Final Report

Improving the functioning of “Land van Ons” soils by inoculation with well-functioning donor soils to support the growth of highbush blueberry (*Vaccinium corymbosum x “Reka”*)

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Abstract

Intensive management leads to a decline in soil functioning and biodiversity. The *Land van Ons* citizens initiative is looking into enhancing farmers profit and biodiversity in the "Oud Ade" region. One of their plans is to make a berry plot that will host highbush blueberries (*Vaccinium corymbosum).* The research project at hand looks at the possibilities to promote soil functioning and target vegetation growth by inoculating intensively managed peat soils from "Oud Ade" (topsoil and lower peat) with well-functioning donors from a forest, a heathland, and a grassland ecosystem. It seemed as if the donor soils lead to a higher root colonization by beneficial mycorrhiza, yet there was little effect on the plant growth. A slight increase might be visible regarding the number of leaves the blueberries developed when inoculated with forest soil, which could correlate to a higher root colonization. The soil respiration measured was elevated in the higher peat soils, which were expected to have a more active microbiome compared to the lower peat. Compared to the control treatments an inoculation with either donor also seemed to increase soil respiration in most cases. Overall, no clear conclusions could be drawn and further research, especially into the soil functioning, is advised. However, there is evidence indicating a slight beneficial effect of the inoculation on both the soil and the blueberry plant. Inoculating especially the lower peat with, for example, the grassland soil could be considered when creating the berry ridges in "Oud Ade".

Table of Contents

List of Figures:

List of Tables:

Title page picture:

Blueberry Botanical Illustration – Douglas Schneider

1. Introduction

Soils are an important part of the ecosystem that host and support global biodiversity. They provide food and other agricultural goods and act as a carbon sink. Furthermore, they are an important part of the global nutrient and water cycles (van Leeuwen et al., 2019). Over the years intensive land management lead to a decrease in soil biodiversity and functioning. Current estimations indicate that about a quarter of soils worldwide are degraded (Wagg et al., 2021).

Soils store about four times the amount of carbon that is present in all vegetative material, making them the third biggest carbon sink after geological matter and the oceans. Carbon is found in either inorganic material (e.g. limestone) or soil organic matter (e.g. plant roots and litter, soil biota). In total, soils store about 3150 Pg of carbon, most of which (1500 Pg C) in the topsoil, in up to 1m of depth (Bell & Lawrence, 2009).

Next to storing, soils also emit carbon, usually in form of CO_2. This process also referred to as soil respiration, has several sources including roots, soil fauna, the rhizosphere as well as microbial respiration as a result of dead organic matter degradation (heterotrophic respiration) (Xu & Shang, 2016).

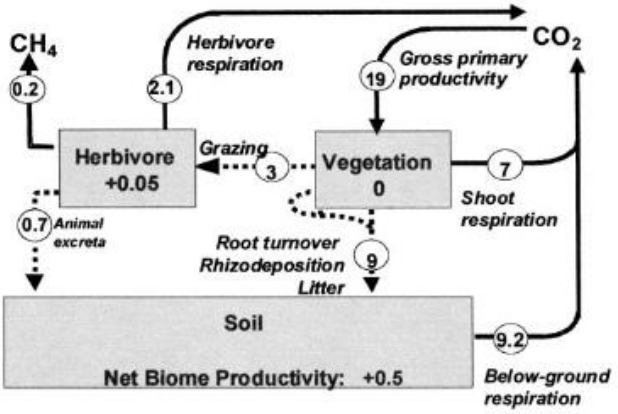

Figure 1 - Illustration of the carbon cycle in a grazed temperate grassland (Soussana et al., 2004)

As indicated in Figure 1 above, below-ground respiration is a considerable contributor to atmospheric CO2 levels and the global and regional carbon cycle (Soussana et al., 2004). It is estimated that annually soils emit 10-times more CO_2 than the burning of fossil fuels does (Phillips & Nickerson, 2015). Overall, the whole process of soil respiration is complex and poorly understood, yet it is a crucial part of the global carbon cycle and relevant when predicting future climate (Xu & Shang, 2016).

Intensive agricultural practices, as applied in the Netherlands from the 1960s onwards, lead to high production rates due to monocultures and the use of pesticides but caused a decrease in biodiversity (Wubs et al., 2016, 2018). The permanent meadow grassland, representative of a Dutch landscape, is often poor in biodiversity and the soils left in an unfavourable condition. (*Ons Plan – Land van Ons*, n.d.).

When a natural grassland is turned into an agricultural plot, it loses 25 – 30% of its carbon stock, a process that is sped up by tilling and growing crops. Converting agricultural land into a grassland has the reverse effect by sequestering carbon at an average rate of 0.5 tC ha^{-1} yr^{-1}. The build-up of soil carbon is consequently much slower than the losses that occur when disturbing a natural ecosystem (Soussana et al., 2004).

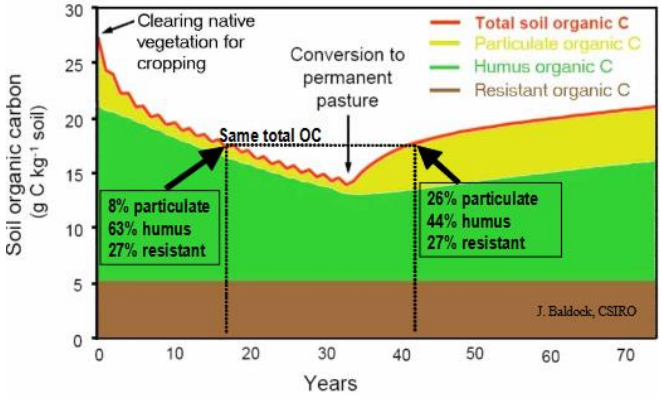

Figure 2 - Loss and recovery of soil organic carbon, J. Baldock, CSIRO (Bell & Lawrence, 2009)

Next to the losses of their carbon stock, intensively managed soils also show a decline in soil biodiversity (Wagg et al., 2021). While it is known that a decline in plant biodiversity impacts soil- and ecosystem functioning, the role the soil microbiome plays is less understood (Hannula & Träger, 2020; Wagg et al., 2021). Evidence is indicating that a diverse microbial community has a positive and stabilizing effect on ecosystem services such as plant biodiversity, nutrient cycling or carbon assimilation (Wagg et al., 2021). As the soil communities and plant diversity are tightly linked, restoration of degraded ecosystems can be assisted by the manipulation of the soil biodiversity (Wubs et al., 2016).

When a degraded soil is left to recover it will go through a process of moving from early succession species to the later succession species whilst increasing in biodiversity both above and below ground. This process can be sped up by inoculating with a soil from the desired target ecosystem such as grassland. Studies have shown that the donor soil will favour its representative vegetation (Wubs et al., 2016).

Land van Ons (Dutch: "Our land") is a citizens initiative which aims to increase biodiversity, improve the soils and local farmers profits while decreasing greenhouse gas emissions. They are buying up land to extensify agriculture and turn the large monocultures and fields into smaller parcels with agricultural practices closer to natural conditions, while also taking actions to actively enhance biodiversity (*Ons Plan – Land van Ons*, n.d.). The research focuses on an area close to the Dutch city of Leiden, where the *Land van Ons* initiative bought up 33ha of former agricultural fields, referred to as "Oud Ade". Together with *Leiden University*, they started a 10-year project on enhancing biodiversity in the area in September 2021. To this date, baseline measurements were taken, and plans are being made on how to shape the area (*Oud Ade – Land van Ons*, n.d.).

One of the goals of the *Land van Ons* initiative is to create ridges that host berry plants, including highbush blueberry (*Vaccinium corymbosum*). These are of high market value and consumer interest, especially when sustainably farmed (Yu et al., 2020). Organic blueberry farming represents some challenges as practices like crop rotation and mechanical weed control are not feasible (Drummond et al., 2009; Yu et al., 2020).

Both in natural and cultivated conditions, blueberries form symbiotic relationships with ericoid mycorrhizal fungi (EMF) (Kasurinen et al., 2001; Scagel, 2005). The group of taxonomically diverse fungi colonizes the roots of ericaceous plants, forming hyphal coils inside the cells (Kasurinen et al., 2001). EMF have the ability to break down organic nitrogen-rich compounds such as amino acids or peptides and facilitate the uptake of nitrogen by the plant (Yang et al., 2002). The benefits the plant has from this relationship are greater than the costs of hosting the fungi. Next to nitrogen, EMF can also assist with the uptake of phosphorus and enhance plant resistance against toxic compounds such as aluminium (Kasurinen et al., 2001). Ericoid fungi are common in heathland soils supporting plants in the often rather acidic and nitrogen-poor environment (Kasurinen et al., 2001; Scagel, 2005). DNA metabarcoding of the *Land van Ons* soils revealed the presence of three ericoid mycorrhizal fungi species, namely *Oidiodendron maius*, *Penzoloma ericae* and *Byssoascus striato*.

The goal of this study is to improve soil functioning and the growth of highbush blueberry as a target species by introducing specifically selected soil microbial communities. They are thought to increase carbon sequestration as well as significantly increase the growth of highbush blueberry. This could potentially reduce future fertilizer and pesticide input and allow for a more sustainable agriculture while supporting soil health and functioning.

2. Research Question

Facing the issues of decreasing soil biodiversity, the wish to enhance and diversify profit and production in the "Oud Ade" and the challenges regarding organic blueberry production mentioned above, lead to the following research question:

Is it possible to improve Land van Ons soil functioning and target vegetation growth via soil inoculation from well-functioning donor soils?

This study also aims to bridge the gap between soil and plant sciences regarding the effect of microbial communities: while in plant sciences the focus is commonly on one single microbial taxon that enhances growth or suppresses diseases, soil scientists have started to acknowledge the importance of microbial communities for biodiversity. The research could help to pave the way to a more sustainable agriculture by utilizing and shaping soil biodiversity to enhance crop production.

The effect on the peat soils will most likely vary: the higher peat is expected a more active microbiome and a higher nutrient content compared to the lower peat soils. Hence, the effect of the inoculation and differences between treatments will probably be more pronounced in the low peat groups, due to the lack of competition and the reliance of the plants on the microbiome for nutrients. It is hypothesised that the low peat control group will show limited growth, biomass generation and root colonization, as fewer microbes are expected in the soil. The soil collected from the Boterhuispolder, an area of the "Oud Ade" that is less disturbed and more representative of a natural grassland, is probably going to support growth, biomass and colonization but less when compared to the heathland and the forest inoculate due to the increased presence of EMF.

The high peat soils on the other hand are expected to show a limited response to the donor, as their microbiome is already active and thriving and will probably compete with the introduced communities. Together, with the higher nutrient availability it will most likely lead to smaller differences between treatments. The high peat control treatment is expected to show better growth and biomass production and a higher colonization than the low peat control.

Furthermore, the CO_2 values measured with the soil respiration are expected to be higher for the topsoil when compared to the low peat groups. Again, an increase from the control to the grassland and finally heathland and forest donor is expected.

3. Materials and Methods

3.1. Experimental setup

General Setup

The experimental setup is loosely based on Silva et al. who performed an experiment on the growth promotion of highbush-blueberry by fungal and bacterial inoculants in 1996 and 1997, *Gliocladium virens* was shown to have a beneficial effect on leaf area when compared to control treatments (Silva et al., 2000).

The experiment takes place in the nursery garden of the Hortus Botanicus in Leiden (52°09'21.1"N 4°29'06.1"E) where the blueberry plants are grown for 15 weeks (end of March until the beginning of July 2022).

The plants are potted in 20L white plastic buckets that are fit in holes to keep the soil temperature representative of natural conditions. Each bucket has drainage holes on the bottom followed by a layer of clay pebbles to avoid the loss of soil. The pots are arranged in an order determined by a random-number generator to minimize bias. The arrangement is changed once to minimize bias due to light conditions.

For the experiment commercially available highbush "Reka" blueberries (*Vaccinium Corymbosum x* Reka) have been chosen. "Reka" blueberries are known for their productivity and quality fruit that have a long shelf life. A study on blueberry farming in the Netherlands, performed in 1998 by J. Bal and his team, recommended "Reka" in particular for commercial open field cultivation (Bal et al., 2006).

The plants used in this study were organically farmed and purchased from *Natural Bulbs* (Hillegom, the Netherlands) and delivered in high-quality potting soil on the 16th of March. They were stored in a pre-determined random order on upside-down plastic buckets to protect them against snails until potting. All blueberry plants are the same age and developmental stage. Notes were taken of possible damage that occurred during transport on the 17th of March.

Soil collection and potting

Peat soils were collected on the 22nd of March close to a future Agroforestry plot in the "Oud Ade" fields (52°11'30.8"N 4°33'18.2"E). The soils have been intensively managed in the past and are now target of restoration. The soil from the upper level hereby referred to as "high peat" (short: "H") is comprised of any layers present between 0-30 cm in depth. Anything extracted from 30-60 cm in depth is labelled as "low peat" (short: "L").

About 10L of forest soil and the heathland soils were collected on the 17th of March close to Wageningen, in an area that is part of other scientific studies and has been sequenced previously. On the 25th of March the grassland soil was collected from the Boterhuispolder (52°11'05.0"N 4°32'20.2"E). It is important to note that due to transport complications the sampling location was changed from the Lakerpolder to the Boterhuispolder. As the labelling had already been established, the Grassland/Boterhuispolder samples are referred to as "L".

On the 24th and 25th of March, the experiment was set up and the blueberries were potted. Each bucket was filled with approximately the same amount of high or low peat respectively. The inoculation of all treatments took place on Friday, the 25th of March. Each donor soil was measured out in the transportation pot of the blueberries to equalize the amount of donor soil added to each treatment. The donor soil was mixed into the peat by hand before re-potting the blueberry plant.

3.2. Measurements

Plant measurements

Every two weeks, starting from March 28th, the plants are measured in size (root crown – end top shoot) and their leaves, buds, flowers and fruits are counted. Furthermore, records are taken of potentially damaged or sick leaves as well as the presence of parasites. A photo was taken of every plant and stored in a OneDrive folder for further reference, if required.

Before potting, a root sample was collected from one randomly selected plant of each treatment and stained using Trypan blue to investigate for the presence or absence of ericoid mycorrhizal fungi. This test was repeated in June with a root sample from every plant. Using the gridline intersect method, the colonization percentage was determined (Giovannetti & Mosse, 1980; Kasurinen et al., 2001).

Soil Measurements

Soil samples were taken twice during the experiment, once in week 2 (April 7th) and once in week 10 (May 30th). The samples were stored in the fridge until further usage. The pH was determined from each sample and a Chloroform fumigation was performed on the week 10 samples on the 2nd and 3rd of June. The results of the chloroform fumigation are not included in the report.

A soil respiration experiment was performed three times: once in Week 5 (April 25th- 26th), in week 9 (May 23rd – 24th) and one last time at the end of the experiment in week 13 (June 20th – 21st). Gas samples were collected for 24h starting from 10am and were analysed with a gas chromatograph.

Furthermore, all soil samples were assessed for their moisture content and an unsuccessful attempt soil organic matter content with the loss of ignition (LOI) test was performed. (Hoogsteen et al., 2015).

4. Data Treatment

The data will be collected in Microsoft Excel in a "tidy" format and transferred to *RStudio 4.1.1* for further data treatment. The data was plotted, and potential models investigated before performing statistical tests.

All models were checked for normally distributed residuals using the *shapiro.test()* function and for equal variance using the non-constant-variance test (*ncvTest()*). Some required transformations to fit the assumptions of normally distributed residuals and homoscedasticity. The according formula was identified with the *boxcox()* function from the R "MASS" package and is mentioned in the explanations of the results. In general, a *log* transformation was used to correct for unequal variance, a *1/y* transformation to adjust for not normally distributed residuals and a square root transformation in the case that both of the assumptions were violated.

Due to the small number of replicates, it was generally avoided to remove outliers but instead accept them as part of the natural variation. This is valid for the plant measurements such as size or number of leaves. Exceptions were made for the soil respiration dataset, as outliers are potentially due to measuring mistakes or errors in the experimental setup. Outliers were investigated visually with the *influenceIndexPlot()* function and statistically with the *outlierTest()*.

The Akaike Information Criterion (AIC) was used to compare models. This was done to, for example, justify the transformation of the data or the inclusion of "time" as response variable. The model with the lowest AIC score was chosen as it explains the most variation with the lowest number of variables.

In most cases, the treatment groups were compared using a factorial ANOVA with the peat and donor soil as categorical explanatory variables, while also including their interaction term. For the growth and biomass generation (leaves and fruit) a curve was plotted by including time as a continuous explanatory variable into the model. The curves were analysed using an ANCOVA.

In case of statistically significant results a post hoc test (*TukeyHSD*) was performed to investigate the differences between treatments further.

For the plant growth, biomass production, root colonization and soil respiration t-test were performed to investigate the differences between the inoculated and the control plants. These results are of questionable statistical quality due to the unequal sample sizes (not inoculated n = 10, inoculated n = 30) but are used to get an indication whether the soil inoculation treatments had any effect on the response variable.

5. Results

Soil pH

The soil pH was measured twice, once from the samples collected in April and once from the samples collected at the end of May. Blueberries prefer soils with a pH of 5.5 or lower. The pH measurements ranged between 4.66 and 5.58, respectively, creating an environment suitable for the berry plants. The results are visualized in Figure 3 and Figure 4 below.

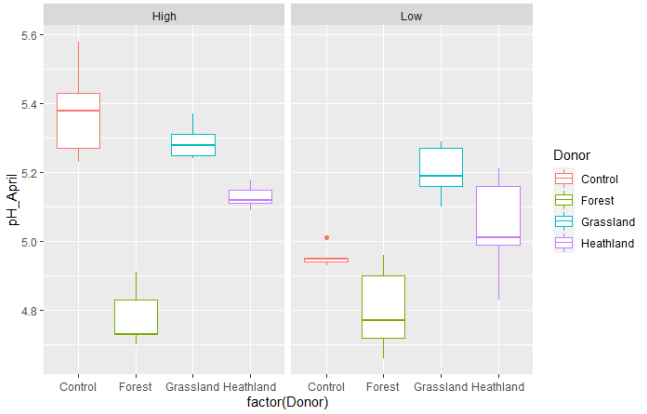

Figure 3 - Boxplots of the pH values from the April soil samples

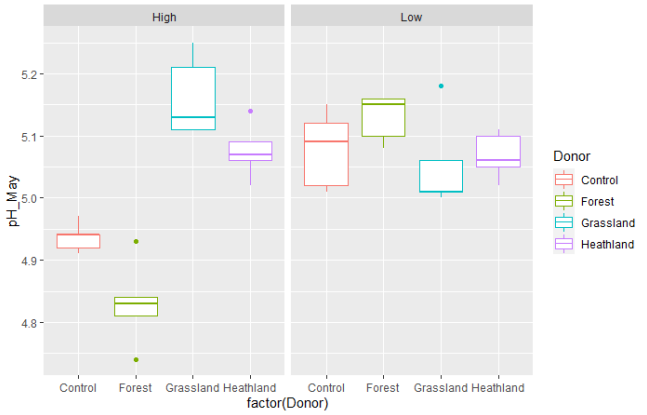

Figure 4 - Boxplots of the pH values from the May soil samples

An ANOVA on both data set revealed a significant effect of the donor (April: $p = 4.1^{-11}$; May: $p = 2.64^{-5}$) the peat soil used (April: $p = 5.1^{-5}$; May: $p = 4.32^{-5}$) as well as the interaction of both (April: p = 0.000108; May: $p = 5.9^{-9}$). The extremely low p-values for the analysis point to potential overfitting of the data, due to the inclusion of the interaction between donor and peat soil. Taking out the interaction term yields lower p-values for both the effect on the donor soil (April: $p = 1.49^{-8}$; May: p = 0.0239) and the peat (April: p = 0.00115; May: p = 0.0128), which are more realistic and remain significant. The deviation between the treatments regarding the soil pH was not expected. However, being in a range suitable for blueberries the variation is pH is accepted. It might have impacted other factors such as the soil respiration but as the soil samples were not taken at the same time they can not be related reliably.

Plant Growth

Measured every 14 days, the growth data contained continuous data points over a period of 15 weeks from April until July of 2022. In Figure 5 the total growth was plotted. This was calculated by subtracting the blueberries initial size (week 1) from the final size (week 15).

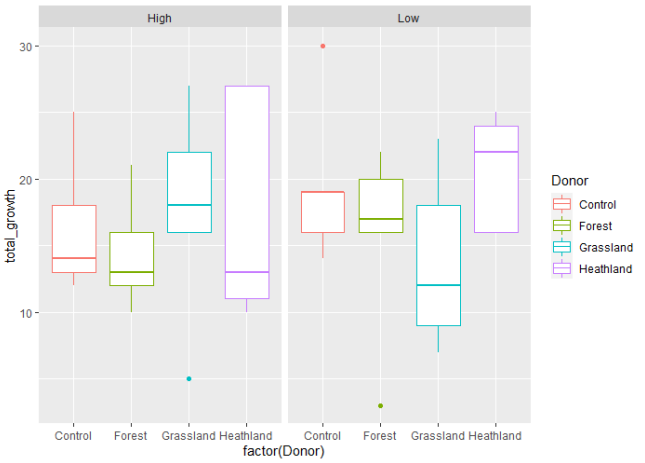

Figure 5 - Total plant growth, boxplot

A factorial ANOVA was performed on the dataset. The model tested for the effects of peat soil (p = 0.67), donor soil (p = 0.47) and their interaction (p = 0.61) yet none of the values was significant.

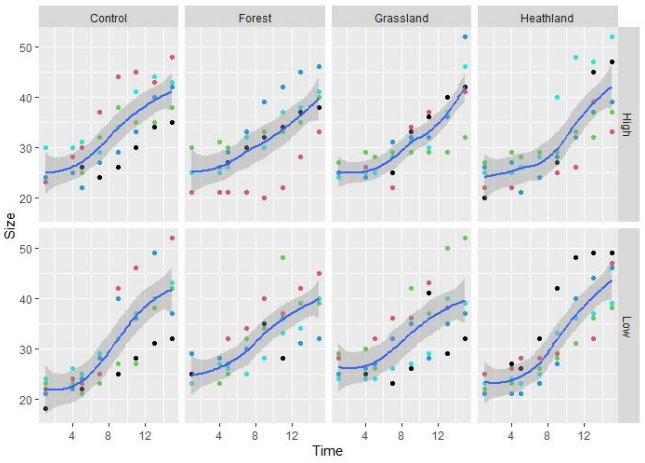

Figure 6 - Plant growth plotted over 15 weeks of observation

In addition to the total growth, the increase in size over time was plotted (Figure 6) and the resulting curves analysed using an ANCOVA. The data required a transformation (*1/y*) to fit the requirement of normally distributed residuals. The analysis yielded significant results for the effect of the peat (p = 0.005) and the interaction of peat and donor (p = 0.02). The only near significant p-value revealed by the post hoc test of 0.09 indicated that the low-peat-control treatment might be growing faster than the high-peat-control treatment.

A t-test on the effect of inoculation as such determined no significant difference (p = 0.84) in growth between the inoculated and the control treatments.

Biomass Generation

The biomass was measured by counting leaves, buds, flowers, old flowers as well as fruits every two weeks. The number of leaves (vegetative biomass) and fruits (reproductive biomass) were selected for further investigation.

<u>Vegetative Biomass</u>

Figure 7 below shows the increase in total number of leaves over the 15 weeks of observations. The occasional dips in the graph can be explained by branches that broke off or lice that consumed part of the biomass, causing a decrease in the total number of leaves.

Based on the graphs shown in Figure 7 a linear model was created, investigating the effect between the number of leaves based on the peat and donor soil used. The model was transformed with a square root function to fit the model assumptions of normally distributed residuals and homoscedasticity before further analysis with an ANCOVA.

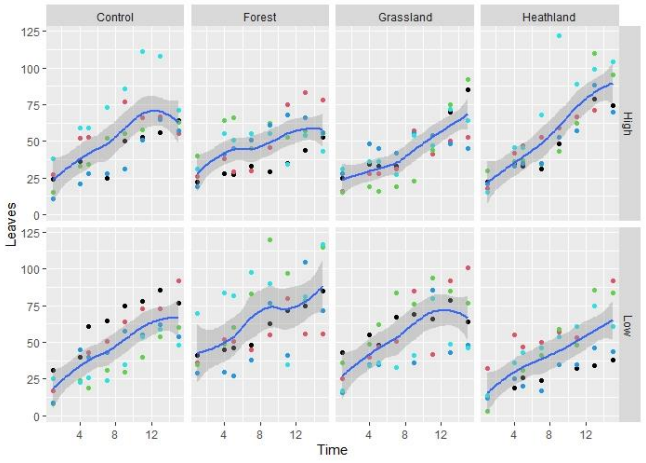

Figure 7 - Increase in the total number of leaves over the 15 weeks of observation

The results indicated a significant effect of the donor (p = 0.005) and the interaction between peat and donor (p = 7.341 e-10). Table 1 below lists the relevant p-values for the donor, and **Error! Reference source not found.** the relevant p-values for the donor and peat interaction.

Table 1 - Post Hoc test on the vegetative biomass ANCOVA, relevant p-values for the donor and donor-peat interaction

Donor	**Difference**	**Adjusted P-value**
Forest – Control	0.521	0.019
Forest – Grassland	0.525	0.018
Forest – Heathland	0.583	0.006
Donor:Peat	**Difference**	**Adjusted P-value**
LF – HC	0.935	0.006
LF – LC	1.249	0.0000327
LF – HF	1.141	0.000229
LF – HL	1.520	0.0000001
LF – LH	1.676	0.0000000
HL – LL	-0.849	0.019
HL – HH	-0.887	0.012
LH – LL	-1.004	0.002
LH - HH	-1.042	0.001

There appears to be a significant difference between the forest and control treatments, with the forest plants having more leaves on average. The same goes for the comparison between the forest and grassland and the forest and heathland soils.

Looking into the interactive effect, it appears as though the plants in the LF treatment group have more leaves on average than in other (HC, LC, HF, HL, LH) treatment groups. It seems as if the effect of the forest donor is more pronounced in the low peat soil. The high-peat-grassland and the low-peat-heathland plants have fewer leaves on average than the low-peat-grassland and high-peat-heathland plants.

Reproductive Biomass

Figure 8 below, shows the number of fruits plotted over time. Both, the high-peat-grassland and low-peat-heathland treatments show one plant (HL1 and LH1) with an outstanding number of berries.

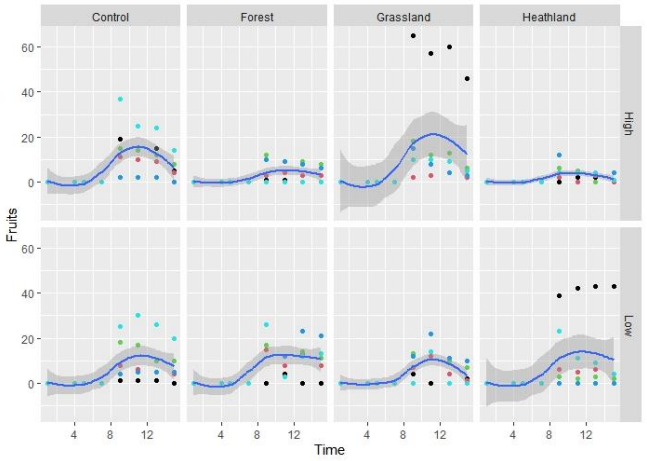

Figure 8 - Increase in the total number of fruits over the 15 weeks of observation

The analysis of the reproductive biomass was much like the analysis of the vegetative biomass. The data required transformation with a square root function to fit the assumption of normally distributed residuals and homoscedasticity. An ANCOVA on the dataset indicated a significant effect on the donor soil ($p = 0.0007$) and the donor and peat interaction ($p = 0.005$).

Looking into detail with the post hoc, the HL treatment had significantly more berries than the HF treatment ($p = 0.04$) and the HH treatment ($p = 0.01$). It is possible, however, that this effect is due to the replicate (HL1) which had an exceptionally high number of berries.

A t-test was performed to investigate the effect of inoculation, regardless of donor or peat, on the plants but found no significant effect on the number of leaves ($p = 0.45$) or fruits ($p = 0.46$).

Root Colonization

Before potting a root sample was taken from one randomly selected plant of each treatment. Using trypan blue, the root samples were stained, making the fungal hyphae visible under a light microscope. It was evident that all eight initial samples were already colonized.

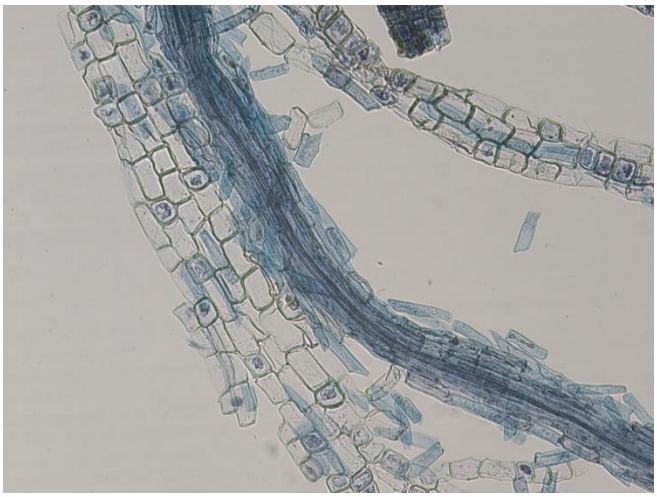

Figure 9 - LF3 March sample, colonized cells, x10 magnification

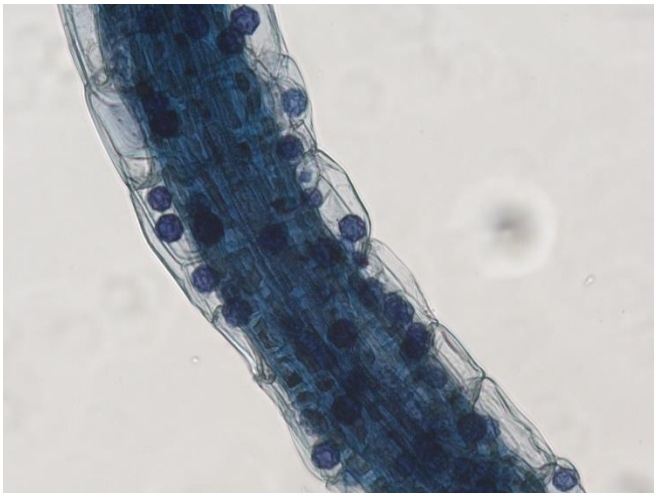

Figure 10 - HH4 March sample, bulbous fungi, x20 magnification

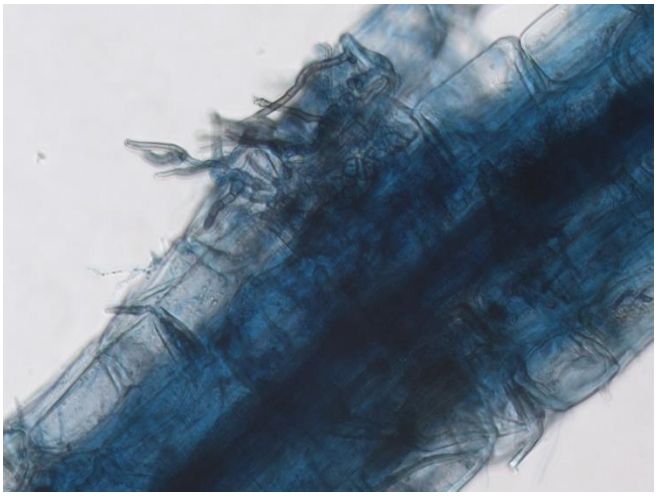

Figure 11 - HF3 March sample, mycorrhizal hyphae, x40 magnification

Most common were hyphae coils inside the cells (Figure 9) or round bulbs (Figure 10). In some places, hyphae between and outside the cells were visible (Figure 11).

In week 12 of the experiment, the staining was repeated with root samples from all plants. Using an adapted version of the Gridline Intersection Method, the colonization percentage was calculated (Giovannetti & Mosse, 1980; Kasurinen et al., 2001).

Figure 12 below visualizes the differences in form of a boxplot. It seems as if the control treatments show a slightly lower percentage of colonization, while with the donor soils the colonization percentage varies. This hypothesis is supported by a t-test investigating the difference between the inoculated and not inoculated plants regarding root colonization. With p = 0.02 there is sufficient evidence to conclude that the control treatments show a lower rate of colonization when compared to the inoculated treatments. It is therefore very likely that the treatment with donor soil leads to a higher level of root colonization by ericoid mycorrhizal fungi.

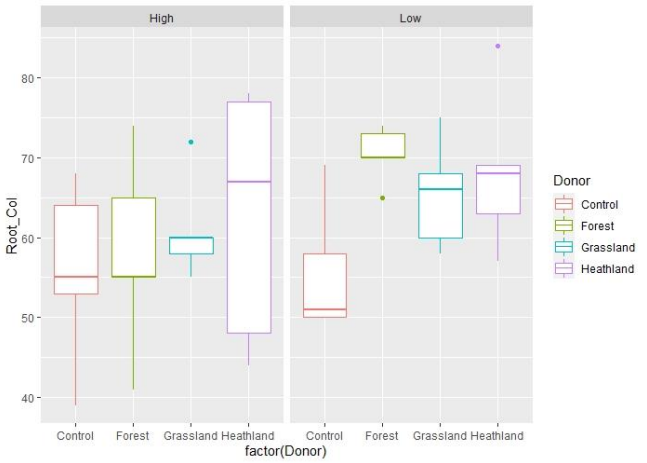

Figure 12 - The colonization percentage by treatment group, boxplot

The data was analysed with a factorial ANOVA but no significant differences between the treatments were found, not for the donor soil (p = 0.14), the peat (p = 0.09) or the interaction between both (p = 0.57).

The post hoc test did not reveal any significant p-values for the comparison of different donors or the combinations of peat and donor. Interestingly, the effect of the peat soils, indicated as near significant with p = 0.09, points to a higher rate of colonization in the low-peat groups.

Soil respiration

Three soil respiration samples per plant were collected at the end of April, May and June. As the temperatures and weather conditions differed between the collection days, no comparison between the different months is possible. The samples are only compared between the treatment for each sampling point.

Table 2 - Weather conditions during soil respiration sampling, data from AccuWeather for Leiden, NL

Day	Average Temperatures	Weather Conditions
April 25th – April 26th	10°C	rainy
May 23rd – May 24th	15°C	rainy
June 20th – June 21st	15°C	dry, partly sunny

The boxplots below show the soil respiration values, normalized by subtracting the blank and to g CO_2 per m^2 of soil per hour.

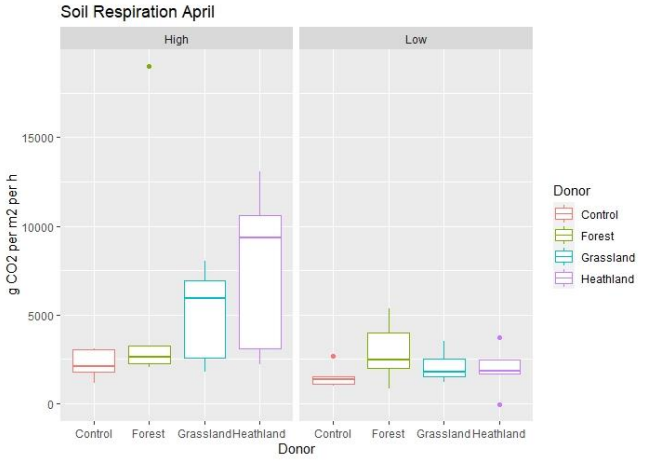

Figure 13 - Soil respiration April, boxplot

Figure 13 shows the values collected during the April soil respiration sampling. Observation LH2 (-59.81 $gCO_2/m^2/h$) was removed as the value was negative after normalization. This is most likely a measurement error and was regarded as an outlier. Furthermore, the HF2 value was removed, as it was very high (19021.84 $gCO_2/m^2/h$) and flagged as an outlier by R. In addition to that, the dataset required a log-transformation due to non-constant variance. The *ncvTest()* remained significant with p = 0.02 but this was accepted.

A factorial ANOVA indicated a significant effect of the peat (p = 0.004) and the donor (p = 0.02) on the soil respiration. The low peat has, on average, lower soil respiration values than the high peat and there is evidence indicating that the heathland donor leads to higher soil respiration values.

More specifically, the HH treatment had higher soil respiration values when compared to the HC treatment (p = 0.06). And the LC treatment had lower respiration values, on average, than the HL (p = 0.06) and HH (p = 0.04) groups.

A t-test indicated a significant effect (p = 0.05) of the soil inoculation when compared to no inoculation, for the soil respiration April dataset.

In Figure 14 the values for the May soil respiration sampling are visualized. The model required a log-transformation and the observations for HF2 (1420.39 $gCO_2/m^2/h$), HL4 (2375.47 $gCO_2/m^2/h$) and LC1 (2142.28 $gCO_2/m^2/h$) were removed as they were flagged as outliers by R.

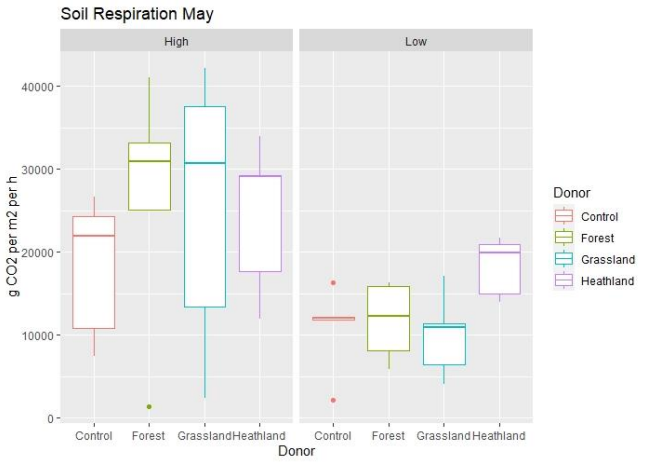

Figure 14 - Soil respiration May, boxplot

The factorial ANOVA returned a highly significant p-value (p = 1.9e-05) for the effect of peat and p = 0.008 for the interaction between peat and donor. Evidence indicated that the soil respiration values were lower for the low peat soils when compared to the topsoil. This trend can also be seen when looking at the interaction between peat and donor, with the HF treatment having higher soil respiration values than the LF treatment (p = 0.008) and the LL treatment (p = 0.001). The same goes for the respiration values measured for the HL group when compared to LF (p = 0.006) and LL (p = 0.001) and the high peat heathland group compared to the low peat grassland group (p = 0.01).

A t-test comparing the inoculated and not inoculated treatments showed no significant results for the effect of soil inoculation on the soil respiration for the May dataset (p = 0.15).

The June respiration samples, as shown in Figure 15 were treated in much the same way as the April and May values were. The model required a log-transformation and the observation for LF5 (19875.78 $gCO_2/m^2/h$) was removed after being indicated as an outlier by R.

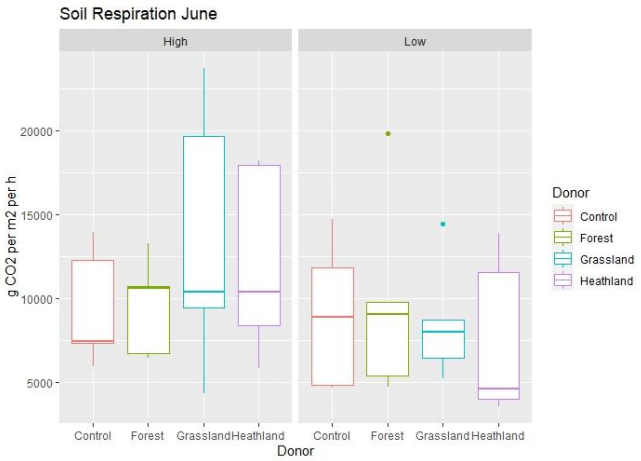

Figure 15 - Soil respiration June, boxplot

The factorial ANOVA performed on the June dataset yielded a significant p-value of 0.05 for the effect of the peat soil. Again, the low-peat treatments appeared to have lower soil respiration values, on average, when compared to the high-peat treatments.

Looking into detail at the different treatments, the LC had lower respiration values than the HH (p = 0.004) and HL group (p = 0.06). A similar trend showed when comparing the LL to the HH treatment (p = 0.04). The comparison of the HC and HH group showed a near significant p-value of 0.06, indicating higher soil respiration for the inoculated soil. This trend was not seen when performing a t-test on the inoculated and uninoculated soils, however, as it yielded a p-value of 0.5 and therefore remained not significant.

6. Discussion

In a study similar to the one described in this paper blueberry saplings were inoculated with different fungi to test their growth-promoting abilities. This ultimately led to an expansion in leaf area and an 80% increase in the number of leaves for the groups treated with *G. virens* (Silva et al., 2000). Contrary to the setup at hand, where young bushes arrived in high-quality potting soil, the team around A. de Silva was working with bare-rooted and 9-month-old blueberries. In addition to that, the use of pasteurized soil and a greenhouse setup were mentioned in the article (Silva et al., 2000).

Another study looked at the effect of mycorrhiza and soil amendments on highbush blueberry growth and nitrogen uptake. The experiment was performed with sterile soil and plants and the inoculate (*O. maius*) a pure laboratory culture (Yang et al., 2002). The same species was detected via metabarcoding in the LvO soils.

In their article Yang and their team mention that across different studies the effect of ericoid mycorrhizal inoculation on blueberry growth varies, which they attribute to the lack of controls as well as the sourcing of the cultures. Overall, they observed a dry weight increase in the inoculated treatments, a parameter that was not tested in this study. This, they explain with the increased nutrient availability due to the infection (Yang et al., 2002). In their article de Silva and their team described the increase in biomass promoted by *P.flourescens Pf5* as non-significant in non-pasteurized soils, which they attribute to competition from other soil microbiota, unfavourable growth conditions and limited root-colonization abilities (Silva et al., 2000).

Both Yang and de Silva point out difficulties when working with unpasteurized soil as the effects on plant growth are not clearly visible or statistically supported (Silva et al., 2000; Yang et al., 2002). It is very likely that the inconclusive evidence derived from this study had similar causes.

Possibly the microbiome in the peat in combination with the donor soil microbiota competed with each other and lead to inconclusive and statistically non-significant results. Also, the blueberry bushes used in this experiment were already inoculated with ericoid mycorrhiza, as was detected in the first root-staining study. Most likely they carried those microbes to their treatment soils, despite the disinfection with H_2O_2 prior to potting. It is possible that the existing microbiome in the blueberry roots and the soil were able to sustain plant growth adequately and no inoculation would have been needed in the first place. This could be deduced from the limited statistically relevant results when comparing the growth or biomass production of the control treatment with the inoculated soils. Furthermore, since the plants were older than 9 months and on the verge of blooming, all their buds and hence the basis for flowers and fruits had already been produced, independent from the donor soils they received. In combination, this could have had a bigger effect on the measurements and results than the actual soil treatments applied. As described previously, there was a significant effect of the donor and the peat on the soil pH. As blueberries prefer acidic soil with a pH of 5.5 or lower, it was within acceptable limits but might have had a bigger impact on the growth and well-being than estimated. As the pH diverged between the two sampling points, it is not possible to reliably correlate it to any observation.

While the null hypothesis regarding the differences between root colonization rates could not be rejected, an interesting factor should be examined more closely: There seems to be a slight effect of the peat soil origin on the root colonization ($p = 0.09$) and looking at the data in detail, the low-control treatment shows a lower colonization than the donor-treatments. A study performed by Kasurinen and Holopainen on the root colonization of highbush blueberry compared to close relatives, namely bog whortleberry (*Vaccinium uliginosum*) and bilberry (*Vaccinium* myrtillus), found slightly lower colonization percentages of 40 – 50% for the highbush blueberry (Kasurinen et al., 2001). These values observed in this study were higher, around 50 – 60%, especially for the inoculated soil treatments, but around 50% for the control group grown in low peat soil. This observation is backed up by the significant difference between the average colonization of the control groups when compared to the inoculated groups. So regardless of the peat applied, there was a difference in the soils that were treated with different donors.

This does reflect one hypothesis stated at the beginning of the study, namely, that the effect of the donor might be highest in the low-peat group, as the soils have supposedly fewer microbes. This is supported by a study from Wubs et al. concluding that soil inoculation is a tool to enhance biodiversity which can be applied in the field. Its effect is most pronounced when the topsoil is removed (equivalent to the low peat group), but it does show an effect on the topsoil as well (Wubs et al., 2016).

Furthermore, it was also hypothesised that the forest soil contained the most beneficial microbes, followed by the heathland and the grassland soil. And looking at the boxplot of the colonization, the means follow the same trend. Additionally, it was observed that in the lower peat, which supposedly harboured fewer microbes, the effect of the forest soil, which was hypothesised to be most beneficial for the blueberry plant, was significant for the number of leaves developed. This is also reflected by the root colonization being the highest, with

around 70% on average in this treatment group. As the size did not show a clear significant difference among treatments, it is possible that the plant had a desire or need to expand photosynthetic capacities due to the costs associated with mycorrhizal infection. This result would align with the observation of increased leaf number (Silva et al., 2000) and the increased dry weight in inoculated treatments (Yang et al., 2002).

However, despite the evidence seemingly supporting the hypothesis that in the lower peat, the forest soil is leading to increased leaf growth compared to the other treatments, one should not disregard the possibility that the slight increase in leaf growth could be due to natural variation between the plants. An example can be seen in the observations of fruit numbers: looking at the high-peat and grassland donor treatment that was flagged as significantly different from some others, one plant developed much more berries than others, shifting the entire average. A similar observation is found in the low-peat and heathland donor treatment. One replicate developed an extraordinarily large number of fruit while others carried no berries. It is possible that the number of replicates in this study was not chosen correctly to account for the natural variation present in their population relative to the expected effect size of the treatment. Power calculations had been made when designing the experimental setup and based on the study of the growth-promoting abilities of inoculates on blueberries, an increase of 80% in leaves was expected (Silva et al., 2000). As the effect was much less pronounced, the power decreased in hindsight. For a future replication of this study, it would be advisable to either increase the number of plants or decrease the number of treatments.

The soil respiration that is measured on the surface originates from and is impacted by different processes, both biotic and abiotic. It includes root respiration (Rr, growth and maintenance of roots) which also includes rhizospheric respiration (Rz) from microbes living in and around the roots. Next to that, the heterotrophic respiration (Rh, released by microbes breaking down organic matter), soil fauna respiration (Rf) and non-biological sources of CO_2 (Rn) all impact the total CO_2 flux that is measured at the soil surface (Xu & Shang, 2016).

The differences in soil respiration seen among treatments in this study could be explained by varying microbial activity. A higher level of microbial activity leads to higher soil respiration values. Hence, the higher peat soils, which were expected to have more microbes than the lower peat, were showing overall higher values for the soil respiration. Looking at the differences between the control and the inoculated treatments, it seems as if the soil respiration, and consequently the microbial activity, is higher in the soils treated with either donor. No clear gradient or pattern related to the origin of the donor soil is visible. The hypothesis of higher microbial biomass in the inoculated soils and the high peat soils, in general, can potentially be backed up with a chloroform fumigation. While the experiment was performed, the results could not be analysed in time and are therefore not included in this report.

The higher levels of soil respiration within the donor treatments, though not fully statistically supported, could be seen as evidence of an improved soil microbiome and higher root respiration due to improved growth and mycorrhizal colonization (Xu & Shang, 2016). Even though the gas samples were collected away from the bulk root mass in the centre of the pots, it is possible that the root and rhizospheric respiration did impact the measurement. No statistical test has been performed, yet the similarities in root colonization percentage and the amount of soil respiration recorded should be pointed out. Along with the tendency that the root colonization was higher for inoculated treatments it could be deduced that by applying the donor soils, beneficial microbes were introduced to the soil.

On the other hand, the root respiration could also be higher because the donor soils contained more organic matter and hence increased the rate of heterotrophic respiration (Xu & Shang, 2016). Studies concluded a positive correlation between the amount of soil organic carbon present, and the basal soil respiration, regardless of climate (Luo & Zhou, 2006). Herby the kind of substrate provided could have made a difference, simple sugars, as are present in photosynthetic plant materials, are quickly degraded contrary to woody material rich in lignin or cellulose (Luo & Zhou, 2006; Xu & Shang, 2016). The type of carbon source present in the soil is hence just as important as the amount that is present. Based on visual clues alone, the forest donor soil stood out as having a variety of plant material from leaves to pieces of bark mixed in, while both the heathland and grassland donor were of a sandier texture and similar in colour. The soil organic matter content has not been tested successfully but determining the amount as well as the kind of carbon sources present could provide further inside into the soil respiration dynamics.

The overall increase in soil respiration from April to May and June can be related to a number of factors, including an increase in temperature of 5°c, which will boost microbial activity and is positively correlated with an increase in soil respiration (Luo & Zhou, 2006). Generally, the highest respiration values were recorded in May, when the weather was rather warm (15°c on average) and rainy. This could have benefitted the soil microbiome as the optimum moisture conditions are near the soil field capacity where the soluble substrate can diffuse freely but the conditions still allow for aerobic digestion (Luo & Zhou, 2006). Especially during

the May sampling, the weather was determined by heavy periods of rain which would have saturated the soil. The buckets that contained the peat were fitted with holes and a layer of clay pebbles to facilitate drainage so no water retention should have occurred, creating optimum conditions for the soil microbiome.

Tests regarding soil functioning were lacking in this study and a further investigation into that topic is strongly advised. This includes the analysis of the chloroform fumigation samples and the organic matter content of the soils. These results could assist in drawing conclusions on the soil functioning and the impact the donor soils had on that.

It is likely that working with sterile plants and peat soil would have caused a more visible effect of the inoculation. But since the goal was to conduct a field study looking at the soil- and plant functioning it is unfeasible to pasteurize either. The soil would have been disturbed and the conditions incomparable to the Land van Ons pasture. An alternative, cost-effective option to the soil inoculation would be potting newly obtained plants in the soil they are delivered in, as this is likely to contain a microbiome beneficial for the respective plant. The first root staining test showed a substantial amount of colonization in the blueberry roots which is likely from the nursery potting soil.

7. Conclusion

Overall, there is insufficient evidence to draw a clear conclusion on whether the soil functioning or target vegetation growth were improved via soil inoculation. The data suggests that there is a positive effect on leave growth when inoculating with forest soil on low peat. The total size or growth appear to not be affected by the inoculation. The root colonization is improved when inoculating with either donor soil and overall higher in the peat collected from the topsoil. There might be a difference in root colonization in the low peat groups that aligns with the hypothesis but without statistical support. Regarding soil functioning, it could be concluded that based on the higher respiration in the inoculated treatments and the differences between higher and lower peat the microbial activity is higher when the donor is applied. This, in combination with the higher root colonization in the high peat and inoculated treatments, might suggest that beneficial microbes were introduced. As soil respiration is dependent on a variety of factors such as the amount and source of carbon present in the soil further investigations are required.

While there seemed to be a difference in working with the higher or the lower peat soils in most cases, the origin of the donor appeared to have little impact. The lower peat soils with supposedly fewer microbes seemed more responsive to the inoculation. The higher peat soils on the other hand appeared to function and support the plants without manipulation of the ecosystem.

Overall, there still seemed to be a slight effect of the soil inoculation which was seen in the root colonization and the slightly elevated levels of soil respiration. Perhaps the effects measured in this experiment are too small to justify a large-scale treatment of the future berry ridges with forest or heathland soil, but a long-term observation of the legacy effects could be beneficial as there might be an effect on next years harvest. Especially if the plants are grown on low peat, soil inoculation may be beneficial in the long run. As the grassland donor did seem to benefit the soil and the plants, an inoculation with the soil from the Boterhuispolder may be sufficient. This would also cut down on transport costs and legal work regarding the removal of heathland or forest soil, while still promoting soil functioning and plant wellbeing.

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