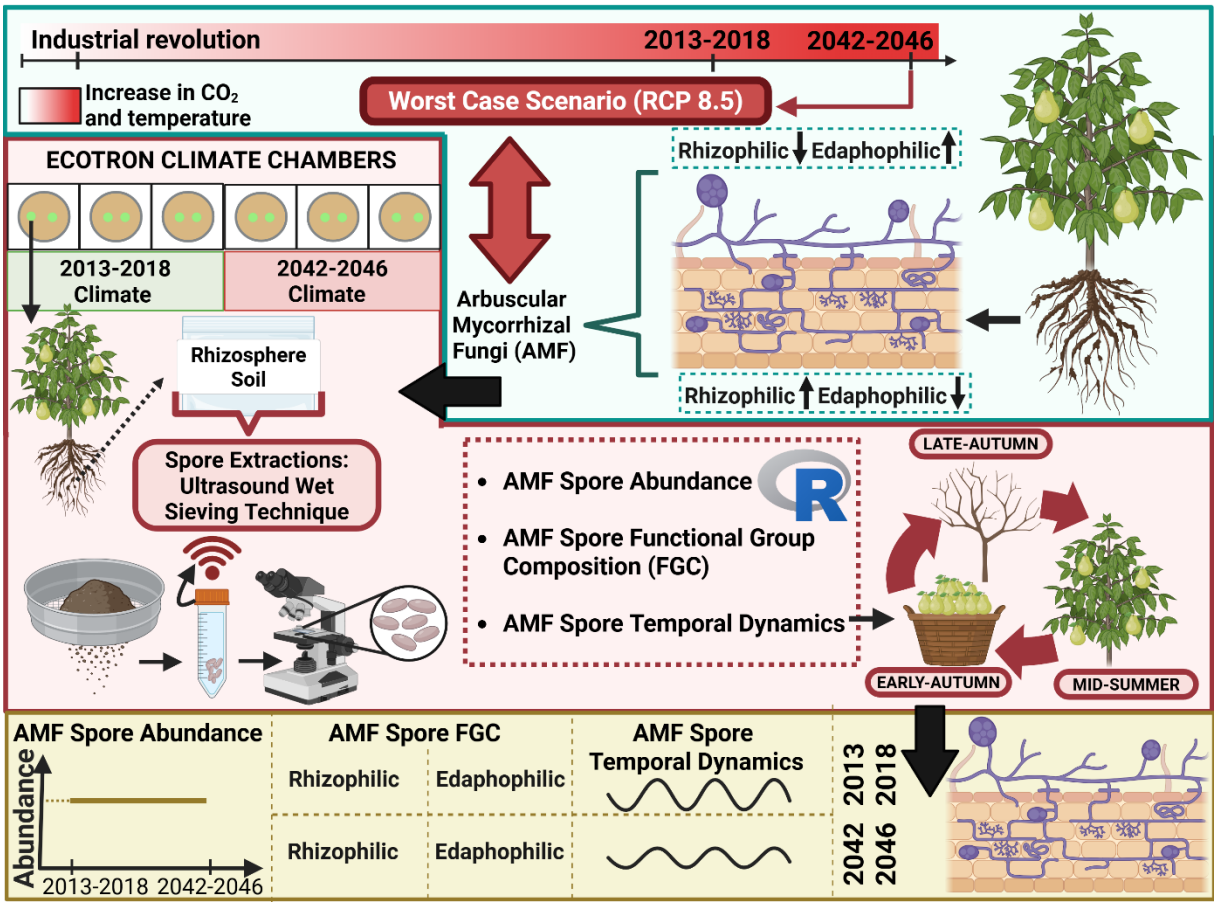
Graphical Abstract



ABSTRACT (240 words)

Climate change affects soil microbial communities, including arbuscular mycorrhizal fungi (AMF), crucial for plant nutrient uptake and resistance to pathogens. This study examines the impact of future climate on the abundance, functional group composition and temporal dynamics of AMF spores in Belgian pear orchards using an advanced Ecotron climate simulation facility. By simulating present (2013-2018) and future (2042-2046) climates based on the "worst-case" scenario (RCP 8.5), the sporulation responses of the two main AMF functional groups (*Glomeraceae* and *Gigasporacea*) to climate change were assessed. *Gigasporaceae* prioritize biomass allocation to slow-growing extraradical hyphae, favoring survival in low-stress, low-disturbance environments. In contrast, *Glomeraceae* fungi adopt a ruderal strategy, prioritizing rapid colonization of roots rather than soil. The results show that overall AMF spore abundance was not affected by climate treatment, suggesting that AMF sporulation is resistant to climate alterations. However, *Glomeraceae* consistently exhibited higher spore abundance than *Gigasporaceae* across all phenological time points. Despite this, the abundances of the functional groups—*i.e.,* functional group composition— remained unaffected by climate treatment. Notably, phenological shifts in spore production were observed, with a mid-summer peak in *Glomeraceae* spore counts in 2013-2018 that diminished towards 2042-2046, leading to a more stable phenological distribution. This outcome suggests that climate change influences AMF phenology rather than overall abundance or functional group composition. 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 (WMO, 2024). 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 (WMO, 2023). Global warming is predicted to lead to major shifts in weather patterns, intensifying heatwaves, prolonging droughts, and increasing both the frequency and severity of heavy rainfall (IPCC, 2022). 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 (IPCC, 2022). These climate-driven shifts pose serious challenges to both natural and agricultural ecosystems. Terrestrial plants are sessile organisms, meaning they cannot migrate to evade environmental stressors. Therefore, 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 ones associated with climate change (*e.g.,* heat and drought), is the symbiotic relationship with arbuscular mycorrhizal fungi (AMF) that can ameliorate these stressors (Baldrian *et al.,* 2022).

These fungi form symbiotic associations with up to 80% of terrestrial plant species, including economically valuable crops, such as fruit trees that hold significant value for the European agricultural industry (Cotton, 2018). 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 (Cotton, 2018). Research indicates that AMF can contribute up to 90% of the plant's P supply (Begum *et al.,* 2019), making this AM fungal symbiosis a critical mechanism for improving crop adaptation to phosphorus-limited conditions. To facilitate symbiosis, AMF develop both extraradical and intraradical hyphal structures (Figure 1). The extraradical hyphae extend beyond the root system, expanding soil exploration and thereby increasing P availability and translocation, ultimately enhancing plant nutrition and growth (Brundrett & Tedersoo, 2018). Additionally, extraradical structures that extend beyond the plant roots into the soil include spores, which are reproductive units dispersed in the soil. 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; vesicles, specialized for lipid storage; and spores (Soudzilovskaia *et al.,* 2015).

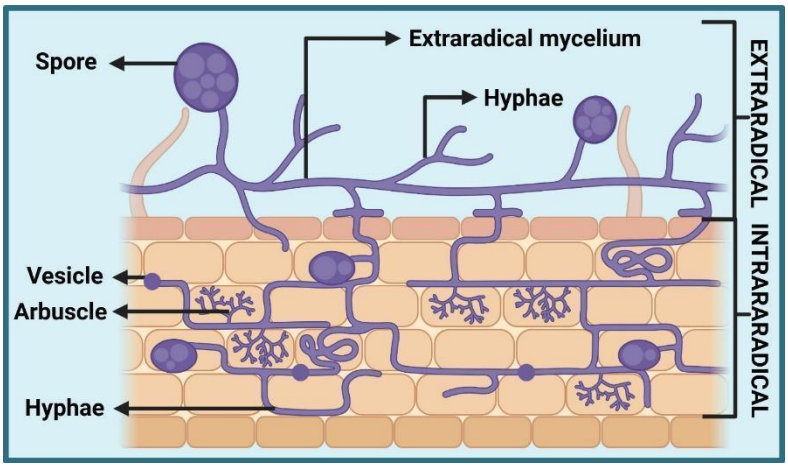


Figure 1: Plant Root Colonization by Arbuscular Mycorrhizal Fungi (AMF): Extraradical and Intraradical Structures.

In particular, spores are essential for the long-term survival of AM fungi and the re-establishment of mycorrhizal networks following disturbances (Horsch, Antunes & Kallenbach, 2023). *Gigasporaceae* and *Glomeraceae*, the two predominant AM fungal functional groups, rely on distinct life-history strategies to fulfill their specific roles in relation to host plants (Horsch, Antunes & Kallenbach, 2023). *Gigasporaceae* adopt an edaphophilic (*i.e.,* soil-loving) strategy, prioritizing biomass allocation to extraradical hyphae for long-term persistence in stable environments, acquiring growth-limiting resources to recolonize when conditions become favorable. These fungi ensure 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 (Hart & Reader, 2002; Hart & Reader, 2005; Staddon *et al.,* 2003; Maherali & Klironomos, 2007; Chagnon *et al.,* 2013). In contrast, *Glomeraceae* fungi employ a rhizophilic (*i.e.,* root-loving) and ruderal strategy, prioritizing rapid intraradical colonization. These fungi are adapted for rapid colonization in both 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 rapid plant colonization and nutrient acquisition, particularly in high-disturbance environments (Alguacil *et al.,* 2010; Verbruggen & Kiers, 2010; Ma *et al.,* 2018).

Previous research indicates that AM fungi promote plant growth under abiotic stress conditions. 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 enhanced stress tolerance facilitates higher crop yields per hectare across a wide range of agricultural species (Brundrett & Tedersoo, 2018). 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 annually, which accounts for approximately 36% of the CO_2 emissions from fossil fuels, highlighting the vital role of these fungi in global carbon dynamics and ecosystem stability (Soudzilovskaia *et al.,* 2015; Soudzilovskaia *et al.,* 2019; Hawkins *et al.,* 2023). Given their substantial impact on both plant health and ecosystem functioning, it is crucial to examine how AMF communities are affected by climate change. As climate change becomes a growing concern, research into how shifting climatic variables affect AMF communities is becoming more critical.

Studies indicate that AM fungi exhibit significant sensitivity to changing climatic parameters, which can affect their diversity, distribution, and functions within ecosystems both directly and indirectly. Indirect effects arise from climate-driven changes in host plants, soil properties (*e.g.,* pH), and nutrient availability (Cotton, 2018; Weber *et al.,* 2019). Although research on altered water availability's effects on AMF communities is more abundant than drought-focused studies, findings remain controversial, inconsistent, and context-dependent (Cotton, 2018). Furthermore, the effects of increased rainfall on AMF diversity vary, with increases, decreases, and no change being reported (Cotton, 2018). Nevertheless, both drought and increased rainfall have been shown to influence the AMF community composition (Cotton, 2018). In contrast to increased rainfall, drought has been reported to negatively affect AMF, leading to reduced root colonization, decreased extraradical hyphal density, and altered abundances of rhizophilic and edaphophilic AMF (Weber *et al.,* 2019). Moreover, while most studies show that increased atmospheric CO_2 does not significantly affect AMF richness or diversity, it does alter community composition, thereby changing community structure. However, this shift is primarily driven by changes in carbon allocation to AMF, favoring *Glomeraceae* over *Gigasporaceae*, rather than broader climatic factors (Cotton, 2018).

A critical aspect of AMF dynamics under climate change that has gained increasing attention is the role of AMF spores (Wolf *et al.,* 2003; Zhang *et al.,* 2016; Kilpeläinen *et al.,* 2020). Research suggests that in cold and temperate climates, rising temperatures promote AMF colonization. Spores, as reproductive units, enable AM fungi to establish and maintain contact with plant roots, which is essential for their survival, particularly in harsh environmental conditions (*e.g.,* drought, extreme heat) (Kilpeläinen *et al.,* 2020; Ahammed & Hajiboland, 2024). AMF spores can remain dormant in the soil until favorable conditions trigger their germination, allowing them to recolonize plant roots when conditions are optimal (Giovannetti, 2000). Seasonal climatic variations that affect host plant phenology can, consequently, alter the timing of root colonization and nutrient exchange, impacting plant health and ecosystem productivity (Asato *et al.,* 2023). With projected temperature increases by 2040, enhanced CO_2 assimilation and transport to roots may delay AMF dormancy onset (Gray & Brady, 2016). However, these effects are taxon-dependent, with research indicating that *Glomeraceae* spores are often more resilient to environmental stressors, such as elevated CO_2, suggesting their potential to dominate in future climates (Wolf *et al.,* 2003). Thus, climate change could alter the temporal dynamics of AMF, making it crucial to understand these phenological shifts to predict their future ecological roles in soil ecosystems, as well as their potential implications for agricultural practices in a changing climate.

Previous research on the impacts of climate change has mainly focused on individual climatic parameters, such as temperature increases and changes in precipitation, creating a gap in understanding the joint effects of a realistic climate change scenario that exposes ecosystems to a full suite of environmental stresses (Cotton, 2018; Zhang *et al.,* 2016; Hu *et al.,* 2022). This study aims to address this gap by exploring the response of the AMF community in a pear orchard, an agricultural system, to the projected 2042-2046 climate change scenario in Belgium. To simulate climate change under the predicted Belgian climate 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 enables precise control over key climatic parameters, such as soil temperature, air humidity, and atmospheric CO_2 levels, enabling the replication of both ambient (2013–2018) and future (2042-2046) 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 AMF spore communities (Hasselt University, n.d.).

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

Addressing the gaps in current research on AMF responses to a full climate change scenario, this study assesses how AMF spores, specifically associated with pears (*Pyrus communis L.*), respond to the projected 2042-2046 climate conditions in Belgium, based on the worst-case Representative Concentration Pathway (RCP 8.5 scenario) (Copernicus Climate Change Service, n.d.; IPCC, 2014). We hypothesized that the AMF spore community in a pear orchard will exhibit more rapid and pronounced temporal changes under future climate conditions (RCP 8.5, 2042-2046), as well as a significant shift in AMF spore functional group composition and an increased dominance of *Glomeraceae*, but no change in overall AMF spore abundance.

MATERIAL AND METHODS

The study aimed to assess climate change impacts on AMF spore abundance, functional group composition and temporal dynamics across key pear tree phenophases (*i.e.,* fruit growth, harvest, and dormancy). Therefore, we examined pear tree rhizosphere soil samples under both ambient (2013-2018) and future (2042-2046) climate conditions following the worst-case Representative Concentration Pathway (RCP 8.5) scenario.

Climate manipulations.

This study is part of the broader QPear experiment, which aims to evaluate the impact of climate change expected in Belgian Limburg by 2042-2046 on pear tree growth, fruit quality, and orchard ecosystem functioning, compared to ambient conditions of 2013-2018. Hereto, we employed the state-of-the-art Ecotron facility, operated by Hasselt University (Hasselt University, n.d.). This facility consists of 12 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, atmospheric CO_2 levels, precipitation, groundwater content, and windspeed. Each climate chamber includes an atmospheric compartment and a lysimeter housing a soil-canopy column, enabling real-time monitoring of ecosystem processes (Figures 2 and 3) (Rineau *et al.,* 2019; Roy *et al.,* 2021). A detailed description of the macro-scale Ecotron facility is provided by Rineau *et al.,* (2019). In late autumn 2021, twelve adult pear trees, each approximately three meters tall, 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 four macro-scale lysimeters (two meters in diameter and 1.5 meters in depth), with two trees per lysimeter. Subsequently, the trees were grown for one year in open air within the lysimeters, exposed to ambient climatic conditions. This pre-treatment facilitated the acclimatization of both the trees and the soil communities to the lysimeter environment.

In January 2022, the lysimeters containing the trees were transported to the Ecotron, where they were exposed until 2024 to one of the two climatic treatments for Belgium under the RCP8.5 scenario: a typical climate from the 2013-2018 period or a typical climate from the period of 2042-2046. Each climate treatment was applied to two lysimeters, providing two biological replicates per condition. The climate scenarios were generated by selecting a simulation from an ensemble of dynamically downscaled regional climate model (RCM) outputs, ensuring the representation of present-day climate conditions for the key variables in the region of interest, while also aligning with future multi-model mean projections. 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 (Roy *et al.,* 2021; Vanderkelen *et al.,* 2020). Further details on the simulation of climate projections are provided in Supplementary Methods 1.

Sampling and Sample Processing.
Rhizosphere soil sampling was conducted in 2024 at three key phenological time points of pear tree growth: 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 radius of 60 cm around both tree trunks, with one sample taken near each trunk and a third sample positioned between trunks, yielding a total of 36 soil samples. Rhizosphere soil was collected from both the soil directly surrounding the roots and the soil adhering to the roots. Sampling depths and three phenological stages (i.e., fruit growth [leaves and fruits present], harvest [pears harvested, leaves still present], and dormancy [leaves fallen]) were documented, and sampled areas were labelled after each sampling event 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.

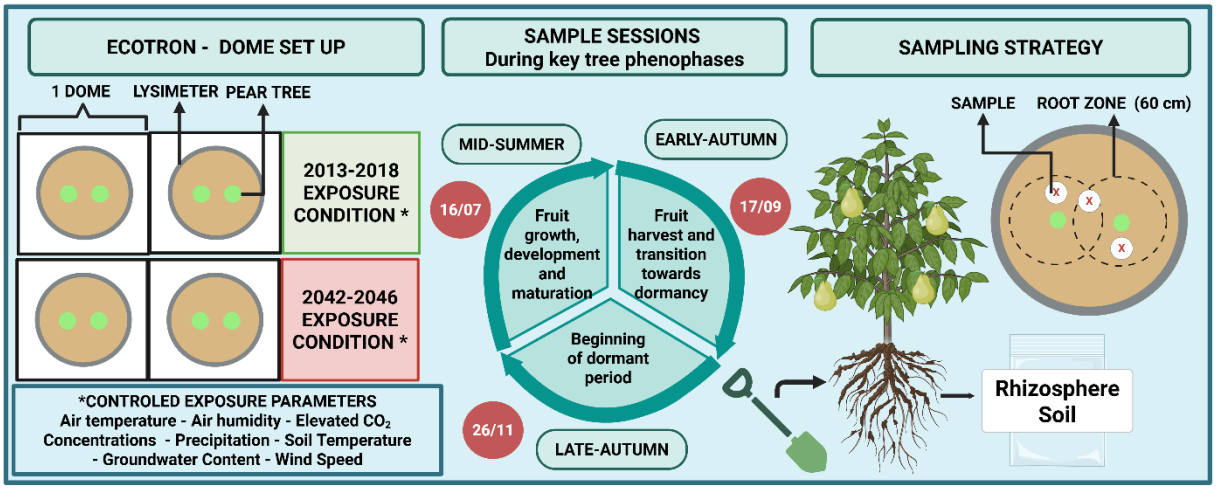


Figure 3: ECOTRON Dome Setup, Sample Sessions, and Sampling *Strategy – White circles with a red cross indicate random sampling locations at each time point to ensure unbiased data collection.*

Pilot Experiments.
To optimize spore extraction techniques, two pilot experiments were conducted comparing the Ultrasound Wet-Sieving Technique (UWST) and Ultrasound Centrifuge Technique (UCT) (Boyno *et al.,* 2023). Both methods were tested using a combination of Mycorrhiza Mix ("Mycorrhiza Mix" Snelkiemende Endomycorrhiza 50Gr—by Dutch Garden Seeds) (Dutch Garden Seeds, n.d.) and bulk soil samples, collected near a pear orchard with low spore density, to evaluate 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, including a detailed description of both methods, is provided in Supplementary Methods 2.

Spore Extractions and Quantification.
AM fungal spores were extracted from the 36 processed rhizosphere soil samples using the UWST method. 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 captured using the Nikon SMZ800N stereomicroscope at 30× or 40× magnification and processed with NIS Elements software (Suppl. Figure 1). Each sample was visualized in triplicate 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 one milliliter, ensuring *W* is also standardized to one milliliter.

Spore Species Identification.
Spore lengths were measured using ImageJ for identification and quantification of functional groups (*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 (University of Kansas, n.d.; Muiruri *et al.,* 2022; Biosci *et al.,* 2021).

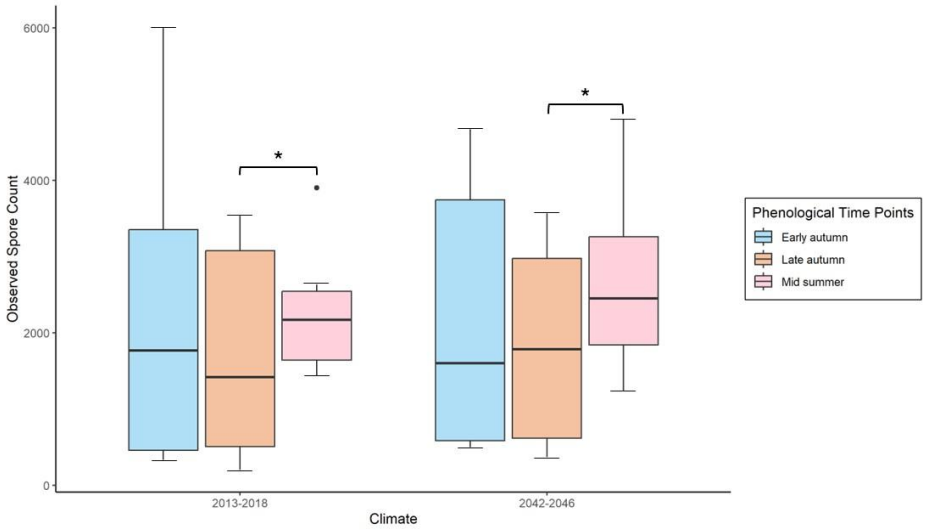
Statistics.
Total AMF spore numbers (TSN) were used as the response variable in the statistical analysis. To assess statistical differences in AMF spore counts between ambient and future climate conditions, as well as between tree phenological time points, data analysis was performed using R (v4.4.2). 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).

Before analysis, data were checked for overdispersion by comparing the mean and variance of TSN, along with evaluating residual patterns using the DHARMa package. Because variance exceeded the mean, a Negative Binomial GLMM (run through nbinom2 function of glmmTMB R package) was chosen. The model included climate condition (ambient vs. future), phenological time points (early-autumn, late-autumn and mid-summer), and spore type (*Gigasporaceae* and *Glomeraceae*) as fixed effects, with Unit (Lysimeter number) as a random effect to account for within-experiment variability. To assess statistical differences in spore counts between climate conditions within each phenological time point, post-hoc pairwise comparisons were conducted using emmeans(), applying Tukey's adjustment for multiple comparisons. Additionally, differences among phenological time points within each climate condition were analyzed using the same approach. Predicted spore counts were estimated using emmeans() and visualized with bar plots to show the adjusted estimates for each climate condition and phenological time point using ggplot2 (version 3.3.5), while observed spore counts represent raw data, shown with boxplots for each climate condition and phenological time point. Additionally, the AMF functional group composition (*Gigasporaceae* and *Glomeraceae*) was visualized using stacked bar plots, boxplots, and line plots. P-values were derived using emmeans() based on the interaction between climate condition, phenological time point, and spore type (*Gigasporaceae* and *Glomeraceae*), while accounting for Unit as a random effect.

RESULTS

Spore counts did not differ significantly ($p > 0.05$) between the ambient (2013–2018) and future (2042-2046) climate conditions at each phenological time point. However, phenological variations were significant ($p < 0.05$) within both climates. Specifically, mid-summer spore counts were significantly higher than those observed in late-autumn ($p = 0.018$ for 2013–2018; $p = 0.016$ for 2042-2046). Additionally, in the future climate, mid-summer spore counts were higher than those in early-autumn ($p = 0.061$), although no significant difference was found between mid-summer and early-autumn in either climate condition ($p = 0.129$ for 2013–2018; $p = 0.061$ for 2042-2046). Furthermore, no significant difference ($p > 0.05$) was found between early- and late-autumn spore counts ($p = 0.712$ for 2013–2018; $p = 0.871$ for 2042-2046) (Figure 4). The predicted spore counts made through the Negative Binomial Generalized Linear Mixed Model (GLMM) show the same results (Suppl. Figure 2).

(A)



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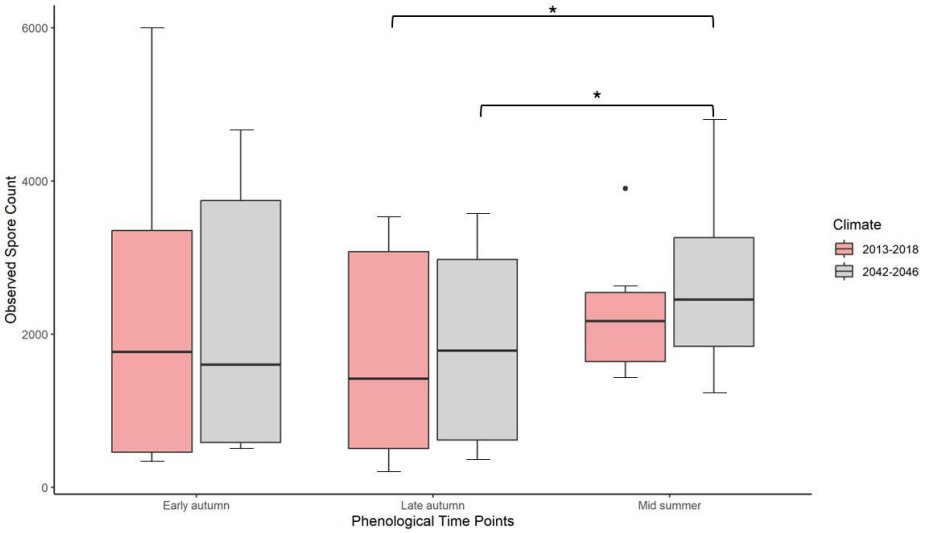

Figure 4. (A) Comparison of observed spore counts between both climates (2013-2018 and 2042-2046) during each of the phenological time points (Early-autumn, Late-autumn, and Mid-summer). (B) Comparison of observed spore counts between key phenological time points for each of the climate conditions. * represents significance ($p < 0.05$) across the time points within each climate.

Next, spore counts were analyzed by functional group. Across both climates and phenological time points, *Glomeraceae* consistently exhibited significantly higher spore counts than *Gigasporaceae*

(Figure 5, Suppl. Figure 3 and 4). The contrast was particularly pronounced in mid-summer, where the spore count difference was largest, with estimates of -3.57 in 2013–2018 and -3.26 in 2042–2046. Notably, climate change did not alter the pattern of higher *Glomeraceae* spore counts compared to *Gigasporaceae*, which remained consistent across both climate conditions. Nevertheless, the phenological dynamics of spore abundance for the *Gigasporaceae* and *Glomeraceae* functional groups differed by climate treatment (Figure 5, Supll. Figure 3 and 4). For *Gigasporaceae*, spore counts remained stable across phenological time points in both climates, with no significant differences detected. In contrast, for *Glomeraceae* mid-summer spore counts were significantly higher than in early- and late-autumn in 2013-2018. However, this pattern weakened by 2042-2046, with differences no longer significant. Specifically, in 2013-2018, *Glomeraceae* spore counts were significantly higher in mid-summer compared to early-autumn ($p = 0.0218$) and late-autumn ($p = 0.0013$). By 2042-2046, these differences were no longer statistically significant ($p = 0.2924$ and $p = 0.1915$).

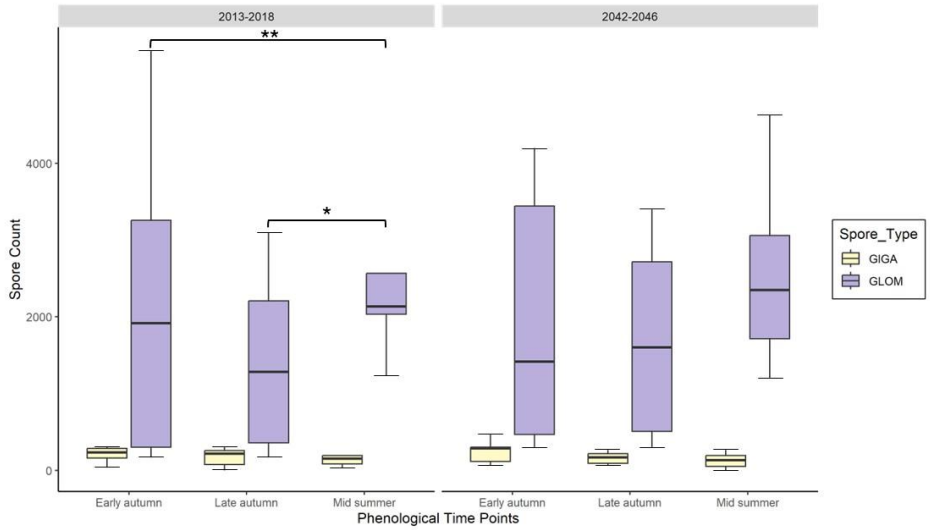

Figure 5: Comparison of spore distribution of *Gigasporaceae* (GIGA) and *Glomeraceae* (GLOM) spore types across phenological time points (Early-autumn, Late-autumn, and Mid-summer) for both climates (2013-2018 and 2042-2046)— * indicates significance at $p < 0.05$ and ** at $p < 0.005$ across time points within each climate condition.

DISCUSSION

Climate change is a global challenge with profound and escalating impacts on ecosystems and agriculture. Its consequences are becoming increasingly evident, driving shifts in weather patterns, ecosystem dynamics, and agricultural practices (WMO, 2024; WMO, 2023; IPCC, 2022). These shifts intensify the need for sessile organisms like plants to adopt effective adaptation strategies. Arbuscular Mycorrhizal Fungi (AMF) play a vital role in plant resilience by enhancing nutrient uptake and protecting against soil pathogens (Cotton, 2018). Understanding how climate change affects AMF spore production is essential for sustainable agriculture, as AMF play a crucial role in maintaining crop productivity (Baldrian *et al.,* 2022; Cotton, 2018). This study therefore investigates the impact of projected climate change on AMF spore production in Belgian pear orchards.

Our results show that while AMF spore production exhibits significant phenological shifts ($p < 0.05$), particularly between mid-summer and late-autumn ($p = 0.0175$ for 2013–2018; $p = 0.0158$ for 2042-2046), these patterns remain consistent across climate conditions, indicating no effect of climate change on overall AMF spore phenology (Figure 4, Suppl. Figure 2). The increase in mid-summer spore counts aligns with previous research suggesting that higher temperatures enhance AMF sporulation and colonization in temperate regions (Smith & Read, 2008; Treseder *et al.,* 2018). However, the lack

of significant differences between current (2013–2018) and future (2042-2046) climate conditions suggests that AMF communities may exhibit notable resilience to climate change, at least within the temporal and regional scope of this study. Long-term studies may be required to detect long-term gradual shifts in AMF communities under changing climate conditions.

This finding stands in contrast to some studies focused on individual climate drivers. For example, a study in northeast China found that warming and elevated CO_2 significantly influenced AM fungal abundance, including spore production. Warming was shown to positively affect AMF spore abundance; however, this effect became negative when temperatures exceeded 4°C. The AMF responses to these factors varied with the degree of warming and CO2: warming effects declined at higher temperatures, while CO_2 effects intensified with increasing concentration (Hu *et al.,* 2022). Similarly, a global meta-analysis examining the impact of warming and elevated CO_2 on AMF abundance found that elevated temperatures reduced AMF spore density and diameter, but increased hyphal length. Although fewer and smaller spores were produced, fungal hyphae exhibited increased growth. This could help plants better tolerate heat by improving nutrient and water absorption (Zhang *et al.,* 2016). However, a review on the effects of altered precipitation and elevated CO_2 on AMF spore density showed varying results, with only elevated CO_2 having no significant effect on AMF abundance (Cotton, 2018). Our findings, which reflect a full climate change scenario rather than individual climatic drivers, support the hypothesis that AMF spore abundance remains stable despite climatic shifts.

Besides overall spore production we examined the effects of climate change upon the AMF spore functional group composition, analyzing both rhizophilic (*Glomeraceae*) and endaphophlic (*Gigasporaceae*) AMF groups. A study in southern California showed that increased aridity reduced AMF root colonization and extraradical hyphal density, highlighting the key role of water availability in shaping AMF communities, with environmental changes influencing spore diversity and composition in complex ways (Weber *et al.,* 2019). It is therefore 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 group composition (*Glomeraceae* and *Gigasporaceae)*, we found that *Glomeraceae* consistently exhibited significantly ($p < 0.0001$) higher spore counts compared to *Gigasporaceae* across all climate and phenological conditions, with the largest differences observed in mid-summer (Figure 5, Suppl. Figure 3 and 4). This result indicates that *Glomeraceae* produce more spores than *Gigasporaceae* regardless of phenological timing or climate period. When assessing the impact of climate on overall AMF spore abundance at each phenological time point, we saw that climate change showed minimal effect on overall spore production for both *Glomeraceae* and *Gigasporaceae* (Figure 5, Suppl. Figure 3 and 4).

Interestingly, while overall spore counts remained stable, climate change did alter the phenological dynamics of *Glomeraceae* spore production. In 2013-2018, *Glomeraceae* exhibited significantly ($p < 0.05$) higher spore counts in mid-summer compared to early- and late-autumn, indicating a phenological peak in spore production. However, by 2042-2046 this pattern was no longer significant ($p > 0.05$). This shift reflects a response of AMF spores to climate change, resulting in a more even distribution of spore production across the growing season in 2042–2046. On the other hand, *Gigasporaceae* demonstrated already a stable spore distribution across phenological time points in both climates, indicating that the spore production of this group is less influenced by phenological changes and potentially more resistant to phenological shifts under climate change. Our results, showing an increased mid-summer dominance of *Glomeraceae* over *Gigasporaceae* supports the study's hypothesis and aligns with previous research on how individual climatic factors influence AMF functional group composition. For example, a study evaluating the impact of rising atmospheric CO_2 on AMF found that elevated CO_2 significantly altered AMF community composition, increasing the *Glomeraceae*-to-*Gigasporaceae* ratio. This shift has been attributed to greater carbon allocation belowground, favoring rhizophilic AM fungi like *Glomeraceae* due to their faster and more efficient carbon uptake (Cotton, 2018). Climate change reduces carbon allocation to AMF, often due to elevated

nitrogen, with *Glomeraceae* being less affected than *Gigasporaceae* (*i.e.,* edaphophilic) due to their lower carbon demands associated with smaller extraradical hyphal networks (Treseder *et al.,* 2018).

Glomeraceae allocate more resources towards absorptive hyphae, resulting in rapid hyphal growth and efficient phosphorus (P) uptake, which makes them more effective at increasing plant biomass (Bruce *et al.,* 1994; Bago *et al.,* 1998; Yang *et al.,* 2017, Gosling *et al.,* 2016). In contrast, *Gigasporaceae* allocate more resources to transport hyphae (Hart & Reader, 2005; Souza *et al.,* 2005) and their investment in extraradical hyphae suggests potential advantages in stable environments (Hart & Reader, 2002; Hart & Reader, 2005; Staddon *et al.,* 2003; Maherali & Klironomos, 2007; Chagnon *et al.,* 2013). Additionaly, *Glomeraceae* demonstrates greater flexibility in forming anastomoses (*i.e.,* connections between different fungal hyphae), allowing them to integrate more effectively into large-scale mycorrhizal networks to further facilitate improved nutrient exchange between plants. In contrast, *Gigasporaceae* tend to form anastomoses within their own hyphae, limiting their capacity to interact with other mycorrhizal networks, potentially constraining their ecological functionality (De La Providencia *et al.,* 2004).

Niche partitioning suggests that *Glomeraceae* preferentially colonize younger roots, while *Gigasporaceae* may establish in older roots over time, particularly in perennial plants (Kil *et al.,* 2014; Vukicevich *et al.,* 2019). Such ecological differentiation may support long-term coexistence of both groups, but in dynamic or frequently disturbed conditions *Glomeraceae* are more likely to stabilize as dominant group, as supported by our findings (Figure 5, Suppl. Figure 3 and 4) (Horsch, Antunes & Kallenbach, 2023). Other factors explaining the increased dominance of *Glomeraceae* are their life history traits, notably their ruderal strategy, which enables rapid colonization and allows them to thrive in frequently disturbed (*e.g.,* agroecosystems) or rapidly changing environments (Alguacil et al., 2010; Verbruggen & Kiers, 2010; Ma *et al.,* 2018; Wolf *et al.,* 2003). In contrast, *Gigasporaceae* grow more slowly and adopt competitive strategies, which are better suited for stable environments and may require longer periods to establish dominance (Bruce *et al.,* 1994; Xu *et al.,* 2017).

A study suggests that combining both fungal groups may enhance root colonization synergistically, but not plant growth or nutrient uptake, likely due to the over-selection of *Glomeraceae* (Horsch, Antunes & Kallenbach, 2023). 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 our results align with this trend, it was hypothesized that climate change would further increase the dominance of *Glomeraceae*, altering AMF functional group composition. However, the findings do not provide strong evidence for this increased trend under climate change, indicating that this shift may be less pronounced than previously expected. These findings suggest that the effects of climate change on AMF functional group dynamics may be more complex than initially predicted, potentially requiring longer-term studies to detect clear shifts in spore abundance. Alternatively, the increased dominance of *Glomeraceae* could lead to more stable phenological patterns over time, buffering the impacts of climatic variation.

Climate change did not affect overall spore abundance or functional group composition. However, a shift in *Glomeraceae* phenology observed between mid-summer and early and late autumn during 2013–2018 disappeared by 2042–2046, suggesting a more continuous spore production pattern.This aligns with previous findings showing that climate change influences soil organism phenology (Asato *et al.,* 2023). Seasonal climate variation also affects host plant phenology, influencing AMF root colonization timing and nutrient exchange, which in turn affects plant health and ecosystem productivity (Cera *et al.,* 2021). Rising temperatures in 2042-2046 may enhance CO_2 assimilation and root transport, potentially delaying AMF dormancy (Gray & Brady, 2016; Keeler *et al.,* 2021) and sustaining spore production across seasons, thereby changing AMF phenology.

These findings suggest that climate change will likely alter AMF phenological dynamics. Although more rapid and pronounced phenological shifts were hypothesized, the results instead indicate a trend toward stabilization by 2042–2046. This stabilization highlights the need to understand AMF responses to better predict their ecological roles and agricultural impacts under future climate scenarios. First, warmer winters may weaken environmental cues, such as temperature drops or resource scarcity, which normally trigger dormancy. This may alter AMF dormancy patterns, leading to more consistent fungal activity and evenly distributed spore production across seasons (Kilpeläinen *et al.,* 2020; Ahammed & Hajiboland, 2024; Giovannetti, 2000; Keeler *et al.,* 2021; Dumbrell *et al.,* 2011). Additionally, *Glomeraceae* may contribute to stabilization due to their resilience to stressors like elevated CO_2 (Wolf *et al.,* 2003). *Glomeraceae* species may dominate the community, leading to more consistent spore production across phenological time points (Cotton, 2018; Horsch *et al.,* 2023). Finally, shifts in host plant phenology may also influence AMF dynamics (Cotton, 2018; Weber *et al.,* 2019; Dumbrell *et al.,* 2011). As plants adapt to longer growing seasons and altered growth patterns due to climate change (Baldrian *et al.,* 2022; Cotton, 2018; Calanca *et al.,* 2023), the timing of AMF root colonization and nutrient exchange could become more consistent, contributing to more consistent spore production.

Understanding AMF phenology is important, as changes in phenology can potentially disrupt the timing of spore production in both ecosystems and agriculture. This can in turn affect plant nutrient uptake, soil health, and crop yields (Cera *et al.,* 2021; Baldrian *et al.,* 2022). Shifts in the timing of spore production may alter the symbiotic relationship between plants and AMF, influencing nutrient exchange, plant health, and overall ecosystem productivity (Cotton, 2018; Baldrian *et al.,* 2022). As phenological shifts in AMF occur, they may also interact with changes in host plant phenology, potentially influencing AMF root colonization patterns and further altering agricultural outcomes (Giovannetti, 2000; Wolf *et al.,* 2003; Baldrian *et al.,* 2022). Such shifts underscore the importance of understanding AMF dynamics in the face of climate change to inform sustainable agricultural practices and ensure crop resilience.

This study, conducted in Belgian pear orchards, is limited by its regional scope, which may constrain the broader applicability of its findings. 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 study's short temporal resolution (*i.e.,* one-year sampling) may not capture long-term effects of climate change on AMF 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.

CONCLUSION

This study provides insights into the effects of projected climate change on the spore production dynamics of Arbuscular Mycorrhizal Fungi (AMF) communities in pear orchards in Belgium, comparing the climate conditions of 2013-2018 with the projected 2042-2046 climate. Overall AMF spore abundance was higher in mid-summer compared to early and late autumn, and this pattern was consistent across both the 2013-2018 and 2042-2046 climate periods, suggesting that climate change did not significantly affect the overall spore abundance. Across both climate conditions, *Glomeraceae* consistently exhibited higher spore abundance than *Gigasporaceae* at all phenological time points, indicating a persistent dominance of *Glomeraceae* in the AMF community. However, no significant differences in spore abundance or functional group composition were observed between the two climate periods, suggesting that climate change had little to no impact on overall AMF abundance or functional group composition during the study period.

In contrast, phenological shifts in spore production were observed. In the 2013-2018 climate *Glomeraceae* spore production peaked in mid-summer, with significantly lower spore abundance

observed in autumn. However, climate change contributed to a more stable temporal pattern *Glomeraceae* spores by 2042-2046, leading to a more continuous spore production. This shift indicates that climate change could influence AM fungal phenology, potentially disrupting the timing of spore production in both ecosystems and agriculture. This can in turn affect plant nutrient uptake, soil health, and crop yields. 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.

The study's climate projections were generated by integrating both large-scale and regional models to estimate local conditions at a 15 km spatial resolution. The climate scenarios were based on a Representative Concentration Pathway (RCP), as defined by the Intergovernmental Panel on Climate Change (IPCC) in their Fifth Assessment Report of 2014 (Copernicus Climate Change Service, n.d.; IPCC, 2014), representing standardized greenhouse gas concentration pathways that project future climate outcomes. The RCP 8.5 scenario (*i.e.,* worst-case emission pathway) is characterized by a continuous rise of greenhouse gas emissions throughout the 21st century, projecting a mean global temperature increase of +2.0°C by 2046-2056 and of +3.7°C by 2081-2100 that leads to more significant climate change (Copernicus Climate Change Service, n.d.; IPCC, 2014). In this study, the RCP8.5 scenario for Belgium in 2042-2046 and 2013-2018 is used, as it is expected to result in the most severe ecological effects. The experiment included two exposure periods: 2022 to 2023 and from 2023 to 2024.

The model output consists of 3-hourly data for air temperature, relative humidity, precipitation, and wind speed, which was downscaled to half-hour intervals through linear interpolation, in accordance with the Ecotron's operational scale. 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.

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) and 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 (Dutch garden seeds, n.d.). The protocols for UWST and UCT were based on previous research (Boyno *et al.,* 2023), 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.

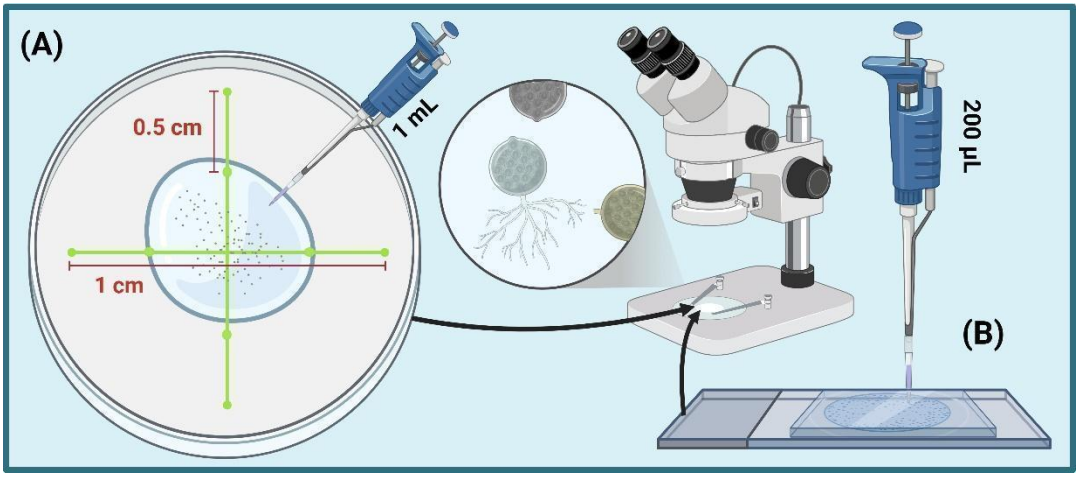
When using the UWST technique, a spore suspension was prepared by suspending one gram of Mycorrhiza Mix or a 1:1 mixture of Mycorrhiza Mix and bulk soil in dH_2O using a magnetic stirrer for 1 min. The suspension was then subjected to an ultrasound bath (30 sec at 28 kHz) and subsequentially filtered 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),

before being 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 determined using ImageJ analysis and quantified as outlined in the *Materials and Method*s section under *Spore Extractions and Spore Quantification*.
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 was obtained by sieving to isolate fine particles, important as subsequent analyses will utilize homogenized soil rather than the aggregate clusters present in the Mycorrhiza Mix. 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 not as effective as UWST.

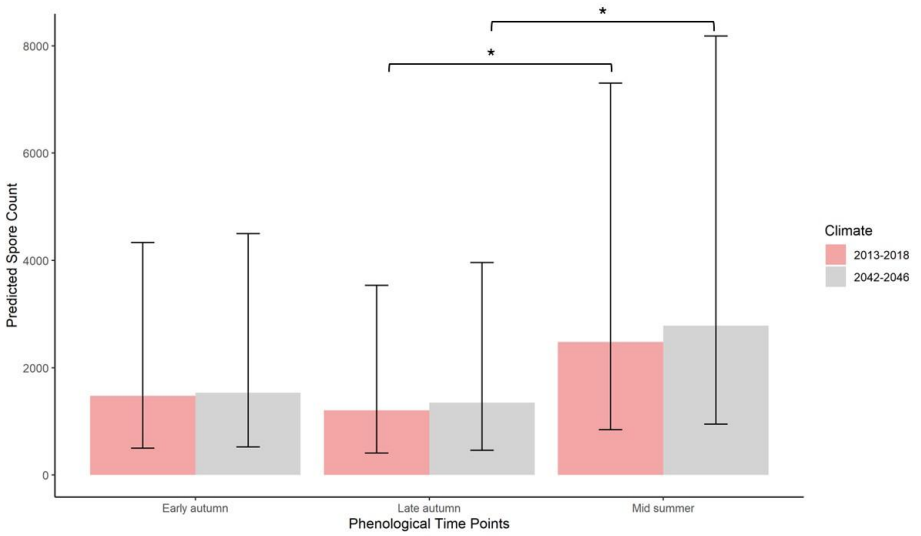
Pilot experiment 2—The second pilot experiment aimed at assessing the impact of ultrasound on spore integrity and recovery. One gram of powdered Mycorrhiza Mix underwent UWST without ultrasound exposure, serving as a control. Additionally, a 1:1 mixture of bulk soil (0.5 g) and powdered Mycorrhiza Mix (0.5 g) was subjected to UWST with ultrasound exposure. Ultrasound exposure showed to enhance spore recovery without compromising 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 observed spores 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.

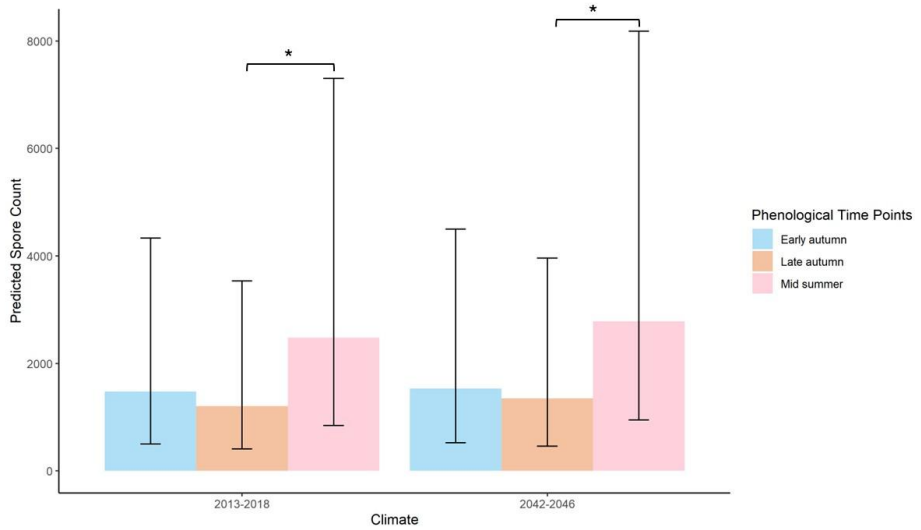


Supplementary Figure 1: Spore visualization methods (A) Measuring grid on Petri dish with 1 mL of spore solution. (B) Microscopic slide with 200 µL of spore solution covered with a slide cover. (A-B) Both are visualized for arbuscular mycorrhizal fungal (AMF) spores using a stereomicroscope.

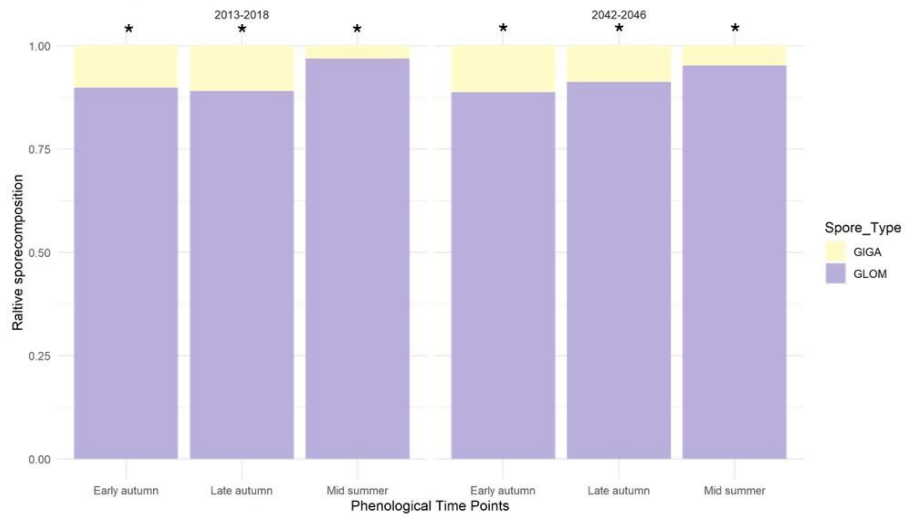
A



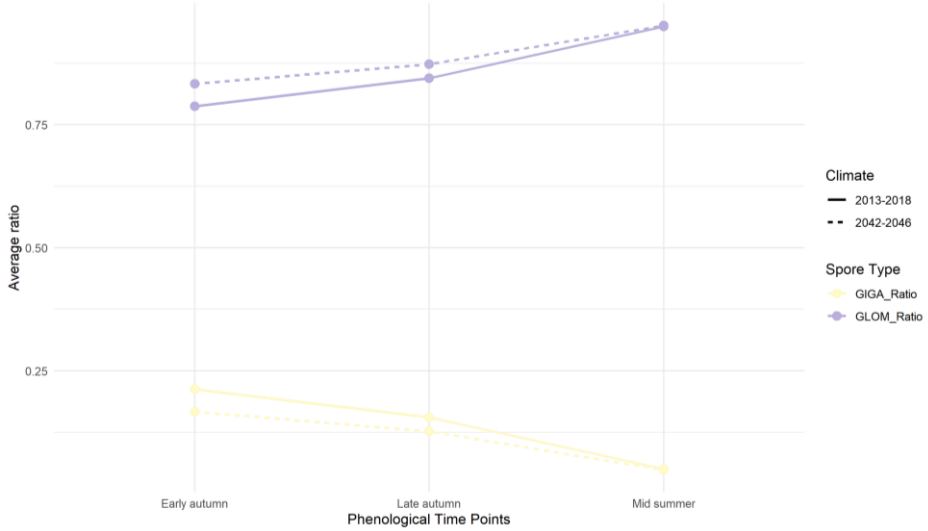
B



Supplementary Figure 2: (A) Comparison of predicted spore counts between both climates (2013-2018 and 2042-2046) during each phenological time points (Early-autumn, Late-autumn, and Mid-summer) (B) Comparison of predicted spore counts between phenological time points for each climate condition—* represents significance ($p < 0.05$) across time points within each climate.



Supplementary Figure 3. Relative AMF functional group composition of *Gigasporaceae* (GIGA) and *Glomeraceae* (GLOM) spore types across the phenological time points (Early-autumn, Late-autumn, and Mid-summer) for both climates (2013-2018 and 2042-2046)— * represents significance ($p < 0.05$) between the functional groups within each phenological time point and within each climate condition.



Supplementary Figure 4. Ratio of *Gigasporaceae* (GIGA) and *Glomeraceae* (GLOM) spore types over the key phenological time points (Early-autumn, Late-autumn, and Mid-summer) across both climates (2013-2018 and 2042-2046).