Plant Traits in relation to Arbuscular Mycorrhizal Mixotrophy

Author: Jochem Sterk

Supervisors: Vincent Merckx, Nick Pruijn

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Naturalis Biodiversity Center

van hall larenstein university of applied sciences

Colophon

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Author:	Jochem Sterk
Educational institution:	Van Hall Larenstein University of Applied Sciences
Supervisors:	Vincent Merckx (Naturalis Biodiversity Center) & Nick Pruijn
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Postbus 9001
6880 GB Velp
Telephone: 026 3695695 (reception)
www.hogeschoolvhl.nl

Abbreviations

AM	Arbuscular mycorrhiza
AGB	Aboveground biomass
BGB	Belowground biomass
BGB:AGB	Belowground to aboveground biomass ratio
C	Carbon
CO_2	Carbon dioxide
DPI	Dots per inch
EM	Ectomycorrhizal
LAR	Leaf area ratio
Lnu	Number of leaves
Lth	Leaf thickness
MH	Myco-heterotrophic
NM	Non-mycorrhizal
PCA	Principal Component Analysis
Pht	Plant height
Rle	Root length
Rtd	Root tissue density
Rth	Root thickness
SLA	Specific Leaf Area
SRL	Specific root length
TRY	Plant Trait Database

Preface

Before you I present my bachelors thesis “plant traits in relation to arbuscular mycorrhizal mixotrophy”. It was written as the final step in my bachelors’ program of the study Bos- en Natuurbeheer (Forest and Nature management) with a specialization in applied ecology at Van Hall Larenstein University of applied sciences. The report was written on behalf of Naturalis Biodiversity Center, contributing to the project called “Mixotrophy: an uncharted carbon flux in the plant world”. My internship started in February 2023 and ended in June 2023, during this period the research and the reporting were done.

Together with Vincent Merckx I formulated the research question. This report is primary written for the team working on the mixotrophy project, however everyone interested in plant traits among mixotrophs may be interested in this report. I really enjoyed conducting this study and broadening my knowledge on mycorrhizal interactions, especially in relation to plant traits. By combining literature and experiments I was able to formulate a conclusion.

I would like to thank Vincent Merckx and Nick Pruijn for their supervision during my internship, the feedback on my work and the advice they gave me throughout the process. Furthermore, I would like to thank Deyi Wang and Cas Verbeek for their assistance during the field- and laboratory work and helping me out with doing the statistics in R. I would like to thank all the others within the MIXOTROPHY team who helped me during the process. Furthermore, I would like to thank Sofia Gomez for letting me use the facilities at the Institute of Biology Leiden (IBL) of Leiden University. I would like to thank Rob Langelaan and Bertie Joan van Heuven for their advice and technical support in the laboratory. Lastly, I would like to thank my family and friends who supported me during this period.

Jochem Sterk
Vlaardingen, June 12, 2023

Abstract

Plants growing in the understory of forests are often light limited. The competition for light has over time evolved in different survival strategies, different survival strategies result in differences in traits between plant species. New findings suggest that green plants might be able to take up carbon from fungi, this strategy is called mixotrophy. A proxy for mixotrophy is colonization with *Paris*-type arbuscular mycorrhiza. Being able to take up carbon from fungi will potentially result in differences in certain plant traits compared to autotrophic (*Arum*-type) plants.

This results in the following main question: In which above- and belowground traits do potential mixotrophic plants (*Paris*-type) differ from autotrophic (*Arum*-type) plants?

To answer this question literature was reviewed to find potential traits which can be used to recognize mixotrophic plants. In addition to the literature review a database analysis was done. The literature review and database analysis resulted in the following traits: belowground to aboveground biomass ratio, number of leaves, plant height, leaf area ratio, leaf thickness, specific leaf area, root length, root thickness, root tissue density, and specific root length.

After selecting the traits plants were sampled on Landgoed de Utrecht. Within the plot, with main tree species *Fraxinus excelsior*, *Acer pseudoplatanus* and *Quercus robur*, 20 species were found in the understory. For 15 of the species the arbuscular mycorrhizal type could be determined, 7x *Arum*-type and 8x *Paris*-type. The plants were dug out and further analyzed in the laboratory, where the traits were measured.

Three of the traits measured showed a significant difference, these traits are plant height, root length and root tissue density. The 24 individuals with *Paris*-type AM (M = 28.161, SD = 15.223) showed a significantly higher plant height compared to the 19 individuals with *Arum*-type AM (M = 18.853, SD = 10.481), t (40) = 2.205, p = .033. The 19 individuals with *Arum*-type AM (M = 3974.939, SD = 2301.107) showed a significantly higher root length compared to the 24 individuals with *Paris*-type AM (M = 2648.926, SD = 1777.328), t (40) = -2.055, p = .046. A Mann-Whitney test indicated that the Rtd was significantly greater for *Paris*-type species (M = .520, SD = .514) than for *Arum*-type species (M = .166, SD = .091), U 127, p = .021. Furthermore, the correlations were tested, using the Pearson's r, between the traits this showed that 3 of the correlations between the traits were moderate (BGB:AGB with Rtd, BGB:AGB with SRL, and Lth with SLA), 11 weak correlations were found, and 32 combinations were not correlated. A principal component analysis (PCA) visualized the traits which have the strongest influence on whether a plant is autotrophic or mixotrophic. The PCA showed that along the X-axis strongest distribution was caused by leaf thickness, leaf number and root thickness. On the Y-axis the strongest distribution was caused by belowground to aboveground biomass ratio and root tissue density.

Content

1 Introduction

1.1 Framework

Light in the understory of a forest is scarce because the dense canopy captures most of the light. Also, the light reaching the forest floor is strongly reduced in quality (Théry, 2001). Therefore, plants in the understory are often light limited. This light limitation leads to competition among the species covering the forest floor. This competition results in different survival strategies among plants. For example, growing in canopy gaps (Esquivel-Muelbert, et al., 2018), relying on sun flecks (Zhang, et al., 2021) or a complete adaptation to low light conditions (Valladares & Niinemets, 2008).

Several aboveground traits have evolved to deal with light limitation. The evolution of plant traits has resulted in shade-tolerant plants, which are common in forest ecosystems. Shade-tolerant species adapted by optimizing their ability to do photosynthesis when light becomes less in quantity and quality. This leads potential to e.g., increased specific leaf area (SLA) and a higher physical defense to minimize damage (Gommers, Visser, St Onge, Voesenek, & Pierik, 2013). These traits may act independently (contributing to the rate or stability of an ecosystem function) or interactively and synergistically (complementing each other and leading to further enhancements to rates or stability (Powell & Rillig, 2018).

However, the fact that some plants may compensate light limitation with carbon (C) take up from fungi (mixotrophy) is a new finding. This way plants have an alternative source for their C and are not fully dependent on photosynthesis.

A proxy for mixotrophy is colonization with *Paris*-type arbuscular mycorrhizae (AM), one of the two morphological forms of AM (Giesemann, Rasmussen, & Gebauer, 2021). Plants interacting with AM fungi give C to the fungi, in exchange the plants get nutrients from the fungi. New findings suggest that plants with *Paris*-type AM are potentially able to take up carbon from the fungi. This carbon originates from surrounding plants, *Paris,* or *Arum*-type, which are simultaneously linked by the fungi.

I hypothesize that the adaptation to the survival in low light conditions through mixotrophy, may be reflected in certain plant traits and therefore lead to a possible difference in traits between autotrophic (*Arum*-type) and mixotrophic (*Paris*-type) plants. A plant trait is any morphological, physical, or phenological feature measurable at the level of individual plants. Plant traits are found in all plant parts: Leaf traits, root traits, whole plant traits, stem traits, regenerative traits.

1.2 Naturalis Biodiversity Center

At Naturalis Biodiversity Center more than one hundred scientists conduct research to understand and protect biodiversity worldwide. An enormous collection (more than 42 million specimens) and driven researchers are trying to map and understand biodiversity (Naturalis Biodiversity Center, 2023).

In September 2022 a research project started studying the symbiotic relationship between plants and arbuscular mycorrhizal fungi. The project focusses on the potential for-type plants to take up C from AM fungi and leading to a mixotrophic mode of life in these plants. The main objective of this project,

"Mixotrophy: an uncharted carbon flux in the plant world", is to investigate which plants can take up carbon from fungi and in which conditions this takes place. Also, above- and belowground plant traits of potential mixotrophic plants are mapped, and the evolutionary history will be reconstructed. This will result in the first empirical dataset which will assess the occurrence of AM mixotrophy in different ecosystems and the identification of characteristic plant traits and their habitats (Merckx, 2021).

Functional plant traits will be measured to get a better understanding of the survival strategies of mixotrophic plants. The obtained trait data will be compared between mixotrophic and autotrophic plants. Merckx (2021) hypothesize that AM mixotrophiz plants can be differentiated from autotrophic plants by a distinct set of above- and below ground traits.

1.3 Problem description

To survive and thrive in their respective niches, plants have developed different strategies to obtain carbon and nutrients. These different strategies are reflected in their traits. Mixotrophic plants found an alternative source of C while autotrophic plants get all their C from photosynthesis. We hypothesize that these different strategies may result in differences in traits. Which traits show a potential difference is not yet known. Identifying the characteristic traits for mixotrophic plants makes it possible to recognize potential mixotrophic plants on species level. Furthermore, it will lead to a better understanding of their habitats and survival strategies and therefore, it might lead to better protection of the ecosystems they are in (Merckx, 2021).

1.4 Problem analysis

The assessment of the potential link between plant functional traits and AM mixotrophy is important to predict future dynamics on ecosystem level. Yet there have not been any large-scale systematic attempts which studies plants strategies associated with the diversity and composition of AM fungal networks (Davison, et al., 2020). AM fungi and plant traits seem to be related (Sweeney, de Vries, van Dongen, & Bardgett, 2020; Semchenko, et al., 2018), although this is not always the case (Leff, et al., 2018).

The ability to get carbon from fungi is useful when growing in the darker conditions of the understory in forests. It gives an advantage to mixotrophic plants over fully autotrophs. But does this strategy result in a difference in traits and what traits are most likely to show a potential difference?

Identifying the traits makes it possible to get a better understanding of the mixotrophic mode of life. The better understanding of mixotrophic plants will make it easier to study the effect of mixotrophy within ecosystems, and more importantly will give insight on how to protect and preserve the habitats they are in. Furthermore, the traits may be used as a proxy for the ability to be mixotrophic. The traits will ideally replace the slower and more expensive isotope analysis.

Merckx (2021) already did a preliminary study on belowground traits, using the Global Root Trait (GRooT) database, identified eight traits which show a significant difference between AM mixotrophic and autotrophic plants. These traits are mean root diameter, specific root length, mycorrhizal colonization, root cortex thickness, root stele fraction, root vessel diameter, root xylem vessel number, and specific root area. The GRooT database (Guerrero-Ramírez, et al., 2020) is a large database which

combines different studies related to root traits. Not for every species the same traits are identified, therefore the database contains gaps.

To get a first indication of the differences in plant traits between mixotrophic and autotrophic plants, 10 plant traits will be selected, measured, and analyzed. This will result in data on potential differences in traits between mixotrophs and autotrophs, and thus give insight in the traits differences between the two survival strategies The obtained data will form the basis for the trait analysis in the Mixotrophy-project.

1.5 Research question

1.5.1 Main question

In which above- and belowground traits do potential mixotrophic plants (*Paris*-type) differ from autotrophic (*Arum*-type) plants?

Because mixotrophic plants likely require less light and have a higher dependency on mycorrhizal fungi than autotrophic plants, we hypothesize that these both above- and belowground traits can be used to distinguish mixotrophic from autotrophic plants.

1.5.2 Sub questions

1. What are potential above- and belowground plant traits differences between mixotrophic and autotrophic plants?
2. How do mixotrophic plants differ from autotrophic plants regarding below- and aboveground biomass ratio, number of leaves, plant height, leaf area ratio, leaf thickness, specific leaf area, root length, root thickness, root tissue density, and specific root length?
3. To what extent does mixotrophy or autotrophy effect the above- and belowground plant traits?
    a. To what extent are the measured plant traits correlated?
    b. Which traits have the strongest influence on the distribution?

1.6 Objective

Select 10 above- and belowground traits with a potential difference between mixotrophic and autotrophic plants. Measure the selected traits to test if there is difference between mixotrophic and autotrophic plants. Also, it will be evaluated how feasible it to measure the traits in the field.

The outcome of this study will contribute to the Mixotrophy-project. Where the traits will be used to get a better understanding of AM mixotrophy worldwide. So, the method used in this project should be useable within the Mixotrophy-project to sample sites worldwide.

1.7 Reading guide

In chapter 2 the selection criteria and the selected traits are being introduced. In chapter 3 the research location is introduced, and the research method is explained. In chapter 4 the results are presented. In

chapter 5 the results are discussed and there is the conclusion. Finally in chapter 5 there are recommendations for further research.

2 Selected Plant traits

We expect only specific traits to vary in relation to arbuscular mycorrhizal mixotrophy. The traits must be widespread throughout the plant communities to be able to draw conclusions, so the traits must not be too species specific. The traits were selected through a literature and database analysis. Based on the following criteria:

- The traits should potentially be related to mixotrophy, Positive or negative.
- The traits must be widespread among the vegetation, so not species specific.
- The selected traits must cover an as broad spectrum of traits as possible.
- No complicated, nor expensive equipment or software is needed to measure the trait.
- The processing time should be as limited as possible in manhours.

The Plant trait database (TRY) (Boenisch & Kattge, 2019) was used to do a database study on the selected traits. The results are shown in appendix 1.

2.1 Whole plant traits

2.1.1 Belowground to aboveground biomass ratio

The ratio of belowground to aboveground biomass (BGB:AGB) is a measure which provides insight in the investment of a plant's biomass between the roots and shoots. The higher the ratio the more a plant invest in its belowground biomass (BGB) (Wang, et al., 2014).

For the BGB mixotrophic plants might have a higher mass compared to autotrophic plants because due to the investment in housing the fungi as potential alternative carbon source (Vannier, Bittebiere, Mony, & Vandenkoornhuyse, 2020).

Hypothesis: Mixotrophic plants have a bigger percentage of belowground (and lower percentage of aboveground) biomass compared to autotrophic plants.

2.1.2 Number of leaves

Leaves are vital organs when it comes to photosynthesis and gas exchange. However, for plants having an alternative carbon source the need to invest in photosynthesis might be reduced. For the mixotrophic plants this may result in the forming of fewer leaves compared to fully autotrophic plants (Leake, 1994).

The reduction of leaves can also be an environmental adaptation. In light limited areas species may have fewer leaves compared to species in open environments (Wu, Gong, & Yang, 2017). When focusing on mixotrophic plants the need for many leaves might even be further reduced because of their potential carbon take up from their arbuscular mycorrhizal partner. For fully myco-heterotrophic plants it is known that they are strongly reduced in the number of leaves (Leake, 1994).

Even though myco-heterotrophic plants are strongly reduced in number, no difference was found between *Arum*- and *Paris*-type plants (see appendix 1 for the boxplot).

The hypothesis is: Autotrophic plants form a larger number of leaves compared to mixotrophic plants because of the dependence on photosynthesis of the latter.

2.1.3 Plant Height

Plants adapt to their surroundings, for instance by adapting to limiting factors. When light availability becomes scarce growing tall gives an advantage in capturing the last bit of sunlight reaching the forest floor. In a dense understory growing taller than your neighbor might be key to survival (Gommers C. M., 2019). Therefore, when photosynthesis is your only source of C the urge to invest in plant height (Pht) might be higher.

When looking at the data available in the TRY database no difference was found between *Arum*- and *Paris*-type species (see appendix 1 for the boxplot).

The hypothesis is: Because of their dependence on sunlight autotrophic plants grow taller compared to mixotrophic plants.

2.2 Leaf traits

2.2.1 Leaf area ratio

Leaf area ratio (LAR) is a plant trait which provides information about the relationship between the total leaf area of a plant and its total biomass. Measuring LAR gives information about the ability of a plant to capture light and CO_2 and to conduct photosynthesis (Poorter & Remkes, 1990).

A high LAR represents a higher leaf area over a given mass. In light limited environments plants with a high LAR might have an advantage over plants with a lower LAR. This is because a higher LAR will outcompete species with a lower leaf area ratio (Caliskan, Odabas, Cirak, Radusiené, & Odabas, 2010). Therefore, autotrophic plants might have a higher demand for a high LAR.

When looking at the database data no difference was found for LAR between *Arum*- and *Paris*-type species (see appendix 1 for the boxplot).

The hypothesis is: The higher light demand for autotrophic plants will result in a higher LAR compared to mixotrophic plants.

2.2.2 Leaf thickness

Leaf thickness (Lth) refers to the distance between the upper and lower epidermis. Furthermore, leaf thickness is determined by mesophyll and vascular bundles (Wit, Tonn, Ackerveken, & Kalkman, 2020). The mesophyll is the primary tissue for photosynthesis and mainly causes the thickness of a leaf.

Since mesophyll is the main driver of Lth it is likely that plants who are more dependent on photosynthesis will develop thicker leaves (Coneva & Chitwood, 2018).

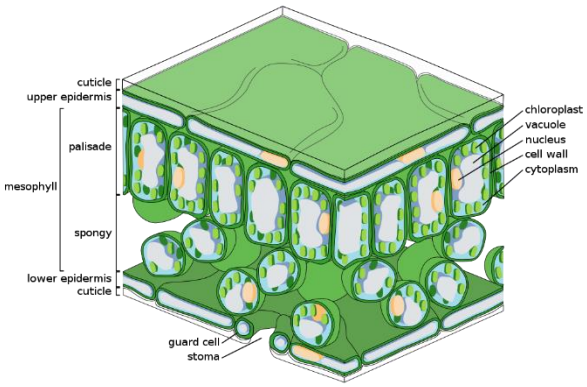

Figure 2.1: Cross section of a leaf (Zephyris, n.d.)

The Database did not show any difference between *Arum*- and *Paris*-type plants (see appendix 1 for the boxplot).

The hypothesis is: The dependency on photosynthesis among autotrophic plants result in thicker leaves compared to mixotrophic plants.

2.2.3 Specific leaf area

On the forest floor light is limited yet important for plants to survive. the shaded conditions result in light intensity, photosynthesis, and plant growth loss (Pérez-Harguindeguy, et al., 2013). Plants react to the shaded conditions by adjusting their morphological, as well as their physiological traits, such as their specific leaf area (SLA). SLA is a parameter used to describe the relationship between leaf area and leaf mass.

Usually, a higher SLA is developed when growing in shaded conditions (Feng & van Kleunen, 2014). The increasing of the SLA might help plants to capture light and maximize carbon gain (Gommers, Visser, St Onge, Voesenek, & Pierik, 2013).

However, if plants have alternative sources for their carbon they might need to invest less in their SLA. This may result in a lower SLA in mixotrophic plants compared to autotrophic plants. The database does not show a significant difference between the two AM types (see appendix 1 for the boxplot).

Therefore, the hypothesis is: SLA is higher in autotrophic plants compared to mixotrophic plants.

2.3 Root traits

2.3.1 Root length

Roots are an important organ for water and nutrient up take, and for the housing of for example mycorrhizal fungi. Plants have different strategies to take resources from the soil (Petruzzello, 2023). Different strategies may result in different root measurements. Roots can be long with a narrow diameter, short with a thicker diameter, or anything in between. The long, thin roots are best associated with direct source take up from the soil. The short, thick roots may indicate an investment in carbon to house mycorrhizal fungi in exchange for limited nutrient acquisition (Bergmann, et al., 2020).

Root length (Rle) is a trait which quantifies the extent and development of roots by measuring a plant's fine roots (< 2 mm) (Freschet & Roumet, 2017).

The Try database also contains data for root length. The root length is significantly higher for *Arum*-type species compared to *Paris*-type species (see appendix 1 for the boxplot).

The hypothesis for root length is: because autotrophic plants invest less in the housing of fungi, they form longer roots to take up nutrients from the soil, while mixotrophic plants invest in fungal partners and will form shorter roots.

2.3.2 Root thickness

Root thickness (Rth) gives information about the diameter of a root. Thinner roots are generally better for exploring the soil, as thin roots penetrate easier through the soil. On the other hand, thicker roots are generally more associated with fungal housing (Kong, et al., 2015).

The data on root thickness in the TRY database is significantly higher for *Paris*-type species compared to *Arum*-type species (see appendix 1 for the boxplot).

The hypothesis for root thickness is: Mixotrophic depend on fungal partners to survive and therefore invest in thicker roots to better house the fungi, autotrophic plants invest less in the housing of fungi and therefore form thinner roots.

2.3.3 Root tissue density

Root tissue density (Rtd) refers to the mass of a root over its volume. Denser root tissues are often associated with slower growth rates and slower up take of nutrients and water (Birouste, Zamora-Ledezma, Bossard, Pérez-Ramos, & Roumet, 2013). Plants with a high Rtd usually invest more in structural tissue. On the other hand, plants with a low Rtd are mostly associated with faster root growth and higher rates of water and nutrients up take.

The database does not show a difference between *Arum*- and *Paris*-type plants (see appendix 1 for the boxplot).

Therefore, the hypothesis is: Mixotrophic plants have a higher Rtd compared to autotrophic plants, because mixotrophic plants invest more in the housing of their fungal partner.

2.3.4 Specific root length

Specific root length (SRL) is a measure which represents the length of a root per given root volume. SRL gives information about the efficiency of resource acquisition and nutrient up take by the roots. A high SRL indicates a finer and more extensive root system, which means that roots are longer in relation to their mass (Kramer-Walter, et al., 2016).

Autotrophic plants form finer roots (see §2.2.2) therefore, the SRL is expected to be higher for *Arum*-type plants. This enhances their ability to explore larger areas to take up nutrients and water. For mixotrophic plants the need to explorer the soil is lower because of their increased dependency on arbuscular mycorrhizal fungi.

The database does show a significant difference between the two AM type. The SRL for *Arum*-type plants being significantly higher compared to *Paris*-type plants (see appendix 1 for the boxplot).

This leads to the following hypothesis: Autotrophic plants have a higher SRL compared to mixotrophic plants, to meet their nutrient demand.

3 Methodology

3.1 Location description

The location used for sampling is Landgoed de Utrecht near Esbeek (see appendix 2). Landgoed de Utrecht is in the Brabantse Kempen, the area is 2,485 hectares of which circa 1,600 hectares of forest (Landgoed de Utrecht, 2013). The forest consists of mixed broadleaf forest, beech forest and pine forest. The species richness in the mixed forest is high, with main tree species as *Fraxinus excelsior*, *Acer pseudoplatanus* and *Quercus robur* (see Figure 3.1).

Figure 3.1: Sampling location Landgoed de Utrecht (Merckx, unpublished).

On the sampling location a plot of 10,5 by 10,5 meters was sampled. Of all the species within the understory in the plot 3 individuals were collected for trait measurements. The plants were carefully dug out with all the roots still on the plant. The plants were put in plastic bags and given a unique code, so they could be further processed in the laboratory. Until further processing the roots were stored chilled. In total 20 species were found within the plot. Of the 20 species 15 could be associated with the arbuscular mycorrhizal type they form using a database. The other 5 species could not be linked to AM fungi, or the AM-type was not yet known. An overview of the species is shown in table 3.1.

Table 3.1: The species found within the plot in Landgoed de Utrecht. The different mycorrhizal types are arbuscular mycorrhiza (AM), ectomycorrhiza (EM), and non-mycorrhizal (NM).

#	Species	Mycorrhiza	AM type
1	*Acer pseudoplatanus*	AM	*Paris*
2	*Amelanchier lamarckii*	AM	*Arum*
3	*Anemone nemorosa*	AM	*Paris*
4	*Athyrium filix-femina*	AM	*Paris*
5	*Carex cespitosa*	NODATA	NODATA
6	*Corylus avellana*	AM/EM	*Paris*
7	*Deschampsia flexuosa*	AM	NODATA
8	*Fagus sylvatica*	EM	NODATA
9	*Fraxinus excelsior*	AM	*Arum*
10	*Geum urbanum*	AM	*Arum*
11	*Hedera helix*	AM	*Arum*
12	*Ilex aquifolium*	AM	*Paris*
13	*Prunus serotina*	AM	*Arum*
14	*Quercus robur*	EM	NO*DATA*
15	*Rubus* spec.	AM	*Arum*
16	*Sorbus aucuparia*	AM	*Paris*
17	*Stellaria holostea*	NM	NODATA
18	*Urtica dioica*	AM	*Arum*
19	*Viburnum opulus*	AM	*Paris*
20	*Viola* spec.	AM	*Paris*

3.2 Research methodology

3.2.1 Measuring the plant traits

Sub question: How do mixotrophic plants differ from autotrophic plants regarding below- and aboveground biomass ratio, number of leaves, plant height, leaf area ratio, leaf thickness, specific leaf area, root length, root thickness, root tissue density, and specific root length?

3.2.1.1 Measuring whole plant traits

For this paragraph, the method of Wigley, et al. (2020) is used. The plant height was measured from the base of the plant to the highest point of the top leave. The measuring was done using a ruler.

The leaves were counted manually per individual. Important to consider was whether a plant formed single or compound leaves, as will be described in §3.2.1.2.

The whole plants were separated into the aboveground biomass (AGB) and the belowground biomass (BGB). The separated parts were washed, to remove all other organic matter and soil. Each sample was oven dried for 72 hours at 60°C. After drying the plant parts were directly weighed. Then, the belowground to aboveground biomass ratio (BGB:AGB) was calculated. This was done for each plant individually. Plants may have a substantial difference in weight because of the different growth forms. Therefore, plants are divided in woody and non-woody (Rowe & Speck, 2005).

3.2.1.2 Measuring the leaf traits

The method for these traits is based on the method of Pérez-Harguindeguy et al. (2013). Specific leaf area (SLA), leaf area ratio (LAR), and leaf thickness (Lth) was measured with the fully expanded, and hardened leaves from the individuals. A leaf can be simple or compound. Compound leaves contain multiple leaflets (Jakinboaz, 2023). The start of the petiole from a bud is the beginning of a leaf (see Figure 3.2)

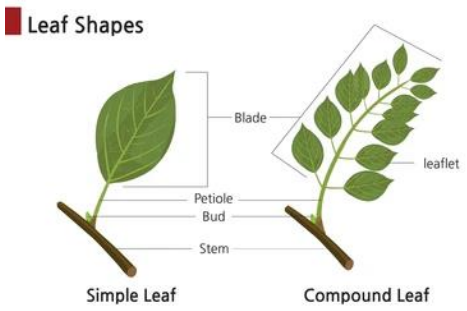

Figure 3.2: A leaf can be simple (left) or compound (right). Compound means several leaflets together form one leaf (Jakinboaz, 2023).

The leaves were separated from the stem (if still on it), patted dry, and weighed to measure the fresh leaf weight. scans were made using a flatbed scanner using a resolution of 800 dpi (see Figure 3.3). The scans were then analyzed using the imageJ software (National Institutes of Health, 2018)to determine the area.

Figure 3.3: Example scan for the leaves of Viola with a resolution of 800 dpi.

To calculate the Lth the total leaf area was multiplied by the mass of all the leaves:

$$Lth = \ One\ sided\ leaf\ area * leaf\ \ fresh\ weight$$

For the SLA the leaf mass was also measured. The leaves were dried at 60°C for a minimum of 72 hours. The dried leaves were weighed to determine the dry mass. The leaves will take up moisture from the air once exposed to it. To avoid rehydration the leaves were directly weighed once out of the oven. For the SLA dived the area of the leaf by its oven dried mass.

$$SLA = \frac{One\ sided\ leaf\ area}{Leaf\ oven\ dry\ mass}$$

For the LAR the total plant biomass was measured as described in §3.2.1.1. The LAR was calculated using the following formula:

$$LAR = \frac{Total\ leaf\ area}{Total\ plant\ biomass}$$

3.2.1.3 Measuring the root traits

The method for these traits is based on the method of Pérez-Harguindeguy et al. (2013). In the field the individual plants were dug out, so that all the roots were still on the plant.

The unwashed roots were stored humidified and refrigerated until further processing. The roots were first washed before further processing, to remove soil and other organic matter. In general, to remove fine heavy particles it is best to clean the roots with running water and a small sieve (0.2-1 mm). larger particles will be removed in a container with water using forceps.

The next step is to digitize the roots using a scanner with a resolution of 1200 dpi (dots per inch). Such a scanner provides images with a resolution of 15 µm, this is half the width of any plant's finest roots. To get crisp root images, a scanner with a transparency adaptor illuminating items on the scanner bed from above was used. The roots were submerged in water to obtain the best images (see Figure 3.2). The images were analyzed using the image-analysis software RhizoVision Explorer (Seethepalli & York, n.d.). The root length, diameter, and volume were automatically determined within the software.

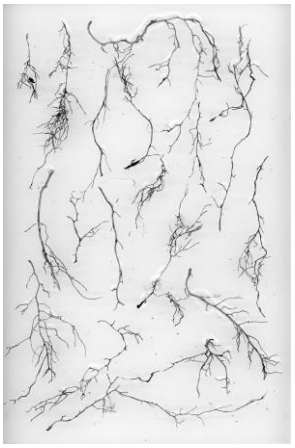

Figure 3.2: Example scan for the roots of Hedera with a resolution of 1200 dpi.

For the SRL and Rtd the dried root weight was needed of the fine roots, to measure the weight of the fine roots (Birouste, Zamora-Ledezma, Bossard, Pérez-Ramos, & Roumet, 2013), the method as described in §3.2.1.1 was used.

The formula used for Rtd was:

$$Rtd = \frac{Total\ root\ biomass}{Total\ root\ volume}$$

The formula used for SRL was:

$$SRL = \frac{Total\ root\ length}{Total\ root\ volume}$$

3.2.1.4 Statistical analysis

The data gathered in the field was statistically analyzed using Excel, Jamovi and R. Differences were tested using independent sample T-test (data normally distributed) or a Mann-Whitney U test (data not normally distributed). To test the traits the plants were split in mixotrophic (*Paris*-type) and autotrophic plants (*Arum*-type). The variables are shown in Table 3.2. Because woodiness may be a driver of the differences between *Arum*- and Paris-type AM, woody plant parts cause a higher plant biomass, this influences for example BGB:AGB and LAR. To test if the woodiness of the plant caused the differences, the traits were also tested for all the woody and all the non-woody species separately.

Table 3.2: Variables for the traits to be measured.

Trait	Independent variable	Dependent variable	Unit
Biomass	Mixotrophic/autotrophic	AGB and BGB	g
Number of leaves	Mixotrophic/autotrophic	Leaf number	#
Plant height	Mixotrophic/autotrophic	Height of the plant	cm
Leaf area ratio	Mixotrophic/autotrophic	Leave area and plant mass	cm^2/g
Leaf thickness	Mixotrophic/autotrophic	Thickness of leaf	mm
Specific Leaf Area	Mixotrophic/autotrophic	Leaf area and mass	mm^2/mg
Root length	Mixotrophic/autotrophic	Root length	cm
Root thickness	Mixotrophic/autotrophic	Root diameter	mm
Root tissue density	Mixotrophic/autotrophic	Root mass and volume	g/mm^3
Specific root length	Mixotrophic/autotrophic	Root length and volume	mm/mg

3.2.2 Trait effect on mixotrophic and autotrophic plants

Sub question: To what extent does mixotrophy or autotrophy effect the above- and belowground plant traits?

3.2.2.1 Relations between the plant traits

A trait might not show any difference between the two groups of AM when looked at separately. However, looking at multiple traits together might give an insight into how a combination of trait values may separate mixotrophic from autotrophic plants. This was tested using the Pearson's r statistical test. For the correlation matrix all the traits were tested for correlation among each other. The correlation between the traits was analyzed using R, Jamovi and Excel.

3.2.2.2 Influence on distribution

The distribution between the different traits was visualized using a Principal component analysis (PCA). A PCA is a technique to reduce the dimensionality of large datasets. It increases the interpretability and visualization while information loss is minimized (Jolliffe & Cadima, 2016). The aim of the PCA was to test which traits have the most influence on whether a plant is mixotroph or autotroph. So ideally in the PCA two clouds will form, with one cloud representing the autotrophic plants and the other cloud representing the mixotrophic plants. An example of a PCA test is shown in figure 2.1.

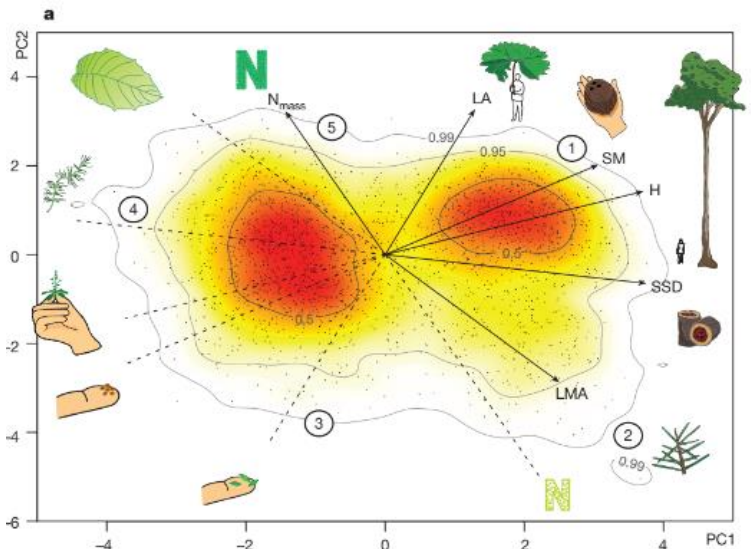

Figure 3.1: PCA made by Díaz, et al. (2015) visualizing the influence of 6 different traits among vascular plants.

4 Results

4.1 Trait measurements

4.1.1 Total plant traits

For the traits BGB:AGB and Lnu no significant difference was found between *Arum*- and *Paris*-type species. The boxplots for these traits are shown in appendix 4, the results of the statistical tests are shown in Table 4.2.

The average biomass ratio of all the *Arum*-type species is 0.662, for the *Paris*-type species this is 0.872. In Table 4.1 a summary of the biomasses for both *Arum*- and *Paris*-type is shown. The table shows that for non woody plants the ratio difference between *Paris*- and *Arum*-type plants is the highest.

Table 4.1: Average biomass for Arum- and Paris-type species. All biomasses are given in grams (g).

AM-type	Division	Avg. BGB	Avg. AGB	Avg. Tot. biomass	Ratio
Arum	Total	0.603	1.315	1.918	0.662
	Woody	0.312	1.401	1.713	0.457
	Non-woody	1.004	1.197	2.201	0.946
Paris	Total	3.017	1.065	4.082	0.872
	Woody	1.282	2.676	3.958	0.352
	Non-Woody	0.729	3.546	4.275	1.682

The maximum plant height is on average higher for *Paris*-type species than for *Arum*-type species. For the *Paris*-type species the average Pht is 28.160 cm compared to the average of 18.852 cm for *Arum*-type species. The 24 individuals with *Paris*-type AM (M = 28.161, SD = 15.223) showed a significantly higher Pht compared to the 19 individuals with *Arum*-type AM (M = 18.853, SD = 10.481), t (40) = 2.205, p = .033.

4.1.2 Leaf traits

For the traits LAR, Lth, and SLA no significant difference was found between *Arum*- and *Paris*-type species. The boxplots for these traits are shown in appendix 4, the results of the statistical tests are shown in Table 4.2.

4.1.3 Root traits

Measurements of the root length of the fine roots shown that *Arum*-type species, with an average root length of 3974.939 cm, have longer roots when compared to *Paris*-type species, where the average root length is 2648.926 cm. The 19 individuals with *Arum*-type AM (M = 3974.939, SD = 2301.107) showed a significantly higher root length compared to the 24 individuals with *Paris*-type AM (M = 2648.926, SD = 1777.328), t (40) = -2.055, p = .046.

Measurements of the root tissue density show that *Paris*-type species have denser root tissue compared to *Arum*-type species. The *Paris*-type species (24 individuals) have an average Rtd of 0.520 mg/mm^3 while the average Rtd for *Arum*-type species (19 individuals) is 0.166 mg/mm^3. A Mann-Whitney test indicated that the Rtd was significantly greater for *Paris*-type species (M = .520, SD = .514) than for *Arum*-type species (M = .166, SD = .091), U 127, p = .021.

For the traits Rth and SRL no significant difference was found between *Arum*- and *Paris*-type species. The boxplots for these traits are shown in appendix 4, the results of the statistical tests are shown in Table 4.2.

For an overview of all the measured data see appendix 3.

Table 4.2: Statistical analysis of all traits. M-W = Mann-Whitney.

Dependent variable	*Arum*-type		*Paris*-type		T-test	M-W	
	M	*SD*	*M*	*SD*	*t (40)*	*U*	*p*
BGB:AGB	.662	.710	.872	1.601		217	.980
Lnu	10.632	7.117	8.652	5.990		181	.342
Pht	18.852	10.481	28.161	15.223	2.205		**.033**
LAR	85.069	67.515	64.197	48.038		174	.783
Lth	1.783	.852	1.942	1.067		207	.783
SLA	43.734	24.250	39.253	15.404		203	.708
Rle	3974.939	2301.107	2648.926	1777.328	-2.055		**.046**
Rth	.477	.094	.473	.113	-.122		.903
Rtd	.166	.091	.520	.514		127	**.021**
SRL	28.011	18.258	24.898	14.822		214	.909

For the non-woody plants 6 species were found (3 *Arum*-type and 3 *Paris*-type). The statistical tests show that 2 traits are significantly different, these traits are Lnu and Rle. The 8 individuals with *Arum*-type AM (M = 14.625, SD = 7.052) showed a significantly higher Lnu compared to the 9 individuals with *Paris*-type AM (M = 5.667, SD = 3.367), t (15) = 3.194, p = .006. A Mann-Whitney test indicated that the Rle was significantly higher for *Arum*-type species (M = 5508.141, SD = 1731.397) than for *Paris*-type species (M = 2399.219, SD = 2127.142), U 10, p = .011. In Table 4.3 an overview for all the traits tested is shown.

Table 4.3: Statistical analysis for the non-woody plants. M-W = Mann-Whitney.

Dependent variable	*Arum*-type		*Paris*-type		T-test	M-W	
	M	*SD*	*M*	*SD*	*t (15)*	*U*	*p*
BGB:AGB	.946	.825	1.682	2.317		32	.743
Lnu	14.625	7.052	5.667	3.367	3.194		**.006**
Pht	17.863	9.808	21.811	14.844	-.600		.558
LAR	106.560	60.417	77.866	51.835	.990		.810
Lth	2.125	.768	2.377	.886		26	.370
SLA	36.844	11.168	43.935	12.794	-1.137		.273
Rle	5508.141	1731.397	2399.219	2127.142		10	**.011**
Rth	.477	.076	.407	.078	1.773		.097
Rtd	.178	.104	.479	.561		33	.815
SRL	29.122	13.626	32.191	6.930	-.559		.585

For the woody plants 9 species were found (4 *Arum*-type and 5 *Paris*-type). The statistical tests show that 2 traits are significantly different, these traits are Pht and Rtd. The 14 individuals with *Paris*-type AM (M = 32.243, SD = 14.017) showed a significantly higher Pht compared to the 11 individuals with *Arum*-type AM (M = 19.573, SD = 10.889), t (23) = -2.368, p = .027. A Mann-Whitney test indicated that the Rtd was

significantly higher for *Paris*-type species (M = 0.547, SD = 0.481) than for *Arum*-type species (M = 0.157, SD = 0.079), U 23, p = .002. In Table 4.4 an overview for all the traits tested is shown.

Table 4.4: Statistical analysis for the woody plants. M-W = Mann-Whitney.

Dependent variable	*Arum*-type		*Paris*-type		T-test	M-W	
	M	*SD*	*M*	*SD*	*t (23)*	*U*	*p*
BGB:AGB	.456	.525	.352	.255		66	.572
Lnu	7.727	5.593	10.571	6.500		57.5	.296
Pht	19.573	10.889	32.243	14.017	-2.368		**.027**
LAR	69.439	68.107	55.410	43.204		66	.572
Lth	1.535	.824	1.662	1.080		74	.893
SLA	48.745	29.417	36.242	16.168		52	.183
Rle	2859.883	2003.233	2809.452	1488.289	.069		.945
Rth	.477	.106	.515	.113	-.833		.413
Rtd	.157	.079	.547	.481		23	**.002**
SRL	27.204	20.957	20.209	16.549		60	.373

4.2 Trait effect

4.2.1 Correlations

All the traits were tested for correlation. No strong correlations were found. SLA and Lth were found to be moderately negatively correlated, r (40) = -.639, p = <.001. BGB:AGB was found to be moderately positively correlated to both Rtd and SRL, r (40) = .545, p = <.001 and r (40) = .552, p = <.001, respectively. Eleven of the correlations were found to be weak. For all the other correlations no correlation was found. See Table 4.5 for all the correlations between the different traits.

*Table 4.5: Pearson's correlation matrix. The upper value represents the Pearson's r, and the lower value representing the p-value. a Pearson's r value of .00-.30 represents no correlation, .30-.50 represents weak correlation, .50-.70 represents moderate correlation, .70-.90 represents strong correlation, and .90-1 represents very strong correlation. * = <.05 ** = <.001*

	BGB: AGB	Lnu	Pht	LAR	Lth	SLA	Rle	Rth	Rtd	SRL
BGB:AGB										
Lnu	-.100									
Pht	-.202	.148								
LAR	-.059	-.011	-.339*							
Lth	-.015	-.111*	.158	-.130						
SLA	.142	-.185	-.202	.394*	**-.639****					
Rle	-.174	.235	.175	-.131	.336*	-.451*				
Rth	-.050	.406*	.301	-.391*	.149	-.423*	.212			
Rtd	**.545****	.079	.266	-.135	.215	-.086	-.337*	.143		
SRL	**.552****	-.297	-.280	.300	-.128	.360*	-.158	-.277	.126	

4.2.2 Influence on distribution

The results of the visualization of the trait data using a PCA can be seen in Figure 4.1. The green points represent the *Arum*-type species while the purple points represent the *Paris*-type species. No obvious clouds are forming showing a difference between the two types as the green circle is almost completely within the purple circle. The bigger purple circle is mainly caused by the three outliers on the bottom of the PCA.

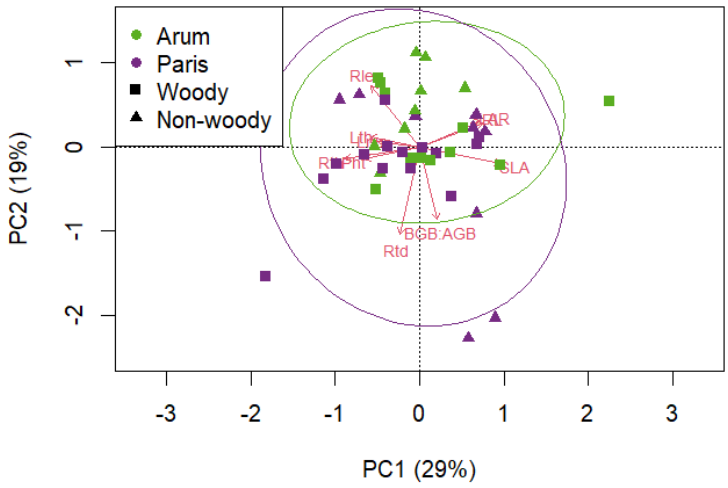

Figure 4.1: PCA

The biggest influence on the X-axis is caused by Leaf thickness, leaf number, and root thickness while on the Y-axis belowground to aboveground biomass ratio and root tissue density have the strongest explanatory power.

5 Discussion and Conclusion

5.1 Discussion

The objective was to select 10 above- and belowground traits with a potential difference between mixotrophic and autotrophic plants. The traits were measured to test if there were difference between mixotrophic and autotrophic plants. These results will contribute to the Mixotrophy-project. Where the traits will be used to get a better understanding of AM mixotrophy worldwide.

Three of the traits show a significant difference between autotrophic and mixotrophic plants, for all the plants in the understory combined. These traits are plant height, root length and root tissue density. For all the other traits no significant difference was found (see chapter 4.1).

BGB:AGB was not found to be significantly different between autotrophic and mixotrophic plants. However, BGB:AGB is correlated with Rtd and SRL. The correlation with both traits is positive, meaning that the higher the mass for Rtd and SRL the higher BGB:AGB. A higher BGB will logically result in a higher BGB:AGB. For the aboveground biomass there is no correlation with the BGB:AGB this can be linked to the lack of weight difference between autotrophic and mixotrophic plants.

Myco-heterotrophic (MH) and mixotrophic (or partial MH) plants are known to have reduced leaf size, reduced number of leaves (sometimes even leaflessness for fully MH plants), variegated leaves, and lower levels of chlorophyll. So, these characteristics suggest that a plant relies on C from their mycorrhizal partner (Simard, et al., 2012) . The number of leaves in the sampled plot did not show any difference between autotrophic and mixotrophic plants (however, for the non-woody plants it did) also when looked at the Try database no significant difference was found.

The plant height is higher for mixotrophic (*Paris*-type) species compared to autotrophic (*Arum*-type) species. This does not follow the hypothesis stated in §3.2.1. An alternative solution to the higher plant height for mixotrophic species is that the alternative carbon source provides the plant with such amounts that their growth rate increases. The increased growth rates will result in the overgrowing of the autotrophic species. Lower photosynthetic rates may cause C to be a limiting factor within plant growth (BBC, n.d.). When carbon from photosynthesis is limited, being able to take up c from an alternative source, such as their fungal partner, gives an advantage over species which cannot.

No difference was found between autotrophic and mixotrophic plants regarding LAR, Lth and SLA. The sampling time and period might have influenced the results, as the best time for sampling is in the morning mid- to late summer (Hanson, 2008). The samples for this project were sampled May 1, 2023, between 11 am and 15 pm.

For the mixotrophic plants it would have been logical if the LAR was lower compared to autotrophic plants. Since mixotrophic plants have a potential alternative carbon source they can put less energy into maintaining a high LAR (Leake, 1994).

Shade leaves are generally thinner compared to sun leaves, this also results in an increase in SLA (Wu, Gong, & Yang, 2017). For Lth a proxy was used, to measure Lth the leaf area was multiplied by the fresh mass of the leaves. The Lth is correlated with SLA, the correlation is negative. The negative correlation

means that for a higher leaf thickness the SLA will be lower. Thicker leaves will generally result in more mass for a given area. SLA will thus get lower.

Root length is as expected higher for autotrophic species. This is shown in database data and own data. The longer roots for autotrophic species can be linked to the nutrient up take. Plants build longer roots in order to 'explore' their surroundings in search for nutrients and water. Despite, *Arum*-type species also get nutrients from the fungi. The roots containing *Arum*-type arbuscules are generally shorter lived compared to the roots with *Paris*-type arbuscules which are longer lived. The constant need of developing new roots and investing in new arbuscules might be an answer to the longer roots (Smith & Read, 2008).

No difference was found in the plot for root thickness. However, the database shows that *Paris*-type plants have thicker roots. A higher root thickness mostly results in a higher colonization rate of arbuscular mycorrhizal fungi (Comas, Callahan, & Midford, 2014). Therefore, it would make convenient for *Paris*-type plants to invest in root thickness.

A low Rtd can be linked to high nutrient availability, nonetheless nutrient availability does not have much influence on SRL. (Kramer-Walter, et al., 2016), however, it does not specify how this can be linked to a mixotrophic mode of live.

In the PCA there is a lot of overlapping between the two type. Most individuals are clustered. However, certainly for the *Paris*-type plants, there are some outliers. The square on the bottom left is *Ilex_02*, this individual was much larger compared to the other *Ilex* individuals, this resulted in higher trait values. The two purple triangles on the bottom right are *Anemone*. *Anemone* forms rhizomes, this resulted in a much higher BGB:AGB ratio, this resulted in the position on the bottom right. The third *Anemone* is the purple triangle on the bottom right within the cluster (positioned on the green circle).

It is important to note that *Paris*-type plant are used as a proxy for mixotrophy. However, it might be possible that only a subset of all the *Paris*-type plants is able to have a mixotrophic mode of live. Meaning that not all the species labelled as *Paris*-type may eventually be mixotrophic. *Paris*-type is used as a proxy because it is known that all fully myco-heterotrophs are *Paris*-type AM, in addition the *Paris*-type shows higher coverage in darker ecosystems, such as forests. Therefore, *Paris*-type AM is best linked to mixotrophy (Murata-Kato, et al., 2022).

Database data contains a combination of both in situ and ex situ specimens. This may result in misleading results as plants growing ex situ may be growing under ideal circumstances. It also may lead to lower or no symbiosis with arbuscular mycorrhiza, as might be the case in pot culture. It could also be possible that the data did not have many species in common with the species of which their AM type is known.

Different survival strategies, for instance, trees shrubs and herbs, are likely to reflect difference in traits based on their strategy. However, the study focusses on the understory of forest. This means that only the individuals in the understory will be sampled to measure their traits. Considering the plot will represent the local vegetation the circumstances within the plot will be homogenous when looking at growing conditions. Therefore, plants will search for survival strategies to outcompete their neighbor and thrive on that location. Also looking at the fact that all plants need to conduct photosynthesis to survive and that the ones which are best adapted will have the highest change of survival. Within all the

different survival strategies, including mixotrophic or autotrophic, patterns and differences are being studied.

Sub sampling leaves into deionized water is recommended, however if the plants are stored chilled and processed within a few days it is also possible to leave the leaves on the plant. That way little degradation takes place and saves a lot of time in the field. For some species (*Athyrium filix-femina* and *Ilex aquifolium*) the leaves on the plant were even better after a few days than the leaves in the test tubes.

The sampling of one location (Landgoed de Utrecht) makes it difficult to already eliminate traits which are not significant. Certainly, since the mixotrophy project will sample a broad range of vegetations. The database will be expanded, more data will result in more reliable results and thus better conclusions can be drawn from the results. This may also cause the low correlations between the traits.

To see how long it takes to measure a trait, the measuring times were timed, see table 5.1 for the time costs to measure the traits. The time for each measurement is strongly dependent on the morphology of a plant. For example, lots of small compound leaves take more time to scan then simple leaves. The same for roots, the finer the roots the more time it takes to wash and scan.

*Table 5.1: Time costs shown in man-hours to measure all the data needed for a trait per species (3 individuals). The drying of the samples is not addressed in this table. *The washing includes the subsampling of the roots for the Mixotrophy-project.*

Measurement	Time	#traits	Trait
Measuring height	3-5 min/species	1	Pht
Counting leaves	3-5 min/species	1	Lnu
Weighing fresh mass	3-5 min/species	1	LAR
Weighing dried leaves	3-5 min/species	3	BGB:AGB, LAR, SLA
Weighing dried roots	3-5 min/species	2	BGB:AGB, Rtd
Weighing dried rest	3-5 min/species	1	BGB:AGB
Scanning leaves	10-15 min/species	3	LAR, Lth, SLA
Scanning roots	15-20 min/species	4	Rle, Rth, Rtd, SRL
Software analysis	8-10 min/species	7	LAR, Lth, SLA, Rle, Rth, Rtd, SRL
Washing*	15-30 min/species	All	All
Total ca.	**65-105 min/species**		

5.2 Conclusion

For the BGB:AGB the hypothesis was: Mixotrophic plants have a bigger percentage of belowground (and lower percentage of aboveground) biomass compared to autotrophic plants. This hypothesis can be rejected because no significance difference was found. Nonetheless, the BGB:AGB ratio was, as expected, higher for mixotrophic plants compared to autotrophic plants.

For the Lnu, the hypothesis was: Autotrophic plants form a bigger number of leaves compared to mixotrophic plants because of the dependence on photosynthesis. No significant difference was found.

So, the hypothesis can be rejected. For the non-woody plants, the Pht was significantly higher for *Arum*-type species.

The hypothesis for Pht was: Because of their dependence on sunlight autotrophic plants grow taller compared to mixotrophic plants. The results were significant, however *Paris*-type species grew taller compared to *Arum*-type species. A possible solution for this is the potential alternative carbon source gives the mixotrophic plants an advantage. Where the C taken up enhances growth rates of the mixotrophic plants over the autotrophic plants where C is limited.

For LAR, the hypothesis was: The need for light among the autotrophic plants will result in a higher LAR compared to mixotrophic plants. No significant different was found. Therefore, the hypothesis can be rejected.

The hypothesis for SLA was: SLA is higher in autotrophic plants compared to mixotrophic plants. The difference is not significant, so, the hypothesis can be rejected.

The hypothesis for root length was: because autotrophic plants invest less in the housing of fungi, they form longer roots to take up nutrients from the soil, while mixotrophic plants invest in fungal partners and will form shorter roots. For the *Arum*-type species a significant higher root length was found. Thus, the hypothesis can be accepted. The longer root system for autotrophic species likely means that they investigate their environment more trying to find nutrients in the soil.

The hypothesis for root thickness was: Mixotrophic depend on fungal partners to survival and therefore invest in thicker roots to better house the fungi, autotrophic plants invest less in the housing of fungi and therefore, form thinner roots. No significant difference was found. So, the hypothesis can be rejected.

For Rtd the hypothesis was: Mixotrophic plants have a higher Rtd compared to autotrophic plants, because mixotrophic plants invest more in the housing of their fungal partner. Rtd was found to be higher in *Paris*-type species. Mixotrophic plants forming a higher Rtd was not expected, the difference was even significant.

For SRL the hypothesis was: Autotrophic plants have a higher SRL compared to mixotrophic plants, to meet their nutrient demand. No significance difference was found. Therefore, the hypothesis can be rejected.

When looking at the set of traits used to distinguish autotrophic from mixotrophic plants as stated in the hypothesis: both above- and belowground traits can be used to distinguish mixotrophic from autotrophic plants. The answer is not straight forward. 3 of the traits show a significant difference between mixotrophic and autotrophic and some traits could potentially be important as de dataset will grow. Other traits, for instance, the BGB to AGB ratio does not show promising results. For this set of traits, the PCA does not show the separating of autotrophic plants from mixotrophic plants.

The main question was: In which above- and belowground traits do potential mixotrophic plants (*Paris*-type) differ from autotrophic (*Arum*-type) plants?

According to the results root length, root tissue density, and plant height can be used within the plot in Landgoed the Utrecht. When looking at data from the database root thickness can be added to the traits showing differences between autotrophic and mixotrophic.

The hypothesis was: Because mixotrophic plants likely require less light and have a higher dependency on mycorrhizal fungi than autotrophic plants, we hypothesize that these both above- and belowground traits can be used to distinguish mixotrophic from autotrophic plants. No unambiguously answer can be given. Based on the separate hypothesis, traits can be used to distinguish autotrophy from mixotrophy. The results show more promising results for the belowground traits compared to the aboveground traits. Further research will be needed to be able to recognize mixotrophic plants using their traits.

6 Recommendations

When comparing the data measured for this project with data from databases it is the recommendation of potential traits is as follows:

*Table 6.1: Potential of each trait for the distinguishing of mixotrophic plants. *SLA is almost significant p = .055 for the data from the database, so it might be a potential aboveground trait when looked only at in situ plants within the same sampling location.*

Potential	Significant difference	Traits
High	Own data and database data	Root length
Medium	Own data or database data	Plant height Root thickness Root tissue density Specific root length
Low	Own data nor database data	BGB:AGB Number of leaves Leaf area ratio Leaf thickness Specific leaf area*

There were more traits which showed potential to be measured but could not be measured due to lack of equipment and/or time. These traits are stomatal density and leaf astringent pigments.

The number of stomatal on a leaf are measured within the trait stomatal density. Stomata have a key role regarding gas-exchange with the atmosphere. Stomata enable the take up of CO_2 required for the photosynthesis. Plants adapt to different environments with different stomatal densities, light intensity is one of these factors (Leake, 1994). So, when a plant is not fully dependent on photosynthesis it might reduce its stomatal density. Reducing stomatal density reduces stress related to water. Stomatal density is calculated by counting the stomata on given leaf surface multiplied by the total leaf surface. It must be kept in mind that plants can form stomata on both sides of a leaf or on only one side. For mixotrophic plants this would mean that they need less stomata per area compared to autotrophic plants.

Leaf astringent pigments are every other pigment then chlorophyl. It is known for fully myco-heterotrophic plants that they are enriched in astringent pigments reflecting in different leaf colors. Ranching from red to pink to blue. The pigments devoid chlorophyll which plays a role in the preventing of herbivory (Leake, 1994).

All the traits can be measured on the same individual. Therefore, the following order of steps is recommended:

1. Measuring plant height in the field and collecting them.
2. Washing of all specimens.
3. Making the root and leave scans.
4. Drying the specimens.
5. Software analysis.
6. Weighing of the dried samples.

When the stomatal density and/or the astringent pigments are added to the trait list, one leaf extra is needed for the analysis. Because the measurements need to be done on fresh leaves and will demolish the leave in such a way that it cannot be used for further biomass analysis.

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Appendix

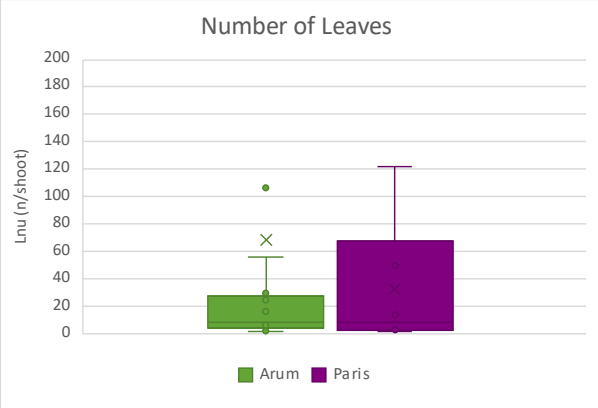
Appendix 1 – Boxplot for data from the Try database

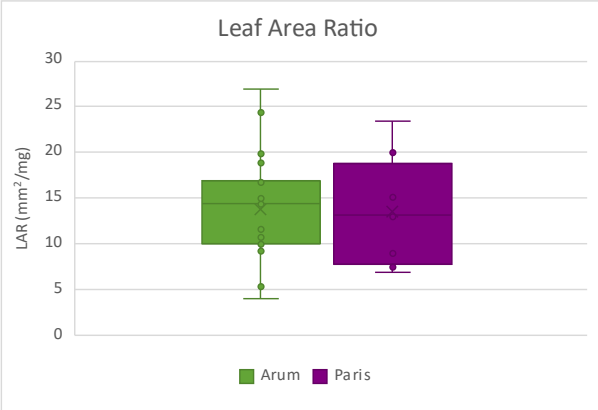
Appendix 2 – Sampling location

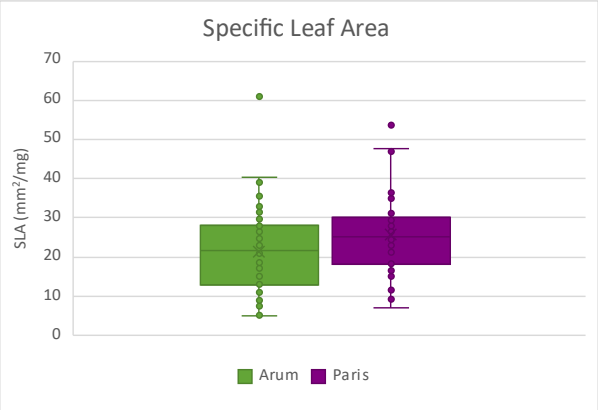
Appendix 3 – Plant measurements and trait values

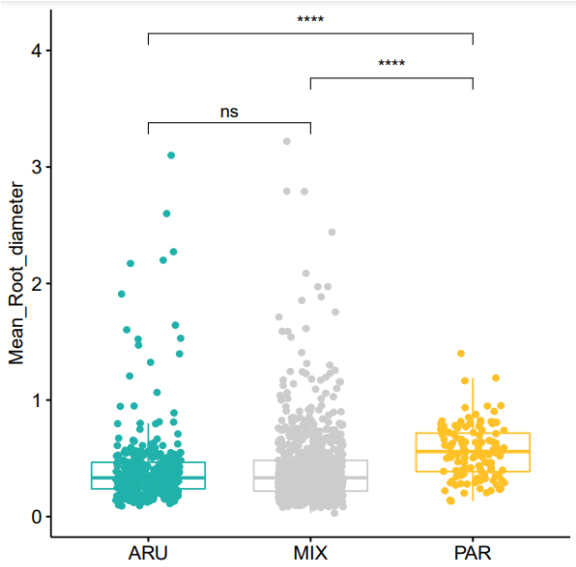
Appendix 4 – Boxplots for the trait values of Landgoed de Utrecht

Appendix 1 – Boxplot for data from the Try database

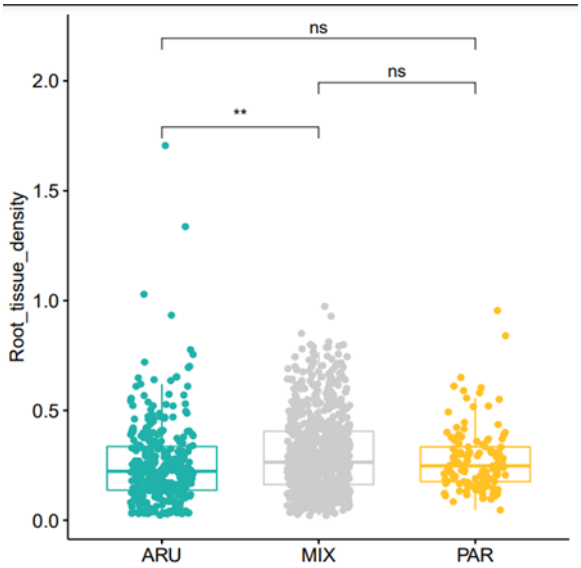




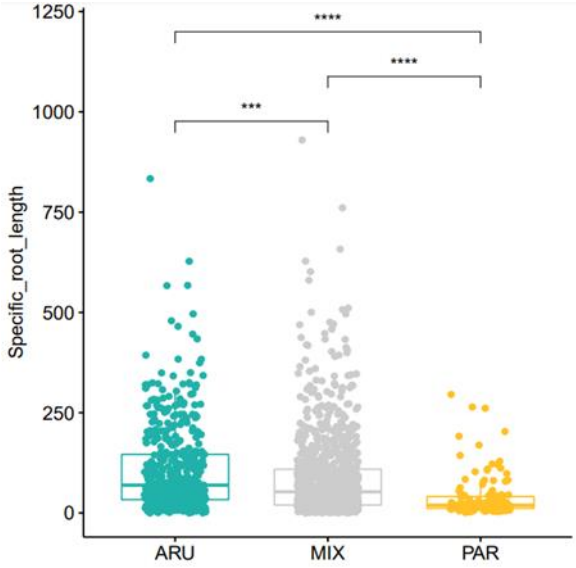




(Merckx, unpublished)

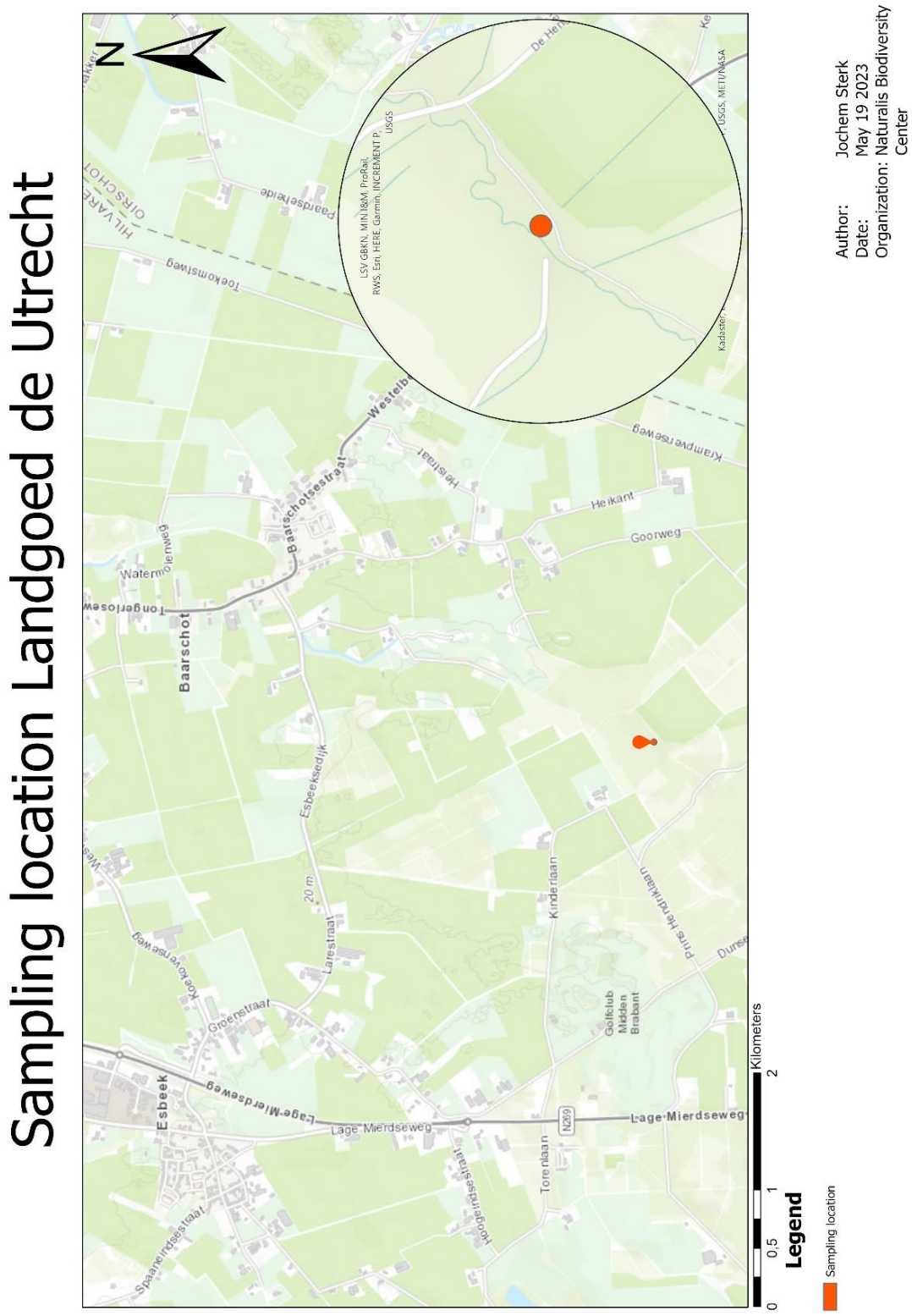


(Merckx, unpublished)



(Merckx, unpublished)

Appendix 2 – Sampling location



Appendix 3 – Plant measurements and trait values

Species	Mycorrhiza	AM Type	Height	#Leaves	Fresh Leaves	Total leaf	Total root
			cm	#	g	g	g
Acer_01	AM	Paris	26.2	4	2.735	0.542	0.461
Acer_02	AM	Paris	25.9	6	5.356	0.771	1.261
Acer_03	AM	Paris	33.6	4	1.891	0.312	0.237
Amelanchier_01	AM	Arum	25.9	13	0.804	0.213	0.150
Amelanchier_02	AM	Arum	31.8	22	1.263	0.329	0.181
Amelanchier_03	AM	Arum	16.5	4	0.127	0.014	0.027
Anemone_01	AM	Paris	6.6	4	0.345	0.038	0.449
Anemone_02	AM	Paris	12	4	0.771	0.063	0.455
Anemone_03	AM	Paris	21.5	4	1.150	0.108	0.286
Athyrium_01	AM	Paris	42.3	3	7.718	1.100	0.243
Athyrium_02	AM	Paris	37.4	3	6.178	0.458	0.103
Athyrium_03	AM	Paris	45.5	2	8.208	0.760	0.232
Corylus_01	AM/EM	Paris	44.5	7	0.424	0.087	0.622
Corylus_02	AM/EM	Paris	25	5	0.351	0.062	0.089
Corylus_03	AM/EM	Paris	23.1	9	0.542	0.131	0.170
Fraxinus_01	AM	Arum	22.4	3	0.515	0.067	0.235
Fraxinus_02	AM	Arum	24	2	0.189	0.024	0.373
Fraxinus_03	AM	Arum	10.6	2	0.259	0.020	0.018
Geum_01	AM	Arum	20.5	14	6.113	0.741	0.318
Geum_02	AM	Arum	18.6	8	4.290	0.512	0.330
Geum_03	AM	Arum	18.7	12	8.188	0.914	0.618
Hedera_01	AM	Arum	5.2	10	1.852	0.482	0.255
Hedera_12	AM	Arum	19.5	7	2.880	1.336	0.269
Hedera_03	AM	Arum	11.4	7	2.869	0.709	0.090
Ilex_01	AM	Paris	7.5	6	0.930	0.370	0.228
Ilex_52	AM	Paris	66	19	14.369	5.942	2.176
Ilex_13	AM	Paris	19.5	10	2.753	1.011	1.894
Prunus_01	AM	Arum	5.5	6	2.006	0.403	0.109
Prunus_02	AM	Arum	42.5	9	0.659	0.147	0.399
Rubus_01	AM	Arum	37.9	11	1.662	0.354	0.313
Rubus_02	AM	Arum	9.9	6	1.687	0.304	0.267
Sorbus_01	AM	Paris	42.8	26	1.180	0.280	1.736
Sorbus_02	AM	Paris	44.3	16	0.874	0.241	1.592
Sorbus_03	AM	Paris	41.2	6	0.331	0.065	0.237
Urtica_01	AM	Arum	7	26	1.653	0.236	0.998
Urtica_02	AM	Arum	6.1	14	0.903	0.123	0.098
Urtica_03	AM	Arum	24.2	26	1.761	0.247	0.665
Viburnum_01	AM	Paris	31.1	18	1.372	0.312	0.098

Viburnum_02	AM	Paris	20.7	12	0.915	0.191	0.226
Viola_01	AM	Paris	5.5	10	0.346	0.051	0.065
Viola_02	AM	Paris	13.5	11	0.924	0.138	0.037
Viola_03	AM	Paris	12	10	0.472	0.046	0.033

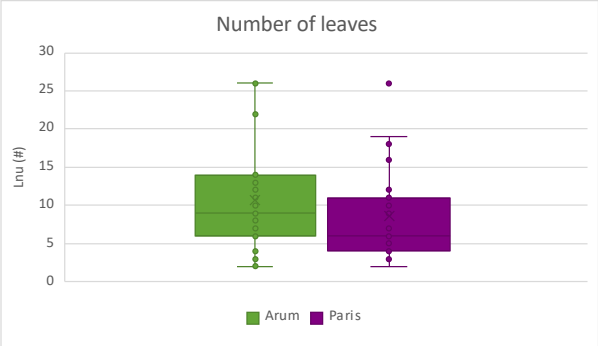
Species	Total Fine Root	Total AGB	Total BGB	Total Biomass	BGB:AGB	Leaf area	Leaf thickness
	g	g	g	g		cm2	µm
Acer_01	0.461	1.302	0.461	1.764	0.354	245.657	671.847
Acer_02	1.261	3.539	1.261	4.800	0.356	392.903	2104.388
Acer_03	0.237	0.921	0.237	1.158	0.257	161.145	304.645
Amelanchier_01	0.150	2.097	0.150	2.248	0.072	105.115	84.460
Amelanchier_02	0.181	1.751	0.181	1.931	0.103	165.475	209.045
Amelanchier_03	0.027	0.783	0.027	0.810	0.035	13.761	1.745
Anemone_01	0.017	0.070	0.449	0.519	6.418	18.356	6.336
Anemone_02	0.021	0.085	0.455	0.540	5.338	40.850	31.508
Anemone_03	0.021	0.153	0.286	0.439	1.865	57.927	66.587
Athyrium_01	0.243	14.748	1.980	16.729	0.134	266.946	2060.369
Athyrium_02	0.103	6.090	0.947	7.037	0.155	219.925	1358.763
Athyrium_03	0.232	10.175	2.310	12.485	0.227	179.123	1470.295
Corylus_01	0.106	1.463	0.622	2.084	0.425	43.640	18.508
Corylus_02	0.030	0.315	0.089	0.404	0.283	33.662	11.829
Corylus_03	0.028	0.511	0.170	0.681	0.333	57.420	31.099
Fraxinus_01	0.100	0.303	0.235	0.538	0.775	38.802	97.374
Fraxinus_02	0.109	0.336	0.373	0.709	1.110	13.340	24.326
Fraxinus_03	0.009	0.058	0.018	0.076	0.320	20.542	81.112
Geum_01	0.111	0.914	0.318	1.233	0.348	189.002	438.798
Geum_02	0.159	0.574	0.330	0.905	0.575	128.916	575.587
Geum_03	0.217	1.014	0.618	1.632	0.609	312.691	668.296
Hedera_01	0.255	2.707	0.255	2.962	0.094	71.780	132.908
Hedera_12	0.269	3.226	0.269	3.495	0.083	134.182	386.458
Hedera_03	0.090	1.779	0.090	1.868	0.050	81.622	234.157
Ilex_01	0.228	0.846	0.228	1.074	0.269	34.076	31.697
Ilex_52	0.101	7.184	2.176	9.360	0.303	308.028	4426.024
Ilex_13	0.483	7.316	8.811	16.127	1.204	82.293	226.586
Prunus_01	0.109	2.139	1.436	3.575	0.671	179.917	360.878
Prunus_02	0.399	0.235	0.399	0.634	1.702	61.546	40.571
Rubus_01	0.313	1.842	4.738	6.580	2.572	85.452	142.047
Rubus_02	0.267	3.810	0.267	4.077	0.070	110.092	185.714
Sorbus_01	0.417	5.597	1.736	7.332	0.310	112.305	132.475
Sorbus_02	0.256	3.825	1.592	5.416	0.416	88.092	76.984
Sorbus_03	0.082	1.508	0.237	1.745	0.157	21.625	7.151
Urtica_01	0.384	0.519	0.998	1.518	1.923	103.341	170.864

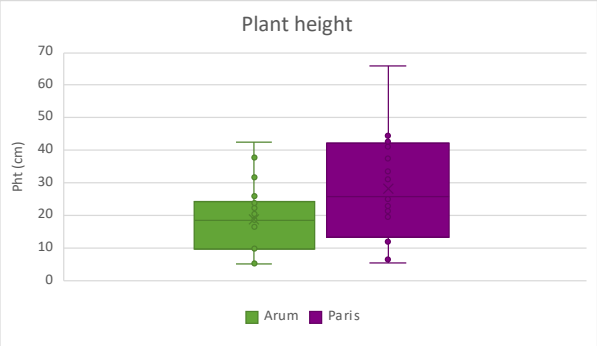
Urtica_02	0.098	0.329	0.098	0.427	0.297	64.216	57.993
Urtica_03	0.273	0.569	0.665	1.234	1.170	131.522	231.663
Viburnum_01	0.098	2.041	0.098	2.139	0.048	119.168	163.439
Viburnum_02	0.226	1.104	0.226	1.330	0.204	76.540	70.019
Viola_01	0.065	0.092	0.065	0.157	0.702	21.336	7.380
Viola_02	0.037	0.323	0.037	0.360	0.115	52.879	48.871
Viola_03	0.033	0.176	0.033	0.209	0.186	23.714	11.193

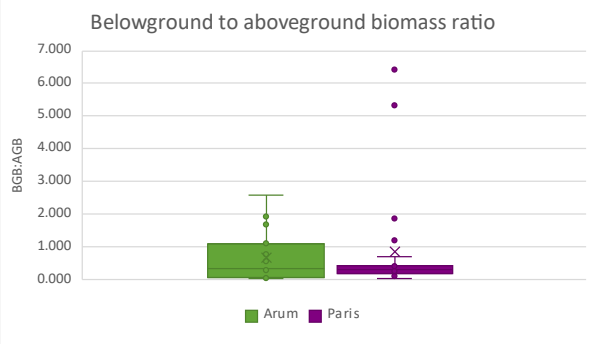
Species	Root length	Root diameter	Root volume	Root tissue density	SLA	LAR	SRL
	mm	mm	mm3	mg/mm3	mm2/mg	cm2/g	mm/mg
Acer_01	2544.136	0.480	1221.511	0.378	45.328	139.299	5.514
Acer_02	3896.241	0.498	1939.417	0.650	50.934	81.862	3.090
Acer_03	780.018	0.378	295.098	0.803	51.658	139.107	3.291
Amelanchier_01	1426.736	0.476	678.586	0.222	49.391	46.767	9.490
Amelanchier_02	1364.232	0.486	663.407	0.272	50.287	85.676	7.552
Amelanchier_03	589.779	0.365	214.992	0.126	98.692	16.981	21.802
Anemone_01	701.054	0.461	323.154	1.389	48.627	35.378	42.232
Anemone_02	860.043	0.365	314.085	1.448	65.045	75.648	41.750
Anemone_03	781.310	0.407	317.949	0.898	53.709	132.087	37.029
Athyrium_01	6385.031	0.504	3220.281	0.075	24.272	15.958	26.283
Athyrium_02	3601.783	0.434	1561.784	0.066	48.004	31.254	34.988
Athyrium_03	5713.374	0.525	3001.613	0.077	23.574	14.347	24.659
Corylus_01	3871.575	0.423	1639.416	0.379	50.389	20.937	36.672
Corylus_02	1133.719	0.377	427.611	0.209	54.626	83.267	38.374
Corylus_03	1490.357	0.347	516.504	0.329	43.814	84.268	53.341
Fraxinus_01	3790.404	0.423	1601.664	0.147	57.798	72.080	37.722
Fraxinus_02	3583.189	0.386	1382.244	0.270	55.255	18.813	33.007
Fraxinus_03	731.719	0.337	246.390	0.075	101.704	269.746	82.630
Geum_01	4082.667	0.411	1679.439	0.189	25.499	153.348	36.880
Geum_02	7747.939	0.437	3382.599	0.098	25.158	142.477	48.668
Geum_03	8106.624	0.442	3586.159	0.172	34.223	191.624	37.427
Hedera_01	6101.466	0.472	2879.611	0.088	14.891	24.237	23.946
Hedera_12	6719.360	0.449	3016.278	0.089	10.041	38.395	25.026
Hedera_03	3626.695	0.545	1976.915	0.045	11.518	43.686	40.386
Ilex_01	966.209	0.489	472.204	0.482	9.207	31.737	4.241
Ilex_52	1596.775	0.650	1037.836	2.097	5.184	32.910	15.745
Ilex_13	4121.830	0.645	2657.698	0.713	8.139	5.103	8.538
Prunus_01	1326.486	0.572	758.323	0.144	44.639	50.323	12.173
Prunus_02	2198.644	0.734	1614.686	0.247	41.976	97.130	5.509
Rubus_01	6036.025	0.432	2609.954	0.120	24.114	12.987	19.314
Rubus_02	3449.503	0.540	1862.367	0.143	36.171	27.003	12.934

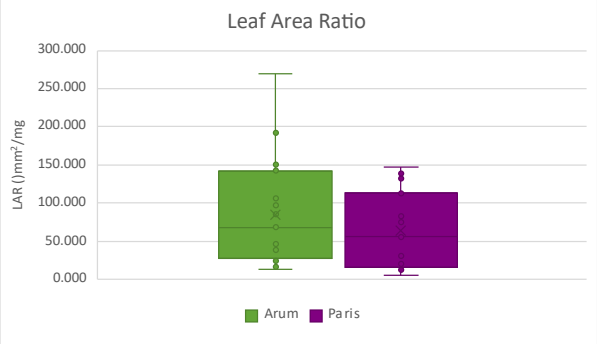
Sorbus_01	4705.027	0.628	2954.963	0.587	40.146	15.317	11.275
Sorbus_02	5179.504	0.526	2724.033	0.584	36.556	16.264	20.216
Sorbus_03	2029.718	0.436	885.608	0.268	33.190	12.392	24.796
Urtica_01	3766.931	0.647	2435.655	0.410	43.849	68.088	9.803
Urtica_02	4292.573	0.493	2115.105	0.046	52.406	150.390	43.865
Urtica_03	6582.866	0.417	2742.561	0.243	53.333	106.562	24.084
Viburnum_01	4643.796	0.674	3128.135	0.031	38.161	55.713	47.307
Viburnum_02	2373.419	0.663	1572.708	0.143	40.058	57.565	10.518
Viola_01	1410.582	0.278	391.512	0.165	42.221	136.042	21.802
Viola_02	1179.543	0.325	383.298	0.097	38.381	146.815	31.794
Viola_03	960.251	0.365	350.326	0.094	51.586	113.264	29.187

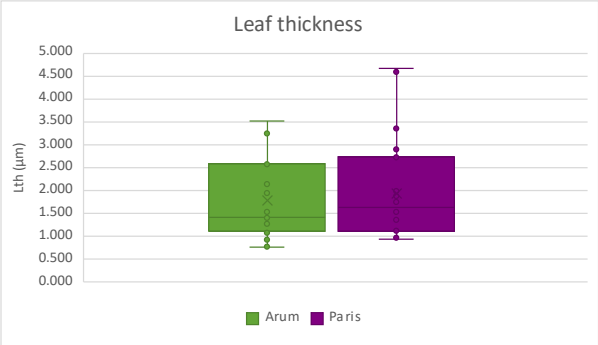
Appendix 4 – Boxplots for the trait values of Landgoed de Utrecht

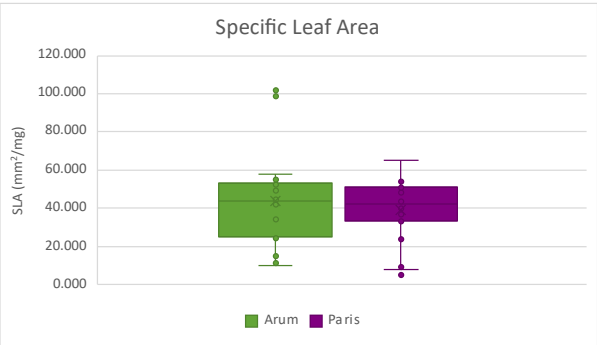


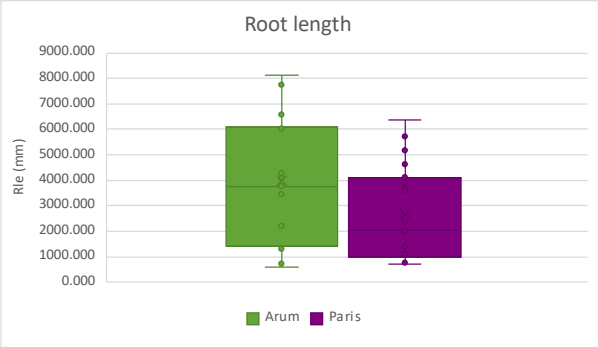


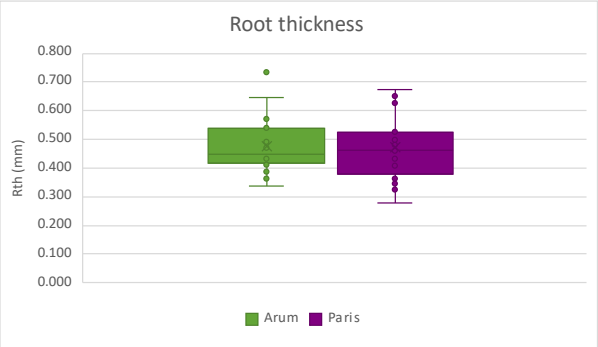


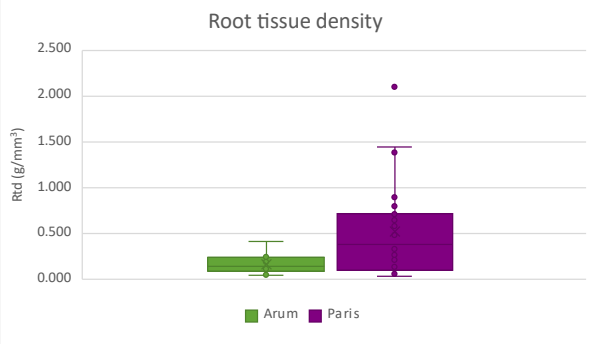


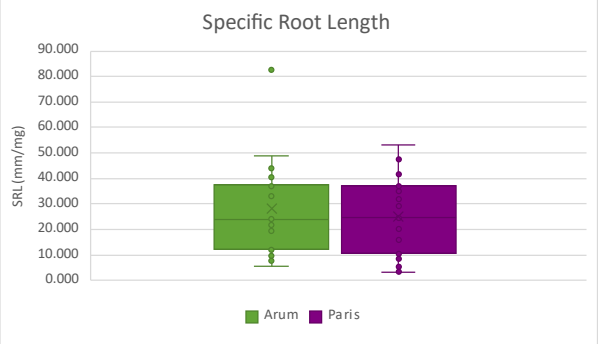





Root length
Rle (mm)
9000.000
8000.000
7000.000
6000.000
5000.000
4000.000
3000.000
2000.000
1000.000
0.000
Arum
Paris


Root thickness
Rth (mm)
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Arum
Paris


Root tissue density
Rtd (g/mm³)
2.500
2.000
1.500
1.000
0.500
0.000
Arum
Paris


Specific Root Length
SRL (mm/mg)
90.000
80.000
70.000
60.000
50.000
40.000
30.000
20.000
10.000
0.000
Arum
Paris