KU LEUVEN

FACULTEIT WETENSCHAPPEN

# Effects of soil compaction in nature management and restoration

## How does compaction affect soil properties, mycorrhizal fungi and plant responses to drought stress in grasslands and heathlands?

Study in the context of LIFE HARWIN ('HAbitat Restoration WINgevalley, ecological restoration and endangered species recovery in a fragmented landscape')

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# Summary

To preserve semi-natural grasslands and their characteristic species, management interventions such as mowing are required. However, the dominant mowing method has shifted from manual mowing towards machine mowing, with the weights of the equipment gradually increasing over time. Despite having benefits related to efficiency and cost, this trend also has adverse side effects such as soil compaction, which can cause soil degradation, but can also have an impact on the vegetation and the soil microbiome. Within this last category, mycorrhizal fungi are key organisms due to their close association with plants. Combined with the observation that they can experience long-lasting impacts of compaction in forestry and agriculture, this highlights the need to investigate their currently poorly known responses to soil compaction in nature management. However, compaction is not the only environmental stressor. It can occur in combination with for example drought, which can affect both mycorrhizas and their hosts. Also the combination of these two influences is poorly understood, especially for organisms with a limited economic importance such as ericoid mycorrhizas and their host plants.

To remediate this lack of knowledge concerning the effects of soil compaction as well as the combined effects of compaction and drought stress in semi-natural grasslands, we conducted a study consisting of two main components. In the first section, we performed an observational field study to compare soil properties and mycorrhizal communities between non-mown, softrak-mown and tractor-mown grasslands. Secondly, we set up a laboratory experiment to investigate the effects of drought stress under compaction on Heather (*Calluna vulgaris*) and its ericoid mycorrhizal symbionts, as well as on Devil's-Bit Scabious (*Succisa pratensis*) and its arbuscular mycorrhizal symbionts.

The results of the observational field study have shown that mechanical mowing is correlated with increasing nutrient concentrations (nitrate, ammonium and phosphorus) and increasing levels of potentially toxic elements (aluminium, iron and manganese) in the soil, as well as with alterations in the community composition of arbuscular mycorrhizal fungi. The observed patterns were likely to be associated with the degree of compaction inflicted by the different types of mowing. In addition, the results of the lab experiments showed that compaction can influence both the survival of specific plant species and their degree of mycorrhization. More specifically, the long-term survival of *S. pratensis* clearly decreased with increasing compaction levels. Furthermore, the degree of mycorrhization of *C. vulgaris* showed a steadily decreasing trend with increasing compaction.

These findings highlight the importance of considering the potential consequences of soil compaction in nature management and restoration. To prevent negative effects, completely avoiding mechanical mowing in the habitats discussed in this study appears to be the most suitable approach.

# 1. Introduction

## 1.1 Mowing

Semi-natural grasslands are extremely valuable habitats whose appearance and species community composition have been shaped by prolonged anthropogenic influences (Pitkänen et al., 2014). These influences consisted primarily of traditional, non-intensive agricultural practices, which were widely applied up until the first decades of the 20$^{th}$ century (Pitkänen et al., 2014). However, later during that century the agricultural system shifted towards more intensive forms of food production, which caused the loss of traditional land management practices (Pitkänen et al., 2014). As a result, the accompanying habitats also disappeared, which explains the current degraded and threatened status of semi-natural grasslands (Kahmen et al., 2002; Pitkänen et al., 2014; Barber et al., 2022). In order to maintain these open landscapes together with their characteristic species, management is required (Barber et al., 2022). In this regard, mowing is one of the most suitable (Tälle et al., 2014) and most used management interventions to preserve extant semi-natural grasslands (e.g. Power et al., 1998; Zwaenepoel et al., 2002b).

Mowing exerts a strong impact on the plant species composition of grasslands (Bernhardt-Römermann et al., 2009; Bai et al., 2022). For example, it ensures a higher plant species richness and diversity compared to non-mowing conditions (Huhta et al., 2001; Józefowska et al., 2018; Mayel et al., 2021; Bai et al., 2022; Vegini et al., 2022; Zubek et al., 2022), which is combined with a better preservation of target species compared to other management interventions such as mulching or burning (Bernhardt-Römermann et al., 2009). This implies that human interference in the form of biomass removal from the ecosystem is key to the conservation and restoration of species-rich grasslands (Honnay et al., 2017; Józefowska et al., 2018; Zubek et al., 2022) and that mowing is the preferred technique to do so (Bernhardt-Römermann et al., 2009). In addition, these positive effects of mowing seem to apply to all types of grasslands, including those characterized by a high abundance of heather (*Calluna vulgaris*), commonly referred to as heathlands (Vegini et al., 2022).

The positive effect of mowing on biodiversity can be attributed to two complementary mechanisms. Firstly, mowing prevents the invasion and settlement of woody species that, when given the chance to grow, would overshadow and thus outcompete the herb species (Huhta et al., 2001; Vegini et al., 2022). Secondly, the removal of biomass by mowing and transporting the plant remains away from the site decreases the availability of nutrients in the grassland system, which provides an opportunity for plant species with limited competitive abilities to persist (Härdtle et al., 2006; Mayel et al., 2021; Bai et al., 2022). When soil nutrients are not limiting, these species would be suppressed by species with stronger competitive abilities that are able to grow fast and monopolize the available sunlight (Hautier et al., 2009; Honnay et al.,

2017). This is especially important in the light of excessive nutrient availability caused by the deposition of nitrogen and phosphorus (Härdtle et al., 2006).

However, the benefits obtained depend on the exact timing of mowing. For the protection of the already occurring plant species and thus the maintenance of a status quo, mowing at a later moment during the growing season appears to be sufficient (Huhta et al., 2001). When attempting to increase species richness, mowing early during the growing season seems to be a better choice because it removes more nutrients from the ecosystem (Huhta et al., 2001). Despite the necessity to distinguish between these different management goals when deciding on the moment of mowing, it is also important to take into account the timing of plant reproduction to ensure the rejuvenation of vegetation (Mayel et al., 2021).

Besides timing, also the mowing method can vary. Labour intensive approaches such as hand mowing have been largely replaced by machines, which allow to mow larger areas in a smaller timespan and at a lower cost (Keller et al., 2017). This trend is obvious in agriculture and will most likely persist there due to the advantages mentioned above, with tractor weights regularly exceeding 10 tons nowadays (Soane et al., 1981b; Alakukku et al., 2003; Keller et al., 2017). Similarly, in forestry machine weights have risen from five tons before the 1960s up to 40-50 tons in Germany and 64 tons in Sweden at present (Horn et al., 2004; von Wilpert & Schäffer, 2006; Roberge et al., 2020). Moreover, the gradual shift towards increasingly heavy equipment is still ongoing (Keller et al., 2017), with for example tests being performed to put into use harvesting gear weighing 74 tons in Swedish wood harvesting areas (Roberge et al., 2020). Importantly, it must be noted that also in nature management a very similar evolution has occurred through time because the area under management has increased whilst the willingness to invest in labour has decreased (T. Ceulemans, pers. comm., 11/06/2023).

## 1.2 Soil compaction

### 1.2.1 Effects on soil properties

Despite the benefits of machine mowing, there are a number of drawbacks that must be taken into account when implementing it in nature management. The largest of these negative impacts potentially arises from soil compaction, of which the level depends on machine characteristics such as weight and type of tyres, as well as on the duration of exposure to compacting forces (Troldborg et al., 2013). Compaction is defined as a reduction in the proportion of pores in the soil (or equivalently, an increase in bulk density) due to the compression of solid soil material (Soane et al., 1981a; Arvidsson, 1998). This change in soil structure also affects other soil properties: the resistance to penetration increases and the movement of water and air through the soil is hampered, which results in poor drainage, stagnation of water on the soil surface and superficial water runoff (Soane et al., 1981a; Horn et al., 2004; Hamza & Anderson, 2005; Chatterjea, 2007; Hartmann et al., 2012, 2014; Schrama et al., 2013; Keller et al., 2017). This in turn may cause the loss of nutrients from the soil, potentially combined with soil erosion (Horn et al., 2004; Hartmann et al., 2012). Also,

compacted soils have been shown to absorb less methane and to release smaller amounts of carbon dioxide and larger amounts of nitrous oxide (Batey & McKenzie, 2006; Beylich et al., 2010; Hartmann et al., 2014), with the latter resulting directly from the lack of oxygen in compacted conditions (Batey & McKenzie, 2006). This illustrates that chemical processes such as greenhouse gas absorption and emission as well as physical soil characteristics such as water infiltration are altered by compaction.

The effects of compaction on soil properties summarized above demonstrate that compaction can have a far-reaching impact on soil quality. As a matter of fact, compaction is one of the factors included in overall soil degradation (Batey, 2009). In addition, the soil environment is crucial to maintain a healthy ecosystem because it provides a habitat for plants and microorganisms, forms a reservoir of nutrients to sustain primary productivity and regulates the water cycle (Milleret et al., 2009; Francisco et al., 2016; Lewandowski et al., 2019). This implies that soil compaction can potentially inflict substantial damage; directly to the soil and indirectly to the entire ecosystem and its inhabitants. However, not all soils are equally vulnerable to compaction and only when a high susceptibility is combined with exposure to compressing forces, soil compaction will occur (Troldborg et al., 2013). These differences in vulnerability between soils can be attributed to the influence of three main factors. The first important factor is the amount of organic matter present in the soil. Soils containing a higher proportion of it are generally less sensitive to compaction (Arvidsson, 1998; Hamza & Anderson, 2005; Kara & Bolat, 2007; Bell et al., 2011), which is reflected by a lower bulk density and a higher air content (Arvidsson, 1998). However, it must be noted that this applies specifically to agricultural situations and that the responses of the soil to compacting forces can be different in nature management because the soil types differ. Soil water content is the second crucial factor affecting a soil's susceptibility to compaction because moist soils are more heavily compacted than drier ones when exposed to compression (Horn et al., 2004; Miransari et al., 2009; Keller et al., 2017). The water content in its turn is influenced by the specific weather conditions, soil properties, geomorphology and vegetation composition of the location under study (Troldborg et al., 2013). Thirdly, despite some contradictory findings, soil texture seems to have a certain influence, both directly and indirectly (Miransari et al., 2007). Whilst Kara & Bolat (2007), Miransari et al. (2007) and Batey (2009) suggest that a higher clay content increases the level of compaction, Arvidsson (1998) found the opposite. This discrepancy concerning the direct impact of soil texture can possibly be explained by the overriding effect of organic matter content on soil behaviour with regard to compacting forces (Arvidsson, 1998; Kara & Bolat, 2007). Concerning its indirect impact, texture affects the soil's drainage capacity: fine-textured soils retain more water and thus have a higher soil water content (Batey, 2009). Considering the importance of the three factors mentioned above in determining a soil's response to compaction, we can conclude that studying these soil characteristics is crucial (Milleret et al., 2009). And indeed, they have been widely studied while considering them to be

unconnected to the rest of the ecosystem (Arvidsson, 1998). However, it must be noted that manipulations of the soil involving changes in its chemical or physical properties also affect its microbial community (Francisco et al., 2016). Therefore, organisms living within or depending upon the soil environment are also very likely to be affected by soil compaction, potentially even more so than the abiotic soil properties (Jensen et al., 1996).

### 1.2.2 Effects on vegetation

On the level of the vegetation, it has been shown that the plant community composition shifts when the soil is compacted (Schrama et al., 2013; Sikorski et al., 2013). This can be attributed to the different capacity of plant species to root in soils with low levels of available oxygen. The species best suited for this will become dominant and eventually displace species that need a well aerated soil (Schrama et al., 2013). However, compaction not only induces changes at the community level, also the morphology of individuals can be affected. More specifically, the plant rooting system usually becomes more shallow and less dense, which can be attributed to two important factors (Hamza & Anderson, 2005; von Wilpert & Schäffer, 2006; Thorne et al., 2013). Firstly, the increased penetration resistance of compacted soil complicates the elongation of roots to deeper soil layers (Thorne et al., 2013). Secondly, compacted soils contain fewer air-filled pores and have a limited level of gas exchange with the aboveground environment, which means that the availability of oxygen is limited (von Wilpert & Schäffer, 2006). In combination with the fact that plant roots require a relatively large amount of oxygen for their growth compared to the rest of the plant body, this implies that the boundary of sufficient available oxygen is already reached very close to the interface with the air (von Wilpert & Schäffer, 2006). Besides morphology and community composition, also nutrient and water uptake by plants are affected (Batey, 2009). The direct impact of compaction on these processes is generally negative, which can be further exacerbated by the compaction-induced morphological changes mentioned above (Batey, 2009). A more superficial root system implies an extraction of water mainly from the topsoil, which becomes depleted of moisture more rapidly, thus impeding the extraction of nutrients from the soil (Batey & McKenzie, 2006; Batey, 2009).

### 1.2.3 Effects on the soil microbiome

On a smaller level compared to the vegetation, we find the bacteria and fungi inhabiting the soil that form the soil microbiome. This microbial community plays an indispensable role in many ecosystems by for example breaking down dead organic matter, maintaining a good soil structure and cycling essential nutrients such as nitrogen, phosphorus and sulphur, thus maintaining soil fertility (Allison & Martiny, 2008; Bell et al., 2011; Zubek et al., 2022). As a matter of fact, most ecosystem services associated with the soil would not be provided in the absence of the microbes that inhabit it (Francisco et al., 2016). In addition to its overall importance, the soil microbiome can be used to forecast perturbations of the natural state of an ecosystem (Hartmann et al., 2012, 2014). This implies detecting changes in an early stage of

the disturbance, preferentially before it causes severe disruptions to the ecosystem functioning (Hartmann et al., 2012). Such a fast response of the microbiome, caused by its sensitivity to disturbances (Allison & Martiny, 2008; Francisco et al., 2016), stands in contrast to the response of plants, whose roots are assumed to have fully reacted to new environmental conditions only after a time lag of several years (von Wilpert & Schäffer, 2006). Furthermore, the soil microbial community does not return to its pre-disturbed state easily as the process of resilience only pays off after several years (Allison & Martiny, 2008). Also, these changes in community composition can potentially inflict damage to the entire ecosystem. Because not all microbial groups have the potential to exert the same functions, the decreased abundance or disappearance of some groups (even when paralleled by an increase in the abundance of other groups) might trigger changes to ecosystem processes (Allison & Martiny, 2008). Taking into account their importance, fast response to changes in the environment and the long-lasting and potentially pervasive impact of disturbance on them, we can conclude that soil microbes are the most appealing organisms to study in the context of soil compaction.

Regarding the ways in which to study these soil microorganisms, relatively recent advancements in molecular techniques have opened up new opportunities. Previously used and less advanced methods such as morphology (Epelde et al., 2017), measuring microbial biomass (Hartmann et al., 2014) or PLFA (phospholipid fatty acid) analysis (Schnurr-Pütz et al., 2006) are prone to several disadvantages. Identifying soil organisms such as mycorrhizal fungi by describing their morphology requires vast amounts of time and can only be performed by specialists (Epelde et al., 2017). In addition, this approach does not appear to have an added value compared to other techniques when used in studies concerning microbial communities (Epelde et al., 2017). For PLFA analysis, Schnurr-Pütz et al. (2006) remarked that the patterns revealed by this technique are quite crude and are not necessarily a truthful reflection of reality. Another shortcoming of most relatively dated approaches, which applies in particular to the determination of microbial biomass, is the lack of differentiation between taxa (Hartmann et al., 2014). However, this is crucial to obtain meaningful information about microbial communities, their composition and how this changes in response to external factors (Hartmann et al., 2014). In contrast to the above-mentioned approaches, recently developed techniques such as next generation sequencing allow us to identify a large number of microbes at once up to the level of operational taxonomic units, which revolutionized our ability to study these otherwise elusive organisms (Hartmann et al., 2012). An example of its possible applications is provided by Vályi et al. (2015), who noted that these new molecular methods revealed for the first time the impact of host plant species on the mycorrhizal communities they associate with.

All above-mentioned techniques have been used to investigate the impact of soil compaction on microorganisms, and they have revealed somewhat ambiguous results. Whilst Keller et al. (2017) found no differences in the abundance of microorganisms between compacted and non-compacted soils, Hartmann et al. (2014) showed that their abundance

decreased in soils with a strongly reduced porosity. However, a less extreme reduction in porosity did not result in significant differences (Hartmann et al., 2014), thus confirming the results obtained by Keller et al. (2017). Similarly, Beylich et al. (2010) found no consistent relationship between compaction level and the biomass of microorganisms. In line with these slightly contradictory results concerning microbe abundance, the effects on species composition also differ between studies. In some cases the species of bacteria and fungi that make up the soil microbiome are significantly different between compacted and non-compacted sites (Hartmann et al., 2012, 2014), whereas others found no impact of compaction specifically on the fungal species assemblage (Kara & Bolat, 2007). Considering these discrepancies and the fact that soil properties are possibly of greater significance for soil microbes than compaction, a more in-depth investigation of the effects of compaction on the soil microbiome that distinguishes between different vegetation and land use types is recommended (Kara & Bolat, 2007).

Arable fields are extremely sensitive to soil compaction due to the mechanization of agriculture (Hamza & Anderson, 2005; Keller et al., 2017). In addition, it has been shown that crop productivity can be negatively affected by compaction (Hamza & Anderson, 2005) and that soil-inhabiting microorganisms are key to nutrient cycling and thus to healthy crops (Grayston et al., 1998). These observations assure that the effects of compaction on soil microorganisms are relatively well-studied (e.g. Hamza & Anderson, 2005; Longepierre et al., 2021). Despite the fact that compaction does not seem to alter the total abundance and activity level of the microbes in the soil (Hamza & Anderson, 2005), the relative abundances of different groups do change (Longepierre et al., 2021). Those requiring oxygen for their survival and growth decrease in abundance, whilst microbes that are able to live without oxygen and those that feed on decaying organic matter become more prevalent (Schnurr-Pütz et al., 2006; Longepierre et al., 2021). Given the fact that the availability of oxygen decreases further in a wet medium (due to its slow diffusion in water versus air), the shift towards anaerobic microorganisms can be expected to be even more pronounced in soils with a higher moisture content (Schnurr-Pütz et al., 2006).

Forests, in particular those used for the production of wood, are another type of environment that is vulnerable to soil compaction caused by heavy machinery. Additionally, the soil and its microbial inhabitants form a crucial component of a healthy and productive forest ecosystem that fully delivers its ecosystem services (Hartmann et al., 2012, 2014; Thees & Olschewski, 2017). Therefore, several studies have focused on the effects of soil compaction on the soil microbiome in forests (e.g. Ponder & Tadros, 2002; Schnurr-Pütz et al., 2006; Hartmann et al., 2012, 2014). The species composition of bacterial and fungal communities has been shown to shift as a result of compaction (whether or not combined with the removal of biomass) (Hartmann et al., 2012, 2014). These changes result in a clearly different microbial species composition for each increment in the level of soil compaction (Hartmann et al., 2014),

which illustrates the potentially large impact of the trend towards heavier and heavier machinery (Keller et al., 2017). Compaction also appears to have a negative impact on the number of microbes in the soil (Hartmann et al., 2014), especially for fungi and eukaryotic unicellular organisms (Schnurr-Pütz et al., 2006). As a result, the relative abundance of prokaryotic organisms (in particular those capable of surviving at low levels of oxygen) increases, which implies a change in the overall community composition towards a predominance of bacteria and archaea (Schnurr-Pütz et al., 2006). This might be explained by the decreased availability of oxygen in compacted soils (Schnurr-Pütz et al., 2006). Despite the decreased abundance of certain groups, the overall biomass of soil-inhabiting microorganisms is not affected by compaction (Ponder & Tadros, 2002), and the diversity of fungal operational taxonomic units even appears to increase (Hartmann et al., 2014). However, the latter effect might indicate the disentanglement of those microbial relationships that are crucial for the functioning of the ecosystem (Hartmann et al., 2014). And importantly, compaction always has an effect on the soil microorganisms, irrespective of environmental differences that give rise to distinct communities (Hartmann et al., 2012). Another consistent aspect seems to be that the effects on fungi are more radical and long-lasting compared to those on bacteria (Hartmann et al., 2012, 2014), which can potentially be explained by their morphology (Francisco et al., 2016). Multicellular fungi form fine, branched networks which can easily be damaged by disruptions to their growth medium, which stands in contrast to the unicellular nature of bacteria (Francisco et al., 2016).

## 1.3 Mycorrhizal fungi

The finding mentioned above combined with the observation that all types of soil disturbance can potentially exert an influence on fungi, suggests that the research concerning soil compaction should focus on this group (Dell, 2002). More specifically, mycorrhizal fungi are key organisms because they play a pivotal role in ecosystems by supporting plants during processes such as nutrient uptake, thus enhancing plant growth and survival and maintaining their productivity under less favourable environmental conditions (Entry et al., 2002; Brundrett & Tedersoo, 2018). This can also be the case specifically for stress imposed by soil compaction (Miransari et al., 2007). Also, in a broader context rather than at the plant level, these fungi are indispensable given their role in the fluent circulation of nutrients through the ecosystem (Dell, 2002). Furthermore, there are indications that at least one type of mycorrhizal fungi is able to reduce compaction (Milleret et al., 2009). This mitigating effect is largely produced by the interaction between fungus and plant roots: the fungal hyphae form a fine-mazed extension of the host's root system, thus enabling it to expand through portions of the soil that lack large pores (Milleret et al., 2009). As a result, carbon compounds secreted by the roots and the hyphae penetrate the soil over a larger volume, where they are utilized as a source of energy by soil-inhabiting bacteria (Milleret et al., 2009; Herman et al., 2012). These microbes in their turn ameliorate soil structure by enlarging soil pores and by improving the cohesion between soil

particles (Milleret et al., 2009). Finally, the importance of mycorrhizas is also illustrated by the potential loss of plant species following a decrease in mycorrhizal species richness and diversity (Ceulemans et al., 2019), which can be caused by elevated concentrations of nutrients in the soil (Ceulemans et al., 2019; Van Geel et al., 2020), heavy metals, agrochemicals or soil compaction (Entry et al., 2002).

Mycorrhizas can be subdivided into four groups: ectomycorrhizal fungi (EcM), arbuscular mycorrhizal fungi (AMF), ericoid mycorrhizal fungi (ErM) and orchid mycorrhizal fungi (OrM) (Brundrett & Tedersoo, 2018). This classification is based on the types of plants they associate with and on the morphological characteristics of the fungal tissue (Brundrett & Tedersoo, 2018). However, considering our focus on grassland ecosystems, only three out of four types must be discussed here (AMF, ErM and OrM). Ectomycorrhizal fungi are left out of account because they mostly associate with trees such as *Pinus*, *Larix*, *Salix*, *Ulmus*, *Betula* and other woody species and are thus associated with forest environments (Amaranthus et al., 1996; Dell, 2002; Brundrett & Tedersoo, 2018).

Arbuscular mycorrhizal fungi on the other hand are very important in grasslands (Francisco et al., 2016; Honnay et al., 2017). Despite their relatively low species diversity compared to other groups of fungi (Lee et al., 2013), they are in general the most prevalent of the four mycorrhizal types (Entry et al., 2002; Brundrett & Tedersoo, 2018; Thangavel et al., 2022; Zubek et al., 2022). This is illustrated by the fact that these fungi (belonging to the subphylum Glomeromycotina within the phylum Mucoromycota) associate with 72% of all angiosperm species (Honnay et al., 2017; Brundrett & Tedersoo, 2018), which corresponds to more than 200,000 plant species (Lee et al., 2013). Furthermore, this specific plant-fungus association already exists since the first plants conquered the terrestrial environment, which highlights its importance for the survival of plants on land (Lee et al., 2013). Despite the facultative nature of the relationship with their host plants, AMF can exert many positive influences on both their hosts and the ecosystem in general (Honnay et al., 2017). Firstly, they improve soil structure by enhancing the cohesion between soil particles (Milleret et al., 2009). Secondly, AMF can influence the plant species composition of an ecosystem by supporting the species that would otherwise be outcompeted by fast-growing plants that do not depend on mycorrhizal symbionts for the acquisition of nutrients (Entry et al., 2002; Honnay et al., 2017). Therefore, the presence of these fungi maintains biodiversity (Lee et al., 2013). Thirdly, plants that live in symbiosis with AMF are less likely to be negatively affected by stressors in their environment (Miransari et al., 2007), including for example pathogenic organisms, damaging agents such as heavy metals and unfavourable pH-values (Dell, 2002). Lastly, the presence of AMF can increase the biomass production of an ecosystem (Lee et al., 2013), especially when the primary producers are subjected to some form of stress (Miransari, 2010). This can be achieved by the production of substances such as glomalin by the fungi themselves (Miransari, 2010), by stimulating the synthesis of growth-promoting molecules by the plants (Thangavel et

al., 2022), but also by providing additional nutrients to the host (Entry et al., 2002; Miransari et al., 2009; Vályi et al., 2015; Thangavel et al., 2022; Zubek et al., 2022). Despite the fact that this type of mycorrhizal fungi is not able to actively decompose organic substances, the last mechanism does seem to be the most important one as it forms the foundation of the symbiosis between AMF and their hosts (Herman et al., 2012). The fungi absorb soil nutrients (especially phosphorus and nitrogen) via a network of hyphae with small diameters that extends beyond the root network (Dell, 2002), after which these nutrients are transferred to the host plant in exchange for assimilated carbon (Entry et al., 2002; Miransari et al., 2007, 2009; Vályi et al., 2015). This exchange takes place at the level of the arbuscules, which are specialized hyphae that form tree-like structures inside the plant root cells (Entry et al., 2002; Miransari, 2010; Herman et al., 2012; Thangavel et al., 2022). The formation of these typical morphological characteristics is governed by biochemicals produced by both the fungus and the plant and used for the molecular recognition of suitable symbiotic partners (Miransari, 2010). When a suitable partner has been found, fungal growth (usually starting from a spore) is initiated and hyphae are formed to grow around and within the plant roots (Entry et al., 2002; Miransari, 2010). This process of recognition followed by growth is not very specific as it is assumed that arbuscular mycorrhizal OTUs are able to from symbioses with multiple plant species (Miransari, 2010; Honnay et al., 2017). However, it has been shown that the species composition of an AMF community is influenced to some extent by the plant species composition of that ecosystem, implying at least a certain level of specificity in the plant-fungus interaction (Horn et al., 2017; Thangavel et al., 2022). Nonetheless, factors such as soil physical properties (Vályi et al., 2015; Sepp et al., 2018; Thangavel et al., 2022), space (Horn et al., 2017) and time (Thangavel et al., 2022) are generally assumed to have an overriding impact on the AMF community. Specifically for the soil characteristics, the pH and the concentration of nitrogen and carbon seem to exert the largest influence on the community composition of the arbuscular mycorrhizal fungi (Sepp et al., 2018).

The relatively limited host specificity of arbuscular mycorrhizas mentioned above stands in stark contrast to the lifestyle of ericoid mycorrhizal fungi. Despite also being strongly influenced by soil properties, the distinct OTUs of these fungi are often restricted to a single plant species (Van Geel et al., 2020). These host plants belong to the families of the Ericaceae and the Diapensiaceae (both subdivisions of the order Ericales) and constitute in total about 1,5% of all angiosperm species (Brundrett & Tedersoo, 2018). Similarly, the number of fungal species involved is limited (Straker, 1996). However, these fungal partners belong to different taxa (Straker, 1996), which makes them more diverse compared to the monophyletic AMF (Lee et al., 2013). Despite the limited number of host and fungal species involved, ericoid mycorrhizas play a crucial role in the survival of their hosts by providing them with additional nutrients such as phosphorus and nitrogen, which they can obtain by breaking down organic compounds (Smith & Read, 2008; Van Geel et al., 2020). This is necessary for the survival of

their hosts as these plant species inhabit environments such as heathlands and peatlands, in which nutrients are scarce (Smith & Read, 2008; Van Geel et al., 2020). Therefore, the symbiotic relationship between ErM fungi and their hosts is obligatory, and the advantages for the fungus most likely consist of carbon compounds combined with a secure place to live (Brundrett & Tedersoo, 2018). The main zone of contact between both partners is formed by dense, circularly shaped hyphal networks inside the root cells that are placed furthest away from the root centre (Smith & Read, 2008; Brundrett & Tedersoo, 2018). The number of hyphae placed around the roots is relatively limited and these do not form any specialized structures (Brundrett & Tedersoo, 2018).

The last mycorrhizal type, the orchid mycorrhizas or OrM, only have plant species belonging to the Orchidaceae as hosts, which makes them even more host-specific than the ericoid mycorrhizal fungi (Brundrett & Tedersoo, 2018). This implies that the OTU community composition of OrM fungi largely depends on the orchid species that are present (Oja et al., 2017). However, the Orchidaceae are a very diverse plant family (Smith & Read, 2008). As a result, OrM fungi are present in 10% of all angiosperms, making them the second most abundant group of mycorrhizas (Brundrett & Tedersoo, 2018). The fungi involved in this relationship obtain their nutrients by breaking down dead organic matter and they mostly belong to the Tulasnellaceae, Ceratobasidiaceae, Serendipitaceae and Pezizales (Brundrett & Tedersoo, 2018). However, also fungi that are normally ectomycorrhizal are able to live in close association with orchids (Smith & Read, 2008; Brundrett & Tedersoo, 2018). The orchid hosts of these EcM fungi usually lack the ability to photosynthesise and entirely depend on their fungal partner to sustain them (Smith & Read, 2008; Brundrett & Tedersoo, 2018). On the other hand, the adult orchids associated with non-EcM fungi photosynthesise and are thus able to at least partly produce the sugar molecules they need themselves (Smith & Read, 2008; Brundrett & Tedersoo, 2018). However, they still require their fungal partner to supply them with nutrients (Smith & Read, 2008; Brundrett & Tedersoo, 2018). As a matter of fact, no orchid is able to germinate and develop into a full-grown individual without the help of a mycorrhizal fungus (Smith & Read, 2008; Smith et al., 2009; Oja et al., 2017). This type of development, termed ‘symbiotic germination’, is inevitable for orchids because their seeds lack reserves and because the plants themselves are not yet capable of photosynthesis during the early stages of growth (Smith & Read, 2008; Oja et al., 2017). It also implies that the fungal partner does not benefit from its relationship with the host plant (Brundrett & Tedersoo, 2018), but that it is rather ‘exploited’ to a variable extent, depending on the degree to which the orchid can sustain itself (Smith & Read, 2008). A part of the orchids energy is actually derived from the digestion of the peletons that are no longer used by the fungus and have been replaced by newly formed ones (Brundrett & Tedersoo, 2018). These peletons are hyphal structures situated inside the host root cells, forming a characteristic feature of orchid mycorrhizal fungi (Smith & Read, 2008; Brundrett & Tedersoo, 2018).

The indispensable role of AMF, ErM and OrM in grassland ecosystems described above also implies that these fungi are potentially affected by disturbances to the soil environment such as compaction. Specifically for arbuscular mycorrhizal fungi, Thorne et al. (2013) found no significant effect of compaction on the extent to which the hyphae grow around and within the plant roots. However, the level of compaction used in this study was relatively limited (maximal bulk density of 1.5 g/cm$^3$; Thorne et al., 2013), and for more severe compaction (maximal bulk density of 1.75 g/cm$^3$; Nadian et al., 1998) the consequences appear to be mostly negative. The fungal biomass, the percentage of the plant roots associated with the fungi, as well as the rate at which new hyphae are formed decline with augmenting compaction levels (Nadian et al., 1998). These responses can, at least partly, be explained by the loss of large soil pores (Nadian et al., 1998). Without these, the fungal hyphae have less space for growth and might struggle to make their way through the soil (Nadian et al., 1998). Another possible explanation is related to the availability of oxygen: compacted soils contain smaller amounts of this gas that is crucial for the growth and survival of all aerobic organisms (Nadian et al., 1998), which results in increased competition for this resource between the mycorrhizal fungi and other soil-inhabiting microbes (Miransari et al., 2009). A final explanation specifically concerns the reduced colonization of roots by AMF. Plants whose root growth is hampered by a poorly penetrable soil produce ethylene, which might negatively affect their mycorrhizal symbionts (Nadian et al., 1998). Unlike for arbuscular mycorrhizas, where some studies are available, the effects of soil compaction on for example the abundance and community composition of ericoid and orchid mycorrhizal fungi are unclear (e.g. Nadian et al., 1998; Thorne et al., 2013). As a matter of fact, to our best knowledge no studies have been performed that investigate this up till now.

Besides the above-mentioned (potentially) negative effects of soil compaction on the plant-fungi symbiosis, the impact might be very persistent. Not only the changes in soil structure caused by compaction (Horn et al., 2004; von Wilpert & Schäffer, 2006; Keller et al., 2017; Longepierre et al., 2021), but also the modifications inflicted to the microbial life appear to be long-lasting (Hartmann et al., 2012, 2014). For the soil physical properties, four years of recovery after a compaction event is not yet sufficient to return to the pre-compacted state (Longepierre et al., 2021) and full recovery might even require tens of years (Keller et al., 2017). This is especially true for soils that do not undergo practices such as tilling that pull apart soil particles and aggregates and rearrange them (Longepierre et al., 2021), which applies to grassland habitats. Apart from the soil structure, also the species composition of soil-inhabiting microorganisms does not recover quickly (Hartmann et al., 2012, 2014). Even fifteen years after compaction the community structure is still significantly different from uncompacted soils (Hartmann et al., 2012).

## 1.4 Drought stress

The potentially long-lasting impact of soil compaction on mycorrhizal fungi might be further aggravated by other stressors in the environment. In particular, drought seems to be an important factor which might induce additional alterations to the symbiosis between plants and fungi (e.g. Fini et al., 2011; Gehring et al., 2017; Li et al., 2021). More specifically, a lack of moisture can change the community composition and abundance, as well as the functioning of several types of mycorrhizas (Gehring et al., 2017). For example, Li et al. (2021) have shown that drought stress causes a decline in mycorrhization in young individuals of the tree species *Quercus acutissima*, which forms associations with ectomycorrhizal fungi. However, such a pronounced negative impact does not seem to be ubiquitous across all species of plants and types of mycorrhizal fungi (e.g. Jeliazkova & Percival, 2003; Fini et al., 2011). In Wild Blueberry (*Vaccinium angustifolium*) for example, the degree of root colonization by ErM fungi did not differ significantly between well-watered and drought-exposed plants (Jeliazkova & Percival, 2003). Similarly, drought did not seem to exert a significant impact on orchid mycorrhizal fungi (Oja et al., 2017) or on the EcM and AMF fungi associated with Littleleaf Linden (*Tilia cordata*) (Fini et al., 2011). Seedlings of Hedge Maple (*Acer campestre*, associates with AMF) and Pedunculate Oak (*Quercus robur*, associates with EcM fungi) grown under experimentally induced drought stress even showed increased mycorrhization compared to well-watered individuals (Fini et al., 2011). This substantial variability concerning the response of mycorrhizas to water deprivation can possibly be attributed to interspecific differences among fungal species or OTUs (Gehring et al., 2017). In relation to this, it has for example been suggested that arbuscular mycorrhizal fungi are in general more tolerant to low moisture conditions compared to ectomycorrhizal fungi (Gehring et al., 2017). However, variability in drought-sensitivity is not limited to the species level. Also within OTUs and within the same host species, differences in response can arise (Gehring et al., 2017). In addition to this inter-and intraspecific variation, also the degree to which the soil is dehydrated might influence the effect on the mycorrhizal fungi present therein (Gehring et al., 2017).

Despite the importance of the (seemingly diverse) impacts of a lack of water on mycorrhizas, the interplay between drought and fungi extends beyond this one-way effect. Given the fact that mycorrhizal fungi appear to be capable of protecting their host plants against the negative influences of drought stress, we can also consider the reverse part of the interaction (the effect of mycorrhizas on drought stress) (Parke et al., 1983; Entry et al., 2002; Jeliazkova & Percival, 2003; Miransari, 2010; Fini et al., 2011; Worchel et al., 2013; Jayne & Quigley, 2014; Gehring et al., 2017; Sebastiana et al., 2018, 2019; Van Geel et al., 2020; Li et al., 2021; Mu et al., 2021; Lou et al., 2022). The positive impact of fungal symbionts on plant resistance against low moisture levels has been demonstrated for arbuscular mycorrhizas (Entry et al., 2002; Miransari, 2010; Gehring et al., 2017), ectomycorrhizas (Parke et al., 1983; Gehring et al., 2017; Sebastiana et al., 2018, 2019; Li et al., 2021) and ericoid mycorrhizas (Jeliazkova &

Percival, 2003; Mu et al., 2021; Lou et al., 2022), which illustrates that it is most likely a common trait for the majority of plant-fungal symbioses. However, it must be noted that, similar to their sensitivity to drought, interspecific differences exist in the protective effect of mycorrhizas (Parke et al., 1983; Mu et al., 2021; Lou et al., 2022). For example, an experiment in which Douglas-Fir (*Pseudotsuga menziesii*) seedlings each associated with one of four different EcM species underwent water deprivation, showed that the level of host protection differed significantly between the fungal species (Parke et al., 1983). Similar variations were found for ErM fungi in drought-stressed Lingonberry (*Vaccinium vitis-idaea*) (Lou et al., 2022) and Velvetleaf Blueberry (*Vaccinium myrtilloides*) (Mu et al., 2021). Despite these interspecific differences in the strength of the response, the generally positive effect can even withstands non-natural conditions such as a lab setting in which plants are kept in containers that confine the otherwise unrestricted growth of the fungal tissue (Parke et al., 1983; Jayne & Quigley, 2014).

The positive effect of mycorrhizas on their hosts under drought stress is reflected by for example a higher net photosynthetic rate, a larger leaf surface area and higher biomass accumulation in both above- and belowground organs compared to non-inoculated plants (Parke et al., 1983; Worchel et al., 2013; Sebastiana et al., 2018; Lou et al., 2022). Despite the fact that all these effects are unambiguous indicators of an increased plant fitness, they can be brought about by a variety of potential mechanisms in which it is often unclear which is the primary one. A first possible mechanism consists of an enhanced uptake of mineral nutrients from the soil when mycorrhizal fungi are present (Sebastiana et al., 2018). This might result in an increased tolerance of the plant to unfavourable environmental conditions such as drought by stimulating the biochemical processes involved in photosynthesis (Sebastiana et al., 2018). Observations of higher rates of photosynthesis, carboxylation and regeneration of key photosynthetic enzymes in mycorrhizal plants subjected to drought might support this idea (Fini et al., 2011). However, clear confirmation for this hypothesis is limited as for example Parke et al. (1983) and Sebastiana et al. (2018) did not find indications for it to be important in Douglas-Fir and Cork Oak respectively. In addition, the rates of carboxylation and regeneration of enzymes are always increased in inoculated plants, also under conditions in which water is not limiting (Fini et al., 2011). Nonetheless, altered nutrient status might still play a role in drought tolerance by stabilizing the structure of chloroplast membranes (Sebastiana et al., 2019). A second mechanism concerns the regulation of stomatal opening (Parke et al., 1983). Preventing the stomata from closing under water shortage could sustain gas exchange to drive carbon assimilation (Sebastiana et al., 2018), which seems to be the case in strongly water-deprived host plants of arbuscular mycorrhizal fungi (Augé et al., 2015). However, this mechanism has been found to be unimportant in Cork Oak trees (Sebastiana et al., 2018). A third possibility states that mycorrhizas reduce the chance of damage to membranes by reactive oxygen species (Sebastiana et al., 2018). Despite the fact that this did not seem to apply to drought-stressed

mycorrhizal Cork Oaks, a fourth mechanism also concerning membrane structure does seem to be crucial in the same tree species (Sebastiana et al., 2018). A higher prevalence of unsaturated lipid molecules in the membranes of mycorrhizal plants might preserve the flexibility of these membranes, thus protecting them against the harmful effects of desiccation (Sebastiana et al., 2018). Similarly for the fifth mechanism, the presence of mycorrhizas appears to alter the relative abundance of different lipid molecules in leaves, which makes them less sensitive to drought (Sebastiana et al., 2019). Another potential mechanism, however also proven to be unimportant in at least one tree species, suggests that symbiotic fungi make it easier for the host plant to store soluble sugars and thus to attract water into their cells via osmosis during periods of water shortage (Sebastiana et al., 2018). A seventh possible mechanism consists of enhanced moisture acquisition in mycorrhizal plants (Parke et al., 1983). Despite the obvious nature of this anti-drought mechanism, it does not appear to play a significant role in all species. For Douglas-Fir trees it is most likely a crucial pathway (Parke et al., 1983), whilst for Cork Oaks no indications towards it were found (Sebastiana et al., 2018). Finally, it has been shown that (at least ectomycorrhizal) fungi are capable of improving the efficiency with which water is used by their host plants (Li et al., 2021). This might be achieved by elevating the calcium concentration in the plant tissue, both by increasing the availability of these ions in the soil and by stimulating their absorption by the plant (Li et al., 2021). However, the exact pathway behind this mechanism remains elusive (Li et al., 2021). Taking into account the large variety of possible mechanisms described above, the lack of agreement between different studies and the fact that the research towards these drought-resistance mechanisms is biased towards trees and their ectomycorrhizal symbionts, it must be noted that different mechanisms might apply to other plant species associated with other types of mycorrhizas (e.g. Parke et al., 1983; Sebastiana et al., 2018, 2019; Li et al., 2021).

Despite the uncertainty regarding the mechanism(s) of drought protection by mycorrhizal fungi, it is crucial to study those mechanisms, the impact of mycorrhizas on drought stress in their hosts, as well as the direct effects of drought on mycorrhizal fungi. This need for additional research is highlighted by the fact that drought is a highly unfavourable environmental condition which impedes the normal growth of plants (Entry et al., 2002; Worchel et al., 2013; Sebastiana et al., 2018). Its negative impacts on this fitness parameter have been demonstrated for a wide range of plant species, ranging from Lingonberry shrubs with a diminished growth (Lou et al., 2022) to young oak trees that remain smaller after a drought treatment (Li et al., 2021). Besides growth, also plant survival (Sebastiana et al., 2018) and physiology (Lou et al., 2022) are affected by a lack of moisture. In addition, these negative impacts will most likely become more pronounced in the future as changes occur in the hydrological cycle due to climate change (Gehring et al., 2017; Sebastiana et al., 2019; Li et al., 2021). More specifically, areas already subjected to drought at present will become even more vulnerable to water deprivation in the future (IPCC, 2014). This trend could be further

aggravated by rising temperatures, which causes an increase in evapotranspiration and thus a loss of water (IPCC, 2014).

## 1.5 Research gap

Despite the importance of mycorrhizas in grassland ecosystems and the potentially pervasive impact of soil compaction on them, the research effort devoted to this topic can be considered substandard (Entry et al., 2002; Oja et al., 2017; Zubek et al., 2022). Already at the basis of the ecosystem, this lack of understanding exists as grassland soils are poorly studied (Newell-Price et al., 2013). The same applies to the soil microbiome in general, and in particular to the impact of compaction on it (Hartmann et al., 2012; Longepierre et al., 2021). Also for mycorrhizal fungi, which form a specific subset of the microorganisms that inhabit the soil, the way in which they are influenced by management techniques such as machine mowing remains elusive (Entry et al., 2002; Oja et al., 2017; Zubek et al., 2022). This could be partly due to the entanglement between mycorrhizal fungi, their hosts and the soil environment, which complicates the research concerning these fungi (Horn et al., 2017). This general lack of research in grasslands stands in sharp contrast with economically more important ecosystems such as forestry systems and agricultural fields, which have been discussed above. However, Longepierre et al. (2021) remarked that the results obtained in one of these environments do not necessarily apply to the other as there are substantial differences between them. Presumably, this is also valid for the comparison between forests and/or arable fields and grassland ecosystems, which further stresses the need for additional research focussing specifically on the latter. Furthermore, reliable information concerning the impact of soil compaction on mycorrhizas (and on the properties of their soil environment) is indispensable to be able to formulate management plans that impose minimal damage to the ecosystem, thus enabling a better conservation of these areas and their inhabitants (Alakukku et al., 2003; Ceulemans et al., 2019; Zubek et al., 2022).

Similar to compaction, the research concerning drought focusses mostly on agricultural and forestry systems in which respectively AMF and EcM fungi are most important (Gehring et al., 2017). As a result, the interaction between ErM and OrM fungi on the one hand and drought on the other hand remains underexposed (Gehring et al., 2017; Mu et al., 2021). Nonetheless, mycorrhizas can shape entire communities by for example affecting competitive interactions between their hosts (Worchel et al., 2013). This implies that knowledge concerning the impact of drought on all types of mycorrhizas is key to an enhanced understanding of the effects of global warming on several ecosystems, including grasslands (Worchel et al., 2013). In addition, the effects of multiple stressors at once should be studied more intensively because their combined impact probably cannot be predicted based on the isolated effects of one factor (Meyer-Grünefeldt et al., 2016). These potentially non-additive effects are currently understudied as for example, to our best knowledge, the combined impact of drought and compaction on mycorrhizas and their host plants has not yet been investigated explicitly.

# 2. Goals

Taking into account the need for additional research mentioned above, this study will focus on the effects of soil compaction in grasslands and heathlands. More specifically, we will investigate the effects on soil properties and mycorrhizal fungi. In addition, the open questions concerning the effects of drought stress under compaction on both plants and mycorrhizas will be tackled. To achieve these objectives, our research consists of two components. In the first section, we performed an observational field study to compare soil properties and mycorrhizal communities between grasslands with contrasting mowing regimes (Figure 1). Secondly, we set up a laboratory experiment to investigate the effects of drought stress under compaction on Heather (*Calluna vulgaris*) and its ericoid mycorrhizal symbionts, as well as on Devil's-Bit Scabious (*Succisa pratensis*) and its arbuscular mycorrhizal symbionts (Figure 2).

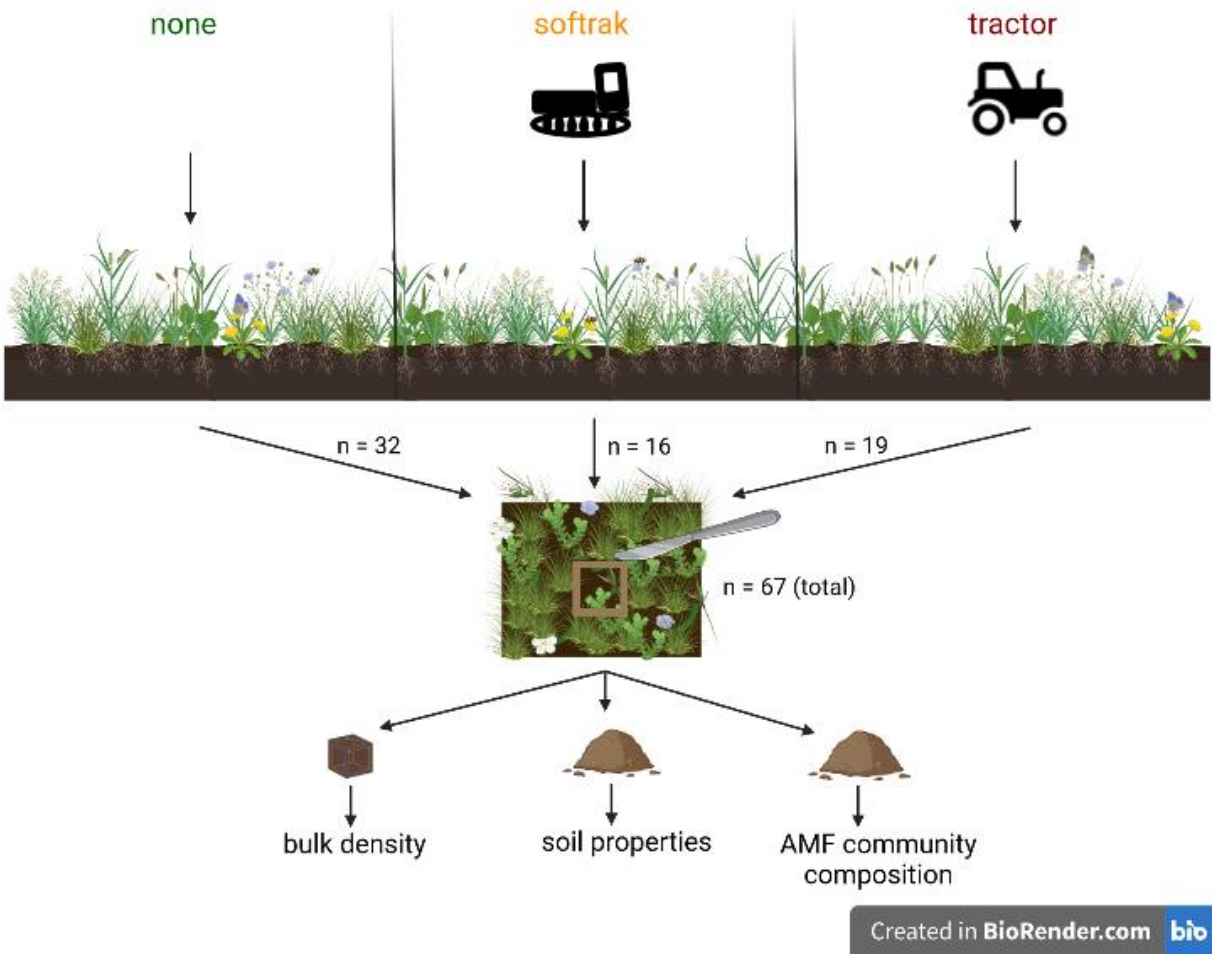


*Figure 1: Schematic overview of the observational field study (created in BioRender.com).*

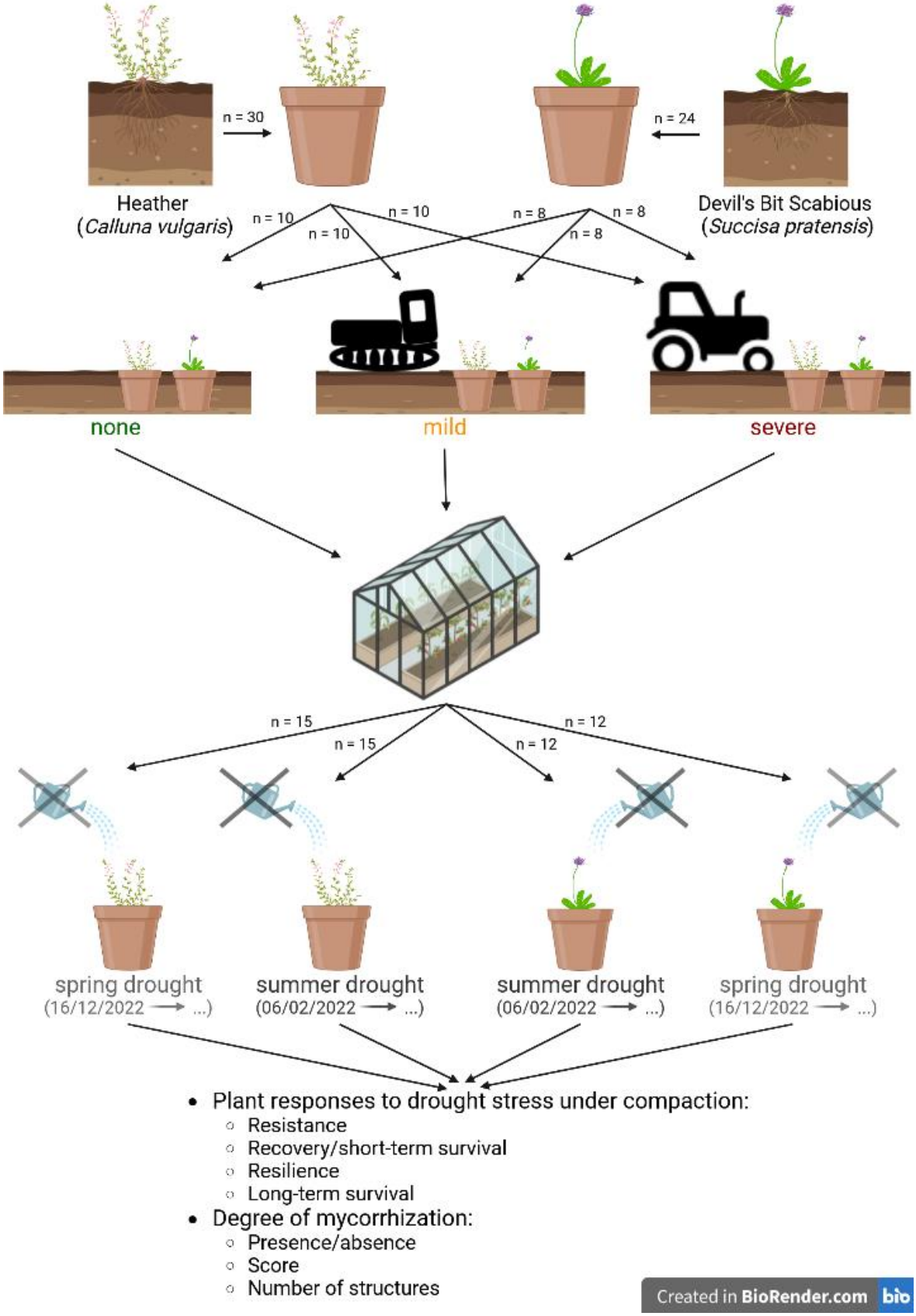


*Figure 2: Schematic overview of the two laboratory experiments on* Calluna vulgaris *on the one hand and* Succisa pratensis *on the other hand (created in BioRender.com).*

# 3. Materials and methods

## 3.1 Observational field study

### 3.1.1 Study area

For the observational part of this study, we collected a total of 67 soil samples from seven locations in which different mowing regimes have been employed over the past years. Three of these locations were situated in Vorsdonkbos-Turfputten (ca. 50.97°N, 4.79°E; Aarschot, Belgium; Figure 3A), an area which is situated approximately 12 m above sea level and where the average annual temperature and rainfall equal 11.0 °C and 807.2 mm respectively (Geopunt; Royal Meteorological Institute Belgium). Here, we collected 40 samples, spread across three locations (Figure 3B). Each sample was either located in a non-mown (20 samples), a softrak-mown (10 samples) or a tractor-mown (10 samples) grassland patch (Figure 3C-E). An additional set of 27 samples was collected across four other locations: three non-mown, three softrak-mown and three tractor-mown samples from Spicht (ca. 50.90°N, 4.85°E), three non-mown and three tractor-mown samples from Zwartenbos (ca. 50.81°N, 4.76°E), three non-mown and three tractor-mown samples from Koebos (ca. 50.86°N, 4.79°E) and three non-mown and three softrak-mown samples from Walenbos (ca. 50.93°N, 4.88°E) (Figure 3A).

The grasslands in the main Vorsdonkbos-Turfputten sampling area belonged to the *Molinion caeruleae*, *Junco-Molinion* or *EU-Molinion* associations (all subdivisions of the collective term 'blauwgraslanden'), or to the *Nardo-Galion* association ('heischrale graslanden'). The first type of associations is marked by a typical blue colour caused by characteristic species such as Devil's-bit Scabious (*Succisa pratensis*), Carnation Sedge (*Carex panicea*), Common Milkwort (*Polygala vulgaris*), Purple Moor Grass (*Molinia caerulea*) and Mountain Heath Grass (*Danthonia decumbens*) (Zwaenepoel et al., 2002b). However, the range of the species mentioned above expands beyond this specific plant association. Meadow Thistle (*Cirsium dissectum*) and the hybrid between Meadow Thistle and Marsh Thistle (*Cirsium x forsteri*) on the other hand, are species that can be considered as unique for this vegetation type (Zwaenepoel et al., 2002b). Concerning the abiotic conditions, these grasslands are nutrient poor and relatively wet, with the latter applying especially during winter when puddles can form (Zwaenepoel et al., 2002b). Even though the soil is (slightly) acidic, there is often an influence of base-rich seepage water (Zwaenepoel et al., 2002b). Management includes mowing and removing the resulting clippings once a year (Zwaenepoel et al., 2002b). The second association occurring at our sampling sites (*Nardo-Galion*) is also characterised by low levels of soil nutrients and by management interventions consisting in most cases of mowing (Zwaenepoel et al., 2002a). However, in contrast to the first association, *Nardo-Galion* grasslands are not necessarily wet and their soil is always characterised by a low pH (Zwaenepoel et al., 2002a). Characteristic species include Tormentil (*Potentilla erecta*),

Mountain Heath Grass (*Danthonia decumbens*), Matgrass (*Nardus stricta*), Heath Bedstraw (*Galium saxatile*) and Brown Bent (*Agrostis vinealis*) (Zwaenepoel et al., 2002a).

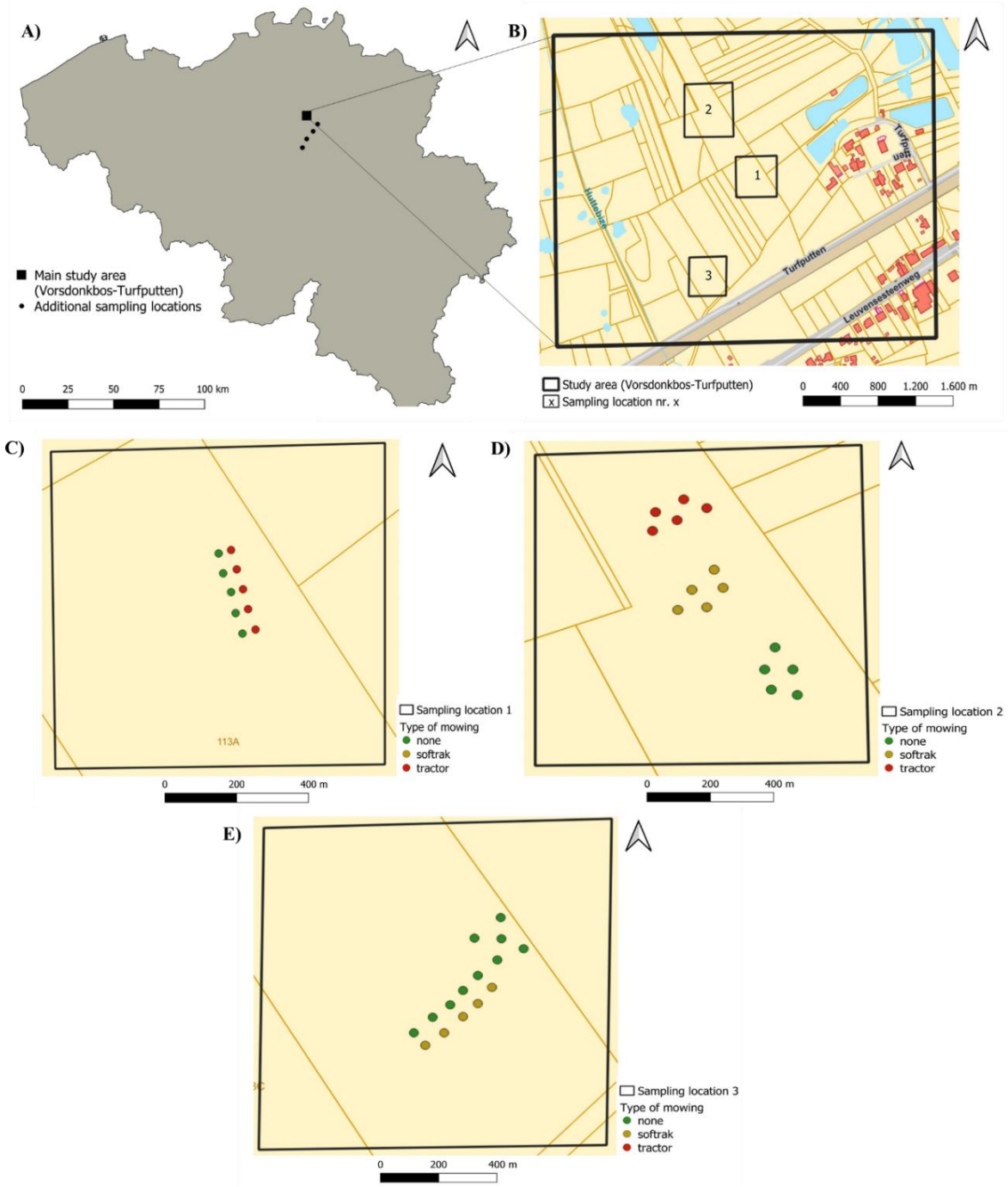


*Figure 3: Maps showing the study area of the observational field study in different degrees of detail. Base maps were obtained from Global Administrative Areas (for map A) or from Geopunt (for maps B-E). All maps were generated with QGIS version 3.22.3. A) Location in Belgium of the main study area containing four out of seven sampling locations. Also the four additional sampling locations are shown (from north to south: Walenbos, Spicht, Koebos, Zwartenbos). B) Overview of the main study area with indication of the three sampling locations. C-E) Positioning of individual samples (represented by dots) in sampling location one (10 samples), two (15 samples) and three (15 samples) respectively. Green dots are samples located in non-mown grassland patches, orange dots are located in softrak-mown patches and red dots are located in tractor-mown patches.*

### 3.1.2 Sampling

At each of the seven locations, we sampled at least two out of the three mowing types considered in this study ('none and softrak', 'none and tractor' or 'none, softrak and tractor'). This allowed us to compare the different mowing types without confounding effects such as soil type and thus to detect causal relationships between mowing type and the measured variables. Sampling was performed according to a standardized procedure in October 2022 on dry days with a mild temperature and without frost during the previous night. To collect a sample, we first placed a quadrant with an area of 1 $m^2$ and recorded the coordinates with a Samsung Galaxy A52. After this, we used a sharp, finely serrated bread knife with a blade length of 20 cm to cut out a roughly cuboid shaped block of soil with top surface dimensions of approximately 8 by 8 cm. Sampling depth varied between 10 and 15 cm. Each soil sample was placed in a plastic zip lock bag for transportation.

For paired samples (compacted versus non-compacted), we maintained a distance of approximately 4 m between both locations. This distance was measured perpendicular to the interface between both mowing types. However, taking into account that large roots could potentially hamper the sampling procedure, slight deviations from the 4 m distance were allowed if one or more large plants were present at the envisaged sampling location. For non-paired samples, we randomly distributed the sampling locations over the areas with a homogeneous mowing regime. The distance between two samples was always at least 3 m. Also, care was taken to avoid ditches and other terrain depressions because these were usually water-logged, which would prevent us from obtaining clear samples. Furthermore, a high water content increases the soil's vulnerability to compaction, which implies that samples from low-lying locations would not be representative for the grassland in general (Horn et al., 2004; Miransari et al., 2009; Keller et al., 2017).

Further processing of the samples was performed in the lab on the same day as the field sampling. For each block of soil, we cut off and discarded the top 1-2 cm to remove the living plant material. Subsequently, the dimensions of each sample were reduced by cutting off slices of soil from the sides and bottom in order to retain a cube with a volume of approximately 27 $cm^3$, which was then placed in a plastic zip lock bag and stored in the freezer at -20 °C to be used for the bulk density measurements. For all cutting operations, we used the same knife as for the field sampling. The remaining part of every sample (containing everything except the top layer and the cube) was manually broken apart and mixed. The resulting relatively homogeneous mass was then divided over two separate plastic zip lock bags. One of these was stored at -20 °C to be used for sequencing of the mycorrhizal OTUs, whilst the other was stored at +4 °C to be used for the analysis of the soil properties.

To prevent compaction of the soil during the sampling and processing, we took several precautions. Firstly, during the sampling procedure in the field, we made an additional incision in the soil adjacent to the actual soil sample. This allowed us to first remove the portion of the

soil between the extra incision and the actual sample with a hand-held metal spade. The space thus created allowed us to cut off the actual soil sample at its base (at a depth of 10-15 cm within the soil). As a result, the sample could be removed from the soil by pulling it upward, which prevented potential compaction by pushing the sides of the sample with the knife during the process of wrenching it loose. A second precaution was to avoid treading on the sampling sites by staying outside of the quadrants. If entering the quadrants was necessary, we stayed close to the outer edges. Thirdly, soil samples were not placed on top of each other during transportation to the lab. Lastly, during cutting in the lab we held on to the samples at places that were not meant to be part of the cube for bulk density measurement. This was done because slight squeezing of the soil was inevitable during processing.

### 3.1.3 Bulk density

Since bulk density is a frequently used measure of soil compaction, it was also applied in this study to quantify the level of compaction (e.g. Hamza & Anderson, 2005; Kara & Bolat, 2007; Newell-Price et al., 2013; Schrama et al., 2013; Sikorski et al., 2013; Longepierre et al., 2021). For this, the cubes of soil stored at -20 °C were used. Taking into account that bulk density is defined as "the mass of dry soil per unit volume", we first determined the exact volume of the frozen cubes by placing them in a glass cup filled to the edge with water (Hamza & Anderson, 2005). The weight of the water that was displaced by the cube and thus flowed out of the cup was recorded, after which this value was divided by the density of water (998.29 $kg/m^3$ at 20 °C). This resulted in the volume of displaced water, which approximates the volume of the soil cube. Secondly, the blocks of soil were dried at 50 °C and weighed after both three and four days in the oven to verify whether the weights remained constant, thus ensuring that all fluid had evaporated. The obtained constant weights were then recorded and used as dry weights. Finally, dividing these dry weights of the soil cubes by their corresponding volumes yielded the bulk density (in $kg/m^3$) at every sampling location.

### 3.1.4 Soil properties

With regard to the soil properties, we determined the soil gravimetric water content, organic matter content and pH, as well as the concentration of ammonium, nitrate and a number of trace elements (Al, Ca, Fe, K, Mg, Mn, Na, P, S and Si) in the soil. These analyses were performed on the soil samples stored at +4 °C, maximally four weeks after sampling.

The gravimetric water content (% moisture) was determined by drying a known weight of soil at ± 45°C for 2-4 days (until stabilization of the weight to ensure that all moisture had evaporated), after which the formula *% moisture = ((g fresh soil)-(g dried soil)) / (g dried soil)* was applied (Ceulemans & van Acker, 2017). Subsequently, these dried soil samples were placed in the oven at 650 °C for two hours to combust the organic matter. Applying the formula *((g dry soil)-(g combusted soil)) * 100 / (g dry soil)* then yielded the soil organic matter content (expressed as a percentage) (Ceulemans & van Acker, 2017). The soil pH($H_2O$) was determined

electrometrically with a glass electrode (WTW SenTix® 950, Weilheim, Germany) on a mixture containing 5 (± 0.25) g of soil and 25 ml of deionised water that was shaken for 20 min at 300 rpm prior to measurement (Ceulemans & van Acker, 2017). To extract the nitrate and ammonium ions, 5 (± 0.25) g of soil was mixed with 25 ml of a 1 M KCl solution by shaking for 30 min at 300 rpm (Ceulemans & van Acker, 2017). Subsequently, the mixture was centrifuged for 5 min at 3500 rpm and poured through a filter paper to obtain a clear extract, which was then analysed spectrophotometrically with the Evolution 201 UV-visible Spectrophotometer (Thermo Scientific, Waltham, MA, USA) to obtain the concentration of nitrate and ammonium in each sample (expressed in mg/kg soil; Ceulemans & van Acker, 2017). For the quantification of the trace elements the same extraction procedure was applied to a mixture of 2 (± 0.20) g of soil and 20 ml of very pure HPLC water. After addition of 200 µl nitric acid ($HNO_3$), the aqueous extract was analysed with the Varian 720-ES ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy) System (Varian/Agilent Technologies, Mulgrave, Victoria, Australia) to obtain the concentration of the trace elements (expressed in mg/kg soil). Finally, these concentrations, as well as the concentrations of ammonium and nitrate, were converted to mg/l soil solution by applying the formula $((concentration\ mg/kg) * (bulk\ density\ kg/m^3))/1000$.

### 3.1.5 Mycorrhizal fungi

To determine the community composition of the arbuscular mycorrhizal fungi at the sampling sites, we performed DNA extractions on the soil samples stored at -20 °C, maximally three months after sampling. This was done only on the subset of samples from Vorsdonkbos-Turfputten (Figure 1). In addition, one sample originating from the non-mown area in location three was omitted due to insufficient quality. Therefore, DNA extractions were performed on 39 samples using the Soil DNA Isolation Plus Kit (Norgen Biotek Corp., Thorold, ON, Canada). We used approximately 250 mg of soil material per sample, which was homogenized with 750 µl of lysis buffer in a Bead Mill Homogenizer (Omni International) for 30 s at 4 M/s. After diluting the isolated genomic DNA five times (to minimize the inhibiting effect of humic acids in the soil on the PCR reaction), PCR amplification of the DNA extracts was performed with the sample-specific barcode-labelled versions of the primer pair AMV4.5NF/AMDGR (Sato et al., 2005). This primer pair is well-suited to characterize arbuscular mycorrhizal communities as it covers the region of the small subunit (SSU) rRNA gene that contains the largest amount of variation (Van Geel et al., 2014). PCR reactions were carried out on a Biometra TAdvanced thermal cycler (Westburg) in a reaction volume of 25 µL (10.8 µL of PCR-grade water, 1 µL of genomic DNA, 0.3 µM of each primer and 12.5 µl ALLIn Hot Start Mastermix (HighQu)). Prior to amplification, we denatured the DNA samples at 95 °C for a period of 2 min. Subsequently, 40 cycles were ran, each made up of 15 s at 95 °C, 15 s at 52 °C and 15 s at 72 °C. Correctly sized amplicons were purified by means of the Agencourt AMPure XP kit (Beckman Coulter Life Sciences, Indianapolis, IN, USA), after which we quantified the

resulting dsDNA amplicons using the Qubit dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA) and the Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). Samples were then pooled in equimolar concentrations to form an amplicon library, which was loaded on an agarose gel. From this gel, we cut out the amplicon with the correct size (350 bp) under UV light and purified it by means of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Finally, after being diluted to 2 nM, the resulting library was sequenced on an Illumina Miseq platform with v2 500 cycle reagent kit (Illumina, San Diego, CA, USA) at the Genomics Core (Gasthuisberg, Leuven, Belgium).

USEARCH (version 11) was used to derive discrete operational taxonomic units (OTUs) from the raw Illumina sequencing data (Edgar, 2013). Firstly, the command 'fastq_mergepairs' was used to generate consensus sequences based on the paired-end reads (Edgar, 2013). During this step, we set the minimally required identity match for alignment to 80 % whilst allowing for maximally 10 mismatches in the alignment. Secondly, reads were truncated to a length of 200 bp and filtered based on their quality using the 'fastq_filter' command with the maximum number of expected errors per read set to one (Edgar, 2013). In the third step, dereplication (identification of the unique sequences) was performed using the 'fastx_uniques' command (Edgar, 2013). During this process we discarded the sequences that could only be found once in the complete set of sequences. If left in the dataset, these very rare sequences could decrease the correctness of diversity measures (Brown et al., 2015). Next, the 'cluster_otus' command was used to remove chimeric sequences and to cluster the remaining sequences into operational taxonomic units based on a sequence similarity threshold of 97 % (Edgar, 2013). Finally, the sequences of the resulting OTUs were queried against the MaarjAM database to match them with an appropriate taxonomic identity. This was done based on a minimal sequence similarity of 95 %. Sequences for which this query did not yield any results were also queried against the NCBI database using the BLAST algorithm, for which only plausible matches with minimally 90 % sequence similarity were retained (Altschul et al., 1990; Sayers et al., 2022).

To reduce the number of OTUs obtained after clustering to a manageable number, we filtered the data using the R package 'phyloseq' to remove low-frequency OTUs (McMurdie & Holmes, 2013). First, the absolute OTU abundances were transformed to relative abundances by dividing the number of counts of each OTU in a sample by its total number of counts across all samples. Next, the 'filter_taxa' function was used to remove the OTUs of which the sum across all samples was smaller than 0.05 % of all OTUs, thus eliminating low-frequency operational taxonomic units.

### 3.1.6 Data analysis

All analyses were performed in R version 4.2.3 (R Core Team, 2022). We used 0.05 as a cut-off value for significance, whilst *p* values between 0.05 and 0.1 were considered to be marginally insignificant.

Concerning the soil properties of the 67 samples collected across seven locations (bulk density, gravimetric water content, organic matter content, pH and the concentrations of nitrate, ammonium, phosphorus, iron, aluminium, manganese and sulphur), we first performed a PCA (Principal Component Analysis) on all properties combined by using the 'prcomp' function in base R. Next, to investigate the relationship between mowing type ('none', 'softrak' and 'tractor') and the soil properties, we constructed a linear mixed-effects model for each property separately with the soil property as the dependent variable and type of mowing as the explanatory variable using the R package 'nlme' (Pinheiro et al., 2023). This was done for bulk density, gravimetric water content, organic matter content, pH, nitrate, ammonium, phosphorus, iron, aluminium, manganese and sulphur. Location was always included as a random factor to account for potential non-independence of samples within the same location. The distribution of the residuals was assessed with a Shapiro-Wilk test and in case the assumption of normality was violated ($W < 0.9$), we transformed the data. This was the case for ammonium, iron, manganese and sulphur (log10 transformed). However, for these elements the analysis was also performed on the non-transformed data to verify that the transformations did not cause excessive distortions of the data and/or strongly divergent results (Appendix 1). Furthermore, an additional (but otherwise identical) model was constructed that allowed the variances to differ between the different levels of mowing type. From the original and additional model, we then selected the most suitable one based on their AIC (Akaike Information Criterion) values. For the final model, the overall relation between mowing type and bulk density was assessed with a type III ANOVA (Fox, 2022). Subsequently, we performed a Tukey post hoc test using the R package 'emmeans' to test for differences between the three levels of the categorical independent variable ('none', 'softrak' and 'tractor') (Lenth et al., 2023). The same approach was used to assess the relation between mowing type and the elements of which the concentrations were relatively unlikely to be affected by compaction (calcium, magnesium, potassium, sodium and silicon) (Appendix 2). However, this was done only for the subset of 40 samples from Vorsdonkbos-Turfputten (Appendix 2). Finally, the hypothesis that type of mowing as well as location are potentially correlated with the concentrations of the main plant nutrients ($NO_3^-$, $NH_4^+$, P) was tested by means of a PERMANOVA (Permutational Analysis of Variance) with the concentrations of nitrate, ammonium and phosphorus as dependent variables and type of mowing, location and their interaction as independent variables. The same approach was used to test the hypothesis that mowing type, location and their interaction are related to the concentrations of elements that are sensitive to changes in their oxidation state (iron, aluminium, manganese and sulphur). For each of these two hypothesis-driven PERMANOVAs,

we used the ‘adonis2’ function (1000 permutations, Bray-Curtis distance matrix) in the R package ‘vegan’ (Oksanen et al., 2022). Prior to each PERMANOVA, we tested whether the assumption of homogeneous multivariate dispersions was fulfilled for both mowing type and location by using the ‘betadisper’ and ‘permutest’ functions in the ‘vegan’ package (99 permutations; Oksanen et al., 2022; Appendix 3). In case the assumption was violated, a Tukey Honest Significant Difference Test was performed to determine which groups significantly differed with respect to their dispersions (Appendix 3).

With regard to the mycorrhizal fungal community composition of the 39 soil samples from Vorsdonkbos-Turfputten, we first performed a Non Metric Multidimensional Scaling (NMDS) on the Bray-Curtis distance matrix of the data concerning the OTU abundances in the 39 samples using the ‘ordinate’ function in the ‘phyloseq’ package (McMurdie & Holmes, 2013). To test the hypothesis that both mowing type and location might be associated with changes in the mycorrhizal fungal community composition, we performed a PERMANOVA with OTU abundances as the dependent variables and mowing type and location as the independent variables using the ‘adonis2’ function (1000 permutations, Bray-Curtis distance matrix) in the ‘vegan’ package (Oksanen et al., 2022). The procedure for testing the assumption of homogeneous multivariate dispersions was identical to the one described above for the PERMANOVAs concerning the soil properties (Appendix 3). Next, we calculated the OTU richness and Shannon diversity (Hill numbers with order q=0 and q=1 respectively) for every sample. Given the fact that the ‘sample-size-based rarefaction and extrapolation sampling curves of the Hill numbers’ did not continue to increase during extrapolation to the double of the observed sample size (Appendix 4), we opted to use the asymptotic estimates of the OTU richness and Shannon diversity in the subsequent analyses (Chao et al., 2014, 2020). These measures were calculated using the R package ‘iNEXT’ (Chao et al., 2014; Hsieh et al., 2022) and their potential correlation with mowing type was investigated by constructing two linear mixed-effects models with either OTU richness or Shannon diversity as the dependent variable in the R package ‘nlme’ (Pinheiro et al., 2023) following the same procedure as described above for the soil properties (Appendix 5). Furthermore, we assessed whether the communities of mycorrhizal OTUs present in the softrak- and tractor-mown grassland patches constitute only a subset of the OTUs present in the non-mown grasslands. For this purpose, we conducted a nestedness analysis using the ‘nestedtemp’ function in the ‘vegan’ package (Oksanen et al., 2022), which calculates a number (the nestedness temperature) expressed in degrees that varies between 0° (maximally nested) and 100° (completely non-nested) (Rodríguez-Gironés & Santamaría, 2006). Non-randomness of the output obtained through this function was evaluated by means of the ‘oecosimu’ function (99 simulations, ‘quasiswap’ null model method) in ‘vegan’ (Oksanen et al., 2022). Subsequently, potential differences in position in the packed data matrix of samples originating from grasslands with different mowing types were investigated by means of a linear mixed-effects model in the R package ‘nlme’ (Pinheiro et al.,

2023), following the same procedure as described above for the soil properties and the OTU richness and diversity (Appendix 6). In this model, the position of the samples in the packed data matrix was the dependent variable, mowing type was the independent variable and location was included as a random factor. Lastly, to investigate whether certain mycorrhizal OTUs are characteristic of grassland soils subjected to specific mowing types, we performed an indicator species analysis using the 'multipatt' function ('IndVal' species-site group association function, 999 permutations) from the R package 'indicspecies' (De Caceres & Legendre, 2009) (Appendix 7). For the indicator species analysis, as well as for the nestedness analysis, the PERMANOVA and the NMDS, we used the filtered, relative abundance data. For the calculation of OTU richness and Shannon diversity, the filtered, absolute abundance data was used. Differences in sequencing depth between the different samples could potentially distort the results of the above described data analysis performed on the OTU abundances (Honnay et al., 2017). Rarefaction, a technique used to mimic an equal sequencing depth across all samples by means of interpolation, is the most conventional approach to tackle this problem (Honnay et al., 2017). However, considering the fact that rarefaction has been shown to involve serious disadvantages such as an increase in the incidence of both Type-I and Type-II errors and loss of information due to interpolation to the smallest sample size (McMurdie & Holmes, 2014; Honnay et al., 2017), we opted not to apply this technique but rather to use the relative OTU abundances for all analysis where this was possible.

## 3.2 Lab experiment on *Calluna vulgaris*

### 3.2.1 Study species

Heather (*Calluna vulgaris* (L.) Hull) is a low-growing evergreen shrub or chamaephyte with small, lancet-shaped leaves that dominates heathlands (Gimingham, 1960; Power et al., 1998; Vegini et al., 2022). More specifically, together with species such as Tormentil (*Potentilla erecta*), Scotch Broom (*Cytisus scoparius*), Dyer's Greenweed (*Genista tinctoria*), German Greenweed (*Genista germanica*) and Sheep's Fescue (*Festuca ovina*), it is an indicator species of European dry heaths (Vegini et al., 2022). However, the range of Heather extends beyond this specific habitat type. Also bogs and certain forest types can accommodate this species, although its importance there is much more limited compared to heathlands (Gimingham, 1960). This relatively broad range of habitats where *C. vulgaris* can occur is consistent with the extensive area it naturally occupies in Europe (Gimingham, 1960). Only the outermost south-eastern part of Europe lies outside of its range (Gimingham, 1960). However, Heather does have relatively strict requirements regarding its abiotic environment. Firstly, the soil must be at least slightly acidic, with the optimal pH ranging from 3.5 to 6.5 (Gimingham, 1960). Secondly, Heather requires soils with a sufficiently high drainage capacity, which is illustrated by the fact that an excessive soil moisture content impairs its root development and consequently also the entire plant's growth (Gimingham, 1960). Finally, *C. vulgaris* only thrives in an oligotrophic environment, which is related to its life history and morphology

(Gimingham, 1960; Power et al., 1998). As a result of its small posture and very slow growth, Heather can only outcompete other plant species when nutrients are limiting (Power et al., 1998). The ability of *C. vulgaris* to survive in such nutrient-poor environments is (at least partly) made possible by its mycorrhizal symbionts (Brundrett & Tedersoo, 2018). Given the fact that *C. vulgaris* is a member of the Ericaceae, it forms a symbiosis with ericoid mycorrhizal fungi that supply their host plant with nutrients obtained from the soil (Brundrett & Tedersoo, 2018). The importance of this association is further stressed by the fact that Heather lacks root hairs (Gimingham, 1960). More specifically, the root network of *C. vulgaris* consists solely of strongly branched vertical and lateral roots with a decreasing diameter towards the extremities (Gimingham, 1960).

### 3.2.2 Experimental design

At the end of October 2022, we collected 30 sods each containing a *Calluna vulgaris* individual from an unmown heathland patch situated in Langdonken (51.02°N, 4.86°E; Herselt, Belgium). These sods had an average gravimetric water content of 18 %, an average organic matter content of 4.5 % and an average pH of 4.86 and were obtained by making use of a sharp spade in order to minimize compaction of the soil. For the duration of the experiment, the Heather plants were then kept in a greenhouse at the university of Leuven (Heverlee, Belgium) where the lighting followed the natural day/light cycles. For every plant, we estimated the soil volume of the sod by measuring its dimensions and we described the plant itself by counting the number of main branches as well as the number of side branches larger than 1 cm. After description, the sods were placed in shallow plastic containers in groups of four or five. The remaining free spaces between the blocks of soil, as well as their side faces were then covered with a layer of nutrient-poor potting soil (Peltracom, Belgium) in order to prevent dehydration without adding nutrients to the soil. Additionally, the plants were sprinkled at least three times a week with tap water.

After four weeks of acclimatization to the greenhouse conditions, the Heather plants were placed in 2 l plastic pots. If needed, some soil material was scraped or broken from the sides of the sods to allow for a smooth placement in the pots. However, care was taken not to damage or compact the sods during manipulation. In order to prevent implosion upon compaction in the next step of the experiment, the remaining free area in the pots was filled up with white sand (Hubo, Belgium). Subsequently, the plants were watered amply to ensure that the sand filled up all pores. To prevent the sand from flowing away through the drainage openings, we placed a rectangular piece of horticultural fleece at the bottom of each pot.

These Heather individuals in pots were then used in a full factorial design combining three levels of compaction and two levels of drought, resulting in six distinct treatments (no compaction/spring drought, mild compaction/spring drought, severe compaction/spring drought, no compaction/summer drought, mild compaction/summer drought, severe compaction/summer drought) with five replicates per treatment. Plants were randomly

distributed over the treatments based on their size by ranking them from small to large based on their total number of branches (number of main branches + number of side branches larger than 1 cm). Next, we divided them in groups of six in which all individuals had a similar size, after which we randomly assigned each of the individuals in a group to one of the six different treatments until each treatment comprised five *C. vulgaris* plants.

Three days after preparation of the Heather plants, we experimentally compacted the soil in which they grew (Figure 4). Firstly, by creating a cylindrical hole with a spade, an empty pot was dug into the ground in such a way that the upper edge of the pot coincided with the ground surface level. Gaps between the pot and the surrounding soil were filled with white sand (Hubo, Belgium) to ensure firm fixation. Subsequently, the pots containing *C. vulgaris* plants were placed one-by-one inside this identical, empty pot. To ensure an even division of pressure during compaction, we placed a large wooden board on top of the pot after covering the plants with wooden cylinders with the same diameter as the pot. Mild compaction was then mimicked by driving back and forth twice over this wooden board with the front wheel of a Volvo XC60. Severe compaction was mimicked by driving back and forth once over the wooden board with the front wheel of a Belarus 1221.5 tractor. Every time the tractor wheel was positioned exactly above the pot, we did not move the tractor for approximately 5 s. After compaction, the pots were returned to the greenhouse. Plants in the 'no compaction' treatment were left undisturbed in the greenhouse during the experimental compaction of the plants in the 'mild' and 'severe compaction' treatments.

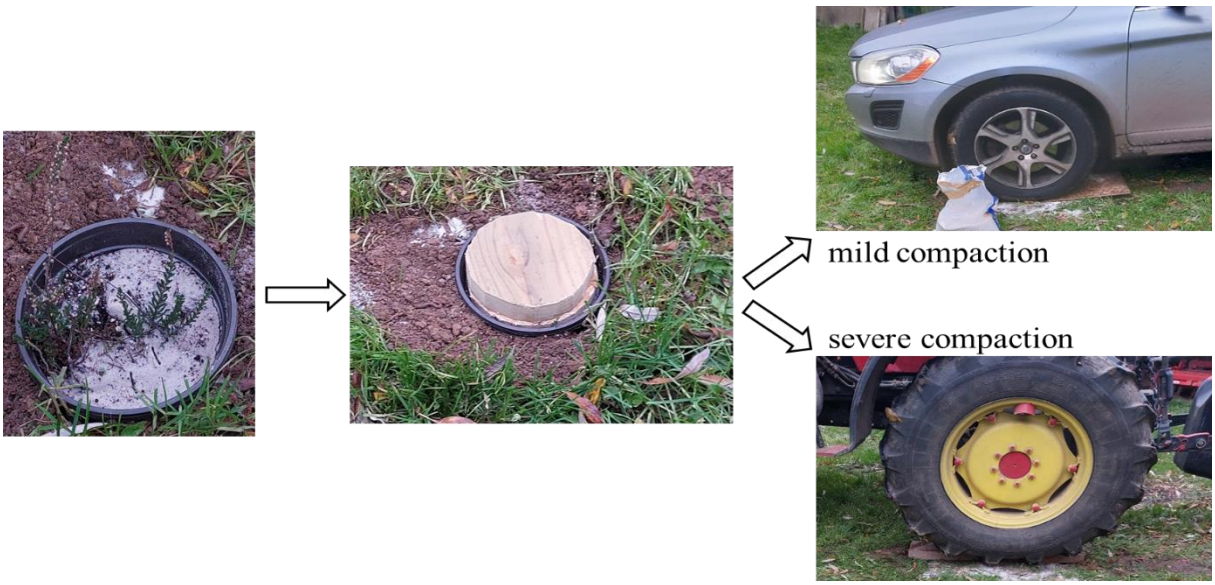


*Figure 4: Setup for experimental compaction of the soil in the 'mild' and 'severe compaction' treatments. See main text for a detailed explanation.*

After experimental compaction, the *C. vulgaris* plants were left undisturbed for two weeks (which also implies we did not water them), after which the 'spring drought' treatment was started. The Heather individuals in this treatment were completely deprived of water starting 14 days after compaction, whereas those in the 'summer drought' treatment received tap water twice a week. To keep the soil approximately at field capacity, these plants were each

time watered until fluid started dripping through the drainage openings at the bottom of the pots. Sixty-six days after experimental compaction, we also started imposing drought stress on the plants in the 'summer drought' treatment by completely ceasing watering. During the 'spring drought' treatment, the average temperature in the greenhouse was 17 ± 3 °C, whilst during the 'summer drought' treatment the average temperature was 25 ± 5 °C. Five times a week, we randomly rearranged all pots in the greenhouse in order to average out any potential differences in environmental conditions. Concurrently with the start of the 'spring drought' treatment, the lighting in the greenhouse was adjusted to an artificial 12 h light/12 h dark cycle in order to stimulate evapotranspiration and growth of the plants. This lighting regime was maintained for the entire course of the experiment.

### 3.2.3 Plant responses to drought stress under compaction

To investigate the impact of drought stress and compaction on *Calluna vulgaris*, we first determined the length of the drought period needed for every individual plant to reach its permanent wilting point during the 'spring drought' and 'summer drought' treatments. This was assessed visually by paying attention to signs such as cessation of growth, changes in colour and loss of turgescence. The length of the drought period was expressed in days, beginning from the start date of the respective drought treatment, and was used as a measure for plant resistance to drought stress under the different compaction levels. On the day that permanent wilting point was reached for a specific plant, we rehydrated this individual by abundantly watering it while placing its pot in a non-draining container for 1-2 h. This was done four to five times a week, during which the plant was closely monitored for signs of recovery including regain of turgescence, shifts in colour towards brighter green and renewed growth. Whether or not the Heather individual recovered was recorded and used as a second measure of the plant's response to drought and compaction. We also recorded the exact day of recovery, which enabled us to use the length of time (expressed in days) needed for a plant to recover after water deprivation as a measure for resilience. Finally, the long-term survival of the plants was assessed by checking whether they survived or not after they were planted back in their natural outdoor environment after recovery. As a result, we used a total of four different measures for the effect of compaction and drought on *Calluna vulgaris*: resistance (the time to permanent wilting point), recovery or short-term survival (whether or not the plants recovered when watering was resumed), resilience (the time to recovery) and long-term survival (whether or not the plants survived in natural conditions after their recovery). Plants that did not recover were given a value of zero days for their resilience. Similarly, two plants belonging to the 'severe compaction/summer drought' treatment that died prior to the start of the drought period were given a value of zero days for their resistance and were omitted from the analysis concerning resilience.

### 3.2.4 Degree of mycorrhization

To investigate the effects of compaction and drought treatment on the mycorrhizal symbionts of *Calluna vulgaris* under drought stress, we stained root samples of all Heather individuals to quantify the degree of mycorrhization. Staining was performed on root samples taken either after recovery for surviving plants or after death of the individual for the plants that did not survive the experiment. To collect the samples, we carefully exposed the root network and cut off fine roots with a pair of scissors. These were then stored in plastic zip lock bags at -20 °C for maximally three weeks.

For every plant, we stained 20 fragments of the finest roots (≤ 1 mm diameter), each having a length of 1 (± 0.2) cm. This was achieved by first letting the clean root fragments soak in a 5 % potassium hydroxide (KOH) solution for 50 minutes (the first 10 minutes at room temperature, followed by 40 minutes in a 60 °C water bath). Next, the roots were rinsed with tap water and placed in a 1 % hydrochloric acid (HCl) solution for 5 minutes at room temperature. After discarding the hydrochloric acid, the roots were covered with a 0.05 % Trypan blue solution consisting of Trypan blue dissolved in lactoglycerol (lactic acid, glycerol and deionized water in equal proportions). The roots covered in dye were then placed in a 60 °C water bath for 10 minutes, after which the Trypan blue solution was discarded and the root fragments were placed in lactoglycerol for at least 40 minutes to extract excess dye. Finally, the stained root pieces were mounted on microscope slides for further investigation.

Following the staining procedure, we assessed the degree of mycorrhization based on three distinct methods. First, we determined for every root fragment whether or not mycorrhizal structures were present, resulting in a score of zero (no structures) or one (structures present) for every fragment. Secondly, each fragment was given a score ranging from zero to five (0: mycorrhizal structures in 0 % of the cells, 1: mycorrhizal structures in < 25 % of the cells, 2: mycorrhizal structures in 25-50 % of the cells, 3: mycorrhizal structures in 50-75 % of the cells, 4: mycorrhizal structures in > 75 % of the cells, 5: mycorrhizal structures in 100 % of the cells). Lastly, we counted the number of distinct mycorrhizal structures per root fragment. Every method was based on visual assessment of the stained samples under a Dialux 20 Leitz Wetzlar light microscope (Leica Microsystems, Belgium) at a magnification of 125x. Only clearly delineated structures that were highly likely to be mycorrhizal were included. In the case of the ericoid mycorrhizal symbionts of *C. vulgaris*, these were mostly hyphal coils (Appendix 8, Figure A.9A) and putative vesicles (Appendix 8, Figure A.9B). Hyphae (Appendix 8, Figure A.9A-B) were not taken into account as these are continuous structures without a clear start- and endpoint, which makes them difficult to quantify. Similarly, structures putatively belonging to dark septate endophytes (Appendix 8, Figure A.9C-D) were also not taken into account. To account for potential observation bias, the assessment of mycorrhization was done blindly (without knowledge concerning the compaction or drought treatment of the plant in question).

### 3.2.5 Data analysis

All analyses were performed in R version 4.2.3 (R Core Team, 2022). We used 0.05 as a cut-off value for significance, whilst *p* values between 0.05 and 0.1 were considered to be marginally insignificant.

Concerning the plant responses to drought stress under compaction, we tested the effect of compaction treatment ('none', 'mild' and 'severe'), drought treatment ('spring' and 'summer') and their interaction on the resistance, recovery (short-term survival), resilience and long-term survival of *Calluna vulgaris*. For resistance, this was done by constructing a generalized linear model with a Poisson distribution and log link function with the plant response expressed in number of days as the dependent variable and drought treatment, compaction treatment and their interaction as explanatory variables. Also plant size was included in the model as an additive independent variable. Based on its total number of branches, every *C. vulgaris* individual was assigned to one of the three size categories delineated based on the median number of branches across all plants (139.5) plus or minus 50 ('small': < 89 branches, 'medium': 89-189 branches, 'large': > 189 branches). For plant resistance, one outlier from the 'severe compaction/spring drought' treatment was omitted from the analysis. For resilience, the model included all 30 plants and was identical to the one for resistance. However, taking into account that the response variable contained many zero's, we opted to use a generalized linear model with a negative binomial distribution and log link function by means of the R package 'MASS' (Venables & Ripley, 2002). For short- and long-term survival, we constructed generalized linear models with a binomial distribution and logit link function with the plant responses ('yes' or 'no') as the dependent variable and drought treatment, compaction treatment and their interaction as explanatory variables. Contrary to the models for resistance and resilience, plant size was not included in the models for survival because its addition did not improve the AIC values. For all four models, we checked whether there was overdispersion and if necessary accounted for this, after which the overall significance of the independent variables was assessed with a type III ANOVA (Fox, 2022). Subsequently, we performed Tukey post hoc tests using the R package 'emmeans' to test for differences between the three compaction levels for every level of drought, as well as for differences between the two drought levels for every level of compaction (Lenth et al., 2023).

With regard to the effects of compaction and drought stress on the degree of mycorrhization of *C. vulgaris*, we tested the effect of compaction treatment ('none', 'mild' and 'severe'), drought treatment ('spring' and 'summer') and their interaction on the presence or absence of mycorrhizal structures and on the number of mycorrhizal structures. The score to quantify the degree of mycorrhization was not statistically analysed because all root fragments were given a score of 0 or 1, which means that the data for this variable were identical to the data concerning the presence or absence of mycorrhizal structures. For the latter, we constructed a generalized linear mixed-effects model with a binomial distribution and logit link function

with the presence or absence of mycorrhizal structures per root fragment as the dependent variable and drought treatment, compaction treatment and their interaction as explanatory variables. Plant size was included in the model as an additive independent variable and plant identity was coded as a random factor to account for non-independence of root fragments originating from the same Heather individual. For the number of mycorrhizal structures per root fragment, we constructed a generalized linear mixed-effects model with a Poisson distribution and log link function that was otherwise identical to the model for the presence/absence data. For every model, we checked whether there was overdispersion and if necessary accounted for this, after which the overall significance of the independent variables was assessed with a type III ANOVA (Fox, 2022). Subsequently, we performed Tukey post hoc tests using the R package 'emmeans' to test for differences between the three compaction levels for every level of drought, as well as for differences between the two drought levels for every level of compaction (Lenth et al., 2023).

## 3.3 Lab experiment on *Succisa pratensis*

### 3.3.1 Study species

Devil's Bit Scabious (*Succisa pratensis* Moench) is a herbaceous, perennial hemicryptophyte that can be found in large parts of Europe, ranging from central Spain in the south to central Scandinavia in the north (Adams, 1955; Vergeer et al., 2003). This wide distribution can partially be explained by the fact that *Succisa pratensis* has a relatively high resistance towards frost and drought (Adams, 1955). However, a lack of moisture can inhibit flowering, and thus reproduction (Adams, 1955). As a result, this species mostly occurs in relatively wet habitats such as oligotrophic bogs, (whether or not grazed) grasslands and heathlands, where the soil pH varies between 4.5 and 7.5 (Adams, 1955). Also the roots are adapted to wet conditions by forming a branched network close to the ground surface, thus providing support (Adams, 1955). This network consists of relatively rigid adventitious roots, with a limited number of less rigid lateral roots attached to them (Adams, 1955). The adventitious roots depart from a short vertical rhizome and first grow vertically, but then turn to grow more or less horizontally at a few centimetres depth (Adams, 1955). Furthermore, the roots of *S. pratensis* are associated with arbuscular mycorrhizal fungi, of which the community composition is affected by geography and soil properties such as pH, moisture content, organic matter content and nitrogen and phosphorus concentration, as well as by the genetic profile of the host plant (Van Geel et al., 2021). Aboveground, 2-28 cm long, elliptic-shaped leaves placed in a rosette are fixed on the rhizome (Adams, 1955). From the end of July until mid-October, the rhizome also carries lateral shoots bearing flower heads at their ends (Vergeer et al., 2003).

### 3.3.2 Experimental design

The experimental design was identical to the one used in the experiment with *Calluna vulgaris* (Section 3.2.2), except for some minor dissimilarities. Firstly, the *Succisa pratensis*

individuals were obtained from a different location, more specifically from an undisturbed grassland patch in Vorsdonkbos-Turfputten (50.97°N, 4.79°E; Aarschot, Belgium). The sods had an average gravimetric water content of 84 %, an average organic matter content of 58 % and an average pH of 4.58. Secondly, we used a total of 24 plants instead of 30, which resulted in four replicates per treatment rather than five. Also, the Devil's Bit Scabious individuals were collected two days before experimental compaction, which implies that there was no prolonged acclimatization period in the greenhouse. The plants were collected by cutting out cuboids of soil with a sharp, finely serrated bread knife and were placed in 1.5 l pots the day before compaction. Similarly to the experiment on *C. vulgaris*, the volume of each block of soil was estimated by measuring its dimensions. However, description of the plants themselves was limited to counting the total number of leaves for each dominant individual. Small seedlings were omitted from the inventory. Subsequently, plant were sorted from small to large based on the number of leaves and divided in groups of six in which all individuals had a similar size. To ensure an unsystematic distribution over the treatments regarding plant size, we then randomly assigned each of the individuals in a group to one of the six different treatments until each treatment comprised four *S. pratensis* plants.

### 3.3.3 Plant responses to drought stress under compaction

To determine the effects of drought and compaction on *Succisa pratensis* plants, we again determined their resistance, recovery or short-term survival, resilience and long-term survival. For this, the same procedure was used as for the experiment on *Calluna vulgaris* (Section 3.2.4).

### 3.3.4 Degree of mycorrhization

To investigate the effects of compaction and drought treatment on the mycorrhizal symbionts of *Succisa pratensis*, we stained root samples of all Devil's Bit Scabious individuals to quantify the degree of mycorrhization. The procedure to obtain root samples for staining, as well as the staining procedure itself and the methods to quantify the degree of mycorrhization were almost identical to those used for the experiment on *Calluna vulgaris* (Section 3.2.4). However, taking into account that *S. pratensis* roots are softer in structure (less lignified) than those of *C. vulgaris*, the roots were immediately placed in the 60 °C water bath and were kept there for only 8 minutes after addition of the KOH solution. During microscopic investigation of the root fragments, the clearly delineated structures that were highly likely to be mycorrhizal were mostly arbuscules, spores and vesicles (Appendix 8, Figure A.10A-C).

### 3.3.5 Data analysis

All analyses were identical to those performed on the data obtained in the experiment on *Calluna vulgaris* (Section 3.2.5). However, taking into account that the size variation among the *Succisa pratensis* individuals was more limited compared to the *Calluna vulgaris* plants, the delineation of the three plant size categories was based on the median number of leaves

across all plants (eight) plus or minus 1 (‘small’: ≤ 6 leaves, ‘medium’: 7-9 leaves, ‘large’: ≥ 10 leaves). In addition, the model for plant resilience had a Poisson distribution with a log link function rather than a negative binomial distribution and was therefore identical to the model for resistance. Considering that all *S. pratensis* plants recovered when watering was resumed after reaching their permanent wilting point, recovery (short-term survival) was not statistically analysed for this species. The scores for the degree of mycorrhization of *S. pratensis* showed more variation than those for *C. vulgaris*, which enabled us to analyse these results. This was done by means of ordinal linear regression in the R package ‘ordinal’ (Christensen, 2022). We constructed a cumulative link mixed-effects model with the score (ranging from 0 to 5) as the dependent variable and drought treatment, compaction treatment and their interaction as explanatory variables. Plant size was included in the model as an additive independent variable and plant identity was coded as a random factor. After checking the proportional odds assumption by means of the ‘nominal_test’ function, the overall significance of the independent variables was assessed by means of likelihood ratio tests (Christensen, 2022). For the interaction term between compaction and drought, this was done by means of single term deletions starting from the full model using the ‘drop1’ function in base R. For the main effects, this was done by means of single term additions starting from the model that only included the random factor using the ‘add1’ function in base R. Subsequently, we performed Tukey post hoc tests using the R package ‘emmeans’ to test for differences between the three compaction levels for every level of drought, as well as for differences between the two drought levels for every level of compaction (Lenth et al., 2023).

# 4. Results

## 4.1 Observational field study

### 4.1.1 Soil properties

The Principal Component Analysis showed that the variation in soil properties was higher for the samples from non-mown grassland patches compared to the samples from softrak- or tractor-mown areas (Figure 5). The first principal component axis (PC1), which explained 39.33 % of the variation in the data, was most strongly negatively correlated with bulk density (Figure 5). The second principal component axis (PC2), which explained 18.57 % of the variation in the data, was most strongly positively correlated with pH and most strongly negatively correlated with the concentrations of sulphur (S) and manganese (Mn) (Figure 5).

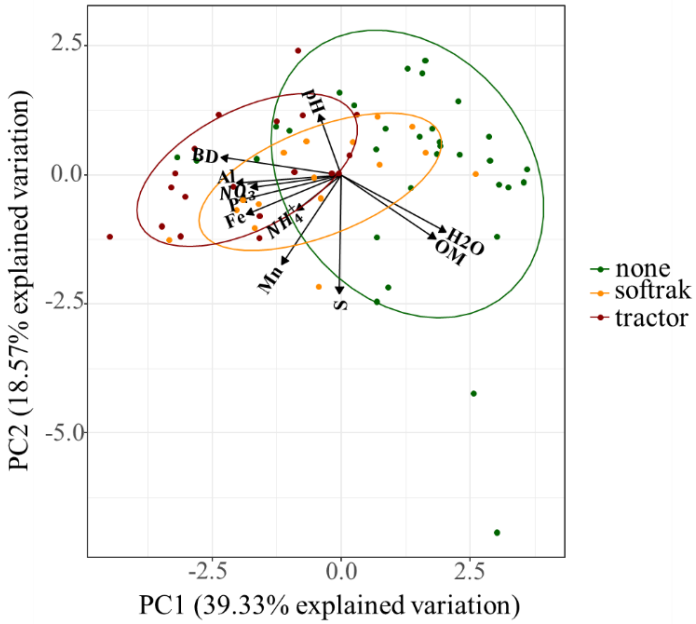


*Figure 5: Biplot that resulted from Principal Component Analysis (PCA) on the data concerning the soil properties of the 67 samples. Dots represent individual soil samples obtained from non-mown ('none'), softrak-mown ('softrak') or tractor-mown ('tractor') grassland patches. For each group (mowing type), the normal ellipse is drawn. Arrows represent the soil properties included in the analysis: bulk density (BD, in kg/m$^3$), pH($H_2O$), gravimetric water content ($H_2O$, in %), organic matter content (OM, in %), nitrate ($NO_3^-$, in mg/l), ammonium ($NH_4^+$, in mg/l), phosphorus (P, in mg/l), sulphur (S, in mg/l), iron (Fe, in mg/l), aluminium (Al, in mg/l) and manganese (Mn, in mg/l).*

The type III ANOVAs performed on the linear mixed-effects models for the 67 soil samples showed that there was a significant correlation between mowing type on the one hand and bulk density (BD), gravimetric water content (% $H_2O$), organic matter content (OM) and the concentrations of nitrate ($NO_3^-$), ammonium ($NH_4^+$), phosphorus (P), sulphur (S), iron (Fe), aluminium (Al) and manganese (Mn) on the other hand (Table 1). The pH was not significantly correlated with mowing type (Table 1, Appendix 9). Bulk density was strongly significantly higher in tractor-mown grasslands compared to non-mown areas (Table 1, Figure 6A). In addition, this soil property was strongly significantly higher in softrak-mown samples than in

non-mown samples (Table 1, Figure 6A). For the gravimetric water content and organic matter content, the same pairs of mowing types were significantly different, but the values were higher in non-mown samples than in softrak-mown samples and higher in softrak-mown samples than in tractor-mown samples (Table 1, Figure 6B-C). For the concentration of nitrate in the soil, the same pattern of significant differences was observed as for the bulk density (Table 1, Figure 6D). The log10-transformed ammonium concentrations were significantly higher in samples originating from tractor-mown areas compared to samples from non-mown areas (Table 1, Figure 6E). The phosphorus concentration was found to be significantly higher in tractor-mown grasslands than in non-mown grasslands and marginally insignificantly higher in tractor-mown areas than in softrak-mown areas (Table 1, Figure 6F). For sulphur, no significant differences were found between the three mowing types (Table 1, Figure 6G). The log10-transformed iron concentrations, the aluminium concentrations and the log10-transformed manganese concentrations were significantly higher in tractor-mown samples than in non-mown samples (Table 1, Figure 6H-J). In addition, these concentrations were significantly higher in softrak-mown areas compared to non-mown areas (Table 1, Figure 6H-J).

*Table 1: Results of the type III ANOVAs and subsequent Tukey post hoc tests that were performed on the linear mixed-effects models constructed for the soil properties of the 67 samples. In case the dependent variable was transformed to achieve a normal distribution of the residuals, the type of transformation used is indicated underneath the variable name. *** $p < 0.001$, ** $0.001 \leq p < 0.01$, * $0.01 \leq p < 0.05$, ~ $0.05 \leq p < 0.1$.*

| | Type III ANOVA | | Tukey post hoc test | | |
|---|---|---|---|---|---|
| | Test statistic $\chi^2_{2,58}$ | *p* value | *p* value for contrast none - softrak | *p* value for contrast none - tractor | *p* value for contrast softrak - tractor |
| BD ($kg/m^3$) | 47.257 | < 0.001 *** | < 0.001 *** | < 0.001 *** | 0.137 |
| $H_2O$ (%) | 32.488 | < 0.001 *** | 0.004 ** | < 0.001 *** | 0.299 |
| OM (%) | 14.803 | < 0.001 *** | 0.005 ** | 0.001 ** | 1.000 |
| pH | 0.658 | 0.720 | 0.916 | 0.714 | 0.952 |
| $NO_3^-$ (mg/l) | 26.589 | < 0.001 *** | 0.008 ** | < 0.001 *** | 0.605 |
| $NH_4^+$ (mg/l) Log10 | 7.287 | 0.026 * | 0.306 | 0.030 * | 0.643 |
| P (mg/l) | 13.812 | 0.001 ** | 0.167 | 0.002 ** | 0.072 ~ |
| S (mg/l) Log10 | 6.830 | 0.033 * | 0.618 | 0.105 | 0.634 |
| Fe (mg/l) Log10 | 11.024 | 0.004 ** | 0.011 * | 0.018 * | 0.989 |
| Al (mg/l) | 12.652 | 0.002 ** | 0.031 * | 0.010 * | 0.959 |
| Mn (mg/l) Log10 | 22.772 | < 0.001 *** | 0.017 * | < 0.001 *** | 0.396 |

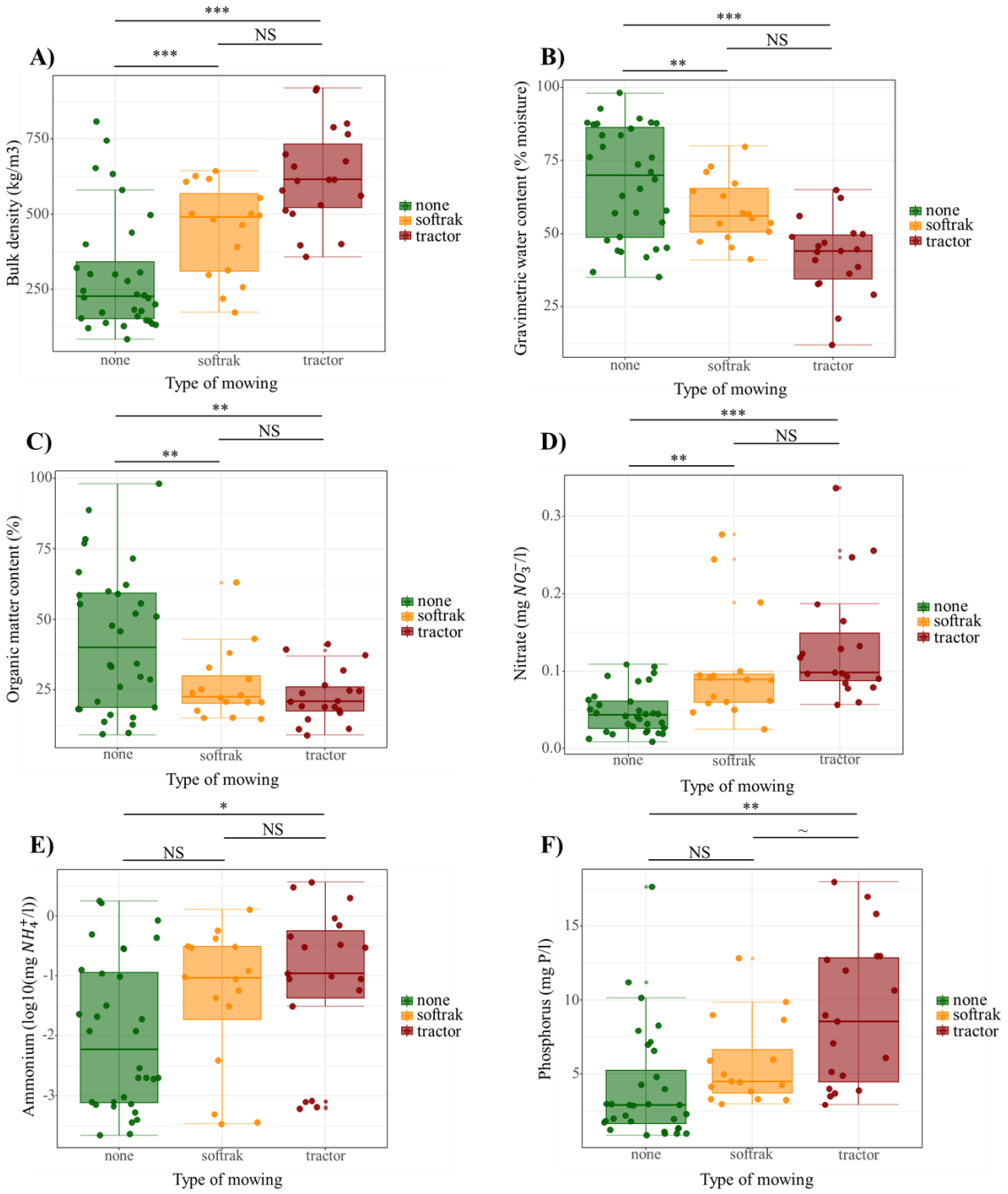


*Figure 6: Boxplots of the soil properties of the 67 samples for which the type III ANOVA yielded a significant result (Table 3). Results of the Tukey post hoc tests are indicated above each boxplot: *** p < 0.001, ** 0.001 ≤ p < 0.01, * 0.01 ≤ p < 0.05, ~ 0.05 ≤ p < 0.1, NS p ≥ 0.1. A) There was an overall significant correlation between mowing type and bulk density ($ANOVA_{2,58}$: $\chi^2$ = 47.257, p < 0.001), with a difference between none and softrak (Tukey: p < 0.001) and between none and tractor (Tukey: p < 0.001). B) There was an overall significant correlation between mowing type and gravimetric water content ($ANOVA_{2,58}$: $\chi^2$ = 32.488, p < 0.001), with a difference between none and softrak (Tukey: p = 0.004) and between none and tractor (Tukey: p < 0.001). C) There was an overall significant correlation between mowing type and organic matter content ($ANOVA_{2,58}$: $\chi^2$ = 14.803, p < 0.001), with a difference between none and softrak (Tukey: p = 0.005) and between none and tractor (Tukey: p = 0.001). D) There was an overall significant correlation between mowing type and nitrate ($ANOVA_{2,58}$: $\chi^2$ = 26.589, p < 0.001), with a difference between none and softrak (Tukey: p = 0.008) and between none and tractor (Tukey: p < 0.001). E) There was an overall significant correlation between mowing type and the log10-transformed ammonium concentrations ($ANOVA_{2,58}$: $\chi^2$ = 7.287, p = 0.026), with a difference between none and tractor (Tukey: p = 0.030). F) There was an overall significant correlation between mowing type and phosphorus ($ANOVA_{2,58}$: $\chi^2$ = 13.812, p = 0.001), with a difference between none and tractor (Tukey: p = 0.002) and a marginal difference between softrak and tractor (Tukey: p = 0.072).*

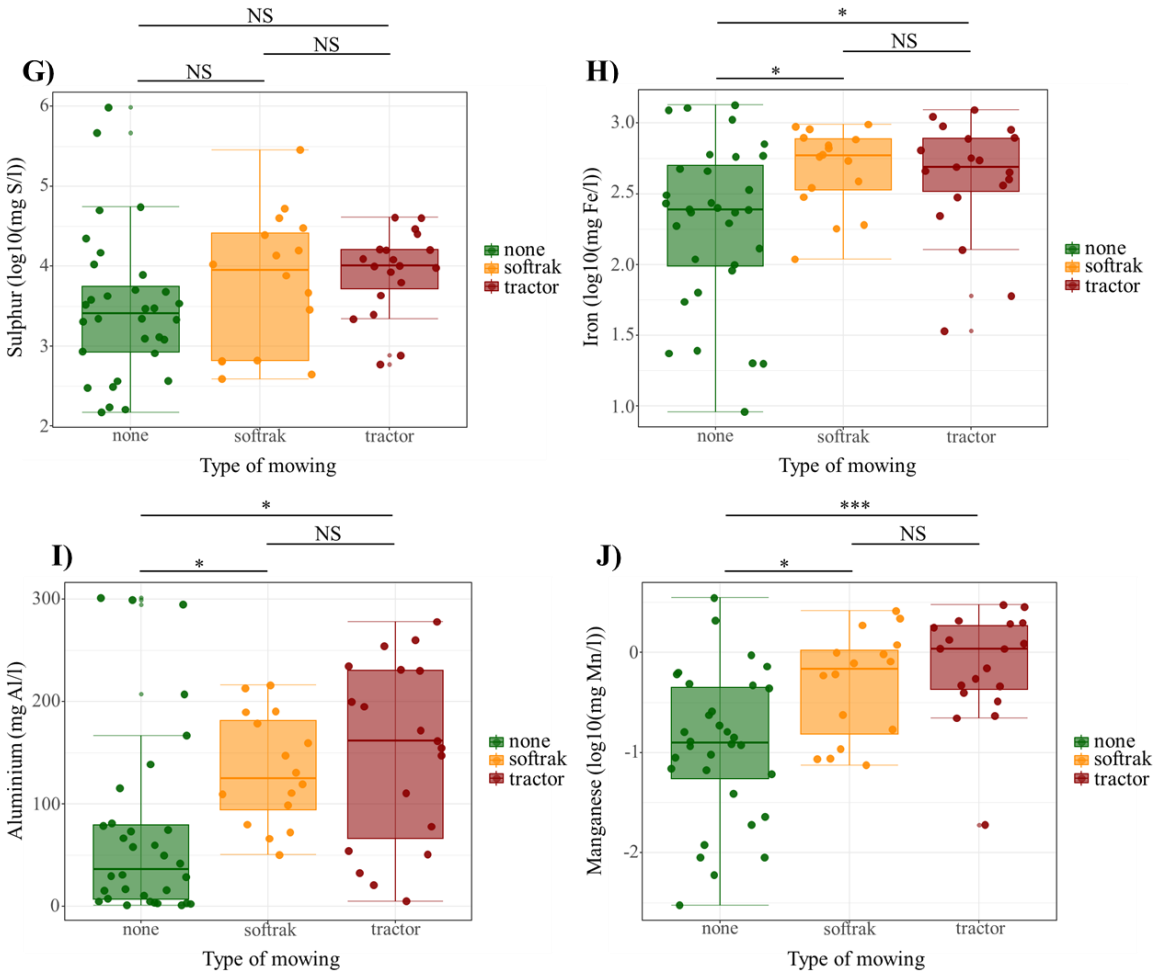


*Figure 6 (continued): Boxplots of the soil properties of the 67 samples for which the type III ANOVA yielded a significant result (Table 3). Results of the Tukey post hoc tests are indicated above each boxplot: *** p < 0.001, ** 0.001 ≤ p < 0.01, * 0.01 ≤ p < 0.05, ~ 0.05 ≤ p < 0.1, NS p ≥ 0.1. G) There was an overall significant correlation between mowing type and the log10-transformed sulphur concentrations (ANOVA$_{2,58}$: $\chi^2$ = 6.830, p = 0.033). H) There was an overall significant correlation between mowing type and the log10-transformed iron concentrations (ANOVA$_{2,58}$: $\chi^2$ = 11.024, p = 0.004), with a difference between none and softrak (Tukey: p = 0.011) and between none and tractor (Tukey: p = 0.018). I) There was an overall significant correlation between mowing type and aluminium (ANOVA$_{2,58}$: $\chi^2$ = 12.652, p = 0.002), with a difference between none and softrak (Tukey: p = 0.031) and between none and tractor (Tukey: p = 0.010). J) There was an overall significant correlation between mowing type and the log10-transformed manganese concentrations (ANOVA$_{2,58}$: $\chi^2$ = 22.772, p < 0.001), with a difference between none and softrak (Tukey: p = 0.017) and between none and tractor (Tukey: p < 0.001).*

The concentrations of the main plant nutrients as well as the concentrations of the redox-sensitive elements were strongly significantly correlated with sampling location, mowing type and the interaction between these factors (Table 2).

*Table 2: Results of the hypothesis-driven PERMANOVAs that investigated the relation between mowing type, location and their interaction on the one hand and two different groups of soil properties on the other hand. *** p < 0.001, ** 0.001 ≤ p < 0.01.*

| | Independent variables | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mowing | | | Location | | | Mowing * Location | | |
| | $F_{2,51}$ | $R^2$ | *p* | $F_{6,51}$ | $R^2$ | *p* | $F_{7,51}$ | $R^2$ | *p* |
| $NO_3^-$ + $NH_4^+$ + P (main nutrients) | 13.207 | 0.222 | < 0.001 *** | 2.974 | 0.150 | 0.005 ** | 3.383 | 0.199 | 0.002 ** |
| Fe + Al + Mn + S (redox-sensitive elements) | 5.925 | 0.102 | < 0.001 *** | 5.395 | 0.279 | < 0.001 *** | 2.941 | 0.178 | < 0.001 *** |

### 4.1.2 Mycorrhizal fungi

Sequencing the DNA extracts of the 39 soil samples from Vorsdonkbos-Turfputten resulted in a total of 3,364,366 AMF sequences and 1468 OTUs. After the filtering procedure, this number was reduced to 93 OTUs. Blasting the putative AMF sequences against the MaarjAM database and the NCBI database showed that the 93 OTUs belonged to four different families, with the Glomeraceae being the most prevalent one (18 OTUs or 19.35 % of the total), followed by the Paraglomeraceae (10 OTUs or 10.75 % of the total), the Acaulosporaceae (9 OTUs or 9.68 % of the total) and the Claroideoglomeraceae (2 OTUs or 2.15 % of the total). However, the majority of OTUs could not be assigned to a specific taxon (54 OTUs or 58.06 % of the total).

The Non Metric Multidimensional Scaling (NMDS) showed that within the same location, the AMF community composition was more similar for soil samples originating from areas with the same mowing type compared to soil samples from grassland patches with a different mowing type (Figure 7). In other words, samples from the same location that are subjected to the same mowing regime seem to cluster together on the NMDS plot (Figure 7). Importantly, the difference between softrak-mown samples on the one hand and both non- and tractor-mown samples on the other hand appears to be intermediate to the difference between non- and tractor-mown samples (Figure 7: middle panel). Across different locations, this aggregation based on mowing type is less clear because the samples also clusters according to location (Appendix 10). In agreement with this, mowing type ($PERMANOVA_{2,32}$: $F = 1.829$, $R^2 = 0.073$, $p = 0.010$), sampling location ($PERMANOVA_{2,32}$: $F = 5.017$, $R^2 = 0.200$, $p = 0.001$) and their interaction ($PERMANOVA_{2,32}$: $F = 2.377$, $R^2 = 0.094$, $p = 0.001$) were all significantly correlated with the relative OTU abundances.

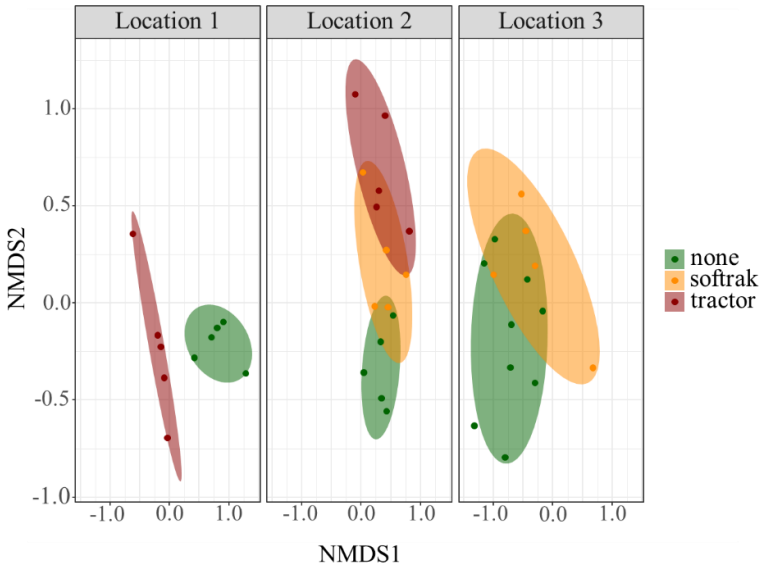


*Figure 7: NMDS ordination plot of the arbuscular mycorrhizal communities from the non-mown, softrak-mown and tractor-mown areas of Vorsdonkbos-Turfputten. The AMF communities differed significantly between the three mowing types ($PERMANOVA_{2,32}$: $F = 1.829$, $R^2 = 0.073$, p = 0.010). Ellipses represent the bivariate normal density ellipses and cover 70 % of the variation within each group.*

## 4.2 Lab experiment on *Calluna vulgaris*

### 4.2.1 Plant responses to drought stress under compaction

For plant resistance of *Calluna vulgaris* (Figure 8A), there was no significant interaction effect between compaction and drought treatment ($ANOVA_{2,21}$: $\chi^2$ = 4.480, $p$ = 0.106). However, there was an overall significant effect of drought treatment ($ANOVA_{1,21}$: $\chi^2$ = 5.083, $p$ = 0.024) and an overall marginal insignificant negative effect of compaction ($ANOVA_{2,21}$: $\chi^2$ = 5.361, $p$ = 0.069). For the plants in the 'severe compaction' treatment, we found a significant difference between the two drought treatments (Tukey: $p$ = 0.036), with the individuals in the 'spring drought' treatment being more resistant than those in the 'summer drought' treatment (Figure 8A). The same pattern was found for the plants that were not subjected to compaction, albeit only marginally insignificant (Tukey: $p$ = 0.060) (Figure 8A). For the 'summer drought' treatment, the plants subjected to mild compaction were marginally insignificantly more resistant than those subjected to severe compaction (Tukey: $p$ = 0.088) (Figure 8A).

For the short-term survival (or recovery) of *C. vulgaris* (Figure 8B), again no significant interaction effect between compaction and drought treatment was found ($ANOVA_{2,22}$: $\chi^2$ = 4.369, $p$ = 0.113). However, there was an overall significant effect of compaction treatment ($ANOVA_{2,22}$: $\chi^2$ = 6.400, $p$ = 0.041) and an overall marginal insignificant effect of drought treatment ($ANOVA_{1,22}$: $\chi^2$ = 2.900, $p$ = 0.089). Nonetheless, the Tukey post hoc tests did not yield any significant differences (Figure 8B).

For plant resilience when watering was resumed (Figure 8C), we found strongly significant overall effects of compaction treatment ($ANOVA_{2,20}$: $\chi^2$ = 76.016, $p$ < 0.001) and drought treatment ($ANOVA_{1,20}$: $\chi^2$ = 49.914, $p$ < 0.001), as well as a strongly significant interaction effect between these treatments ($ANOVA_{2,20}$: $\chi^2$ = 96.312, $p$ < 0.001). For the plants subjected to mild compaction, those in the 'summer drought' treatment took significantly less long to recover compared to those in the 'spring drought' treatment (Tukey: $p$ = 0.002) (Figure 8C). In addition, for the *C. vulgaris* individuals in the 'summer drought' treatment we found a significant difference between the 'none' and 'mild compaction' treatments (Tukey: $p$ = 0.020) and a marginal insignificant difference between the 'none' and 'severe compaction' treatments (Tukey: $p$ = 0.076) (Figure 8C). In both cases, the plants that were not subjected to compaction took longer to recover after reaching their permanent wilting point (Figure 8C).

Concerning the long-term survival of the Heather plants (Figure 8D), no significant overall effects of compaction treatment ($ANOVA_{2,24}$: $\chi^2$ = 2.505, $p$ = 0.286), drought treatment ($ANOVA_{1,24}$: $\chi^2$ = 2.643, $p$ = 0.104) or their interaction ($ANOVA_{2,24}$: $\chi^2$ = 0.112, $p$ = 0.112) were found. Additionally, the Tukey post hoc tests did not reveal any significant differences between the different levels of compaction or drought.

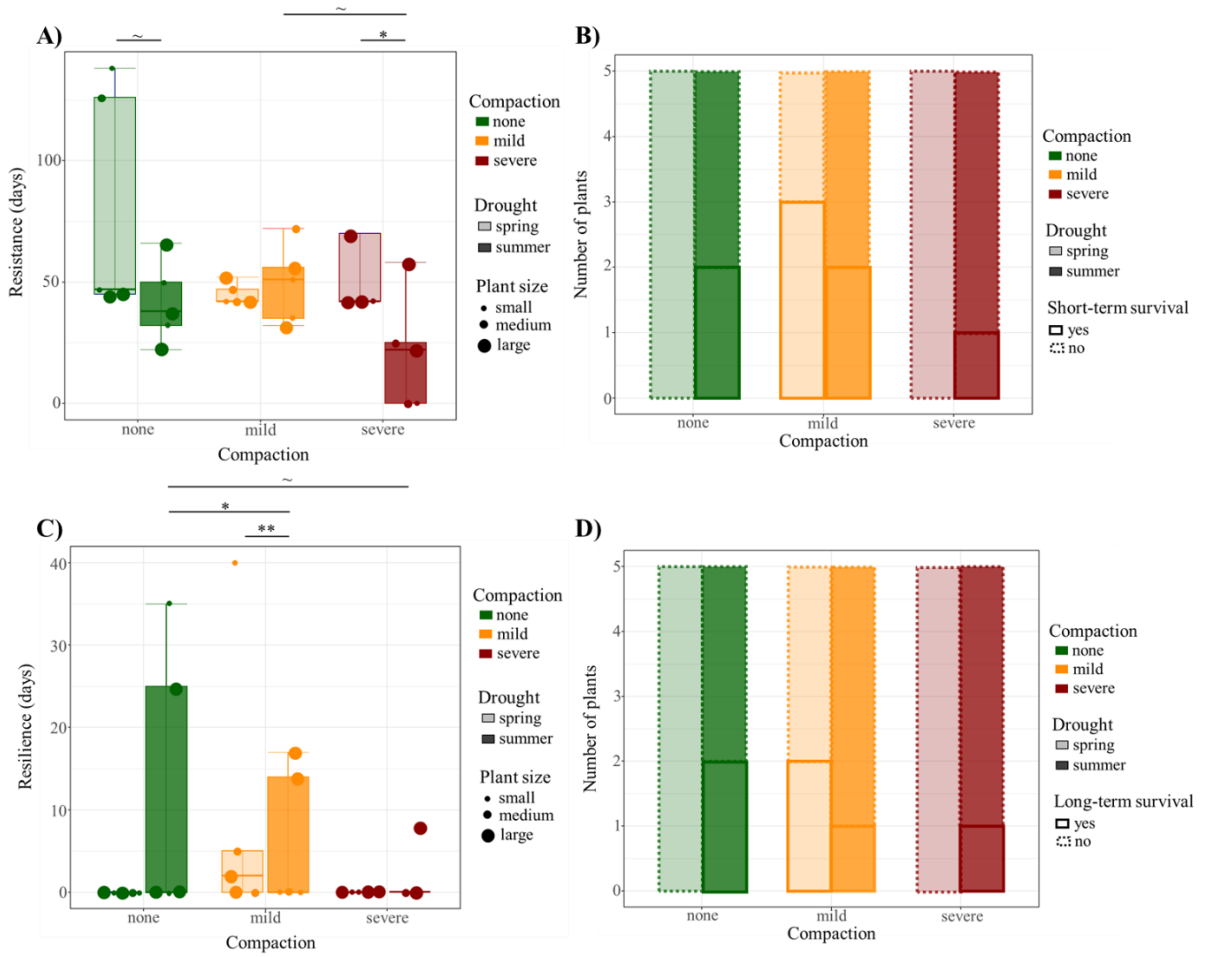


*Figure 8: Boxplots (A and C) and bar plots (B and D) of the responses of* Calluna vulgaris *to spring or summer drought stress under different levels of compaction. (Marginally) significant differences between different levels of the drought or compaction treatments are indicated above the figures: ** $0.001 \leq p < 0.01$, * $0.01 \leq p < 0.05$, ~ $0.05 \leq p < 0.1$. A) There was an overall significant effect of drought treatment ($ANOVA_{1,21}$: $\chi^2 = 5.083$, $p = 0.024$) and an overall marginal insignificant effect of compaction treatment ($ANOVA_{2,21}$: $\chi^2 = 5.361$, $p = 0.069$) on plant resistance, with a significant difference between spring and summer drought for severe compaction (Tukey: $p = 0.036$), a marginal insignificant difference between spring and summer drought for no compaction (Tukey: $p = 0.060$) and a marginal insignificant difference between mild and severe compaction for summer drought (Tukey: $p = 0.088$). B) There was an overall significant effect of compaction treatment ($ANOVA_{2,22}$: $\chi^2 = 6.400$, $p = 0.041$) and an overall marginal insignificant effect of drought treatment ($ANOVA_{1,22}$: $\chi^2 = 2.900$, $p = 0.089$) on the short-term survival (or recovery) of the plants. 20 % of the plants in the 'no compaction' treatment, 50 % of the plants in the 'mild compaction' treatment and 10 % of the plants in the 'severe compaction' treatment survived on the short term. C) There were overall significant effects of compaction treatment ($ANOVA_{2,20}$: $\chi^2 = 76.016$, $p < 0.001$), drought treatment ($ANOVA_{1,20}$: $\chi^2 = 49.914$, $p < 0.001$) and their interaction ($ANOVA_{2,20}$: $\chi^2 = 96.312$, $p < 0.001$) on plant resilience, with a significant difference between spring and summer drought for mild compaction (Tukey: $p = 0.002$), a significant difference between no compaction and mild compaction for summer drought (Tukey: $p = 0.020$) and a marginal insignificant difference between no compaction and severe compaction for summer drought (Tukey: $p = 0.076$). D) There were no significant overall effects of compaction treatment ($ANOVA_{2,24}$: $\chi^2 = 2.505$, $p = 0.286$), drought treatment ($ANOVA_{1,24}$: $\chi^2 = 2.643$, $p = 0.104$) or their interaction ($ANOVA_{2,24}$: $\chi^2 = 0.112$, $p = 0.112$) on the long-term survival of the plants. 20 % of the plants in the 'no compaction' treatment, 30 % of the plants in the 'mild compaction' treatment and 10 % of the plants in the 'severe compaction' treatment survived on the long term.*

### 4.2.2 Degree of mycorrhization

Concerning the presence/absence data of mycorrhizal structures in the root fragments of Heather (Figure 9A), we found no significant overall effect of compaction treatment ($ANOVA_{2,589}$: $\chi^2 = 2.925$, $p = 0.232$) or of the interaction between compaction and drought ($ANOVA_{2,589}$: $\chi^2 = 0.058$, $p = 0.971$). However, there was a significant overall effect of drought treatment ($ANOVA_{1,589}$: $\chi^2 = 8.681$, $p = 0.003$). In addition, the Tukey post hoc tests revealed marginally insignificant differences between the two drought treatments for the 'no compaction' (Tukey: $p = 0.097$) and 'severe compaction' (Tukey: $p = 0.069$) treatments, with in both cases more root fragments being mycorrhizal in the 'summer drought' treatment (Figure 9A).

For the number of mycorrhizal structures per root fragments (Figure 9B), there was no overall significant effect of compaction ($ANOVA_{2,588}$: $\chi^2 = 2.134$, $p = 0.344$), nor of the interaction between compaction and drought ($ANOVA_{2,588}$: $\chi^2 = 0.737$, $p = 0.692$). However, we did find a strongly significant overall effect of drought treatment ($ANOVA_{1,588}$: $\chi^2 = 17.526$, $p < 0.001$). In addition to a marginal insignificant difference between the two drought treatments for the 'mild compaction' treatment (Tukey: $p = 0.090$), there were significant differences between spring and summer drought for the 'no compaction' (Tukey: $p = 0.005$) and 'severe compaction' treatments (Tukey: $p = 0.011$) (Figure 9B). In all three cases, the root fragments from plants in the 'summer drought' treatment contained a larger number of mycorrhizal structures compared to those from plants in the 'spring drought' treatment (Figure 9B).

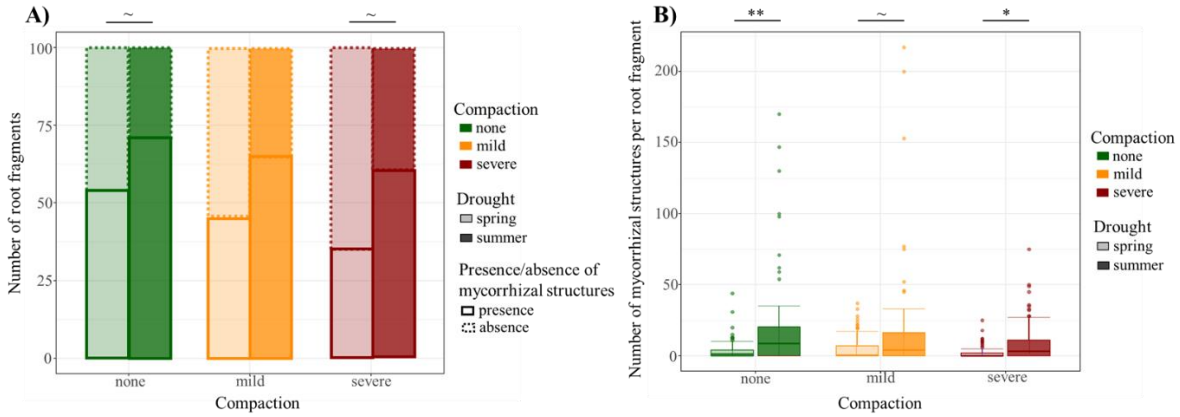


*Figure 9: Bar plot (A) and boxplot (B) of the degree of mycorrhization of* Calluna vulgaris *in response to spring or summer drought stress under different levels of compaction. (Marginally) significant differences between different levels of the drought or compaction treatments are indicated above the figures: ** 0.001 ≤ p < 0.01, * 0.01 ≤ p < 0.05, ~ 0.05 ≤ p < 0.1. A) There was a significant overall effect of drought treatment ($ANOVA_{1,589}$: $\chi^2 = 8.681$, p = 0.003) on the presence or absence of mycorrhizal structures in the root fragments, with marginal insignificant differences between spring and summer drought for the 'no compaction' (Tukey:* p = 0.097*) and 'severe compaction' (Tukey:* p = 0.069*) treatments. 62 % of the root fragments in the 'no compaction' treatment, 55.28 % of the root fragments in the 'mild compaction' treatment and 48.24 % of the root fragments in the 'severe compaction' treatment contained mycorrhizal structures. B) There was a significant overall effect of drought treatment ($ANOVA_{1,588}$: $\chi^2 = 17.526$, p < 0.001) on the number of mycorrhizal structures per root fragment, with significant differences between spring and summer drought for no compaction (Tukey:* p = 0.005*) and severe compaction (Tukey:* p = 0.011*), and a marginal insignificant difference between the drought treatments for mild compaction (Tukey:* p = 0.090*).*

## 4.3 Lab experiment on *Succisa pratensis*

### 4.3.1 Plant responses to drought stress under compaction

For plant resistance of *Succisa pratensis* (Figure 10A), we found no significant overall effects of compaction treatment ($ANOVA_{2,16}$: $\chi^2$ = 0.983, $p$ = 0.612), drought treatment ($ANOVA_{1,16}$: $\chi^2$ = 0.890, $p$ = 0.346) or their interaction ($ANOVA_{2,16}$: $\chi^2$ = 0.033, $p$ = 0.984). Similarly, the Tukey post hoc tests did not yield any significant results (Figure 10A).

When watering was resumed after either the spring or summer drought treatment, all *S. pratensis* individuals recovered. Therefore, survival on the short term equalled 100 % for all treatments.

For plant resilience (Figure 10B), there were no significant overall effects of compaction treatment ($ANOVA_{2,16}$: $\chi^2$ = 2.694, $p$ = 0.260), drought treatment ($ANOVA_{1,16}$: $\chi^2$ = 1.234, $p$ = 0.267) or their interaction ($ANOVA_{2,16}$: $\chi^2$ = 3.347, $p$ = 0.188). Nonetheless, the Tukey post hoc tests revealed a marginal insignificant difference between spring and summer drought for mild compaction (Tukey: $p$ = 0.051), with the plants from the spring drought treatment recovering more slowly (Figure 10B). In addition, the plants in the summer drought treatment that were subjected to severe compaction recovered marginally more slowly than those subjected to mild compaction (Tukey: $p$ = 0.087) (Figure 10B).

Regarding the long-term survival of the *S. pratensis* plants (Figure 10C), there were no significant overall effects of drought treatment ($ANOVA_{1,16}$: $\chi^2$ = 0.214, $p$ = 0.644) or of the interaction between drought and compaction ($ANOVA_{2,16}$: $\chi^2$ = 3.393, $p$ = 0.183). However, there was an overall significant negative effect of compaction ($ANOVA_{2,16}$: $\chi^2$ = 7.993, $p$ = 0.018). We did not find any significant differences between the different levels of the compaction or drought treatments (Figure 10C).

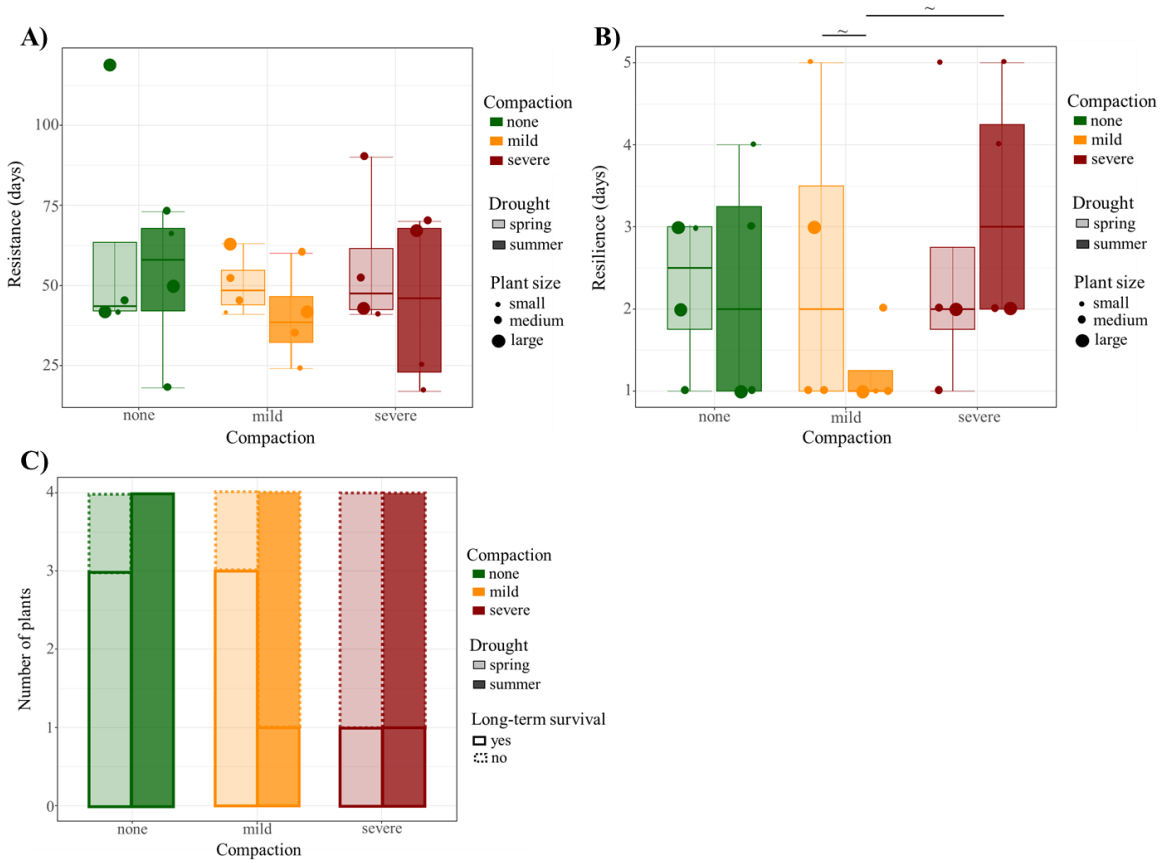


*Figure 10: Boxplots (A and B) and bar plot (C) of the responses of* Succisa pratensis *to spring or summer drought stress under different levels of compaction. (Marginally) significant differences between different levels of the drought or compaction treatments are indicated above the figures: ~ 0.05 ≤* p *< 0.1. A) There were no significant overall effects of compaction treatment (*$ANOVA_{2,16}$*:* $\chi^2$ *= 0.983,* p *= 0.612), drought treatment (*$ANOVA_{1,16}$*:* $\chi^2$ *= 0.890,* p *= 0.346) or their interaction (*$ANOVA_{2,16}$*:* $\chi^2$ *= 0.033,* p *= 0.984) on plant resistance. B) There were no significant overall effects of compaction treatment (*$ANOVA_{2,16}$*:* $\chi^2$ *= 2.694,* p *= 0.260), drought treatment (*$ANOVA_{1,16}$*:* $\chi^2$ *= 1.234,* p *= 0.267) or their interaction (*$ANOVA_{2,16}$*:* $\chi^2$ *= 3.347,* p *= 0.188) on plant resilience. There were marginal insignificant differences between spring and summer drought for mild compaction (Tukey:* p *= 0.051) and between mild and severe compaction for summer drought (Tukey:* p *=0.087). C) There was an overall significant effect of compaction treatment (*$ANOVA_{2,16}$*:* $\chi^2$ *= 7.993,* p *= 0.018) on the long-term survival of the plants. 87.5 % of the plants in the 'no compaction' treatment, 50 % of the plants in the 'mild compaction' treatment and 25 % of the plants in the 'severe compaction' treatment survived on the long term.*

### 4.3.2 Degree of mycorrhization

Concerning the presence/absence data of mycorrhizal structures in the root fragments of the *Succisa pratensis* plants (Figure 11A), we found no significant overall effects of compaction treatment ($ANOVA_{2,470}$: $\chi^2 = 0.520$, $p = 0.771$), drought treatment ($ANOVA_{1,470}$: $\chi^2 = 0.005$, $p = 0.945$) or their interaction ($ANOVA_{2,470}$: $\chi^2 = 0.123$, $p = 0.940$). In addition, the Tukey post hoc tests did not reveal any significant differences (Figure 11A).

For the scores describing the degree of mycorrhization (Figure 11B), there were no significant overall effects of compaction treatment ($LRT_{2,13}$: $\chi^2 = 4.053$, $p = 0.132$), drought treatment ($LRT_{1,13}$: $\chi^2 = 1.441$, $p = 0.230$) or their interaction ($LRT_{2,13}$: $\chi^2 = 2.368$, $p = 0.306$). However, there was a significant difference between no compaction and mild compaction for the 'spring drought' treatment (Tukey: $p = 0.022$), with the score being higher for root

fragments of plants subjected to mild compaction. Additionally, in the 'mild compaction' treatment, the roots from plants in the 'spring drought' treatment had a significantly higher score than those in the 'summer drought' treatment (Tukey: $p = 0.025$).

For the number of mycorrhizal structures per root fragments (Figure 11C), there was no overall significant effect of drought treatment ($ANOVA_{2,469}$: $\chi^2 = 2.619$, $p = 0.106$), nor a significant overall effect of the interaction between compaction and drought ($ANOVA_{1,469}$: $\chi^2 = 0.693$, $p = 0.707$). Nonetheless, we found a marginally insignificant overall effect of compaction treatment ($ANOVA_{2,469}$: $\chi^2 = 5.946$, $p = 0.051$). For the 'spring drought' treatment, the number of mycorrhizal structures per root fragment was marginally insignificantly higher for mild compaction than for no compaction (Tukey: $p = 0.076$) (Figure 11C).

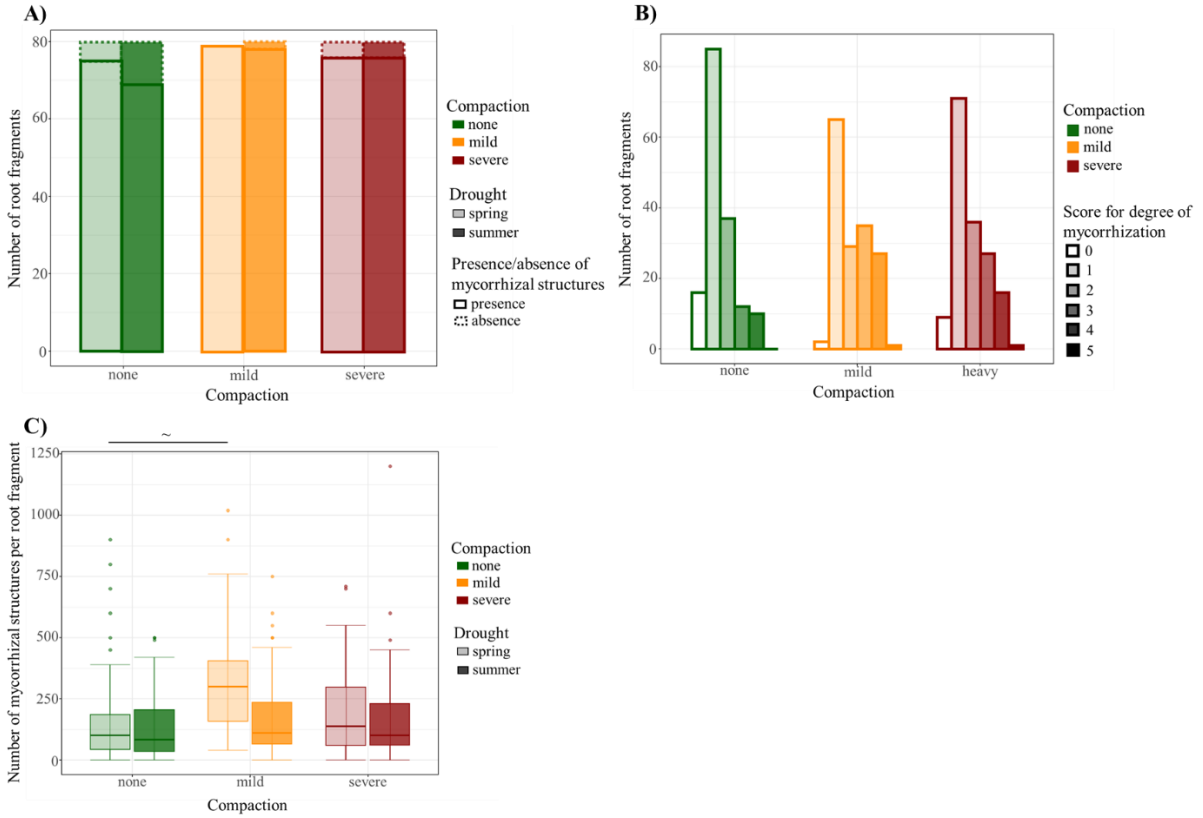


*Figure 11: Bar plots (A and B) and boxplot (C) of the degree of mycorrhization of* Succisa pratensis *in response to spring or summer drought stress under different levels of compaction. (Marginally) significant differences between different levels of the drought or compaction treatments are indicated above the figures: ~ 0.05 ≤ p < 0.1. A) There were no significant overall effects of compaction treatment ($ANOVA_{2,470}$: $\chi^2 = 0.520$, p = 0.771), drought treatment ($ANOVA_{1,470}$: $\chi^2 = 0.005$, p = 0.945) or their interaction ($ANOVA_{2,470}$: $\chi^2 = 0.123$, p = 0.940) on the presence or absence of mycorrhizal structures in the root fragments. 90 % of the root fragments in the 'no compaction' treatment, 98.74 % of the root fragments in the 'mild compaction' treatment and 95 % of the root fragments in the 'severe compaction' treatment contained mycorrhizal structures. B) There were no significant overall effects of compaction treatment ($LRT_{2,13}$: $\chi^2 = 4.053$, p = 0.132), drought treatment ($LRT_{1,13}$: $\chi^2 = 1.441$, p = 0.230) or their interaction ($LRT_{2,13}$: $\chi^2 = 2.368$, p = 0.306) on the scores for the degree of mycorrhization. C) There was a marginally insignificant overall effect of compaction treatment ($ANOVA_{2,469}$: $\chi^2 = 5.946$, p = 0.051) on the number of mycorrhizal structures per root fragment, with a marginal difference between none and mild for the 'spring drought' treatment (Tukey:* p = 0.076*).*

# 5. Discussion

## 5.1 Observational field study

### 5.1.1 Soil properties

The Principal Component Analysis showed that the samples from non-mown grassland patches were more variable concerning their soil properties compared to samples from softrak- or tractor-mown areas (Figure 5). This can potentially be explained by the fact that the largest number of analysed samples originated from non-mown locations (32 samples versus 16 from softrak-mown and 19 from tractor-mown areas; Figure 1), which increases the chance that natural variation occurs in that portion of the samples. However, also the opposite pattern could be expected. Compaction compresses the soil and thus brings deeper soil layers relatively closer to the surface (Jaafari et al., 2014). Combined with the fact that the depth of all soil samples was very similar, this implies that compacted samples consist of slightly deeper soil layers together with the more superficially situated soil material that was also sampled in the non-compacted areas. Consequently, a larger variability in soil properties could be expected for samples from locations that are likely to be more compacted (softrak- or tractor mown). Nonetheless, the likelihood of this explanation depends on the composition and structure of those deeper soil layers, which was not investigated in this study. As a result, this does not necessarily contradict our findings.

For the bulk density, strongly significant differences were found between both non-mown and tractor-mown conditions and non-mown and softrak-mown conditions (Figure 6A), which indicates that the passing of increasingly heavy equipment is indeed associated with increasing levels of soil compaction. This is in accordance with our expectations and with the majority of comparable studies (e.g. Alakukku et al., 2003; Horn et al., 2004; Schnurr-Pütz et al., 2006; Miransari et al., 2007; Schrama et al., 2013; Keller et al., 2017). Importantly, despite these pronounced differences, no significant difference was found between softrak- and tractor-mown grasslands (Figure 6A). This implies that passing of a softrak, a machine designed to minimize compacting forces, might actually result in a similar level of compaction as passing of a tractor. If correct, this could potentially have important implications for nature management by undermining the usefulness of specifically adapted machinery. However, the lack of a significant difference between ‘softrak’ and ‘tractor’ might also have been brought about by the (to us unknown) weather conditions under which the mowing equipment passed over the grassland plots (Soane et al., 1981a). Taking into account that a high moisture content increases the soil’s sensitivity to compaction, it is possible that mowing with a softrak during or after a period of ample rainfall causes a similar degree of compaction as mowing with a tractor under drier conditions (Horn et al., 2004; Batey, 2009; Miransari et al., 2009; Troldborg et al., 2013; Keller et al., 2017; Longepierre et al., 2022). Furthermore, other methods to quantify soil compaction might be more suitable, or could at least provide additional information. Possible

alternatives for bulk density include the fast and easy use of a penetrometer to measure the resistance of the soil to penetration (Soane et al., 1981a; Gerrard, 1982), determination of the soil redox potential (Schrama et al., 2013) and the utilization of visual assessment methods (Newell-Price et al., 2013).

Nonetheless, taking into account that the same pattern of significant differences as for bulk density was found for most of the other soil properties (e.g. gravimetric water content, organic matter content, nitrate, iron, aluminium and manganese; Figure 6), these drawbacks of our study are relatively unlikely to have affected the outcomes. More specifically, most soil properties were significantly different between both non- and tractor-mown areas and non- and softrak-mown areas, but not between softrak- and tractor-mown grasslands. Therefore, also the supposedly lower levels of compaction inflicted by a softrak are still correlated with significant changes in soil properties.

For example, regarding the gravimetric water content and organic matter content, the relevant linear mixed-effects models demonstrated that the values in samples from non-mown grasslands were significantly higher compared to those in samples from softrak- or tractor mown areas (Figure 6B-C). For the gravimetric water content, our results are in accordance with the reduced moisture content of soils compacted by trampling (Makuch-Pietraś et al., 2017). However, our finding stands in contrast to the study of Schrama et al. (2013), who found that mowing of grasslands with heavy machinery results in waterlogged soils. Nonetheless, compaction reduces the speed at which water infiltrates into the soil (Chatterjea, 2007). As a result, the accumulation of moisture probably only applies to the uppermost portion of the soil, which limits its relevance for the samples in this study of which the upper 1-2 cm was excluded from analysis (Section 3.1.2). For the organic matter content, our results imply that compaction-induced disturbance could accelerate the mineralization of organic carbon in the soil.

In addition, this increased mineralization of organic matter could explain the trend towards higher nutrient concentrations in softrak- and tractor mown areas (nitrate, ammonium and phosphorus; Figure 6D-F). This result is of particular importance as it might impact the plant community composition by favouring species with stronger competitive abilities (Hautier et al., 2009). Furthermore, this finding strongly contradicts the objective of mowing as a nature management technique, which is mainly the removal of nutrients from the ecosystem (Härdtle et al., 2006; Mayel et al., 2021; Bai et al., 2022). Therefore, the beneficial effects of mowing might be attenuated by performing this management intervention with compaction-inducing heavy machinery.

Furthermore, the same pattern of increasing concentrations with increasing compaction level was found for three out of the four redox-sensitive elements (iron, aluminium and manganese; Figure 6H-J). This corresponds to the study of Nawaz et al. (2016), who remarked that the levels of iron in compacted soils are elevated compared to soils with a lower bulk density. Similar to the nutrient concentrations, these findings might also have implications for

the vegetation as these elements can be toxic when present abundantly (Connolly & Guerinot, 2002; Panda et al., 2009; Abedi et al., 2013; Li et al., 2019). For aluminium, the negative effects are expressed through for example the stunted growth of roots and the abnormal swelling and discoloration of root tips (Abedi et al., 2013). Therefore, too high concentrations of this element can hamper the development and growth of plant roots, which is of particular importance for the establishment of seedlings (Abedi et al., 2013). Similarly, elevated concentrations of iron usually lead to an underdeveloped root system, often combined with discoloration (so-called 'bronzing') of the leaves (Becker & Asch, 2005). Manganese on the other hand can cause local necrosis in fully grown leaves, chlorosis in developing leaves and can even stunt the growth of the entire plant (Li et al., 2019). Importantly, the majority of plants in grasslands and heathlands is sensitive to elevated concentrations of these three elements (T. Ceulemans, pers. comm., 11/06/2023). Some exceptions include Lesser Spearwort (*Ranunculus flammula*), Common Bent (*Agrostis capillaris*) and White Clover (*Trifolium repens*) in grasslands (T. Ceulemans, pers. comm., 11/06/2023). In heathlands, Heather (*Calluna vulgaris*) is a plant species with relatively high resistance against iron and aluminium, but not necessarily against manganese (T. Ceulemans, pers. comm., 11/06/2023). As a matter of fact, these particular species with a limited sensitivity for toxic elements have been observed to increase in abundance after mowing with heavy machinery (T. Ceulemans, pers. comm., 11/06/2023). Nonetheless, all endangered plant species in these ecosystems do not tolerate high concentrations of aluminium, iron or manganese (T. Ceulemans, pers. comm., 11/06/2023).

For the soil pH, no significant correlation with mowing type was found (Appendix 9). This stands in contrast to the majority of studies, which have detected a higher pH in compacted soils (e.g. Schnurr-Pütz et al., 2006; Bhandral et al., 2007; Jaafari et al., 2014). Nonetheless, taking account that soil compaction is not always associated with changes in pH, this does not necessarily contradict our overall finding that compaction inflicted by mowing with heavy machines is correlated with changes in soil properties in nature management (e.g. Nawaz et al., 2016).

### 5.1.2 Mycorrhizal fungi

Compaction can potentially influence fungal richness and/or diversity (Hartmann et al., 2014; Appendix 5). However, these variables might have a limited role in the functioning of the ecosystem, especially for specific plant species associated with mycorrhizal fungi. It has for example been shown that AMF richness and diversity are not correlated with respectively the population growth and viability of *Succisa pratensis* (Van Geel et al., 2021). As a matter of fact, increases in these diversity measures could even reflect the gradual loss of functional organization in the studied community, which implies that changes in these variables can produce misleading results and should thus be interpreted with great caution (Hartmann et al., 2014).

Therefore, it might be recommended to focus on the mycorrhizal fungal community composition rather than on diversity measures. With regard to this component, our results have shown a strong correlation with sampling location. This is in agreement with the significant association between geography and the composition of different fungal communities found in other studies (Hartmann et al., 2014; Van Geel et al., 2021). Nonetheless, both the NMDS and PERMANOVA confirmed that, when accounting for the overriding effect of location, mowing type was also significantly related to the mycorrhizal community composition (Figure 8). This association could possibly be caused by the removal of biomass in softrak- and tractor-mown areas compared to the lack of such removal in non-mown grasslands. However, no information is currently available concerning the connection between biomass removal resulting from mowing on the one hand and fungal community composition on the other hand (Zubek et al., 2022). Moreover, if the observed pattern would indeed be caused by mowing itself, we would not expect to find differences in community composition between softrak- and tractor-mown areas. Therefore, we can assume that the differences in AMF community composition are related to the compaction level of the soil inflicted by the different types of machinery used. In addition, the gradual transition in AMF community composition from non-mown over softrak-mown to tractor-mown areas (Figure 8: middle panel) provides a strong indication that increasing levels of compaction provoke increasingly strong shifts in community composition. This larger overlap in community composition between non- and softrak-mown areas and softrak- and tractor-mown areas compared to non- and tractor-mown grasslands is further confirmed by the indicator species analysis (Appendix 7). More specifically, only one OTU was found to be significantly indicative of both non- and tractor-mown grasslands, whilst respectively seven OTUs were characteristic of both non- and softrak-mown grasslands and nine OTUs were characteristic of both softrak- and tractor-mown areas (Appendix 7). Furthermore, the gradual shift in community composition is supported by the observation that different AMF species respond differently to compaction owing to their contrasting sensitivity to for example the decreased availability of oxygen (Nadian et al., 1998). In forests, similar results have been found following experimental compaction of the soil (Hartmann et al., 2012, 2014). More specifically, at every distinct level of compaction, clearly different fungal communities exist (Hartmann et al., 2014). In addition, these changes in forest soil communities composed of ectomycorrhizal and saprophytic fungi continue to exist for prolonged periods after application of compacting forces (Hartmann et al., 2014). However, taking into account that the community composition of microfungi is not affected by compaction, these changes do not appear to be ubiquitous across all fungal groups (Kara & Bolat, 2007). Nonetheless, when we assume that the changes observed in this study are related to the compaction associated with the different types of mowing, the changes do seem to apply to arbuscular mycorrhizal communities.

Notwithstanding that the performed analyses resulted in the findings described above, also the shortcomings associated with these analyses should be taken into account. For example, many OTUs could not be assigned to a specific taxonomic group, which complicates the interpretation of for example the indicator species analysis. More specifically, this prevents us from drawing any conclusions regarding the distribution of common (e.g. Glomeraceae) and less common (e.g. Acaulosporaceae) AMF families across the different mowing categories (e.g. Honnay et al., 2017; Van Geel et al., 2021). As a result, it is not possible to confirm or invalidate our hypothesis that OTUs belonging to more rare families are relatively more likely to be characteristic of non-compacted grasslands.

Although it is important to take the above-mentioned shortcoming into account, it is also indispensable to explore the potential implications of our finding that the AMF community composition is correlated with mowing type and therefore possibly also with soil compaction. Taking into account that the plant species composition of a site has a major impact on the mycorrhizal species present in the soil (Grayston et al., 1998; Horn et al., 2017), it might be plausible to assume that the opposite is also true (Lee et al., 2013). It has for example been shown that an artificial reduction of the mycorrhizal symbionts in the soil caused significant shifts in plant community composition in tallgrass prairies (Hartnett & Wilson, 1999). Consequently, mowing-induced compaction can potentially cause changes in the community composition of plants through an alteration in the community composition of their mycorrhizal symbionts. However, specifically for grasslands, this effect might be attenuated by the limited host-specificity of their arbuscular mycorrhizas (Lee et al., 2013). Nonetheless, in addition to being crucial for the survival of plant species with a high stress tolerance (Honnay et al., 2017), different AMF species can have slightly different impacts on the same host plant (e.g. Gange et al., 2005). Therefore, to gain a better insight into the potential effects of changes in mycorrhizal communities on plant communities, future studies concerning soil compaction should ideally include a component that focuses on plant species composition by for instance performing vegetation surveys at different time points following compaction. Besides possibly affecting the plant community composition, our findings might also have ramifications on other components of the ecosystem by for example influencing the performance of individual host plants, changing the flow of carbon (Swaty et al., 2004) or altering the community composition of other soil microorganisms by means of changing their microhabitats (Dell, 2002) or changing competitive interactions (McCormick et al., 2006; Miransari et al., 2007, 2009; Kivlin et al., 2013). Taking into account that both food quantity and quality are important for pollinators (Ceulemans et al., 2017) and that both of these factors could be influenced by changes in mycorrhizal communities, our findings might even have an impact on these nectar- and pollen-feeding insects, together with other insects that depend on plants for their nutrition (Gange et al., 2005).

## 5.2 Lab experiment on *Calluna vulgaris*

### 5.2.1 Plant responses to compaction under drought stress

*Calluna vulgaris* is known to start using the available water resources more efficiently when subjected to drought stress, which implies that this species can adjust relatively well to conditions involving a lack of moisture (Gordon et al., 1999). Nonetheless, this ability depends on the morphology of the individual plants, as well as on the particular conditions under which the drought stress occurs (Gimingham, 1960). More specifically, Heather does not seem to have a high tolerance to drought imposed during warmer periods (Gimingham, 1960). Indeed, concerning the resistance of *C. vulgaris* to drought stress under compaction, our results demonstrated that plants in the 'no compaction' and 'severe compaction' treatments took significantly less long to reach their permanent wilting point when they were subjected to summer drought stress compared to spring drought stress (Figure 8A). In addition, we found that plants in the 'summer drought' treatment that were subjected to severe compaction had a significantly lower resistance than those in the 'mild compaction' treatment, with the same trend being present when comparing plants from the 'severe' and 'no compaction' treatments (Figure 8A). These findings indicate that mowing with heavy machinery might be more harmful for Heather during summer than during spring. However, it must be noted that the severity of compaction strongly depends on the soil moisture content when the compacting forces are exerted (Hamza & Anderson, 2005; Hatley et al., 2005; Batey, 2009; Bell et al., 2011). Therefore, mowing during spring, when the soil is likely to be more wet, is not necessarily less harmful than mowing during summer. This assumption is supported by our observation that two plants from the 'severe compaction' treatment died before drought stress was imposed (Figure 8A), which suggests that compaction on its own can also exert a negative influence on *C. vulgaris*.

For the short-term survival of *C. vulgaris*, we found an overall significant effect of compaction treatment. Despite the fact that no significant differences were found between the different levels of compaction, it is remarkable that only 10 % of the plants from the 'severe compaction' treatment survived (Figure 8B). Nonetheless, the number of Heather individuals that survived on the short term was relatively limited for all compaction treatments (Figure 8B). This confirms that *C. vulgaris* is a plant species with a limited potential for recovery (Roovers et al., 2004), which might be explained by the fact that its shoots stay green during periods of water scarcity, thus keeping the rate of water loss higher compared to species of which the shoots wilt more rapidly (Albert et al., 2011).

With regard to resilience, we found strongly significant overall effects of drought, compaction and their interaction, with plants from the 'no compaction' treatment subjected to summer drought stress taking longer to recover than those from the 'mild' or 'severe compaction' treatments (Figure 8C). However, these findings contradict our expectation that plants growing in more compacted soil would recover more slowly. This unexpected result

could possibly be explained by considering the mycorrhizal symbionts of the Heather plants. Because the symbiosis also has a cost for the host plant through the loss of photosynthates to the fungal partner (Lee et al., 2013; Sebastiana et al., 2018), it has been suggested that mycorrhizas can become harmful for their host plant under drought stress (Worchel et al., 2013). Combined with the observation that stronger compaction appears to slightly reduce the degree of mycorrhization of *C. vulgaris* (Figure 9A, Section 5.2.2), this might explain the slower recovery of plants from the 'no compaction' treatment. However, whether the effect of mycorrhizas on their hosts indeed varies from positive to negative depending on the availability of water remains highly speculative (Worchel et al., 2013). Therefore, alternative explanations for our aberrant results might be more plausible. It is for example possible that the very low number of plants that recovered, especially for the 'severe compaction' treatment, distorted the results. Alternatively, shortcomings in our experimental set-up might have contributed to the observed patterns. More specifically, the exact days that the plants recovered (as well as the days they reached their permanent wilting point) were determined visually, which makes the measures for these plant responses subjective estimates. Therefore, more objective measures such as determination of the shoot water potential might be more precise and reliable (e.g. Power et al., 1998). Furthermore, some sods contained not only the Heather individual of interest but also plants belonging to other species that might have competed for moisture with the focal plant. Considering that the number of additional plants per sod, nor the amount of water they used was quantified or included in the models, the potential confounding effects it caused remain elusive.

Although no significant effects of compaction or drought on the long-term survival of *C. vulgaris* were found, it must be noted that only 10 % of the plants belonging to the 'severe compaction' treatment survived the experiment (Figure 8D). Across all treatments, only 20 % of the plants survived on the long term (Figure 8D). Combined with the high mortality on the short term (Figure 8B), this suggests that drought stress, whether or not combined with soil compaction, can have a detrimental impact on Heather. However, the severity of this impact might depend on the location of origin of the plants. More specifically, *C. vulgaris* plants originating from the centre of the species' distribution range are usually more sensitive to drought than those from more southern or eastern areas (Meyer-Grünefeldt et al., 2016).

### 5.2.2 Degree of mycorrhization

As shown by the presence/absence data of mycorrhizal structures in the root fragments of *Calluna vulgaris*, there seems to be a trend towards a gradual decrease in the degree of mycorrhization for increasing compaction levels (Figure 9A). Possible mechanisms that could explain this negative effect of compaction on the ericoid mycorrhizal symbionts of Heather include direct damage inflicted to the roots containing mycorrhizal structures, as well as potential changes in the flow of carbon from the host plant to the fungi (Miransari et al., 2007). Despite the clear trend, there was no overall significant effect of compaction, nor significant

pairwise differences between the three compaction levels. Nonetheless, this lack of significance might be explained by the relatively limited number of root fragments investigated per plant. Whereas most studies use 30 root fragments per plant to assess the degree of mycorrhization, we used only 20 (e.g. Giovannetti & Mosse, 1980; Zubek et al., 2022).

Another striking pattern that was revealed by the presence/absence data concerned the contrasting effects of spring and summer drought on the degree of mycorrhization. The number of mycorrhizal root fragments was consistently higher for the plants in the 'summer drought' treatment compared to those in the 'spring drought' treatment, with marginally insignificant differences between the two treatments for 'severe' and 'no compaction' (Figure 9A). This was further confirmed by an overall significant effect of drought treatment on the number of mycorrhizal structures per root fragment, as well as by more strongly significant differences between spring and summer drought for this variable (Figure 9B). Consequently, more severe drought stress appears to result in a more extensive colonization of the root system of *C. vulgaris* by ErM fungi and in a higher number of mycorrhizal structures per unit of length. However this corresponds to results found for the mycorrhization of oak and maple (Fini et al., 2011), other studies report that moisture levels do not affect mycorrhization (Jeliazkova & Percival, 2003) or that drought can even decrease the degree of mycorrhization (Li et al., 2021). This confirms the review study of Mohan et al. (2014), where the effects of drought on the abundance of mycorrhizal symbionts were found to be strongly divergent across different studies, with approximately half of the reviewed research works finding decreases in mycorrhization and the other half finding increases. Therefore, these responses to drought are most likely species- and context-dependent. In addition, we did not investigate the degree of mycorrhization of *C. vulgaris* in the absence of drought stress, which could possibly reveal different patterns.

## 5.3 Lab experiment on *Succisa pratensis*

### 5.3.1 Plant responses to compaction under drought stress

Taking into account that the main goal of nature management is to preserve target ecosystems and species on the long term, considering only the short-term effects of compaction can be misleading. Therefore, the long-term survival of species is a key indicator of the potential deleterious effects of compaction. Importantly, in this study we found a significant overall negative effect of compaction on the long-term survival of *S. pratensis*, with respectively 87.5 %, 50 % and 25 % of the plants surviving in the 'no', 'mild' and 'severe compaction' treatments (Figure 10C). Despite the fact that no significant differences between the different levels of compaction could be demonstrated for every drought treatment, there was a clear trend towards lower survival for increasing levels of compaction (Figure 10C). This indicates that Devil's Bit Scabious is indeed sensitive to the effects of compaction on the long term, which stresses the need to avoid compacting forces in nature management. This finding also highlights the

importance of studying other plant species that occur in areas subjected to compacting forces, even when they are assumed not be vulnerable on the short term.

In addition to the negative effect on long-term survival, we found a trend towards slower recovery for the *S. pratensis* individuals in the 'summer drought' treatment that were subjected to severe compaction compared to those subjected to mild or no compaction (Figure 10B). Although non-significant for the contrast 'none-severe' and marginally insignificant for the contrast 'mild-severe' (possibly caused by a too limited number of replicates), this trend corresponds to the results found for the resistance of *Calluna vulgaris* in which the combination of severe compaction and summer drought seemed to have the most pronounced negative impact on the plants (Figure 8A, Section 5.2.1).

As opposed to the findings for long-term survival and resilience, our results did not show any significant effects of compaction, drought treatment or their interaction on the resistance of *Succisa pratensis*. Additionally, we could not discern a trend towards lower resistance for severe compaction (Figure 10A). This is further confirmed by the fact that all plants recovered when watering was resumed. Therefore, Devil's Bit Scabious seems to be relatively unaffected by prolonged drought periods, which is confirmed by the existing information concerning this species (Adams, 1955). A potential explanation for the absence of an effect of compaction specifically on the resistance and short-term survival of *S. pratensis* might be related to its habitat. Taking into account that this species commonly grows in areas subjected to trampling such as grazed meadows and (the edges of) paths, it can most likely recover relatively well form very mild compacting forces on the short term (Adams, 1955; Zwaenepoel et al., 2002a). In addition, this might partially explain why *Calluna vulgaris*, which occurs in less disturbed areas (Zwaenepoel et al., 2002a), showed a more pronounced trend towards a negative impact of compaction on its resistance compared to *S. pratensis*.

Nonetheless, the most important parameter in nature management, long-term survival, is negatively affected by compaction. In addition, this damaging effect on adult plants, which can have a negative influence on the entire population, might be further complemented by a potentially adverse impact on the recruitment of new individuals. It has for example been shown that the germination of pea and wheat can be obstructed by soil compaction (Longepierre et al., 2022). In addition, plants that germinated in compacted soils appear to have a slightly underdeveloped, more shallow root system compared to plants that germinated in non-compacted soils (Longepierre et al., 2022). This might have implication for their future survival under drought stress because a less developed root system makes it more difficult to take up a sufficient amount of moisture. Therefore, future research focussing on the effects of compaction on recruitment of new plant individuals might provide novel insights concerning the effects of compaction in nature management.

### 5.3.2 Degree of mycorrhization

For the presence/absence of mycorrhizal structures in the root fragments of *Succisa pratensis*, no significant effects of drought or compaction treatment were found. Moreover, the number of mycorrhizal root fragments was remarkably high for all treatments (Figure 11A). Although highly speculative whether applicable to *S. pratensis*, this result could potentially be explained by fact that some plant species are capable of protecting their fungal symbionts against drought stress by the release of moisture from their roots into the adjacent soil (Querejeta et al., 2007). Alternatively, the method used to determine the presence or absence of mycorrhizal structures might overestimate the actual degree of mycorrhization by giving a value of one to every root fragment that contains at least one mycorrhizal structure even though the remaining portion of the fragment is not colonized (Giovannetti & Mosse, 1980; McGonigle et al., 1990). To minimize this overestimation, it might be recommended to use shorter root fragments (McGonigle et al., 1990). Nonetheless, most studies use fragments with a length of 1 cm (e.g. Diaz et al., 2006; Bernhardt-Römermann et al., 2009; Zubek et al., 2022). Irrespective of the cause behind the observed results, our findings suggest that the degree of mycorrhization of Devil's Bit Scabious does not differ between different levels of soil compaction, nor between spring or summer drought stress.

Nonetheless, the scores quantifying the degree of mycorrhization did show a difference between 'mild' and 'no compaction' for spring drought (Figure 11B), which was confirmed by the observation that in the 'spring drought' treatment the root fragments from plants subjected to mild compaction contained marginally insignificantly more mycorrhizal structures than those in the 'no compaction' treatment (Figure 11C). This pattern of a higher degree of mycorrhization at an intermediate compaction level could be linked to the level of stress in the host plant. When the host experiences increasing levels of stress, more energy will be allocated to its mycorrhizal symbionts, which can increase the degree of mycorrhization compared to stress-free conditions (Swaty et al., 2004). However, when stress levels continue to rise, the host plant will no longer be able to invest (additional) energy in the fungal partner, which leads to decreased levels of mycorrhization (Swaty et al., 2004).

Despite the potential correlation between host stress levels and mycorrhizal abundance, the results for *Succisa pratensis* do not match with those for *Calluna vulgaris*. More specifically, the arbuscular mycorrhizal symbionts of *S. pratensis* do not seem to be negatively affected by soil compaction. This can potentially be explained by the relatively limited duration of our study. It is possible that on the long term, compaction does have negative consequences on the symbionts of Devil's Bit Scabious comparable to those on the symbionts of Heather. However, it must be noted that it is not necessarily meaningful to compare differences in the degree of mycorrhization between different plant species (Bernhardt-Römermann et al., 2009). In addition, it cannot be excluded that other aspects of the mycorrhizal symbiosis, such as community composition, could be affected by compaction. Therefore, it might be recommended to investigate this in future studies. Moreover, it must be noted that obtaining the

sods containing the plant individuals and their symbionts already caused unintentional disturbance of the soil, which could also affect the mycorrhizas. To avoid this, field experiments might be more appropriate. Furthermore, less subjective and more precise approaches to quantify the degree of mycorrhization, such as relative qPCR (quantitative Polymerase Chain Reaction), could provide novel insights into the effects of compaction of the symbionts of Devil's Bit Scabious (Bodenhausen et al., 2021).

## 5.4 Implications for nature management and restoration

Our results demonstrated that soil compaction can indeed impact several aspects of the ecosystem in nature management. More specifically, it can increase the concentrations of nutrients and potentially toxic elements in the soil solution, it can induce shifts in the community composition of arbuscular mycorrhizal fungi and it can diminish the long-term survival of *Succisa pratensis*. Therefore, it is of paramount importance not to underestimate the potential effects of compaction in nature management. In addition, compaction is not the only anthropogenic factor that impacts grasslands and heathlands. In this study, we specifically addressed the effects of compaction and compaction under drought stress, but these two agents can further interact with for example changes in temperature and nitrogen deposition (Gordon et al., 1999; Kivlin et al., 2013; Mohan et al., 2014; Meyer-Grünefeldt et al., 2016). It has for instance been demonstrated that elevated levels of nitrogen in the soil increase the susceptibility of *C. vulgaris* to drought stress (Meyer-Grünefeldt et al., 2016). Taking into account that studies focussing on a single factor cannot elucidate the combined effects of multiple environmental change factors, it is still unclear whether such an additive effect also applies to other combinations of stressors (Meyer-Grünefeldt et al., 2016). Therefore, additional research addressing the effects of soil compaction, both on its own and in combination with other anthropogenic influences, is required.

Despite the need for additional research, our findings have clearly demonstrated that it is advisable to take the potential effects of compaction into consideration during the execution of interventions related to nature management or restoration. Considering that prevention is preferred above remediation, it might be recommended to completely replace machine mowing by manual mowing in the environments our study focussed on (Alakukku et al., 2003; Spoor et al., 2003). The desirability of such a drastic change is highlighted by the fact that most interventions to attenuate soil compaction are appropriate for agricultural purposes, but cannot be applied in nature management (for example tillage; Keller et al., 2017). However, in case such a change is made impossible by financial or time constraints, less far-reaching (but most likely also less effective) alternatives are available. Firstly, it is important to avoid mowing with heavy machines during or directly after wet periods because a high soil moisture content strongly increases the risk of compaction (Hamza & Anderson, 2005; Hatley et al., 2005; Batey, 2009; Bell et al., 2011). However, also the potential impact of drought periods after mowing

should be taken into account as plant resistance and/or resilience could be affected by compaction (Section 5.2.1, Section 5.3.1). A second set of potential measures consists of modifying the used equipment to minimize the compacting forces exerted on the soil. This can be achieved by lowering the inflation pressure of the tyres (Alakukku et al., 2003; Chatterjea, 2007; Filipovic et al., 2016; Thees & Olschewski, 2017), by increasing the size and especially the width of the tyres (Alakukku et al., 2003; Chatterjea, 2007) or by replacing wheel-based machines with track-based ones (Horn et al., 2004; Filipovic et al., 2016; Mudarisov et al., 2020). However, the latter option appears to be somewhat less effective than using machines with multiple tyres placed next to each other (Arvidsson & Keller, 2014), which indicates that dual or triple wheels could also be a good option (Alakukku et al., 2003; Chatterjea, 2007; Filipovic et al., 2016). In addition to these technical alterations, it is also recommended to limit the passing of heavy machinery to well-delineated trails (von Wilpert & Schäffer, 2006) and to identify the zones with the highest sensitivity to compaction (Alakukku et al., 2003; Troldborg et al., 2013). Finally, there are a number of recommendations related to policy. Making it mandatory for the constructors of mowing equipment to provide information on the exerted soil pressures would facilitate the estimation of the potential degree of compaction, thus creating more awareness concerning this issue (Comparetti et al., 2010). Also, it is indispensable that soil compaction is taken into account in the cost-effectiveness analyses used to determine the most suitable management interventions (Müller, 2003). Lastly, policy could benefit from the determination of compaction thresholds above which the damage to the soil becomes too pronounced (Beylich et al., 2010; Hartmann et al., 2014). However, we should be cautious about the implementation of such thresholds. Despite the fact that they can be informative, the effects of different levels of soil compaction will most likely depend on for example the nature of the ecosystem and soil type, as well as on the portion of the ecosystem under study (soil properties, micro-organisms, plants…). Therefore, levels of compaction that are not yet harmful for one part of the ecosystem might already inflict severe damage to another part of it.

# 6. Conclusions

In the observational field study, we found the lowest bulk densities for non-mown areas, the highest for tractor-mown areas and intermediate values for softrak-mown grasslands. This suggests that mechanical mowing indeed causes soil compaction, with more pronounced effects for heavier machinery. Combined with the fact that the values of the soil properties observed in softrak-mown areas were always intermediate between those measured in non-mown and tractor-mown areas, this indicates that the observed changes in soil properties are most likely associated with the soil compaction caused by mechanical mowing. Therefore, our results have shown that compaction is correlated with increases in the concentration of nutrients (nitrate, ammonium and phosphorus) and potentially toxic elements (aluminium, iron and manganese), as well as with alterations in the community composition of arbuscular mycorrhizal fungi. Importantly, each of these three changes can potentially alter the plant species composition. In addition, the differences between softrak- and tractor-mown areas were mostly non-significant, which indicates that the supposedly lower levels of compaction inflicted by a softrak are still correlated with similar changes in soil properties as those inflicted by a tractor.

Furthermore, as shown by the results of the lab experiments, also specific plant species seem to be influenced by compaction. Most importantly, the long-term survival of *Succisa pratensis* was strongly reduced under increasing compaction levels, with respectively 87.5 %, 50 % and 25 % of the plants surviving in the 'no', 'mild' and 'severe compaction' treatments. This highlights the importance of considering the long-term effects of soil compaction in nature management. In addition, *Calluna vulgaris* appears to be less resistant to summer drought stress when the soil is severely compacted and *Succisa pratensis* tends to recover more slowly under the same conditions. Moreover, there was a trend towards a decreased mycorrhization under increasing compaction levels for the ericoid mycorrhizas of *C. vulgaris*.

Taking into account that the soil compaction caused by machine mowing can negatively impact grasslands and heathlands through the four main mechanisms mentioned above (nutrient enrichment, increased soil toxicity, shifts in mycorrhizal community composition and long-term survival of plants), it is strongly recommended to take its potential effects in nature management and restoration into account. Considering that softrak-mowing seems to inflict similar damage to the ecosystem as tractor-mowing, avoiding mechanical mowing altogether in the habitat types our study focussed on appears to be the most suitable approach to deal with the potential negative effects of compaction.

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# 8. Addendum

## 8.1 Risk analysis

During the laboratory analyses involved in this study, several potentially harmful substances were used. Therefore, appropriate precautionary measures were taken to limit the risks. Firstly, during the analysis of the soil samples, we used nitric acid ($HNO_3$) to acidify the aqueous extracts. Taking into account that $HNO_3$ causes severe burns upon contact with the skin and that it also has a corrosive effect on the respiratory tract upon inhalation, we always worked under a fume hood and wore gloves and a lab coat when using this substance. Secondly, for the DNA extractions involved in the process of determining the mycorrhizal fungal community composition in the soil samples, several reagents could potentially have adverse health effects upon contact with the skin or the eyes. This necessitated the wearing of a lab coat, gloves and protective glasses. Thirdly, the plant root staining procedure involved hydrochloric acid (HCl), potassium hydroxide (KOH) and lactic acid ($C_3H_6O_3$; to make lactoglycerol), which can all cause skin irritations. In addition, the dye (Trypan blue) is potentially carcinogenic. All these risks involved in the staining procedure emphasised the importance of wearing a lab coat and gloves during the entire process.

However, not only measures to protect our own health were crucial during all laboratory analyses. It was also important to consider the potentially harmful effects of the chemicals used on the environment. For example, Trypan blue can cause severe water pollution when discharged into the environment. As a result, it was of paramount importance to dispose the residues of each substance into the appropriate waste container (‘acid inorganic waste liquids’ for nitric acid and hydrochloric acid, ‘basic inorganic waste liquids’ for potassium hydroxide, ‘non-halogenated organic waste liquids’ for lactic acid and a specifically adapted waste container for Trypan blue).

Not only during the laboratory analyses, but also during the field work some precautionary measures had to be taken. Given the fact that we used a very sharp knife to obtain the soil samples (to limit unintentional compaction during sampling), it was important to handle this with care to avoid cuts. In addition, it was recommended to thoroughly wash our hands after sampling and contact with the soil because some soil bacteria can potentially infect humans through already existing skin damage and thus cause disease.

Finally, it must be noted that during the fieldwork and the experiments also the health of the involved ecosystems was taken into account. A minimal impact on the environment was assured by only accessing areas where we needed to obtain samples, by avoiding the trampling of sensitive plant species, by collecting no more soil material and plants than we actually needed for the study and by replanting the plants that survived the experiment on the same location as where they were originally obtained.

# 8.2 Appendix 1

*Table A.1: Results of the type III ANOVAs and subsequent Tukey post hoc tests that were performed on the linear mixed-effects models constructed for the non-transformed soil properties of the 67 samples (assumption of normality is violated). Differences in the level of significance compared to the transformed data (Table 1) are placed between red brackets. ** 0.001 ≤ p < 0.01.*

| | Type III ANOVA | | Tukey post hoc test | | |
|---|---|---|---|---|---|
| | Test statistic $\chi^2_{2,58}$ | *p* value | *p* value for contrast none - softrak | *p* value for contrast none - tractor | *p* value for contrast softrak - tractor |
| $NH_4^+$ (mg/l) | 2.991 | 0.224 () | 0.956 | 0.204 () | 0.257 |
| S (mg/l) | 0.060 | 0.971 () | 0.987 | 0.968 | 1.000 |
| Fe (mg/l) | 4.572 | 0.102 () | 0.191 () | 0.229 () | 1.000 |
| Mn (mg/l) | 12.031 | 0.002 (**) | 0.148 () | 0.004 (**) | 0.473 |

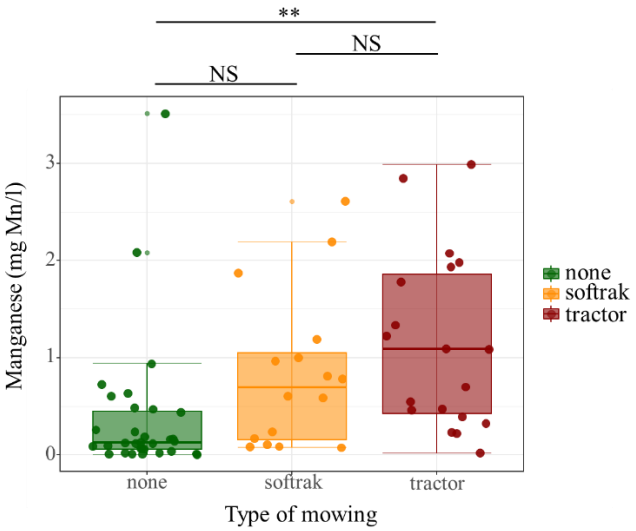


*Figure A.1: Boxplot of the untransformed data for the soil property of the 67 samples for which the type III ANOVA yielded a significant result (Table A.1). Results of the Tukey post hoc test are indicated above the boxplot: ** 0.001 ≤ p < 0.01, NS p ≥ 0.1. There was an overall significant correlation between mowing type and manganese ($ANOVA_{2,58}$: $\chi^2 = 12.031$, p = 0.002), with a difference between none and tractor (Tukey: p = 0.004).*

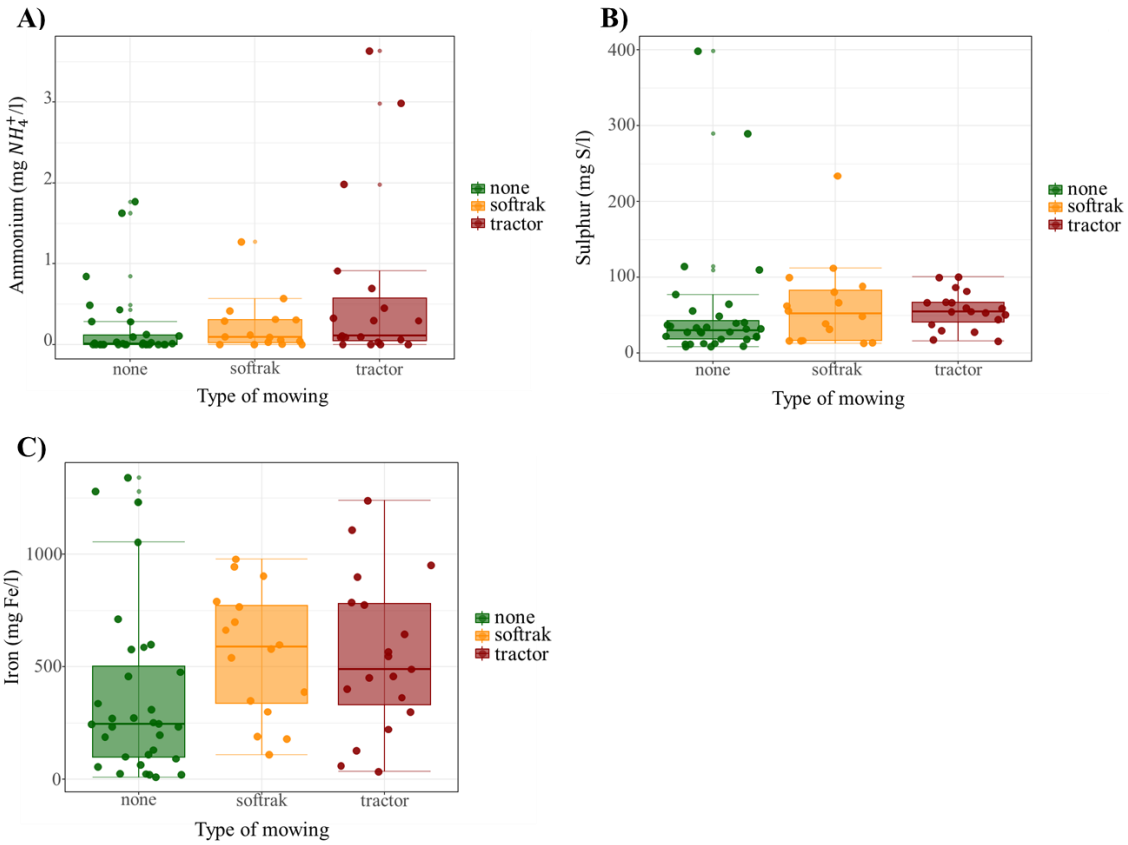


*Figure A.2: Boxplots of the untransformed data for the soil properties of the 67 samples for which the type III ANOVA did not yield a significant result (Table A.1). A) There was no overall significant correlation between mowing type and ammonium ($ANOVA_{2,58}$: $\chi^2 = 2.991$, p = 0.244). B) There was no overall significant correlation between mowing type and sulphur ($ANOVA_{2,58}$: $\chi^2 = 0.060$, p = 0.971). C) There was no overall significant correlation between mowing type and iron ($ANOVA_{2,58}$: $\chi^2 = 4.572$, p = 0.102).*

# 8.3 Appendix 2

*Table A.2: Results of the type III ANOVAs and subsequent Tukey post hoc tests that were performed on the linear mixed-effects models constructed for the cations (potassium, calcium, magnesium, sodium and silicon) in the 40 samples from Vorsdonkbos-Turfputten. In case the dependent variable needed transformation to achieve a normal distribution of the residuals, both the results for the transformed and non-transformed data are shown. The type of transformation used is indicated underneath the variable name. Differences in the level of significance compared to the transformed data are placed between red brackets. *** p < 0.001, ** 0.001 ≤ p < 0.01, * 0.01 ≤ p < 0.05, ~ 0.05 ≤ p < 0.1.*

| | Type III ANOVA | | Tukey post hoc test | | |
|---|---|---|---|---|---|
| | Test statistic $\chi^2_{2,35}$ | *p* value | *p* value for contrast none - softrak | *p* value for contrast none - tractor | *p* value for contrast softrak - tractor |
| K (mg/l) | 0.622 | 0.733 | 0.810 | 0.951 | 0.733 |
| Ca (mg/l) Inverse | 1.699 | 0.428 | 0.826 | 0.816 | 0.404 |
| Ca (mg/l) | 5.214 | 0.074 (~) | 0.271 | 0.823 | 0.148 |
| Mg (mg/l) | 0.007 | 0.997 | 0.997 | 1.000 | 0.997 |
| Na (mg/l) Log10 | 10.868 | 0.004 ** | 0.286 | 0.718 | 0.006 ** |
| Na (mg/l) | 8.339 | 0.015 (*) | 0.148 | 0.938 | 0.046 (*) |
| Si (mg/l) | 0.483 | 0.786 | 0.770 | 0.992 | 0.894 |

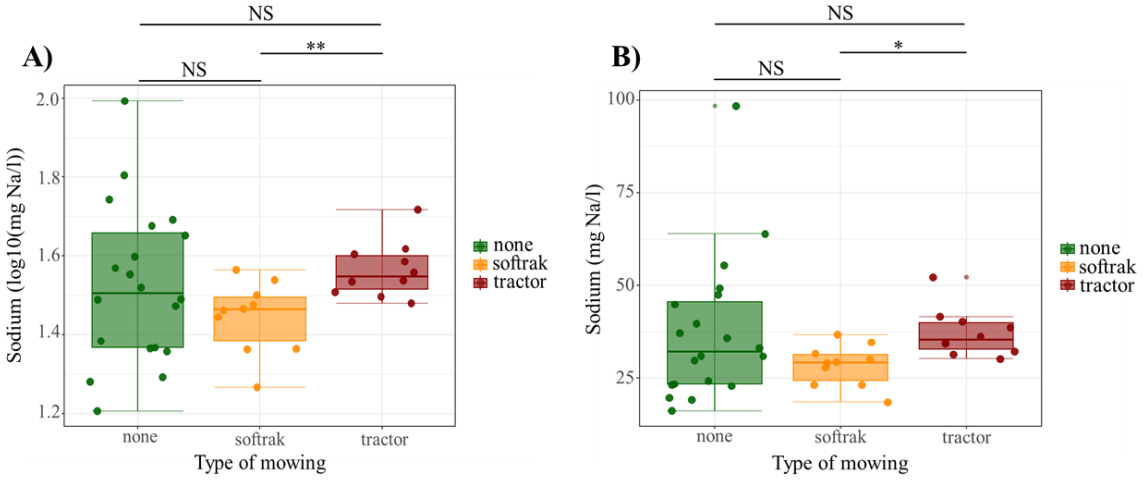


*Figure A.3: Boxplots of the data of the cations from Vorsdonkbos-Turfputten for which the type III ANOVA yielded a significant result (Table A.2). Results of the Tukey post hoc tests are indicated above each boxplot: ** 0.001 ≤ p < 0.01, * 0.01 ≤ p < 0.05, ~ 0.05 ≤ p < 0.1, NS p ≥ 0.1. A) There was an overall significant correlation between mowing type and the log10-transformed concentrations of sodium ($ANOVA_{2,35}$: $\chi^2$ = 10.868, p = 0.004), with a difference between softrak and tractor (Tukey: p = 0.006). B) There was an overall significant correlation between mowing type and sodium ($ANOVA_{2,35}$: $\chi^2$ = 8.339, p = 0.015), with a difference between softrak and tractor (Tukey: p = 0.046).*

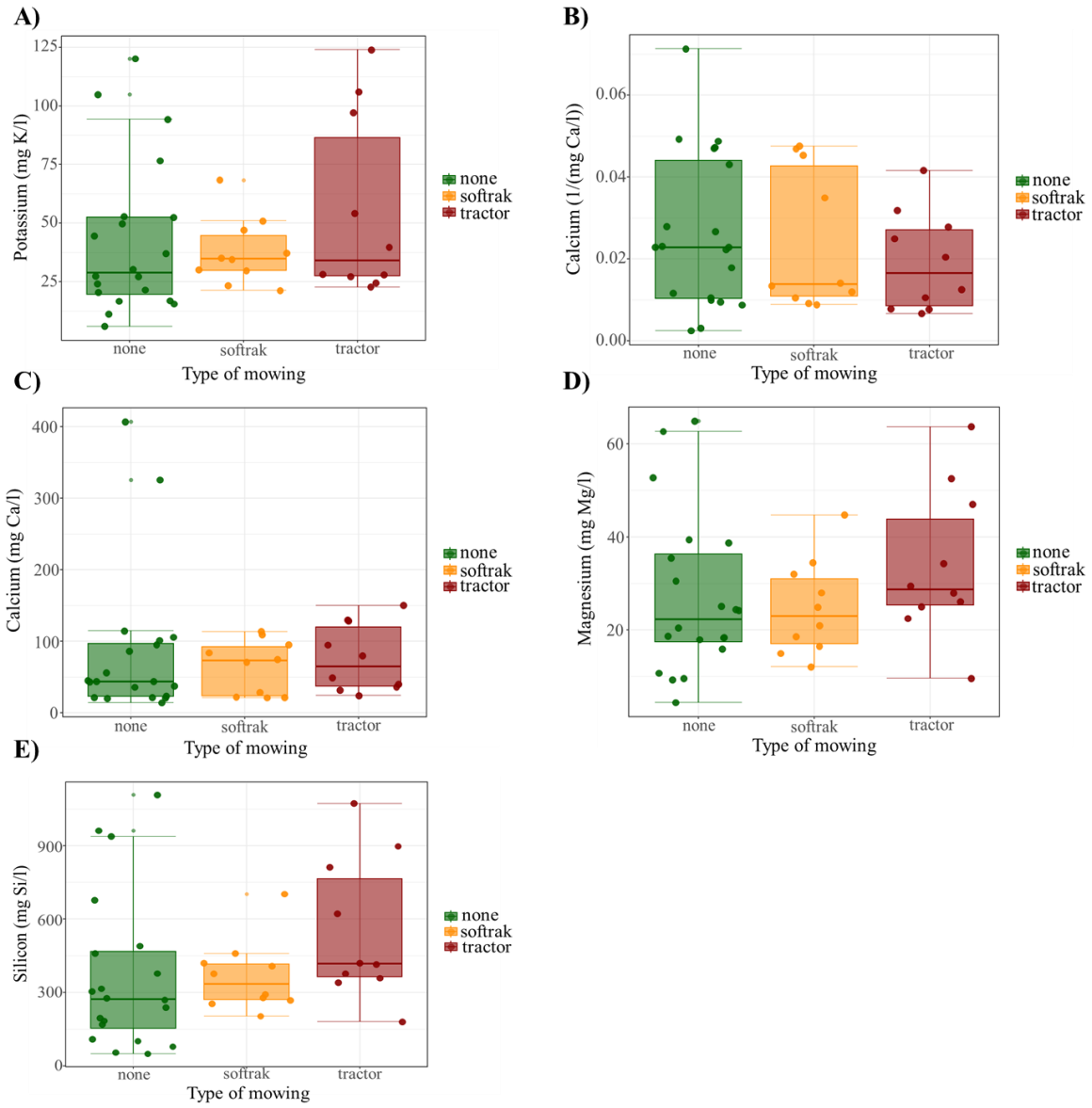


*Figure A.4: Boxplots of the data of the cations from Vorsdonkbos-Turfputten for which the type III ANOVA did not yield a significant result (Table A.2). A) There was no overall significant correlation between mowing type and potassium ($ANOVA_{2,34}$: $\chi^2 = 0.622$, p = 0.733). B) There was an overall marginal insignificant correlation between mowing type and the inverse-transformed concentrations of calcium ($ANOVA_{2,34}$: $\chi^2 = 5.214$, p = 0.074). C) There was no overall significant correlation between mowing type and calcium ($ANOVA_{2,34}$: $\chi^2 = 1.699$, p = 0.428). D) There was no overall significant correlation between mowing type and magnesium ($ANOVA_{2,34}$: $\chi^2 = 0.007$, p = 0.997). E) There was no overall significant correlation between mowing type and silicon ($ANOVA_{2,34}$: $\chi^2 = 0.483$, p = 0.786).*

# 8.4 Appendix 3

*Table A.3: Results of the procedure to test the assumption of homogeneous multivariate dispersions for both mowing type and location for each PERMANOVA. A significant* p *value for 'permutest' indicates that the assumption is violated. For groups that are significantly different with respect to their dispersions as determined by the Tukey Honest Significant Difference Test ('TukeyHSD'), the group with the largest dispersions is displayed in red underneath the* p *value. For location, only the contrasts that were found to be significant in the TukeyHSD tests are shown in the table. ** 0.001 ≤* p *< 0.01, * 0.01 ≤* p *< 0.05.*

| | Mowing | | | | | Location | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 'permutest' | | 'TukeyHSD' | | | 'permutest' | | 'TukeyHSD' | | |
| | $F_{2,64}$ | *p* | *p* value for contrast none - softrak | *p* value for contrast none - tractor | *p* value for contrast softrak - tractor | $F_{6,60}$ | *p* | *p* value for contrast Vorsdonk 1 - Spicht | *p* value for contrast Vorsdonk 2 - Spicht | *p* value for contrast Vorsdonk 2 - Vorsdonk 3 |
| $NO_3^-$ + $NH_4^+$ + P (main nutrients) | 4.380 | 0.016 * | 0.012 * none | 0.435 | 0.250 | 3.750 | 0.005 ** | 0.025 * Spicht | 0.006 ** Spicht | - |
| Fe + Al + Mn + S (redox-sensitive elements) | 5.164 | 0.017 * | 0.017 * none | 0.049 * none | 0.870 | 2.830 | 0.015 * | - | - | 0.022 * location 3 |
| | $F_{2,36}$ | *p* | *p* value for contrast none - softrak | *p* value for contrast none - tractor | *p* value for contrast softrak - tractor | $F_{2,36}$ | *p* | *p* value for contrast location 1 - location 2 | *p* value for contrast location 1 - location 3 | *p* value for contrast location 2 - location 3 |
| OTU abundances | 2.156 | 0.136 | - | - | - | 0.576 | 0.568 | - | - | - |

## 8.5 Appendix 4

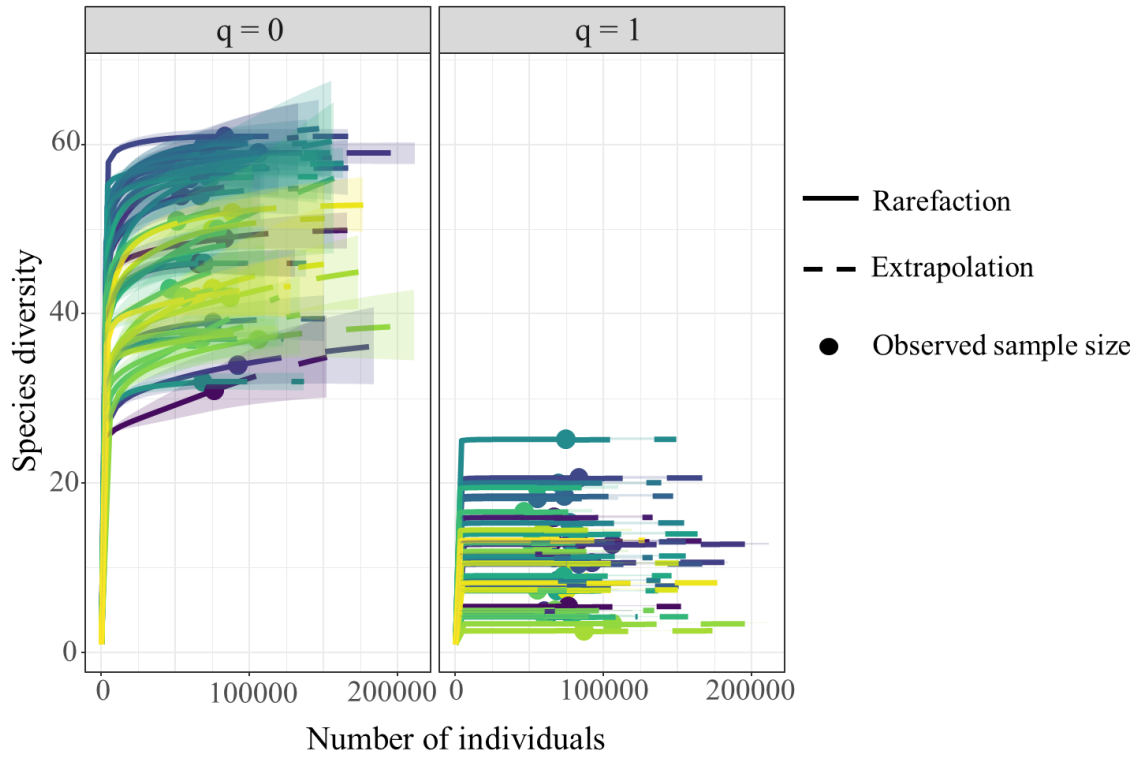


*Figure A.5: Sample-size-based rarefaction and extrapolation sampling curves of the Hill numbers with order q=0 (OTU richness) and q=1 (OTU Shannon diversity). Every coloured line represents a soil sample.*

# 8.6 Appendix 5

*Table A.4: Results of the type III ANOVAs and subsequent Tukey post hoc tests that were performed on the linear mixed-effects models constructed for the OTU richness and Shannon diversity of the 39 samples from Vorsdonkbos-Turfputten. * 0.01 ≤ p < 0.05, ~ 0.05 ≤ p < 0.1.*

| | Type III ANOVA | | Tukey post hoc test | | |
|---|---|---|---|---|---|
| | Test statistic $\chi^2_{2,34}$ | *p* value | *p* value for contrast none - softrak | *p* value for contrast none - tractor | *p* value for contrast softrak - tractor |
| OTU richness | 7.694 | 0.021 * | 0.036 * | 0.984 | 0.072 ~ |
| OTU Shannon diversity | 4.561 | 0.102 | 0.178 | 0.247 | 0.992 |

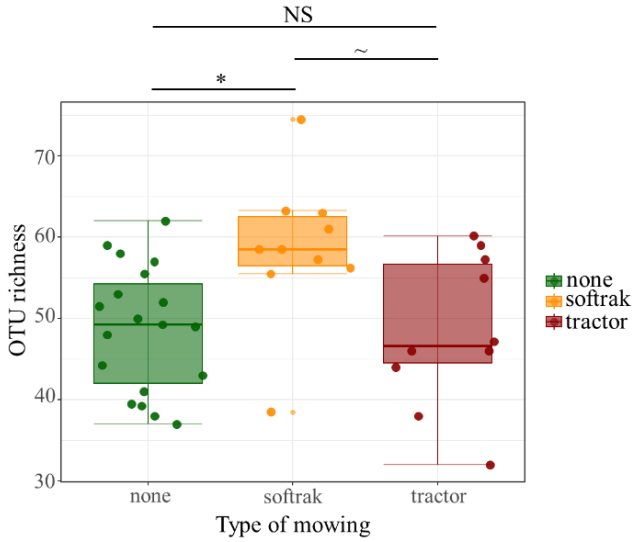


*Figure A.6: Boxplot of the data concerning the OTU richness (Hill number with order q=0) of the 39 samples from Vorsdonkbos-Turfputten. Results of the Tukey post hoc test are indicated above the boxplot: * 0.01 ≤ p < 0.05, ~ 0.05 ≤ p < 0.1, NS p ≥ 0.1. There was an overall significant correlation between mowing type and OTU richness (ANOVA$_{2,34}$: $\chi^2$ = 7.694, p = 0.021), with a difference between none and softrak (Tukey: p = 0.036) and a marginal difference between softrak and tractor (Tukey: p = 0.072).*

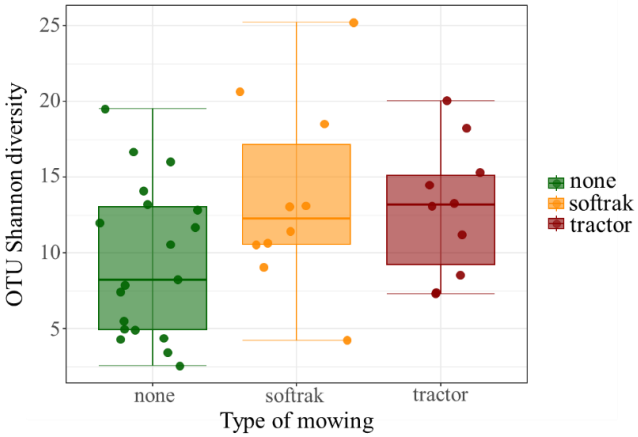


*Figure A.7: Boxplot of the data concerning the OTU Shannon diversity (Hill number with order q=1) of the 39 samples from Vorsdonkbos-Turfputten. There was no overall significant correlation between mowing type and OTU Shannon diversity (ANOVA$_{2,34}$: $\chi^2$ = 4.561, p = 0.102).*

# 8.7 Appendix 6

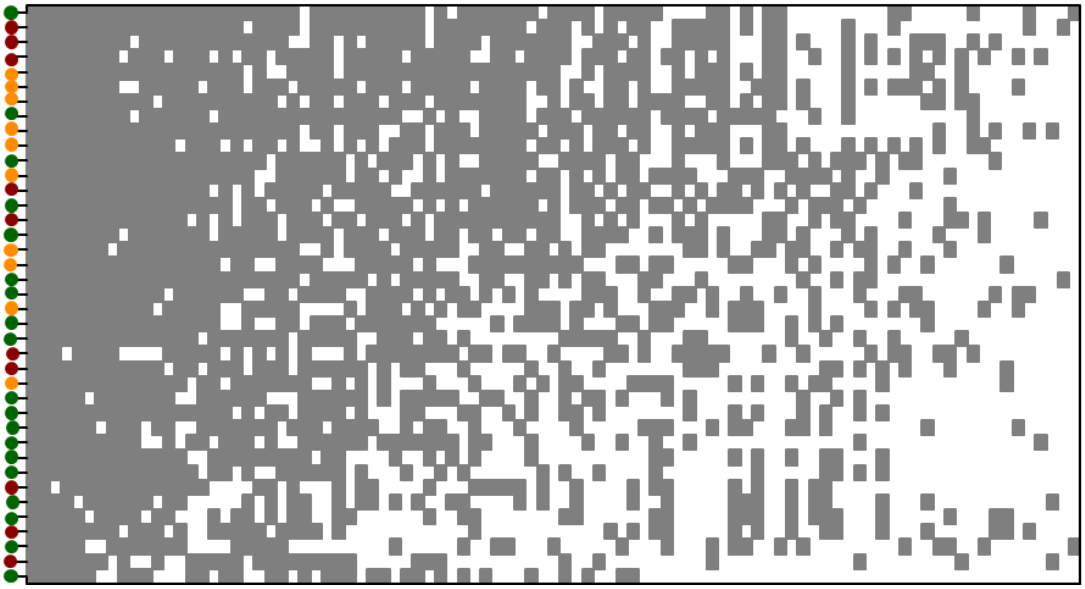

*Figure A.8: Packed data matrix created by nestedness analysis. Rows represent the 39 soil samples from Vorsdonkbos-Turfputten and columns represent the 93 distinct OTUs. Coloured dots indicate the mowing type that applies to the soil sample in question (red = 'tractor', orange = 'softrak', green = 'none'). Presence of an OTU in a soil sample is indicated by a grey square. The arbuscular mycorrhizal fungal communities showed a significantly nested pattern (nestedness analysis: p = 0.010, 'quasiswap' null model), with the nestedness temperature being equal to 40.74°. There was an overall marginally insignificant correlation between mowing type and the position of the samples in the data matrix (*$ANOVA_{2,34}$*: $\chi^2 = 4.960$, p = 0.084), with the softrak-mown samples being placed marginally insignificantly higher in the packed data matrix than the non-mown samples (Tukey:* p = 0.083*).*

# 8.8 Appendix 7

*Table A.5: Results of the indicator species analysis for the different mowing types, performed on the 39 samples from Vorsdonkbos-Turfputten, with indication of the taxonomic identity (family and genus) of every indicator OTU. ** 0.001 ≤ p < 0.01, * 0.01 ≤ p < 0.05, ~ 0.05 ≤ p < 0.1.*

| | Indicator OTUs | *p* value | Family | Genus |
|---|---|---|---|---|
| 'none' | OTU 38 | 0.011 * | Unassigned | Unassigned |
| | OTU 46 | 0.021 * | Glomeraceae | *Glomus* |
| | OTU 27 | 0.022 * | Glomeraceae | *Glomus* |
| | OTU 94 | 0.024 * | Unassigned | Unassigned |
| 'softrak' | OTU 12 | 0.001 ** | Glomeraceae | *Glomus* |
| | OTU 13 | 0.006 ** | Paraglomeraceae | *Paraglomus* |
| | OTU 50 | 0.011 * | Glomeraceae | *Glomus* |
| 'tractor' | OTU 56 | 0.007 ** | Unassigned | Unassigned |
| | OTU 55 | 0.016 * | Unassigned | Unassigned |
| | OTU 30 | 0.023 * | Glomeraceae | *Glomus* |
| | OTU 33 | 0.045 * | Unassigned | Unassigned |
| 'none' + 'softrak' | OTU 112 | 0.003 ** | Unassigned | Unassigned |
| | OTU 172 | 0.004 ** | Unassigned | Unassigned |
| | OTU 52 | 0.007 ** | Unassigned | Unassigned |
| | OTU 60 | 0.013 * | Unassigned | Unassigned |
| | OTU 194 | 0.027 * | Glomeraceae | *Glomus* |
| | OTU 66 | 0.028 * | Unassigned | Unassigned |
| | OTU 20 | 0.029 * | Unassigned | Unassigned |
| 'none' + 'tractor' | OTU 24 | 0.026 * | Unassigned | Unassigned |

*Table A.5 (continued): Results of the indicator species analysis for the different mowing types, performed on the 39 samples from Vorsdonkbos-Turfputten, with indication of the taxonomic identity (family and genus) of every indicator OTU. ** 0.001 ≤ p < 0.01, * 0.01 ≤ p < 0.05, ~ 0.05 ≤ p < 0.1.*

| | Indicator OTUs | *p* value | Family | Genus |
|---|---|---|---|---|
| 'softrak' + 'tractor' | OTU 9 | 0.001 ** | Paraglomeraceae | *Paraglomus* |
| | OTU 17 | 0.002 ** | Unassigned | Unassigned |
| | OTU 156 | 0.025 * | Acaulosporaceae | *Acaulospora* |
| | OTU 59 | 0.029 * | Unassigned | Unassigned |
| | OTU 696 | 0.043 * | Paraglomeraceae | *Paraglomus* |
| | OTU 31 | 0.045 * | Acaulosporaceae | *Acaulospora* |
| | OTU 81 | 0.045 * | Paraglomeraceae | *Paraglomus* |
| | OTU 735 | 0.046 * | Unassigned | Unassigned |
| | OTU 11 | 0.050 ~ | Glomeraceae | *Glomus* |

# 8.9 Appendix 8

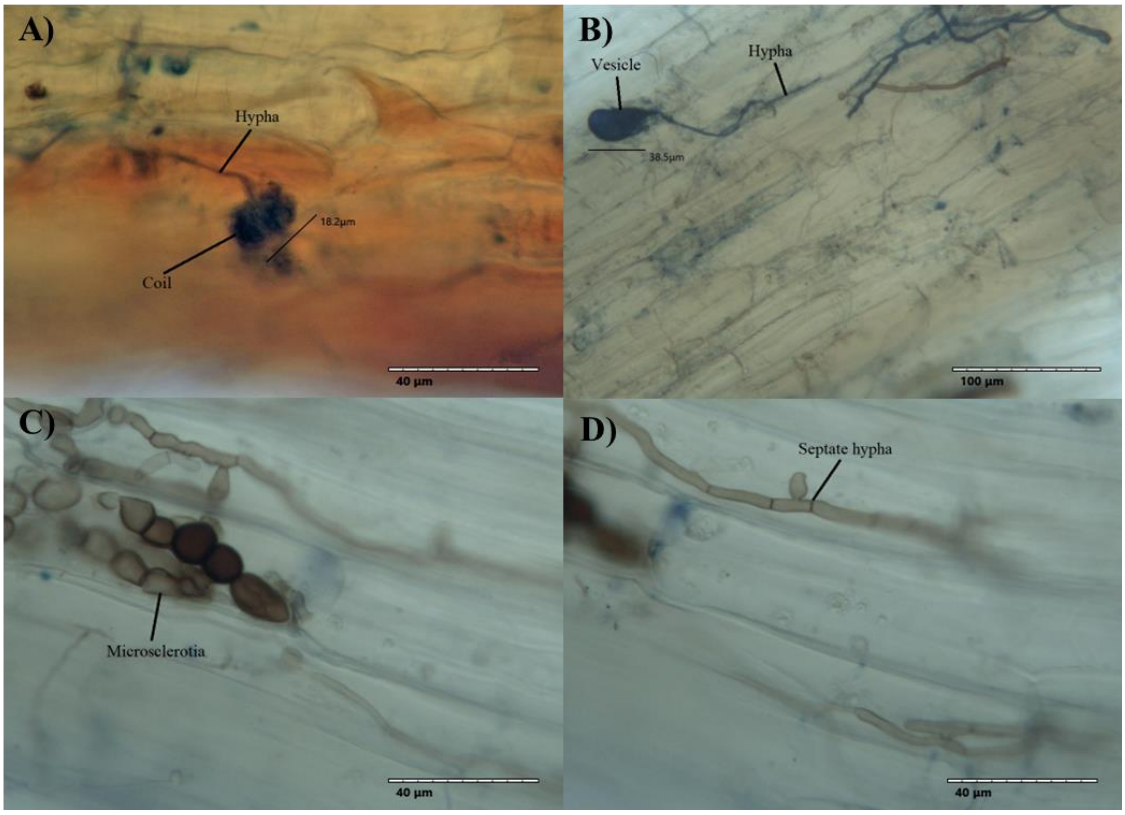


*Figure A.9: Photographs of Trypan blue-stained root samples of* Calluna vulgaris. *All pictures were taken by means of a PixeLink microscope camera. A) Hypha and putative hyphal coil. B) Hypha and putative vesicle. C) Microsclerotia of a dark septate endophyte (DSE). D) Septate hyphae of a dark septate endophyte (DSE).*

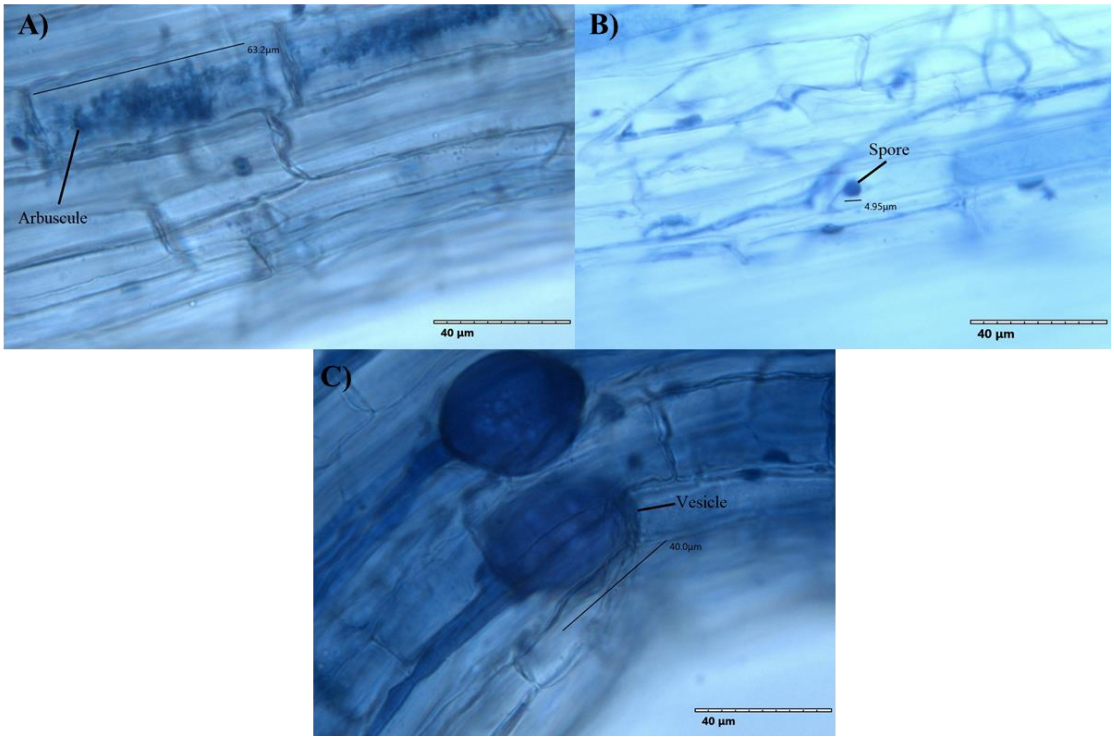


*Figure A.10: Photographs of Trypan blue-stained root samples of* Succisa pratensis. *All pictures were taken by means of a PixeLink microscope camera. A) Putative arbuscule. B) Putative spore. C) Putative vesicle.*

# 8.10 Appendix 9

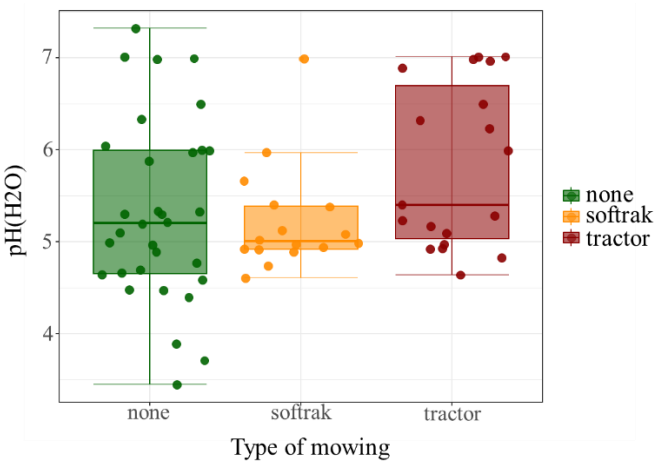


*Figure A.11: Boxplot of the soil property of the 67 samples for which the type III ANOVA did not yield a significant result (Table 1). There was no overall significant correlation between mowing type and pH (*$ANOVA_{2,58}$*:* $\chi^2$ *= 0.658,* p *= 0.720).*

# 8.11 Appendix 10

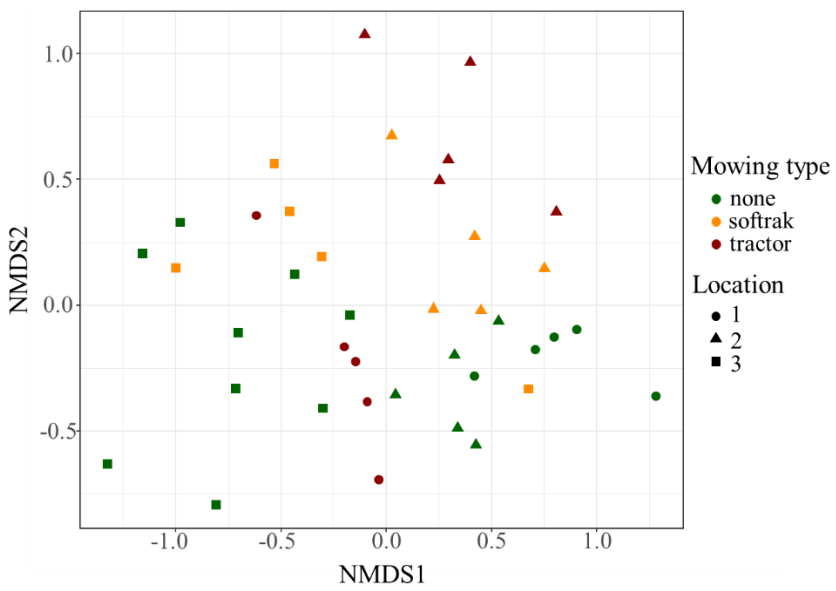


*Figure A.12: NMDS ordination plot of the arbuscular mycorrhizal communities from the non-mown, softrak-mown and tractor-mown areas in Vorsdonkbos-Turfputten. The AMF communities differed significantly between the three mowing types (PERMANOVA$_{2,32}$: F = 1.829, $R^2$ = 0.073,* p *= 0.010), as well as between the three locations (PERMANOVA$_{2,32}$: F = 5.017, $R^2$ = 0.200,* p *= 0.001).*

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