University of Groningen
Faculty of Science and Engineering

Master Thesis:

The effect of mycorrhizal fungi and soil pH on the performance of common juniper, *Juniperus communis.*

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Abstract

Western European populations of common juniper, *Juniperus communis*, are currently suffering from a reduced regeneration. This reduction in seed viability is for various reasons, notably amongst them are the impacts of climate change and nitrogen deposition. However, the effect of soil acidification due to nitrogen deposition upon juniper performance is yet to be determined. Mycorrhizal fungi form a symbiotic relationship with juniper and are assumed to aid their performance. Whether this mycorrhizal fungi symbiosis aids juniper performance under acidifying soil conditions isn't currently known. To investigate this, semi-permeable cores with juniper cuttings were transplanted into the field allowing for mycorrhizal inclusion and exclusion treatments across soil pH gradients. Measurements of juniper growth, juniper needle phosphorus concentration, soil pH, and mycorrhizal colonisation were recorded, and investigated. There was a clear, and significant trend of increased juniper growth as juniper needle phosphorus concentration increased, and under the inclusion core treatment. There was also a significant interactive effect between core treatment and soil pH upon juniper growth. Meanwhile, there was a significant negative effect of decreasing soil pH on arbuscular mycorrhizal colonisation. These findings show a performance increase in the common juniper due to the facilitative effects of mycorrhizal fungi that increases as soil pH decreases. This suggests that as arbuscular mycorrhizal fungi are reduced in the mycorrhizal fungi community structure at low soil pH other mycorrhizal fungi increase their colonisation of juniper providing a superior performance increase. As such we would not recommend the liming of areas as a short-term, remedial management practice. Due to the long lasting effects of soil acidification and lack of reasonable management solutions to remove the abundance of nitrogen and carbon in the soil the reduction of anthropological nitrogen deposition should remain a focus for the conservation of the juniper. It is also recommended that more research be conducted into the effect of nitrogen deposition on the mycorrhizal fungi community and the potential indirect effects this will have upon juniper.

Introduction

Amidst the ongoing crisis of global change, a plethora of plant species are under increasing threat due to climate change and nitrogen deposition. While there is consistent public attention drawn to the dangers of climate change, the effects of nitrogen deposition seem to have slipped out of focus in some Western European countries. This could be due to the reduction of nitrogen emissions and deposition from their peak in the 1980's. However, nitrogen deposition is an ongoing issue contributed to by industrial and agricultural nitrogen fixation, and combustion related emissions (Fowler et al., 2015). With future decreases in nitrogen emissions predicted to be minimal it is important to understand the implications of nitrogen deposition on plant species and their ecosystems.

Previous studies have related nitrogen deposition to an abundance of nitrogen and carbon in soils (Chang et al., 2019) which can increase inter-specific competition from fast growing species and alter both plant and mycorrhizal community structure. However, the impact of nitrogen deposition is not only limited to an increase in nitrogen and carbon in soil. Long-term nitrogen deposition also impacts upon soil ecology by acidifying soil (Bowman et al., 2008) causing the displacement of base cations (calcium, magnesium, potassium and sodium), commonly known as cation leaching.

The common juniper, *Juniperus communis,* is a dioecious conifer which inhabits the ecotone between forest and grassland (Ward & Shellwell, 2017) making it susceptible to the competition pressure that nitrogen deposition can cause. With declining populations in Western Europe (United Kingdom, The Netherlands, Belgium, and Western Germany), and mountainous areas of the Mediterranean due to a lack of recruitment (Gruwez et al., 2013; Gruwez et al., 2016) studies have begun into causes. One such study by Gruwez et al. (2016), has shown that increased nitrogen deposition is linked to reduced seed viability. Gruwez et al. (2016) found an association between acidifying soil depositions and lower calcium concentrations in juniper needles, a deficiency of which is linked to low seed viability.

The vast majority of plants form symbiotic relationships with mycorrhizal fungi which can help to increase plant performance by protecting against drought stress (Calvo-Polanco, 2016; Santander, et al., 2017), and facilitate the intake of nutrients (Hodge & Storer, 2014). The common juniper also forms relationships with mycorrhizal fungi which could present opportunities for resistance against environmental stressors such as that caused by nitrogen deposition and the nutrient poor, sandy soils juniper inhabits in the Netherlands. However, the benefits of mycorrhizal symbiosis can be affected by the mycorrhizal community structure and soil ecology. All of which have varying responses to environmental

stress. Nitrogen deposition has been shown to change mycorrhizal community structure, selecting for less beneficial mycorrhizal fungi (Van Diepen, Lilleskov & Pregitzer, 2011). This effect of nitrogen deposition on mycorrhizal fungi is often overlooked in studies into environmental stress upon plant fitness and performance or results in studies which employ the use of chemical treatments such as fungicides to study this interaction. Johnson, Leake and Read (2001), however, made use of semi-permeable membrane rotating cores to control the presence and absence of mycorrhizal fungi, a methodology that is applicable to in-situ research.

In this study we sought to investigate the effect of mycorrhizal fungi and soil acidification on juniper performance. To this end, we investigated the effect of mycorrhizal fungi colonisation along a gradient of soil pH on the performance, using growth as a proxy, of common juniper at two natural populations in the Netherlands. Making use of semi-permeable membrane cores, male juniper cuttings were transplanted into the field in pairs with mycorrhizal exclusion and inclusion treatments induced by the rotation or lack of to cut mycorrhizal hyphae passing through the core membrane. Drawing on the knowledge of previous studies into plant-mycorrhizal interactions and soil acidity, we hypothesise that the absence of mycorrhizal fungi and increase in soil acidity would result in a compounding effect of decreased performance of the juniper cuttings and that these factors would also individually significantly decrease juniper performance.

Study Design

Juniper Sourcing

Male juniper cuttings were harvested in April 2019 from Mantingerzand (50.917186, 5.917731) in the province of Drenthe, Netherlands, planted in Lentse potgrond potting soil, sterilised by gamma radiation, and provided with a mist-house for the first 3 months of their housing in a greenhouse at 18°C. In November 2019, an investigation into mycorrhizal colonisation of the roots of the cuttings found no presence of mycorrhizal fungi.

Field Experiment

The Natura 2000 sites Mantingerzand (50.917186, 5.917731) and Drouwenerzand (50.917186, 5.917731), Drenthe, The Netherlands (Fig. 1), were chosen as suitable study sites. Mantingerzand is a 780 hectare area characterised by drifting sand, heathland, and scattered coniferous and deciduous trees. Drouwenerzand is a 222 hectare area characterised by drifting sand, heathland and the invasive Scots pine, *Pinus sylvestris*.

These sites were chosen due to their current populations of common juniper and previous soil pH data collected at these sites providing a basis for suitable selection of

planting locations for this experiment. 34 locations, 16 in Drouwenerzand and 18 in Mantingerzand, were selected throughout the range of soil pH at each site (4.46 - 6.48pH and 3.56 - 4.92pH respectively) and were distributed widely throughout each area. Each location had three replicate sub-locations, 5m North, South-East, and South-West of the locations co-ordinates with two juniper cores at each sub-location.

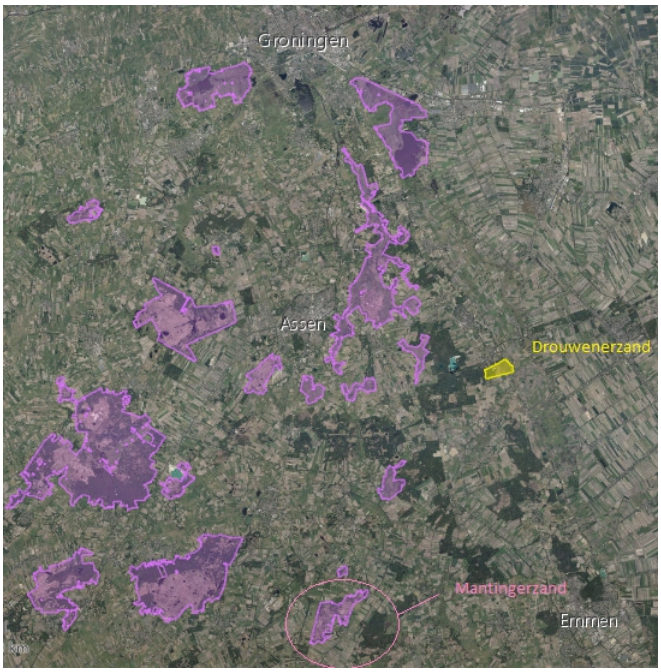

Figure 1. A map of the two field locations (annotated with labels) with Assen, and Groningen visible for reference. All highlighted areas are Natura 2000 areas (Edited: Natura 2000, 2021).

In early April 2020, two soil cores (8.5cm diameter, 20cm depth) 20-100cm apart were taken from each sub-location. Soil was sieved (5mm) and underwent two cycles of autoclave steam sterilisation, to remove possible mycorrhizal fungi already present in the soil, before filling semi-permeable membrane cores (Fig. 2) up to 2cm below the top of the core.

Semi-permeable membrane cores were used to provide two treatments (inclusion and exclusion) of mycorrhizal fungi at each sub-location. These cores were created following the design of Johnson, Leake and Read (2001), using PVC pipe (8cm diameter, 22cm height, 1.6mm thickness) with 2 panels (15cm height, 6cm circumference) cut out and covered with nylon mesh (38µm) glued into place. Two holes drilled at the top of each core allow for bolts to be threaded through and a fencing collar to be attached to prevent herbivore grazing. The bottom of the core is closed using a plug of 2cm depth.

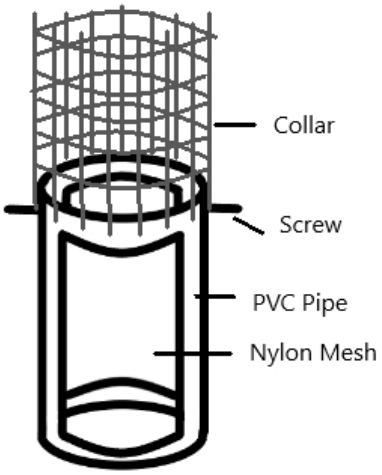

Figure 2. Semi-permeable membrane core including two screws with an attached collar and polyester mesh (38µm) over panels cut from PVC piping.

Juniper cuttings were measured for their stretched height (soil to maximum length height) and planted into the semi-permeable membrane cores before transplantation to their respective soil core sub-locations, in mid to late April, no more than ten days after collection of the soil cores. Excess sieved soil was used to fill space between the juniper cores and the surrounding soil to ensure connectivity of the membrane and soil.

Every 6-8 days the cores designated for mycorrhizal exclusion treatment were manually rotated 180^{o} to disrupt potential mycorrhizal hyphae growth through the membrane. Bi-monthly measurements of unstretched juniper height (soil to highest natural point) and colour were recorded, with colour recorded on a 5 point scale of percentage of green needle coverage: 1 - 0-20%, 2 - 21-40%, 3 - 41-60%, 4 - 61-80%, 5 - 81-100%.

In October 2020, after 5 months, the field experiment was terminated and cores were removed from the field following final field measurements.

Performance & Environmental Measurements

In the laboratory, the juniper cuttings were measured for their final extended above-ground height prior to fresh mass, dry mass, and needle dry mass measurements. Soil moisture and organic content was calculated by taking fresh, dry, and desiccated soil mass measurements. A water extraction was performed using solutions of 17.5g fresh soil with 50ml distilled water. The solutions were rotated for 2 hours at 60rpm before testing for pH and filtering using Rhizons SMS (Eijkelkamp Agrisearch Equipment, the Netherlands) connected to vacuum bottles. Filtered solutions were stored at -20^{o}C until 15ml of each was

analysed using an auto-analyser for soil element concentrations. Juniper needles were ground and tested for element concentrations.

Mycorrhizal Colonisation

Roots systems were brushed clear of sediment and had approximately 1cm lengths of root cut from the end of each root accumulating 10 sections per plant. An adapted version of the protocol by Grace and Stribley (1991) was then followed to prepare the roots for microscopic investigation. The root samples were cleared (10% KOH in a 90C water bath for 1 hour), bleached (10% H2O2 at room temperature for 1 hour), acidified (1% HCL at room temperature overnight), stained (0.05% Trypan Blue glycerol staining solution at room temperature for 30 minutes), and de-stained (70% glycerol de-staining solution at room temperature for 2 days) before storage in a 4°C fridge prior to investigation.

A subset of 89 juniper root samples (44 Drouwenerzand, 45 Mantingerzand) were investigated for mycorrhizal presence. Microscopic investigation of the roots scored mycorrhizal colonisation of 10 roots (except for samples in which small root size resulted in loss of roots during staining) and 5 intersections of each root based on the methods of McGonigle et al. (1990). Roots were placed on microscope slides with cover slips and de-staining solution was added. Intersections were determined using the cross-hair of the CX23 microscope and mycorrhiza presence was scored following discovery of hyphae, vesicles or arbuscules (Fig. 3). No attempt to identify and score species of mycorrhiza was made. However, photographic evidence was taken to document potential variation in mycorrhizal species colonising roots.

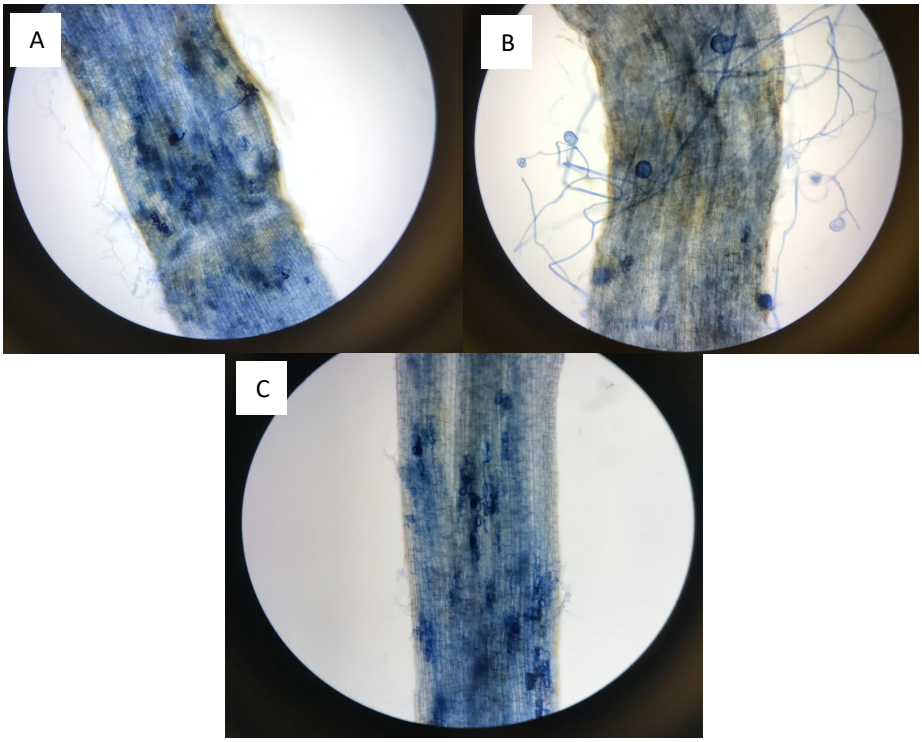

Figure 3. Images showing structures of mycorrhizal fungi on root sections. External hyphae (A & B), internal hyphae (A & C), vesicles (B), arbuscules (C).

Statistical Analysis

Statistical analysis was performed in R (version 4.0.2, 2020) using packages readx1, lme4, qqplotr, ggthemes, ggpubr, nortest, rstatix, plyr, and pbkrtest. Summary statistics and normality tests were performed on data following the exclusion of 31 individuals that were dead or had damage to the mesh of the cores. The 25 junipers that weren't removed due to this criteria but were paired to those removed were also excluded from the data. Mycorrhizal root colonisation data underwent an angular transformation prior to input into a linear mixed effects model using AIC values and null model significance testing for model simplification to test the effect of core treatment, soil pH, initial juniper cutting length, and other measured factors on mycorrhizal root colonisation (Table 1). Juniper growth data was input into a generalised linear mixed effects model using AIC values and null model significance testing for model simplification to test the effect of core treatment, soil pH, initial juniper cutting length, and other measured factors on juniper performance (Table 1). Needle phosphorus concentration data was input into a generalised linear mixed effects model using AIC values and null model significance testing for model simplification to test the effect of core treatment, soil pH, initial juniper cutting length, and other measured factors on juniper performance (Table 1). Sub-location pairing of junipers was used as a random effect for all models.

Effect	Effect Type
Core Treatment	Fixed Effect
Soil pH (pH)	Fixed Effect
Initial Juniper cutting length (mm)	Fixed Effect
Study Site	Fixed Effect
Location	Fixed Effect
Sub-location	Fixed Effect
Pairing	Random Effect
Needle Phosphorus Concentration (µmol/g)	Fixed Effect
Soil Phosphorus Concentration (µmol/g)	Fixed Effect
Soil Moisture Content	Fixed Effect
Mycorrhizal Root Colonisation(%)	Fixed Effect

Table 1. The fixed and random effects analysed in the (generalised) linear mixed models testing for significant effects upon mycorrhizal root colonisation, juniper growth, and needle phosphorus concentration.

Results

Juniper Performance

There was a clear, and significant trend of increased juniper growth as initial juniper cutting length increased ($X^2(df=4) = 4.0782$, $p=0.04$), juniper needle phosphorus concentration increased ($X^2(df=1) = 32.381$, $p<0.001$), and under the inclusion core treatment ($X^2(df=4) = 27.455$, $p<0.001$). While there was no significant effect of soil pH directly upon juniper growth ($X^2(df=1) = 0.3718$, $p=0.54$), there was a significant interactive effect between core treatment and soil pH upon juniper growth ($X^2(df=4) = 8.5285$, $p=0.003$).

Juniper Cutting Growth			
	Chi-Square	Degrees of Freedom	Pr(>Chisq)
Core Treatment	27.455	4	<0.001
Soil pH	0.3718	1	0.54
Initial Cutting Length	4.0782	4	0.04
Needle Phosphorus Concentration	32.381	1	<0.001
Core Treatment: Soil pH	8.5285	4	0.003

Table 2. Results from the significance testing of generalised linear mixed models against their null model for the effect of selected factors on juniper cutting growth.

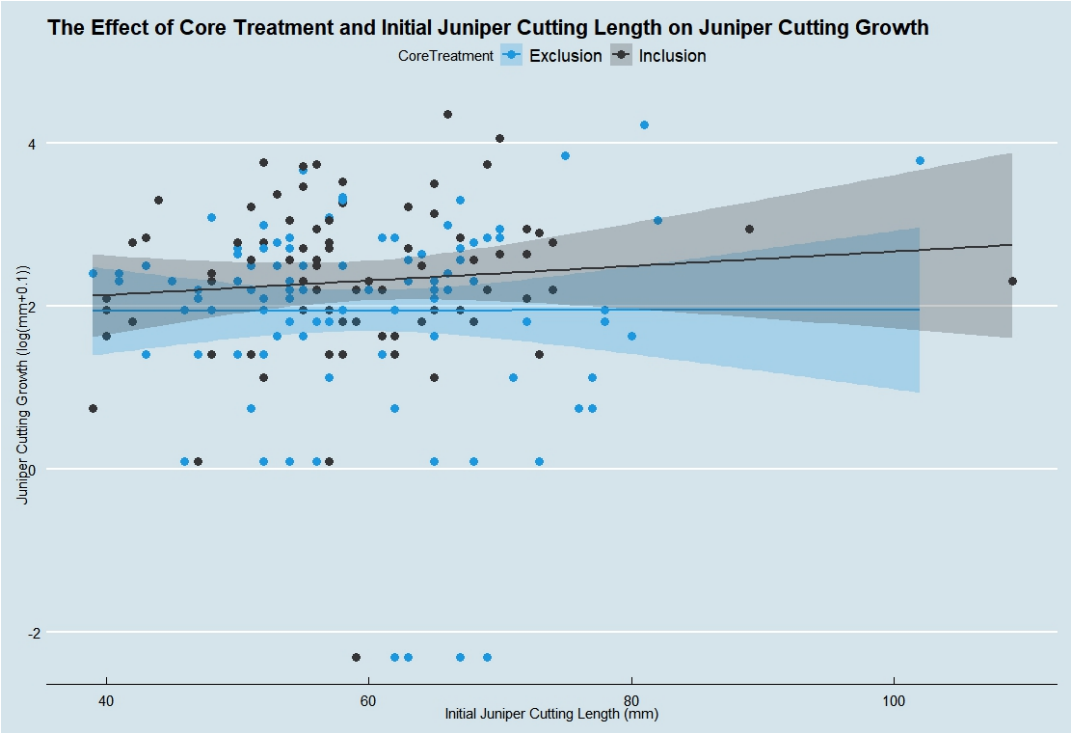

Figure 4. The effect of core treatments of inclusion (grey) and exclusion (blue), and initial juniper cutting length (mm) on juniper cutting growth (log(mm)+0.1).

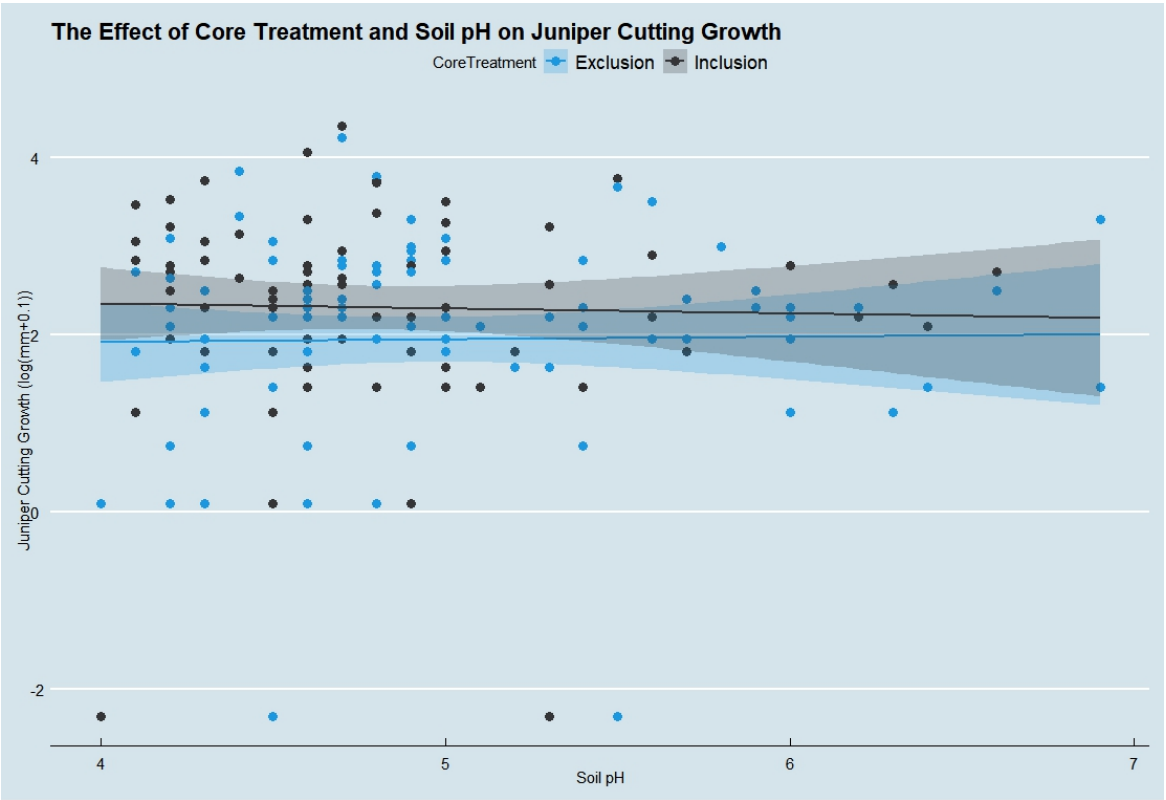

Figure 5. The effect of core treatments of inclusion (grey) and exclusion (blue), and soil pH on juniper cutting growth (log(mm)+0.1).

Both an increase in juniper needle phosphorus concentration (X^2(df=1) = 32.381, p<0.001; Fig. 6), and inclusion core treatment (X^2(df=4) = 27.455, p<0.001) correlate to increased juniper growth. However, the phosphorus concentration in juniper needles is also affected by core treatment with a significant increase in phosphorus concentration in the inclusion core treatment (X^2(df=1) = 4.0105, p=0.045; Fig. 7).

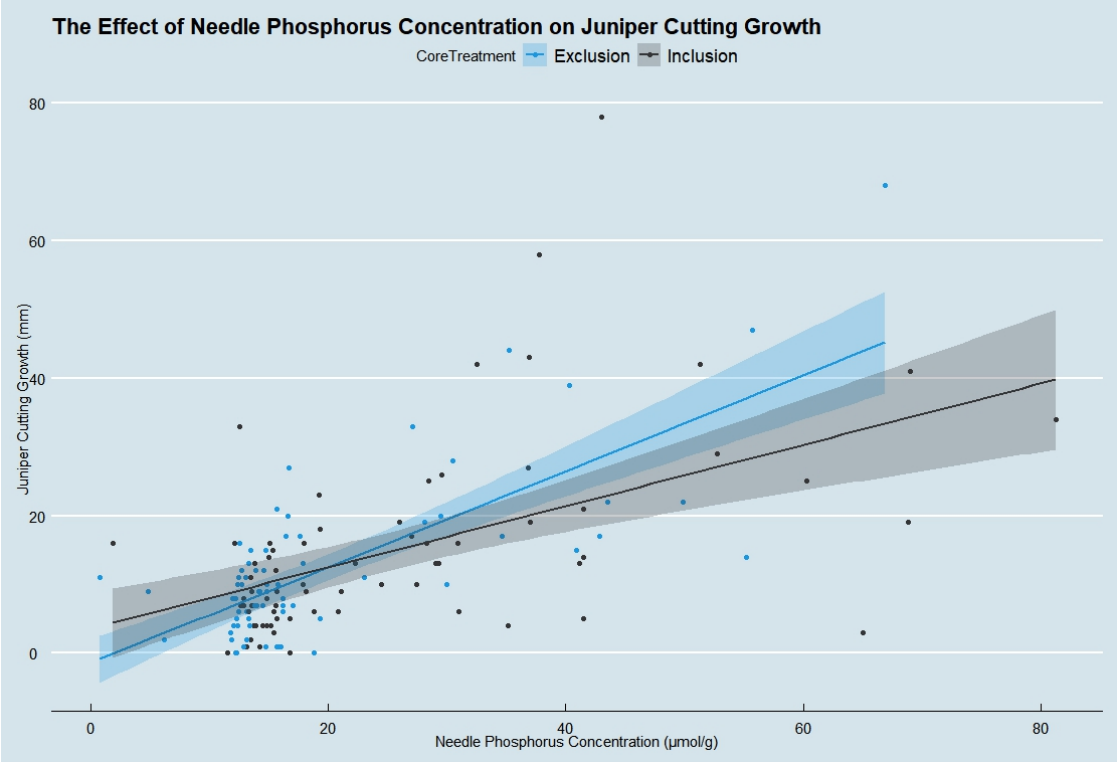

Figure 6. The effect of core treatments of inclusion (grey) and exclusion (blue), and needle phosphorus concentration (µmol/g) on juniper cutting growth (mm).

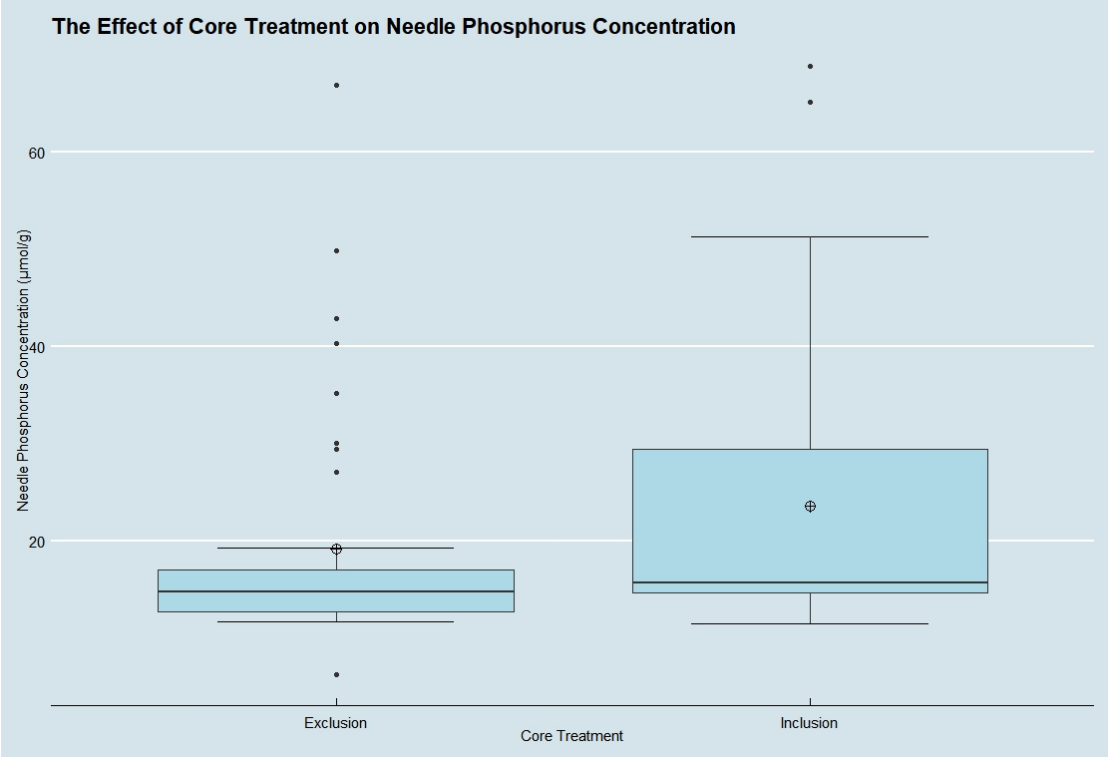

Figure 7. The effect of core treatments of inclusion and exclusion on needle phosphorus concentration (µmol/g).

Mycorrhizal Root Colonisation

There was a significant positive effect of increasing soil pH (X^2(df=1) = 5.8522, p=0.015) on mycorrhizal colonisation and no significant effect of core treatment (X^2(df=1) = 1.0778, p=0.299) upon mycorrhizal colonisation of juniper roots (Table 3; Fig. 8).

This was reflected in the mean mycorrhizal root colonisation being only 5.25% greater in the exclusion core treatment (50.067%, SE = 4.038) compared to that of the inclusion core treatment (44.817%, SE = 3.131).

Mycorrhizal Root Colonisation			
	Chi-Square	Degrees of Freedom	Pr(>Chisq)
Core Treatment	1.0778	1	0.299
Soil pH	5.8522	1	0.015

Table x3. Results from the significance testing of linear mixed models against their null model for the effect of selected factors on mycorrhizal root colonisation.

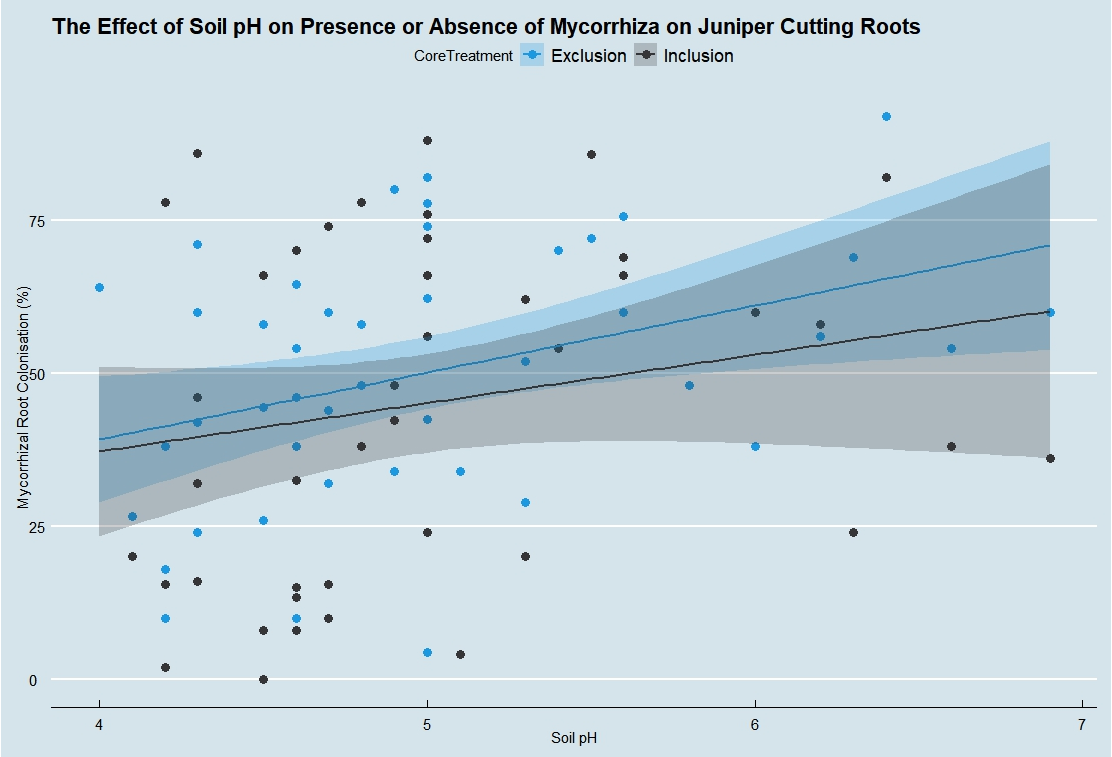

Figure 8. The effect of core treatments of inclusion (grey) and exclusion (blue), and soil pH on the mycorrhizal colonisation root colonisation of juniper cuttings (%).

Discussion

This study aimed to investigate the effect of nitrogen deposition on juniper performance by focusing on the effect of mycorrhizal fungi and soil pH on juniper performance. We also investigated the effect of soil pH upon arbuscular mycorrhizal colonisation of juniper roots. The results of our study indicate that mycorrhizal fungi increases juniper performance and that this symbiosis becomes increasingly important to juniper performance over a gradient of increasingly acidic soil conditions. We also discovered that arbuscular mycorrhizal colonisation significantly reduces under such acidifying soil conditions.

There was a consistent increase in juniper growth in the inclusion treatment which was accentuated at a lower soil pH. This provides support to our hypotheses that mycorrhizal inclusion treatments would result in increased juniper performance, and that there would be a compounding negative interactive effect of decreased soil pH and mycorrhizal exclusion on juniper growth. It was important to determine whether there was a positive interaction in juniper as studies into mycorrhizal-plant interactions across varying species have shown that not all plant species respond with improved performance due to mycorrhizal fungi symbiosis (Koske & Gemma, 1995; Siqueira & Saggin-Júnior, 2001). However, our study findings relate to the vast majority of mycorrhizal-plant interactions in

which similar increases in plant growth have been reported (Fisher & Jayachandran, 2002; Mohandas, 2012; Bona et al., 2017).

Increased uptake of phosphorus, detected in the inclusion treatment by measuring juniper needle phosphorus concentration, directly correlated to an increase in plant growth. Alongside high root colonisation this would suggest that phosphorus is limiting growth at our field sites rather than carbon (Schroeder & Janos, 2005). These findings support a link to the increased abundance of nitrogen and carbon in soils following nitrogen deposition despite our investigation's focus upon soil acidity. The results of higher phosphorus uptake in the inclusion treatment also allows us to understand our root colonisation results.

Our findings of no significant effect of core treatment on mycorrhizal fungi root colonisation conflict with those of Johnson, Leake and Read (2001) and what we initially expected. It also posed the question of how we could confirm that our exclusion treatments were restricting the beneficial effect of mycorrhizal fungi. A possible explanation for this conflict is the root colonisation scoring method used in our study (McGonigle et al., 1990) which has come under recent scrutiny as it overestimates colonisation and is unreliable between observers (Kokkoris, Pogiatzis & Hart, 2019). Despite our colonisation results, the increased uptake of an immobile nutrient such as phosphorus in the inclusion treatment indicates connectivity to the mycelial network in the surrounding soil. This is clearly witnessed in the increased phosphorus concentration in the inclusion treatment while this connectivity and increased phosphorus uptake has been prevented in the exclusion treatment due to weekly severance of hyphae passing through the core membranes.

The reduction of arbuscular mycorrhizal root colonisation of juniper under acidifying conditions found in this study reinforces that of previous studies (Van Aarle, Olsson & Soderstrom, 2002) and could be due to a change in mycorrhizal community structure selecting for different mycorrhizal fungi (Van Diepen, Lilleskov & Pregitzer, 2011). Mycorrhizal fungi species provide varying performance increases to different species of plants and it is possible that a change in the mycorrhizal community structure could confer differing levels of performance enhancement to junipers.

While investigating the effect of soil pH upon juniper growth we only found an interactive effect of this with treatment. In mycorrhizal fungi inclusion treatments growth increased more at lower pH soils in comparison to the exclusion treatment. Alongside a decrease in arbuscular mycorrhizal colonisation at lower soil pH, this could suggest that as arbuscular mycorrhizal colonisation decreases other mycorrhizal fungi, such as ectomycorrhizal fungi, that are more beneficial to juniper colonise the roots.

Indications that the mycorrhizal community structure could be altering in response to soil acidification are concerning due to the ability of mycorrhizal fungi to limit invasive species growth (Klironomos, 2003), and the alteration of the mycorrhizal community caused by invasive species themselves (Zubek et al., 2016). The current management at these sites involves the limited use of liming. However, such a management strategy is unlikely to confer immediate remedial benefits for juniper as their growth is currently higher at lower soil pH. A long term strategy for management should revolve around the reduction of nitrogen deposition as continued deposition of nitrogen will cause an increase in nitrogen and carbon available in the soil and promote constant soil acidification.This could result in soil acidification pressure at a lower soil pH than tested in this study and the removal of mycorrhizal fungi beneficial to juniper populations while providing invasive species with performance benefits over a longer period of time (Klironomos, 2003; Zubek et al., 2016).

In response to the findings of this study we recommend continued political pressure to reduce nitrogen deposition from industry in Western Europe with a focus on agricultural practice and combustion emissions (Fowler et al., 2015). We also advise further investigation of the effect of nitrogen deposition on mycorrhizal community structure in these landscapes and on juniper performance over varying life-stages of the species.

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