Universität für Bodenkultur Wien
University of Natural Resources
and Life Sciences, Vienna

Master Thesis

Screening of Environmentally Diverse Soils to Discover New Beneficial Microbes for Use in Sustainable Agriculture

Submitted by

Marlene NIEDERMAYER

in the framework of the Master programme

Phytomedizin

in partial fulfilment of the requirements for the academic degree

Diplom-Ingenieurin

Vienna, October 2022

Supervisor:

Priv.-Doz. DI[in] Dr.[in] Angela Sessitsch
Institute of Soil Research
Department of Forest and Soil Sciences

UNIVERSITY OF COPENHAGEN

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Affidavit

I hereby declare that I have authored this master thesis independently, and that I have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or in basic content from unpublished sources or from published literature are duly identified and cited, and the precise references included.

I further declare that this master thesis has not been submitted, in whole or in part, in the same or a similar form, to any other educational institution as part of the requirements for an academic degree.

I hereby confirm that I am familiar with the standards of Scientific Integrity and with the guidelines of Good Scientific Practice, and that this work fully complies with these standards and guidelines.

Vienna, 24.10.2022 Marlene Niedermayer *(manu propria)*

Preface

The research for this master thesis was carried out at Dynamo Centre of the University of Copenhagen from September 2021 until April 2022. The project was funded by novo nordisk fonden and Danmarks Grundforskningsfond.

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Abstract

As the human population is growing the need for food is also increasing, but in the face of climate change this should be done in the most sustainable way. This puts pressure on agriculture to achieve high yield with at least impact on the climate as possible. In recent years the possibility to use microbes to reach this goal gained more attention. During this project twelve natural Danish soils have been tested as a source for new beneficial microbes. Four soils showed the possibility of containing beneficial microbes after a first phenotypic screening. A nutrient rescue experiment showed that plants are nutrient deprived when they were grown in those candidate soils, especially when the microbes, that are naturally occurring in those soils, are absent. With one soil a transplantation experiment was done, to locate the microbes (either in bulk soil, rhizosphere or endosphere) that are positively impacting *Arabidopsis thaliana*. A so-called soil dilution experiment was done to reduce the microbes in the soil. Therefore, sterile soil was inoculated with a soil extract of the same site and the plant growth and phenotype was monitored and compared. Previously one soil was found to contain beneficial, endophytic bacteria, which have already been isolated. These were used in a mono-association assay with *Arabidopsis thaliana* where the root growth was compared to a control. One bacterium was found which significantly enhanced the plant weight and root length of the plant. For future experiments also four *Arabidopsis thaliana* mutants have been genotyped during this project. Those can be used to study the molecular mechanism of the plant-microbe interaction.

Kurzfassung

Da die menschliche Population wächst, steigt auch der Bedarf an Nahrung. ~~Mit~~ Aufgrund der aktuellen Klimakrise sollte dies jedoch auf die nachhaltigste Weise erfolgen, um die Umwelt zu schonen. Das Potential der Nutzung von Mikroorganismen in der Landwirtschaft, rückte in den letzten Jahren immer mehr in den Fokus. Um neue effektive Mikroorganismen zu finden, wurden während diesem Projekt zwölf dänische Bodenproben untersucht. Während einem ersten Screening wurden vier Böden gefunden die potenziell, effektive Mikroorganismen enthalten. Weitere Experimente zeigten, dass die Nährstoffversorgung von *Arabidopsis thaliana* in diesen Böden eingeschränkt ist, vor allem wenn die natürlich vorkommenden Mikroorganismen nicht präsent sind. Während eines Transplantationsversuches mit einer Probe wurde versucht die Mikroorganismen zu lokalisieren (entweder in der Erde, Rhizosphäre oder als Endophyten). Auch wurde ein Verdünnungsexperiment durchgeführt, bei dem Extrakte der Bodenproben genutzt wurden, um die sterile Erde derselben Probe zu inokulieren. Dies sollte dazu dienen die Menge an Mikroorganismen zu reduzieren und dadurch die Kandidaten für zukünftige Versuche einzuschränken. Für eine Probe wurden vorhergehend bereits endophytische Bakterien isoliert. Diese waren Teil eines Monoassoziations-Assays bei dem das Wachstum von *Arabidopsis thaliana* mit einer Kontrollgruppe verglichen wurde. Dabei wurde ein Bakterium entdeckt, welches das Gewicht der Pflanze und die Wurzellänge erhöhte. Außerdem wurden vier *Arabidopsis thaliana* Mutanten genotypisiert um in zukünftigen Experimenten verwendet zu werden. Diese können dazu dienen die molekularen Mechanismen der Pflanzen-Mikroorganismen Interaktion zu verstehen.

1. Introduction

In the society, problems, that arise from the use of pesticides, become more attention and therefore people want a more sustainable agriculture (Knapp & van der Heijden, 2018). A goal for the future should be a more environmentally friendly production of food, while also ensuring the global food security (Knapp & van der Heijden, 2018). As the population grows and will probably reach a level of 10 billion people in the near future, it is a huge challenge for this century to provide enough food and combat climate change at the same time, therefore a sustainable and high-yielding agriculture is needed (Lynch, 2019; Lynch et al., 2021).

1.1. Agrochemicals Used in Conventional Agriculture

Mandal et al. (2020) defines agrochemicals as "chemical products comprised of fertilizers, plant-protection chemicals or pesticides, and plant-growth hormones used in agriculture". The general purpose of those agrochemicals is to enhance the yield (Mandal et al., 2020). Many of those agrochemicals were major drivers of the green revolution (Lino-Neto & Baptista, 2022).

The usage of pesticides increases constantly on a global scale (FAO/WHO, 2018). The annual use of pesticides is around two million tons worldwide (De et al., 2014). The main group of pesticides used are herbicides (47.5%) followed by insecticides (29,5%) and fungicides (17,5%) (De et al., 2014). Several negative characteristics of diverse pesticides on the environment are reported (Sarkar et al., 2021). Those harmful effects are affecting non-target organisms such as honeybees, birds, humans and other animals (De et al., 2014). With improving analytical methods, more and more information about pesticides gained and with that more negative aspects of synthetic pesticides were found (Seiber & Kleinschmidt, 2011).

A 2018 published report from the WHO/FAO about pesticide management showed that globally 66% of the countries participating in their questionnaire, encounter problems with pesticide resistance in the target organisms (FAO/WHO, 2018). These resistances will become a major problem in the future as it will become harder to fight pests and pathogens (Hawkins et al., 2019), which will therefore threaten food security.

But because pesticides are an easy to use tool and are still effective they will also play an important role in the future of agriculture, but the application should and will shift to less harmful substances (Sarkar et al., 2021; Seiber & Kleinschmidt, 2011).

Another big group of agrochemicals are fertilizers. Those can generally be divided in organic and inorganic, depending on their source and production (Sabry, 2015). Whereas organic fertilizers usually have their origin in biotic sources like animals, plants or others, inorganic fertilizers are derived from abiotic sources and often derived from mines and/or industrially manufactured (Towhid Osman, 2013).

The consumption of fertilizers is increasing and was in 2018 at a level of 136,824 kilograms per hectare of arable land (Data World Bank, n.d.). The consumption for Austria is at 135,1 and for Denmark at 108,1 kilograms fertilizer per hectare of arable land (Data World Bank, n.d.). As the agricultural used soils usually need additional nutrients to be able to produce yield in an adequate amount, fertilizers are needed (Angus, 2012). Fertilizers that are mainly used in industrial agriculture in developed countries, also contribute to environmental pollutions (Lynch, 2019). This is because the nutrients supplied with fertilizers, which are not taken up by plants, are leaking into groundwater and with time contaminate also surface water (Towhid Osman, 2013). This leakage can lead to negative effects like hypoxia, algal bloom and death of pelagic fish (Angus, 2012). It is known that the run off mainly comes

from nitrate and phosphate-chemical compounds (Mandal et al., 2020). For example the increase of nitrogen(N)-fertilizer application in the 20th century also led to a higher N-content in the groundwater (Angus, 2012).

The use of fertilizers over many years can also lead to an acidification of the soil, which will influence plant growth and productivity (Mandal et al., 2020).

Most commonly the macronutrients, nitrogen (N), phosphate (P) and potassium (K) are supplied with fertilization (Angus, 2012; Towhid Osman, 2013). During the process of producing Nitrogen fertilizer, the Haber-Bosch procedure, a high pressure (200atm) and high heat (450°C) environment is needed (Tomer et al., 2016). Phosphate and potassium are gained from ores with mining (Angus, 2012). In the near future, the now used phosphate supplies will be fully exploited (Angus, 2012) and the need for alternatives will arise.

The enhanced use of agrochemicals following the green revolution led to a negative shift in the environment, including loss of biodiversity and reduction of soil quality (Lino-Neto & Baptista, 2022). The use of agrochemicals along with other environmental changes caused by agricultural practices, such as crop varieties or ploughing also heavily influence the soil microbiome in its structure and composition (Dubey et al., 2019; Mandal et al., 2020).

1.1.1. Can Organic Agriculture be a Sustainable Alternative for the Future?

Organic agriculture is widely seen as a sustainable alternative to conventional agriculture. But even though synthetic agrochemicals are prohibited in this type of farming, there are still properties that lower its sustainability.

Worldwide 71,5 million hectares were used for organic agriculture in 2018, this accounts for 1,5% of the worlds area which is used for agriculture (Willer et al., 2020). In Denmark 256.711ha, which account for 9,8% of the total agricultural area, is used for organic agriculture (Willer et al., 2020).

One of the main issues, that organic agriculture has to deal with, compared to conventional agriculture, is its major yield instability and reduced yield (Knapp & van der Heijden, 2018). This sums up to a lower production of crops with organic farming of around 19,2% (±3,7%) (Ponisio et al., 2015). The yield gap between organic and conventional farming is smaller than it was assumed due to former studies (Ponisio et al., 2015), as there were previous reports stating a yield reduction of 25-50% (Kirchmann et al., 2008). This yield reduction can be explained through pest and pathogen attacks, but also through limited nutrient availability (Kirchmann et al., 2008).

As a result to the yield reduction there would be a need to transform more (natural) land into agricultural fields to achieve a similar amount of yield when all agricultural production is changed to organic farming (Kirchmann et al., 2008), which should be avoided, because with the current climate crisis and biodiversity loss, as much land as possible should stay in the most natural way (Bhattacharyya et al., 2016; Kirchmann et al., 2008) and more land that could be used in agriculture is often not even available (Kirchmann et al., 2008).

To avoid or reduce the use of synthetic agrochemicals and improve the yield in organic agriculture it is necessary to develop more sustainable and alternative approaches like the use of beneficial microbes (Jacoby & Kopriva, 2019; Mandal et al., 2020).

1.2. The Soil Microbiome

The term soil microbiome includes the entity of microbes in soil. It is suggested that soil contains 4x 10^6 to 2x 10^9 prokaryotes per gram of dry soil depending on the soil type (Whitman et al., 1998). The highest activity of soil organisms can be found in the upper 15 cm (Towhid Osman, 2013).

Microorganisms play a major role in nutrient cycles that take place in the soil and have therefore a significant effect on their habitat (Dubey et al., 2019). They can be often found in close proximity to plant roots, where they utilize the metabolites secreted by the plants (Towhid Osman, 2013). Plants translocate up to a fourth of their photosynthetically fixed carbon to their roots where it is excreted (Towhid Osman, 2013). The interaction of plants with microbes can either have a positive impact on the plant or can have a negative impact in form of pathogens (Lino-Neto & Baptista, 2022). In exchange for the metabolites, beneficial microbes can enhance plant nutrition by help in uptake or enhancing nutrient availability (Tomer et al., 2016) or increase the resistance or defence against pests and pathogens (Lino-Neto & Baptista, 2022). As of now a lot of information about the interaction of plants with beneficial microbes, e.g. attraction of beneficial microbes while inhibiting pathogens, are still missing (Lino-Neto & Baptista, 2022). The composition of microbes in the soil varies throughout the year (Hansen et al., 2001). The strongest effect on the soil community have the local environmental conditions (Hansen et al., 2001; Thiergart et al., 2020). With changing abiotic soil properties throughout the year, like pH-value and associated environmental variables, also the bacterial community changes heavily (Thiergart et al., 2020).

The species of the soil microbiome are still scarcely known due to the fact that most of them have not yet been isolated and/or cultivated, which also results in a large knowledge gap about the molecular mechanism and function in their natural environment (Jansson & Hofmockel, 2018).

1.2.1.1. Common Soil Bacteria

Different bacteria genus occur very commonly in soil, which are *Pseudomonas, Arthrobacter, Clostridium, Achromobacter, Bacillus, Micrococcus, Flavobacterium, Corynebacterium, Sarcina, Azospirillum* and *Mycobacterium* (Towhid Osman, 2013). Each of those soil microbes has its own purpose in this community that could be potentially utilized in agriculture (see Table 1).

Table 1: Examples of common soil microorganisms and their potential beneficial trait

Genus	**Trait**	**Source**
Arthrobacter sp.	bioremediation of pesticides in soil	(Labana et al., 2005; Wang & Xie, 2012)
Bacillus sp.	biocontrol agent phosphate solubilization	(Herrmann & Lesueur, 2013)
Azospirillum sp.	nitrogen fixation	(Herrmann & Lesueur, 2013).
Pseudomonas sp.	biocontrol agent potential for bioremediation of pesticides in soil phosphate solubilization	(Mauchline & Malone, 2017) (Tonelli Fernandes et al., 2018) (Nagpal et al., 2021)
Clostridium sp.	potential of production of antimicrobial substances	(Pahalagedara et al., 2020)

Achromobacter sp.	potential for bioremediation of pesticides in soil	(Sviridov et al., 2012; Tonelli Fernandes et al., 2018)
Micrococcus sp.	phosphate solubilization	(Nagpal et al., 2021)
Flavobacterium sp.	phosphate solubilization	(Nagpal et al., 2021)
Corynebacterium sp.	bioremediation production of amino acids	(B.-N. Kim et al., 2014)

Not all microbes in the soil directly influence the plant and enhance growth or immunity, but are indirectly involved in shaping the microbe community and through that acts beneficial for the plant (Bai et al., 2022). Also the pool of soil microbes is the source for the root associated microbes (Vandenkoornhuyse et al., 2015).

1.2.2. Rhizosphere

The rhizosphere is the zone in very close proximity to the plant root, which is commonly inhabited by microbes (Tomer et al., 2016; Vandenkoornhuyse et al., 2015). In the rhizosphere the microbial diversity is increased 10-1.000 times compared to the bulk soil (M. Choudhary et al., 2018), but the inhabitants of the rhizosphere are comparable to the ones in the bulk soil (Bai et al., 2022). The rhizosphere microbiome heavily influences the plant (Jacoby & Kopriva, 2019) and those interactions have been shaped by coevolution of over 450 million years (Zhalnina et al., 2018).

Plant associated microbes in the rhizosphere, not only have beneficial effects on the plants but can also be pathogenic, and their impact on the plant can also vary depending on environmental conditions (Jacoby et al., 2021). Knowledge about the influence of the microbes on the plant can be used, for example when the rhizosphere microbiome is transplanted from a resistant to a susceptible crop variety. The receiving plant can show higher resistance to pathogen infections, depending on the different organisms used in the experimental and environmental conditions (G. Jiang et al., 2022).

1.2.3. Endosphere

Often microbes are called endophytes if they colonize the inside of the plant and do not negatively impact their host, but Hardoim et al. (2015) proposed that under the term endophyte all microbes, which fulfill at least part of their life cycle inside of plants, are combined, without including their influence on plants. This is important as most plant-endophyte interactions are not yet well studied, especially in natural conditions, and it is seldomly known if the interaction is beneficial or pathogenic or if it even has an effect on the plant (Compant et al., 2021; Hardoim et al., 2015). Especially as many existing information's about the interaction were gained in very artificial laboratory conditions and not in nature (Compant et al., 2021).

The plant endosphere is a unique environment, and microbes that are inhabiting it, usually have a very specific and adapted metabolome, which also largely differs from microbes living in the rhizosphere (Brader et al., 2014). But also the endosphere consists of many microenvironments as the characteristics of the habitat changes depending on the plant organ (Compant et al., 2021).

To successfully colonize the plant, microorganisms have to pass several barriers (Berg, 2009). One very important one is to overcome the plant immune responses, which still poses many open questions (Vandenkoornhuyse et al., 2015).

Hardoim et al. (2015) found that when summarizing 16S rRNA sequences from published endophytes, most of them belong to one of the following four phyla: *Proteobacteria* (54%), *Actinobacteria* (20%), *Firmicutes* (16%) and *Bacteroidetes* (6%).

1.2.4. Plant-Microbe Interactions

The plant does not exist and survive on its own but works together with its associated microbes. Therefore it is important to gain more information about this interaction to have a full understanding of the plant holobiont (plant with all associated microbes) (Vandenkoornhuyse et al., 2015).

Up until today most of the microbial diversity is still unknown (>90%) and has to be further investigated to understand its full potential (Dubey et al., 2019). It is estimated that 98% of the soil microbes are not culturable (M. Choudhary et al., 2018), which complicates the potential of studying those microbes and gain more knowledge about their function and purpose (de Souza et al., 2020; Jansson & Hofmockel, 2018). But even though only a small percentage grow in artificial conditions, using culture-dependent approaches still generate a lot of information (Hill et al., 2000). The understanding which mode of action plant growth promoting rhizobacteria (PGPR) use, and identifying those traits could help to forecast which microbes might have a beneficial effect on plants (Akinrinlola et al., 2018).

Most of the plant-microbe interactions are based on the composition of root exudates (Bakker et al., 2018). As a reaction to their biotic or abiotic environment plants exude different metabolites (Bhattacharyya et al., 2016), and therefore change its microbiome to its current needs (Vandenkoornhuyse et al., 2015). Especially different forms of Carbon (C), that was previously fixed by photosynthesis is exuded (5-30% of produced C) (M. Choudhary et al., 2018). Those root exudates are known to shape the respective microbiome of the plant (Dubey et al., 2019) as they are the key tool of communication between plants and microbes (Lino-Neto & Baptista, 2022). Especially the secondary metabolites are often used for communication of different organisms (Brader et al., 2014) and are therefore known to influence the microbiome of the plant (Jacoby et al., 2021). The soil microbes use the metabolites secreted by the plant roots as a nutrient source (Tomer et al., 2016).

The beneficial effect microbes have on their host can either be direct or indirect (Berg, 2009). This can be, by actively helping against pathogen or pest attacks or more passively by transforming nutrients from the soil into their plant available form (Dubey et al., 2019).

The different secondary metabolites, which have been identified in recent years to have an influence on the microbiome structure and community, have diverse mechanisms but are often part of the plant immunity and pathogen response (Jacoby et al., 2021). The concept of “cry for help”, when plants that experience pathogen pressure attract certain microbes that help with the defence, is often described as proof that the plants can actively change their root microbiome (Bakker et al., 2018). Those microbes, which assist the plant during defence, can be used as biological control agents (BCA) (Bhattacharyya et al., 2016). For example *Bacillus sp.* is often used as a BCA (Herrmann & Lesueur, 2013).

There are also hints that the plant shapes its root microbiome when experiencing nutrient starvation (Bakker et al., 2018). Some plant growth promoting rhizobacteria have the ability to ensure availability and uptake of nutrients for plants (Vacheron et al., 2013).

When studying the plant microbiome it is also necessary to define the core members (present throughout many environmental conditions) of the community to gain a transferrable understanding of the plant microbe interactions (Vandenkoornhuyse et al., 2015).

1.2.5. Metabolites Play an Important Role in Plant-Microbe Interactions

The belowground communication happens through the exchange of certain chemicals (Rizaludin et al., 2021). In recent years more and more metabolites and their functions have been identified. But this was mainly done in controlled environments and the effect in *in vivo* or *in planta* conditions might be different (Brader et al., 2014).

In the review from Jacoby et al. (2021), the purpose of different secondary metabolites in regard to their impact on (beneficial) microbes is well summarized.

Glucosinolates are commonly known as a plant-defence mechanism against herbivorous attacks (Jacoby et al., 2021) and a lot of information about them has already been gained (Halkier & Gershenzon, 2006). But they have been shown to have the potential of studying them as signal molecules for (beneficial) microbes as well (Jacoby et al., 2021).

Camalexin is secreted by *Arabidopsis thaliana* often as a response to pathogen attacks (Glawischnig, 2007). When studying the role of camalexin with the *Arabidopsis thaliana* mutant CYP71A27 it became clear that also beneficial bacteria were affected by the loss of function of this gene involved in the camalexin pathway (Koprivova et al., 2019).

Triterpenes could play a role in attracting plant species specific microbes (Jacoby et al., 2021).

Coumarins have also been shown to change the microbes associated with *Arabidopsis thaliana* plants and are often secreted in iron deficient environments, showing that the plant reacts to an insufficient nutritional environment by shaping its microbiome (Harbort et al., 2020; Voges et al., 2019).

Salicylic acid is involved in the plant immune response. But it has been also shown to affect the composition of plant associated microbial families (Lebeis et al., 2015).

1.2.6. Utilizing Plant-Microbe Interactions in Agriculture in Form of Biofertilizers

Berg describes in her review from 2009 several advantages of microbial inoculants in comparison with agrochemicals, those include being less dangerous (not only to humans, but also to the environment and non-target organisms), less persistence in the environment and lead to less resistance development. A diverse and healthy soil microbiome has a major impact on crops, and can therefore, if properly understood, be used to ensure a more sustainable food production (Dubey et al., 2019). Two ways of utilizing microbes in agriculture are commonly known: one is in form of biocontrol agents (increase plant defence against pathogens and pests) and the other is via biofertilizers. Utilizing beneficial microbes, which supply the plants with nutrients, can be a more sustainable alternative to commonly used mineral fertilizers (Dubey et al., 2019; Jacoby & Kopriva, 2019).

Under the term biofertilizers, microbes are combined, which enhance plant nutrition by increasing nutrient availability and uptake (Tomer et al., 2016). The number of biofertilizers sold to farmers increased over the past years (Herrmann & Lesueur, 2013). Whereas North America (28%) and Europe (27%) have the worldwide highest market share of biofertilizer (Sansinenea, 2021). Also the impact of increasing restrictions on pesticide and fertilizer applications in agriculture, lead to a higher interest in alternatives, such as beneficial microbes (Sessitsch & Mitter, 2014). A key factor to successfully use biofertilizers is to understand their mechanism and behaviour in soil and their natural environment (Celador-Lera et al., 2018).

At the moment it is common practice to use a single strain as an inoculant in agriculture (de Souza et al., 2020). Those microbes use either one of two major modes of action. One is by enhancing nutrient availability and uptake and the other is through production of phytohormones (Lino-Neto & Baptista, 2022).

Many microbes are known that change the nutrients in the bulk soil. For example some microorganisms in soil have the ability to transform P that is inaccessible to plants, into a soluble form and are called P-solubilizing microorganisms (PSMs) (Tomer et al., 2016). This ability has been reported in several bacterial genera, like *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas* and *Microbacterium*, and many more (Tomer et al., 2016). Generally the most common microbes on the market belong to the genus *Bacillus* sp. and *Pseudomonas* sp. (Berg, 2009).

A big challenge for a widely usable biofertilizer in agriculture is that many interactions are hugely depending on host and microbe/strain participating and might be very specific (Herrmann & Lesueur, 2013). The plant genotype plays a major role in this interaction, particularly on interactions with fungi (Bergelson et al., 2019). Environmental conditions influence the efficiency of (beneficial) microbes (Herrmann & Lesueur, 2013). Those variances in efficiency have to be minimized in order to widely apply microbes in agriculture (Akinrinlola et al., 2018).

To ensure a common use of biofertilizer in farms they must fulfill certain requirements. Those prerequisites are a stable formulation, being easy to apply and handle, have a reasonable cost-benefit factor and achieve the promised effect (Herrmann & Lesueur, 2013). But they should also be safe to handle for the farmer, it is important to study the potential pathogenicity of the microbes on humans (Berg, 2009). As these requirements pose a huge challenge, many biofertilizers on the market are not of high quality (Sansinenea, 2021). Also the regulations to ensure high-quality products in the EU, are missing (Celador-Lera et al., 2018).

Microbial products are often „available as liquid based formulations, water-dispersible granules or wettable powder or pellets" (Berg, 2009). Inoculants face many challenges (existing microbes, stable plant colonization, etc.) and only overcoming all of them, makes them successful (de Souza et al., 2020).

Using a combination of several microbes in a product can enhance the plant-growth-promoting effect (Bulgarelli et al., 2013). Especially as in natural soil one microbe alone is not able to maintain a healthy soil (Hill et al., 2000). This is the reason why the focus also shifted to synthetic communities (SynComs).

1.2.7. Synthetic Community Building

The interest in Synthetic Communities (SynCom) is rising as their importance is more and more recognized (de Souza et al., 2020). There are studies showing that even a SynCom not specific to its host, enhances plant biomass compared to plants grown in an axenic condition (Wippel et al., 2021). Another major advantage of SynComs lies in the preservation of already existing microbe-microbe interactions and through that potentially enhancing the stability to be implemented in field conditions (Lino-Neto & Baptista, 2022), as those multiple interactions are more similar to the natural circumstances in the rhizosphere (Vacheron et al., 2013).

Also using SynComs in terms of a more functional approach is used. This can and will be an important approach as many taxa might fulfil the same purpose in a soil community and are therefore interchangeable (Hill et al., 2000). Those taxa have a similar effect in the soil and are combined to a functional group, which have specific marker genes (Vacheron et al., 2013). In this approach it is believed that it is easier to adapt a SynCom to certain conditions by exchanging members of the community to more suited strains for the condition, but also adapting the communities to the traits desired from agriculture (de Souza et al., 2020). That can be possible by understanding the functions of the genes of the microbes and what effect they have on the plant (Compant et al., 2021).

The so-called core microbes, could probably positively affect the efficiency of SynComs, by also making the SynCom more stable to environmental changes (de Souza et al., 2020).

When studying the effect of a SynCom on a host, an important tool, can be the phenotyping of plants (de Souza et al., 2020), which was also the approach during this project.

1.3. Plant Nutrition

Fourteen elements play a major role in plant nutrition and have to be taken up by the roots from soil (Giehl et al., 2014). A not optimal supply of nutrients lead to phenotypic changes in plants (see Table 2), which differ depending on the species and nutrient (de Bang et al., 2021). Nutrients are a key factor in plant growth, if limited the plants adapt to this starvation and try to optimize the nutrient uptake, which can also effect the root system architecture (Giehl et al., 2014). One of the major nutrient for plants is nitrogen (N), as it is an essential part of chlorophyll synthesis (Tomer et al., 2016), as well as it is an essential component of proteins and nucleic acids (Sahu et al., 2018). For a more sustainable approach of supplying crops with nitrogen, targeting the root phenotype in breeding programs could be a solution. As roots with the so called shape "steep, cheap and deep" have the greatest success in reaching nitrogen in maize plants (Lynch, 2019).

Another essential element for plants is Phosphorus (P) and it is often applied via fertilizers (M. Choudhary et al., 2018). Two forms of P are commonly present in soil, organic and inorganic phosphorus (M. Choudhary et al., 2018; Tomer et al., 2016). As only 0,1% of P is available to plants, there is commonly a P deficiency in soil used for high-yielding agriculture (Tomer et al., 2016).

Also bioavailability of Iron (Fe) often challenges the plants and is often provided by microbes (Tomer et al., 2016). But too much iron in plants can also be toxic and negatively affect biological processes inside of cells (Giehl et al., 2014). Generally is iron necessary for plants for their role in photosynthesis and respiration (Harbort et al., 2020).

Sulphur occurs in soil mainly in its organic form, but can be made plant available by microorganisms (M. Choudhary et al., 2018). Another form of uptake can be of the plants above ground parts, which are able to take up atmospheric S (de Bang et al., 2021). In agriculture and food production sulphur supply of plants is important, as deficiency also lead to a poorer quality of the product (de Bang et al., 2021).

Magnesium plays an important role in plants as it is a part of many enzymatic pathways and is part of the chlorophyll (Kamiya et al., 2012). Generally Mg deficient symptoms (interveinal chlorosis) are seen on the oldest leaves because Mg is mobile in plants and is allocated from the older to the youngest leaves (de Bang et al., 2021).

The deficiency of Potassium in plants lead to a variety of phenotypic symptoms. For example leave chlorosis on the oldest ones that over time might turn into necrosis (de Bang et al., 2021). As K is a very mobile element in plants, already developed symptoms might get reduced after supplying the plant with sufficient K (de Bang et al., 2021).

Table 2: Example of phenotypic nutrient deficiency symptoms of plants. Table adapted from de Bang et al. (2021).

Nutrient	Symptoms of Deficiency	Phloem Mobile
Nitrogen (N)	general chlorosis of oldest leaves stunted growth, small leaves, reduces shoot branching and early flowering often anthocyanosis on leaf and stem	yes
Phosphorus (P)	anthocyanosis	yes

	dark-green and/or purple leaves	
Potassium (K)	chlorosis on tip of oldest leaves that develop into marginal necrosis bronzing	yes
Sulphur (S)	chlorosis of young leaves stunted growth anthocyanosis	conditional
Magnesium (Mg)	intervenous chlorosis on oldest leaves that eventually develop into necrosis accumulation of sucrose and starch in chloroplast	yes
Calcium (Ca)	disintegration of root tissue necrotic lesions on leaf edges and tips meristem death necrotic spots on fruits and vegetables leaf deformity	no

1.4. *Arabidopsis thaliana* (Columbia-0) as model organism

Arabidopsis thaliana is commonly used in science as a model organism to study various research questions (Koornneef & Meinke, 2010). It belongs to the family of Brassicaceae. *Arabidopsis thaliana* can be found in nature in various places on earth, it naturally occurred on the northern hemisphere in Europe, Asia and Africa, but by now it has been found also in the Southern Hemisphere (USDA et al., 2022). Several characteristics made it an important model organism, like the short life cycle, the simple handling, early sequencing of the genome and a small genome with five chromosomes, etc. (Koornneef & Meinke, 2010). Commonly used is the Columbia-0 variety as a wildtype plant (Koornneef & Meinke, 2010), but also many mutants exist with often already known and specified mutations (Koornneef & Meinke, 2010; Rédei, 1975). Those mutants can be used as a tool to study the molecular mechanisms of the plant microbe interactions during this project. Many of the mutants are produced by either T-DNA insertion or EMS mutation (Koornneef & Meinke, 2010). During T-DNA mutation, a random gene is altered by inserting the *Agrobacterium tumefaciens* transfer DNA (T-DNA) (O'Malley et al., 2015). EMS mutagenesis uses the chemical mutagen ethyl methanesulfonate (EMS), which results in point mutations (Unan et al., 2022).

1.4.1.1. Arabidopsis thaliana *root architecture*

The parts of the root system architecture, which usually are elongation, branching and spacing, are constantly adapting to environment throughout the life of the plants (Giehl et al., 2014; Gruber et al., 2013). Especially the nutrient availability in the surrounding soil, and therefore also the nutritional status of the plant, seem to influence the root system architecture, but the molecular mechanisms behind this, are not yet known (Giehl et al., 2014).

The length of the primary root in dicotyledonous plants is the result of the cells in the root apical meristem (Giehl et al., 2014). The characteristics of lateral roots, number, position and length, are

important to evaluate the properties of the root system architecture (De Smet et al., 2012; Giehl et al., 2014), as those characteristics can be caused by plant genetic but also environmental factors (Gruber et al., 2013). As the (leaf) development is tightly linked with root growth and especially development of lateral root primordia of *Arabidopsis thaliana,* disturbances during development can be later seen in the lateral root density of the plants (De Smet et al., 2012).

Even though it is known that the root architecture depends on many abiotic and biotic factors, how the roots react to different environmental conditions is often not clearly understood (De Smet et al., 2012). What is known is that the root shape influences the ability to take up and reach nutrients in soil (Lynch, 2019).

1.4.1.2. Nutrient Starvation in Arabidopsis thaliana

Nutrient starvation has a major effect on the phenotype of plants (see Figure 1 and Table 2).

In *Arabidopsis thaliana* a nutrient starvation of Sulphur (S), Iron (Fe) or Potassium (K) leads to a 6-fold reduced shoot fresh weight compared to a control with full nutrient availability (Forieri et al., 2017). A plant only starved with Fe also develops chlorosis, beside the reduced rosette size (Garcia-Molina et al., 2020). Also the root fresh weight of *Arabidopsis thaliana* is reduced 6-fold in the Fe and K starved conditions, but the S starved plants only show a 2-fold reduction (Forieri et al., 2017).

Starvation of certain nutrients (P, Ca, K, Mn and B) can lead to reduce length of the primary root in *Arabidopsis thaliana* (Gruber et al., 2013). Also the length of the lateral roots can be affected by nutrient availability, as P, Mg or K deficiency can lead to a reduced length of lateral roots in different orders (Gruber et al., 2013).

When plants are grown in low P environment the growth of the lateral roots is enhanced while at the same time the growth of the primary root is inhibited (de Bang et al., 2021; Giehl et al., 2014). But P starvation does not only lead to a change in root growth, it also leads to increased storage of anthocyanins in rosettes, which causes purple coloration of leaves (de Bang et al., 2021).

Sulphur starvation of *Arabidopsis thaliana* plants negatively affects their mitochondria, but also the general size of the plants were decreased (Ostaszewska et al., 2014).

Magnesium deficient *Arabidopsis thaliana* plants show interveinal chlorosis about two weeks after removal of magnesium. In magnesium starved plants a decrease of the above-ground plant biomass can be detected (Hermans & Verbruggen, 2005).s

In general a phenotypic change is often hard to link to one specific nutrient deficiency, as an excess or deficiency of one nutrient might also effect the uptake of another (Garcia-Molina et al., 2020).

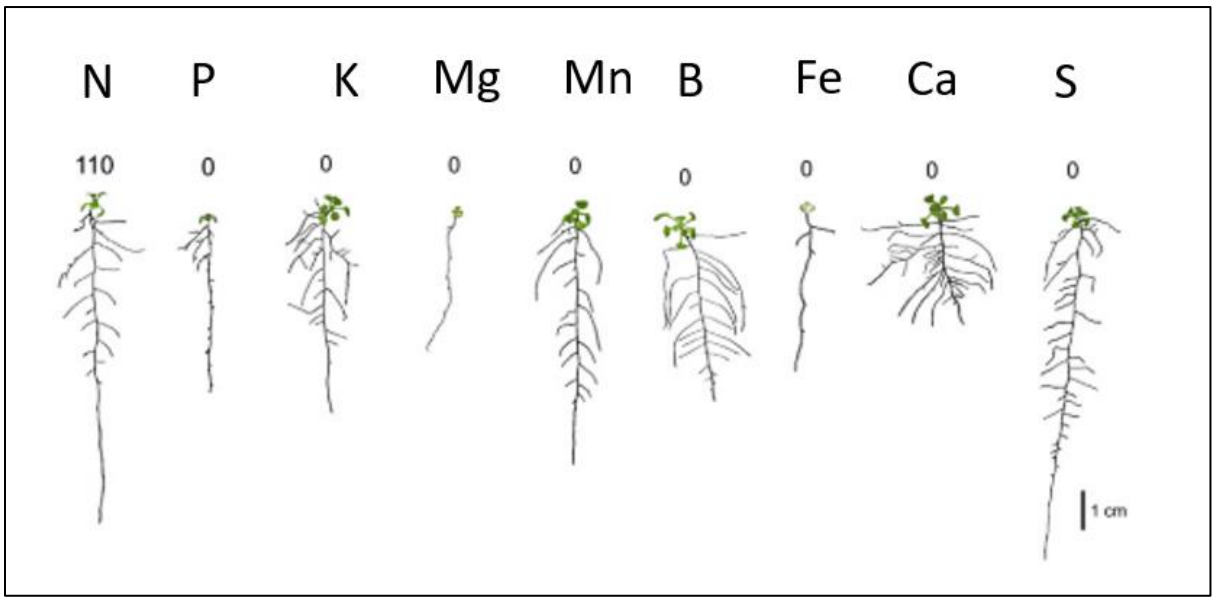

Figure 1: *Arabidopsis thaliana* root phenotype under nutrient deficiency. Adapted from Gruber et al. (2013)

1.4.1.3. Microbes Associated with Arabidopsis thaliana

It is important to define the core microbes and their function to fully understand their importance for the plants (Vandenkoornhuyse et al., 2015). The biodiversity of microbes decreases in *Arabidopsis thaliana* from bulk soil to rhizosphere and to endosphere (Lundberg et al., 2012), but the bacterial composition of the *Arabidopsis thaliana* endosphere across different sites in Europe varies less than the one in the bulk soil, as it is more plant specific (Thiergart et al., 2020).

The most common and widespread operational taxonomic units (OTUs) found in the *Arabidopsis thaliana* endosphere across 13 different sites in Europe belong to the genera *Bradyrhizobium, Pseudomonas, Polaromonas, Acidovorax, Ralstonia, Massilia, Burkholderia, Kineospora* and *Flavobacterium* (Thiergart et al., 2020). Most of those OTUs were also found in grasses harvested in close proximity to the tested *Arabidopsis thaliana* plants (Thiergart et al., 2020). In the *Arabidopsis thaliana* endosphere Actinobacteria, Proteobacteria and Firmicutes were predominant (Lundberg et al., 2012).

1.5. Current State of the Project

1.5.1. Background of the Project

From 2014 to 2018 a survey of 130 sites in Denmark was done by Brunbjerg et al. to observe the biodiversity. The sites were chosen based on different factors. One of those was to analyze as many ecologically diverse areas as possible, not only according to the soil type but also to the land use. On 40 x 40m on each site, data of vascular plants, bryophytes, macrofungi, lichens, gastropods and arthropods were collected. Also, abiotic factors of the sites were measured, e.g. soil pH, total soil carbon, total soil nitrogen, total soil phosphate, soil moisture, soil surface temperature, leaf CNP, humidity, air temperature, light density and boulder density. During the project around 5500 species were observed and of those 143 species were newly recorded in Denmark (Brunbjerg et al., 2019).

Based on the data provided by the Biowide project, sites were chosen to be further investigated in terms of activity/presence of beneficial microbes, which could have a value in future agricultural practices. The focus of the decisions was based on sites containing low nutrient contents and choosing those which potentially cause nutrient starvation in plants.

1.5.2. Findings of the Project so far (Soil 82)

During a pilot experiment in 2019 twelve sites were analysed by Rasmus Plantener Jespersen (unpublished data). Based on a phenotypic initial screening (described in 2.1), comparing *Arabidopsis thaliana* Col-0 grown in sterile and unsterile soil, Rasmus Plantener Jespersen found three sites, where the plants showed a poorer phenotype, when grown in sterile soil compared to unsterile soil. This led to the conclusion that those samples potentially contain (beneficial) microbes causing those phenotypic differences. Based on this finding he further investigated those soils. During a nutrient rescue experiment (described in 2.3.5) Rasmus Plantener Jespersen found that the phenotypic differences visible in the initial screen, were not present anymore when a full nutrient solution was added, indicating that the plants were indeed nutrient starved in the sterile soil and microbes are providing nutrients in the unsterile soil. During this experiment the sample with the name soil 82 showed the most promising phenotype and was therefore subject to further investigations in this project. Plants grown in sterile soil 82 showed leaftip and at times also interveinal chlorosis, this phenotype cannot be seen in plants grown in unsterile soil 82.

Since 2021, Ph.D. student Laura Dethier is working to further understand the mechanisms behind the beneficial effect seen in unsterile soil 82 (unpublished data). During a novel transplantation experiment (described in 2.2) Laura Dethier and student Maja Schmidt Pedersen found indications that endophytic microbes contribute majorly to the beneficial phenotype in unsterile soil 82. As a result of this finding Laura Dethier isolated fungi and bacteria from the *Arabidopsis thaliana* endosphere after growing them in unsterile soil 82. The isolation of bacteria was based on the protocol from Zhang et al. (2021).

Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) analysis of the plants was used to determine the nutritional status in the rosette. The results showed a reduced magnesium and calcium content when the plants were grown in sterile soil 82. Based on this, the student Maja Schmidt Pedersen did a nutrient rescue experiment supplying a nutrient solution with $Mg(NO_3)_2$ and/or $Ca(NO_3)_2$ to the pots. Plants supplied with Ca^{2+} and a combination of Mg^{2+} with Ca^{2+} showed significantly less leaftip chlorosis. But supplying Ca^{2+} alone led to enhanced interveinal chlorosis. All treatments in common had an increased rosette size at the end of the experiment, whereas plants supplied with Mg^{2+} alone or in combination with Ca^{2+} showed the strongest increase in rosette size. But this size increase could be caused by the NO_3 which was added along with the nutrient solutions and needs therefore to be furth investigated.

Based on the ICP-MS analysis it also became clear that the sodium (Na) content of plants grown in sterile soil 82 is higher compared to plants grown in presence of microbes. Based on this finding the goal was to further investigate the possibility of enhanced saline tolerance in the unsterile condition. Because microbes are able to help the plant to deal with salinity stress (Tomer et al., 2016), which will become an important trait as in many areas the amount of saline soil increases (Bai et al., 2022).

A so-called soil-dilution experiment with soil 82 was carried out by Laura Dethier and Camilla Timmerman-Krogh. This experiment revealed that when a soil extract, derived from unsterile soil 82 was added to sterile soil 82 the plants had not only no visible chlorosis on the leaves like the ones in unsterile soil, but they also developed a stronger increase in their growth rate and became overall bigger compared to the control, which suggest that a reduction of the microbes by preparing a soil extract can be used to achieve a beneficial effect on plant growth. This method in combination with a transplantation experiment can help to reduce the microbial candidates for a beneficial SynCom.

1.6. Project Introduction

The research on soil microbiology can generally be split into two approaches: culture-dependent and culture-independent methods (Hill et al., 2000). This project tries to use both, to reach the goal of finding new beneficial microbes in natural Danish soil.

1.6.1. Identification of Potential New Sources for Beneficial Microbes in Different Danish Soils

During this project 12 new soil samples from different sites in Denmark were collected. Half of the amount of each sample was sterilized with irradiation, which is known to be one of the soil sterilization techniques that causes only little change in mineral and organic properties of the sample (Salonius et al., 1967). *Arabidopsis thaliana* Col-0 was used as a model plant to determine phenotypic differences when grown in sterile or unsterile conditions of each sample site. Comparing those two conditions, with the assumption that the only difference is the presence of microbes, it might be possible to find candidate sources for beneficial microbes. As a high-throughput way to phenotypically analyse the *Arabidopsis thaliana* plants, the programme "ara**deep**opsis" was used. The results from the software can be used to evaluate the plant health by measuring anthocyanin-rich area and senescent area (Hüther et al., 2020). To gain more information about the possible presence of beneficial microbes, the samples which resulted in a "improved" plant phenotype in the unsterile condition and an unhealthy/nutrient starved phenotype in the sterile soil were chosen for further investigations. The plants in those candidate soils were also analysed with ICP-MS, to evaluate the nutrient status.

The candidate soils will then be further investigated, trying to ensure that the microbial presence in the unsterile soil caused the "improved" plant phenotype, as well as trying to reduce the number of present microbes to also reduce the number of candidates for identification of beneficial microbes. The further experiments were a so-called transplantation and a soil-dilution process, which were already established for soil 82.

1.6.2. Future Strategies to Unravel the Molecular Mechanism of the Beneficial Microbes in Soil 82

To ensure a stable beneficial effect from microbes, it is necessary to understand their mode of action. This can help to evaluate which host plants and environmental conditions are necessary to achieve the highest effect.

One strategy to unravel the molecular mechanism can be the use of *Arabidopsis thaliana* mutants, which are limited in pathways for secondary metabolite production (Jacoby et al., 2021). Growing those mutants in presence and absence of microbes in the same soil sample and comparing their phenotype will give a better understanding of which pathways in the plant are influenced by the soil microbiome. A culture-depending approach could be the inoculation of the sterile soil with the SynCom of soil 82, if the beneficial microbes are culturable in laboratory conditions. If it is not possible a culture-independent approach in form of either the unsterile soil or a soil-extract (which has a reduced microbial load) is necessary.

The first step of this process is the genotyping of the *Arabidopsis thaliana* mutants. It is necessary that the supposed mutants have a mutation in the correct gene. This can be done with PCR-based methods.

1.6.3. Culture-Depending Approach to Further Investigate the Beneficial Microbes from Soil 82

Laura Dethier adapted the protocol from Zhang et al. (2021) to isolate endophytic microbes from *Arabidopsis thaliana* Col-0 plants grown in unsterile soil 82. The adaptations included a sterilization of the root surface after washing them to only isolate the endophytes, as well as a lower dilution (74x, 148x, 222x) because of the reduced number of microbes because of the root surface sterilization. From this process >300 glycerol stocks, containing bacteria were derived and stored at -80°C. 63 of those glycerol stocks were part of this project. To ensure that every bacterium is only present once, they needed to be identified by 16S rRNA sequencing. Analysing the bacterial 16S rRNA gene for identification of the bacteria has become common practice and is widely used (Janda & Abbott, 2007), because it is highly conserved in all bacterial genera and can therefore be compared among them (Rossi-Tamisier et al., 2015). From those identified samples new glycerol stocks were prepared for further investigations.

To understand which bacteria might be involved in a plant growth promotion even under nutrient sufficient conditions, mono-association assays were conducted with the samples. Also, saline conditions were tested during the mono-association assays, based on the ICP-MS results of plants from soil 82. The plates (with half-strength MS-media) were inoculated with a single bacterium and *Arabidopsis thaliana* Col-0 plants were grown on them for 10 days. Comparison of root length, root architecture and plant fresh weight with the controls gave an indication which bacteria had a beneficial effect on the plants.

1.6.4. Research Questions

How is the phenotype of *Arabidopsis thaliana* plants impacted, when they are grown in sterile and unsterile conditions of 12 Danish soil samples?

Is it possible to localize the microbes impacting the phenotype of *Arabidopsis thaliana* in soil 117 to either bulk soil, endosphere or rhizosphere through a transplantation experiment?

Can a soil extract be used to dilute the microbiome while still showing the beneficial effect on *Arabidopsis thaliana*?

Is nutrient starvation the reason for the impaired phenotype of *Arabidopsis thaliana* when grown in the sterile soils 71, 109 and 116?

Which cultivable endophytes from soil 82 cause a phenotypic improvement in mono-association with *Arabidopsis thaliana* Col-0? Is this effect also present in saline stress conditions?

2. Materials and Methods

The experiments which were performed with the soils 71, 109, 116 and 117 were done in equal parts by Ydun Kalsbeek-Hansen and Marlene Niedermayer. The data analysis and writing of the thesis were done alone.

2.1. Initial Soil Screening

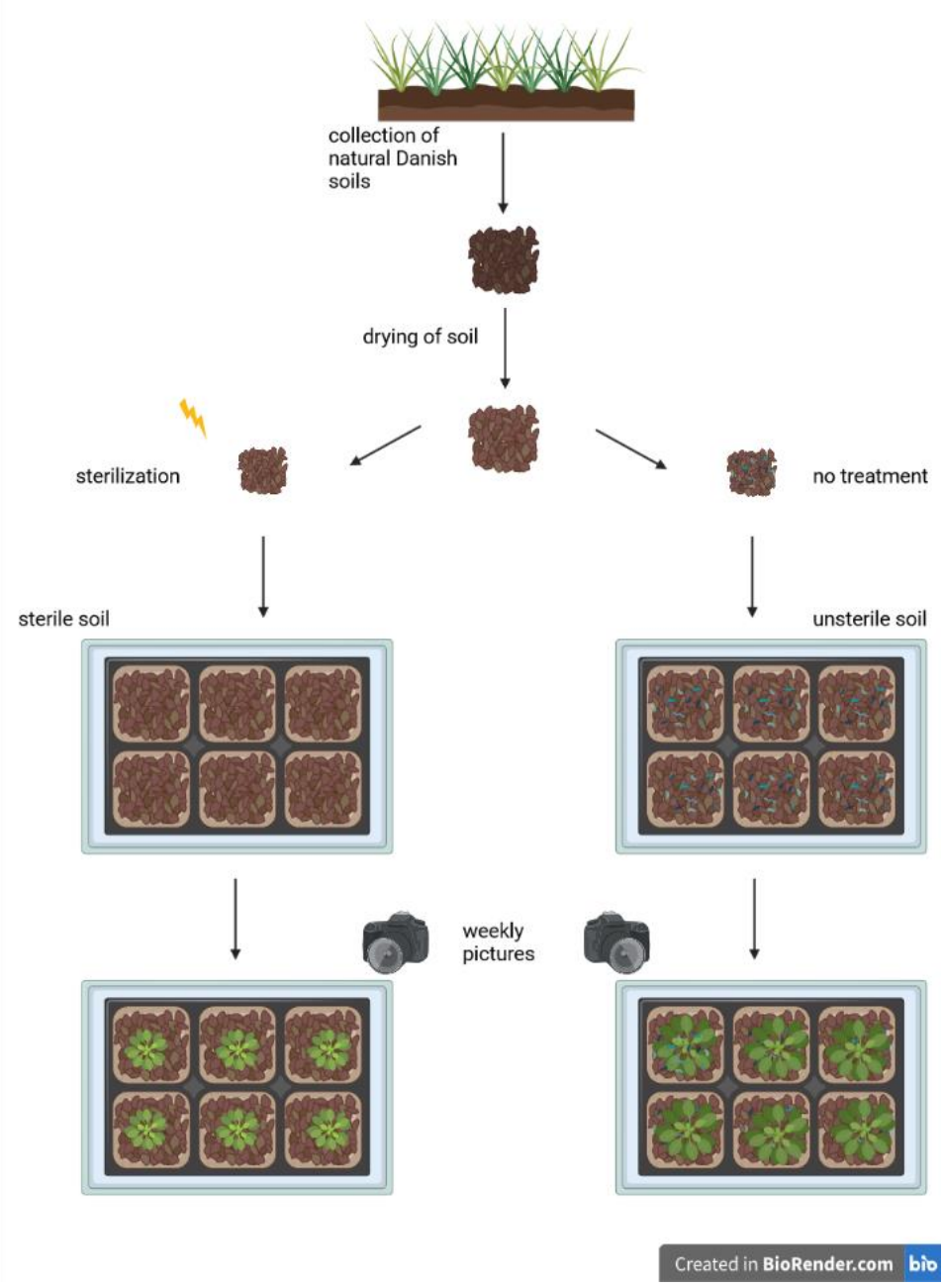

Figure 2: Experimental design of the initial soil screening. Created in Biorender

2.1.1. Sample Sites

The soils were collected based on the GPS data provided by the researchers of the Biowide project (see Table 3).

Table 3: Information about the soil sampling process and location of the 12 tested soils

Soil-ID	Region	GPS	Day of Collection	Days of Drying	Site Information
29	Western Jutland	56°17'42.1"N 8°11'42.2"E	7.-9.7.2021	3	field
37	Western Jutland	55°46'21.5"N 8°10'58.3"E	7.-9.7.2021	4	
41	Western Jutland	55°33'46.2"N 8°04'49.5"E	7.-9.7.2021	2	
71	Vejle	55°40'33.0"N 9°51'02.5"E	7.-9.7.2021	4	
75	Vejle	55°40'01.3"N 9°32'43.5"E	7-.9-7.2021	3	open grass with shrubs, bushes and trees, goats roaming around
107	Fyn	55°07'59.7"N 10°15'12.4"E	1.9.2021	5	forest
109	Fyn	55°06'04.0"N 10°29'58.3"E	1.9.2021	5	open grass area
115	Lolland	54°45'16.7"N 11°29'16.7"E	2.9.2021	2	field (previously cereal on it)
116	Lolland	54°43'29.7"N 11°32'13.2"E	2.9.2021	11	forest
117	Lolland	54°45'07.3"N 11°33'15.7"E	2.9.2021	11	forest
119	Lolland	54°42'52.9"N 11°38'44.5"E	2.9.2021	14	grass with bushes and shrubs
125	Moen	54°57'37.8"N 12°27'19.6"E	2.9.2021	11	

2.1.1.1. *Properties of Soils based on Biowide Data*

The Biowide project also provided data about the abiotic properties, such as pH value or nutrient contents, of all the sites (see Table 4). Based on this data the sites for the experiments were selected. The chosen soils should represent the natural variety of Danish soils, as well as being mostly nutrient poor.

Table 4: Information about the soil properties which were provided from the Biowide project

Soil-ID	Soil Class	Succession	Moisture	Fertility	pH-value
29	Sand	Field			5,9
37	Organic	Early	Wet	Rich	5,1
41	Sand	Mid	Dry	Rich	6,8
71	Clay	Late	Moist	Rich	8,1
75	Sand_Clay	Oldfield			5,4
107	Sand_Clay	Plantation			5,0
109	Sand_Clay	Mid	Dry	Poor	4,8
115	Clay	Field			6,5
116	Organic	Late	Wet	Poor	6,0
117	Organic	Late	Wet	Rich	6,0
119	Organic	Mid	Moist	Rich	4,6
125	Sand_Clay	Early	Wet	Rich	5,6

2.1.2. Soil Collection and Processing

The GPS coordinates (see Table 3), provided by the Biowide project, were used to collect soil samples as close to the location, analyzed by Biowide, as possible. For documentation pictures of the area were taken before collecting soil. The upper layer of soil, containing litter and plant material, was removed and around 25 kg of the upper 30 cm of soil were collected using a spade. Until further processing the samples were stored at 4°C.

In the next step the soil samples were dried. Therefore, the soil was spread out in a thin layer on a table and dried at room temperature. Every few days the soil was mixed to improve the drying process. Depending on the initial water content and the soil type the drying process took between 2 to 14 days (see Table 3). To remove twigs, stones, debris, etc. the samples were sieved using a 1 cm mesh.

Afterwards the soils were prepared for the irradiation process. The whole soil sample was weighed and then split in two equal amounts of soil. One half of each sample was filled in small plastic bags (30x60x3 cm), with maximum of 3kg soil per bag. Then the plastic bags were sealed properly using a heat-sealer. The irradiation was done by Sterigenics located in Denmark. Each bag was irradiated with 18 kGy, using gamma-irradiation, on both sides to sterilize the soil samples. This resulted in a treatment with 36 kGy of gamma-irradiation of each bag. Both, sterile and unsterile, soil samples were stored at 4°C until further use.

2.1.2.1. Confirmation of Soil Sterility

To confirm that the irradiation process resulted in sterile soil samples, five samples were tested for their sterility. Samples of sterile and unsterile soil were taken in the greenhouse while preparing the pots for the initial screening in 15 ml plastic tubes. Those samples were stored at 4°C until the sterility test was conducted.

Petri dishes (plates) with 9 cm diameter of potato dextrose agar (PDA) and tryptic soy broth agar (TSA) were prepared (recipes in Table 21 and Table 23). Two g of soil (sterile and unsterile) of each site were weighed into a 15 ml plastic tube and filled up with sterile milliQ water to 12 ml. Because working in the sterile bench was not possible with the dry soil, a control was included. For this analysis, 12 ml of sterile milliQ water was poured into a 15 ml plastic tube and left open on the lab bench for 1 min. The samples were incubated at 28°C and shaking at 250 rpm for at least 2 hours. Then they were centrifuged at 5000 g for 1 min. In the sterile bench 750 µl supernatant of each sample was plated on 3 PDA and 3 TSA plates. The plates were then sealed with Parafilm and incubated at 25°C for a maximum of 1 week. Afterwards the microbial growth for each sample was evaluated and pictures were taken.

2.1.2.2. pH Measurement of Soil

To test the pH value of the samples, 5 ml of soil was measured in a 15 ml plastic tube and 5 ml of milliQ water was added. The samples were shaken for 1 min. To allow the soil particles to move to the bottom of the tube, the samples were left to rest for at least 30 min. Then the pH value was measured by dipping a color-based pH value indicator strip into the supernatant. The pH value was then determined by comparing the color of the test strip to the manufacturer's information.

2.1.3. Phenotypic Soil Screening Process

2.1.3.1. Preparation of Pots

For each soil 14 pots of unsterile und 14 pots of sterile samples were prepared. Therefore, the soil was mixed 1:3 (V/V) with sand (size no. 2). 250 ml of soil was measured, and 750 ml of sand was added. Then, depending on the sample, 150-200 ml deionized H_2O was added. This was mixed thoroughly and then split into 14 pots with 55 mm diameter. The trays with the pots were put in a plastic bag and stored at 4°C until further use, but at least 2 days, to ensure thorough rehydration of the soil.

2.1.3.2. Preparation of Seedlings

Arabidopsis thaliana Columbia-0 (Col-0) seeds were first surface sterilized to avoid contamination. The seeds were placed in a 1,5 ml Eppendorf tube and 1ml of 70% Ethanol (EtOH) was added and then shaken at 250 rpm or by hand for 20 min. The 70% EtOH was removed and 1 ml of sterile milliQ water was added. The tube was shortly inverted and then centrifuged in a table centrifuge. The supernatant was removed and 1 ml of milliQ water was added again. This washing step was repeated four times to ensure that all the 70% EtOH was removed. To synchronize the germination the *Arabidopsis thaliana* seeds were stratified in milliQ water at 4°C for at least 48 hours.

Square plates with half strength Murashige and Skoog (MS) medium were prepared. Using a pipet, 30 Col-0 seeds were placed on each plate. The plates were sealed with a microporous tape and placed

vertically in a climate chamber. The growing conditions in the climate chamber can be seen in Table 5.

Table 5: Growth conditions in climate chamber

temperature	22°C
relative humidity	55%
day length	16 h
light intensity	140 µmol/m²sec

After 11 days of incubation, the seedlings were moved into the prepared pots with the different soil samples. Using tweezers, one plant was carefully moved from the MS plate into one pot. Similar sized plants were chosen for the sterile and unsterile pots of the same soil. The trays were placed in the greenhouse and covered with plastic foil, which was removed after 1 week.

2.1.3.3. Phenotypic Soil Screening for Beneficial Microbes

The trays containing pots with sterile soil were placed on a different table in the greenhouse than trays containing pots with unsterile soil, to avoid contamination. The *Arabidopsis thaliana* plants were grown in long day conditions and the temperature in the greenhouse was set to 17-19°C (see Table 6). To avoid a location bias all the trays were moved at least once a week randomly on the table. The plant phenotype was evaluated on several time points and pictures of the trays were taken once a week. In pots containing unsterile soil, seeds, which were not *Arabidopsis thaliana,* germinated, those plants were removed continuously. The pots were watered using osmotic water which was filled into the bottom of the tray for the soil to soak it up. Some pots containing different soil samples needed to be watered from the top, because they were not able to soak up the water from the bottom. The trays were watered when needed, so the plants do not experience drought stress.

Table 6: Growth conditions in greenhouse

temperature	17-19°C
day length	16 h
light intensity	140 µmol/m²sec

2.1.4. Picture Analysis

The *Arabidopsis thaliana* plants in the different trays were numbered for identification in the pictures during the experiment as seen in Figure 3.

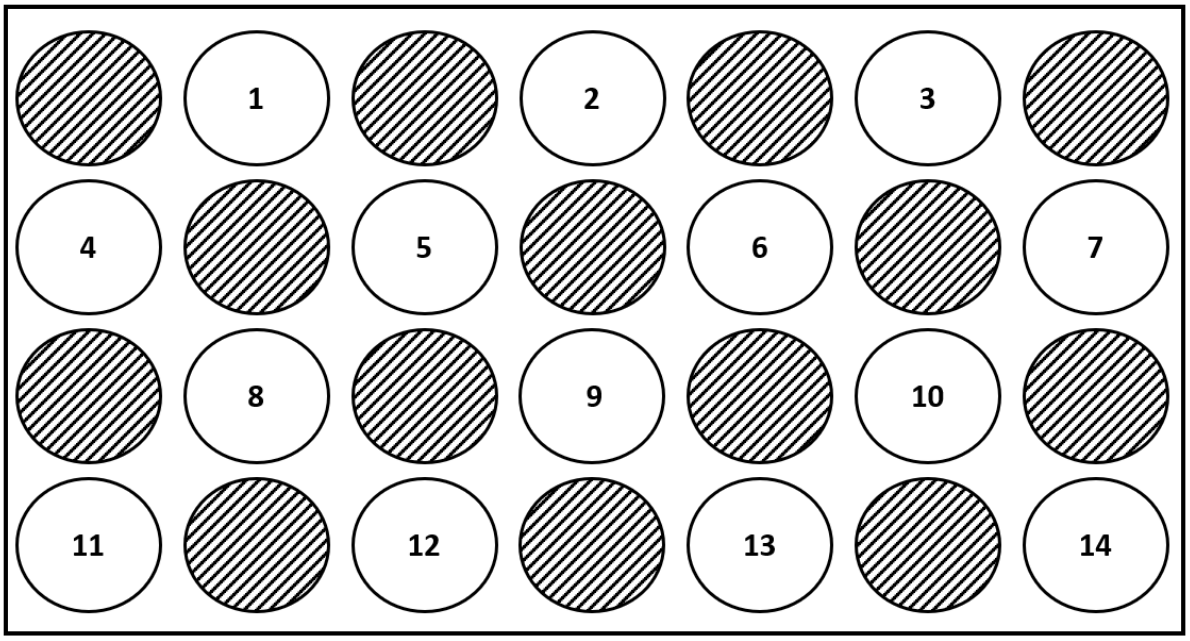

Figure 3: Arrangement of the pots in the tray for the initial phenotypic soil screening

2.1.4.1. Correction for Barrel Lens Distortion

During all experiments a Canon EOS 450D with an EFS 18-55mm lens was used to take pictures.

Due to the camera and lens that were used, a lens barrel distortion occurred on the pictures. To correct for that, the program ACDSee Photo Studio Ultimate 2022 was used. The weekly pictures taken in the phenotypic screening experiment were automatically corrected for barrel lens distortion by the program.

2.1.4.2. Stacking and Cropping of Pictures

The distortion corrected pictures were then processed in FIJI (Fiji Is Just ImageJ). To ease the following process the first step was to align all pictures. This means that the pictures are placed in a way that all trays overlap and have the same size. Using the ROI (region of interest) manager the 14 pots containing plants in each tray were marked (see Figure 3). By running the macro script (see Figure 4) single pots were cut out of the pictures and saved individually.

```
dir=getDirectory("image");
list= getFileList(dir);
  for (j=0; j< list.length; j++){
name= getTitle();
name=replace(name, ".tif", "");
for(i=0; i<roiManager("count"); ++i) {
  run("Duplicate...", "title=crop");
  roiManager("Select", i);
  run("Crop");
  saveAs("png", dir+"cropped_plants/"+name+"_"+(i+1)+".png");
  close();
  //Next round!
  selectWindow(getTitle());
}
run("Open Next");
}
```

Figure 4: Code in ImageJ to automatically cut single pot pictures from whole tray pictures

2.1.4.3. Aradeepopsis

The pictures were analyzed using the Aradeepopsis Software developed by Hüther et al. (2020) (https://github.com/Gregor-Mendel-Institute/aradeepopsis). This software is a high-throughput method to analyze single-pot pictures of *Arabidopsis thaliana*. It includes morphological features like plant size and senescent plant region. In total it analyses 78 different morphological traits which are either independent or dependent (e.g. ratios) of each other.

2.1.5. Harvest and Preparation of Rosettes for ICP-MS analysis

For the soils 71, 109 and 116, *Arabidopsis thaliana* rosettes were harvested to send for nutrient analysis with ICP-MS (Inductively Coupled Plasma – Mass Spectrometry). The ICP-MS was carried out by Thomas Hesselhøj Hansen. Three rosettes from the sterile and three from the unsterile pots were chosen for each site. The rosette was cut with a scalpel. If the plant already had a shoot, it was removed. For each rosette the number of leaves was counted. The rosette was put into a 15 ml Falcon tube and immediately placed in liquid nitrogen. The rosettes were stored at -80°C until freeze drying.

For freeze drying the lid of the tubes was replaced with parafilm. A few small holes were cut into the parafilm to ensure evaporation of water during the process. The rosette samples were freeze dried for 2 days.

The dry weight for the rosette samples was then determined and ICP-MS analysis was conducted. During the ICP-MS analysis the nutrient content for 19 elements in the plant material was determined.

The difference between sterile and unsterile conditions was calculated. From this dataset a heat map is then drawn using the pheatmap package (Kolde, 2019) in R Studio 4.1.0. (R Core Team, 2021) for visualization of how the unsterile conditions had a different impact on *Arabidopsis thaliana* nutrient status compared to sterile conditions. A Welch t-test was done to evaluate the statistical difference between the conditions.

2.1.6. Statistical Analysis of the Aradeepopsis Data

To evaluate the statistical differences between the sterile and unsterile conditions R Studio 4.1.0. was used (R Core Team, 2021), including the packages tidyverse (Wickham et al., 2019), ggpubr (Kassambara, 2020), rstatix (Kassambara, 2021) and readxl (Wickham & Bryan, 2019).

For each trait only the plants from the respective soil in sterile and unsterile conditions were compared, never different soils with each other. As a statistical test a Welch t-test was used to compare the results of Aradeepopsis. The characteristics that were compared are the total plant area, the senescent plant area and the anthocyanin plant area.

2.2. Transplantation Experiment

The experimental setup and design of the transplantation experiment were previously developed by Deyang Xu, Laura Dethier and Maja Schmidt Pedersen. It is an innovative method to localize beneficial microbes in plant roots or surroundings and therefore potentially reduce candidates for isolation and building a future SynCom/BenCom.

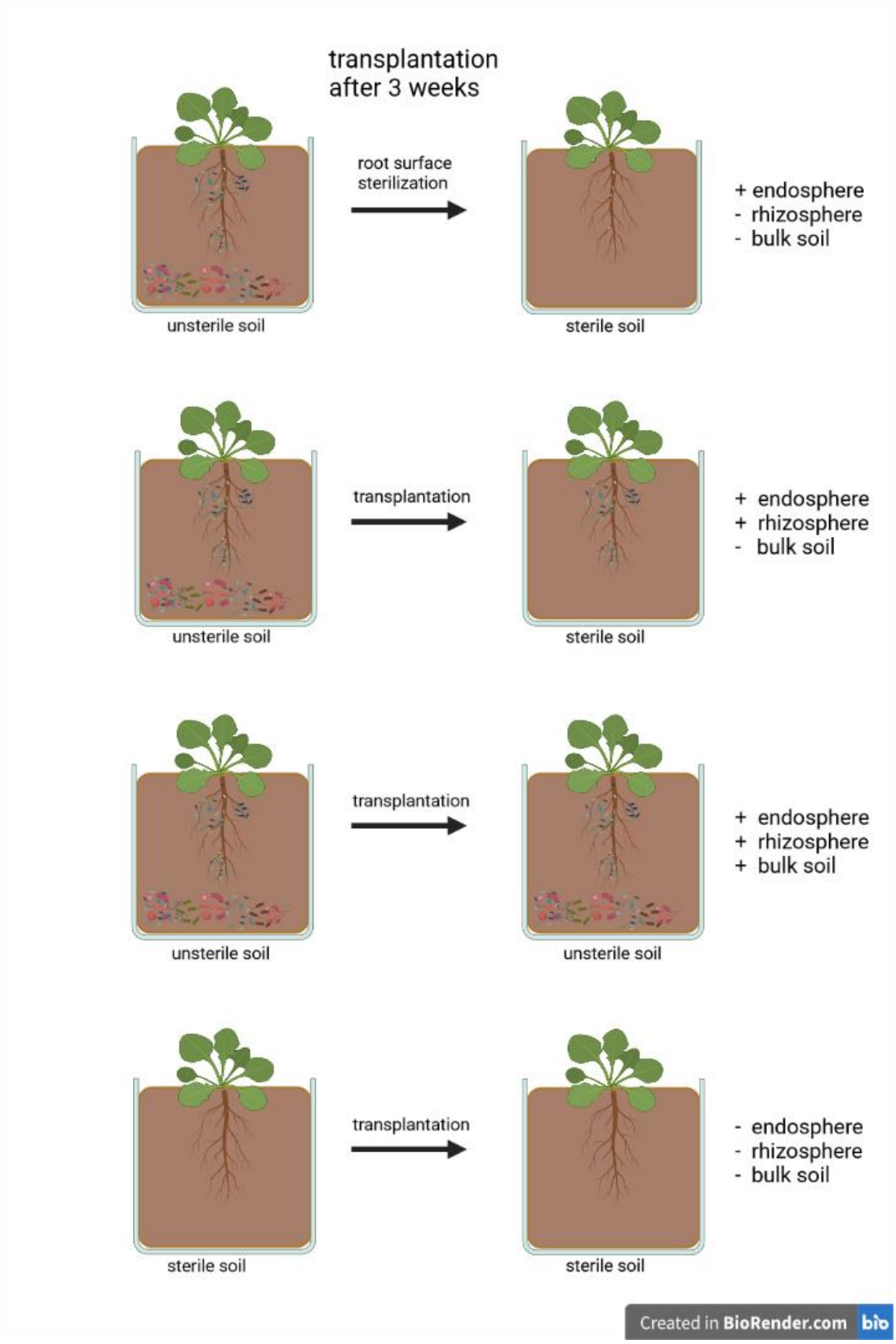

Figure 5: Experimental Design of the Transplantation Experiment for Soil 117. On the left side are the conditions in which the plants were growing for the first three weeks, either in presence of microbes or not. After three weeks the plants were moved into new pots. The group containing only endophytes underwent a root sterilization. The plant roots of the other three treatments were washed in sterile milliQ H_2O to remove big soil particles (concept of figure was made by Laura Dethier). Created in Biorender

2.2.1. Experimental Design

Natural soil contains many microbes, and it is hard to determine the purpose of every strain in the soil community. To have a better understanding where the microbes, providing the beneficial effect on the plants, are located and therefore reduce the number of candidates. This innovative transplantation experiment was carried out for soil 117. It is assumed that there are three distinct locations the microbes occur: the bulk soil, the rhizosphere and the endosphere. The experiment contains two controls: the negative control should contain no soil microbes and the positive control should contain all microbes present in the tested soil. Two treatments are done to either remove the microbes only in the bulk soil or in addition also microbes in the rhizosphere. In the end of the experiment, it is evaluated in which treatment the plants show a beneficial phenotype and it is therefore possible to localize the beneficial microbes. The Figure 5 visualizes the experimental process of the transplantation.

2.2.2. Preparation of Pots and Seeds

A sample size of 28 plants per treatment were intended. Therefore, a total of 112 pots containing unsterile soil 117 and 112 pots containing sterile soil 117 were prepared. The soil was mixed with sand (size No.2) in relation 1:3 and enough ddH_2O added to moisten the soil. The pots were stored at 4°C until further use, but for at least 2 days.

Col-0 seeds were surface sterilized as described in 2.1.3.2 and stratified at 4°C for at least 2 days.

2.2.3. Transplantation Process

28 pots containing sterile soil 117 and 84 pots containing unsterile soil 117 were used in the first step. In each pot 3-5 seeds of Col-0 were pipetted. The trays were covered with plastic film for two weeks. The growth conditions for the plants can be seen in Table 5.

After three weeks the seedlings were transferred to new pots. For the negative control (containing no microbes) 28 plants growing in sterile soil 117 were moved to 28 new pots containing sterile soil 117 as well (called neg. control). For the positive control (containing “all” microbes) 28 plants from unsterile soil 117 were moved to 28 new pots containing unsterile soil 117 (called pos. control). To avoid the microbes in the bulk soil, 28 plants from unsterile soil 117 were moved to sterile soil 117 and the roots were shortly washed with sterile milliQ water to remove bigger soil particles (called endo+rhizo). The treatment, containing only endophytic microbes, included a sterilization step while transplanting. Therefore 28 plants growing in unsterile soil were incubated for 30 sec in 0,25% sodium hypochlorite and thoroughly rinsed with sterile milliQ water afterwards. The plants were then transplanted into sterile soil 117 (called endo). After the transplantation the new trays were covered with plastic film for one week.

During the growing period the plants were watered consistently with ddH_2O, to avoid drought stress. Plants that were not *Arabidopsis thaliana* that germinated in the unsterile soil were constantly removed. The trays containing the different experimental conditions were also moved inside the climate chamber at least once a week to avoid a location bias.

Pictures of the trays were taken right before the transplantation and afterwards once a week.

2.2.4. Harvest of Plants

To analyze the Col-0 plants in addition to the pictures, the plants from the transplantation experiment were harvested. The number of leaves was counted for each plant as well as the number of leaves showing yellow leaf edges. The rosette was cut, and the fresh weight determined. The rosette was then moved in a 15 ml Falcon tube and placed in liquid nitrogen. The roots from the plants were harvested as well. Therefore, the whole content of the pot was poured into a petri dish. One ml of soil was taken as a sample. Then sterile milliQ water was poured on the soil-root mix to be able to carefully remove soil particles from the root system. The clean roots were then dried on filter paper and placed in liquid nitrogen. Until further use the samples were stored at -80°C. The rosette samples were freeze dried.

2.2.5. Picture and Statistical Analysis

The pictures of the trays were cropped into single pot pictures with ImageJ (see Figure 4). Then the pictures were analyzed using the Aradeepopsis software.

The statistical tests were done in R Studio 4.1.0 (R Core Team, 2021). The packages tidyverse (Wickham et al., 2019), readxl (Wickham & Bryan, 2019), rstatix (Kassambara, 2021) and ggpubr (Kassambara, 2020) were used. The data from Aradeepopsis, the counted leaves and the rosette fresh weight were analyzed. In the first step the prerequisites (normality of data and equal variances) for a one-way ANOVA were tested. Depending on the results of pretesting either a one-way ANOVA, a Welch ANOVA or a Kruskall-Wallis test was carried out.

2.3. Soil Dilution Experiment

The experimental design of the so-called “soil dilution experiment” was developed by Deyang Xu, Laura Dethier and Camilla Timmermann-Krogh and adapted for this experiment. During the soil dilution experiment the sterile soil is inoculated with a soil extract derived from the unsterile soil of the same site to introduce a reduced number of microbes to the sterile soil.

2.3.1. Preparation of Soil Extracts and Pots

Per sample three different soil extracts were prepared (see Figure 6). The soil extracts are derived from sterile, unsterile and heat-treated unsterile soil from the respective sites (see Table 7). For the heat-treated unsterile soil extract 20 g of unsterile soil were mixed with a tenth of the volume with sterile milliQ water and incubated at 80°C for 1 hour. Afterwards 200 ml sterile milliQ water was added. For the sterile and unsterile soil extract 20 g of each were mixed with 200 ml sterile milliQ water. After adding the water all samples were soaked for at least 1 hour to ensure extraction of microbes. A certain amount of soil extract solution (avoiding big soil particles) was added to sterile soil of this respective site and mixed thoroughly. Then 3 parts of sand were added, as well as ddH_2O to moisten the soil. This was mixed thoroughly and split up into pots (9 cm diameter). For exact volumes see Table 7. The pots were stored in the cold room at 4°C for at least 4 days.

Table 7: Preparation of soil for the soil dilution experiment

soil	soil extract	soil extract [ml]	sterile soil [ml]	sand [ml]	ddH_2O [ml]
117	sterile	7	100	300	50
	heat-treated	7	100	300	50
	unsterile	17,5	250	750	150
116	sterile	17,5	250	750	150
	heat-treated	17,5	250	750	150
	unsterile	31,5	450	1350	220
109	sterile	17,5	250	750	150
	heat-treated	17,5	250	750	150
	unsterile	31,5	450	1350	220
71	sterile	17,5	250	750	150
	heat-treated	17,5	250	750	150
	unsterile	31,5	450	1350	220

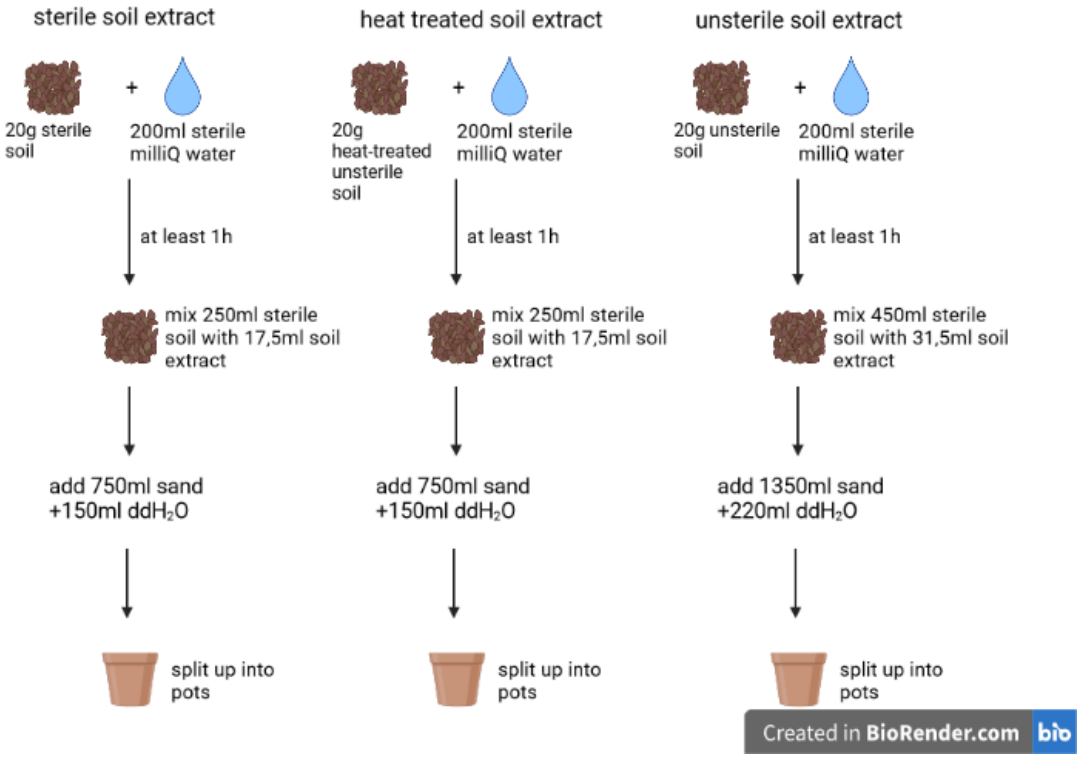

Figure 6: Experimental design of the soil dilution experiment. For each soil three different soil extracts were prepared. Figure was created in Biorender.

2.3.2. Growing Conditions

For the experiment Col-0 seeds were surface sterilized as described above (see 2.1.3.2). After 2 days of stratification at 4°C, 4-5 seeds were sown into each pot. The trays with the pots were then placed in a walk-in chamber (settings see Table 8) and covered with plastic film for one week. The pots were watered consistently with deionized H_2O to avoid drought stress. Pictures of each tray were taken at least once a week. The trays were moved around in the walk-in chamber to avoid a location bias.

Table 8: Growth condition in the walk-in chamber

temperature day	21°C
temperature night	19°C
day length	16 h
light intensity	140 µmol/m²sec

2.3.3. Phenotype Evaluation for the Soil Dilution Experiment

The phenotypic evaluation was carried out with ImageJ. Aradeepopsis could not be used in this case as up to four Col-0 plants were in one pot and the software needs single plant pictures. For the Col-0 plants in soil 71 and 109 the total area was measured. For plants grown in soil 116 additionally the yellow plant area was measured. The first evaluation of the phenotype was done 44 days after germination.

The statistical analysis was carried out in R Studio (R Core Team, 2021). For statistical test either Kruskall-Wallis test followed by a Games Howell test or a Welch-ANOVA followed by a Wilcoxon test was carried out, depending on the prerequisites of the data set.

2.3.4. Additional Transplantation Experiment for Soil 117

As a possibility to further reduce the number of microbes, the soil dilution experiment was combined with the transplantation experiment for soil 117. Col-0 plants from the soil dilution experiment were therefore moved into new pots with soil containing the different soil extracts (sterile or unsterile) following the procedure described in 2.2.3. As it was a preliminary pilot experiment and the initial number of Col-0 plants was low, only 5 replicates per treatment were done. Another adaption was that the Col-0 plants were already moved after two weeks. The phenotype was evaluated with ImageJ, measuring total and yellow area, after 21 days after germination.

2.3.5. Nutrient Rescue Experiment for Soil Dilution Col-0 Plants from Soils 71, 109 and 116

After six weeks the soil dilution experiment did not show an obvious phenotypic difference between Col-0 plants from the different treatments. To gain more information about the effect the soil has on the Col-0 plants a nutrient rescue experiment (NRE) was carried out. Therefore half of the Col-0 plants (7) of the pots treated with sterile and heat-treated soil extract for the soils 71, 109 and 116 were moved to a new tray. They were watered once a week with 500ml of a full nutrient solution. Therefore 1 ml of the four different prepared nutrient solutions (see Table 25, Table 26, Table 27 and Table 28) were added to 1 l of sterile milliQ water. The remaining Col-0 plants were treated as before as a control

and just watered with deionized water. The goal of this experiment is to see if the impaired phenotype of Col-0 plants in sterile soil comes from nutrient deprivation and if it is possible to rescue the phenotype.

2.3.6. Chlorophyll Measurement for Col-0 Plants Grown in Soil 116

The chlorophyll measurement was adapted from the protocols from Li et al. (2022), Ni et al. (2009) and Qin et al. (2015). Three representative Col-0 plants each, grown in sterile soil dilution pots and in NRE sterile soil dilution pots from soil 116 were chosen for chlorophyll measurements. The shoot, if present, was cut off and discarded. The rosette was weighed and immediately placed on ice in the dark. The rosette sample was homogenized with liquid nitrogen in a mortar. Afterwards the chlorophyll was extracted with 5 ml of 80% Acetone. The samples were placed in the dark and on ice at any possible time. Then they were centrifuged at maximum speed for two minutes. For measuring the chlorophyll content in the rosettes, the supernatant was diluted 1:10 and the absorbance measured at 603 nm, 645 nm and 663 nm. The content of chlorophyll a and b was then calculated with the following equations:

$$Chl.a\ [\mu g/g] = (12{,}7 * Abs_{663} - 2{,}69 * Abs_{645}) * (v * \frac{1}{m})$$

$$Chl.b\ [\mu g/g] = (22{,}9 * Abs_{645} - 4{,}86 * Abs_{663}) * (v * \frac{1}{m})$$

Where v is the volume of the extraction solution in ml and m is the weight of the fresh rosette in g.

2.3.7. Anthocyanin Measurement for Col-0 Plants Grown in Soil 71

To determine the anthocyanin content of Col-0 plants grown in soil 71, the protocols from Laby et al. (2000), Rabino & Mancinelli (1986) were adapted. For soil 71 three Col-0 plants from sterile soil dilution and NRE sterile soil dilution were chosen for anthocyanin measurements. The shoot was removed, and the rosette was cut off. After weighing the rosette, it was immediately placed on ice in the dark. The plants were homogenized with liquid nitrogen in a mortar. Afterwards 5 ml of methanol + 1% (v/v) formic acid was used to extract anthocyanins. The solutions were centrifuged at maximum speed for 2 min and kept on ice and in the dark as much as possible. The supernatant was diluted 1:10 and the absorbance measured at 530 nm and 657 nm. The following equation was used to determine the anthocyanin content of each sample:

$$Anthocyanin\ [\mu g/g] = (Abs_{530} - (0{,}25 * Abs_{657}) * (v * \frac{1}{m})$$

Where v is the volume of the extraction solution in ml and m is the weight of the fresh rosette in g.

2.3.8. Picture and Statistical Analysis for the NRE

The pictures were analyzed after 44 days (for SD) and after 51 days (for NRE) measuring the plant area, and for soil 116 also the yellow area, with ImageJ. The software Aradeepopsis could not be used in this experiment because more than one plant was in one pot, which is a situation that is not supported by the program.

The statistical analysis was done in R Studio 4.1.0 (R Core Team, 2021) supported by the packaged tidyverse (Wickham et al., 2019), readxl (Wickham & Bryan, 2019), rstatix (Kassambara, 2021) and ggpubr (Kassambara, 2020).

For the chlorophyll and anthocyanin content a Welch two sample t-test, comparing plants grown in sterile soil dilution soil and plants grown in the nutrient supplied soil, was carried out.

Depending on the prerequisites of the data either a one-way ANOVA, Welch ANOVA or a Kruskall-Wallis test was carried out.

2.4. *Arabidopsis thaliana* Mutant Genotyping

2.4.1. *Arabidopsis thaliana* Mutants

2.4.1.1. mrs2-5 and mrs2-7 mutant

The *Arabidopsis thaliana* mutants mrs2-5 (SALK_105475C) and mrs2-7 (SALK_090559) are derived from T-DNA insertion and obtained from the Nottingham Arabidopsis stock center (www.arabidopsis.org). For the mrs2-5 *Arabidopsis thaliana* mutant, no significant phenotypic changes compared to the wildtype can be observed and the gene plays and important role of magnesium transport especially in early plant development (Gebert et al., 2009). In comparison, the mutant line mrs2-7 shows growth inhibition under magnesium deprived growth conditions, but not under regular growth conditions (Gebert et al., 2009). There are clear indications that the mrs2-7 transporter is localized only in roots during early development, especially in the ER and probably other membranes (Gebert et al., 2009). The mrs2-5 transporter seems to be located in the vascular tissue of the expanded cotyledons (Gebert et al., 2009). Primers for these mutants were derived through TAG Copenhagen. Both mutants were developed in the background of Col-0 (Arabidopsis Biological Resource Center, n.d.-c, n.d.-b).

2.4.1.2. rbohF mutant

The T-DNA insertion *Arabidopsis thaliana* mutant line rbohF (SALK_059888) was obtained from the Nottingham Arabidopsis stock center (www.arabidopsis.org). The rbohF mutant is phenotypically very similar to the Col-0 wildtype and has only marginally smaller rosettes (Angel Torres et al., 2002). The rbohF gene seems to be important for hypersensitive response and is involved in the production of reactive oxygen intermediates (ROI) which is particularly important for the plant defense (Angel Torres et al., 2002). Primers for this mutant were derived through Tag Copenhagen. The rbohF mutation was introduced in a Col-0 background (Arabidopsis Biological Resource Center, n.d.-a).

2.4.1.3. ein2-1/pad4-1/sid2-2 mutant

The ein2-1/pad4-1/sid2-2 (N66006) mutant was developed in the background on Col-0 and derived through standard genetic crossing (Arabidopsis Biological Resource Center, n.d.-d). The triple mutant is mostly affected in its defense mechanisms. The mutation on the ein2-1 allele results in ethylene insensitivity and therefore also in a resistance to cytokinin in dark growing conditions (Vandenbussche et al., 2007). But ein2 is also known as a Nramp (Natural resistance-associated macrophage proteins) metal-ion transporter (Hammond-Kosack & Parker, 2003). The ein2-1 mutation leads to a premature stop codon. The mutation in the pad4-1 gene, which is involved in the plant defense (Tsuda et al., 2009), especially in resistance mediated by salicylic acid, is a lipase-like protein (Hammond-Kosack & Parker, 2003). The sid2-2 mutation affects the synthesis of salicylic acid, which is required for local and systemic acquired resistance (Wildermuth et al., 2001).

In contrast to the other two mutations was the sid2-2 mutation derived through fast-neutrons. Mutagenesis through this method results in a gene deletion which causes a loss of transcription (Wildermuth et al., 2001). The other two mutations were derived through ems mutation (Arabidopsis Biological Resource Center, n.d.-d).

2.4.2. Preparation of Seedlings

Twenty-30 seeds of each mutant were placed in a 1,5 ml Eppendorf tube and 1 ml of sterile milliQ water was added. Those tubes were placed at 4°C to stratify the seeds. After 3 days the mutant seeds were placed on a square MS-plate (half strength, 0,8% agar, pH 5,7), which was closed with microporous tape. The mutant plants were grown under controlled conditions for 16-18 days (see Table 9 for growth conditions).

Table 9: Growth conditions for *Arabidopsis thaliana* mutants in the climate chamber

temperature	22°C
relative humidity	55%
day length	16 h
light intensity	140 µmol/m²sec

2.4.3. Growing of *Arabidopsis thaliana* Mutants in Greenhouse

One pot (9 cm diameter) per plant containing regular greenhouse soil as growth medium was prepared. Using tweezers, the mutant seedlings were transferred to a pot. As the germination rate varied between the mutants, for each mutant between two to nine plants were grown. The trays were covered with plastic film for one week to ease the process for the seedlings. The plants were grown in greenhouse conditions (for settings see Table 10). The plants were watered with tap water when required, to avoid drought stress. When the first siliques were developed the plant was packed in a paper bag and stabilized by a bamboo stick, so the seeds were not lost.

Table 10: Growth conditions in greenhouse for *Arabidopsis thaliana* mutants

temperature	17-19°C
day length	16 h
light intensity	140 µmol/m²sec

2.4.4. Genotyping using Polymerase Chain Reaction (PCR)

2.4.4.1. DNA-Extraction of Arabidopsis thaliana *Mutant-Leaves*

After 15-23 days, depending on the plant size, the DNA was extracted from leaves using a rapid protocol. Using a scalpel and a tweezer one leaf or leaf piece (~1 cm²) was cut from each plant and put in a 1,5 ml Eppendorf tube, which was immediately placed on ice. In the next step 400 µl lysing buffer (see Table 30) and 2 metal beads were added to each Eppendorf tube. Those were then placed

in a Retsch Mixer Mill for 1 min 10 sec and 30 1/s frequency. The samples were then centrifuged for 10 min at 13.000 rpm in room temperature. In the meantime, 300 µl of ice-cold isopropanol was pipetted in a new 1,5 ml Eppendorf tube in the fume hood. After the centrifugation 300 µl of the supernatant were added to the isopropanol and mixed by inversion. The samples were then again centrifuged for 10 min at 13.000 rpm in room temperature. The supernatant was removed, and the pellet was then washed twice with 70% EtOH. Therefore, 300 µl of 70% EtOH were added to the pellet and centrifuged for 5 min at 13.000 rpm in room temperature. The supernatant was again discarded and the Eppendorf tube with the pellet was placed in an incubator at 37°C to let the rest of the EtOH evaporate. The pellet was then resuspended in 30 µl sterile milliQ water. Until further use the DNA samples were stored at -20°C.

2.4.4.2. Polymerase Chain Reaction for Arabidopsis thaliana *Mutant Genotyping*

Different polymerase chain reactions (PCR) were needed to determine the mutation of each *Arabidopsis thaliana* mutant. All the following steps were carried out on ice. A master mix was produced (see Table 13) and 8 µl of it was pipetted in a PCR-tube. Also, 5 µl of the required primer mix (see Table 12) and 2 µl of the sample DNA were added. For every primer pair two controls were included, one was a negative control (milliQ water) and the other was DNA from Col-0. The tubes were then placed in a thermos-cycler, and the program seen in Table 11 was used.

Table 11: PCR program for *Arabidopsis thaliana* mutant genotyping

temperature	**time**	
94°C	3 min	
94°C	30 sec	repeated 35 times
60°C	30 sec	
72°C	2 min	
72°C	10 min	
10°C	hold	

Table 12: Recipe for primer mix used in *Arabidopsis thaliana* genotyping

substance	**amount in µl for 10 reactions**
milliQ	44
forward primer (10µM)	3
reverse primer(10µM)	3

Table 13: Mastermix for *Arabidopsis thaliana* genotyping

substance	**amount in µl for 1 reaction**
milliQ	4,9
10x PCR buffer	1,5

dNTPs	1,2
Taq polymerase	0,4

2.4.4.3. *Genotyping of T-DNA* Arabidopsis thaliana *Mutants*

The mutants mrs2-5, mrs2-7 and rbohF were derived through T-DNA insertion. For genotyping T-DNA *Arabidopsis thaliana* mutants two primer pairs are needed. The first one is LP+RP (Left Primer + Right Primer), the LP primer only binds in the wildtype and the RP binds in the wildtype as well as in the mutant. The second is LB+RP (Left Border Primer + Right Primer), were the LB only binds in the mutant. For this reason, the homozygous mutant DNA would only be amplified with the LB+RP primer pair. The heterozygous mutant DNA would be amplified with both primer pairs, and the wildtype DNA only with the LP+RP primers (see Table 14) (O'Malley et al., 2015).

Table 14: Table to evaluate the genotype of *Arabidopsis thaliana* T-DNA mutants

	LP+RP	**LB+RP**
wildtype	yes	no
homozygous mutant	no	yes
heterozygous mutant	yes	Yes

The LP and RP primers which are specific for each mutant are derived with the online tool from the SALK institute (*T-DNA Primer Design*, n.d.). As LB primer a SALK line specific primer (Lb3.1) was used.

2.4.4.4. *Genotyping of ein2-1/pad4-1/sid2-2* Arabidopsis thaliana *Mutants*

The ein2-1/pad4-1/sid2-2 mutant was derived through ems and fast-neutron mutations. For each mutation a different primer set was used to identify the mutation.

To verify the ein2-1 mutation, sequencing was required after the PCR. The sequencing was carried out by Eurofins. For sample preparation (according to the company requirements) 5 µl PCR product and 5 µl of 5 µM sequencing primer were pipetted in the tube provided by the company. In addition to the mutants also one sample of Col-0 PCR product was sent. The sequence was then analyzed using Geneious Prime 2022.0.1.

The pad4-1 PCR product needed to be digested with a restriction enzyme. The mastermix from Table 15 was prepared. Four µl of PCR product and 6 µl of mastermix were added to an Eppendorf tube. The pad4-1 PCR product was then digested with BsmFI from New England BioLabs for 1 hour at 65°C. The cut site of BsmFI is GGGAC(10/14) (New England BioLabs, 2022). This was followed by a deactivation of 20 min at 80°C. To evaluate the result, the digested PCR product was stained with GelRed and analyzed with electrophoresis on a 1% agarose gel.

Table 15: Recipe for digestion of PCR products with restriction enzymes for *Arabidopsis thaliana* mutant genotyping

substance	**amount in µl for 1 reaction**
milliQ	2,5

buffer Cut Smart	1
restriction enzyme BsmFI	0,5

To analyze the sid2-2 mutant the PCR product was directly used for gel electrophoresis.

2.4.4.5. Gel Electrophoresis

To evaluate the result of the genotyping, gel electrophoresis was used. Therefore a 1% agarose gel was prepared. To the tubes from the PCR 1 µl of GelRed was added. Three µl of base pair ladder was pipetted in the outer slots. Fifteen µl of each PCR was loaded on the gel. This was then run at 120 V for 20-60 min. A GelDoc XR+ system (Bio-Rad laboratories) was used to take a picture of the gel.

2.4.5. Harvesting *Arabidopsis thaliana* Mutant Seeds

Seeds were collected for plants which were identified as either homozygous or heterozygous. When the first siliques were produced by the plants, a paper bag was imposed over the plant stabilized by a bamboo stick, to avoid losing seeds. When the plant was dry the seeds were harvested. The content of the paper bag was sieved, and the mutant seeds were collected. The mutant seeds are stored in labelled paper bags until further use.

2.5. Identification and Purification of Endophytic Bacteria Isolated from Col-0 Plants grown in unsterile Soil 82

2.5.1. Isolation of Endophytic Bacteria from Unsterile Soil 82

The endophytic bacteria from *Arabidopsis thaliana* plants grown in unsterile soil 82 were previously isolated by PhD student Laura Dethier following the protocol from Zhang et al. (2021). As the goal of her isolation was to isolate only endophytic bacteria, the protocol was adapted accordingly. The focus on endophytes is based on results from a previous transplantation experiment with soil 82 and *Arabidopsis thaliana* Col-0 as plant model. The findings of the transplantation experiment suggested that endophytes are primarily responsible for the beneficial effect in unsterile soil 82. This allowed a reduction of the bacteria by root sterilization before the homogenization. Due to this reduction of microbes, also the dilutions were changed to 222x, 148x and 74x. As in the 74x dilution approximately ⅓ of the wells showed microbial growth, those plates were used to produce glycerol stocks. Laura Dethier managed to acquire over 300 glycerol stocks following this protocol, those samples were stored at -80°C until further use.

2.5.2. Purification of Endophytic Bacteria from Unsterile Soil 82

In total 63 glycerol stocks containing isolated bacteria had to be identified in this project. The content of the stocks were unknown, not only the bacterial strain but also if it is a mixture or a single strain, they had to be further analyzed. The glycerol stock was streaked out on a plate with tryptic soy broth agar (TSA) using a pipet tip and sealed with parafilm. Those plates were incubated at 25°C until colonies were visible. A single colony was then restreaked on a new TSA plate and again incubated at 25°C. If there was more than one bacteria species growing, they were separated to avoid mixtures, by

continuously restreaking them. If a glycerol stock did not show bacterial growth after 10 days, it was streaked out again on a TSA plate from the glycerol stock. When there was no growth again, it was considered as not containing bacteria.

2.5.3. Identification by Colony PCR and subsequent Sequencing of Endophytic Bacteria from Unsterile Soil 82

2.5.3.1. Colony PCR

To identify the bacteria a colony PCR was performed to amplify the 16S rRNA region. A single bacteria colony was picked from the plate and transferred to an Eppendorf tube containing 50 µl sterile milliQ water and properly mixed. Then a mastermix (see Table 16) was prepared on ice. For the amplification of the 16S rRNA region the universal primers 27F and 1492R were used. This primer pair outcompeted two other primer sets, in detecting phyla and individual taxa (de Lillo et al., 2006). Twenty-four µl of mastermix was pipetted in each reaction tube and 1 µl of the bacteria colony mix was added. As negative control 1 µl of milliQ was used. The PCR amplification program can be seen in Table 17. The derived PCR product should have a length of approximately 1500 bp.

Table 16: Mastermix for Colony PCR of endophytic bacteria from soil 82

Substance	**amount in µl for 1 reaction**
10x PCR buffer	2,5 µl
dNTPs (2,5 mM)	2 µl
$MgCl_2$ (50 mM)	1 µl
27 Forward Primer 27F (10 µM)	0,75 µl
1492 Reverse Primer 1492R (10 µM)	0,75 µl
Taq Polymerase	0,6 µl
milliQ water	16,4 µl

Table 17: PCR program for colony PCR of endophytic bacteria from soil 82

temperature	**time**	
94°C	2 min	
94°C	30 sec	x30
55°C	30 sec	
72°C	2 min	
72°C	10 min	
10°C	hold	

To visualize the result from the colony PCR a gel electrophoresis was carried out. Twenty-five µl of each PCR product, stained with GelRed, were loaded onto a 1% agarose gel. After approximately 25 min at 120 V the result was evaluated with a GelDoc XR+ system (Bio-Rad laboratories).

2.5.3.2. DNA extraction from Agarose Gel

For each bacterium where a PCR product was visible after the gel electrophoresis an Eppendorf tube was pre-weighed. With the help of red light, the band was cut out of the agarose gel with a scalpel and placed in the Eppendorf tube. Then the Eppendorf tube containing the piece of gel was weighed again and the difference calculated. The following DNA extraction from the agarose gel was done with the E.Z.N.A gel extraction kit from Omega Bio-Tek (Omega BioTek, 2012). In the last step the pellet was diluted in 30 µl sterile milliQ water.

2.5.3.3. Sanger Sequencing

The Sanger sequencing was carried out with the Mix2Seq kit from Eurofins. In the tubes provided by the company 15 µl of the extracted DNA and 2 µl of the 1492R primer (10 µM) were added and the barcode number noted. During this project, Eurofins changed the requirements to 5 µl of extracted DNA mixed with 5µl of 1492R primer (5 µM). The remaining bacterial DNA was then stored at -20°C.

The received sequence was then analyzed using Geneious Prime 2022.0.1. As a quality mark the %HQ was used, if the %HQ value was low the PCR and all following steps were repeated. The sequence was then blasted against the internal database of already isolated and identified bacteria. If there was no similar sequence, the new sequence was blasted in the NCBI database, and the new strain added to the internal database. Those new strains were also used to prepare new glycerol stocks in the following step. The bacteria strains that were already present in the internal database were not continued.

2.5.4. Preparing Liquid Cultures and Glycerol Stocks from Bacteria

In total eleven of the tested bacteria strains, that were not already part of the internal collection, were used to prepare new glycerol stocks. Therefore, 4 ml of TSB were pipetted into a 15 ml plastic tube. With a pipet tip a single colony was picked up from the TSA plate and transferred to the plastic tube. This tube was then incubated at 28°C and 200 rpm until the TSB became cloudy. To make the glycerol stock 750 µl of the liquid culture and 750 µl of 80% glycerol were mixed in a screw-cap tube and stored at -80°C.

2.6. Mono-association Assays of Identified Endophytic Bacteria from Unsterile Soil 82

The endophytic bacteria from *Arabidopsis thaliana* grown in unsterile soil 82, that were identified, purified and new to the internal collection from the previous experiment were now tested for their effect as a single bacterium on the model plant *Arabidopsis thaliana* Col-0 (see Figure 7). During this *in vitro* experiment also the effect in saline conditions on *Arabidopsis thaliana* was examined. Previous results from soil 82 suggest a higher salt tolerance of plants grown in unsterile soil 82 (see 1.5.2).

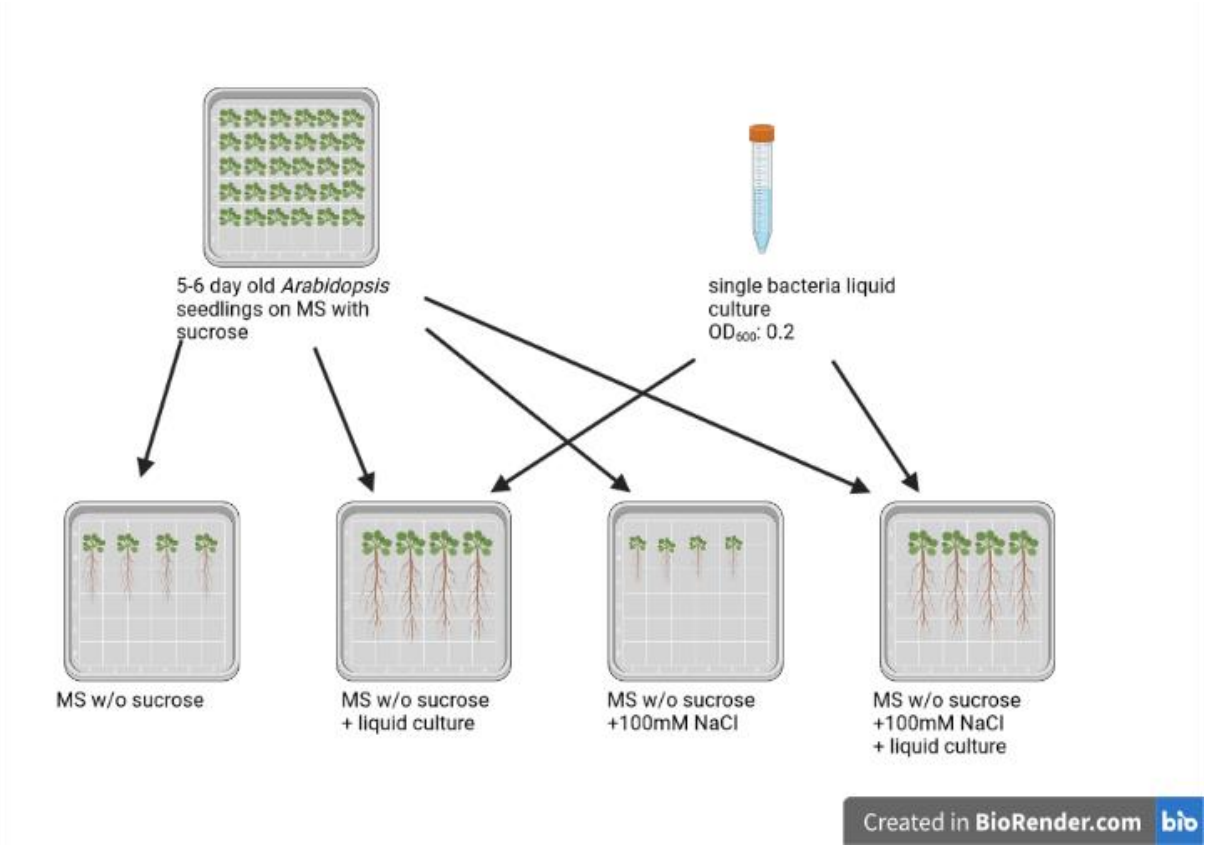

Figure 7: Experimental design of mono-association assay with endophytic bacteria from soil 82. Created in Biorender.

2.6.1. Preparation of *Arabidopsis thaliana* Col-0 seedlings

For the mono-association assay *Arabidopsis thaliana* Col-0 seeds were surface sterilized following the protocol previously described in 2.1.3.2. To synchronize the germination the seeds were stratified at 4°C for 48 hours. Using a pipet tip approximately 30 seeds per plate (½ MS + 5% sucrose) were sown out. The plates were sealed with microporous tape and grown for 6 days under controlled settings in a climate chamber (settings see Table 18).

Table 18: Growth conditions of *Arabidopsis thaliana* seedling for the mono-association assay

temperature	22°C
relative humidity	55%
day length	16 h
light intensity	140 µmol/m²sec

2.6.2. Preparation of Single Bacterial Liquid Cultures

Depending on the growth rate of the bacteria, a liquid culture was started 1 to 7 days prior to the start of the mono-association assay. Around 3 ml of TSB were inoculated with a single bacterial glycerol stock, derived from the identification process described previously. The tubes were incubated at 28°C and 200 rpm. A total of 9 different bacteria strains were tested in the mono-association assay. Two

bacteria of different genera (*Paenibacillus* sp. and *Cohnella* sp.) did not show growth in the liquid culture, despite several tries and change of growing conditions (e.g. oxygen availability).

2.6.3. Mono-association Assay

2.6.3.1. Preparation of Plates

When the bacterial liquid cultures show growth, 1 ml of this liquid culture was added to 3 ml of fresh TSB and incubated at 28°C and 200 rpm for 4-6 hours. If the liquid culture showed only little signs of growth, the cultures were not diluted freshly. After this incubation time at least 2 ml of the liquid culture were centrifuged at 8000 rpm for 5 min. The supernatant was discarded and 1 ml of 10 mM $MgCl_2$ was added, and the pellet dissolved. After another centrifugation step the washing was repeated. In the last step the pellet was diluted in 350-1000 µl of 10 mM $MgCl_2$, depending on the size of the pellet. Hundred µl of the bacterial solution was diluted 1:10 with the buffer and the absorbance at 600 nm (OD_{600}) measured. The bacterial solutions were then diluted to reach a final OD_{600} of 0,2. A heat-killed bacteria was added to the experiment as a control. Therefore, one diluted bacteria solution was incubated at 99°C for 20 min.

Two different growth media were prepared. The first one is a half-strength MS-media without sucrose and for the second one 100 mM of NaCl was added. The media were cooled to around 50°C and divided into 50 ml plastic tubes. Bacterial solution (125 µl, OD_{600} = 0,2) were added to 50 ml of medium (final OD_{600} of 0,0005), which was then poured into one square petri dish avoiding air bubbles. As controls the heat-treated bacterial solution and a control with only 10 mM $MgCl_2$ were used.

2.6.3.2. Growing Conditions and Evaluation of Col-0 Plants

Onto each plate 8 of the 6-day old Col-0 seedlings were carefully transferred. The end of the root was marked to evaluate the root growth at the end of the experiment. The plates were sealed with microporous tape and grown vertically in a climate chamber (for growth conditions see Table 18). The plates were moved regularly in the climate chamber to avoid a location bias. Pictures of the plates were taken 5 and 10 days after the plants were transferred on the plate containing bacteria. After 10 days the whole plant was weighed. If there were obvious phenotypic differences compared to the controls without living bacteria, also the shoot weight alone was determined. Those Col-0 plants were also kept at -80°C for further use.

2.6.3.3. Data analysis and Statistical Tests

The pictures of the plates from day 5 and 10 were used to analyze the root growth. The primary root length was measured with ImageJ.

The measured root length (in cm) and the rosette weight (in mg) were then further analyzed in RStudio version 4.1.0 (R Core Team, 2021) by statistical testing. The packages tidyverse (Wickham et al., 2019), rstatix (Kassambara, 2021), readxl (Wickham & Bryan, 2019), ggpubr (Kassambara, 2020) and datarium (Kassambara, 2019) were used.

A one-way ANOVA (analysis of variance) was chosen as statistical test to determine if the bacteria influenced the Col-0 plant growth compared to the controls for both growth conditions (with or without NaCl). This was followed by a pairwise comparison with a t-test against the neg. control to determine which of the bacteria had an impact on the plant. As *Arabidopsis thaliana* traits, the root

length on day 5, plant weight and for some treatments also rosette and root fresh weight were compared.

3. Results

3.1. Phenotypic Screening

3.1.1. Test of Soil Sterility

The soil sterility after the irradiation process was tested for five different samples. After incubating the plates with different media for a maximum of one week, the microbial growth was compared between sterile and unsterile soil samples.

The sterility of the irradiated soil samples was confirmed for all tested samples. Only one plate with TSA medium of sterile soil 117 showed bacterial growth (see Appendix B: Initial Screening), but the number of colonies seen on this plate is significantly less compared to the unsterile soil 117. The bacterial growth could be explained by the way the sample was taken, as this was done in unsterile conditions and contamination could not be prevented.

3.1.2. pH Value of Soil Samples

The pH for sterile and unsterile soil samples from all sites was tested with pH indicator strips. The values can be seen in Table 19.

Table 19: pH-value of the 12 tested soils

Soil ID	Treatment	pH Value
29	sterile	6
	unsterile	6
37	sterile	7
	unsterile	7
41	sterile	6
	unsterile	6
71	sterile	8
	unsterile	8
75	sterile	6
	unsterile	6
107	sterile	6
	unsterile	6
109	sterile	6
	unsterile	6-7
115	sterile	7
	unsterile	7

116	sterile	7
	unsterile	6
117	sterile	7-8
	unsterile	7-8
119	sterile	6
	unsterile	6
125	sterile	8
	unsterile	8

3.1.3. Phenotypic Differences of Col-0 Plants Grown in Sterile and Unsterile Soil Samples

The phenotypic differences of the *Arabidopsis thaliana* Col-0 plants were evaluated with the Aradeepopsis Software. The morphological of the Col-0 plants were compared between sterile and unsterile soil of the same site. As values the measurements for rosette size, chlorotic/senescent area and anthocyanin area were used for the comparisons. Sites were the Col-0 plants grown in the unsterile soil seemed to have a more regular phenotype in comparison to the Col-0 plants grown in sterile soil were collected and send for nutrient analysis via ICP-MS. As the color of the soils varied a lot, Aradeepopsis did not always manage to evaluate only the plant area, but also included pot and soil in its measurements. It also had many problems to correctly evaluate the purple plant area. The choice of which soils were continued in further experiments was mostly done by visual examination of the plants, and not necessarily based on the Aradeepopsis results. As each phenotype developed on a different timepoint, pictures from varying days were used for comparison, depending on the soil.

For four soil samples an interesting phenotype was detected during the initial soil screening. Those samples were collected in sites 71, 109, 116 and 117. *Aradeepopsis thaliana* plants grown in these soils seemed healthier/had a regular phenotype, whereas when grown in sterile conditions the plants seemed negatively influenced by its growth media. For the remaining 8 soils no such phenotype was detected. In those samples Col-0 plants either looked similar in sterile and unsterile conditions, worse in unsterile compared to sterile conditions or did not grow at all (see Appendix B: Initial Screening).

3.1.3.1. Phenotype of Col-0 Plants grown in Soil 71

Figure 8: Phenotype of Col-0 plants grown in soil 71 after 47 days.

In soil 71 plants grown in sterile conditions developed completely purple-red leaves. Col-0 plants grown in unsterile soil 71 stayed green for longer and the leaves only showed a slightly shift to purple and dark green (see Figure 8). This phenotype is not resembled in the results from the Aradeepopsis data. Based on this data set the plants differed significantly in total size ($p = 0{,}00016$) and senescent area ($p = 0{,}0027$) on day 47 after germination (see Figure 9).

The result of the ICP-MS analysis showed some significant differences in the nutrient content of Col-0 plants grown in sterile and unsterile soil 71. The Col-0 plants grown in presence of the microbes had an increased content of B, Ca, K, Mg, P and S compared to plants grown without the microbes (see Figure 10).

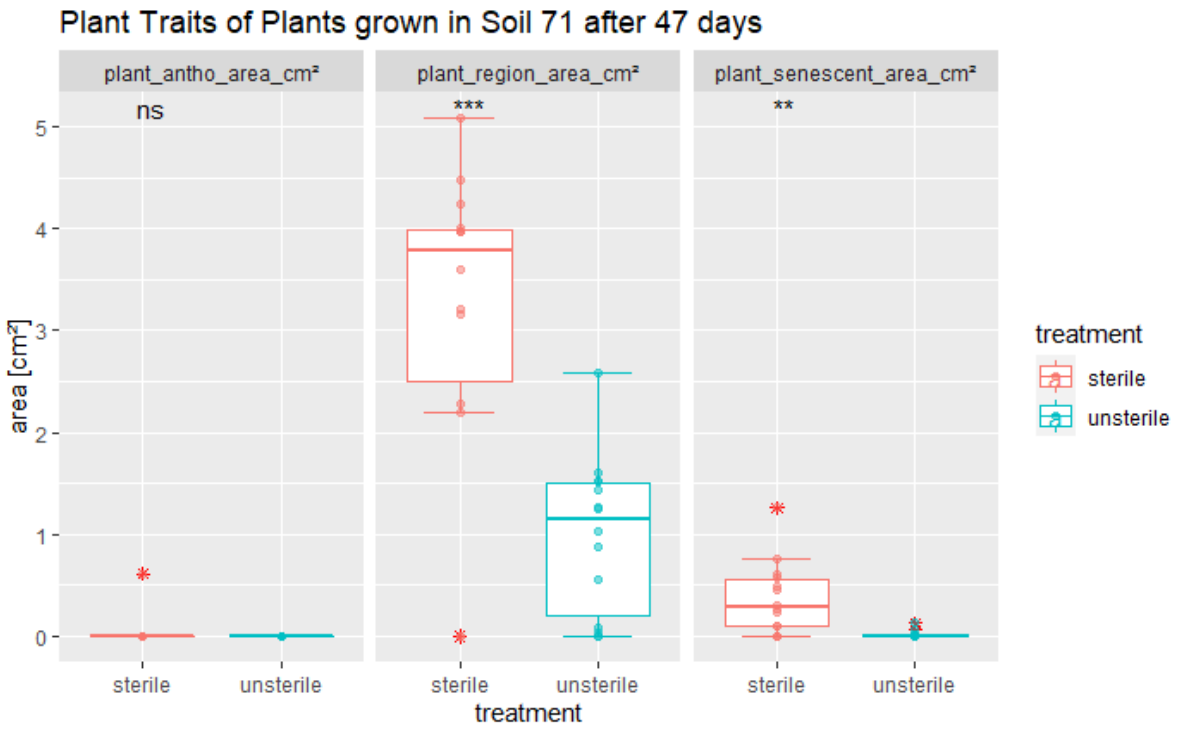

Figure 9: Phenotypic plant traits measured by Aradeepopsis of plants grown in soil 71 after 47 days. The measurements of anthocyanin area, rosette area and senescent areas were used.

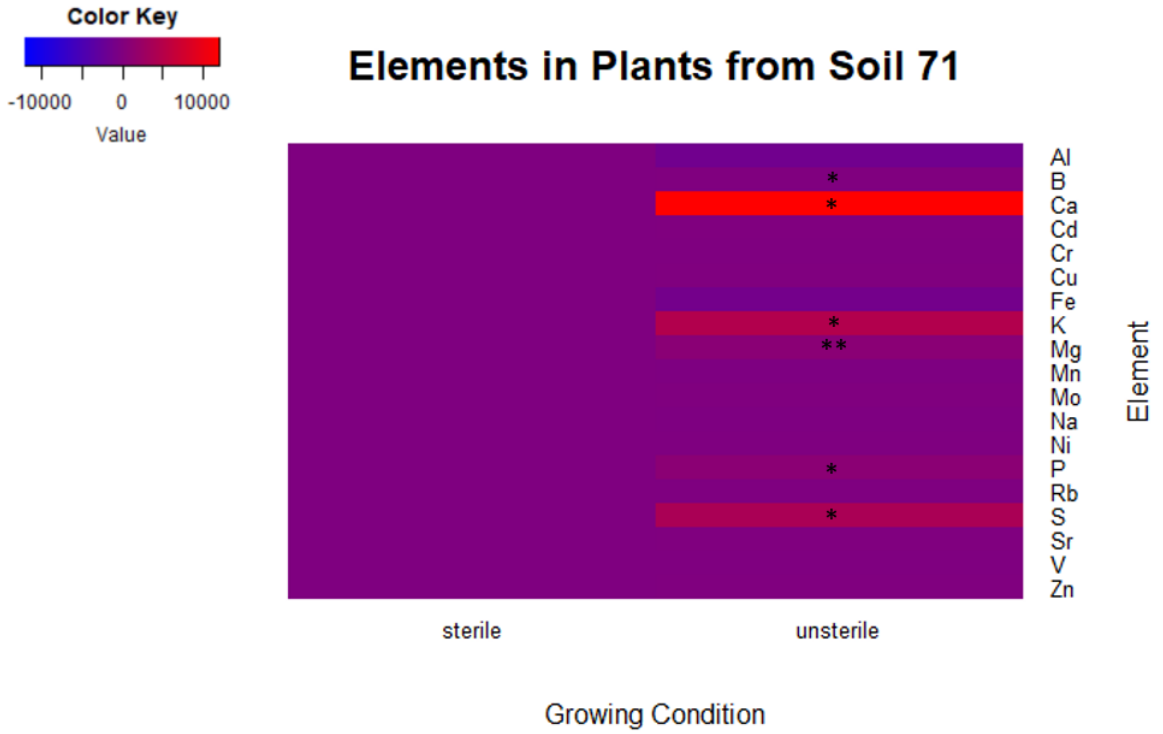

Figure 10: Element content of *Arabidopsis thaliana* grown in soil 71 in sterile and unsterile conditions. The value for sterile conditions is set to 0, while the unsterile column shows the difference in the element content. The statistical difference was calculated with a Welch t-test. * = <0,05, ** = < 0,005

3.1.3.2. Phenotype of Col-0 Plants grown in Soil 109

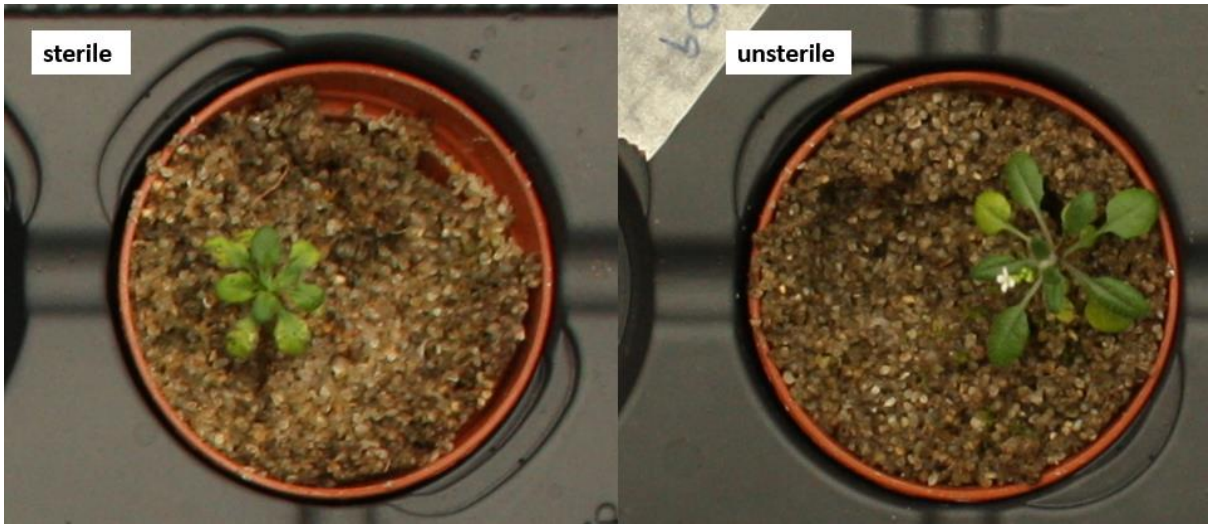

Figure 11: Phenotype of Col-0 plants grown in soil 109 after 47 days

The Col-0 plants grown in sterile soil 109 showed a phenotype only seen in this soil during the screening. The Col-0 plants had a stunted growth with short petioles and an increased number in leaves. Col-0 plants grown in unsterile conditions showed a normal phenotype (see Figure 11). As this is a very specific phenotype the Aradeepopsis software was not able to show this difference. None of the tested traits showed a significant difference between sterile and unsterile soil on day 47 after germination (see Figure 12).

To analyze the difference between the two growth conditions also the nutrient content of the rosettes was measured by ICP-MS analysis. This showed significant differences of seven of the tested elements. The amount of Ca, Cd and Mg was higher in unsterile conditions, while the amount of Cu, Mn, Mo and P was higher in sterile conditions (see Figure 13).

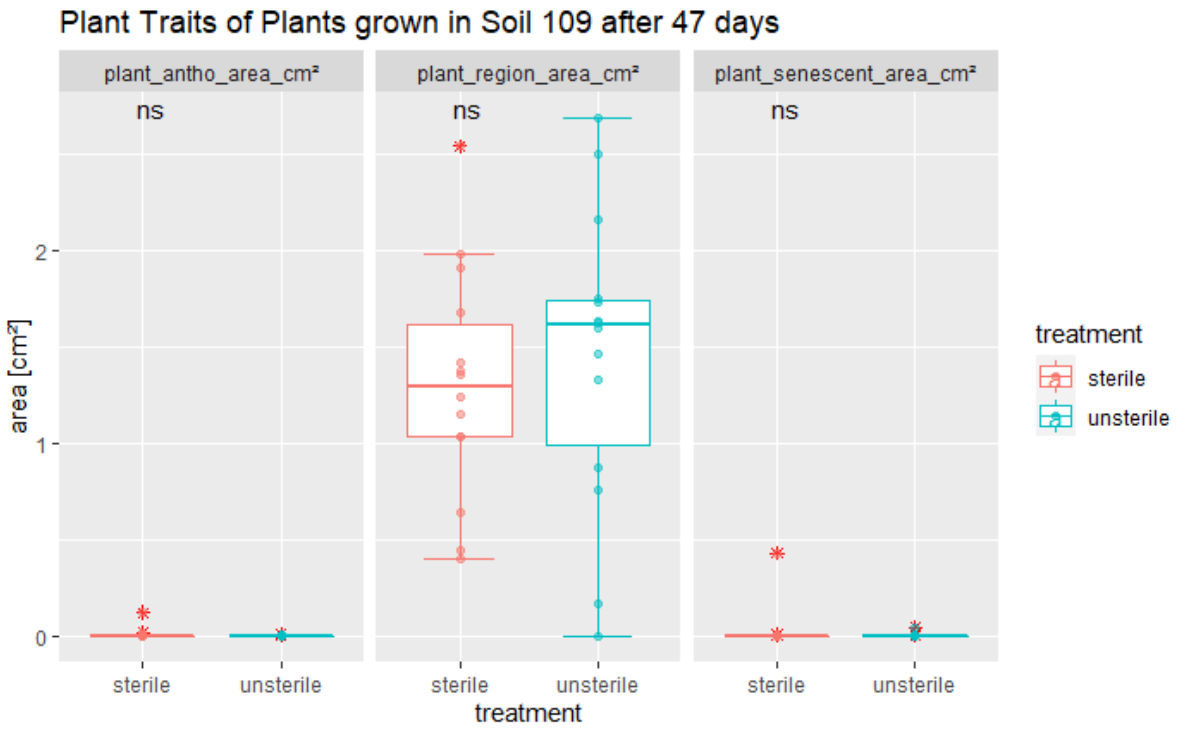

Figure 12: Phenotypic plant traits measured by Aradeepopsis of plants grown in soil 109 after 47 days. The measurements of anthocyanin area, rosette area and senescent areas were used.

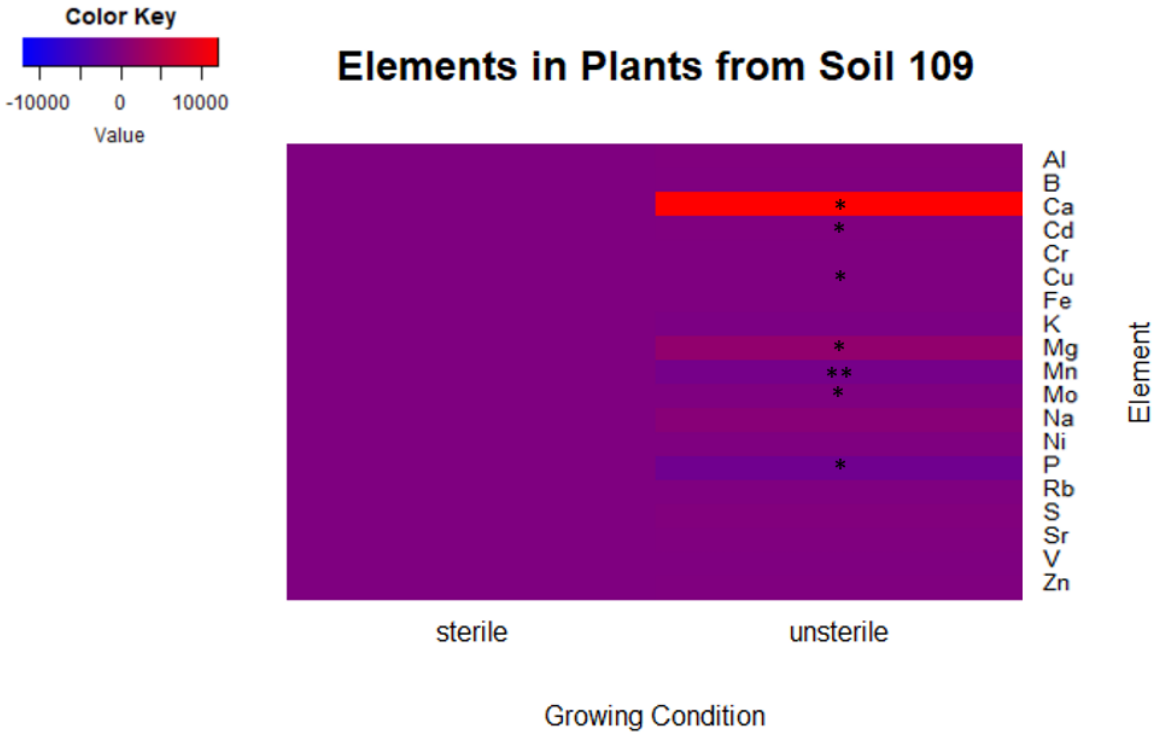

Figure 13: Element content of *Arabidopsis thaliana* grown in soil 109 in sterile and unsterile conditions. The value for sterile conditions is set to 0, while the unsterile column shows the difference in the element content. The statistical difference was calculated with a Welch t-test. * = <0,05, ** = < 0,005

3.1.3.3. Phenotype of Col-0 Plants grown in Soil 116 and Soil 117

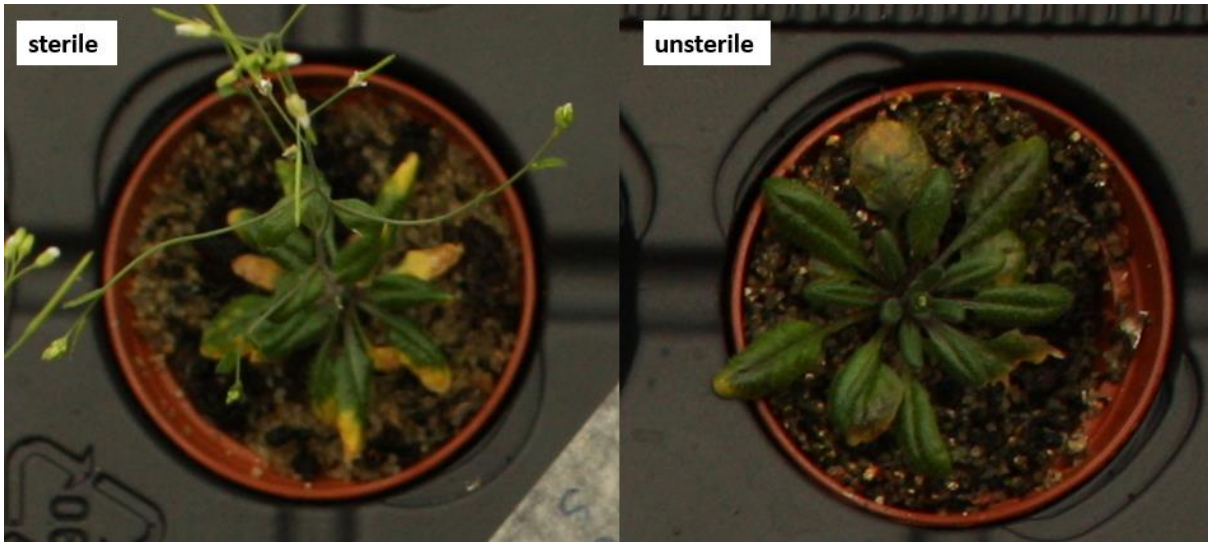

Figure 14: Phenotype of Col-0 plants grown in soil 116 after 55 days

Figure 15: Phenotype of Col-0 plants grown in soil 117 after 47 days

The soil samples from the sites 116 and 117 have a very similar phenotype (see Figure 14, Figure 15). After about 55 (soil 116) and 47 (soil 117) days after germination (DAG) a yellow coloration of the leaf edges became visible on Col-0 plants grown in sterile conditions. On Col-0 plants grown in unsterile conditions this yellow color was not visible in soil 117 and only later and less in soil 116. In both soils the Col-0 plants in the unsterile conditions developed a slight purple coloration in the later growing period.

In soil 116, based on the Aradeepopsis results, the Col-0 plants grown in the two conditions differed in all three traits (see Figure 16). Whereas the Col-0 plants grown in unsterile soil had a bigger size ($p= 0,0041$), a bigger senescent area ($p= 0,026$) and a bigger anthocyanin area ($p= 0,036$).

Only for soil 116 the element content of the Col-0 plants was measured by ICP-MS analysis. In five of the tested elements a significant difference could be detected by comparing the sterile and unsterile growth conditions. The nutrients Cd, Cu, Mn, Mo and P were higher in sterile soil 116 (see Figure 17).

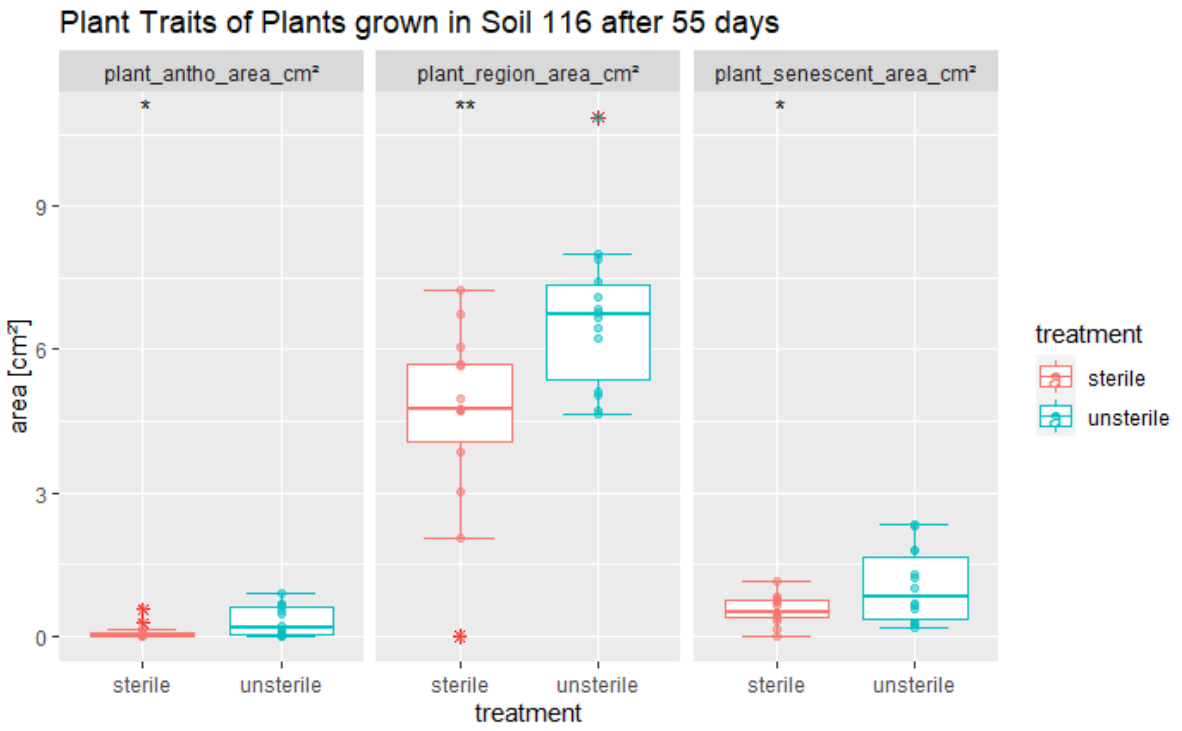

Figure 16: Phenotypic plant traits measured by Aradeepopsis of plants grown in soil 116 after 55 days. The measurements of anthocyanin area, rosette area and senescent areas were used.

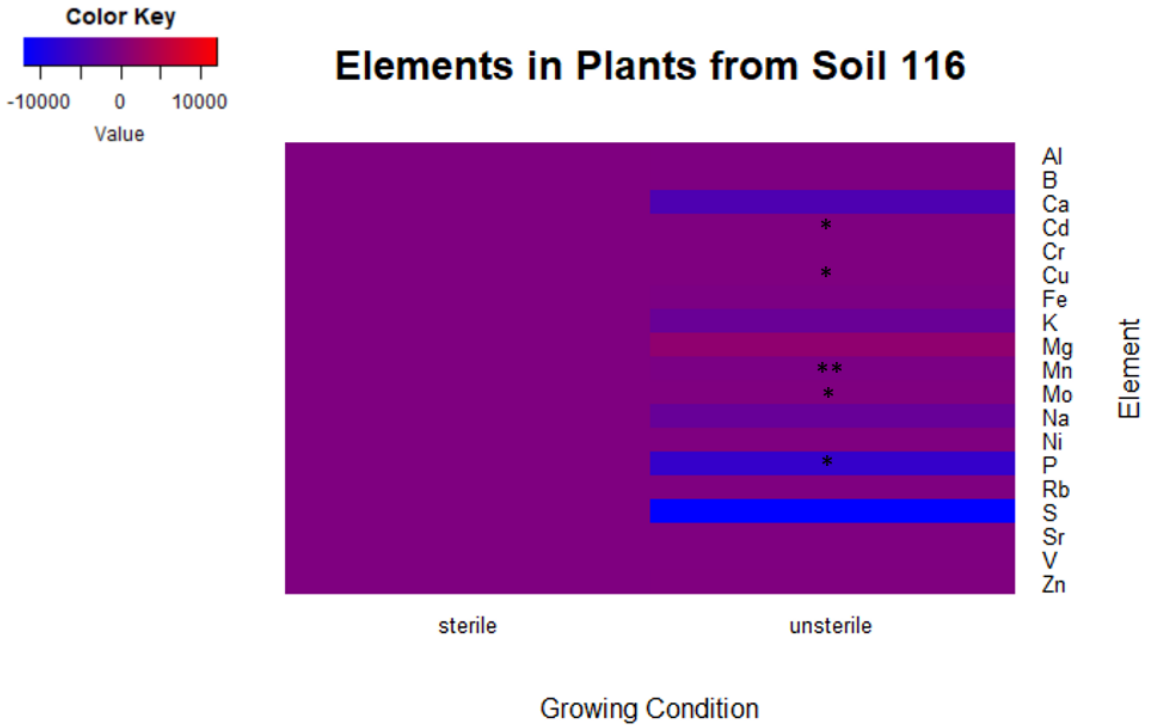

Figure 17: Element content of *Arabidopsis thaliana* grown in soil 116 in sterile and unsterile conditions. The value for sterile conditions is set to 0, while the unsterile column shows the difference in the element content. The statistical difference was calculated with a Welch t-test. * = <0,05, ** = < 0,005

Also, the Col-0 plants grown in soil 117 differed in all three tested traits, based on the data derived from Aradeepopsis (see Figure 18). In this case the Col-0 plants grown in sterile soil 117 were bigger

(p= 4,085e-05) but also had a bigger senescent area (p= 0,00197). The Col-0 plants grown in unsterile soil had a bigger anthocyanin area (p= 0,034).

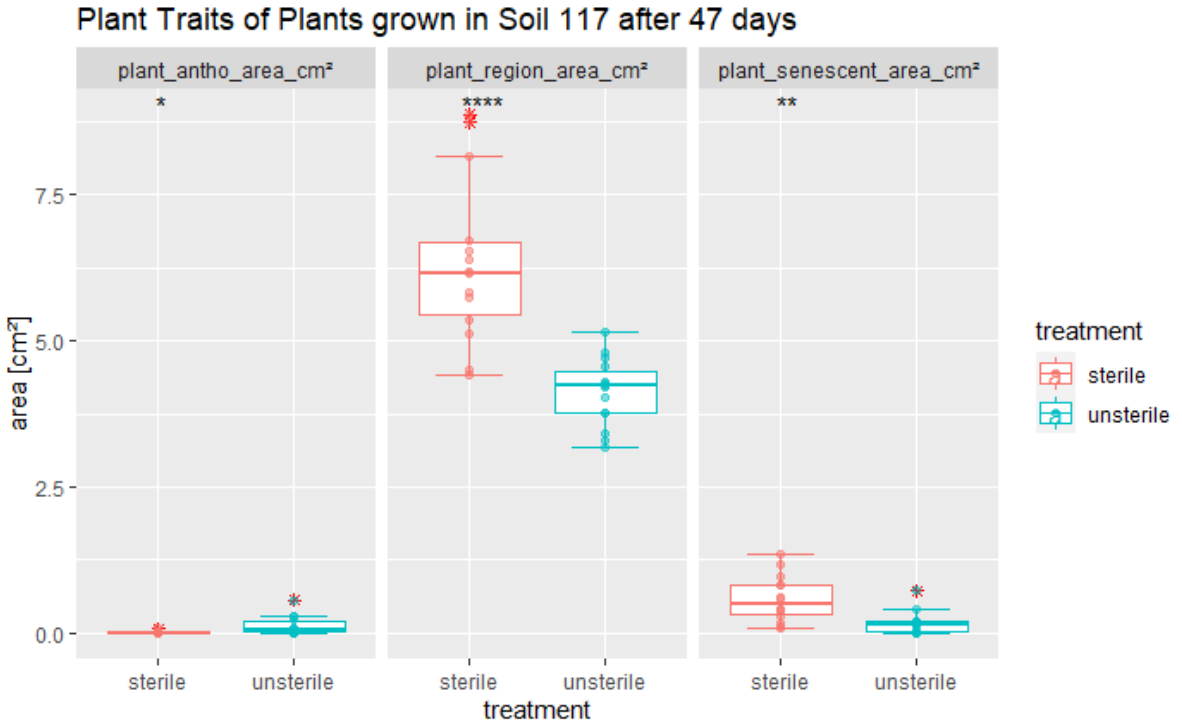

Figure 18: Phenotypic plant traits measured by Aradeepopsis of plants grown in soil 117 after 47 days. The measurements of anthocyanin area, rosette area and senescent areas were used.

3.2. Transplantation Experiment of Soil 117

3.2.1. Issues occurring during the Transplantation Experiment

During this experiment two issues occurred that influenced the results of the experiment. The first problem is that the root sterilization seemed to negatively influence the Col-0 plants grown with only endophytes. The root sterilization procedure was previously tested for soil 82 by Maja Schmidt Pedersen (unpubl.) but seemed to be too harsh for plants grown in soil 117. Those Col-0 plants were smaller and shortly after the sterilization treatment showed very red/purple leaves.

As a second obstacle the soil 117 seemed to be inhabited by mites that were seen on the Col-0 plants in later stages of the experiment (see Figure 19). This infection led to discoloration of the leaves. This was the reason to harvest the Col-0 plants earlier than planned and resulting in not fully developed symptoms in the plants, as was seen during the initial screening.

Those two issues heavily influenced the outcome of the experiment and made it very hard to draw conclusions about the location of the beneficial microbes.

Col-0 plants from all four treatments were harvested after 30 days after germination. For the negative control 23 Col-0 plants were harvested, for the positive control 25 Col-0 plants, for the endo treatment 24 and for the endo+rhizo treatment 20 Col-0 plants.

Figure 19: Mite contamination on Col-0 plant grown in the soil 117 transplantation experiment

3.2.2. Overall Col-0 Plant Phenotype in the Soil 117 Transplantation Experiment

The Col-0 plants from the soil 117 transplantation experiment had a very similar phenotype in all tested treatments as it was too early to fully develop the symptoms seen during the initial screening. The endo treatment was smaller compared to the other groups (see Figure 20). When the leaves were cut of the rosette the yellow coloration, especially in the negative control, can be seen (see Figure 21).

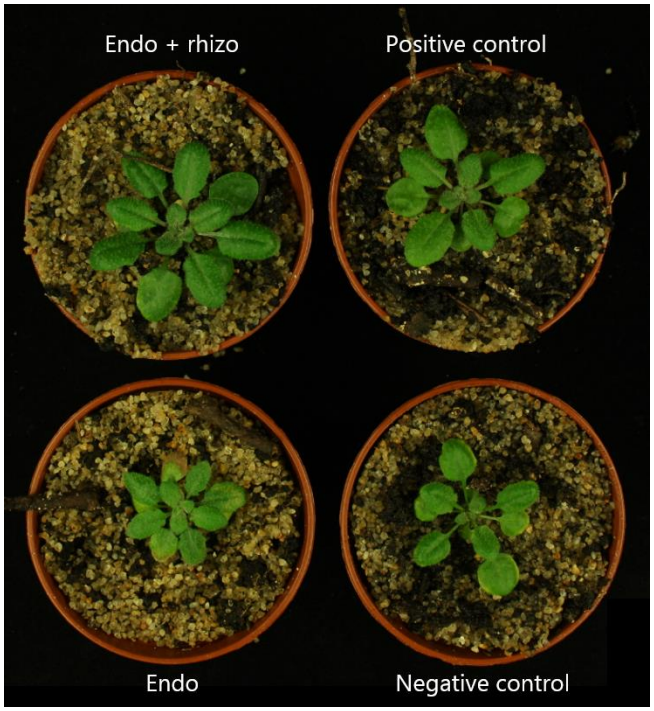

Figure 20: Plant Phenotype of *Arabidopsis thaliana* grown in the soil 117 transplantation experiment. On the left side the two treatments are seen. On the right side the two controls are seen. Endo+Rhizo = microbes from endosphere and rhizosphere are present, Endo = microbes from endosphere are present, Positive Control = microbes from bulk soil, endosphere and rhizosphere are present, Negative Control = no soil related microbes are present.

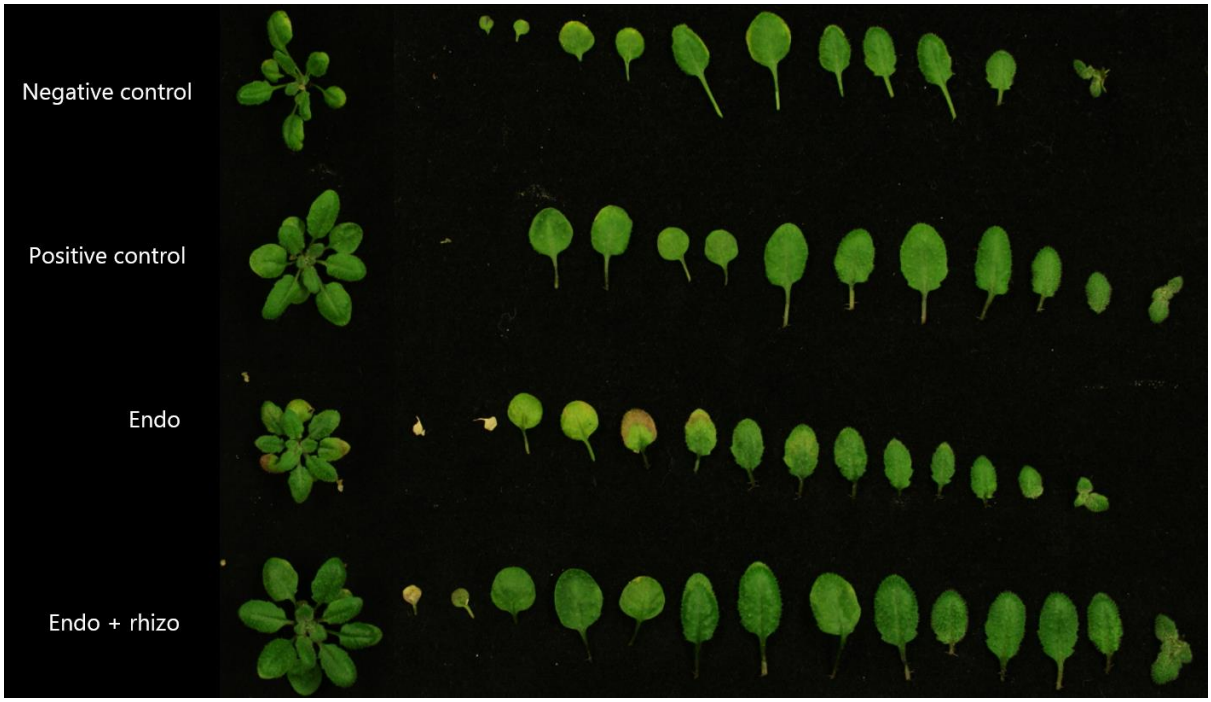

Figure 21: Col-0 plants from the soil 117 transplantation experiment. On the left side the whole rosette is shown. On the right side the leaves of the respective rosette are cut and placed by its position on the rosette. . Endo+Rhizo = microbes from endosphere and rhizosphere are present, Endo = microbes from endosphere are present, Positive Control = microbes from bulk soil, endosphere and rhizosphere are present, Negative Control = no soil related microbes are present.

3.2.3. Rosette Fresh Weight

At the end of the experiment the rosettes from all Col-0 plants were harvested. After cutting them they were weighed to determine the fresh weight. To compare all four treatments a Welch one-way ANOVA was carried out, followed by a Games-Howell test. The positive control has significantly heavier rosettes compared to the negative control and the treatment containing only endophytes (endo). But no difference could be found compared to the treatment containing endophytes and the rhizosphere microbes (endo + rhizo). The endo + rhizo treatment is also significantly heavier than the negative control and the endo treatment. The endo treatment shows a similar plant fresh weight to the negative control (see Figure 22).

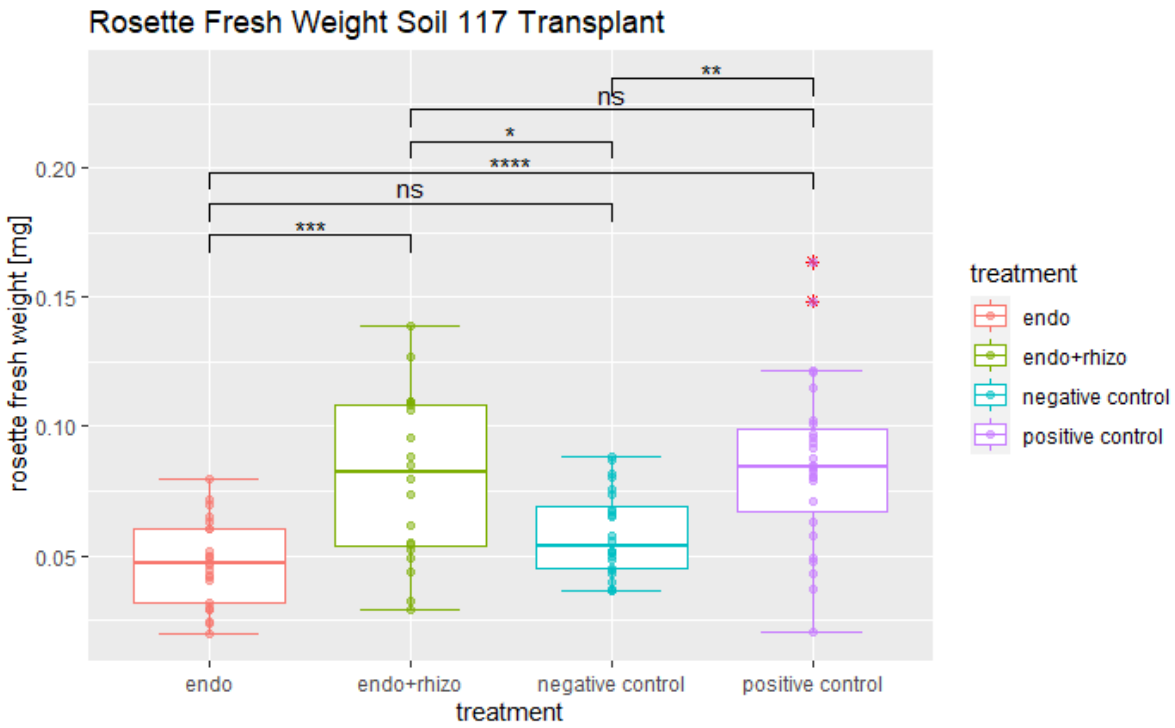

Figure 22: Comparison of the rosette fresh weight of Col-0 plants grown in the soil 117 transplantation experiment. Endo+Rhizo = microbes from endosphere and rhizosphere are present, Endo = microbes from endosphere are present, Positive Control = microbes from bulk soil, endosphere and rhizosphere are present, Negative Control = no soil related microbes are present. As a statistical test a Kruskall-Wallis test, followed by a Games-Howell test was carried out to compare the four treatments.

3.2.4. Total Number of Leaves and Yellowing Leaves

During the harvesting process the total amount of leaves were counted as well as leaves showing yellow coloration. Also the ratio of yellow leaves to total leaves was calculated (see Appendix C: Transplantation Experiment of Soil 117). All three traits were analyzed by a Kruskall-Wallis test followed by a Wilcoxon test (see Figure 23).

The total number of leaves showed no significant difference between all groups. The differences between the groups were seen in the number of leaves with yellow edges as well as in the ratio of yellow to total leaves. For the ratio the negative control had significantly more yellowing leaves than all other treatments. The endo treatment neither showed a significant difference to the endo+rhizo and positive control. But the endo+rhizo and the positive control were significantly different from each other.

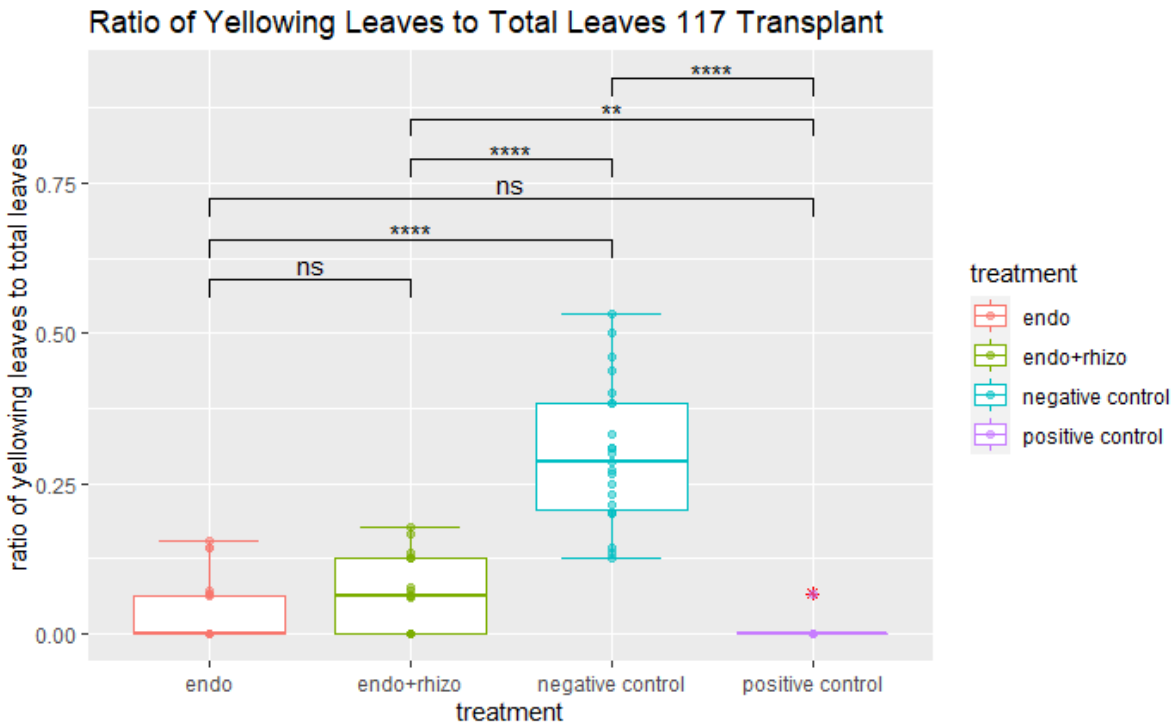

Figure 23: Ratio of leaves having yellow edges to total number of leaves of Col-0 plants grown in the soil 117 transplantation experiment. Endo+Rhizo = microbes from endosphere and rhizosphere are present, Endo = microbes from endosphere are present, Positive Control = microbes from bulk soil, endosphere and rhizosphere are present, Negative Control = no soil related microbes are present. The statistical significance was tested with a Kruskall-Wallis test followed by a Wilcoxon test.

3.2.5. Aradeepopsis Traits

As traits derived from the Aradeepopsis software, the total plant area and the senescent plant area were used for comparison of the four treatments.

As the group containing only endophytes had a slower growth due to the root sterilization, the decreased plant size could also be seen after a Welch-ANOVA, followed by a Games-Howell test. The other groups differed not significantly from each other (see Figure 24).

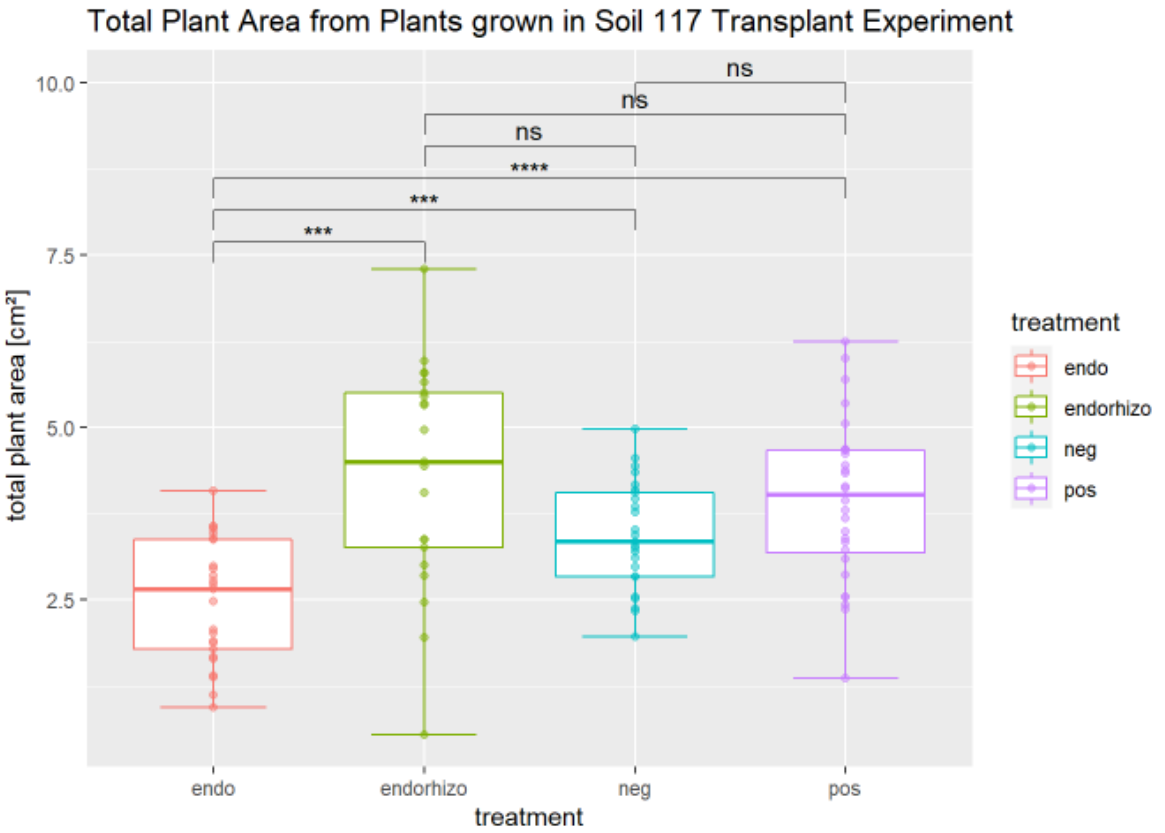

Figure 24: Total plant area from the soil 117 transplantation experiment, determined by the Aradeepopsis software. Endo+Rhizo = microbes from endosphere and rhizosphere are present, Endo = microbes from endosphere are present, Positive Control = microbes from bulk soil, endosphere and rhizosphere are present, Negative Control = no soil related microbes are present. Statistical comparison was done by a Welch-ANOVA followed by a Games-Howell test.

Based on the Aradeepopsis data no differences in the senescent area could be detected (see Figure 25). This could be the result of the not perfect evaluation of plant area by the software as the number of leaves having yellow edges was higher in the sterile condition compared to the others. The statistical comparison was done by a Kruskall-Wallis test followed by a Wilcoxon test.

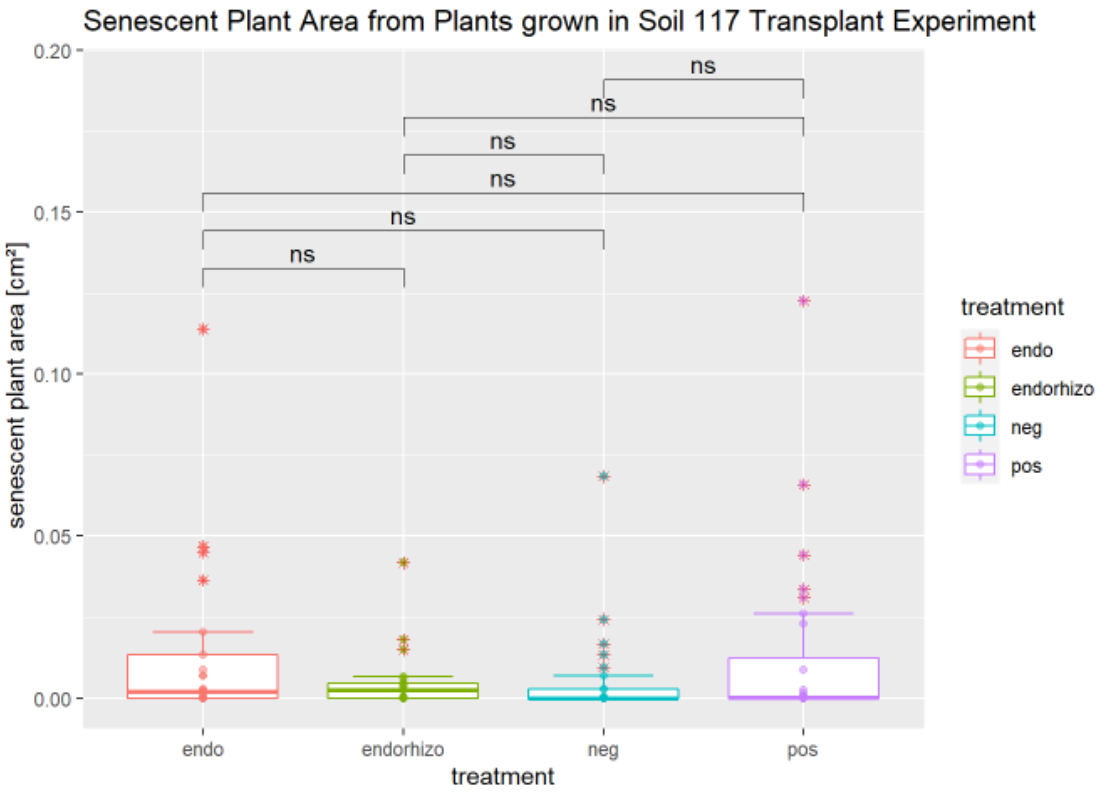

Figure 25: Senescent leaf area of Col-0 plants grown in the soil 117 transplantation experiment. Data was derived by the Aradeepopsis software. Endo+Rhizo = microbes from endosphere and rhizosphere are present, Endo = microbes from endosphere are present, Positive Control = microbes from bulk soil, endosphere and rhizosphere are present, Negative Control = no soil related microbes are present. Statistical comparison was done by a Kruskall-Wallis test followed by a Wilcoxon test.

3.3. Soil Dilution Experiment

The results of the soil dilution experiment were evaluated 44 DAG, by measuring the total plant area and for soil 116 also the yellow area in ImageJ.

3.3.1. Soil 71 in the Soil Dilution Experiment

For Col-0 plants grown in soil 71 treated with different soil extract no significant difference was found using a Kruskall-Wallis test (p= 0,957) (see Figure 27). Also for the dark green to red coloration of the plants previously seen in soil 71 no clear difference was seen by eye (see Figure 26).

Figure 26: Phenotype of Col-0 plants grown in the soil dilution experiment in soil 71. a= heat-treated, b= sterile, c= unsterile

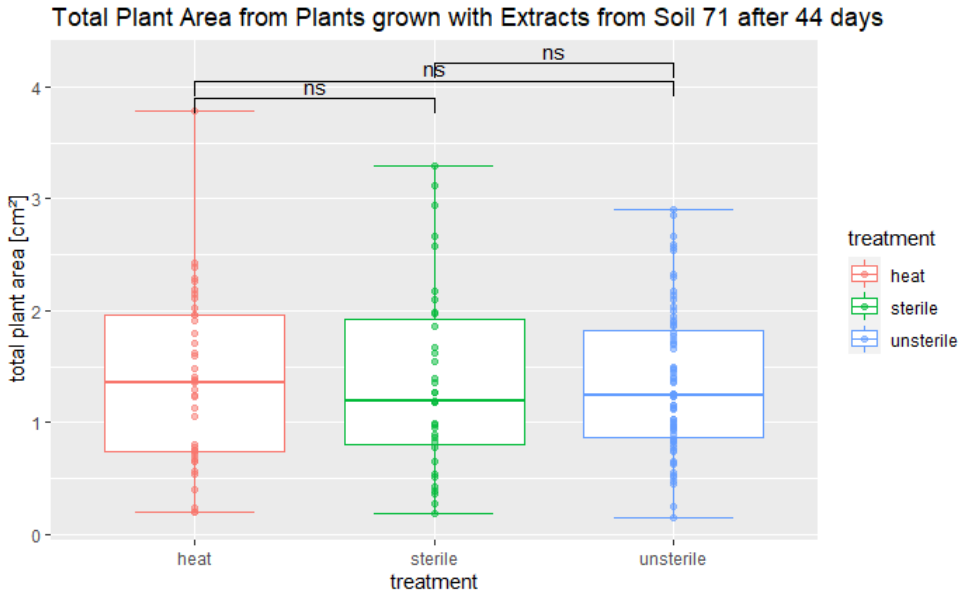

Figure 27: Comparison of the total rosette area of Col-0 plants grown in the soil dilution experiment in soil 71. Measured with ImageJ

3.3.2. Soil 109 in the Soil Dilution Experiment

The Col-0 plants grown in sterile soil 109 was compared by their size, as previously Col-0 plants grown in sterile soil 109 showed a more compact phenotype (see Figure 29). The sample number differs for each group as up to five plants are in one pot. For the sterile soil extract 35 plants were measured, for the heat-treated soil extract 28 plants and for the unsterile conditions 80 plants. The statistical analysis to compare the plant area was done with a Kruskall-Wallis test followed by a Wilcoxon test. The analysis showed a significant difference (p= 0,002) between the groups heat and sterile, which are both supposed to not contain any microbes. There was also a statistical difference between the sterile and unsterile group (p = 0,028) (see Figure 28).

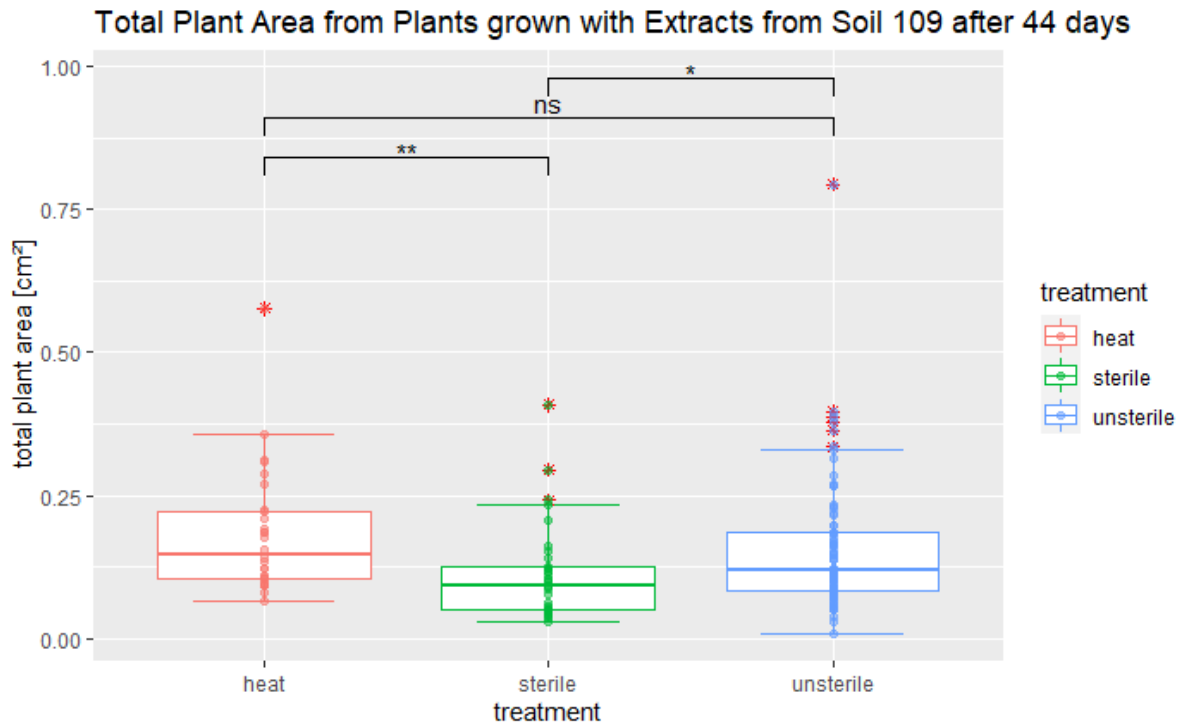

Figure 28: Comparison of the total rosette area of Col-0 plants grown in the soil dilution experiment in soil 109. Measured with ImageJ. Statistical differences were calculated with a Kruskall-Wallis test followed by a Wilcoxon test.

Figure 29: Phenotype of Col-0 plants grown in the soil dilution experiment in soil 109. a= heat-treated, b= sterile, c= unsterile

3.3.3. Soil 116 in the Soil Dilution Experiment

For the Col-0 plants grown in soil 116 treated with different soil extracts, not only the total plant area but also the yellow plant area was measured in ImageJ. For both measurements no significant difference between the groups could be found. For the total plant area a Kruskall-Wallis test was used (p= 0,754) and for the yellow plant area a Welch-ANOVA (p= 0,989) (see Figure 30, Figure 31, Figure 32).

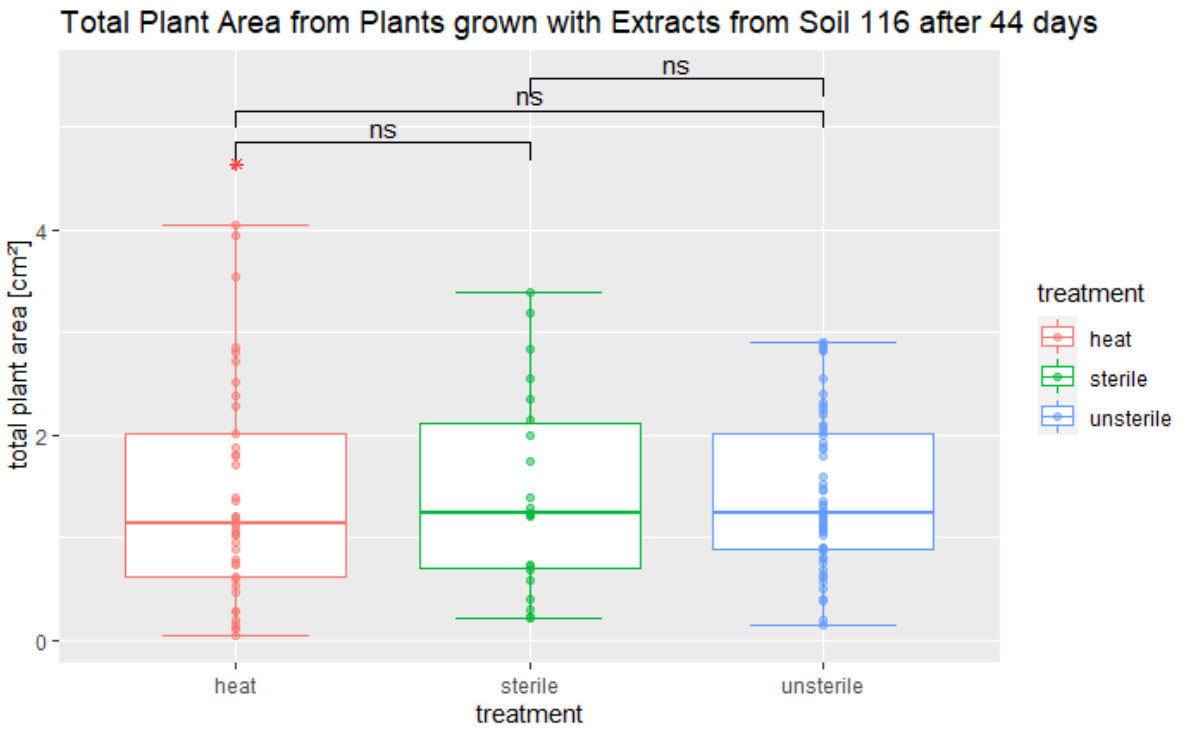

Figure 30: Comparison of the total rosette area of Col-0 plants grown in the soil dilution experiment in soil 116. Measured with ImageJ. Statistical differences were calculated with a Kruskall-Wallis test followed by a Wilcoxon test.

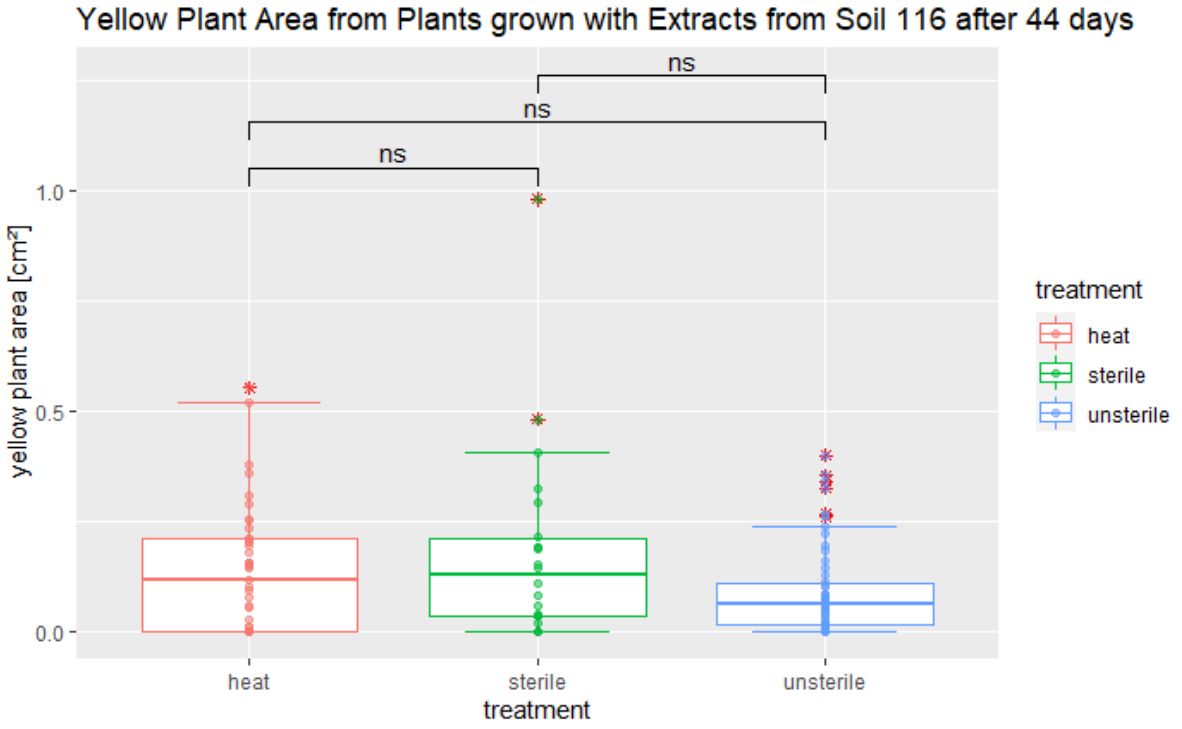

Figure 31: Comparison of the yellow rosette area of Col-0 plants grown in the soil dilution experiment in soil 116. Measured with ImageJ. Statistical differences were calculated with a Welch ANOVA followed by a Games-Howell test.

Figure 32: Phenotype of Col-0 plants grown in the soil dilution experiment in soil 116. a= heat-treated, b= sterile, c= unsterile

3.4. Combined Transplantation and Soil Dilution Experiment for Soil 117

The Col-0 plants grown in the combined experiment of transplantation and soil dilution in soil 117 were analyzed after 21 DAG (see Figure 35). This was generally too early to see differences, but the experiment had to be stopped after this time because, as already seen in the transplantation experiment, mites were attacking the plants, giving the impression that they are occurring in the soil 117 naturally.

The total plant area was analyzed by an one-way ANOVA followed by a Tukey post-hoc test. In this case the plants grown with only endophytes were smaller than the plants in the other treatments (see Figure 33). The yellow area was analyzed with a Kruskall-Wallis test showing no significant differences (p= 0,355) (Figure 34).

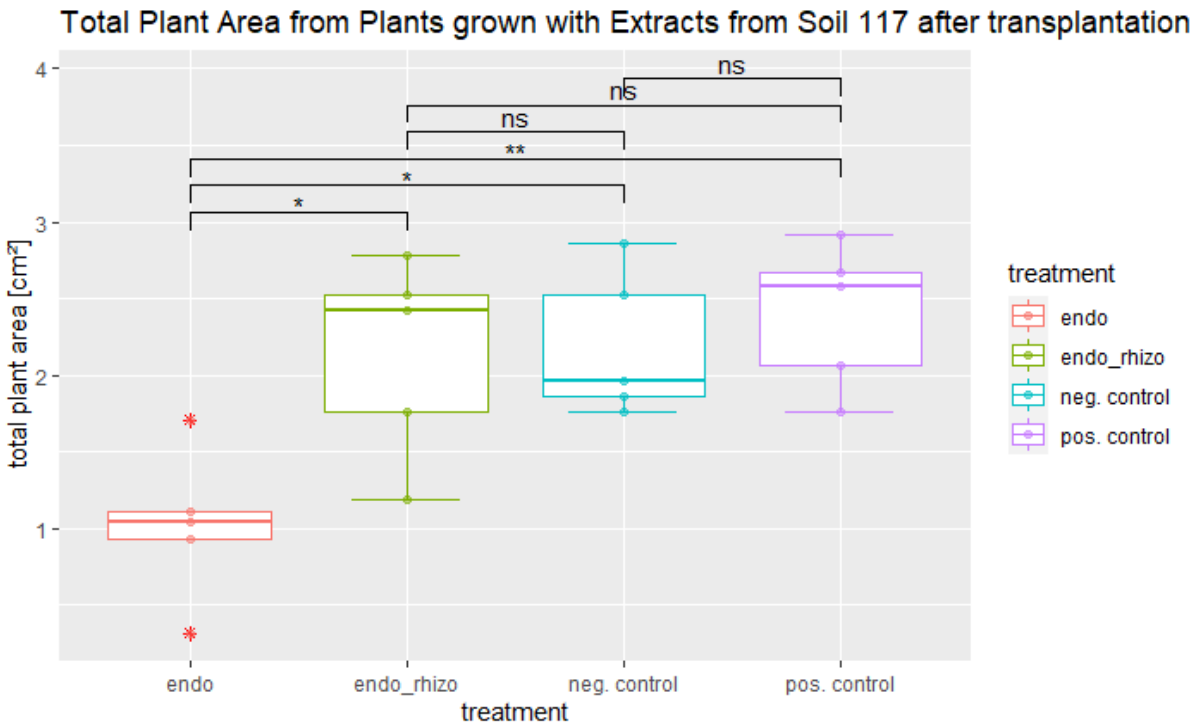

Figure 33: Comparison of the total rosette area of Col-0 plants grown in the soil dilution experiment combined with a transplantation experiment in soil 117. Endo+Rhizo = microbes from endosphere and rhizosphere are present, Endo = microbes from endosphere are present, Positive Control = microbes from bulk soil, endosphere and rhizosphere are present, Negative Control = no soil related microbes are present. Measured with ImageJ. Statistical differences were calculated with a one-way ANOVA followed by a Tukey post-hoc test.

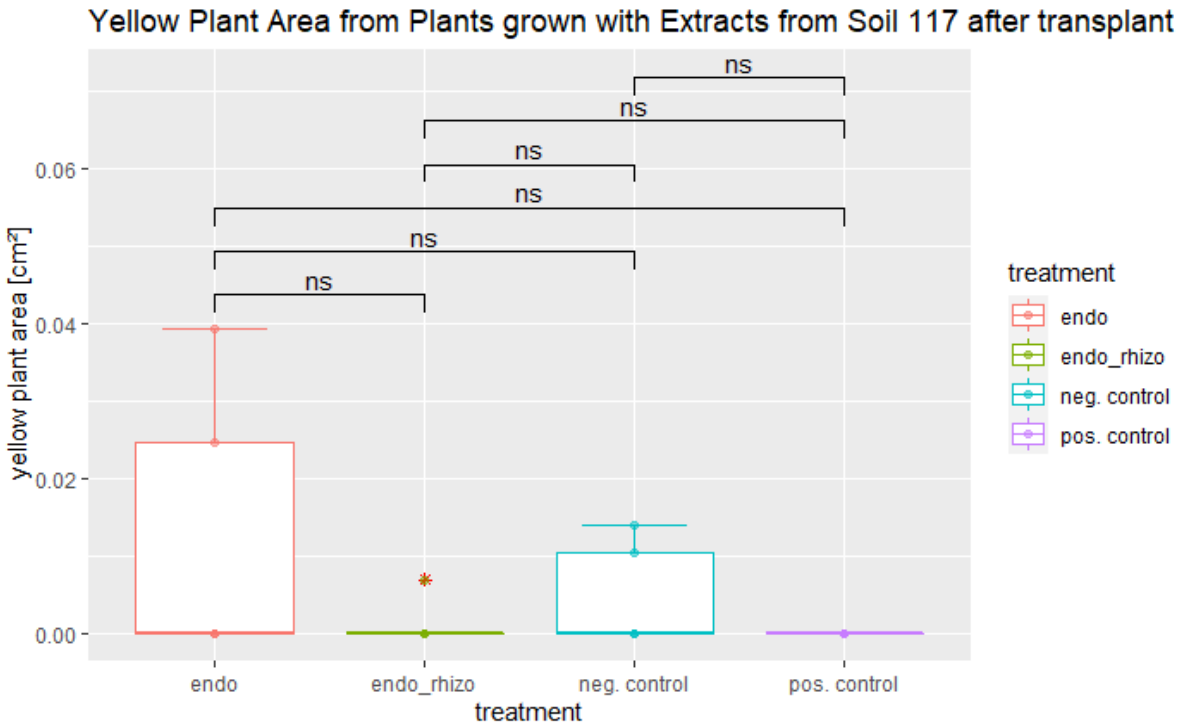

Figure 34: Comparison of the yellow rosette area of Col-0 plants grown in the soil dilution experiment combined with a transplantation experiment in soil 117. Endo+Rhizo = microbes from endosphere and rhizosphere are present, Endo = microbes from endosphere are present, Positive Control = microbes from bulk soil, endosphere

and rhizosphere are present, Negative Control = no soil related microbes are present. Measured with ImageJ. Statistical differences were calculated with a Kruskall-Wallis test followed by a Wilcoxon test.

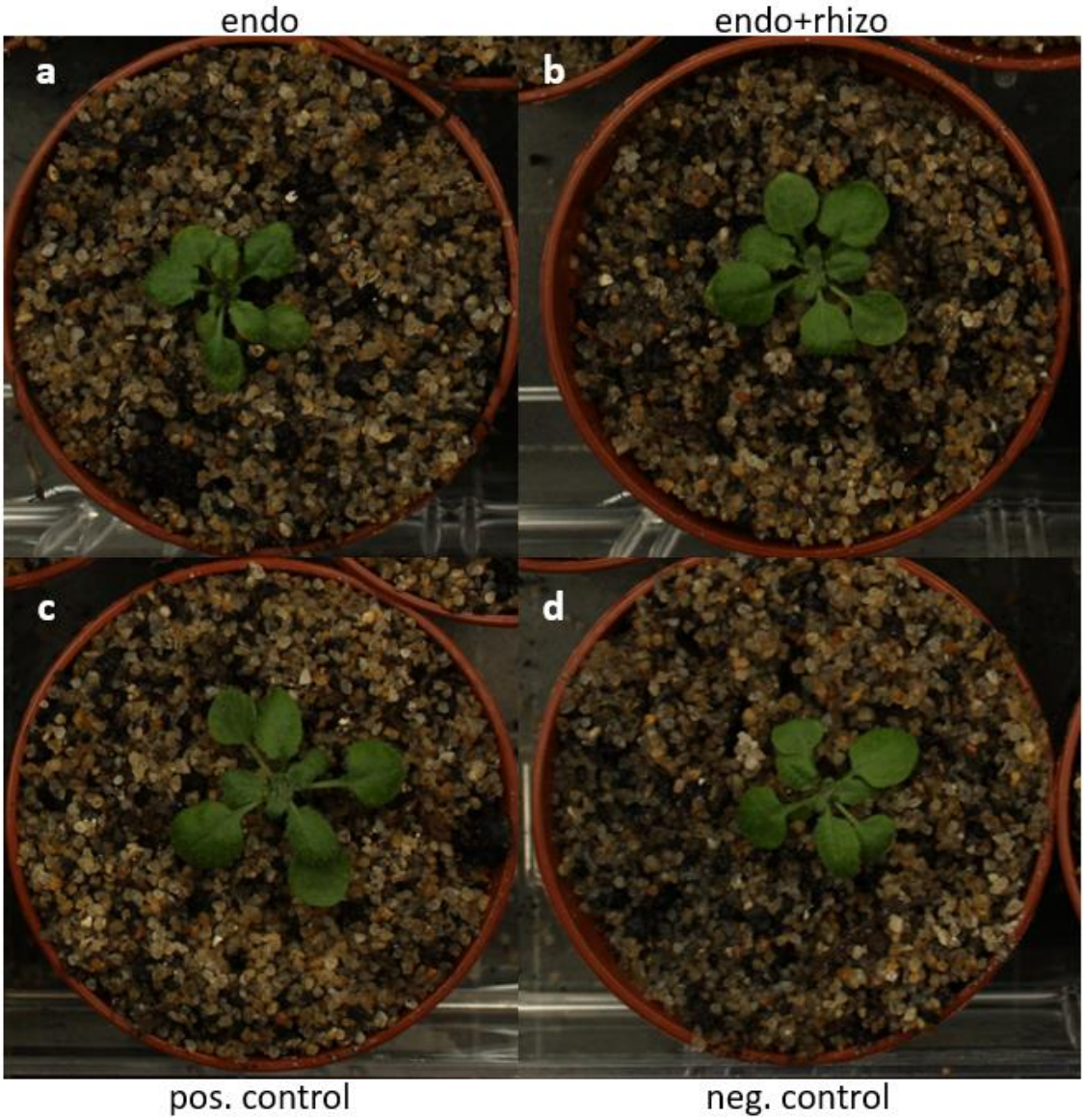

Figure 35: Phenotype of Col-0 plants grown in the soil dilution experiment combined with a transplantation experiment in soil 117. Endo+Rhizo = microbes from endosphere and rhizosphere are present, Endo = microbes from endosphere are present, Positive Control = microbes from bulk soil, endosphere and rhizosphere are present, Negative Control = no soil related microbes are present.

3.5. Nutrient Rescue Experiment

3.5.1. Soil 109 in the Nutrient Rescue Experiment

Figure 36: Phenotype of Col-0 plants grown in soil 109 in the nutrient rescue experiment. a= sterile, b= heat-treated, c= unsterile, d=sterile with nutrients, e= heat with nutrients

For soil 109 the relevant phenotypic traits (see Figure 36) were the rosette size and the number of leaves, both were determined and compared using a Welch or normal one-way ANOVA followed by a post hoc test (either Games Howell or Tukey). For the plant area the only group differing from the others were only treated with sterile soil extract (see Figure 37). No significant difference could be found for the other treatments. For the leaf number differences could be found between the groups sterile and unsterile (p= 3,15x10^-2), sterile and heat with nutrients (p= 8,01x10^-5) and heat with nutrients and unsterile (p= 2,49x10^-2) (see Figure 38).

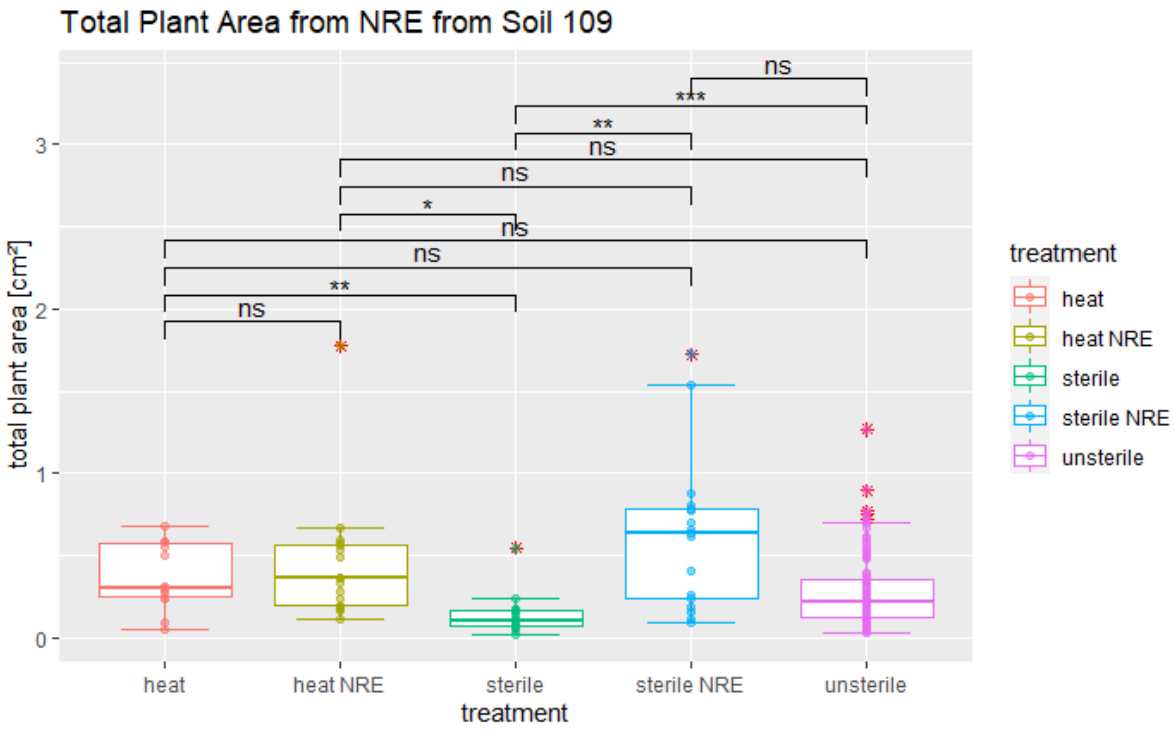

Figure 37: Total plant area of Col-0 plants grown in soil 109 in the nutrient rescue experiment. Measured in ImageJ. Statistical differences were calculated with a Welch ANOVA followed by a Games-Howell test.

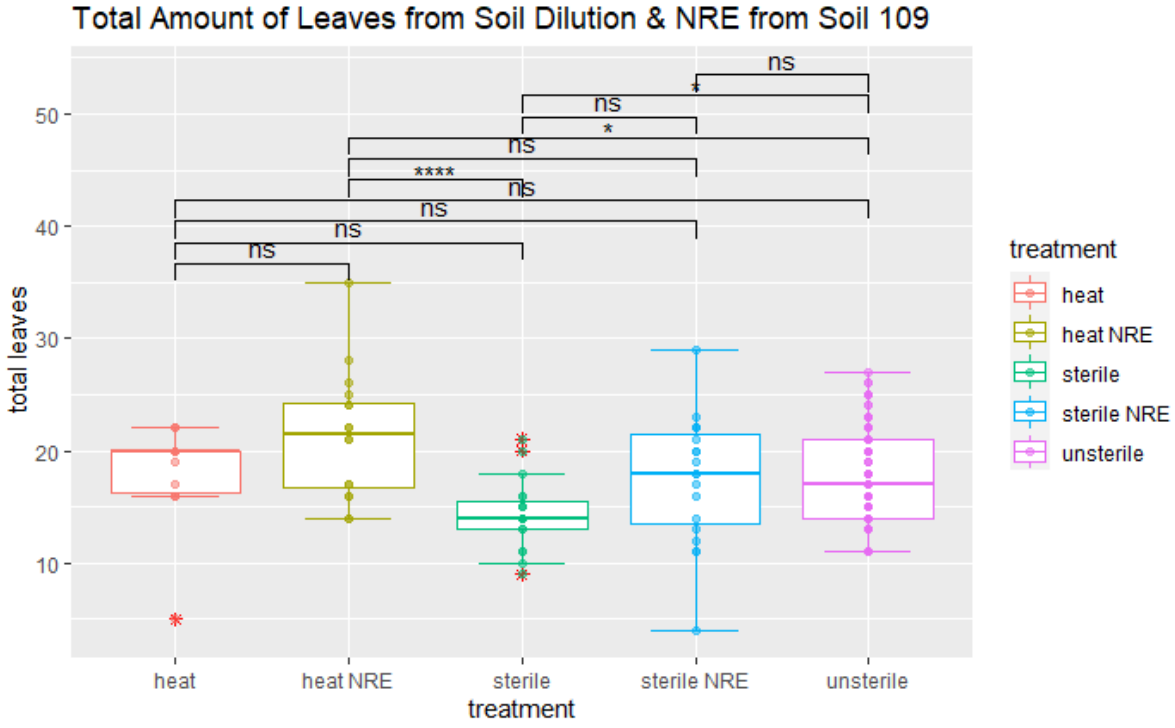

Figure 38: Total amount of leaves of Col-0 plants grown in soil 109 in the nutrient rescue experiment. Statistical differences were calculated with a one-way ANOVA followed by a Tukey post-hoc test.

3.5.2. Soil 71 in the Nutrient Rescue Experiment

All three groups (sterile, heat-treated and unsterile) which did not receive the nutrient solution developed purple/dark-green rosettes (see Figure 39). The Col-0 plants grown in the two groups which were treated with the nutrient solution stayed green. The leaves which were previously purple turned greener, also more senescent leaves appeared.

Figure 39: Phenotype of Col-0 plants grown in soil 71 in the nutrient rescue experiment. a= sterile, b= heat-treated, c= unsterile, d=sterile with nutrients, e= heat with nutrients

3.5.2.1. Anthocyanin Content of Col-0 Plants grown in Soil 71

At the end of the nutrient rescue experiment, Col-0 plants grown in sterile soil dilution soil 71 and nutrient supplied soil 71 were compared in their anthocyanin content (see Figure 40). To compare those two groups a Welch two-sample t-test was carried out. Those two groups were significantly different from each other (p-value = 0,01), with the Col-0 plants grown in sterile soil dilution soil having a higher content in anthocyanin compared to the nutrient supplied Col-0 plants.

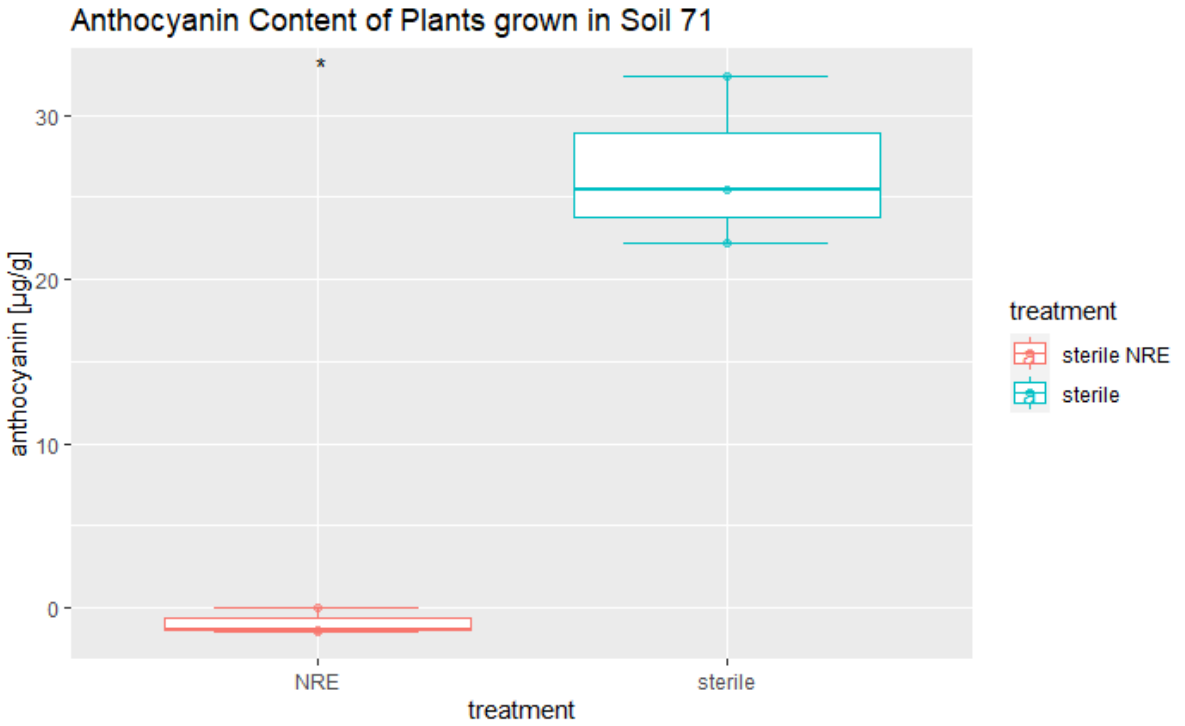

Figure 40: Anthocyanin content inCol-0 plants grown in sterile soil 71 either treated with a nutrient solution or not. Statistical differences were calculated with a Welch t-test.

3.5.2.2. Total Plant Area

The plant area did not show many differences between the groups, except for sterile with sterile supplied with nutrients, were a statistically significant difference (p= 0,045) was found with a Welch-ANOVA followed by a Games Howell test (see Figure 41).

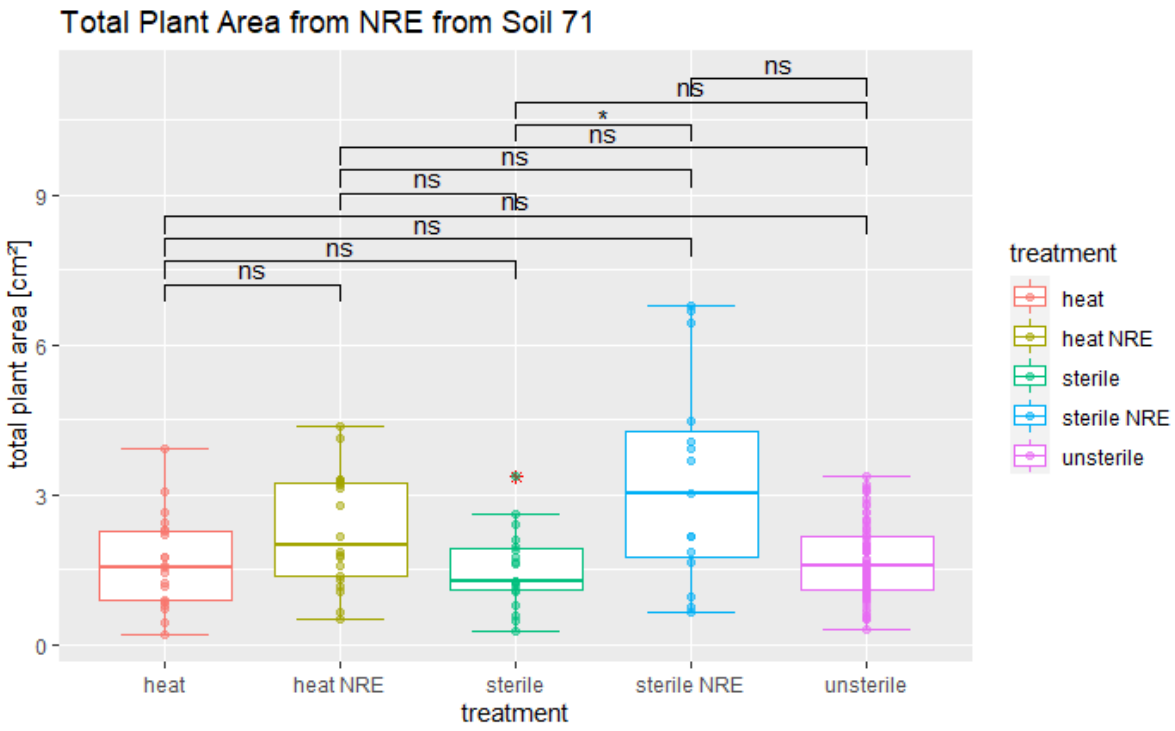

Figure 41: Total plant area of Col-0 plants grown in soil 71 in the nutrient rescue experiment. Measured in ImageJ. Statistical differences were calculated with a Welch ANOVA followed by a Games-Howell test.

3.5.2.3. Branches

The number of branches of the Col-0 plants grown in soil 71 either supplied with nutrients or not differed significantly from each other. Both group which received the nutrient solution had more branches compared to the three other groups using a Welch ANOVA followed by a Games-Howell test (see Figure 42).

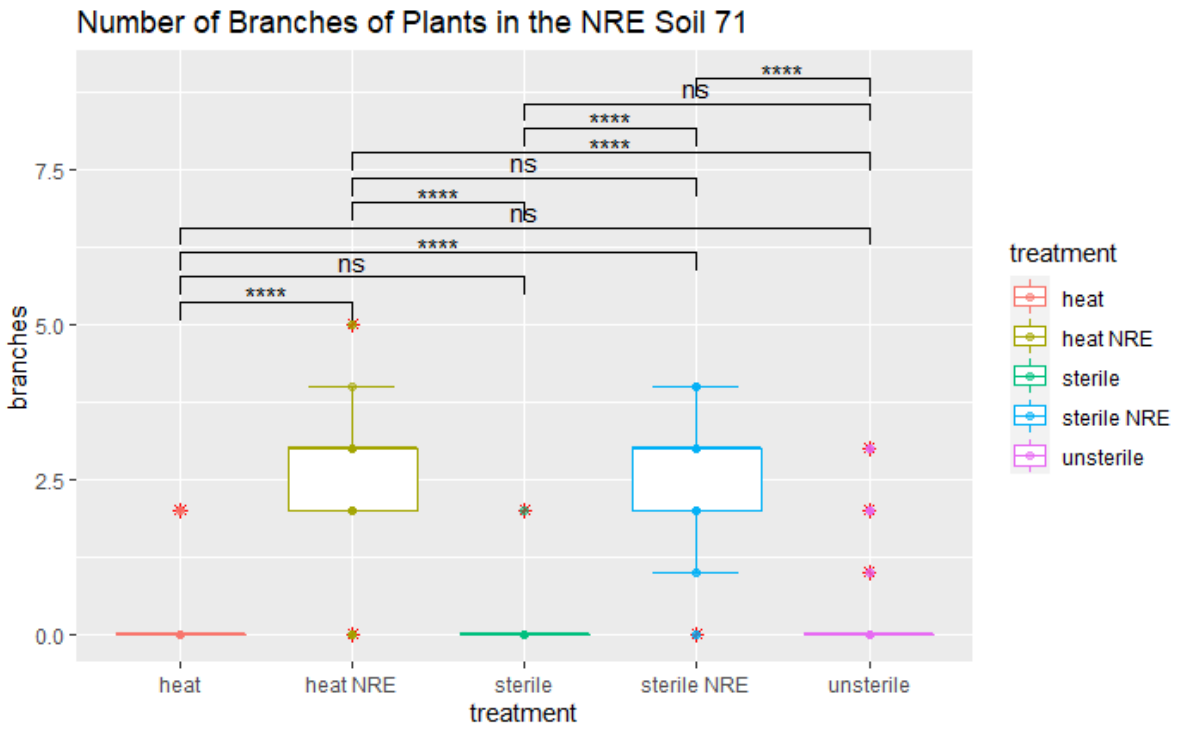

Figure 42: Number of branches of Col-0 plants grown in soil 71 in the nutrient rescue experiment. Statistical differences were calculated with a Welch ANOVA followed by a Games-Howell test.

3.5.2.4. Plant Height

The plant height was measured and compared with a one-way ANOVA followed by a Tukey post-hoc test. It was found that the sterile group differed from both groups which were supplied with nutrients (see Figure 43).

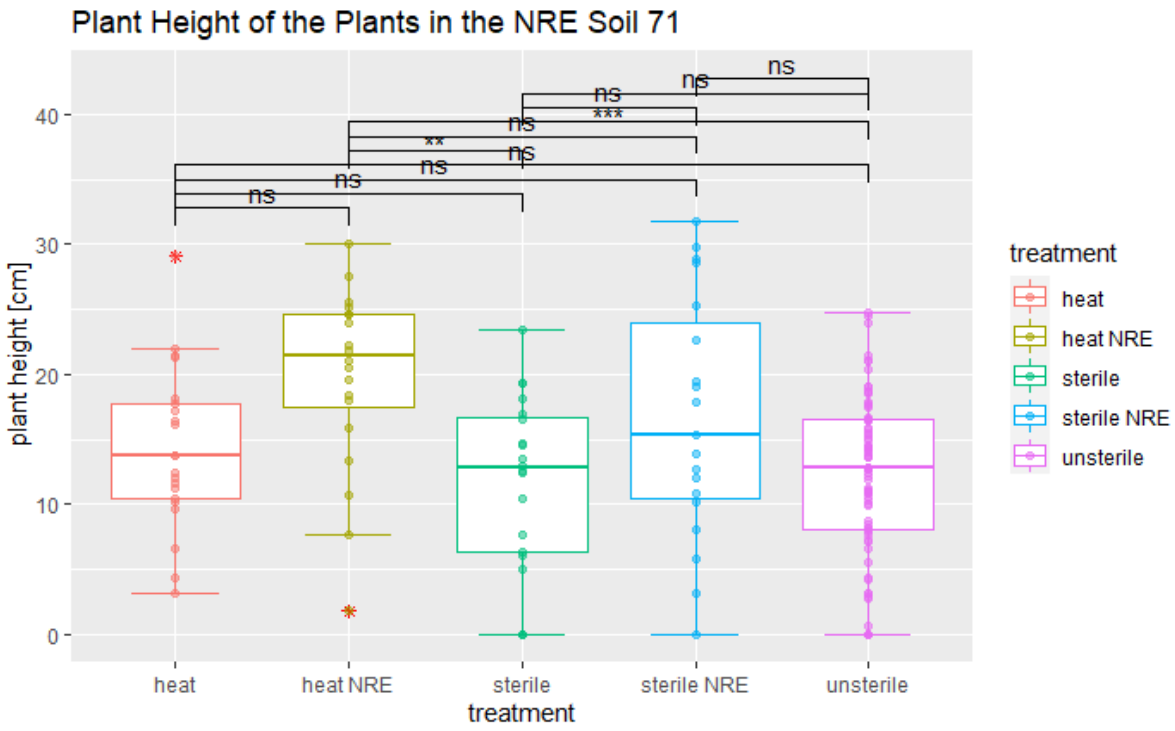

Figure 43: Plant height of Col-0 plants grown in soil 71 in the nutrient rescue experiment. Statistical differences were calculated with a one-way ANOVA followed by a Tukey post-hoc test.

3.5.3. Silique Number

The silique number was compared as a phenotypic trait with a Welch ANOVA. The only significant difference could be found between the unsterile group and the heat group supplied with nutrients (p= 0,048) (see Figure 44).

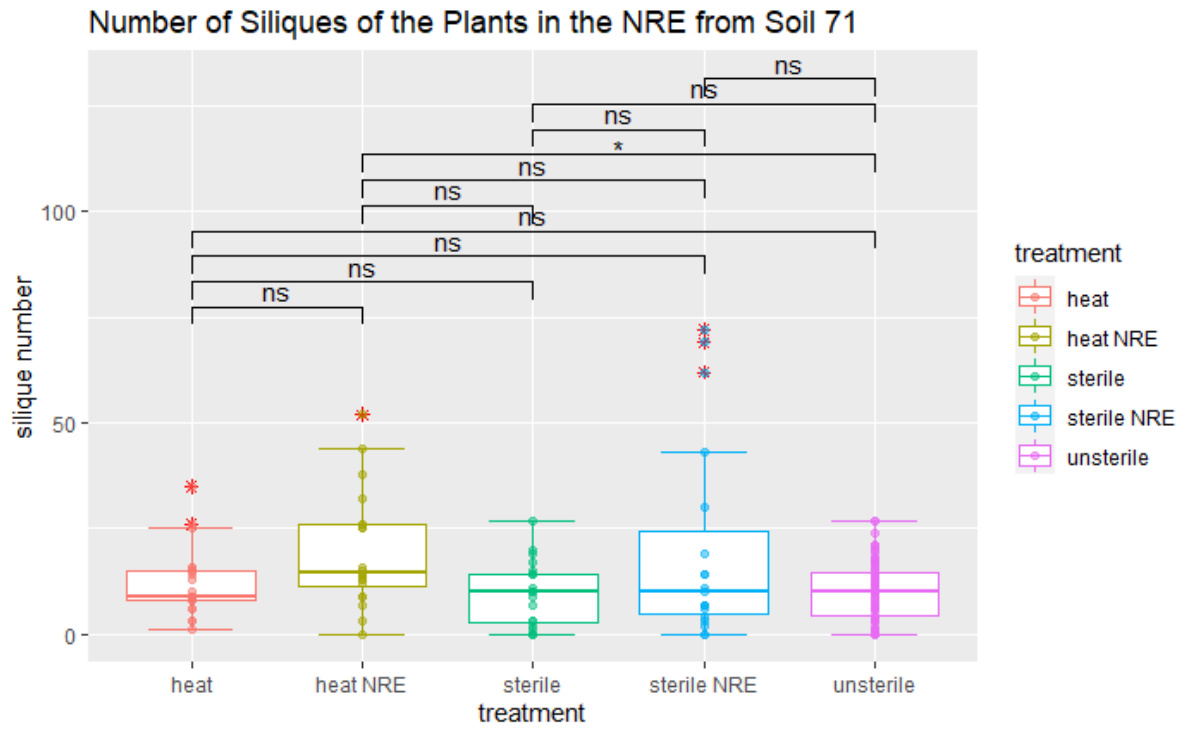

Figure 44: Number of siliques of Col-0 plants grown in soil 71 in the nutrient rescue experiment. Statistical differences were calculated with a Welch ANOVA followed by a Games-Howell test.

3.5.4. Soil 116 in the Nutrient Rescue Experiment

Col-0 plants grown in soil 116 in the nutrient experiment differed from each other when they received nutrients or not. The groups without nutrients developed yellow/senescent leaves, while the leaves of plants supplied with nutrients stayed green and already yellow parts turned green again when they were not yet senescent (see Figure 45).

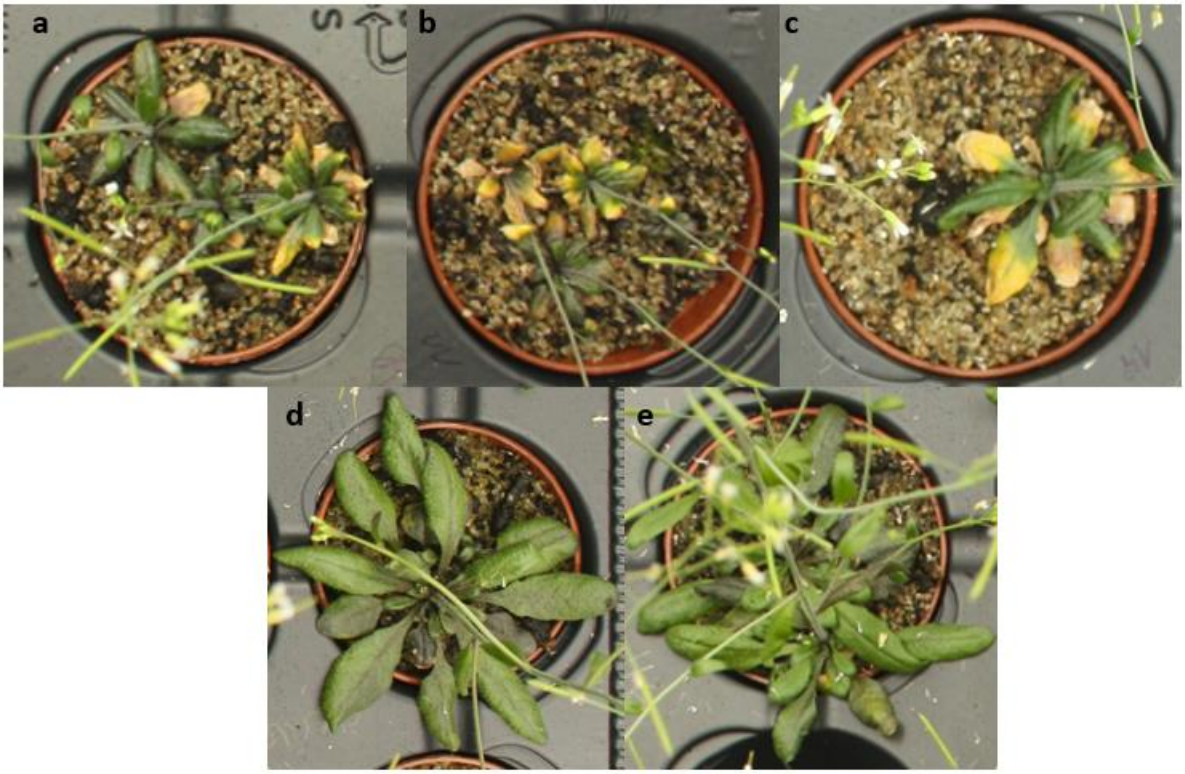

Figure 45: Phenotype of Col-0 plants grown in soil 116 in the nutrient rescue experiment. a= sterile, b= heat-treated, c= unsterile, d=sterile with nutrients, e= heat with nutrients

3.5.4.1. Total Plant Area

The total plant area was significantly bigger for the groups which were supplied with nutrients compared to all other groups, when compared with a Welch ANOVA followed by a Games Howell test (see Figure 46).

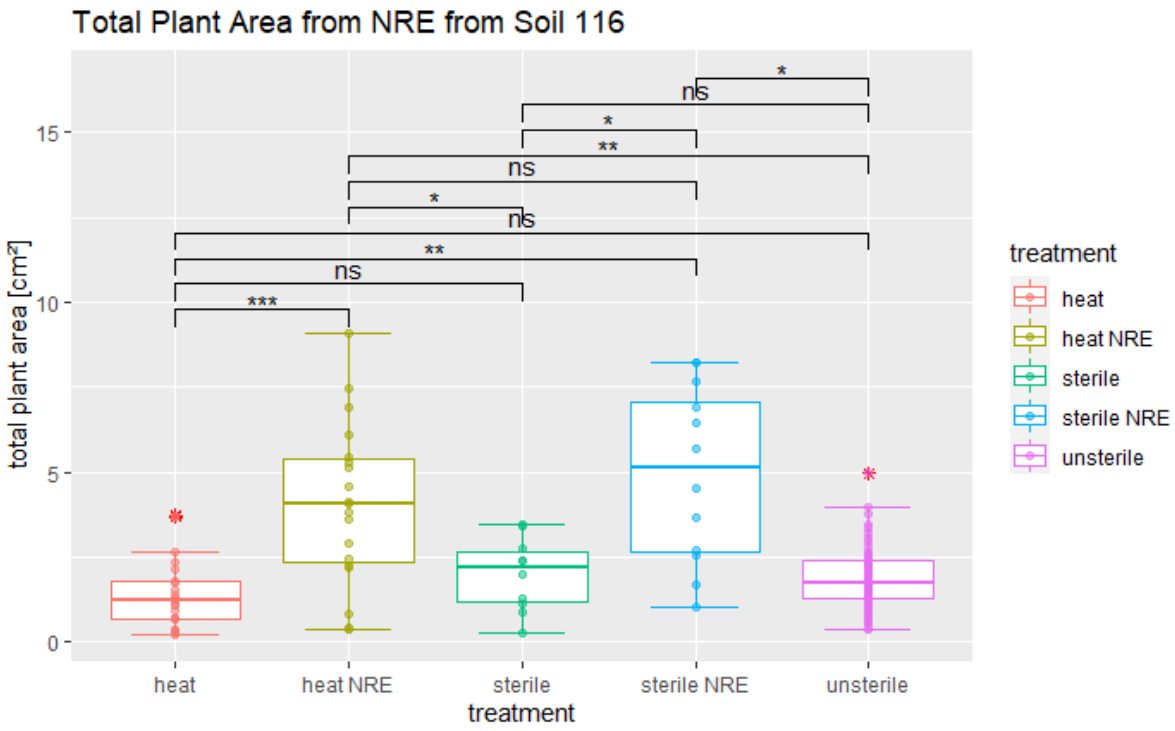

Figure 46: Total plant area of Col-0 plants grown in soil 116 in the nutrient rescue experiment. Measured in ImageJ. Statistical differences were calculated with a Welch ANOVA followed by a Games-Howell test.

3.5.4.2. Yellow Plant Area

No difference could be found between the groups when comparing the size of the yellow/senescent area of the Col-0 plants with a Kruskall-Wallis test followed by a Wilcoxon test (see Figure 47).

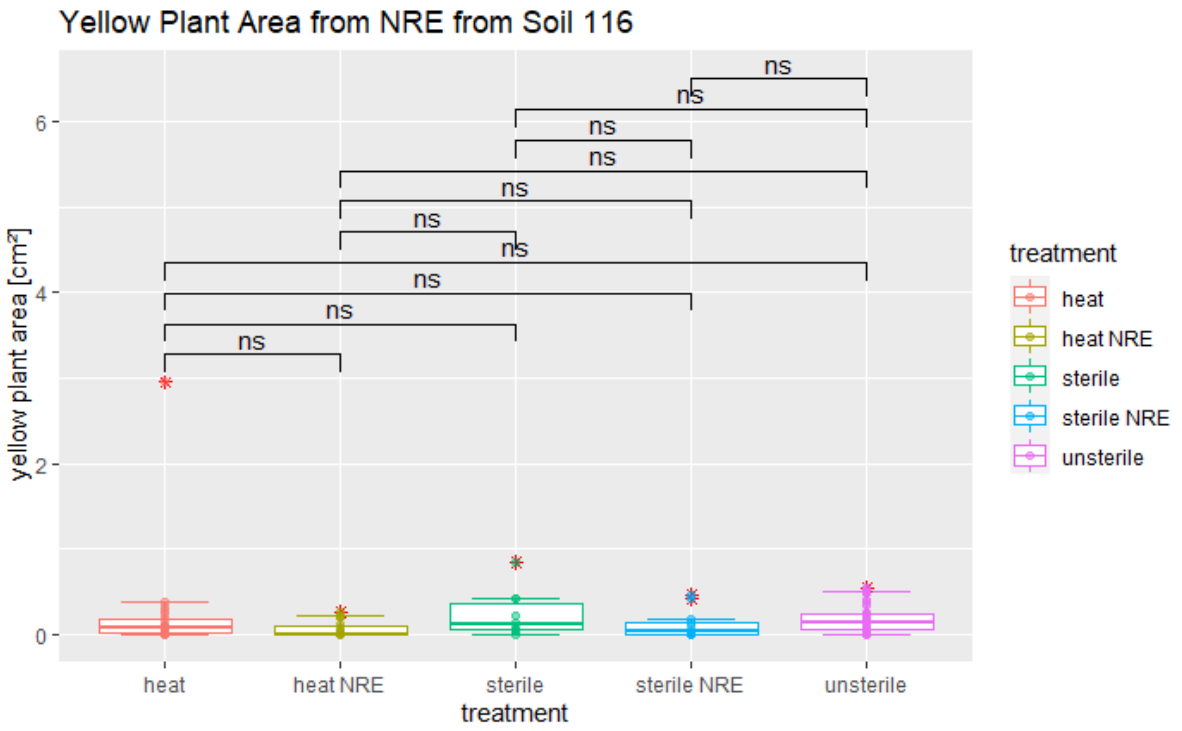

Figure 47: Yellow plant area of Col-0 plants grown in soil 116 in the nutrient rescue experiment. Measured in ImageJ. Statistical differences were calculated with a Kruskall-Wallis test followed by a Wilcoxon test.

3.5.4.3. Number of Leaves

The number of leaves differed significantly for the groups when tested with a Kruskall-Wallis test followed by a Wilcoxon test. The groups with nutrients were significantly different from the sterile and heat group. The unsterile group was only different to the heat group (see Figure 48).

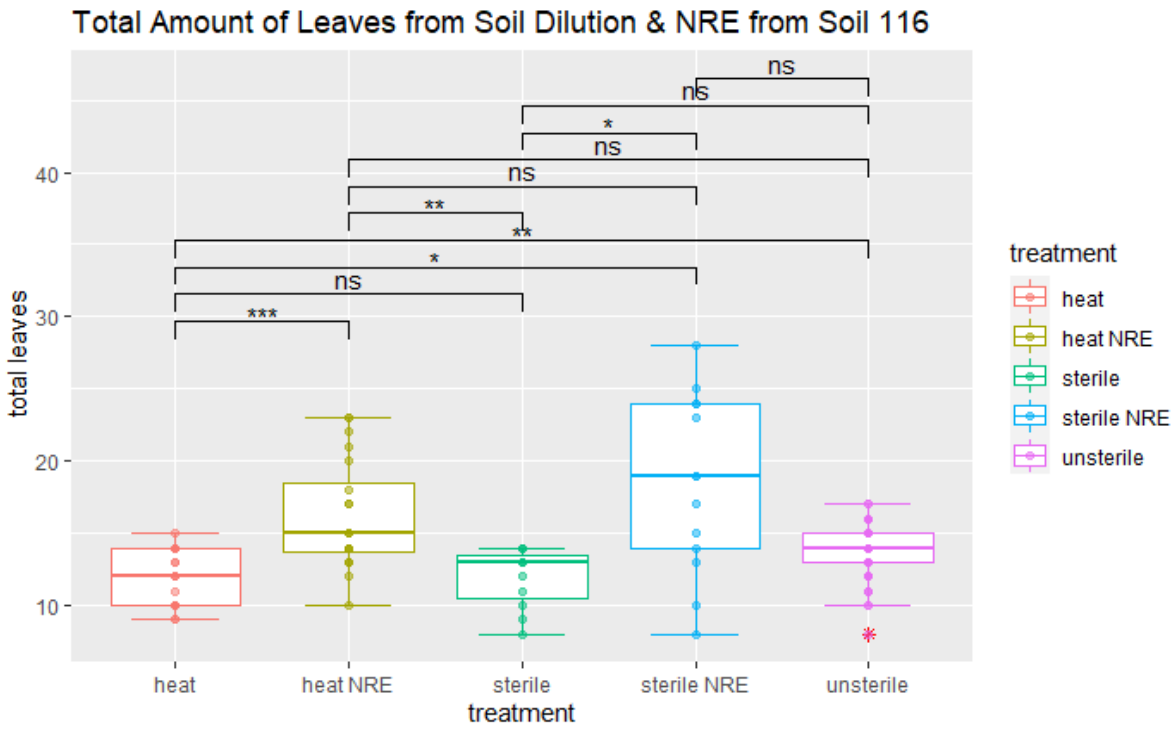

Figure 48: Number of leaves of Col-0 plants grown in soil 116 in the nutrient rescue experiment. Statistical differences were calculated with a Kruskall-Wallis test followed by a Wilcoxon test.

3.5.4.4. Number of Leaves with Yellow Area

The number of leaves which had a yellow area was compared as well. In the groups supplied with nutrients significantly less leaves had a yellow area compared to the groups which did not receive a nutrient solution (see Figure 49).

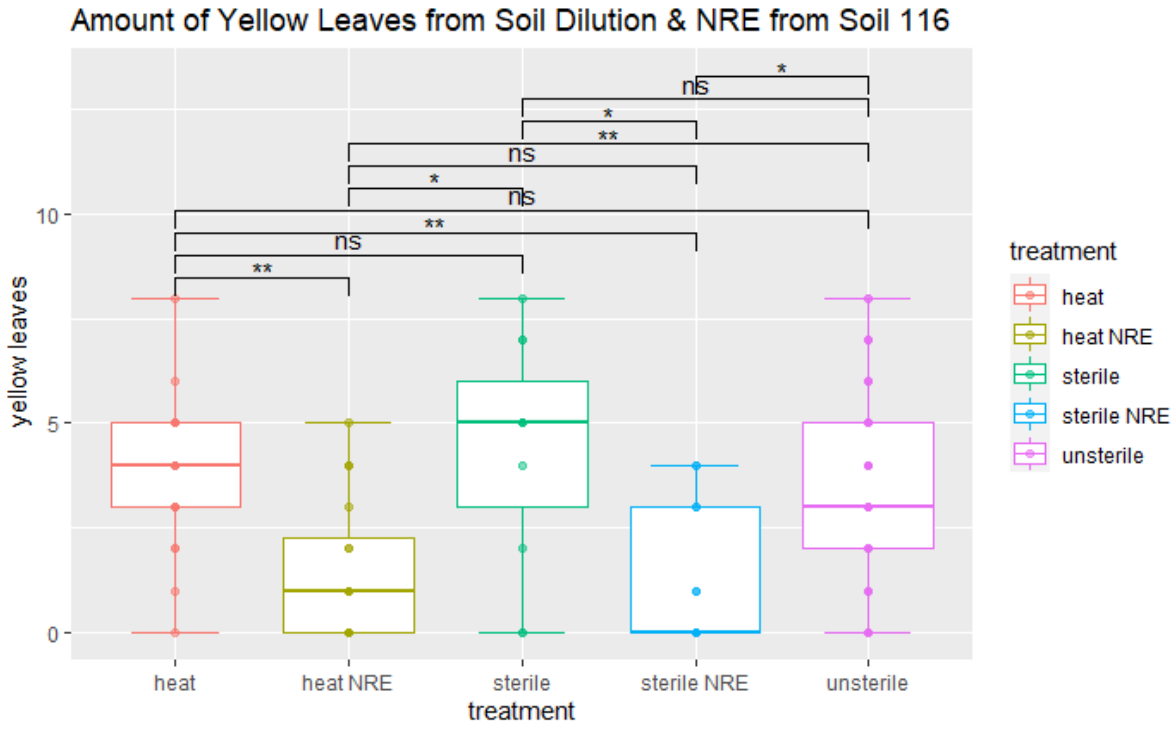

Figure 49: Number of leaves with yellow area of Col-0 plants grown in soil 116 in the nutrient rescue experiment. Statistical differences were calculated with a Welch ANOVA followed by a Games-Howell test.

3.5.4.5. Number of Siliques and Plant Height

No significant differences between the groups (see Appendix D: Soil Dilution and Nutrient Rescue Experiment).

3.5.4.6. Number of Branches

Only a few groups differed significantly from each other in the number of branches with a Welch ANOVA followed by a Games Howell test. The group with heat-treated soil extract which received nutrients was different from the group with heat-treated soil extract which did not receive nutrients and the unsterile group. Also, the heat-treated group without nutrients differed significantly from the unsterile group (see Figure 50).

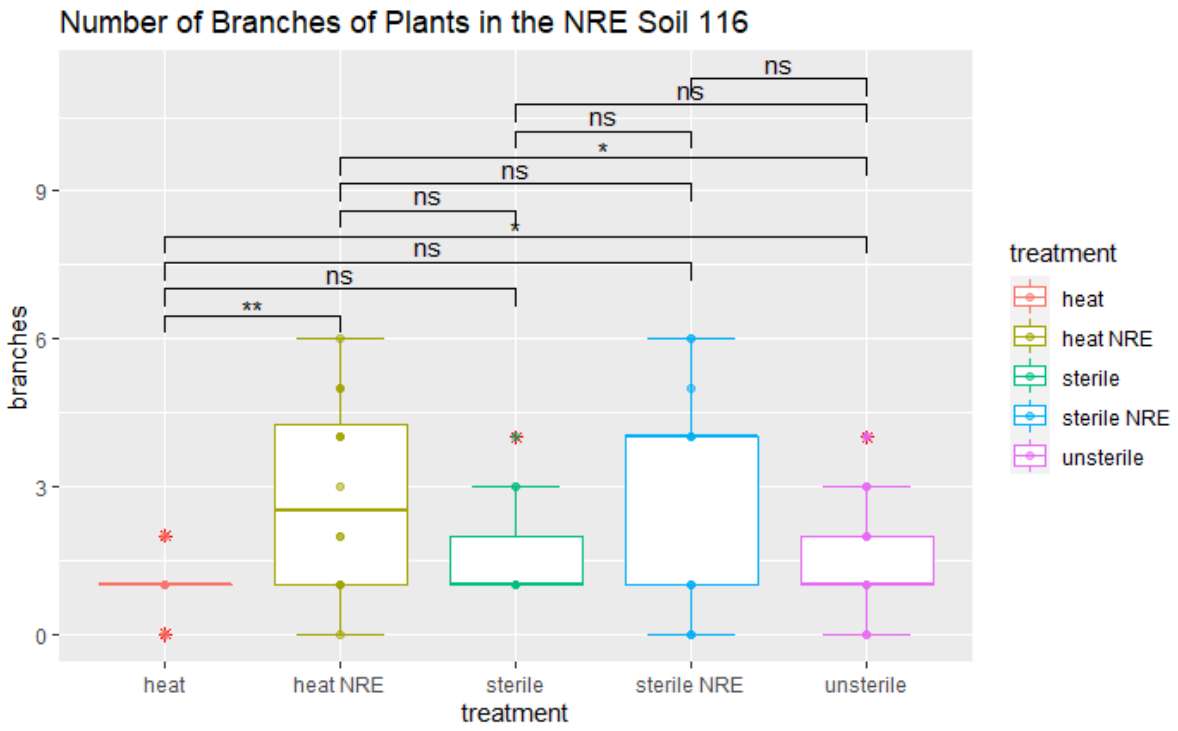

Figure 50: Number of branches of Col-0 plants grown in soil 116 in the nutrient rescue experiment. Statistical differences were calculated with a Welch ANOVA followed by a Games-Howell test

3.5.4.7. Chlorophyll Content of Plants grown in Soil 116

At the end of the nutrient rescue experiment Col-0 plants grown in soil 116 supplied with sterile soil extract or full nutrient solution were harvested. For those Col-0 plants the content of chlorophyll a and b was measured (see Appendix D: Soil Dilution and Nutrient Rescue Experiment), and the combined content calculated. A Welch two sample t-test was used to determine if the samples showed a difference in the chlorophyll content (see Figure 51). No significant difference of the two groups could be determined (p- value = 0,44).

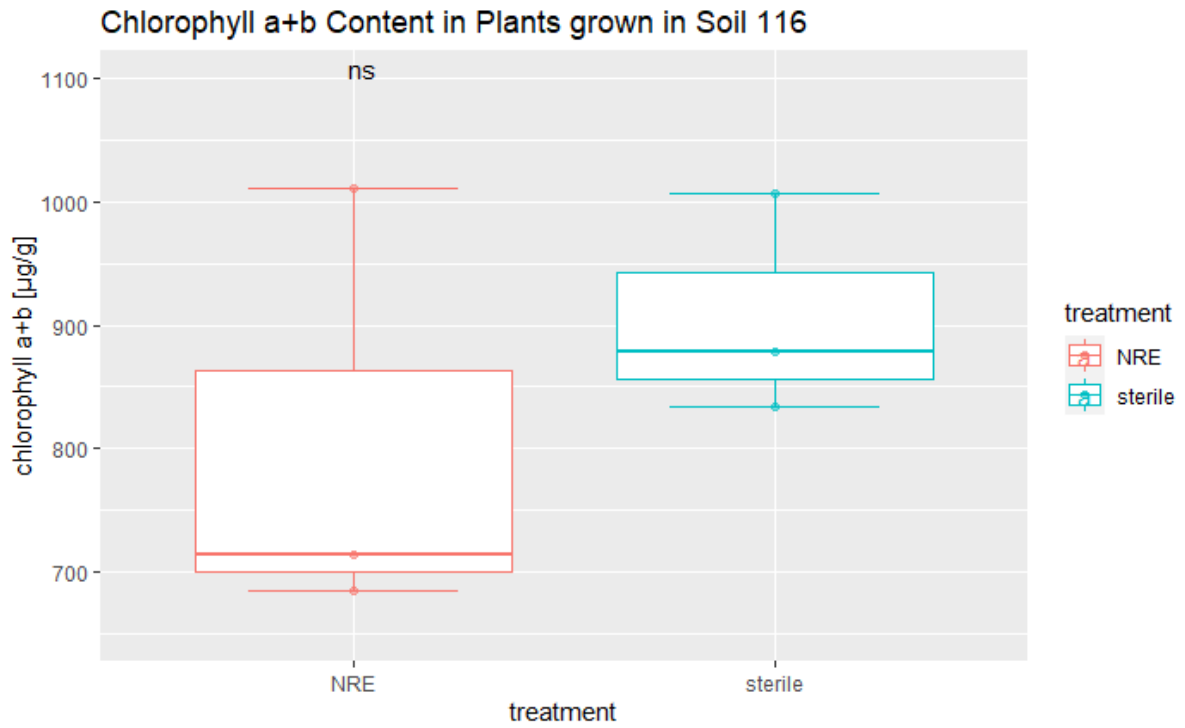

Figure 51: Content of chlorophyll a+b of Col-0 plants grown in sterile soil 116 either supplied with nutrients or not. Statistical differences were calculated with a Welch t-test

3.6. *Arabidopsis thaliana* Mutant Genotyping

3.6.1. mrs2-5 Mutant

Two plants were selected for the mrs2-5 *Arabidopsis thaliana* mutant and DNA isolation was carried out. For the PCR based genotyping two primer pairs were used. The primer pair "a" was LB+RP and the primer pair "b" was LP+RP. The negative control was negative in both reactions. The Col-0 control shows a band with primer pair "b" but not with "a". Both mutants show only a band in the reaction with primer pair "b", which suggests that the mutants carry no mutation in the mrs2-5 gene (see Figure 52).

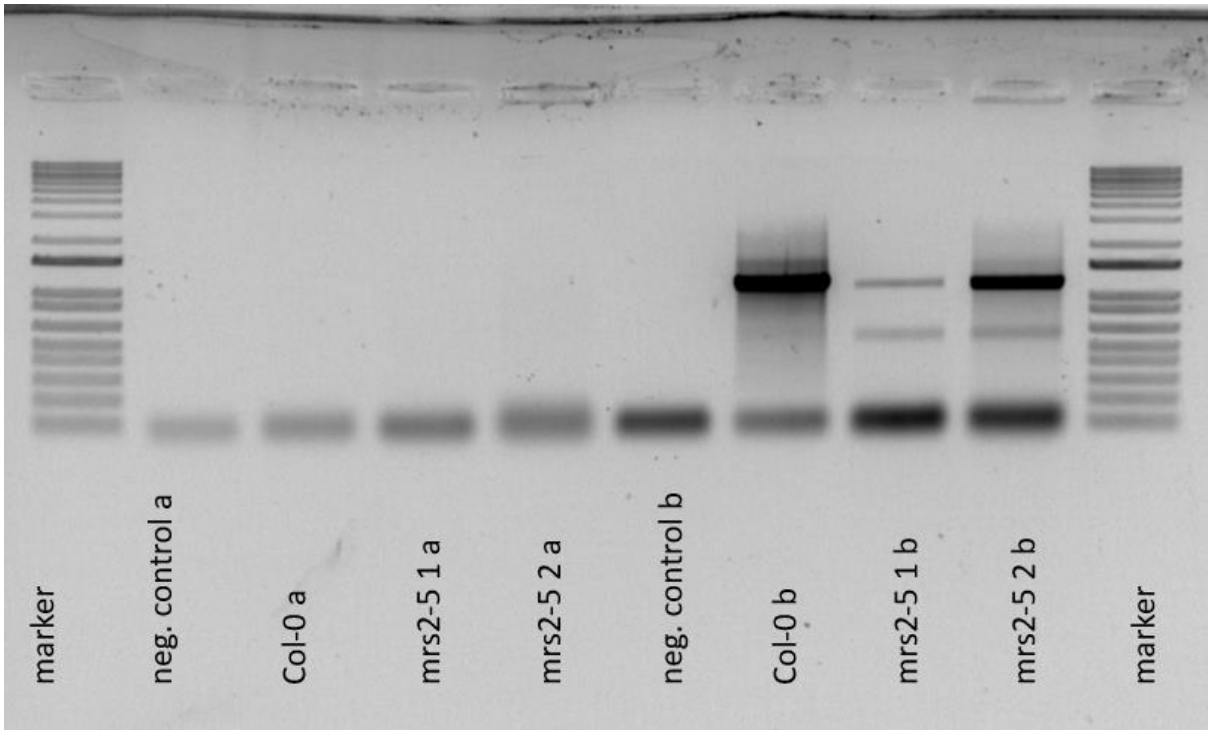

Figure 52: Electrophoresis gel after the PCR of the mrs2-5 mutant with the expected bands at ~950bp with primer pair b and no bands with primer pair a. a= LB+RP primer, b= LP+RP primer

3.6.2. mrs2-7 Mutant

Five plants for the mrs2-7 *Arabidopsis thaliana* T-DNA mutant were selected for genotyping. After successful DNA isolation the PCR with two primer pairs was carried out. The primer pair "a" was LP+RP and the primer pair "b" was LB+RP. The negative control was negative in both reactions. The Col-0 control shows a band with primer pair "a" but not with "b". For the five tested mutants it is the other way around, which leads to the conclusion that all plants carry a homozygous mutation in the mrs2-7 gene (see Figure 53). Seeds were therefore harvested for all five mutants.

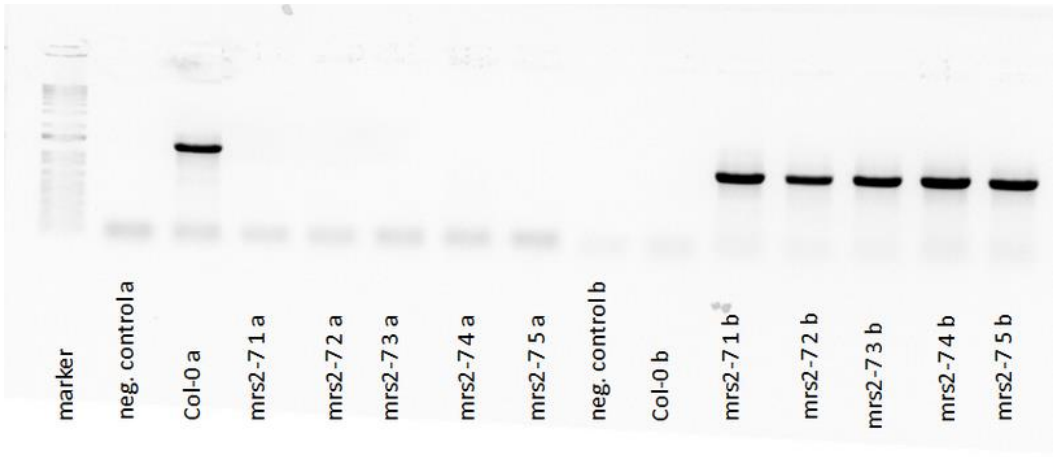

Figure 53: Electrophoresis gel after the PCR for the mrs2-7 mutant with the expected bands between 592-892bp with primer pair b. a= LP+RP primer, b= LB+RP primer

3.6.3. rbohf Mutant

In total 9 plants which supposedly have a mutation in the rbohf gene were selected for genotyping. After the DNA extraction the PCR was carried out with primer pair “a”, which contains the LB and RP primers that bind in the mutated gene, and the primer pair “b”, which contains the LP and RP primer which bind in the wild type gene. Plants 1, 3 and 6 show a band with both primer pairs, which suggests that they are heterozygous in the mutation. Plant 4 only shows a band when amplified with the primer pair “a”, which leads to the conclusion that it is homozygous in the mutation (see Figure 54, Figure 55). Seeds were harvested for plants 1, 3, 4 and 6. The remaining samples (2, 5, 7, 8, 9) carry no mutation in the rbohf gene.

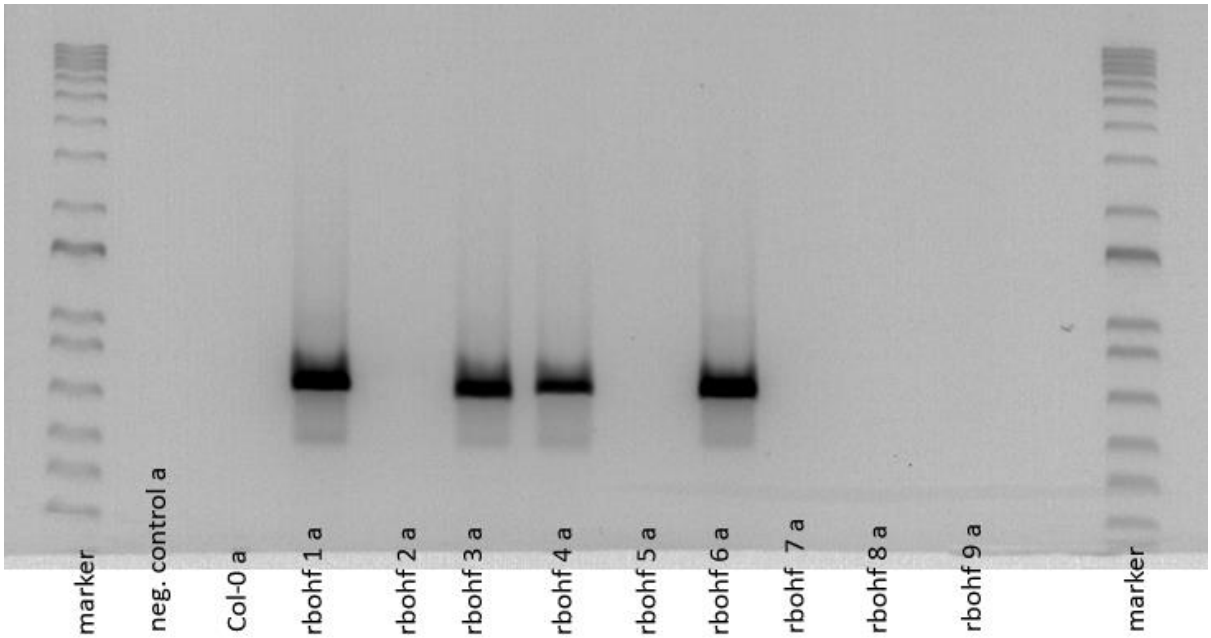

Figure 54: Electrophoresis gel after the PCR for the rbohf mutant. The gel electrophoresis shows band between 524-824bp. a= LB+RP primer

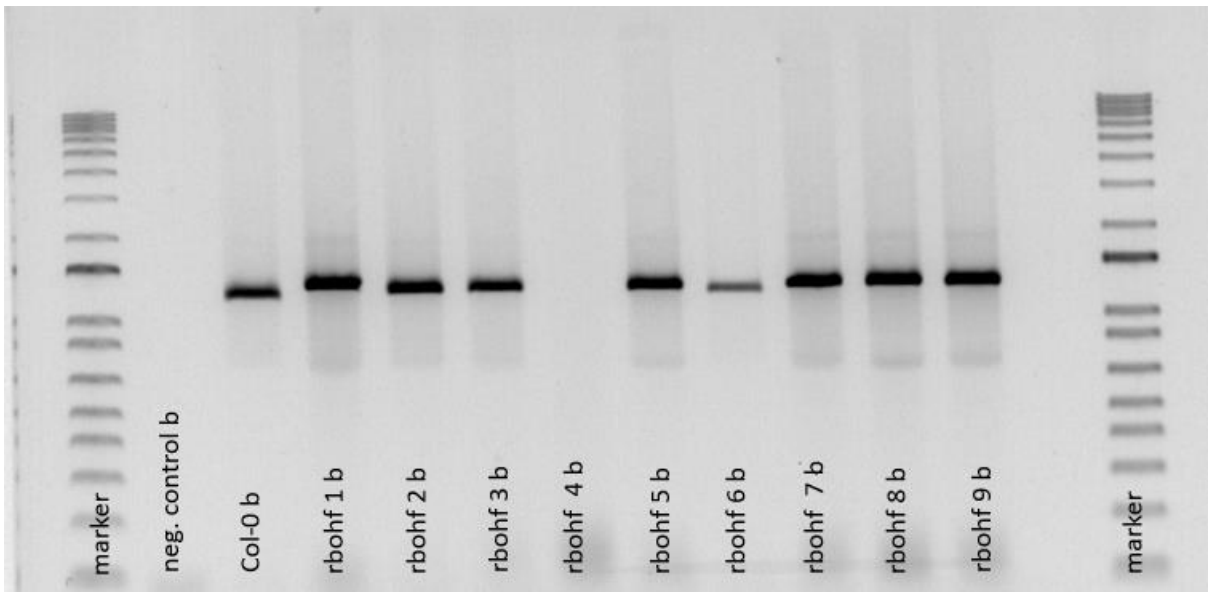

Figure 55: Electrophoresis gel after the PCR for the rbohf mutant. The bands are at around 960bp. b= LP+RP primer

3.6.4. ein2-1/pad4-1/sid2-2 Mutant

To genotype of the ein2-1/pad4-1/sid2-2 *Arabidopsis thaliana* mutant, DNA was isolated from 6 plants. For the triple mutant three different reactions had to be completed to confirm all three mutations. The ein2-1 mutation was analyzed with sequencing of the genomic region of interest. The mutants show in comparison to the Col-0 control a single base pair mutation from cytosine (C) to thymine (T) on position 213 of the analyzed region (see Figure 56).

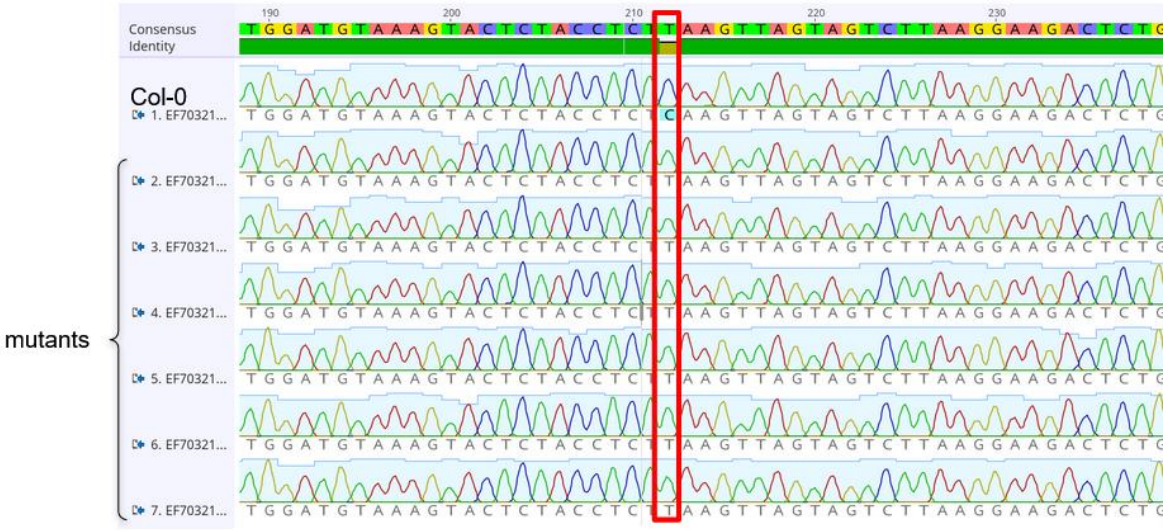

Figure 56: Sequencing result for the ein2-1/pad4-1/sid2-2 mutant to determine a mutation in the ein2-1 gene.

The pad4-1 mutation had to be confirmed by a combination of PCR and digestion with the restriction enzyme BsmF1. After the PCR with a pad4-1 specific primer pair a digestion was done with BsmF1. The digested products were then loaded on an agarose gel and made visible by a GelDoc XR+ setup. In total 6 mutants were genotyped for the mutation in the pad4-1 region. The Col-0 control shows 2 bands and has therefore been cut by the restriction enzyme. Whereas the mutants only show one band (see Figure 57).

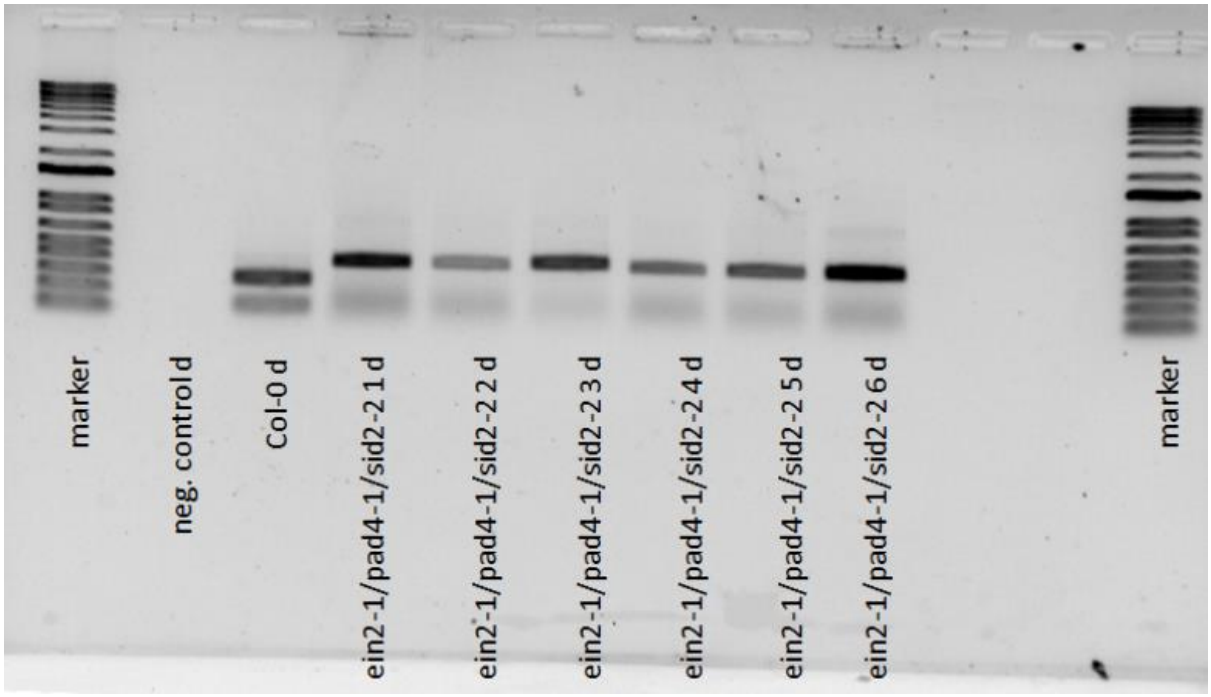

Figure 57: Electrophoresis gel after PCR for the ein2-1/pad4-1/sid2-2 mutant to determine a mutation in the pad4-1 gene after digestion with BsmF1. The Col-0 sample shows two bands as they were cut from the restriction enzyme, while the mutant samples only show one band, which was not cut.

The sid2-2 mutation is confirmed by only a PCR. Therefore, the reaction was carried out for all 6 mutants. The primer should be able to bind only in the Col-0 sample but not in the mutant DNA. The Col-0 control shows a strong band at around 900bp, which is missing in the mutants (see Figure 58). This could suggest that all six plants have indeed a mutation in the sid2-2 gene, as none of them show a band of this size.

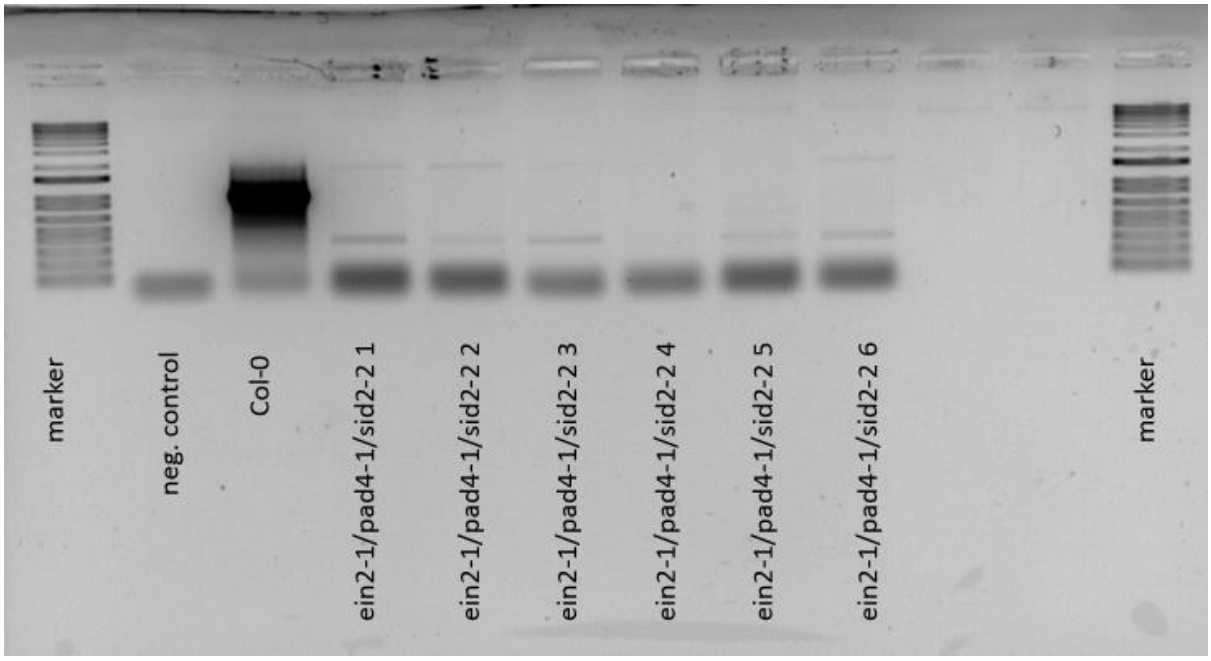

Figure 58: Electrophoresis gel after PCR for the ein2-1/pad4-1/sid2-2 mutant to determine a mutation in the sid2-2 gene. The control shows a strong band at ~900bp while no band at this size is visible for the mutants.

3.7. Identification of Endophytic Bacteria Isolated from Soil 82

While streaking out the glycerol stocks it became clear that 16 of these stocks did not contain bacteria, as they did not show growth even after several attempts. The other 48 bacteria were successfully identified by Sanger sequencing carried out by Eurofins and subsequent comparison with the NCBI database. The identified bacteria belonged to 12 different genera (*Bacillus* sp., *Variovorax* sp., *Rhodococcus* sp., *Mycolicibacterium* sp., *Tetrasphaera* sp., *Caballeronia* sp., *Paenibacillus* sp., *Cohnella* sp., *Psychrobacillus* sp., *Diaminobutyricibacter* sp. and *Cellulomonas sp.)* see Figure 59. Most of the identified bacteria (20) belonged to one of the two *Variovorax* strains. For the other species there are usually only 1 or 2 individual bacteria belonging to this strain. After comparing them with the database of the internal collection, 11 bacteria were new to the collection (see Table 20). For those a new glycerol stock was prepared.

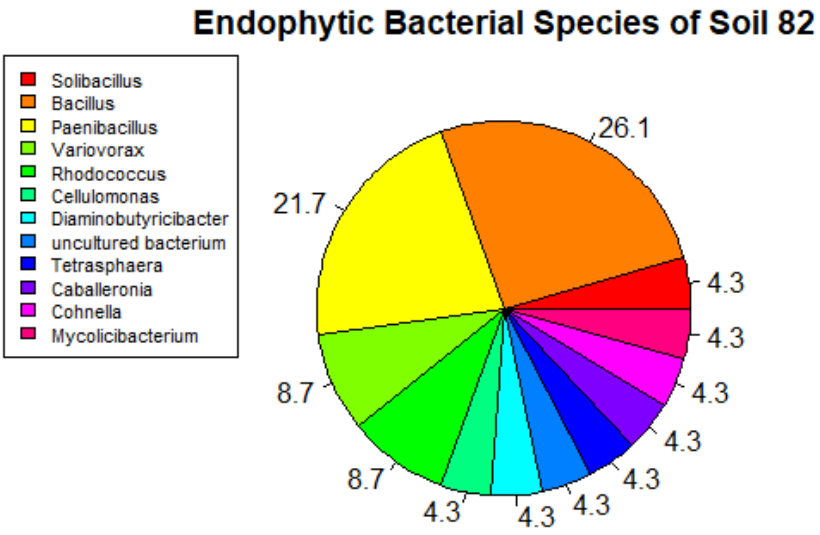

Figure 59: Different genera of endophytic bacteria of soil 82.

Table 20: Result of blasting the sequence of the endophytic bacteria from soil 82 against the NCBI database. In this table the strains new to the internal soil collection are shown. The results in the NCBI database were sorted with highest similarity first and the first four matches are shown in this table (1st to 4th match).

final bacteria ID	**original bacteria ID**	**%HQ**	**Genus**	**1st match**	**2nd match**	**3rd match**	**4th match**
E4	C8	86,4	*Bacillus* sp., *Psychrobacillus* sp.	99,88%: *Bacillus* sp. M2_6	99,65%: uncultured bacterium clone TCCC-A30-8	99,65%: *Bacillus* sp. cryopeg_4b	99,65%: *Psychrobacillus psychrodurans HBT12*
F3	H4	71,8	*Micrococcus* sp.	100%: *Micrococcus luteus* strain AB321	100%: *Micrococcus luteus* strain MA3	100%: *Micrococcus aloeverae* strain Hana49	100%: *Micrococcus aloeverae* strain Hana47

F4	C9	70,1	*Rhodococcus* sp.	99,93%: *Rhodococcus* sp. NEAU-Alolitan	99,88%: *Rhodococcus* sp. R137-12	99,81%: *Rhodococcus* sp. MA116	99,81%: *Rhodococcus canchipurensis* MBRL 353 (more with 99,81% similarity)
F7	C5	87,6	*Bacillus* sp.	99,79%: *Bacillus* sp. NHI-14T	99,79%: *Bacillus* sp. CAU 54-1-2	99,69%: *Bacillus* sp. NH.2	99,69%: *Peribacillus loiseleuriae* HBG29
F8	F7	43,7	*Paenibacillus* sp.	98,51%: *Paenibacillus glycanilyticus* AAR-220	98,51%: *Paenibacillus endophyticus* BMCH-IB-ONF 7	98,37%: *Paenibacillus glycanilyticus* NRCB008	98,37%: *Paenibacillus catalpae* BT428 More with 98,37%
G1	F8	81,9	*Cohnella* sp.	99,43%: *Cohnella luojiensis* HY-22R	99,32%: *Cohnella* sp. HS21	99,32%: *Cohnella* sp. HS21	99,32%: *Cohnella abietis* HS21 More with 99,32%
G2	F9	80,0	*Paenibacillus* sp.	99,76%: uncultured bacteria GJ16S2_G11	99,39%: *Paenibacillus* sp. ARSS51-1	99,02%: *Paenibacillus alginolyticus* BJC15-C16	98,66%: *Paenibacillus* sp. PhyCEm-108
G3	H9	76,3	*Cellulomonas* sp.	100%: *Cellulomonas aerilata* ZSGR31	100%: *Cellulomonas* sp. MDT2-38	99,88%: *Cellulomonas* sp. CC5R	99,88%: uncultured bacterium PEKCLN032 Many more with 99,88%
G4	H7.1	52,5	*Paenibacillus* sp.	99,09%: *Paenibacillus* sp. R20-25	99,09%: *Paenibacillaceae bacterium* MC1-Q	98,57%: *Paenibacillus* sp. BC050	97,27%: *Paenibacillus* sp. BJC16-D12 One more with 97,27%

G6	C9	83, 4	*Bacillus* sp.	100%: *Bacillus thuringiensis* strain S38	100%: *Bacillus cereus* strain NBUAS66958	100%: *Bacillus cereus* strain SA275C1	100%: *Bacillus thuringiensis* strain KF1
G9	C2	71, 9	*Bacillus* sp.	100%: *Bacillus mycoides* 2861	100%: *Bacillus* sp. 206	100%: *Bacillus* sp. L24	100%: *Bacillus* sp. UFSC-20S3 Many many more with 100%

3.8. Mono-association Assays

Mono-association experiments were carried out for nine out of the eleven bacteria. The two missing ones (final bacteria ID: G1 and G2) did not show any growth in the liquid cultures, even in different growing conditions. The effect of the other bacteria on *Arabidopsis thaliana* Col-0 was analyzed in conditions with and without 100 mM NaCl. Pictures were taken 5 and 10 days after the start of the experiment. Because the roots of many plants reached the bottom of the plate after 10 days, which makes evaluation of the root length impossible, the length after 5 days was compared. After 10 days the plants were weighed individually and if the size or root architecture was obviously different to the controls, the rosette was weighed individually, and rosette and root are stored at -80°C.

3.8.1. Mono-association Assay without NaCl

The comparison of the total plant weight showed that three bacteria showed a significant effect (see Figure 60). Both the bacteria F4 and F7 showed a reduced plant weight, while F3 did increase the plants weight compared to the neg. control. For the bacteria F4, F8 and G3 also the rosette and root weight were evaluated individually. But none of the bacteria showed an increased weight of root or rosette compared to the neg. control (see Appendix E: Mono-association Experiment).

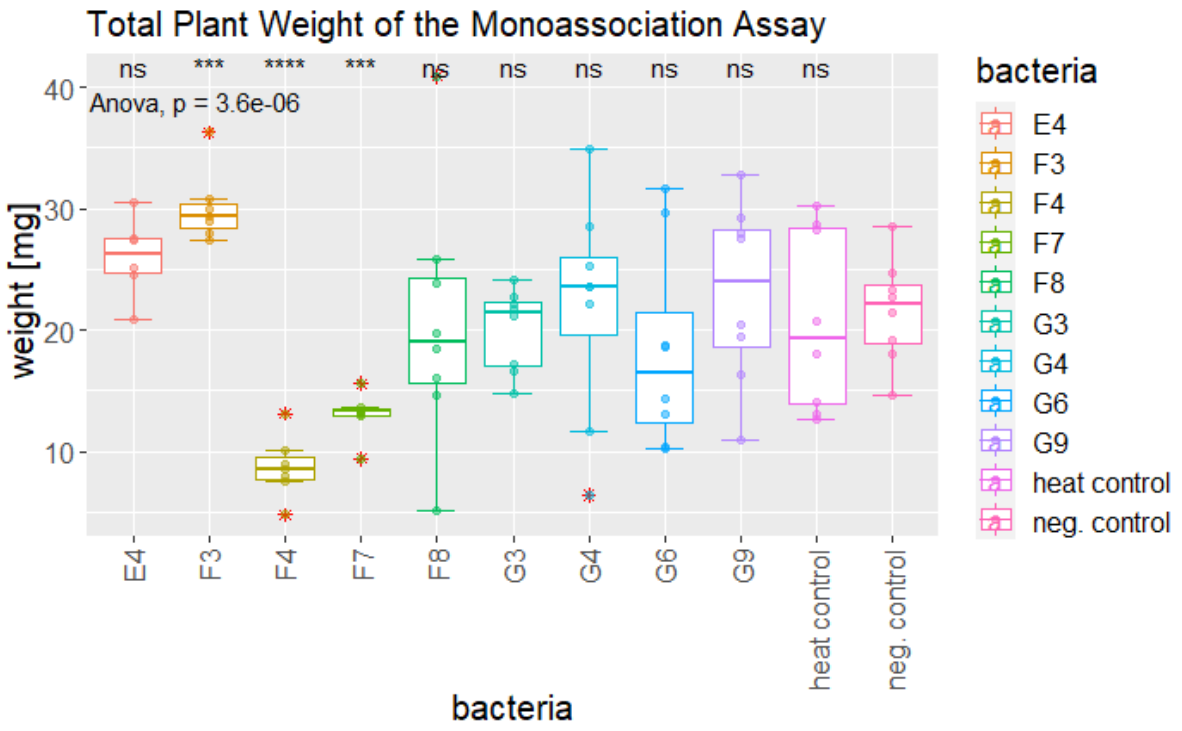

Figure 60: Total plant weight of Col-0 plants in the mono-association assay. Pairwise comparison against the neg. control were done with a t-test.

The root length was evaluated after 5 days. Only bacteria F7 showed a significant difference to the neg. control, but it reduced it (see Figure 61).

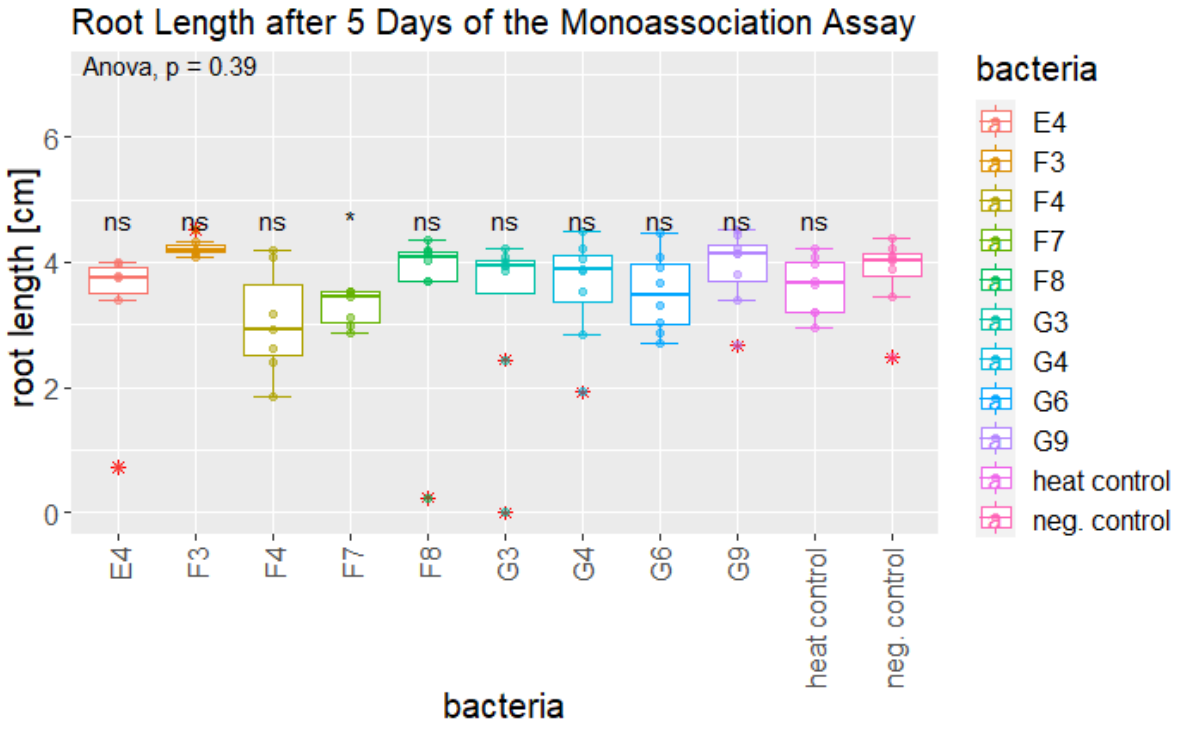

Figure 61: Root length after 5 days of Col-0 plants in the mono-association assay. Pairwise comparison against the neg. control were done with a t-test.

3.8.2. Mono-association Assay with 100 mM NaCl

The comparison of the total plant weight of the plants in the mono-association assay with 100 mM NaCl showed that three bacteria significantly changed the plant weight compared to the neg. control. The bacteria F7 and F8 did reduce the weight, while bacteria F3 did increase it (see Figure 62).

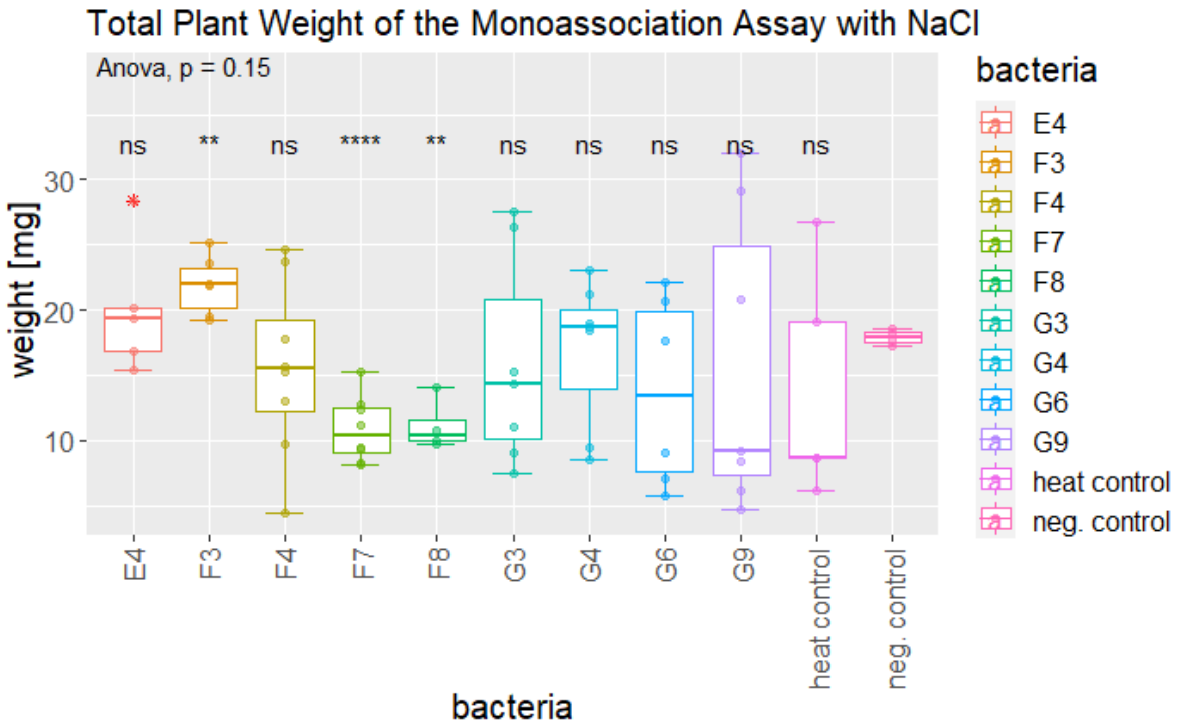

Figure 62: Total plant weight of Col-0 plants in the mono-association assay with NaCl. Pairwise comparison against the neg. control were done with a t-test.

Two bacteria resulted in an increased root length after 5 days in presence of NaCl. Those bacteria are F3 and G3 (see Figure 63).

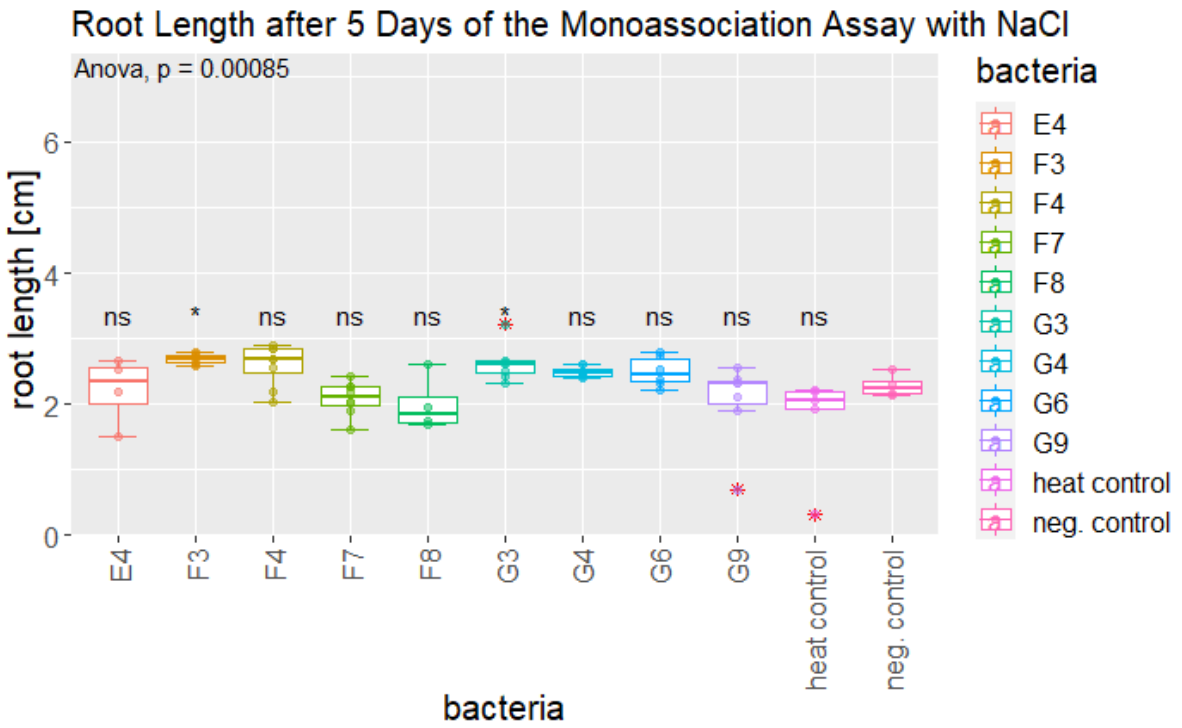

Figure 63: Root length after 5 days of Col-0 plants in the mono-association assay with NaCl. Pairwise comparison against the neg. control were done with a t-test.

The bacteria F3 did not only increase the plant weight in both conditions (with and without NaCl) but it also increased the root length in the presence of NaCl compared to the neg. control. The other bacteria do not have a positive effect on plant growth under the tested conditions.

4. Discussion

4.1. Phenotypic Screening of 12 Danish Soils

On average soil has a pH ranging from 4-10, but ideal pH for plants lies between 6 and 7 (Towhid Osman, 2013), which resembles the pH of the 12 tested soil samples. This is important as the soil pH not only affects the nutrient availability but also the microbiome (Towhid Osman, 2013).

The soil sterility test showed no growth of sterile soil in four out of the five tested samples. For soil 117 on one plate containing TSA media microbial growth was visible. The visible colonies looked phenotypical different to the ones of the unsterile samples as well as the number of CFU was only a fraction on what was seen in unsterile samples. It seems likely that a contamination was introduced when taken the samples for the sterility test, as this was done in non-sterile greenhouse conditions while preparing the pots for the initial screening.

During the screening four soils were selected which showed a plant phenotype that seemed less stressed when the natural microbiome of this sample was present. Those four soils were collected in very different parts of Denmark. Only the soils 116 and 117 were collected close to each other. Both locations are in a forest in Lolland. This proximity is also resembled by a very similar phenotype. The Col-0 plants grown in sterile conditions of both soils developed yellow leave edges, while the Col-0 plants in unsterile conditions did not (soil 117) or not that much (soil 116) develop this symptom. Even though a difference of the yellow area of the leaves was seen by eye and the Aradeepopsis software was not able to detect a significant difference. Interestingly the data showed that Col-0 plants grown in unsterile conditions of soil 116 had a higher senescent area compared to sterile conditions. This can be explained by the way how the software analyzed the pictures. It should detect the plant area on its own but sometimes it also mistook the pot or the surrounding soil for parts of the plants and often for senescent or anthocyanin area. This problem occurred for all soils and depending on the color of soil and plant and plant size it was more imprecise. In the future this could be prevented by using a homogenous background instead of the soil. Even though those problem occurred the software still often resembled what could be seen by eye and the mistakes introduced are only minor for the days chosen.

The element content was not measured for soil 117 as the Col-0 plant phenotype and sampling location are very similar to soil 116. The results for soil 116 showed that five nutrients occurred less in Col-0 plants grown in unsterile conditions. Those nutrients are Cd, Cu, Mn, Mo and P. Cadmium is a heavy metal often occurring in agricultural soils as it is applied with phosphate fertilizers and does probably not play an essential role for plant survival (Herbette et al., 2006). In high amounts Cd can have a negative impact on plant growth and leads to symptoms like chlorosis and growth reduction (Herbette et al., 2006). Therefore, an increased uptake when plants are grown in unsterile soil 116 might not be desirable. In contrast an increased Cu content might be a favorable trait as it is an essential element and it is often not plant available (Garcia-Molina et al., 2020). But it was shown that insufficient Cu supply of *Arabidopsis thaliana* did not lead to a visibly changed phenotype even though the molecular response patterns are changed (Garcia-Molina et al., 2020). The bioavailability of manganese depends on the soil type, e.g. soils with a lot of organic matter reduced bioavailability (Rodríguez-Celma et al., 2016). Soil 116 was classified by the Biowide project as an organic soil (Brunbjerg et al., 2019). An increased amount of Mn in plants grown in unsterile soil 116 might be a trait to look further into. Symptoms of Mn deficiency are inhibited growth and chlorosis on leaves (Rodríguez-Celma et al., 2016). Molybdenum (Mo) is essential for plants growth but it can also be toxic if the levels are too high, more often a deficiency occurs (Gupta, 2009; Ide et al., 2011). If plants are not sufficiently supplied with Mo the phenotypic symptoms often occur on the younger leaves, which could be chlorosis (Gupta, 2009). The symptoms also affect the leaf structure and leads to scorching,

curling, rolling and reduced growth (Gupta, 2009; Ide et al., 2011). A P deficiency leads to purple coloration of leaves (de Bang et al., 2021). In soil 116 not a single element which is increased in unsterile conditions could clearly explain the phenotypic difference. None of the typical deficiency symptoms of those highlighted resemble the plants grown in sterile conditions of soil 116. Therefore it might be the combination of nutritional status leading to the chlorotic leaves or it is not primarily caused by nutritional deficiencies.

Another interesting soil was soil 71. Col-0 plants grown in sterile conditions turned completely purple/red while the Col-0 plants grown in unsterile conditions stayed greener. Even though this difference is very clearly seen by eye (see Figure 8) the Aradeepopsis software was not at all able to detect the area due to high levels of anthocyanins. Also the element content detected by ICP-MS showed interesting results as many important plant nutrients showed a higher content in Col-0 plants grown in unsterile conditions. Those elements are B, Ca, K, Mg, P and S. Boron is often applied as a fertilizer, but as it quickly can shift from supplying the plant with enough boron to being toxic for the plant, dosage can be difficult (Duran et al., 2018). Finding beneficial microbes which supply the plant with enough boron but not too much to become toxic could be a good replacement for synthetic fertilizers that are difficult to dose. When *Arabidopsis thaliana* is grown in a boron deficient environment it develops several symptoms. Many of them concern the root (Duran et al., 2018), which was not analyzed in the initial screening. But boron deficiency also leads to a decreased leaf size (Duran et al., 2018). A study on *Brassica napus* also showed that the plant boron content affects the hormonal system (Eggert & von Wirén, 2017). A boron concentration which exceeds the necessary amount in *Arabidopsis thaliana* leads to chlorotic leaves and inhibited root growth (Duran et al., 2018). A typical symptom of magnesium deficiency are chlorotic leaves (Hermans et al., 2010). Plants with potassium deficiency develop chlorosis followed by marginal necrosis (de Bang et al., 2021). Both Mg and K were increased in plants grown in unsterile soil 71, but for plants grown in soil 71 neither chlorosis nor marginal necrosis were a typical symptom. The key phenotypical symptom of Col-0 plants grown in sterile soil 71 was a very purple rosette color. This anthocyanosis is a typical result of poor phosphorus supply (de Bang et al., 2021). But also other nutrient deficiencies lead to increased anthocyanin content in the leaves, e.g. nitrogen and sulphur starvation (de Bang et al., 2021). Nitrogen was not measured in the ICP-MS analysis, but sulphur was and showed a significant higher amount in plants grown in unsterile soil 71 compared to sterile soil 71. But other symptoms of S-deficiency are chlorotic leaves (de Bang et al., 2021), which was not seen in the initial screening. Therefore the phenotype in combination with the results of the ICP-MS analysis most likely show that the phenotypic differences are caused by a low P-supply of Col-0 plants grown in sterile soil 71.

Col-0 plants grown in soil 109 had a phenotype that seemed more natural in unsterile conditions. The Col-0 plants grown in sterile soil 109 showed a very stunted growth, small leaves, short petioles and a high number of leaves. While Col-0 plants grown in unsterile 109 resembled more the natural phenotype of *Arabidopsis thaliana*. Even though this phenotype seems more likely caused by a hormonal change, the ICP-MS also showed significant differences of the nutrients content. Col-0 plants grown in sterile soil 109 had a higher content of Cu, Mn, Mo and P, while Col-0 plants grown in unsterile soil 109 had a higher content in Ca, Cd and Mg. During the ICP-MS analysis the N content could not be measured. Plants with low N content often show stunted growth as well as reduced leave expansion (de Bang et al., 2021). The color of the leaves (chlorosis, anthocyanins) was not affected in soil 109. The effect that Ca has on cell wall and membranes makes it an important element in terms of plant growth (de Bang et al., 2021). None of the symptoms of a deficiency of one of the other significantly different elements match the phenotype of Col-0 plants grown in sterile soil 109. Therefore, this unique phenotype might not be explained by plant nutrition.

In the next step also the nutritional status of the sterile and unsterile soils should be evaluated to gain further information about which nutrients are involved in the phenotype. Furthermore, we aimed to exclude the possibility that the sterilization had a significant impact on the nutrients in the soil samples and are therefore influencing the plant nutritional status.

4.2. Transplantation Experiment of Soil 117

Due to negative circumstances the results of the transplantation experiment could not show their full potential. The Col-0 plants had to be harvested earlier due to a mite contamination. The pest attack also resulted in yellow leaves which highly resembled the phenotype of Col-0 plants grown in sterile soil 117 during the initial screening. This made it difficult to fully determine which symptom was derived by the soil and which by the insects. Also, a pesticide treatment to remove the mites was not taken in consideration as the chemical might also influence the microbes in the soil. The source of the mites is not known, as they were not seen during the initial screening. Two potential sources exist, one is that the Col-0 plants were infected either by mites in the climate chamber or by the people in contact with soil 117, or it naturally occurs in soil 117 and the mites will appear also in future experiments.

The root sterilization treatment heavily affected the Col-0 plant phenotype of the endo treatment. But in general transplantation processes face other difficulties as well like taking a lot of time or the potential to damage the plants (Hachiya et al., 2021). The Col-0 plants in the endo treatment grew slower and developed a very purple phenotype right after the sterilization. The purple phenotype slowly disappeared again but as the Col-0 plants were harvested early, they could not recover. This was not expected as Maja Schmid Pedersen previously tested different sterilization treatments (unpubl.). Her results showed that the treatment with 0,25% sodium hypochlorite had the best sterilization result while not harming the plant. The effect of the root sterilization seen in the soil 117 transplantation experiment could suggest that the treatment must be adapted to every soil. Different techniques should be tested previously to a transplantation experiment.

Even though the circumstances to draw conclusions from the experiment were not easy, it still gave an indication on where potential beneficial microbes in soil 117 are located. The comparison of the rosette fresh weight showed that when the microbes in the endosphere and the rhizosphere are present the Col-0 plants resemble the positive control. The group containing only endophytes could not be trustfully compared. The total area detected by the Aradeepopsis software did not show a significant difference between the endo+rhizo, pos. control and neg. control. Compared to Figure 21, it seems that the leaves of the neg. control are smaller compared to the endo+rhizo and pos. control. This could be the reason for the difference in the result of comparing fresh weight and plant area.

As seen in the initial screening the major difference of Col-0 plants grown in sterile and unsterile soil 117 is the development of yellow leaf edges when grown without microbes. This was also resembled in the number of leaves with yellow edges during the transplantation experiment, even though no difference in the senescent area derived from the Aradeepopsis data could be seen. This could be caused by the software not fully recognizing yellow plant area, especially when it is not that clearly separated from the green plant area.

Overall, it seems that the bulk soil microbes do not contribute to the phenotype in the unsterile soil. If it is only the endophyte or in combination with the rhizosphere could not be surely said due to the problems that occurred during the transplantation experiment.

4.3. Soil Extracts of Soils 71, 109 and 116 and 117

After 44 days of growing in presence of soil extracts the different groups did not show a difference in any of the tested soils. When comparing the plant size no group containing the unsterile soil extract differed from the two controls. The only case where a slight difference was detected was that in soil 109 the group that received the sterile soil extract had a smaller rosette area. This could suggest that the sterilization technique affects the soil quality.

The dilution of soil is an effective way to reduce the amount of microbes in soil (Lachaise et al., 2017).

There are many reasons why sterile soil treated with unsterile soil extract does not lead to the same phenotype as was seen in the initial screening. One might be that either the microbes would need more time to replicate and be present in a number large enough to fulfill their effect on the plant. Another reason could be that non beneficial microbes replicate faster than the beneficial microbes and therefore replace them. But it could also be that the beneficial effect, that was seen in the initial screening, was not derived by microbes at all.

4.3.1. Combination of Soil Dilution Experiment with Transplantation Experiment in Soil 117

For the soil 117 the soil dilution experiment was combined with the transplantation experiment. Following the same principle as in the previously described transplantation experiment, the goal was to highly reduce the microbial candidates for potential SynCom building. As the combination of these two experiments was new, it was set up as a small pilot experiment, with a limited number of replicates. This set up was also driven by the fact that the supply of soil 117 was low. For each treatment five *Arabidopsis thaliana* plants were used. A difference to the transplantation experiment was that the transplantation procedure was already carried out after 14 days.

The results showed again that the endo group was highly affected by the root sterilization procedure. This confirmed the fact that the sterilization technique, which was found optimal for soil 82, does not properly work for Col-0 plants grown in soil 117.

The mites, which were seen in the transplantation experiment, attacked the plants grown in soil 117 again. This proved the assumption that the insects are naturally occurring in the sampled soil 117 to be true. This was the main reason to not continue any work with soil 117.

4.4. Nutrient Rescue Experiment of Soils 71, 109 and 116

As the amount of each soil is limited, the goal was to still gain as much information of each experiment as possible. A new experiment was started with the 6-week-old Col-0 plants from the soil dilution experiment. Therefore, the sterile and heat group were split in half. One of them was then treated with a full nutrient solution and the other not. Supplying the Col-0 plants now with nutrients gives information if the phenotype seen in the initial screening was based on a better nutrient supply of Col-0 plants grown in the unsterile soils. If the phenotype recovers it might also give hints on if the nutrient involved is mobile in the Col-0 plant or not, as maybe only the newly developed leaves are symptom free or also the older leaves recover. At the end of the experiment not only the ImageJ software was used but also the total leaves, leaves with yellow edges, stem height, branch number and silique number were counted.

4.4.1. Soil 71 in the Nutrient Rescue Experiment

For soil 71 the major phenotypic difference, which was seen in the initial soil screening, was the purple color of the Col-0 plants in sterile soil. Therefore, the anthocyanin content of the Col-0 plants in the NRE was determined. Three Col-0 plants without nutrients and three Col-0 plants which received nutrients were compared. The results show significantly more anthocyanin in Col-0 plants which did not receive the nutrient solution.

For the other phenotypic traits, the most significant was the number of branches. Both groups which did receive a nutrient solution had more branches than the other groups.

When comparing the plant height the groups which received nutrients were higher than the sterile group. Even though plant height and branch number differed from groups without nutrients, the number of siliques showed only two groups which were significantly different from each other, which is that the heat-treated group that received nutrients had more siliques than the unsterile group.

In the total rosette area, which was measured with ImageJ, a significant difference could be detected when comparing the sterile group with and without nutrients.

Overall the results show that the anthocyanin content in Col-0 plants with enough available nutrients is way less. Other phenotypic traits did not show that clear difference of Col-0 plants in the different groups.

4.4.2. Soil 109 in the Nutrient Rescue Experiment

The phenotype of soil 109 seen in the initial screening were Col-0 plants with stunted growth in the sterile conditions. This was also seen during this experiment.

None of the Col-0 plants could yet develop a stem, therefore only the data derived from ImageJ could be used as well as the total leaf number. Therefore only two traits could be compared for soil 109, which are rosette area and total leaf number. In the rosette area the group treated with sterile soil extract without nutrients differed from the other groups. Already in the soil dilution this group was smaller compared to the rest. But it still seemed that the newly developed leaves of Col-0 plants that received nutrients are bigger compared to Col-0 plants without nutrient supplementation. Also, the older leaves did not increase in size after the plants received nutrients.

The total leaf number showed no trend in the different groups. Still the sterile group had less leaves than unsterile and heat-treated with nutrients. Also, the heat-treated group with nutrients still had more leaves than the unsterile group.

4.4.3. Soil 116 in the Nutrient Rescue Experiment

In soil 116 the plant growth did increase after receiving nutrients. The Col-0 plants in the groups which were supplied with a full nutrient solution had bigger rosettes and had more leaves compared to their sterile and heat-treated controls. The characteristic phenotypic trait of Col-0 plants grown in sterile soil 116 were yellow leaf edges. Even though no difference in the yellow area of the rosette could be detected, the number of leaves which had those yellow edges was significantly less in the groups which received the nutrients.

4.4.4. Overall Conclusions of the Nutrient Rescue Experiment

In all soils there are phenotypic traits that improved after receiving a full nutrients solution. This shows that in all soil a nutrient deficiency is implemented in the Col-0 plants growing.

What was generally seen in different soils for different traits is that the heat-treated group acted better than the sterile group, suggesting that the sterilization technique had a significant impact on the soil quality. Maybe the heat made nutrients better plant available and were introduced into the sterile soil by the soil extract.

The results of the NRE suggest that the phenotypic differences in the initial screening are very likely caused by a nutrient deficiency of the Col-0 plants.

4.5. Genotyping *Arabidopsis thaliana* Mutants

Four different *Arabidopsis thaliana* mutants were genotyped in this project. The mutations have been confirmed for three of them. Those mutants should in the future be used to further understand the beneficial effect gained by the microbes found in the Plant-Microbe-Microbe-Interaction project at Dynamo. In the future the homozygous mutants will be grown in sterile soil with and without the beneficial SynCom and the growth will be compared. If they show different reactions, it might suggest that the pathway the mutation is in, is relevant for the beneficial effect of the microbes. This knowledge can then be used to further understand the molecular mechanism of the microbe-plant interaction.

From the four mutants analyzed one, the mrs2-5 mutant, seemed to have no mutation in the relevant gene, therefore it cannot be used for further investigations. All plants analyzed for the mutation in the mrs2-7 gene were found to be homozygous and seeds from all plants were harvested. Those two mutants could be used to analyze the influence of the microbes from the soil samples on the Mg-transport inside the plant (Gebert et al., 2009; Kamiya et al., 2012), which is especially relevant for soil 82, as plants growing in sterile soil 82 have less Mg in their shoots.

For the rbohf mutant 3 heterozygous and 1 homozygous plants were found and seeds from them were harvested. As the mutation is in a gene relevant for plant defense, because rbohf encodes a respiratory burst oxidase protein (Pogány et al., 2009), it can be used to analyze how the microbes can influence the plant defense in *Arabidopsis thaliana*.

The triple mutant ein2-1/sid2-2/pad4-1 was homozygous for all three genes and seeds from all tested plants were harvested. This mutant is deprived in its defense mechanisms (Vandenbussche et al., 2007; Wildermuth et al., 2001) and can be used to gain information how the microbes affect the plants response to microbes, when lacking certain abilities in its defense.

4.6. Effects of Endophytic Bacteria on *Arabidopsis thaliana* Col-0 in Mono-association Assays

The bacteria E4 (*Bacillus* sp., *Psychrobacillus* sp.; strain cryopeg_4b) was identified and the 16S rRNA published by a study of bacteria isolated from permafrost in Siberia, Russia (Bakermans et al., 2003). This published strain has a sequence similarity of 99,65% with the bacteria E4. Only the *Bacillus* sp. strain M2_6 had a higher sequence similarity (99,88%), but there is no published study yet. Also for the other strains with a sequence similarity of 99,65% (TCCC-A30-8; HBT12), no publication was found online. Even though *Bacillus* sp. strains are often associated with plant growth promotion (Akinrinlola et al., 2018), no effect on the Col-0 plants were visible in this mono-association assay.

The bacteria F3 belongs to the *Bacillus* sp. genus. Several *Bacillus* sp. strains are known to have a plant growth promoting effect (Akinrinlola et al., 2018). Also one of the most similar hits of the NCBI database was connected to be plant growth promoting (Sherpa et al., 2021). But others were also closely connected to insects (Choubdar et al., 2021; Gunathilaka et al., 2020). In this project the bacteria F3 was the only one which had a positive significant impact on Col-0 plant growth as weight and root length were enhanced compared to the negative control in saline conditions and plant growth in non-saline conditions.

For the strain F4 (*Rhodococcus* sp., strain NEAU-Alolitan) the highest sequence similarity (99,93%) was reached with a strain isolated from soil, which is still unpublished. The strain with the second highest similarity (99,88%) R138-12 was isolated from the endosphere of tuberous roots of a sweet potato plant, but its effect on the plant was not examined (Marques et al., 2015).

The strain F7 belongs to the *Bacillus* sp. family, with the highest sequence similarity to strains NHI-14T and CAU 54-1-2 (both 99,79%). For the strain NHI-14T no further information was found. The strain CAU-54-1-2 was isolated from a river sediment of the Yong-San River and the sequence submitted to NCBI by Park M.-H. and Kim W.Y., but also no experiments or background of the study was published.

The strain F8 has the highest sequence similarities (98,51%) with two different *Paenibacillus* species. The first one is *Paenibacillus glycanilyticus* strain AAR-220, which was isolated from an Indian paddy soil by Rani, V. et. al, but not yet published in a study. The second is *Paenibacillus endophyticus* strain BMC-IB-ONF 7, which was entered in the NCBI database as part of a project called "Bioseptilon and biocompost 21- products of household biotechnology" by Rafikova, G. et. al. But also for this strain no published paper was found.

Even with adaptations of the liquid culture incubation settings, the bacteria G1 did not result in a cloudy liquid culture, and therefore no OD_{600} was measurable. The highest sequence similarity (99,43%) had the bacterium G1 with a *Cohnella luojiensis* strain HY-22R. This strain was isolated from soil of a Euphrates poplar forest. When first described by Cai et al. (2010), the growth conditions were stated similar to the ones used during this experiment, (TSA, 10-37°C) and they could isolate the bacterium after 2 days. If the strain should be continued as part of a SynCom, the optimal growth conditions must be evaluated more thoroughly, to ensure growth of the bacteria in a liquid culture. The strain HY-22R had high sequence similarity (93,7-96,3%) with other tested *Cohnella* strains, but was most closely related to *Cohnella phaseoli* GSPC1 (Cai et al., 2010). Also the strain with the second highest sequence similarity (99,32%) HS21 was published. It has been isolated from the rhizosphere of the Korean fir (*Abies koreana*) (L. Jiang et al., 2019). As comparable with the strain G1, the strain HS21 has the highest sequence similarity (97,9%) with the *Cohnella* strain HY-22R (L. Jiang et al., 2019). Also this strain could be grown with TSB and at 25°C (L. Jiang et al., 2019), which was not possible in this experiment. The genus *Cohnella* was already found in environmentally diverse habitats and is part of the *Paenibacillaceae* family (L. Jiang et al., 2019).

It was also not possible to achieve a growth in a liquid culture of the bacterium G2. This bacterium had the highest sequence similarity (99,76%) with the bacterial strain GJ16S2_G11, which was not cultured but identified of a sample from a lava-formed Gotjawal forest in Jeju, Korea. The study used pyrosequencing as an analytical method to identify the bacterial diversity of soil of this area (J. S. Kim et al., 2015). The strain with the second highest sequence similarity (99,39%) belongs to the genus *Paenibacillus* sp. and was isolated in Vietnam and is an agarolytic bacteria.

The strain G3 has a 100% sequence similarity with two different *Cellulomonas* sp. strains. The first one is *Cellulomonas aerilata* ZSGR31 and was found in a snow pit in Zangser Kangri. The colony was described as yellowish, green, small and smooth (Yan et al., 2017), which is similar to the phenotype in this project. The second strain with 100% similarity was *Cellulomonas* sp. MDT2-38. This strain was already found in the paper of Yan et al. (2017) to have the highest sequence similarity with their strain. MDT2-38 was isolated from a glacier environment in China, but no further information of this strain could be found.

The strain G4 has the highest sequence similarity (99,09%) with two different *Paenibacillus* sp. strains. The strain R20-25 was isolated from an Alpine forest soil, as part of a study analyzing the effect of altitude and season on the microbial community (França et al., 2016). The strain MC1-Q isolated from a lava tube ice cave and tested for its ability to survive in an artificial martial environment (O'Connor et al., 2021).

The bacterium which was labelled G6 belongs to the *Micrococcus* sp. genus. It had many very similar (100%) hits when blasting it in the NCBI database. The first view were often connected to soils and plants and one of them might even act as a biocontrol agent against *Ralstonia solanacearum* in Banana (Creencia et al., 2022).

The strain G9 belongs to the genus of *Bacillus* sp. When blasting the sequence of G9 in the NCBI database, it results in >100 hit with 100% similarity. This is due to a high sequence similarity in the *Bacillus* sp. genus (Janda & Abbott, 2007). Many of the hits hat a source stated in NCBI as derived from soil.

Even though the sequence similarity in some samples is very high (>99,5%), this does not mean the sample is the same species or strain to the hit in the NCBI database (Janda & Abbott, 2007). In this case the sequencing and identification was mainly used to build the internal collection of endophytes from soil 82 without including the same bacteria several times.

5. Outlook

The four soils which showed a promising phenotype when growing in unsterile soil during the initial screening need to be further investigated to proof that beneficial microbes cause the different plant phenotypes. When the plants received a full nutrient solution during the NRE, the plant phenotype improved (e.g. fewer yellow leaves, bigger rosettes). This led to the conclusion that the plants are nutrient deprived when grown in some of the sterile soils. To further investigate this nutrient deprivation, also the nutrient content of the soils themselves should be measured. Afterwards the nutrient content of the sterile and unsterile soil of the same site should be compared to exclude the possibility that the sterilization impacted the nutrient content. This is an important step to prove that beneficial microbes impact the plants.

As *Arabidopsis thaliana* is not an agricultural crop, the soils which led to an improved plant growth in the unsterile conditions should also be tested with relevant crops (e.g. barley, rapeseed). If the beneficial microbes also have a positive impact on those crops they should be further investigated. Another option could also be to retest the collected samples with crops, as maybe the microbes are more host specific and would not impact the growth of *Arabidopsis thaliana.*

Gruber et al. (2013) showed that also the root shape is impacted by the nutrient supply of *Arabidopsis thaliana.* Therefore, so-called rhizoboxes could be used to monitor the root shape. This would add another phenotypic criterion to compare the plant growth in sterile und unsterile soil. It would also be important as the microbes tested during these experiments are soil habitants and probably colonize the plant roots.

If the evidence suggests that microbes in the soil are causing the beneficial effect on the plant phenotype, they should be isolated, even though the chances are small that the beneficial microbes are culturable. As a method to show if the beneficial microbes have been isolated, the microbial isolates can be used to inoculate the sterile soil. If the plants grown in this inoculated soil show the same phenotype as the plants grown in the unsterile soil of the same site, the likelihood that the beneficial microbes have been isolated is high. Future experiments and possibly development of a microbial inoculant for agricultural use, would be easier to do if the microbes are cultivable in the laboratory.

For soil 82 more experiments should be carried out with the isolated bacteria in sterile soil 82. During the mono-association assay MS-plates were used which are rich in nutrients. This does probably not show the real effect the endophytic bacteria have on *Arabidopsis thaliana* as they might act in a community and not alone. For future assays with the endophytic bacteria, root samples of the plants should be taken to prove the presence of the microbes. If the combined bacteria show a beneficial effect on *Arabidopsis thaliana* when inoculated in sterile soil 82, the next step should be to find the smallest beneficial SynCom. This can be done dividing the SynCom as often as possible, as long as the beneficial effect is seen. If this is established, the mutants which were genotyped in this project could be used to gain further information about the molecular mechanism of the microbes.

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List of abbreviations

DAG = days after germination

PDA = potato dextrose agar

TSB = tryptic soy broth

TSA = tryptic soy broth agar

MS = Murashige and Skoog Medium

KOH = potassium hydroxide

HCl = Hydrochloride

MES = 2-(N-morpholino)ethanesulfonic acid

EDTA = Ethylenediaminetetraacetic acid

EtOH = Ethanol

TAE = Tris-actetate-EDTA

H_2O = water

NaOCl = Sodium Hypochlorite

KH_2PO_4 = Potassium phosphate monobasic

K_2SO_4 = Potassium sulfate

$MgSO_4*7H_2O$ = Magnesium sulfate heptahydrate

NaCl = sodium chloride

$Mg(NO_3)_2*6H_2O$ = Magnesium nitrate hexahydrate

$Ca(NO_3)_2*4H_2O$ = Calcium nitrate tetrahydrate

KNO_3 = Potassium nitrate

Fe(3)-EDTA-Na = Ethylenediaminetetraacetic acid ferric sodium salt

$MnCl_2*4H_2O$ = Manganese(2)chloride tetrahydrate

$ZnCl_2$ = Zinc chloride

$CuSO_4*5H_2O$ = Copper(2)sulfate pentahydrate

H_3BO_3 = Boric acid

$Na_2MoO_4*2H_2O$ = Sodium molybdate dihydrate

SDS = Sodium Dodecyl Sulfate

Tris = Tris(hydroxymethyl)aminomethane

Col-0 = Columbia-0

ROI = reactive oxygen intermediate

HR = hypersensitive response

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Appendix A: Recipes for Media and Solutions

All media and nutrient solutions were autoclaved prior to use.

Table 21: Recipe for TSA

	amount
Tryptic Soy Broth	7,5 g
BactoAgar	7,5 g
milliQ water	500 ml

Table 22: Recipe for TSB

	amount
Tryptic Soy Broth	7,5 g
milliQ water	500 ml

Table 23: Recipe for PDA

	amount
potato dextrose agar	19,5 g
milliQ water	500 ml

Table 24: Recipe for half-strength MS media with sucrose

	amount
Murashige and Skoog medium including vitamins	2,2 g
MES	1 g
Sucrose	5 g
Agar	15 g
milliQ water	1000 ml
adjust pH to 5,7 using 1M KOH	

Table 25: Recipe for Nutrient Solution 1

	concentration [mol/l]	amount
KH_2PO_4	0,2	27,22g
K_2SO_4	0,2	34,85 g

Table 26: Recipe for Nutrient Solution 2

	concentration [mol/l]	amount
$MgSO_4*7H_2O$	0,3	73,94 g
NaCl	0,1	5,84 g

Table 27: Recipe for Nutrient Solution 3

	concentration [mol/l]	amount [g/l]
$Mg(NO_3)_2*6H_2O$	0,3	76,92 g
$Ca(NO_3)_2*4H_2O$	0,9	212,53 g
KNO_3	0,6	60,67 g

Table 28: Recipe for Nutrient Solution 4

	concentration [mol/l]	amount [g/l]
Fe(3)-EDTA-Na	0,05	18,35 g
$MnCl_2*4H_2O$	0,001	0,20 g
$ZnCl_2$	0,0007	0,10 g
$CuSO_4*5H_2O$	0,0008	0,20 g
H_3BO_3	0,002	0,12 g
$Na_2MoO_4*2H_2O$	0,0008	0,19 g
adjust pH to 2 using 4 M HCl		

Table 29: Recipe for 0,25% NaOCl Solution

	amount
14% NaOCl	1,786 ml
milliQ water	100 ml
adjust pH to 5,6 using 4 M HCl	

Table 30: Recipe for Lysing Buffer

	concentration	amount
Tris-HCl (pH 8)	200 mM	40 ml
NaCl	250 mM	2,92 g
EDTA	25 mM	1,86 g
SDS	0,5%	1 g
milliQ water		160 ml

Appendix B: Initial Screening

Sterility Test

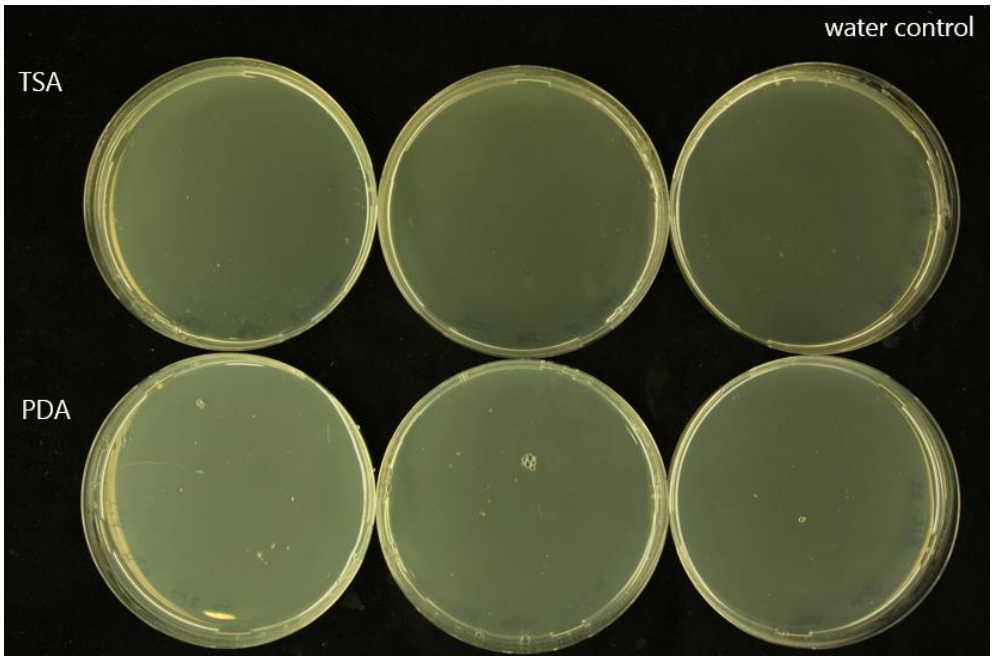

Figure 64: TSA and PDA plates of the water control in the soil sterility test, to analyse the environmental microbial influence when weighing in the samples.

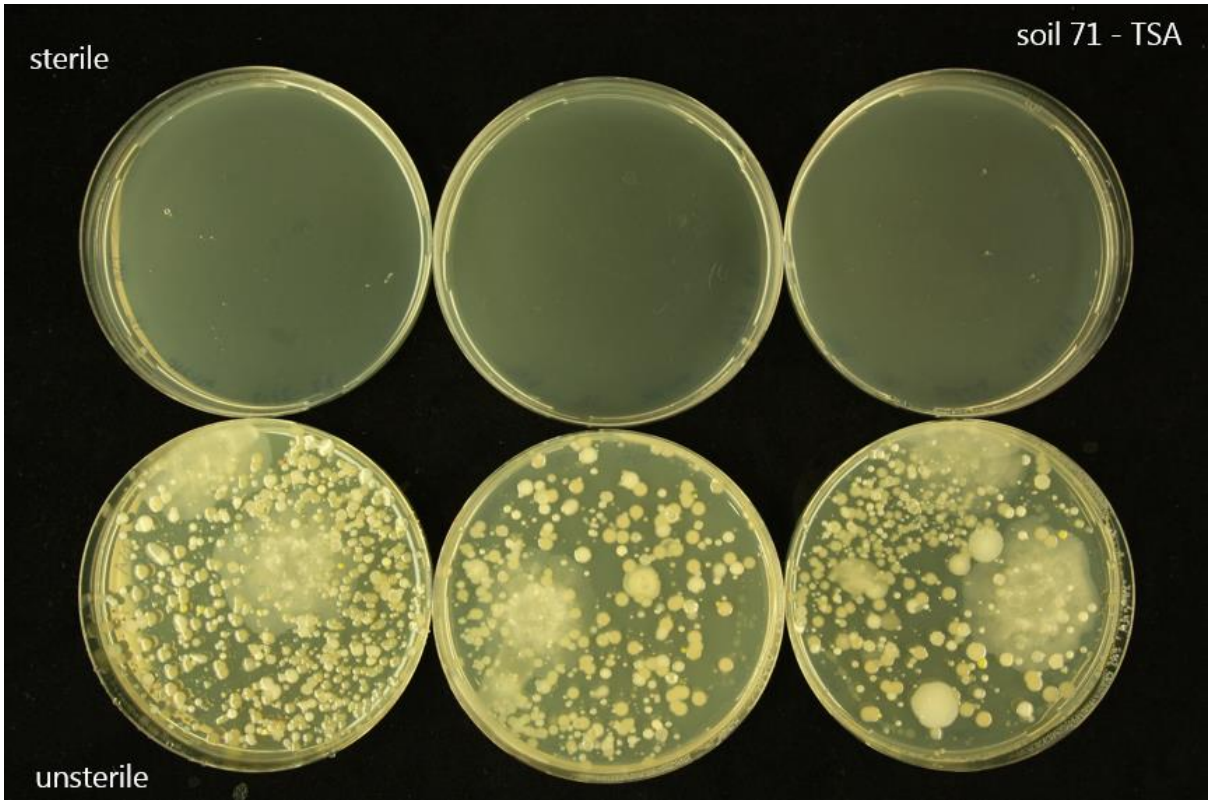

Figure 65: Results of the soil sterility test for soil 71 on TSA media to analyse bacterial infection

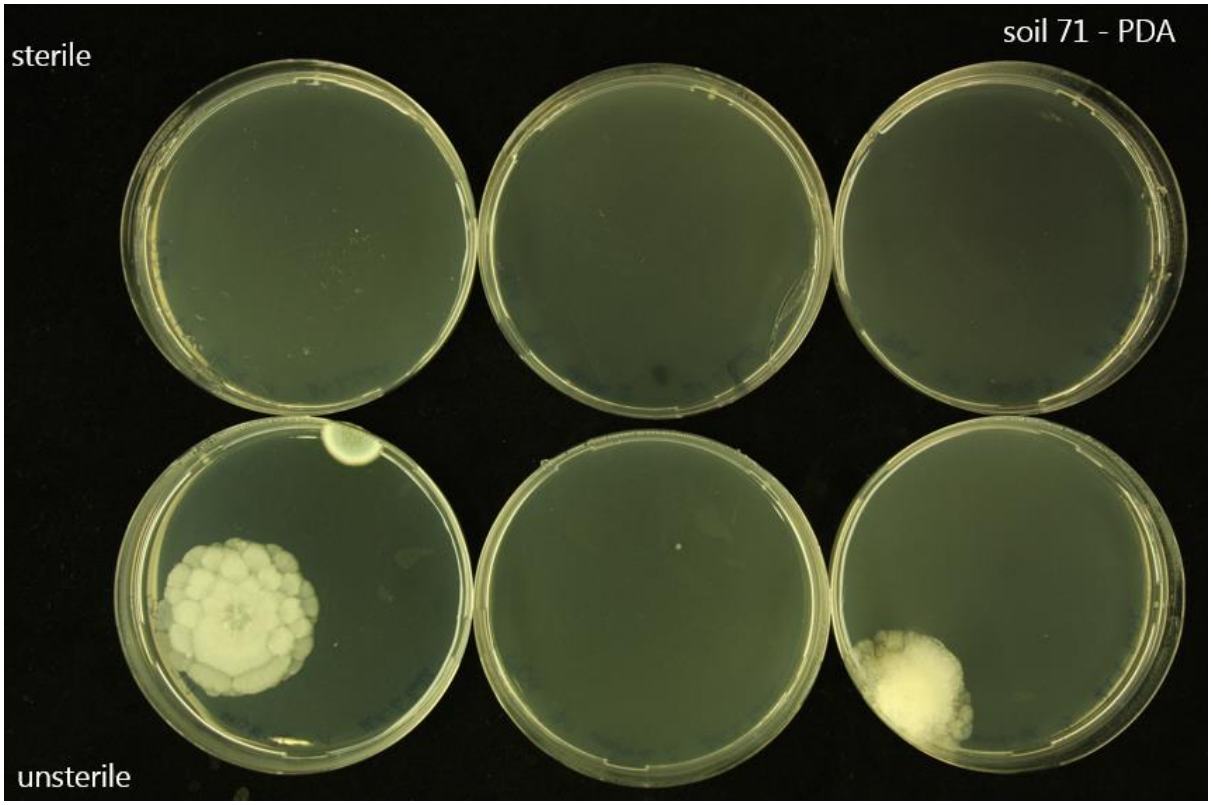

Figure 66: Results of the soil sterility test for soil 71 on PDA media to analyse fungal infection

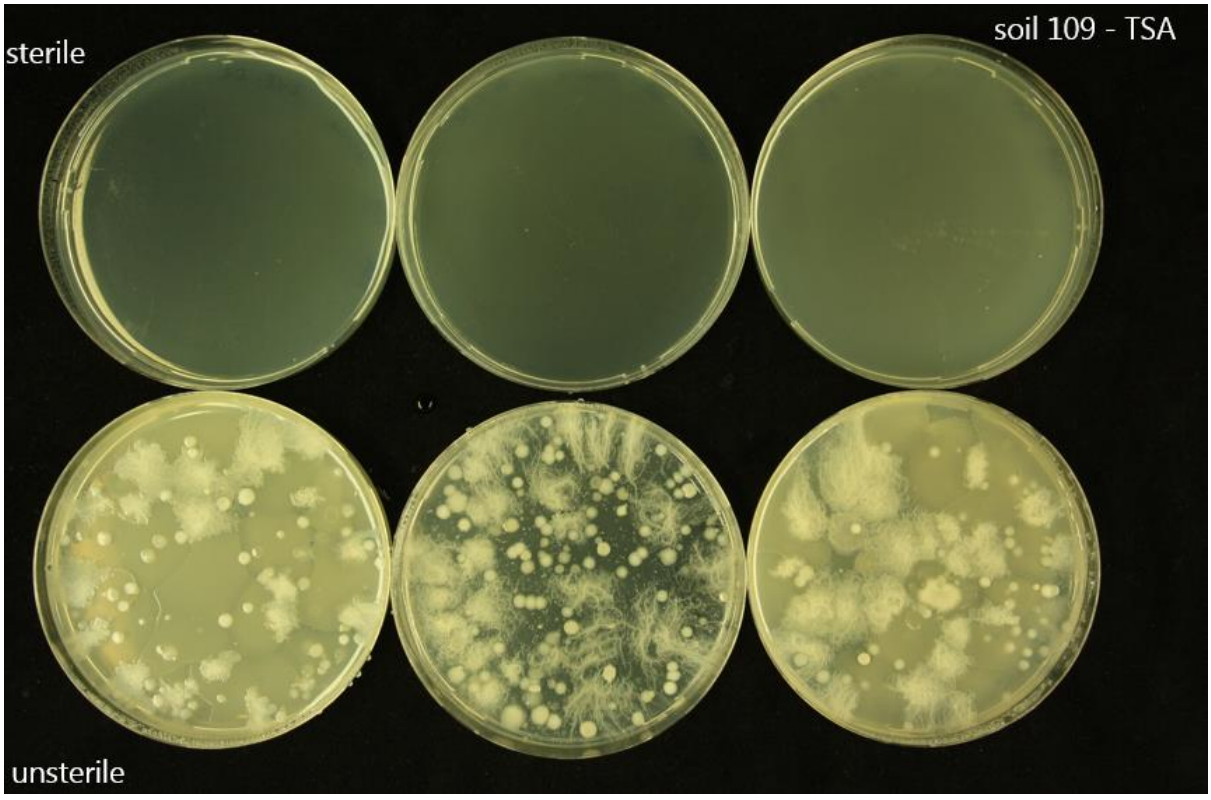

Figure 67: Results of the soil sterility test for soil 109 on TSA media to analyse bacterial infection

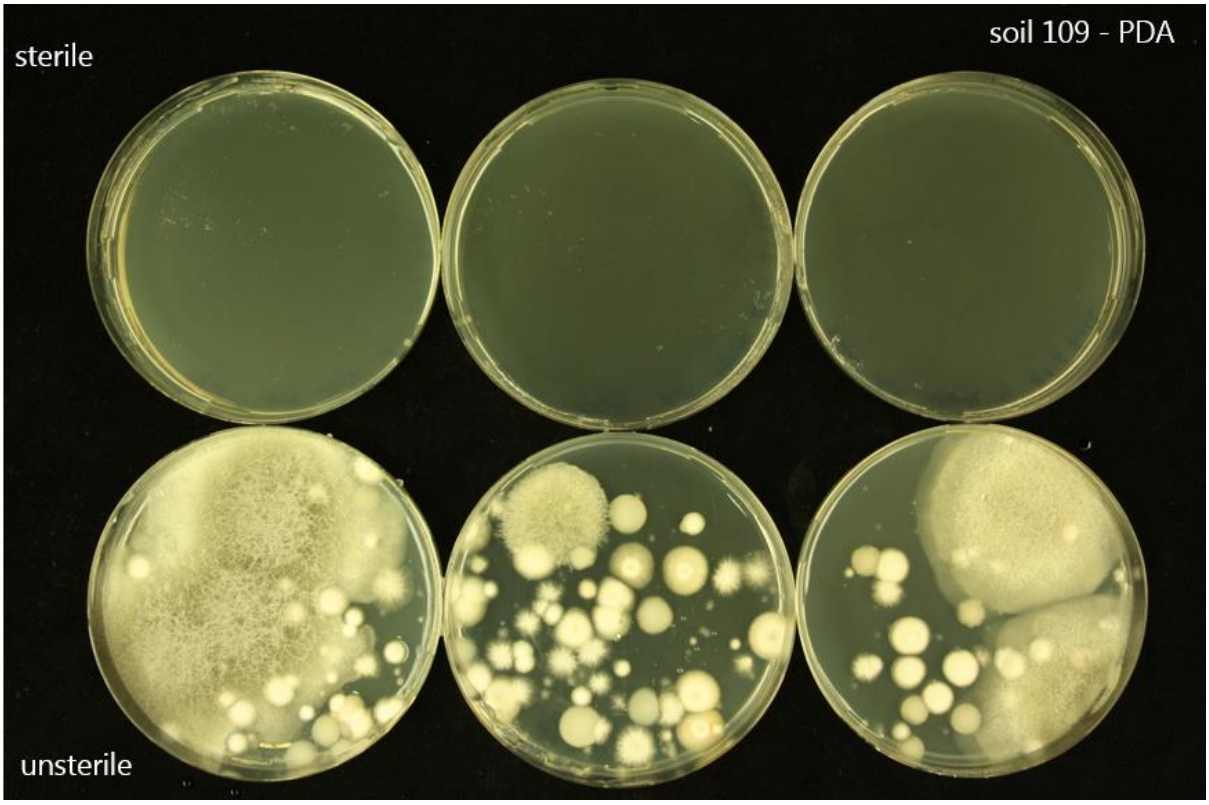

Figure 68: Results of the soil sterility test for soil 109 on PDA media to analyse fungal infection

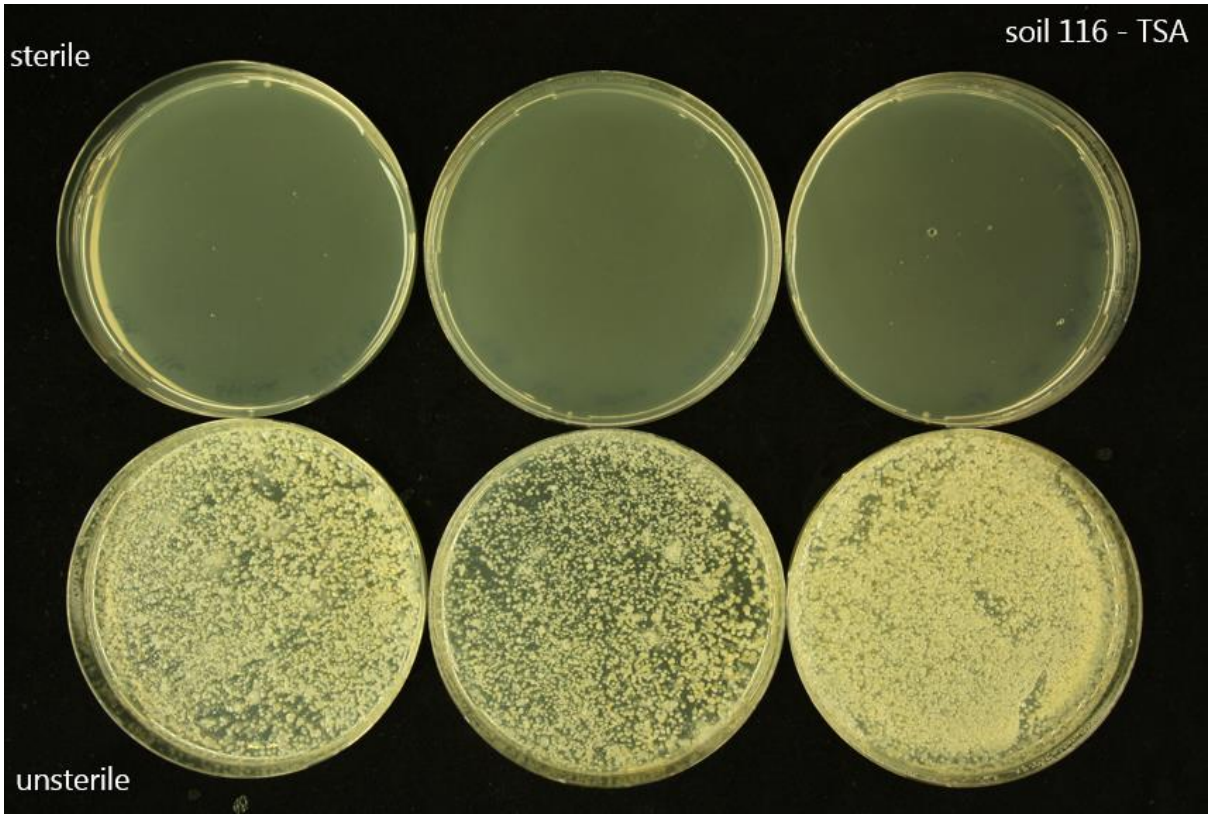

Figure 69: Results of the soil sterility test for soil 116 on TSA media to analyse bacterial infection

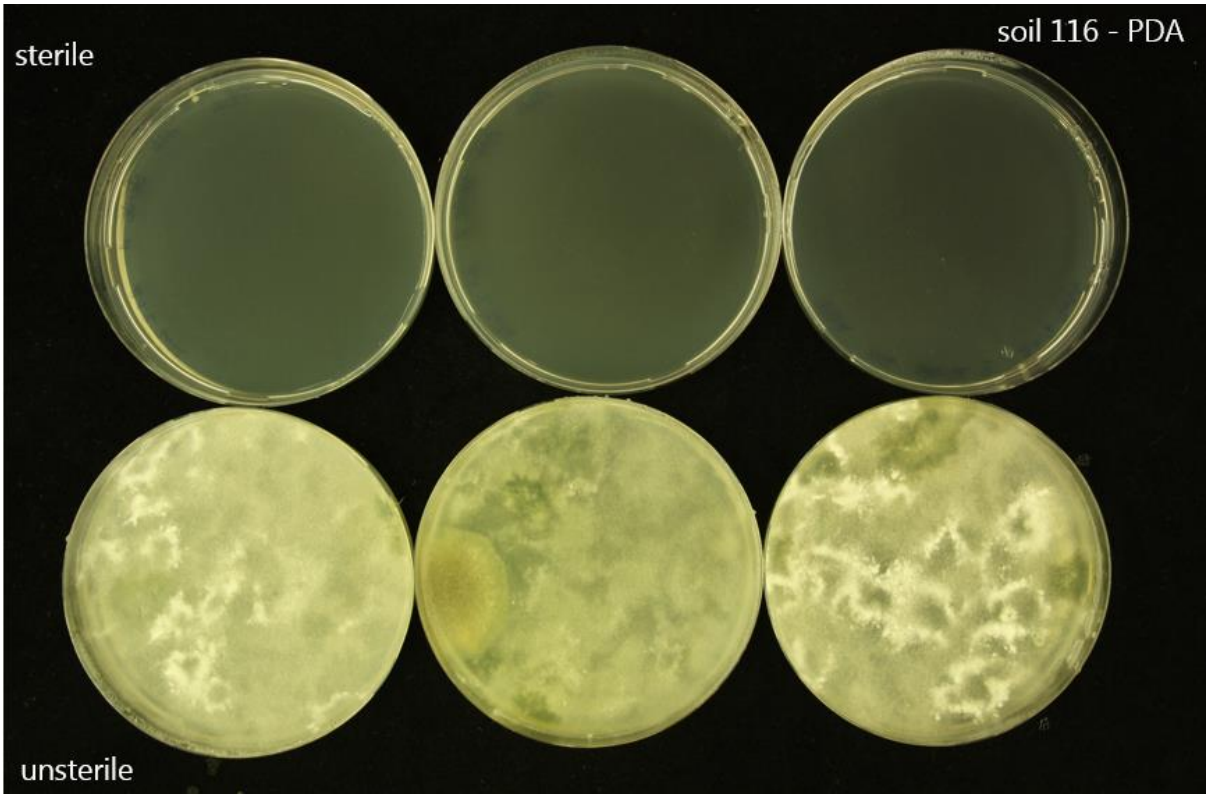

Figure 70: Results of the soil sterility test for soil 116 on PDA media to analyse fungal infection

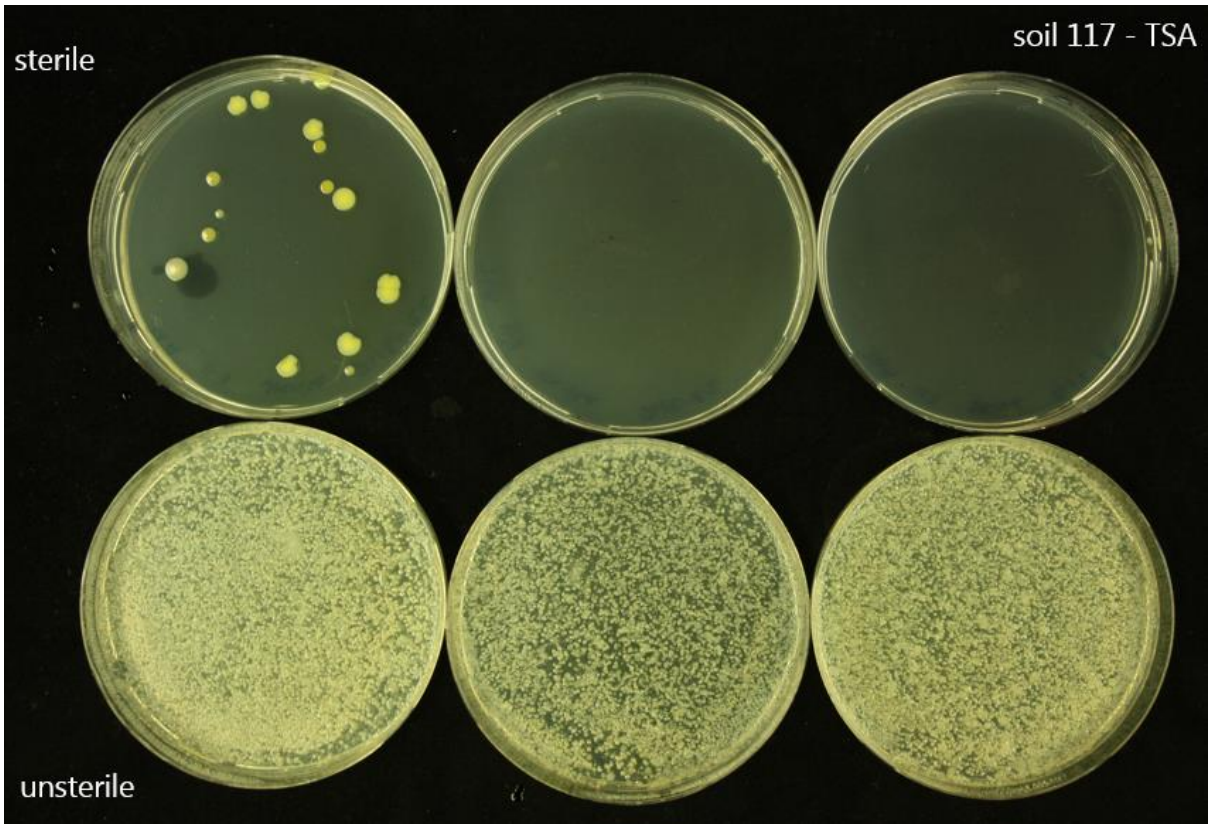

Figure 71: Results of the soil sterility test for soil 117 on TSA media to analyse bacterial infection

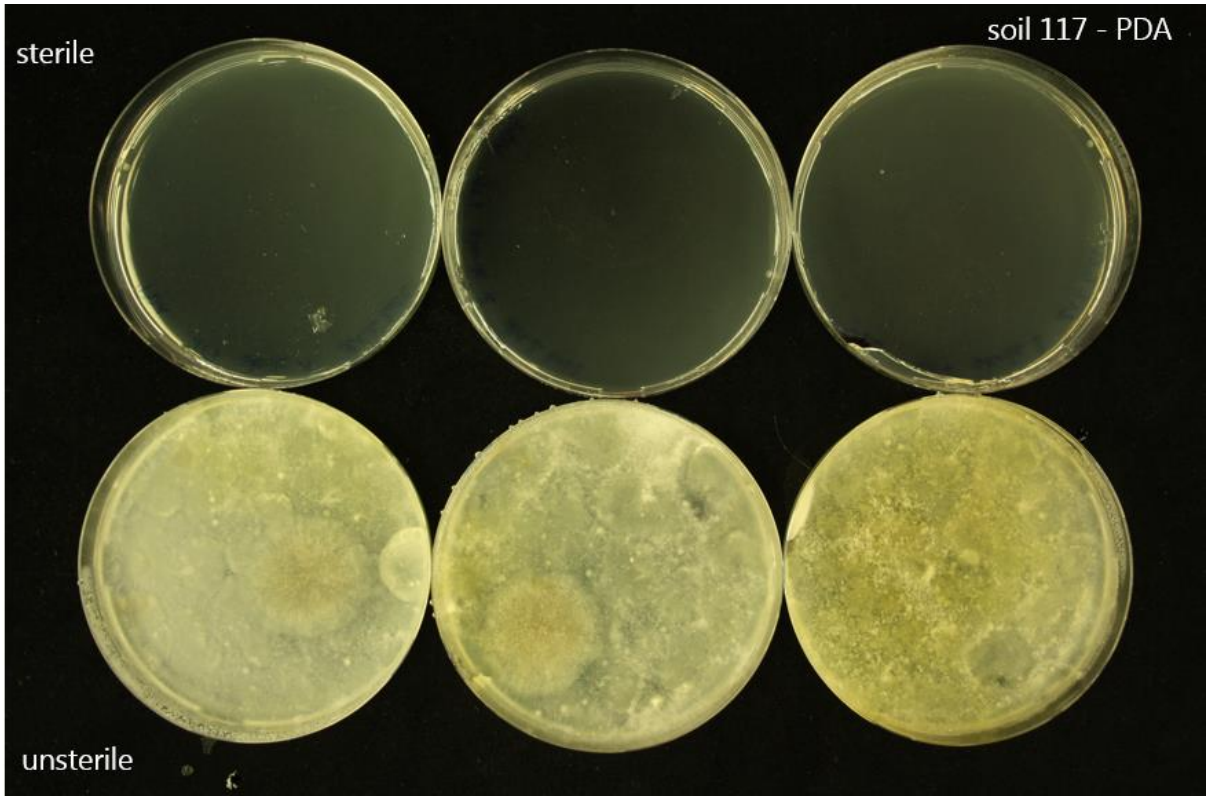

Figure 72: Results of the soil sterility test for soil 117 on PDA media to analyse fungal infection

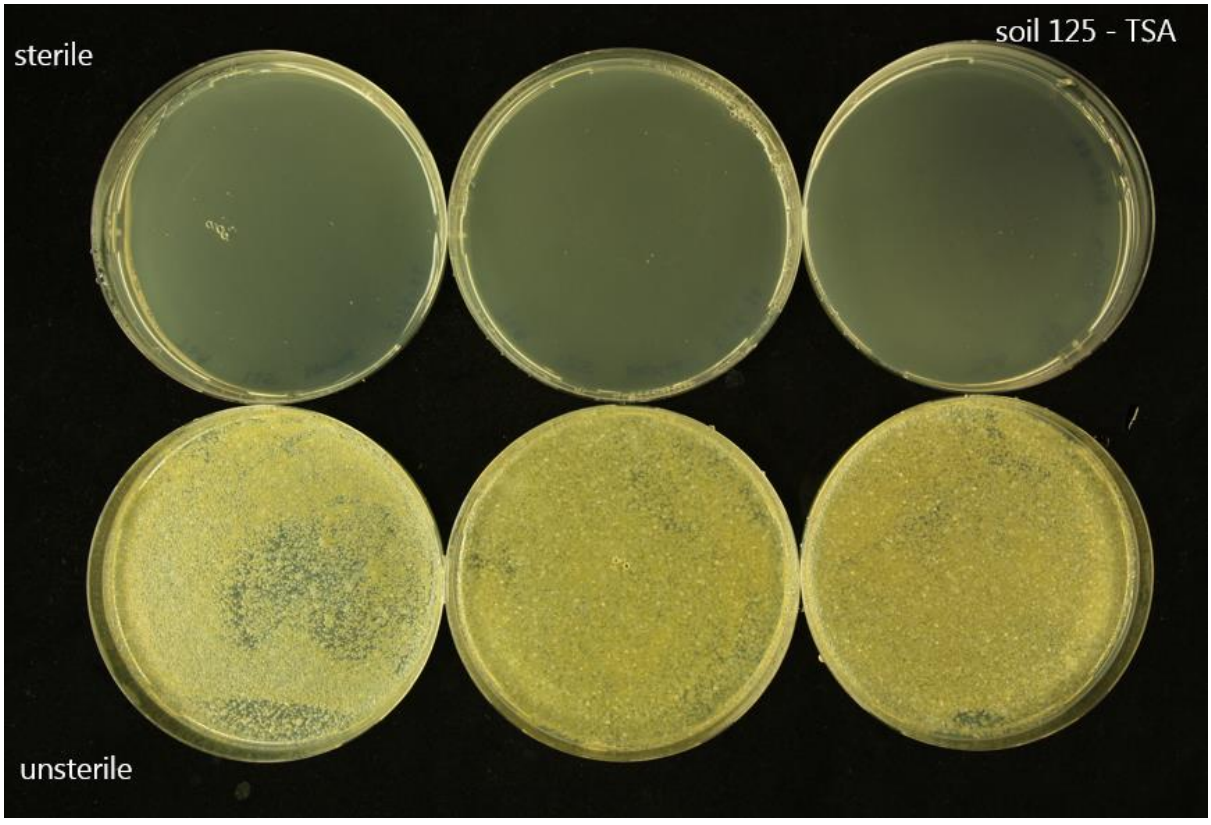

Figure 73: Results of the soil sterility test for soil 125 on TSA media to analyse bacterial infection

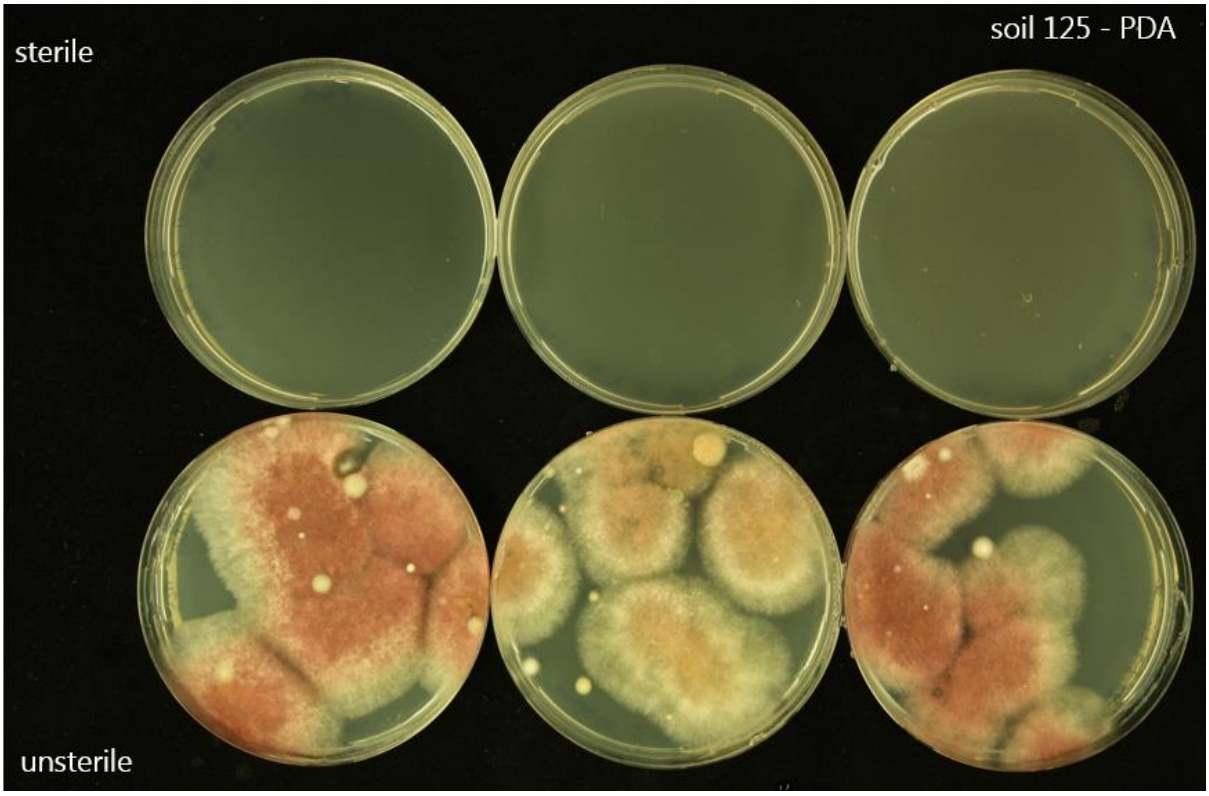

Figure 74: Results of the soil sterility test for soil 125 on PDA media to analyse fungal infection

Phenotypic Screening

The results of eight soils during the initial phenotypic screening did not show a sign of containing microbes that benefit *Arabidopsis thaliana* in this experimental design. Those soils are listed and described in the following pages.

Soil 29

In soil 29 no favoured phenotype could be detected. The Col-0 plants grown in sterile conditions were significantly bigger, while no differences could be detected based on senescent or anthocyanin area by Aradeepopsis (Figure 75, Figure 76). The results of the Welch t-test can be seen in Figure 77.

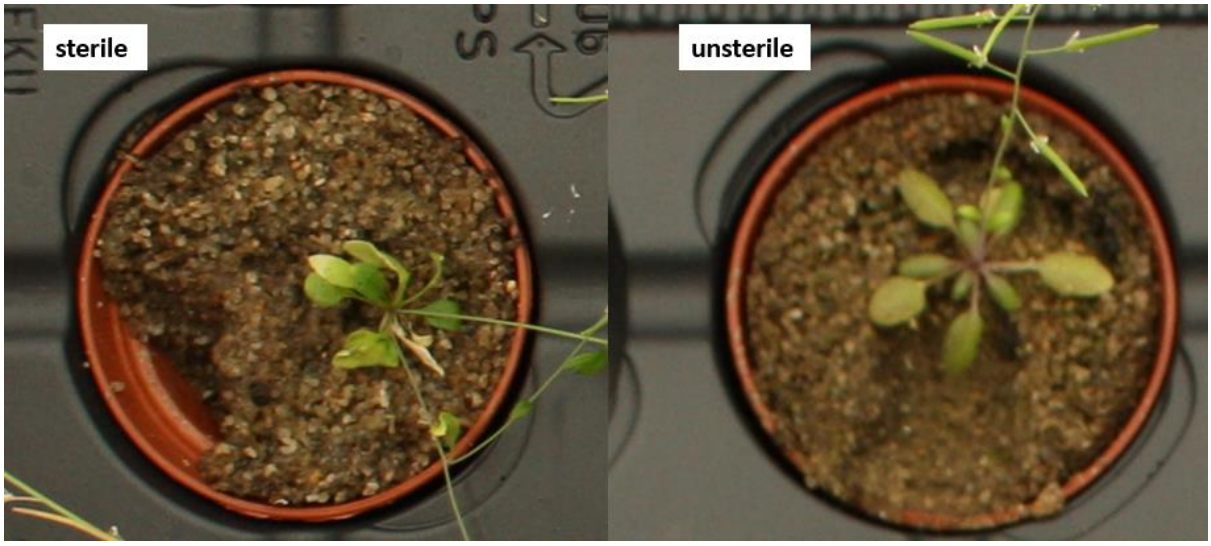

Figure 75: Phenotype of soil 29 47DAG

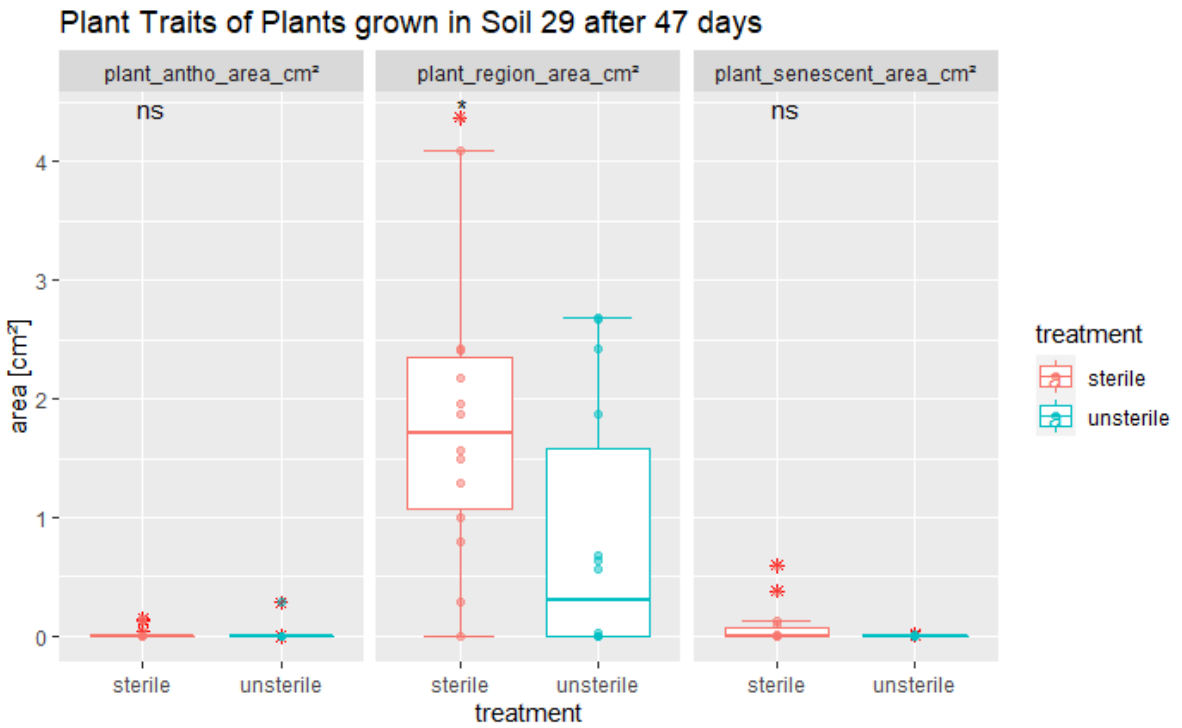

Figure 76: Results of analysis of plant phenotype of soil 29 with Aradeepopsis 47DAG. The measurements of anthocyanin area, rosette area and senescent areas were used. Statistical comparison of plants grown in sterile and unsterile soil 29 by Welch t-test.

```
new_soil29%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = 2.2841, df = 25.51, p-value = 0.03094
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  0.1002824 1.9209402
## sample estimates:
##   mean in group sterile mean in group unsterile
##               1.8365379               0.8259266
```

```
new_soil29%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = 0.067469, df = 22.064, p-value = 0.9468
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.04839945  0.05165506
## sample estimates:
##   mean in group sterile mean in group unsterile
##              0.02198968              0.02036187
```

```
new_soil29%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = 1.809, df = 13.009, p-value = 0.09361
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.01686417  0.19058262
## sample estimates:
##   mean in group sterile mean in group unsterile
##            0.0878587572            0.0009995308
```

Figure 77: Welch t-test of soil 29

Soil 37

The Col-0 plants from soil 37 showed a similar phenotype to soil 29 (Figure 78). The total plant area was bigger in the sterile conditions while no significant difference could be detected Figure 79 for the senescent and anthocyanin area with a Welch t-test (Figure 80).

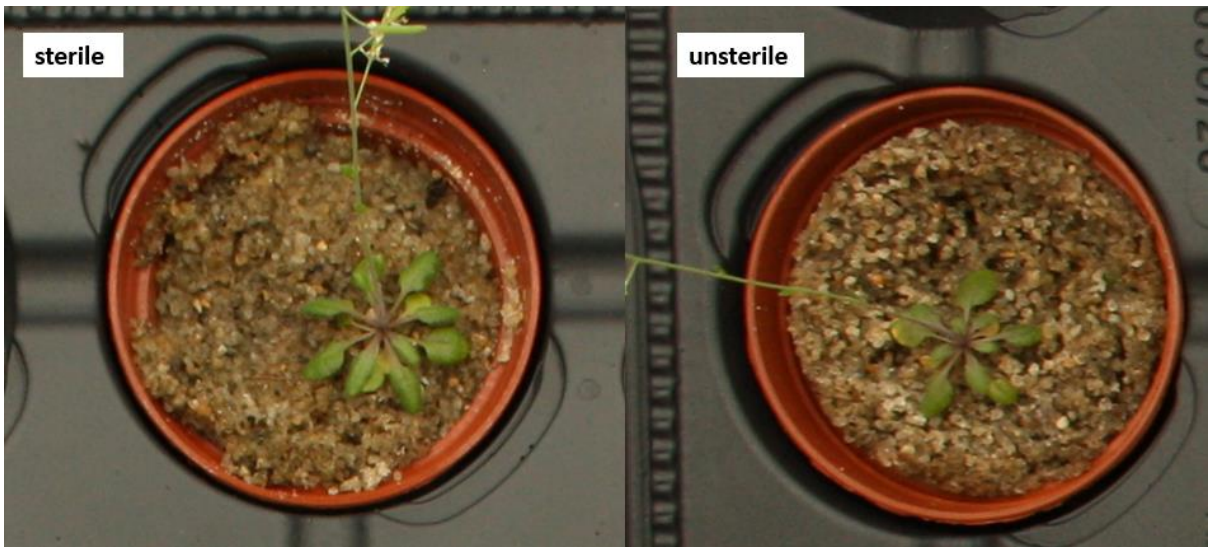

Figure 78: Phenotype of Soil 37

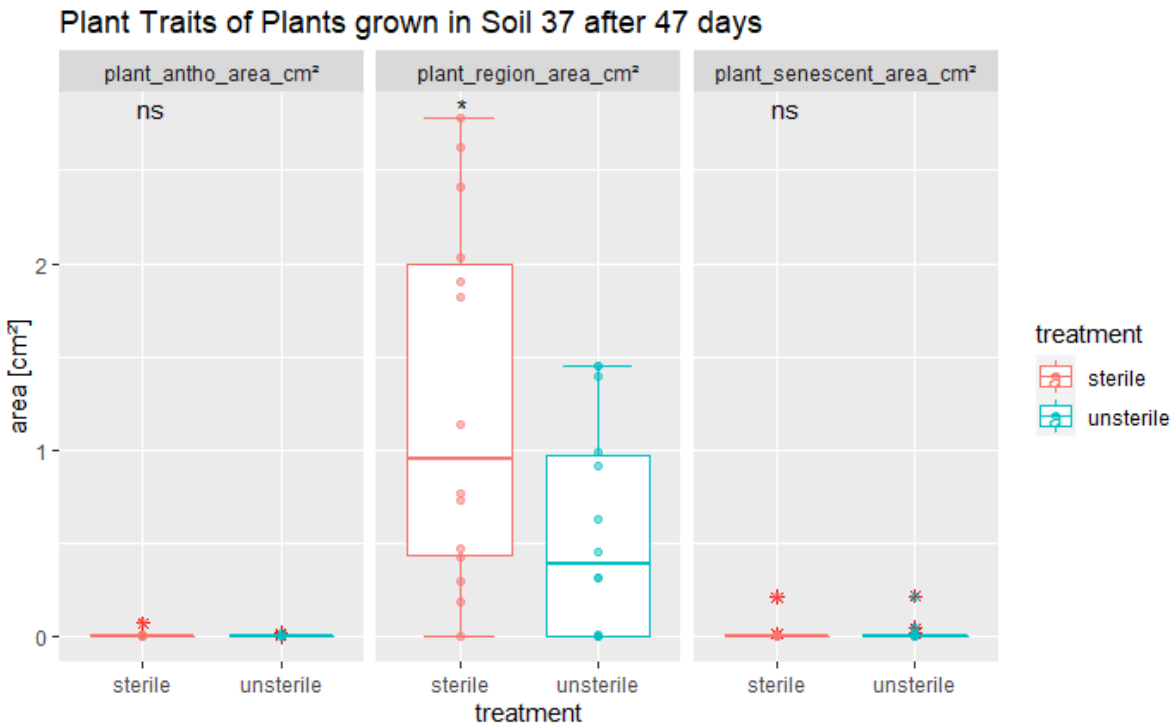

Figure 79: Results of analysis of plant phenotype of soil 37 with Aradeepopsis 47DAG. The measurements of anthocyanin area, rosette area and senescent areas were used. Statistical comparison of plants grown in sterile and unsterile soil 37 by Welch t-test.

```
new_soil37%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = 2.2821, df = 21.029, p-value = 0.033
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  0.06131124 1.31972619
## sample estimates:
##   mean in group sterile mean in group unsterile
##               1.2552679               0.5647492
```

```
new_soil37%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = 0.97008, df = 14.396, p-value = 0.348
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.005902966  0.015698368
## sample estimates:
##   mean in group sterile mean in group unsterile
##            0.006054301             0.001156600
```

```
new_soil37%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = -0.16946, df = 25.983, p-value = 0.8667
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.04762056  0.04036682
## sample estimates:
##   mean in group sterile mean in group unsterile
##              0.01636375              0.01999062
```

Figure 80: Welch t-test of soil 37

Soil 41

Also in soil 41 the Col-0 plants grown in sterile conidtions had a bigger rosette area, while no differences in senescent and anthocyanin area occurred (Figure 81, Figure 82, Figure 83) .

Figure 81: Phenotype of soil 41

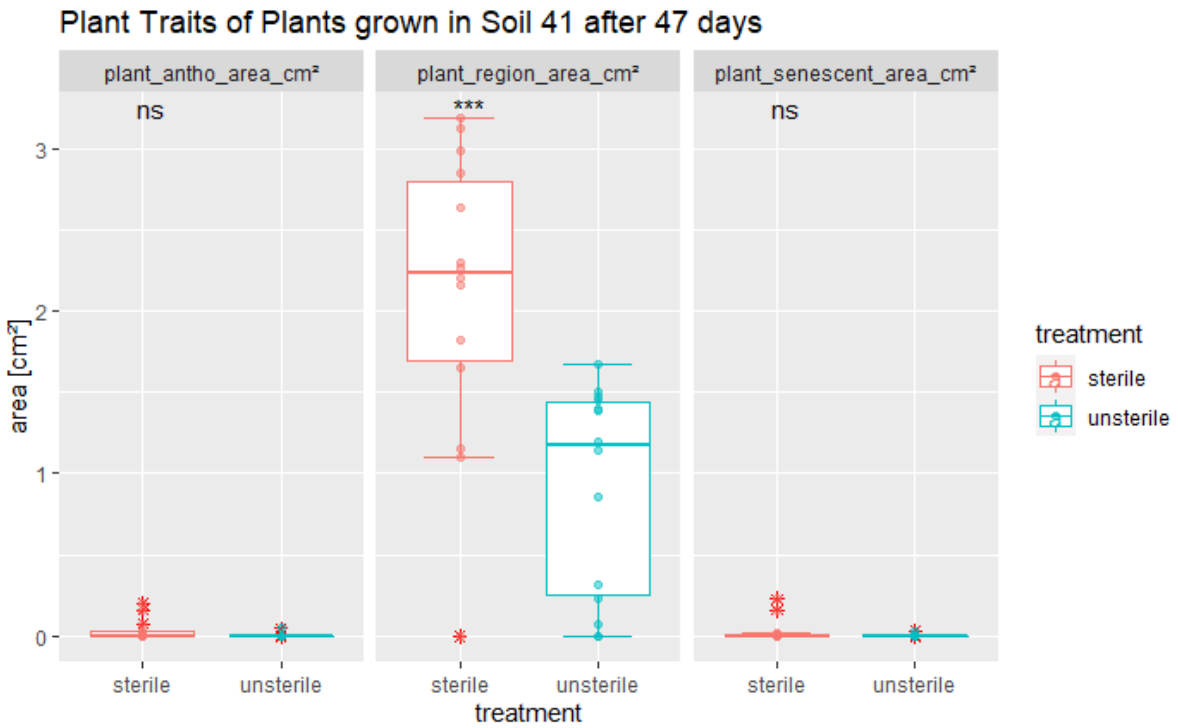

Figure 82: Results of analysis of plant phenotype of soil 41 with Aradeepopsis 47DAG. The measurements of anthocyanin area, rosette area and senescent areas were used. Statistical comparison of plants grown in sterile and unsterile soil 41 by Welch t-test.

```
new_soil41%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = 4.0441, df = 23.457, p-value = 0.0004886
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  0.5841996 1.8050217
## sample estimates:
##   mean in group sterile mean in group unsterile
##               2.1016563               0.9070456
```

```
new_soil41%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = 1.8285, df = 14.009, p-value = 0.08885
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.005595219  0.070307699
## sample estimates:
##   mean in group sterile mean in group unsterile
##             0.035840319             0.003484079
```

```
new_soil41%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = 1.4778, df = 13.467, p-value = 0.1625
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.01292613  0.06952813
## sample estimates:
##   mean in group sterile mean in group unsterile
##             0.031285314             0.002984313
```

Figure 83: Welch t-test of soil 41

Soil 71

The results of the Welch t-test can be seen in Figure 84.

```
new_soil71%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = 4.6951, df = 19.048, p-value = 0.0001567
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  1.203016 3.137718
## sample estimates:
##   mean in group sterile mean in group unsterile
##               3.1531341               0.9827672
```

```
new_soil71%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = 1.0403, df = 13, p-value = 0.3172
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.04899452  0.14000894
## sample estimates:
##   mean in group sterile mean in group unsterile
##              0.04550721              0.00000000
```

```
new_soil71%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = 3.6797, df = 13.267, p-value = 0.002689
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  0.1464303 0.5608092
## sample estimates:
##   mean in group sterile mean in group unsterile
##              0.36908389              0.01546417
```

Figure 84: Welch t-test of soil 71

Soil 75

In soil 75 no significant difference between the tested conditions could be detected for the traits 40DAG (Figure 85, Figure 86, Figure 87).

Figure 85: Phenotype of soil 75

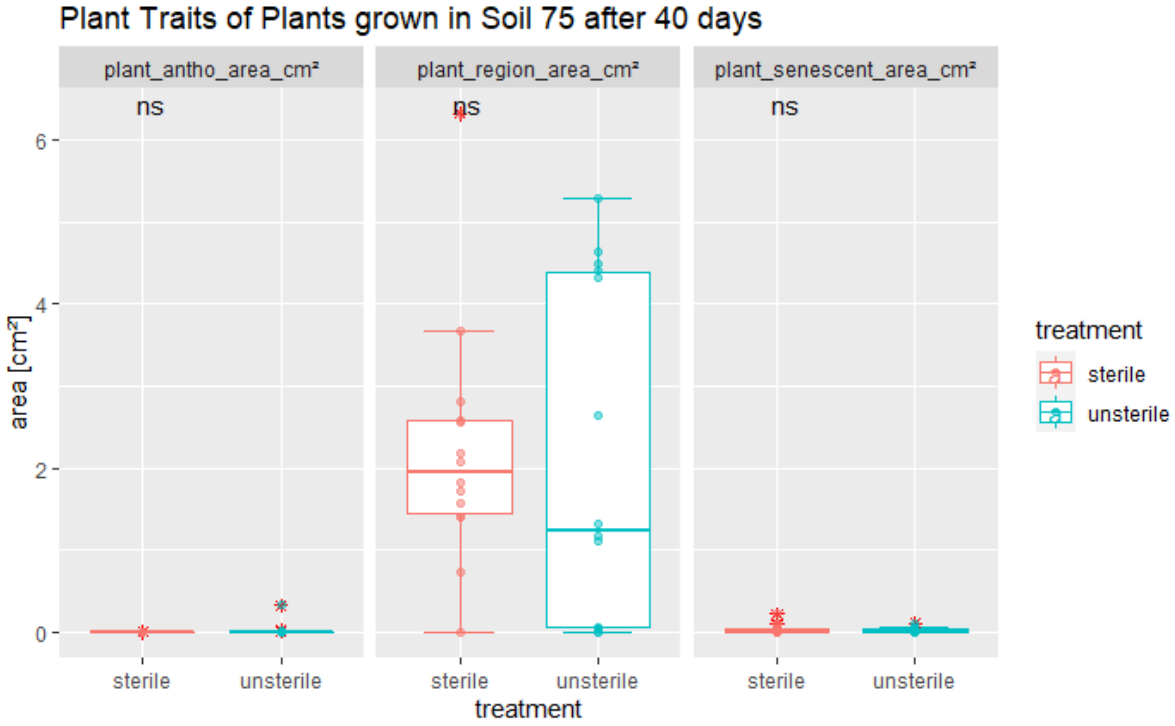

Figure 86: Results of analysis of plant phenotype of soil 75 with Aradeepopsis 40DAG. The measurements of anthocyanin area, rosette area and senescent areas were used. Statistical comparison of plants grown in sterile and unsterile soil 75 by Welch t-test.

```
new_soil75%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = 0.13738, df = 23.496, p-value = 0.8919
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -1.321197  1.509395
## sample estimates:
##   mean in group sterile mean in group unsterile
##                2.203823                2.109724
```

```
new_soil75%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = -1.0441, df = 13.009, p-value = 0.3155
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.07410360  0.02581198
## sample estimates:
##   mean in group sterile mean in group unsterile
##            0.0007139506            0.0248597589
```

```
new_soil75%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = 0.81206, df = 19.269, p-value = 0.4267
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.02516571  0.05712214
## sample estimates:
##   mean in group sterile mean in group unsterile
##              0.03446953              0.01849132
```

Figure 87: Welch t-test of soil 75

Soil 107

In soil 107 the Col-0 plants grown in sterile soil had a bigger rosette area compares to unsterile conditions. Also, the senescent area was slightly bigger in sterile conditions (Figure 88, Figure 89, Figure 90).

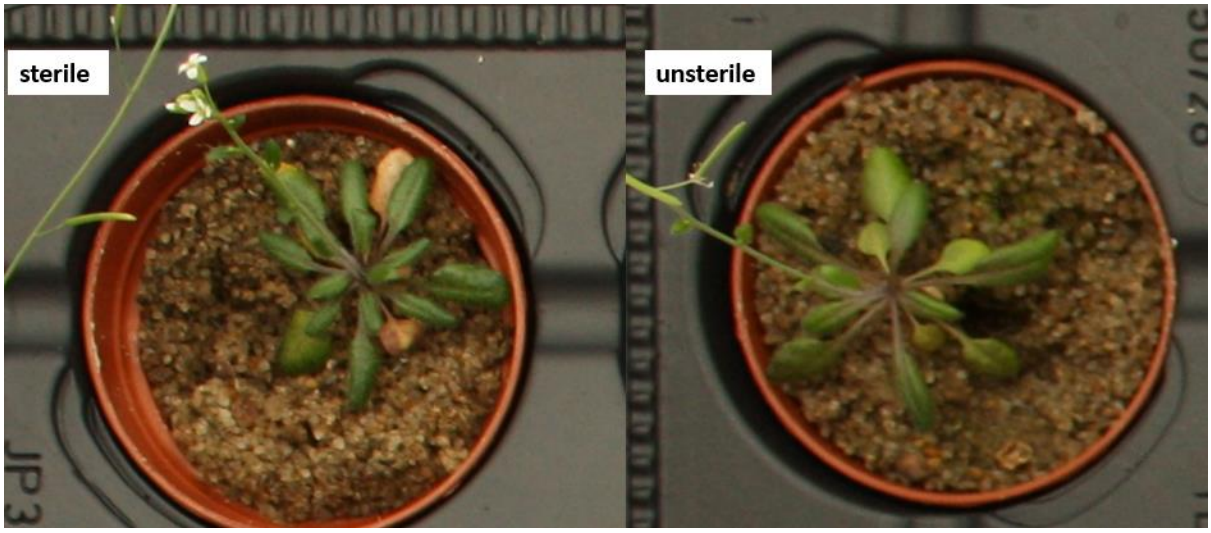

Figure 88: Phenotype of soil 107

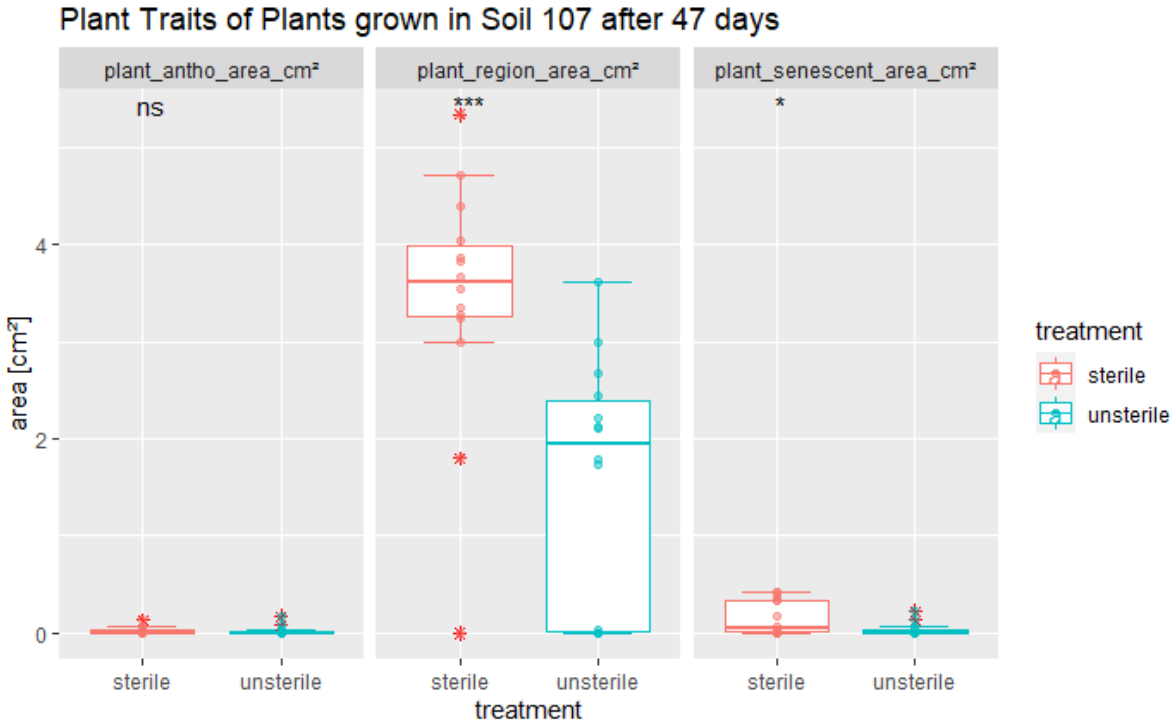

Figure 89: Results of analysis of plant phenotype of soil 107 with Aradeepopsis 47DAG. The measurements of anthocyanin area, rosette area and senescent areas were used. Statistical comparison of plants grown in sterile and unsterile soil 107 by Welch t-test.

```
new_soil107%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = 3.8596, df = 26, p-value = 0.0006735
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  0.8785274 2.8804795
## sample estimates:
##   mean in group sterile mean in group unsterile
##                3.432446                1.552942
```

```
new_soil107%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = 0.16906, df = 24.641, p-value = 0.8671
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.03148119  0.03710712
## sample estimates:
##   mean in group sterile mean in group unsterile
##              0.02694449              0.02413153
```

```
new_soil107%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = 2.4463, df = 16.601, p-value = 0.0259
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  0.01698613 0.23289657
## sample estimates:
##   mean in group sterile mean in group unsterile
##              0.15948228              0.03454093
```

Figure 90: Welch t-test of soil 107

Soil 109

The results of the Welch t-test for soil 109 can be seen in Figure 91.

```
new_soil109%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = -0.55681, df = 24.355, p-value = 0.5827
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.6912689  0.3973497
## sample estimates:
##   mean in group sterile mean in group unsterile
##                1.303174                1.450134
```

```
new_soil109%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = 1.116, df = 13.272, p-value = 0.2842
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.009127649  0.028718453
## sample estimates:
##   mean in group sterile mean in group unsterile
##             0.011109071             0.001313669
```

```
new_soil109%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = 0.90377, df = 13.3, p-value = 0.3822
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.03864111  0.09444349
## sample estimates:
##   mean in group sterile mean in group unsterile
##             0.031842195             0.003941007
```

Figure 91: Welch t-test of soil 109

Soil 115

In soil 115 the Col-0 plants grown in sterile conditions were bigger and had also a little more senescent area as detected by Aradeepopsis (Figure 93, Figure 94). Overall, even though Col-0 plants in sterile soil 115 had yellow leave edges the still looked “healthier” than Col-0 plants in unsterile soil 115, as those had a browner leave colour (Figure 92).

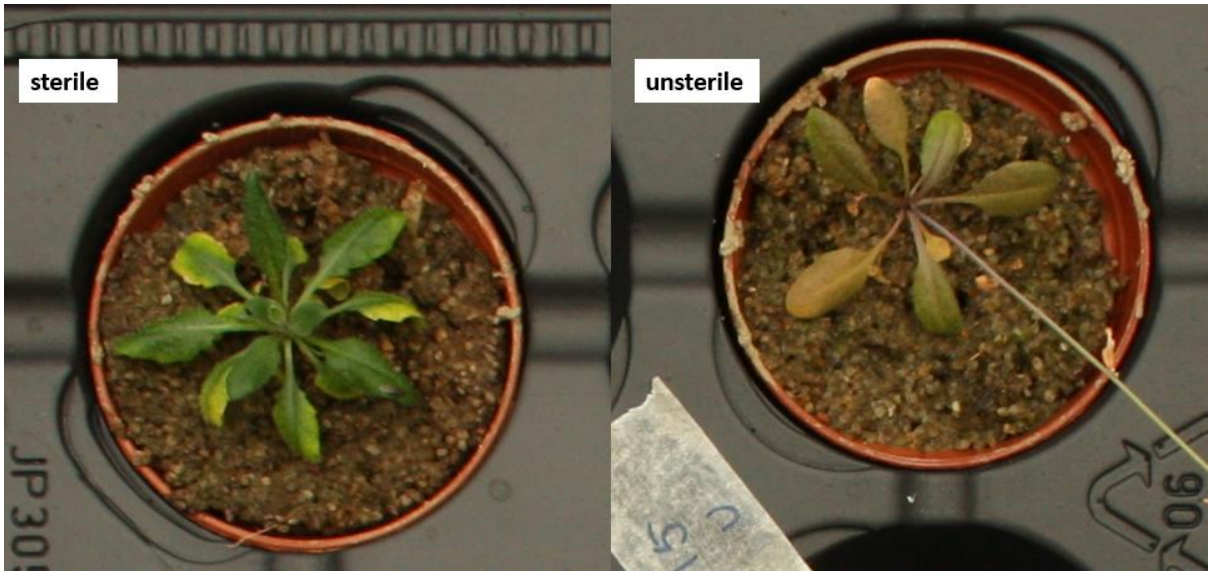

Figure 92: Phenotype of soil 115

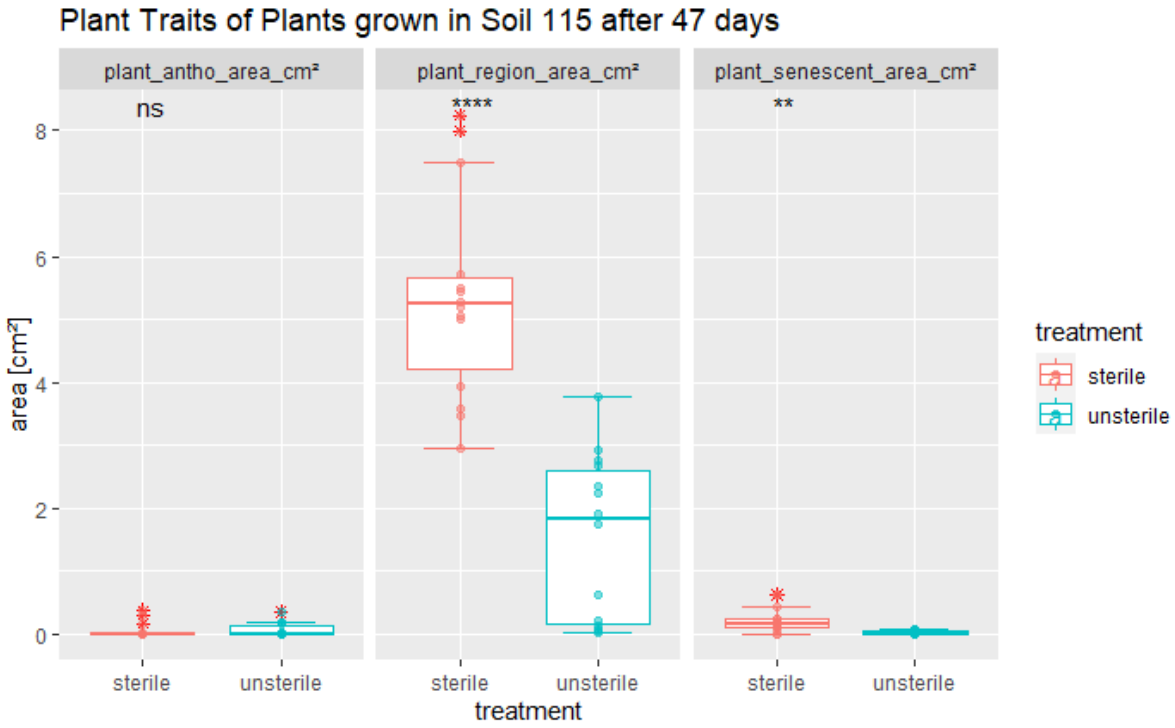

Figure 93: Results of analysis of plant phenotype of soil 115 with Aradeepopsis 47DAG. The measurements of anthocyanin area, rosette area and senescent areas were used. Statistical comparison of plants grown in sterile and unsterile soil 115 by Welch t-test.

```
new_soil115%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = 6.8157, df = 24.821, p-value = 3.976e-07
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  2.659999 4.964908
## sample estimates:
##   mean in group sterile mean in group unsterile
##                5.347147                1.534694
```

```
new_soil115%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = -0.039255, df = 25.71, p-value = 0.969
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.09529829  0.09172854
## sample estimates:
##   mean in group sterile mean in group unsterile
##              0.06619750              0.06798237
```

```
new_soil115%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = 3.7093, df = 13.663, p-value = 0.002425
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  0.08486593 0.31883028
## sample estimates:
##   mean in group sterile mean in group unsterile
##              0.22476592              0.02291781
```

Figure 94: Welch t-test of soil 115

Soil 116

The results of the Welch t-test of soil 116 can be seen in Figure 95.

```
new_soil116%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = -3.1503, df = 25.457, p-value = 0.004139
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -3.484653 -0.731082
## sample estimates:
##   mean in group sterile mean in group unsterile
##                4.584991                6.692858
```

```
new_soil116%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = -2.4138, df = 18.898, p-value = 0.02611
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.42543934 -0.03020392
## sample estimates:
##   mean in group sterile mean in group unsterile
##              0.08737327              0.31519490
```

```
new_soil116%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = -2.2784, df = 16.893, p-value = 0.03599
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.95808061 -0.03656676
## sample estimates:
##   mean in group sterile mean in group unsterile
##                0.550156                1.047480
```

Figure 95: Welch t-test of soil 116

Soil 117

The results of the Welch t-test of soil 117 can be seen in Figure 96.

```
new_soil117%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = 5.436, df = 17.421, p-value = 4.085e-05
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  1.357755 3.075022
## sample estimates:
##   mean in group sterile mean in group unsterile
##                6.340524                4.124135
```

```
new_soil117%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = -2.3689, df = 13.409, p-value = 0.03347
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.205163541 -0.009764139
## sample estimates:
##   mean in group sterile mean in group unsterile
##              0.01513575              0.12259959
```

```
new_soil117%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = 3.5769, df = 19.196, p-value = 0.001986
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  0.1756179 0.6702136
## sample estimates:
##   mean in group sterile mean in group unsterile
##               0.5841401               0.1612243
```

Figure 96: Welch t-test of soil 117

Soil 119

Even thouhg the results of the Welch t-test suggest that Col-0 plants in unsterile soil 119 are bigger (Figure 97, Figure 98, Figure 99), this is not very impactful as only a few Col-0 plants survived in each condition. It therefore seems that soil 119 in general is not favourable to grow *Arabdiopsis thaliana*.

Figure 97: Phenotype of soil 119

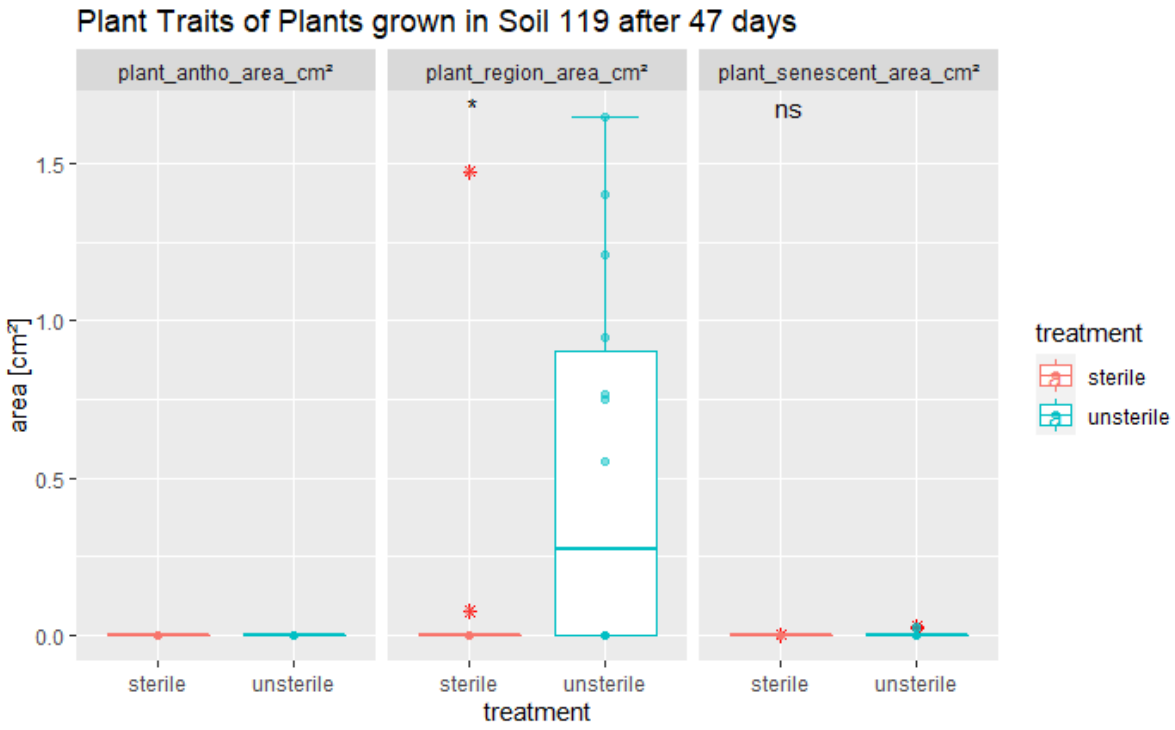

Figure 98: Results of analysis of plant phenotype of soil 119 with Aradeepopsis 47DAG. The measurements of anthocyanin area, rosette area and senescent areas were used. Statistical comparison of plants grown in sterile and unsterile soil 119 by Welch t-test.

```
new_soil119%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = -2.1307, df = 22.355, p-value = 0.04435
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.80868017 -0.01130634
## sample estimates:
##   mean in group sterile mean in group unsterile
##               0.1108051               0.5207984
```

```
new_soil119%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = NaN, df = NaN, p-value = NA
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  NaN NaN
## sample estimates:
##   mean in group sterile mean in group unsterile
##                       0                       0
```

```
new_soil119%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = -1.4264, df = 13.004, p-value = 0.1773
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.008365743  0.001711723
## sample estimates:
##   mean in group sterile mean in group unsterile
##           2.855802e-05            3.355568e-03
```

Figure 99: Welch t-test of soil 119

Soil 125

Also soil 125 is not a good growth media for *Arabidopsis thaliana* as only 2 plants in sterile and 4 in unsterile conditions survived. The Col-0 plants that did survive seemed to be bigger in unterile conditions (Figure 100, Figure 101, Figure 102). Also the fact that more survived in unsterile soil 125 might be a hint that it is somewhat better for growing *Arabidopsis thaliana*.

Figure 100: Phenotype of soil 125

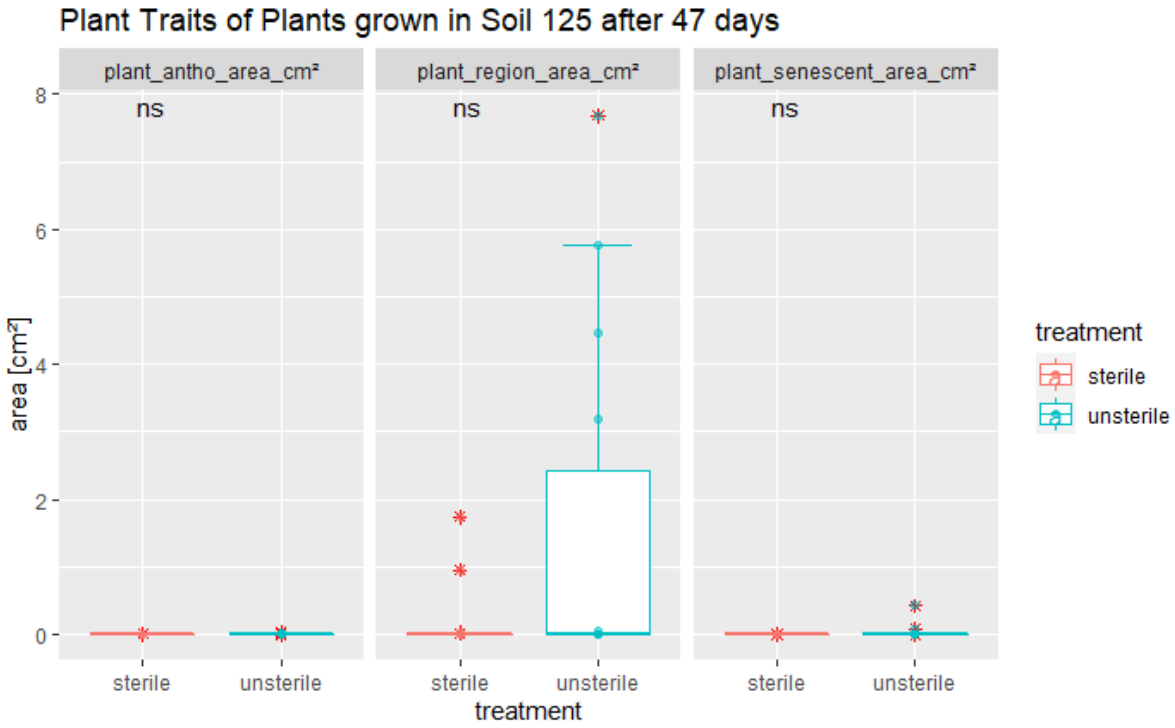

Figure 101: Results of analysis of plant phenotype of soil 125 with Aradeepopsis 47DAG. The measurements of anthocyanin area, rosette area and senescent areas were used. Statistical comparison of plants grown in sterile and unsterile soil 125 by Welch t-test.

```
new_soil125%>%
  drop_na(plant_region_area_cm²)%>%
  t.test(plant_region_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_region_area_cm² by treatment
## t = -1.8358, df = 13.975, p-value = 0.08776
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -2.8572117  0.2220487
## sample estimates:
##   mean in group sterile mean in group unsterile
##               0.1935948               1.5111763
```

```
new_soil125%>%
  drop_na(plant_antho_area_cm²)%>%
  t.test(plant_antho_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_antho_area_cm² by treatment
## t = -1.0472, df = 13.017, p-value = 0.3141
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.007259580  0.002518948
## sample estimates:
##   mean in group sterile mean in group unsterile
##            5.711605e-05            2.427432e-03
```

```
new_soil125%>%
  drop_na(plant_senescent_area_cm²)%>%
  t.test(plant_senescent_area_cm²~treatment, data =.)
```

```
##
##  Welch Two Sample t-test
##
## data:  plant_senescent_area_cm² by treatment
## t = -1.1824, df = 13, p-value = 0.2582
## alternative hypothesis: true difference in means between group sterile and group unsterile is not equal to 0
## 95 percent confidence interval:
##  -0.10051789  0.02940841
## sample estimates:
##   mean in group sterile mean in group unsterile
##            1.427901e-05            3.556902e-02
```

Figure 102: Welch t-test of soil 125

Appendix C: Transplantation Experiment of Soil 117

The statistical analysis of the soil 117 transplantation experiment can be found in Figure 103 to Figure 110.

Welch ANOVA welch anova

```
welch_117_area <- new_transplant%>% welch_anova_test(`plant_region_area_cm²`~treatment)
welch_117_area
```

```
## # A tibble: 1 x 7
##   .y.                      n statistic   DFn   DFd          p method
## * <chr>                <int>     <dbl> <dbl> <dbl>      <dbl> <chr>
## 1 plant_region_area_cm²    99      12.5     3  49.8 0.00000336 Welch ANOVA
```

```
games_117_area <- new_transplant %>% games_howell_test(`plant_region_area_cm²` ~ treatment)
games_117_area
```

```
## # A tibble: 6 x 8
##   .y.             group1 group2 estimate conf.low conf.high   p.adj p.adj.signif
## * <chr>           <chr>  <chr>     <dbl>    <dbl>     <dbl>   <dbl> <chr>
## 1 plant_region_a~ endo   endor~    1.84     0.760     2.92  3.72e-4 ***
## 2 plant_region_a~ endo   neg       0.942    0.310     1.57  1   e-3 ***
## 3 plant_region_a~ endo   pos       1.46     0.702     2.21  2.82e-5 ****
## 4 plant_region_a~ endor~ neg      -0.900   -1.96      0.162 1.19e-1 ns
## 5 plant_region_a~ endor~ pos      -0.383   -1.51      0.748 7.98e-1 ns
## 6 plant_region_a~ neg    pos       0.517   -0.207     1.24  2.41e-1 ns
```

Figure 103: Results of Welch ANOVA and Games-Howell test of the plant area in the soil 117 transplantation experiment

Kruskall-wallis

```
kruskall_117_senescent_area <- new_transplant %>% kruskal_test(`plant_senescent_area_cm²`~treatment)
kruskall_117_senescent_area
```

```
## # A tibble: 1 x 6
##   .y.                          n statistic    df     p method
## * <chr>                    <int>     <dbl> <int> <dbl> <chr>
## 1 plant_senescent_area_cm²    99      4.77     3 0.189 Kruskal-Wallis
```

```
wilcox_117_senescent_area <- new_transplant %>% wilcox_test(`plant_senescent_area_cm²` ~ treatment, p.adjust.meth
od = "bonferroni")
wilcox_117_senescent_area
```

```
## # A tibble: 6 x 9
##   .y.              group1 group2    n1    n2 statistic     p p.adj p.adj.signif
## * <chr>            <chr>  <chr>  <int> <int>     <dbl> <dbl> <dbl> <chr>
## 1 plant_senescent_~ endo   endor~    25    21      300. 0.401 1     ns
## 2 plant_senescent_~ endo   neg       25    25      412  0.045 0.268 ns
## 3 plant_senescent_~ endo   pos       25    28      426. 0.162 0.972 ns
## 4 plant_senescent_~ endor~ neg       21    25      318. 0.203 1     ns
## 5 plant_senescent_~ endor~ pos       21    28      322. 0.556 1     ns
## 6 plant_senescent_~ neg    pos       25    28      308. 0.424 1     ns
```

Figure 104: Results of Kruskall Wallis test and Wilcoxon test of the senescent area in the soil 117 transplantation experiment

welch one way anova

```
weight_welch_aov <- X117_transplant_fresh_weight %>% welch_anova_test(weight~treatment)
weight_welch_aov
```

```
## # A tibble: 1 x 7
##   .y.        n statistic   DFn   DFd          p method
## * <chr>  <int>     <dbl> <dbl> <dbl>      <dbl> <chr>
## 1 weight    95      13.0     3  47.4 0.00000246 Welch ANOVA
```

```
welch_post_weight <- X117_transplant_fresh_weight %>% games_howell_test(weight~treatment)
welch_post_weight
```

```
## # A tibble: 6 x 8
##   .y.    group1          group2 estimate conf.low conf.high   p.adj p.adj.signif
## * <chr>  <chr>           <chr>     <dbl>    <dbl>     <dbl>   <dbl> <chr>
## 1 weight endo            endo+~  0.0338   0.0120   0.0555   1   e-3 ***
## 2 weight endo            negat~  0.0116  -0.00111  0.0242   8.5 e-2 ns
## 3 weight endo            posit~  0.0386   0.0197   0.0575   1.58e-5 ****
## 4 weight endo+rhizo      negat~ -0.0222  -0.0439  -0.000469 4.4 e-2 *
## 5 weight endo+rhizo      posit~  0.00482 -0.0206   0.0303   9.57e-1 ns
## 6 weight negative contr~ posit~  0.0270   0.00810  0.0459   2   e-3 **
```

Figure 105: Results of the Welch ANOVA and Games-Howell test of the plant fresh weight in the soil 117 transplantation experiment

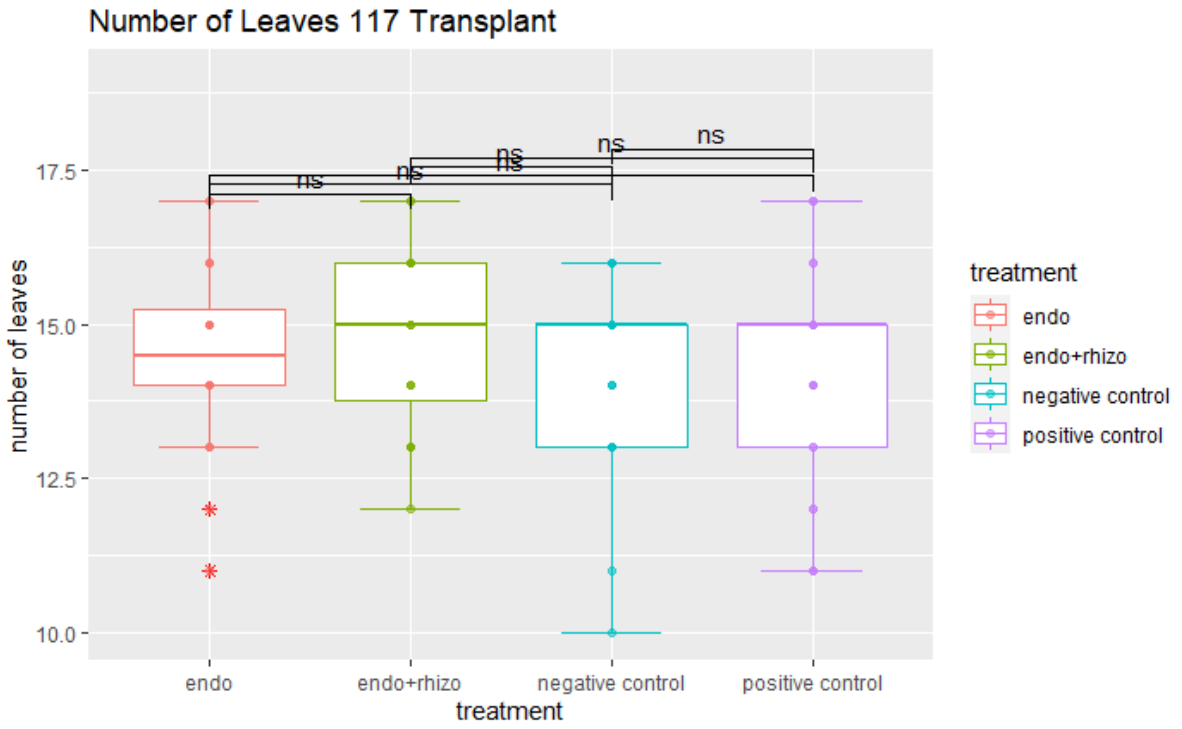

Figure 106: Total number of leaves of the plants in the soil 117 transplantation experiment. Statistical significance was determined by a Kruskall-Wallis test

kruskall-wallis test

```
leaves_kruskall <- X117_transplant_leaves %>% kruskal_test(`total amount of leaves`~treatment)
leaves_kruskall
```

```
## # A tibble: 1 x 6
##   .y.                     n statistic    df     p method
## * <chr>                 <int>     <dbl> <int> <dbl> <chr>
## 1 total amount of leaves    92      2.12     3 0.548 Kruskal-Wallis
```

Figure 107: Results of the Kruskall-Wallis test of the total number of leaves of the plants in the soil 117 transplantation experiment

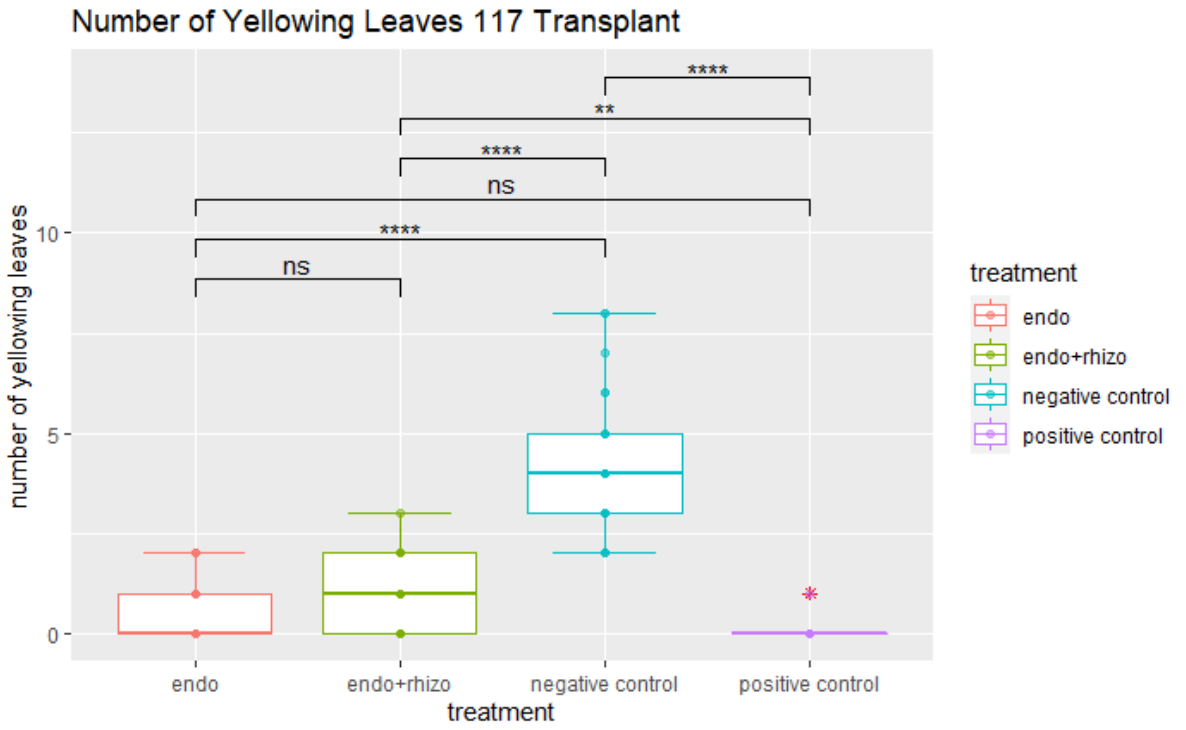

Figure 108: Number of leaves with yellow area of plants in the soil 117 transplantation experiment. Statistical significance was determined by a Welch ANOVA followed by a Games-Howell test

welch one way anova

```
welch_aov_leave <- X117_transplant_leaves %>% welch_anova_test(`chlorotic leaves`~treatment)
welch_aov_leave
```

```
## # A tibble: 1 x 7
##   .y.                n statistic   DFn   DFd        p method
## * <chr>            <int>     <dbl> <dbl> <dbl>    <dbl> <chr>
## 1 chlorotic leaves    92      46.6     3  37.8 8.76e-13 Welch ANOVA
```

```
chl_welch_post_leave <- X117_transplant_leaves %>% games_howell_test(`chlorotic leaves`~treatment)
chl_welch_post_leave
```

```
## # A tibble: 6 x 8
##   .y.            group1 group2 estimate conf.low conf.high    p.adj p.adj.signif
## * <chr>          <chr>  <chr>     <dbl>    <dbl>     <dbl>    <dbl> <chr>
## 1 chlorotic lea~ endo   endo+~    0.533   -0.160    1.23   1.81e- 1 ns
## 2 chlorotic lea~ endo   negat~    3.80     2.71     4.89   1.32e- 9 ****
## 3 chlorotic lea~ endo   posit~   -0.377   -0.793    0.0393 8.6 e- 2 ns
## 4 chlorotic lea~ endo+~ negat~    3.27     2.11     4.42   3.85e- 8 ****
## 5 chlorotic lea~ endo+~ posit~   -0.91    -1.51    -0.309  2   e- 3 **
## 6 chlorotic lea~ negat~ posit~   -4.18    -5.21    -3.14   6.83e-10 ****
```

Figure 109: Results of the Welch ANOVA and Games-Howell test of the number of leaves with yellow area of the plants in the soil 117 transplantation experiment

welch one way anova

```
ratio_welch_aov_leave <- X117_transplant_leaves_r %>% welch_anova_test(ratio~treatment)
ratio_welch_aov_leave
```

```
## # A tibble: 1 x 7
##   .y.       n statistic   DFn   DFd        p method
## * <chr> <int>     <dbl> <dbl> <dbl>    <dbl> <chr>
## 1 ratio    92      54.2     3  37.7 9.59e-14 Welch ANOVA
```

```
ratio_welch_post_leave <- X117_transplant_leaves_r %>% games_howell_test(ratio~treatment)
ratio_welch_post_leave
```

```
## # A tibble: 6 x 8
##   .y.   group1          group2 estimate conf.low conf.high    p.adj p.adj.signif
## * <chr> <chr>           <chr>     <dbl>    <dbl>     <dbl>    <dbl> <chr>
## 1 ratio endo            endo+~   0.0329  -0.0138   0.0795  2.48e- 1 ns
## 2 ratio endo            negat~   0.269    0.198    0.341   1.52e-10 ****
## 3 ratio endo            posit~  -0.0268  -0.0567   0.00310 9.1 e- 2 ns
## 4 ratio endo+rhizo      negat~   0.236    0.161    0.311   3.28e- 9 ****
## 5 ratio endo+rhizo      posit~  -0.0596  -0.0988  -0.0204  2   e- 3 **
## 6 ratio negative contr~ posit~  -0.296   -0.363   -0.229   1.23e-10 ****
```

Figure 110: Results of the Welch ANOVA and Games-Howell test of the ratio of total to yellow leaves of the plants in the soil 117 transplantation experiment

Appendix D: Soil Dilution and Nutrient Rescue Experiment

The statistical analysis and the remaining figures of all soils in the soil dilution and nutrient rescue experiments are shown in the following figures.

Soil 71

Kruskall-wallis

```
kruskall_71_SD_area <- NRE71 %>% filter(experiment=="SD")%>% kruskal_test(total~treatment)
kruskall_71_SD_area
```

```
## # A tibble: 1 x 6
##   .y.       n statistic    df     p method
## * <chr> <int>     <dbl> <int> <dbl> <chr>
## 1 total   146    0.0875     2 0.957 Kruskal-Wallis
```

```
wilcox_71_SD_area <- NRE71 %>% filter(experiment == "SD") %>% wilcox_test(total ~ treatment, p.adjust.method = "bonferroni")
wilcox_71_SD_area
```

```
## # A tibble: 3 x 9
##   .y.   group1  group2       n1    n2 statistic     p p.adj p.adj.signif
## * <chr> <chr>   <chr>     <int> <int>     <dbl> <dbl> <dbl> <chr>
## 1 total heat    sterile      40    35       723 0.812     1 ns
## 2 total heat    unsterile    40    71      1422 0.993     1 ns
## 3 total sterile unsterile    35    71      1201 0.783     1 ns
```

Figure 111: Results of the Kruskall-Wallis and Wilcoxon test for the total plant area for the soil dilution experiment of soil 71

```
## # A tibble: 1 x 7
##   .y.        n statistic   DFn   DFd     p method
## * <chr>  <int>     <dbl> <dbl> <dbl> <dbl> <chr>
## 1 leaves   154      3.19     4  43.2 0.022 Welch ANOVA
```

```
welch_post_71_leaves <- SD71 %>% games_howell_test(leaves~treatment)
welch_post_71_leaves
```

```
## # A tibble: 10 x 8
##    .y.    group1      group2      estimate conf.low conf.high p.adj p.adj.signif
##  * <chr>  <chr>       <chr>          <dbl>    <dbl>     <dbl> <dbl> <chr>
##  1 leaves heat        NRE heat       2.8     -0.409     6.01  0.111 ns
##  2 leaves heat        NRE sterile    3.16    -1.69      8.00  0.334 ns
##  3 leaves heat        sterile        1.9     -0.974     4.77  0.337 ns
##  4 leaves heat        unsterile      0.311   -1.61      2.23  0.989 ns
##  5 leaves NRE heat    NRE sterile    0.358   -4.83      5.54  1     ns
##  6 leaves NRE heat    sterile       -0.9     -4.41      2.61  0.947 ns
##  7 leaves NRE heat    unsterile     -2.49    -5.35      0.372 0.109 ns
##  8 leaves NRE sterile sterile       -1.26    -6.28      3.76  0.947 ns
##  9 leaves NRE sterile unsterile     -2.85    -7.50      1.80  0.381 ns
## 10 leaves sterile     unsterile     -1.59    -4.05      0.874 0.341 ns
```

Figure 112: Results of the Welch ANOVA and Games-Howell test for the number of leaves in the nutrient rescue experiment of soil 71

welch one way anova

```
branches_71_NRE_welch_aov <- SD71 %>% welch_anova_test(Branches~treatment)
branches_71_NRE_welch_aov
```

```
## # A tibble: 1 x 7
##   .y.          n statistic   DFn   DFd        p method
## * <chr>    <int>     <dbl> <dbl> <dbl>    <dbl> <chr>
## 1 Branches   154      24.1     4  48.5 5.24e-11 Welch ANOVA
```

```
welch_post_branches_71_NRE <- SD71 %>% games_howell_test(Branches~treatment)
welch_post_branches_71_NRE
```

```
## # A tibble: 10 x 8
##    .y.      group1      group2  estimate conf.low conf.high   p.adj p.adj.signif
##  * <chr>    <chr>       <chr>      <dbl>    <dbl>     <dbl>   <dbl> <chr>
##  1 Branches heat        NRE he~   2.16      1.08      3.25  3.05e-5 ****
##  2 Branches heat        NRE st~   2.19      1.23      3.15  3.2 e-6 ****
##  3 Branches heat        sterile  -0.186    -0.721     0.349 8.53e-1 ns
##  4 Branches heat        unster~  -0.0290   -0.544     0.486 1   e+0 ns
##  5 Branches NRE heat    NRE st~   0.0237   -1.25      1.30  1   e+0 ns
##  6 Branches NRE heat    sterile  -2.35     -3.39     -1.31  8.35e-6 ****
##  7 Branches NRE heat    unster~  -2.19     -3.22     -1.16  2.32e-5 ****
##  8 Branches NRE sterile sterile  -2.37     -3.28     -1.46  8.81e-7 ****
##  9 Branches NRE sterile unster~  -2.22     -3.12     -1.32  2.76e-6 ****
## 10 Branches sterile     unster~   0.157    -0.217     0.530 7.58e-1 ns
```

Figure 113: Results of the Welch ANOVA and Games-Howell test for the number of branches in the nutrient rescue experiment of soil 71

normal one way anova -use

```
height_71_NRE_aov <- SD71 %>% anova_test(Height~treatment)
```

```
## Coefficient covariances computed by hccm()
```

```
height_71_NRE_aov
```

```
## ANOVA Table (type II tests)
##
##      Effect DFn DFd    F        p p<.05   ges
## 1 treatment   4 149 6.17 0.000127     * 0.142
```

```
post_height_71_NRE <- SD71 %>% tukey_hsd(Height~treatment)
post_height_71_NRE
```

```
## # A tibble: 10 x 9
##    term      group1      group2   null.value estimate conf.low conf.high   p.adj
##  * <chr>     <chr>       <chr>         <dbl>    <dbl>    <dbl>     <dbl>   <dbl>
##  1 treatment heat        NRE heat          0    5.64    -0.316    11.6   7.27e-2
##  2 treatment heat        NRE ste~          0    2.38    -3.66      8.41  8.13e-1
##  3 treatment heat        sterile           0   -2.72    -8.67      3.23  7.15e-1
##  4 treatment heat        unsteri~          0   -2.00    -6.71      2.71  7.66e-1
##  5 treatment NRE heat    NRE ste~          0   -3.26    -9.36      2.84  5.8 e-1
##  6 treatment NRE heat    sterile           0   -8.36   -14.4      -2.33  1.74e-3
##  7 treatment NRE heat    unsteri~          0   -7.64   -12.4      -2.84  2.02e-4
##  8 treatment NRE sterile sterile           0   -5.09   -11.2       1.01  1.49e-1
##  9 treatment NRE sterile unsteri~          0   -4.38    -9.28      0.520 1.04e-1
## 10 treatment sterile     unsteri~          0    0.716   -4.08      5.52  9.94e-1
## # ... with 1 more variable: p.adj.signif <chr>
```

Figure 114: Results of the one-way ANOVA and Tukey post-hoc test for the plant height in the nutrient rescue experiment of soil 71

welch one way anova

```
silique_71_NRE_welch_aov <- SD71 %>% welch_anova_test(Siliques~treatment)
silique_71_NRE_welch_aov
```

```
## # A tibble: 1 x 7
##   .y.          n statistic   DFn   DFd     p method
## * <chr>    <int>     <dbl> <dbl> <dbl> <dbl> <chr>
## 1 Siliques   154      3.01     4  44.6 0.028 Welch ANOVA
```

```
welch_post_silique_71_NRE <- SD71 %>% games_howell_test(Siliques~treatment)
welch_post_silique_71_NRE
```

```
## # A tibble: 10 x 8
##    .y.      group1      group2    estimate conf.low conf.high p.adj p.adj.signif
##  * <chr>    <chr>       <chr>        <dbl>    <dbl>     <dbl> <dbl> <chr>
##  1 Siliques heat        NRE heat      7.55    -2.75    17.9    0.237 ns
##  2 Siliques heat        NRE ster~     7.90    -9.12    24.9    0.647 ns
##  3 Siliques heat        sterile      -2.30    -9.49     4.90   0.89  ns
##  4 Siliques heat        unsterile    -1.92    -7.72     3.88   0.869 ns
##  5 Siliques NRE heat    NRE ster~     0.350   -17.8     18.5    1     ns
##  6 Siliques NRE heat    sterile      -9.85    -20.1      0.358  0.063 ns
##  7 Siliques NRE heat    unsterile    -9.47    -18.9     -0.0663 0.048 *
##  8 Siliques NRE sterile sterile     -10.2     -27.2      6.77   0.406 ns
##  9 Siliques NRE sterile unsterile    -9.82    -26.4      6.74   0.41  ns
## 10 Siliques sterile     unsterile     0.376    -5.21     5.96   1     ns
```

Figure 115: Results of the Welch ANOVA and Games-Howell test for the silique number in the nutrient rescue experiment of soil 71

```
##
##  Welch Two Sample t-test
##
## data:  antho_µg/g by treatment
## t = -9.0915, df = 2.1009, p-value = 0.01015
## alternative hypothesis: true difference in means between group NRE and group sterile is not equal to 0
## 95 percent confidence interval:
##  -40.02772 -15.10172
## sample estimates:
##     mean in group NRE mean in group sterile
##            -0.9088075            26.6559113
```

Figure 116: Results of the Welch t-test of the anthocyanin content of plants grown in sterile soil 71 either with or without nutrients

Soil 109

Kruskall-wallis

```
kruskall_109_SD_area <- NRE109 %>% filter(experiment=="SD")%>% kruskal_test(total~treatment)
kruskall_109_SD_area
```

```
## # A tibble: 1 x 6
##   .y.       n statistic    df       p method
## * <chr> <int>     <dbl> <int>   <dbl> <chr>
## 1 total   143      12.8     2 0.00167 Kruskal-Wallis
```

```
wilcox_109_SD_area <- NRE109 %>% filter(experiment == "SD") %>% wilcox_test(total ~ treatment, p.adjust.method =
"bonferroni")
wilcox_109_SD_area
```

```
## # A tibble: 3 x 9
##   .y.   group1  group2       n1    n2 statistic        p p.adj p.adj.signif
## * <chr> <chr>   <chr>     <int> <int>     <dbl>    <dbl> <dbl> <chr>
## 1 total heat    sterile      28    35      732. 0.000816 0.002 **
## 2 total heat    unsterile    28    80     1370  0.08     0.241 ns
## 3 total sterile unsterile    35    80      971  0.009    0.028 *
```

Figure 117: Results of the Kruskall-Wallis and Wilcoxon test for the plant area in the soil dilution experiment of soil 109

welch anova

```
welch_109_NRE_area <- NRE109 %>% filter(experiment =="NRE") %>% welch_anova_test(total~treatment)
welch_109_NRE_area
```

```
## # A tibble: 1 x 7
##   .y.       n statistic   DFn   DFd         p method
## * <chr> <int>     <dbl> <dbl> <dbl>     <dbl> <chr>
## 1 total   148      9.85     4  39.0 0.0000132 Welch ANOVA
```

```
welch_post_109_NRE_area <- NRE109 %>% filter(experiment =="NRE") %>% games_howell_test(total~treatment)
welch_post_109_NRE_area
```

```
## # A tibble: 10 x 8
##    .y.   group1      group2      estimate conf.low conf.high p.adj p.adj.signif
##  * <chr> <chr>       <chr>          <dbl>    <dbl>     <dbl> <dbl> <chr>
##  1 total heat        heat NRE      0.0710  -0.244     0.386  0.963 ns
##  2 total heat        sterile      -0.242   -0.421    -0.0631 0.005 **
##  3 total heat        sterile NRE   0.244   -0.125     0.614  0.318 ns
##  4 total heat        unsterile    -0.0855  -0.262     0.0914 0.605 ns
##  5 total heat NRE    sterile      -0.313   -0.605    -0.0207 0.032 *
##  6 total heat NRE    sterile NRE   0.173   -0.251     0.597  0.761 ns
##  7 total heat NRE    unsterile    -0.156   -0.448     0.135  0.504 ns
##  8 total sterile     sterile NRE   0.486    0.135     0.838  0.004 **
##  9 total sterile     unsterile     0.156    0.0506    0.262  0.001 ***
## 10 total sterile NRE unsterile    -0.330   -0.680     0.0211 0.071 ns
```

Figure 118: Results of the Welch ANOVA and Games-Howell test for the plant area in the nutrient rescue experiment of soil 109

Soil 116

Kruskall-wallis

```
kruskall_116_SD_area <- NRE116 %>% filter(experiment=="SD")%>% kruskal_test(total~treatment)
kruskall_116_SD_area
```

```
## # A tibble: 1 x 6
##   .y.       n statistic    df     p method
## * <chr> <int>     <dbl> <int> <dbl> <chr>
## 1 total   118     0.564     2 0.754 Kruskal-Wallis
```

```
wilcox_116_SD_area <- NRE116 %>% filter(experiment == "SD") %>% wilcox_test(total ~ treatment, p.adjust.method
 = "bonferroni")
wilcox_116_SD_area
```

```
## # A tibble: 3 x 9
##   .y.   group1  group2       n1    n2 statistic     p p.adj p.adj.signif
## * <chr> <chr>   <chr>     <int> <int>     <dbl> <dbl> <dbl> <chr>
## 1 total heat    sterile      41    22       429 0.758     1 ns
## 2 total heat    unsterile    41    55      1023 0.441     1 ns
## 3 total sterile unsterile    22    55       588 0.852     1 ns
```

Figure 119: Results of the Kruskall-Wallis test and Wilcoxon test for the total plant area in the soil dilution experiment of soil 116

welch anova

```
welch_116_SD_yellow_area <- NRE116 %>% filter(experiment=="SD")%>% welch_anova_test(yellow~treatment)
welch_116_SD_yellow_area
```

```
## # A tibble: 1 x 7
##   .y.        n statistic   DFn   DFd     p method
## * <chr>  <int>     <dbl> <dbl> <dbl> <dbl> <chr>
## 1 yellow   118      2.95     2  46.0 0.062 Welch ANOVA
```

```
games_116_SD_yellow_area <- NRE116 %>% filter(experiment == "SD") %>% games_howell_test(yellow ~ treatment)
games_116_SD_yellow_area
```

```
## # A tibble: 3 x 8
##   .y.    group1  group2    estimate conf.low conf.high p.adj p.adj.signif
## * <chr>  <chr>   <chr>        <dbl>    <dbl>     <dbl> <dbl> <chr>
## 1 yellow heat    sterile     0.0423  -0.0879    0.172  0.706 ns
## 2 yellow heat    unsterile  -0.0493  -0.113     0.0140 0.156 ns
## 3 yellow sterile unsterile  -0.0915  -0.216     0.0326 0.178 ns
```

Figure 120: Results for the Welch ANOVA and Games-Howell test for the yellow plant area in the soil dilution experiment of soil 116

welch anova

```
welch_116_NRE_area <- NRE116 %>% filter(experiment =="NRE") %>% welch_anova_test(total~treatment)
welch_116_NRE_area
```

```
## # A tibble: 1 x 7
##   .y.       n statistic   DFn   DFd         p method
## * <chr> <int>     <dbl> <dbl> <dbl>     <dbl> <chr>
## 1 total   118      8.58     4  30.2 0.0000949 Welch ANOVA
```

```
welch_post_116_NRE_area <- NRE116 %>% filter(experiment =="NRE") %>% games_howell_test(total~treatment)
welch_post_116_NRE_area
```

```
## # A tibble: 10 x 8
##    .y.   group1      group2      estimate conf.low conf.high p.adj p.adj.signif
##  * <chr> <chr>       <chr>          <dbl>    <dbl>     <dbl> <dbl> <chr>
##  1 total heat        heat NRE       2.63     0.893     4.37  0.001 ***
##  2 total heat        sterile        0.575   -0.658     1.81  0.624 ns
##  3 total heat        sterile NRE    3.52     1.05      5.98  0.005 **
##  4 total heat        unsterile      0.470   -0.267     1.21  0.37  ns
##  5 total heat NRE    sterile       -2.06    -3.94     -0.177 0.027 *
##  6 total heat NRE    sterile NRE    0.884   -1.87      3.64  0.873 ns
##  7 total heat NRE    unsterile     -2.16    -3.84     -0.483 0.008 **
##  8 total sterile     sterile NRE    2.94     0.405     5.48  0.02  *
##  9 total sterile     unsterile     -0.105   -1.27      1.05  0.998 ns
## 10 total sterile NRE unsterile     -3.05    -5.48     -0.612 0.013 *
```

Figure 121: Results for the Welch ANOVA and Games-Howell test for the total plant area in the nutrient rescue experiment of soil 116

Kruskall-wallis

```
kruskall_116_NRE_yellow_area <- NRE116 %>% filter(experiment=="NRE")%>% kruskal_test(yellow~treatment)
kruskall_116_NRE_yellow_area
```

```
## # A tibble: 1 x 6
##   .y.        n statistic    df      p method
## * <chr>  <int>     <dbl> <int>  <dbl> <chr>
## 1 yellow   118      10.5     4 0.0327 Kruskal-Wallis
```

```
wilcox_116_NRE_yellow_area <- NRE116 %>% filter(experiment == "NRE") %>% wilcox_test(yellow ~ treatment, p.adjus
t.method = "bonferroni")
wilcox_116_NRE_yellow_area
```

```
## # A tibble: 10 x 9
##    .y.    group1      group2         n1    n2 statistic     p p.adj p.adj.signif
##  * <chr>  <chr>       <chr>       <int> <int>     <dbl> <dbl> <dbl> <chr>
##  1 yellow heat        heat NRE       21    19     266   0.066 0.663 ns
##  2 yellow heat        sterile        21    10      79.5 0.289 1     ns
##  3 yellow heat        sterile NRE    21    12     142.  0.544 1     ns
##  4 yellow heat        unsterile      21    56     486.  0.245 1     ns
##  5 yellow heat NRE    sterile        19    10      42   0.013 0.133 ns
##  6 yellow heat NRE    sterile NRE    19    12      98   0.505 1     ns
##  7 yellow heat NRE    unsterile      19    56     311   0.006 0.064 ns
##  8 yellow sterile     sterile NRE    10    12      81.5 0.162 1     ns
##  9 yellow sterile     unsterile      10    56     296.  0.774 1     ns
## 10 yellow sterile NRE unsterile      12    56     246.  0.144 1     ns
```

Figure 122: Results for the Kruskall-Wallis test and Wilcoxon test for the yellow plant area in the nutrient rescue experiment of soil 116

welch one way anova -> use

```
leaves_116_welch_aov <- SD116 %>% welch_anova_test(leaves~treatment)
leaves_116_welch_aov
```

```
## # A tibble: 1 x 7
##   .y.        n statistic   DFn   DFd         p method
## * <chr>  <int>     <dbl> <dbl> <dbl>     <dbl> <chr>
## 1 leaves   122      9.54     4  32.5 0.0000326 Welch ANOVA
```

```
welch_post_116_leaves <- SD116 %>% games_howell_test(leaves~treatment)
welch_post_116_leaves
```

```
## # A tibble: 10 x 8
##    .y.    group1      group2    estimate conf.low conf.high   p.adj p.adj.signif
##  * <chr>  <chr>       <chr>        <dbl>    <dbl>     <dbl>   <dbl> <chr>
##  1 leaves heat        NRE heat     4.35     1.55      7.15  8.84e-4 ***
##  2 leaves heat        NRE ster~    6.58     1.03     12.1   1.7 e-2 *
##  3 leaves heat        sterile      0.109   -2.22      2.44  1   e+0 ns
##  4 leaves heat        unsterile    2.03     0.574     3.48  3   e-3 **
##  5 leaves NRE heat    NRE ster~    2.23    -3.57      8.04  7.71e-1 ns
##  6 leaves NRE heat    sterile     -4.24    -7.35     -1.14  4   e-3 **
##  7 leaves NRE heat    unsterile   -2.32    -4.96      0.318 1.03e-1 ns
##  8 leaves NRE sterile sterile     -6.48   -12.1      -0.821 2.1 e-2 *
##  9 leaves NRE sterile unsterile   -4.56   -10.1       0.944 1.25e-1 ns
## 10 leaves sterile     unsterile    1.92    -0.234     4.07  9   e-2 ns
```

Figure 123: Results for the Welch ANOVA and Games-Howell test for the total number of leaves in the nutrient rescue experiment of soil 116

Kruskall-wallis

```
kruskall_116_NRE_yellow_leaves <- SD116 %>% kruskal_test(chlorosis~treatment)
kruskall_116_NRE_yellow_leaves
```

```
## # A tibble: 1 x 6
##   .y.           n statistic    df         p method
## * <chr>     <int>     <dbl> <int>     <dbl> <chr>
## 1 chlorosis   122      26.8     4 0.0000221 Kruskal-Wallis
```

```
wilcox_116_NRE_yellow_leaves <- SD116 %>% wilcox_test(chlorosis ~ treatment, p.adjust.method = "bonferroni")
wilcox_116_NRE_yellow_leaves
```

```
## # A tibble: 10 x 9
##    .y.       group1      group2    n1    n2 statistic       p p.adj p.adj.signif
##  * <chr>     <chr>       <chr>  <int> <int>     <dbl>   <dbl> <dbl> <chr>
##  1 chlorosis heat        NRE h~    20    20     332.  2.87e-4 0.003 **
##  2 chlorosis heat        NRE s~    20    13     222.  6.53e-4 0.007 **
##  3 chlorosis heat        steri~    20    11      88.5 3.76e-1 1     ns
##  4 chlorosis heat        unste~    20    58     668.  3.09e-1 1     ns
##  5 chlorosis NRE heat    NRE s~    20    13     140.  6.94e-1 1     ns
##  6 chlorosis NRE heat    steri~    20    11      41.5 4   e-3 0.039 *
##  7 chlorosis NRE heat    unste~    20    58     279   5.02e-4 0.005 **
##  8 chlorosis NRE sterile steri~    13    11      24   5   e-3 0.05  *
##  9 chlorosis NRE sterile unste~    13    58     166.  2   e-3 0.016 *
## 10 chlorosis sterile     unste~    11    58     402.  1.69e-1 1     ns
```

Figure 124: Results for the Kruskall-Wallis test and Wilcoxon test for the number of yellow leaves in the nutrient rescue experiment of soil 116

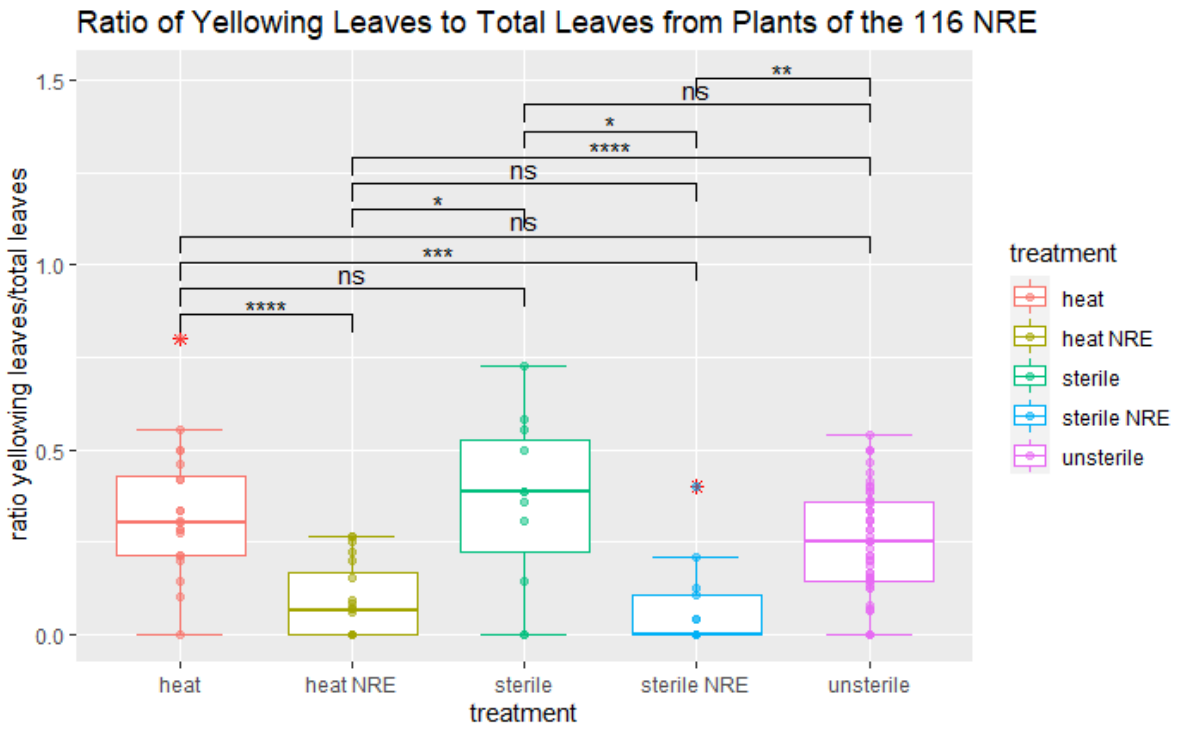

Figure 125: Ratio of yellow leaves to total number of leaves of plants grown in the soil 116 nutrient rescue experiment. Statistical significance was determined by a Welch ANOVA followed by a Games-Howell test

welch one way anova - use

```
ratio_116_NRE_welch_aov <- SD116 %>% welch_anova_test(ratio~treatment)
ratio_116_NRE_welch_aov
```

```
## # A tibble: 1 x 7
##   .y.       n statistic   DFn   DFd           p method
## * <chr> <int>     <dbl> <dbl> <dbl>       <dbl> <chr>
## 1 ratio   122      13.8     4  35.1 0.000000766 Welch ANOVA
```

```
welch_post_ratio_116_NRE <- SD116 %>% games_howell_test(ratio~treatment)
welch_post_ratio_116_NRE
```

```
## # A tibble: 10 x 8
##    .y.   group1      group2     estimate conf.low conf.high   p.adj p.adj.signif
##  * <chr> <chr>       <chr>         <dbl>    <dbl>     <dbl>   <dbl> <chr>
##  1 ratio heat        NRE heat    -0.245   -0.379    -0.112  8.63e-5 ****
##  2 ratio heat        NRE steri~  -0.261   -0.411    -0.111  1.82e-4 ***
##  3 ratio heat        sterile      0.0264  -0.222     0.275  9.97e-1 ns
##  4 ratio heat        unsterile   -0.0904  -0.220     0.0396 2.81e-1 ns
##  5 ratio NRE heat    NRE steri~  -0.0155  -0.134     0.103  9.95e-1 ns
##  6 ratio NRE heat    sterile      0.272    0.0347    0.509  2.2 e-2 *
##  7 ratio NRE heat    unsterile    0.155    0.0695    0.240  4.57e-5 ****
##  8 ratio NRE sterile sterile      0.287    0.0442    0.530  1.7 e-2 *
##  9 ratio NRE sterile unsterile    0.170    0.0562    0.284  2   e-3 **
## 10 ratio sterile     unsterile   -0.117   -0.353     0.119  5.32e-1 ns
```

Figure 126: Results for the Welch ANOVA and Games-Howell test for the ratio of yellow to total number of leaves in the nutrient rescue experiment of soil 116

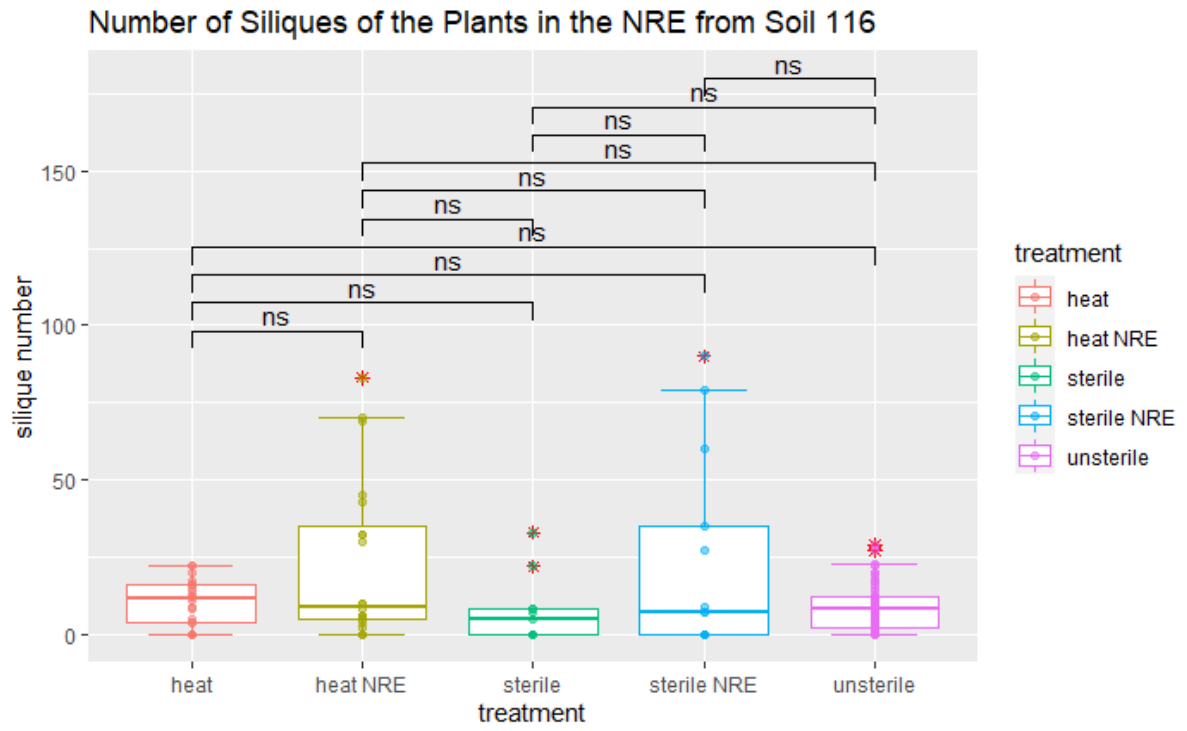

Figure 127: Silique number of plants grown in the soil 116 nutrient rescue experiment. Statistical significance was determined by a Welch ANOVA followed by a Games-Howell test

welch one way anova

```
silique_116_NRE_welch_aov <- SD116 %>% welch_anova_test(Siliques~treatment)
silique_116_NRE_welch_aov
```

```
## # A tibble: 1 x 7
##   .y.          n statistic   DFn   DFd     p method
## * <chr>    <int>     <dbl> <dbl> <dbl> <dbl> <chr>
## 1 Siliques   122      2.22     4  31.4 0.089 Welch ANOVA
```

```
welch_post_silique_116_NRE <- SD116 %>% games_howell_test(Siliques~treatment)
welch_post_silique_116_NRE
```

```
## # A tibble: 10 x 8
##    .y.      group1      group2    estimate conf.low conf.high p.adj p.adj.signif
##  * <chr>    <chr>       <chr>        <dbl>    <dbl>     <dbl> <dbl> <chr>
##  1 Siliques heat        NRE heat     12.7     -5.45     30.8  0.265 ns
##  2 Siliques heat        NRE ster~    13.8    -14.9      42.5  0.571 ns
##  3 Siliques heat        sterile      -2.80   -14.0       8.38 0.935 ns
##  4 Siliques heat        unsterile    -1.76    -7.32      3.79 0.888 ns
##  5 Siliques NRE heat    NRE ster~     1.10   -30.7      32.9  1     ns
##  6 Siliques NRE heat    sterile     -15.5    -35.1       4.08 0.172 ns
##  7 Siliques NRE heat    unsterile   -14.5    -32.3       3.38 0.149 ns
##  8 Siliques NRE sterile sterile     -16.6    -46.0      12.8  0.438 ns
##  9 Siliques NRE sterile unsterile   -15.6    -44.1      13.0  0.452 ns
## 10 Siliques sterile     unsterile     1.04    -9.73     11.8  0.998 ns
```

Figure 128: Results for the Welch ANOVA and Games-Howell test for the number of siliques in the nutrient rescue experiment of soil 116

welch one way anova

```
branches_116_NRE_welch_aov <- SD116 %>% welch_anova_test(Branches~treatment)
branches_116_NRE_welch_aov
```

```
## # A tibble: 1 x 7
##   .y.          n statistic   DFn   DFd        p method
## * <chr>    <int>     <dbl> <dbl> <dbl>    <dbl> <chr>
## 1 Branches   122      6.98     4  33.5 0.000335 Welch ANOVA
```

```
welch_post_branches_116_NRE <- SD116 %>% games_howell_test(Branches~treatment)
welch_post_branches_116_NRE
```

```
## # A tibble: 10 x 8
##    .y.      group1      group2    estimate conf.low conf.high p.adj p.adj.signif
##  * <chr>    <chr>       <chr>        <dbl>    <dbl>     <dbl> <dbl> <chr>
##  1 Branches heat        NRE heat    1.8      0.512     3.09   0.004 **
##  2 Branches heat        NRE ster~   1.77    -0.287     3.83   0.106 ns
##  3 Branches heat        sterile     0.636   -0.490     1.76   0.415 ns
##  4 Branches heat        unsterile   0.483    0.0274    0.938  0.032 *
##  5 Branches NRE heat    NRE ster~  -0.0308  -2.31      2.25   1     ns
##  6 Branches NRE heat    sterile    -1.16    -2.73      0.405  0.224 ns
##  7 Branches NRE heat    unsterile  -1.32    -2.62     -0.0172 0.046 *
##  8 Branches NRE sterile sterile    -1.13    -3.33      1.06   0.539 ns
##  9 Branches NRE sterile unsterile  -1.29    -3.35      0.775  0.334 ns
## 10 Branches sterile     unsterile  -0.154   -1.29      0.982  0.992 ns
```

Figure 129: Results for the Welch ANOVA and Games-Howell test for the number of branches in the nutrient rescue experiment of soil 116

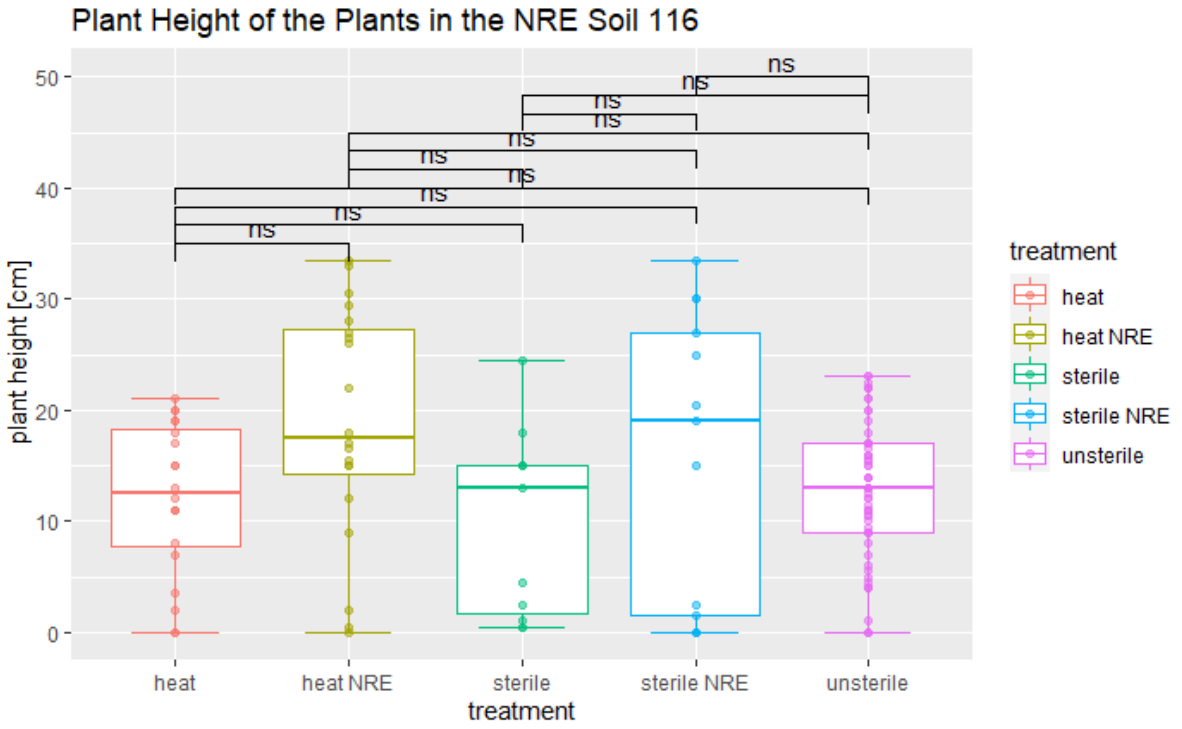

Figure 130: Plant height of plants grown in the soil 116 nutrient rescue experiment. Statistical significance was determined by a Welch ANOVA followed by a Games-Howell test

welch one way anova

```
height_116_NRE_welch_aov <- SD116 %>% welch_anova_test(Height~treatment)
height_116_NRE_welch_aov
```

```
## # A tibble: 1 x 7
##   .y.        n statistic   DFn   DFd     p method
## * <chr>  <int>     <dbl> <dbl> <dbl> <dbl> <chr>
## 1 Height   122      2.01     4  32.4 0.116 Welch ANOVA
```

```
welch_post_height_116_NRE <- SD116 %>% games_howell_test(Height~treatment)
welch_post_height_116_NRE
```

```
## # A tibble: 10 x 8
##    .y.    group1      group2      estimate conf.low conf.high p.adj p.adj.signif
##  * <chr>  <chr>       <chr>          <dbl>    <dbl>     <dbl> <dbl> <chr>
##  1 Height heat        NRE heat        6.7     -1.40     14.8  0.145 ns
##  2 Height heat        NRE sterile     3.57    -8.57     15.7  0.893 ns
##  3 Height heat        sterile        -2.17   -11.2       6.81 0.946 ns
##  4 Height heat        unsterile       0.608   -4.43      5.64 0.997 ns
##  5 Height NRE heat    NRE sterile    -3.13   -16.1       9.80 0.95  ns
##  6 Height NRE heat    sterile        -8.87   -19.0       1.29 0.108 ns
##  7 Height NRE heat    unsterile      -6.09   -13.4       1.26 0.138 ns
##  8 Height NRE sterile sterile        -5.74   -19.0       7.55 0.701 ns
##  9 Height NRE sterile unsterile      -2.96   -14.7       8.82 0.929 ns
## 10 Height sterile     unsterile       2.78    -5.69     11.2  0.831 ns
```

Figure 131: Results for the Welch ANOVA and Games-Howell test for the plant height in the nutrient rescue experiment of soil 116

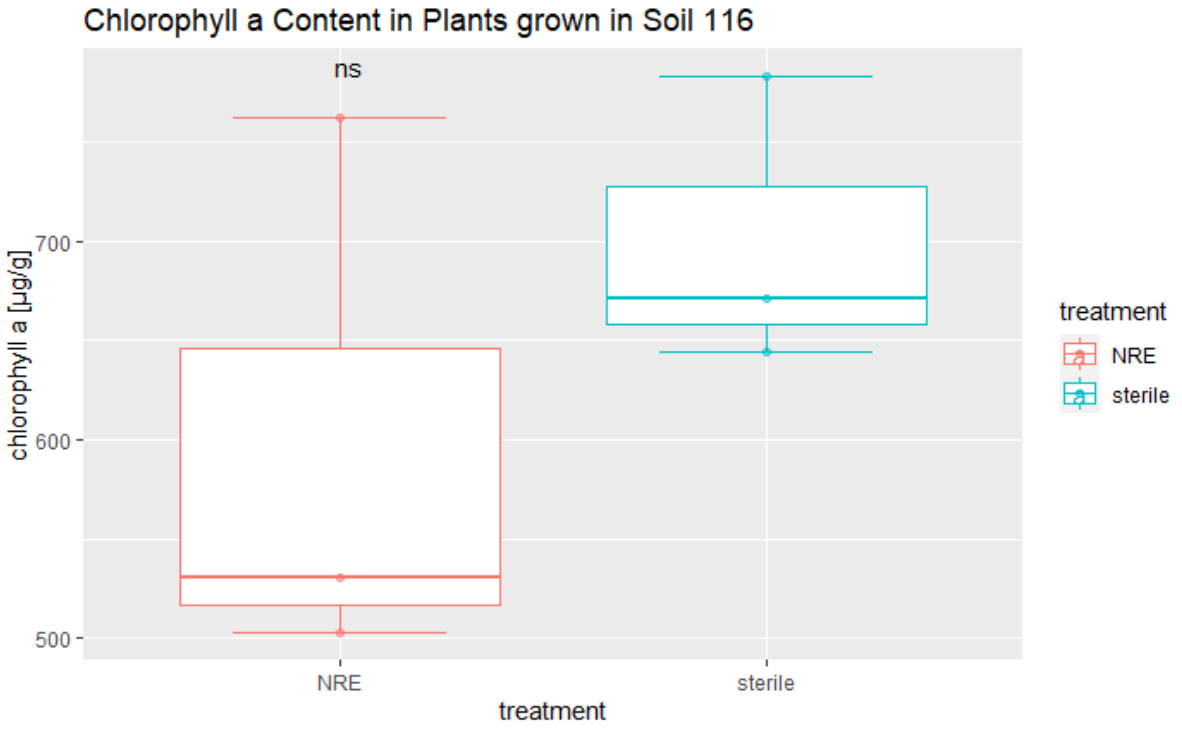

Figure 132: Chlorophyll a content of plants grown in sterile soil 116 either with or without nutrients. Statistical significance was determined by a Welch t-test

```
##
##  Welch Two Sample t-test
##
## data:  Chl._a_µg/g by treatment
## t = -1.0928, df = 2.9981, p-value = 0.3544
## alternative hypothesis: true difference in means between group NRE and group sterile is not equal to 0
## 95 percent confidence interval:
##  -394.5836  192.9237
## sample estimates:
##     mean in group NRE mean in group sterile
##              598.4969              699.3269
```

Figure 133: Results of the Welch t-test of the Chlorophyll a content of plants grown in sterile soil 116 either with or without nutrients

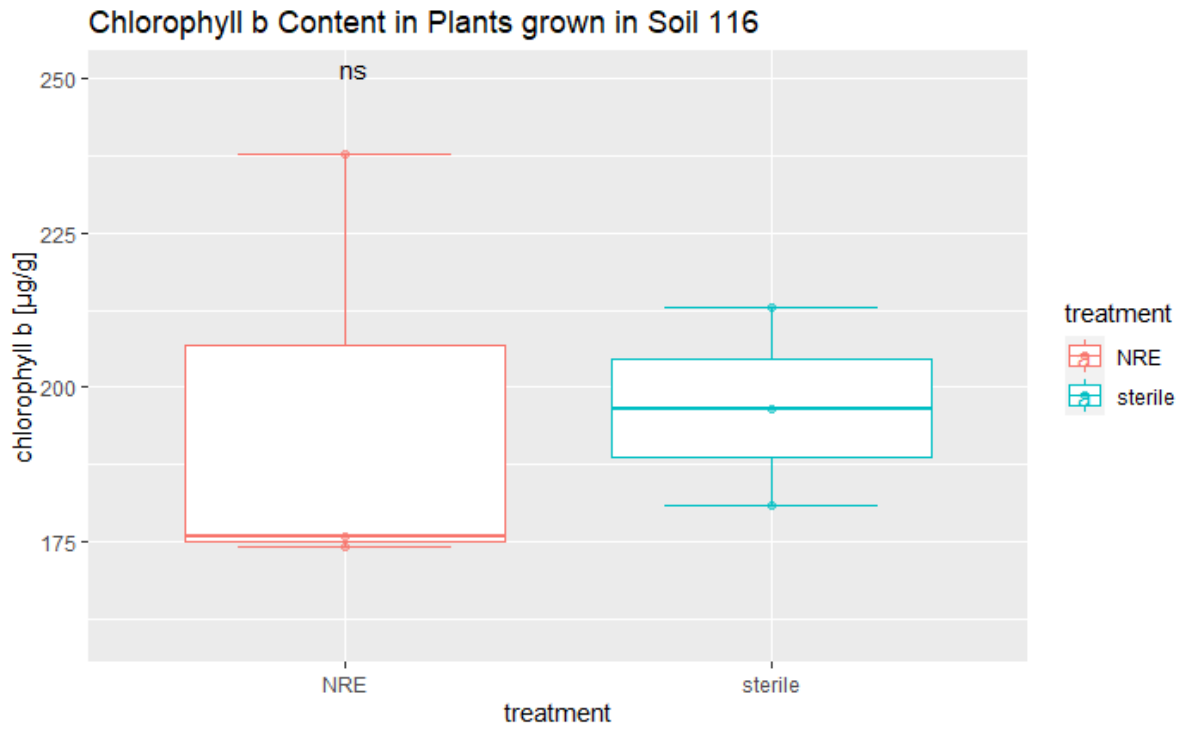

Figure 134: Chlorophyll b content of plants grown in sterile soil 116 either with or without nutrients. Statistical significance was determined by a Welch t-test

```
##
##  Welch Two Sample t-test
##
## data:  Chl._b_µg/g by treatment
## t = -0.034602, df = 2.7569, p-value = 0.9747
## alternative hypothesis: true difference in means between group NRE and group sterile is not equal to 0
## 95 percent confidence interval:
##  -77.36722  75.78400
## sample estimates:
##     mean in group NRE mean in group sterile
##              195.9234              196.7150
```

Figure 135: Results of the Welch t-test of the Chlorophyll b content of plants grown in sterile soil 116 either with or without nutrients

```
##
##  Welch Two Sample t-test
##
## data:  Chl._a+b_µg/g by treatment
## t = -0.88669, df = 2.9368, p-value = 0.4418
## alternative hypothesis: true difference in means between group NRE and group sterile is not equal to 0
## 95 percent confidence interval:
##  -477.6905  271.4879
## sample estimates:
##     mean in group NRE mean in group sterile
##              803.4494              906.5508
```

Figure 136: Results of the Welch t-test of the Chlorophyll a+b content of plants grown in sterile soil 117 either with or without nutrients

Soil 117

anova

```
anova_117_SD_area <- NRE117 %>% anova_test(total~treatment)
```

```
## Coefficient covariances computed by hccm()
```

```
anova_117_SD_area
```

```
## ANOVA Table (type II tests)
##
##      Effect DFn DFd     F     p p<.05   ges
## 1 treatment   3  16 6.875 0.003     * 0.563
```

```
tukey_117_SD_area <- NRE117 %>% tukey_hsd(total ~ treatment)
tukey_117_SD_area
```

```
## # A tibble: 6 x 9
##   term  group1 group2 null.value estimate conf.low conf.high  p.adj p.adj.signif
## * <chr> <chr>  <chr>       <dbl>    <dbl>    <dbl>     <dbl>  <dbl> <chr>
## 1 trea~ endo   endo_~          0   1.11      0.156      2.07 0.02   *
## 2 trea~ endo   neg. ~          0   1.17      0.215      2.13 0.014  *
## 3 trea~ endo   pos. ~          0   1.38      0.418      2.33 0.0041 **
## 4 trea~ endo_~ neg. ~          0   0.0591   -0.899      1.02 0.998  ns
## 5 trea~ endo_~ pos. ~          0   0.262    -0.696      1.22 0.861  ns
## 6 trea~ neg. ~ pos. ~          0   0.203    -0.755      1.16 0.929  ns
```

Figure 137: Results for the one-way ANOVA and Tukey post-hoc test of the soil 117 combined soil dilution and transplantation experiment

Kruskall-wallis

```
kruskall_117_SD_yellow_area <- NRE117 %>%  kruskal_test(yellow~treatment)
kruskall_117_SD_yellow_area
```

```
## # A tibble: 1 x 6
##   .y.        n statistic    df     p method
## * <chr>  <int>     <dbl> <int> <dbl> <chr>
## 1 yellow    20      3.25     3 0.355 Kruskal-Wallis
```

```
wilcox_117_SD_yellow_area <- NRE117  %>% wilcox_test(yellow ~ treatment, p.adjust.method = "bonferroni")
wilcox_117_SD_yellow_area
```

```
## # A tibble: 6 x 9
##   .y.    group1       group2         n1    n2 statistic     p p.adj p.adj.signif
## * <chr>  <chr>        <chr>       <int> <int>     <dbl> <dbl> <dbl> <chr>
## 1 yellow endo         endo_rhizo      5     5      16   0.441     1 ns
## 2 yellow endo         neg. contr~     5     5      14.5 0.724     1 ns
## 3 yellow endo         pos. contr~     5     5      17.5 0.18      1 ns
## 4 yellow endo_rhizo   neg. contr~     5     5       9   0.441     1 ns
## 5 yellow endo_rhizo   pos. contr~     5     5      15   0.424     1 ns
## 6 yellow neg. control pos. contr~     5     5      17.5 0.18      1 ns
```

Figure 138: Results for the Kruskall-Wallis and Wilcoxon test of the soil 117 combined soil dilution and transplantation experiment

Appendix E: Mono-association Experiment

The statistical analysis for the mono-association assay can be found from Figure 139 to Figure 152.

```
#anova test without equal variance


oneway.test(weight~bacteria, weight_no)
```

```
##
##  One-way analysis of means (not assuming equal variances)
##
## data:  weight and bacteria
## F = 25.718, num df = 10.000, denom df = 28.482, p-value = 1.208e-11
```

s

Figure 139: One-way ANOVA of total plant weight of plants in the mono-association assay

```
compare_means(weight~bacteria, data = weight_no, ref.group ="neg. control", method = "t.test")
```

```
## # A tibble: 10 x 8
##    .y.    group1       group2              p   p.adj p.format p.signif method
##    <chr>  <chr>        <chr>           <dbl>   <dbl> <chr>    <chr>    <chr>
##  1 weight neg. control heat control 0.781     1       0.78120  ns       T-test
##  2 weight neg. control F4           0.0000134 0.00013 1.3e-05  ****     T-test
##  3 weight neg. control G9           0.630     1       0.63048  ns       T-test
##  4 weight neg. control G6           0.342     1       0.34176  ns       T-test
##  5 weight neg. control G4           0.907     1       0.90696  ns       T-test
##  6 weight neg. control E4           0.0502    0.35    0.05022  ns       T-test
##  7 weight neg. control F3           0.000638  0.0051  0.00064  ***      T-test
##  8 weight neg. control G3           0.442     1       0.44227  ns       T-test
##  9 weight neg. control F8           0.802     1       0.80179  ns       T-test
## 10 weight neg. control F7           0.000485  0.0044  0.00049  ***      T-test
```

Figure 140: Pairwise comparison of total plant weight of plants in the mono-association assay against the neg. control

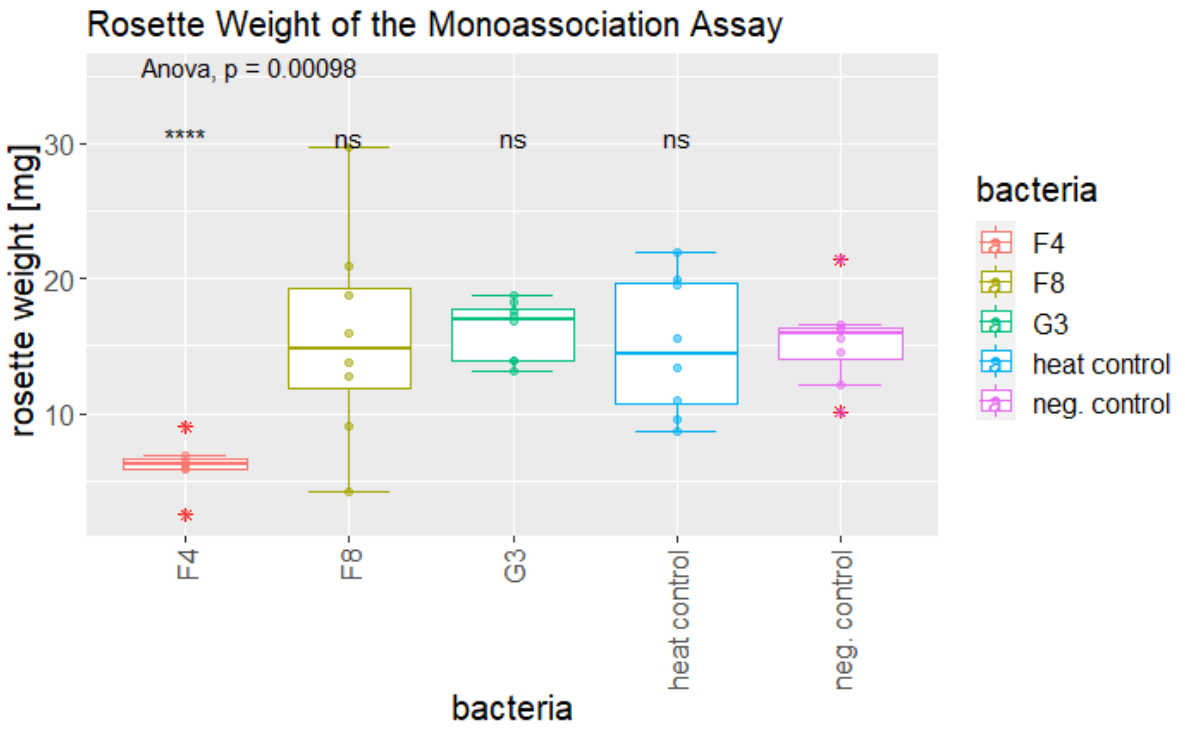

Figure 141: Rosette weight of plants in the mono-association assay. Pairwise comparisons were done with a t-test against the neg. control.

```
#anova test without equal variance


oneway.test(weight_rosette~bacteria, weight_no)
```

```
##
##  One-way analysis of means (not assuming equal variances)
##
## data:  weight_rosette and bacteria
## F = 23.945, num df = 4.000, denom df = 16.598, p-value = 1.032e-06
```

Figure 142: One-way ANOVA of rosette weight of plants in the mono-association assay

```
compare_means(weight_rosette~bacteria, data = weight_no, ref.group ="neg. control", method = "t.test")
```

```
## # A tibble: 4 x 8
##   .y.            group1       group2           p  p.adj p.format p.signif method
##   <chr>          <chr>        <chr>        <dbl>  <dbl> <chr>    <chr>    <chr>
## 1 weight_rosette neg. control heat cont~ 8.47e-1 1  e+0 0.85     ns       T-test
## 2 weight_rosette neg. control F4         2.99e-5 1.2e-4 3e-05    ****     T-test
## 3 weight_rosette neg. control G3         5.77e-1 1  e+0 0.58     ns       T-test
## 4 weight_rosette neg. control F8         9.26e-1 1  e+0 0.93     ns       T-test
```

Figure 143: Pairwise comparison of rosette weight of plants in the mono-association assay against the neg. control

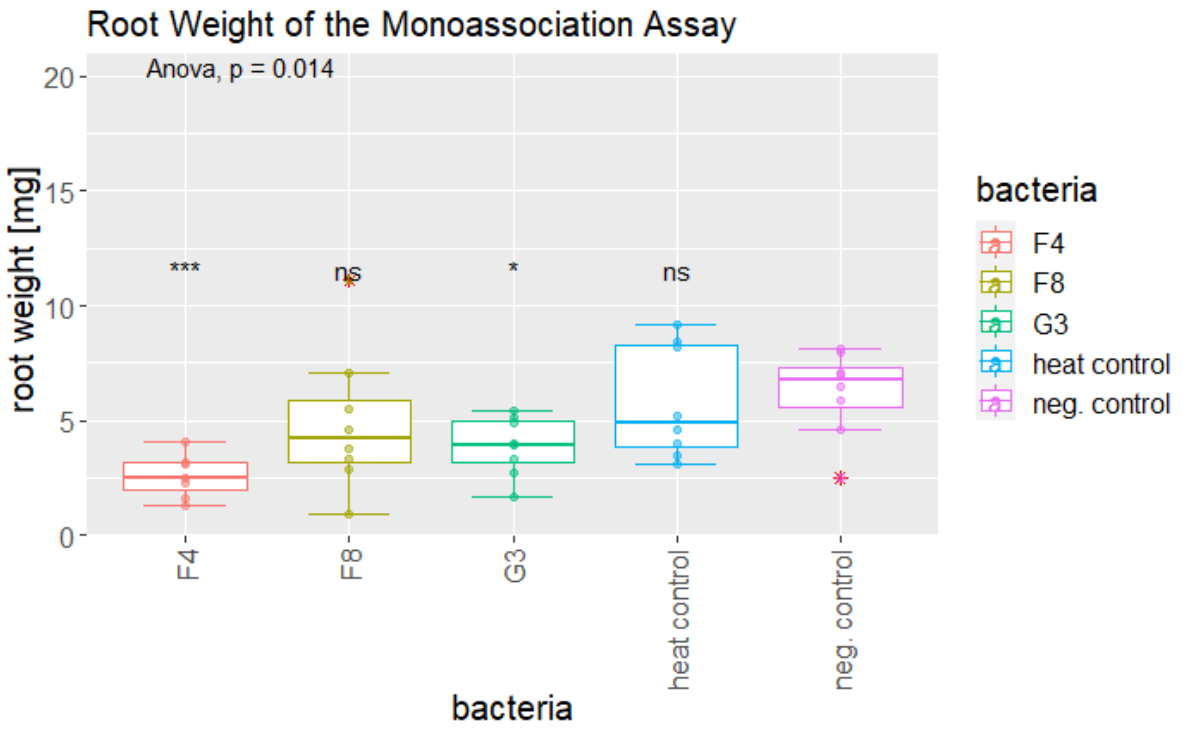

Figure 144: Root weight of plants in the mono-association assay. Pairwise comparisons were done with a t-test against the neg. control.

```
#anova test without equal variance


oneway.test(weight_root~bacteria, weight_no)
```

```
##
##  One-way analysis of means (not assuming equal variances)
##
## data:  weight_root and bacteria
## F = 6.7869, num df = 4.000, denom df = 16.663, p-value = 0.001959
```

Figure 145: One-way ANOVA of root weight of plants in the mono-association assay

```
compare_means(weight_root~bacteria, data = weight_no, ref.group ="neg. control", method = "t.test")
```

```
## # A tibble: 4 x 8
##   .y.         group1       group2             p  p.adj p.format p.signif method
##   <chr>       <chr>        <chr>          <dbl>  <dbl> <chr>    <chr>    <chr>
## 1 weight_root neg. control heat control 0.694    0.69   0.69435  ns       T-test
## 2 weight_root neg. control F4           0.000611 0.0024 0.00061  ***      T-test
## 3 weight_root neg. control G3           0.0127   0.038  0.01272  *        T-test
## 4 weight_root neg. control F8           0.327    0.65   0.32743  ns       T-test
```

Figure 146: Pairwise comparison of root weight of plants in the mono-association assay against the neg. control

```
#anova test without equal variance


oneway.test(root_length_day5~bacteria, weight_no)
```

```
##
##  One-way analysis of means (not assuming equal variances)
##
## data:  root_length_day5 and bacteria
## F = 6.9841, num df = 10.000, denom df = 27.757, p-value = 2.292e-05
```

Figure 147: One-way ANOVA of root length after 5 days of plants in the mono-association assay

```
compare_means(root_length_day5~bacteria, data = weight_no, ref.group ="neg. control", method = "t.test")
```

```
## # A tibble: 10 x 8
##    .y.              group1       group2         p p.adj p.format p.signif method
##    <chr>            <chr>        <chr>      <dbl> <dbl> <chr>    <chr>    <chr>
##  1 root_length_day5 neg. control heat con~ 0.460   1    0.460    ns       T-test
##  2 root_length_day5 neg. control F4        0.0674  0.61 0.067    ns       T-test
##  3 root_length_day5 neg. control G9        0.761   1    0.761    ns       T-test
##  4 root_length_day5 neg. control G6        0.320   1    0.320    ns       T-test
##  5 root_length_day5 neg. control G4        0.562   1    0.562    ns       T-test
##  6 root_length_day5 neg. control E4        0.358   1    0.358    ns       T-test
##  7 root_length_day5 neg. control F3        0.101   0.81 0.101    ns       T-test
##  8 root_length_day5 neg. control G3        0.379   1    0.379    ns       T-test
##  9 root_length_day5 neg. control F8        0.634   1    0.634    ns       T-test
## 10 root_length_day5 neg. control F7        0.0493  0.49 0.049    *        T-test
```

Figure 148: Pairs wise comparison of root length after 5 days of plants in the mono-association assay against the neg. control

```
#anova test without equal variance


oneway.test(weight~bacteria, weight_NaCl)
```

```
##
##  One-way analysis of means (not assuming equal variances)
##
## data:  weight and bacteria
## F = 9.4712, num df = 10.000, denom df = 20.751, p-value = 1.019e-05
```

Figure 149: One-way ANOVA of total plant weight of plants in the mono-association assay with NaCl

```
compare_means(weight~bacteria, data = weight_NaCl, ref.group ="neg. control", method = "t.test")
```

```
## # A tibble: 10 x 8
##    .y.    group1       group2              p   p.adj p.format p.signif method
##    <chr>  <chr>        <chr>           <dbl>   <dbl> <chr>    <chr>    <chr>
##  1 weight neg. control heat control 0.364     1       0.3645   ns       T-test
##  2 weight neg. control F4           0.355     1       0.3553   ns       T-test
##  3 weight neg. control G9           0.642     1       0.6421   ns       T-test
##  4 weight neg. control G6           0.221     1       0.2207   ns       T-test
##  5 weight neg. control G4           0.650     1       0.6502   ns       T-test
##  6 weight neg. control E4           0.408     1       0.4083   ns       T-test
##  7 weight neg. control F3           0.00693   0.055   0.0069   **       T-test
##  8 weight neg. control G3           0.531     1       0.5312   ns       T-test
##  9 weight neg. control F8           0.00454   0.041   0.0045   **       T-test
## 10 weight neg. control F7           0.0000451 0.00045 4.5e-05  ****     T-test
```

Figure 150: Pairwise comparison of total plant weight of plants in the mono-association assay with NaCl against the neg. control

```
#anova test without equal variance


oneway.test(root_length_day5~bacteria, weight_NaCl)
```

```
##
##  One-way analysis of means (not assuming equal variances)
##
## data:  root_length_day5 and bacteria
## F = 5.6516, num df = 10.000, denom df = 18.932, p-value = 0.0006238
```

Figure 151: One-way ANOVA of root length after 5 days in the mono-association assay with NaCl

```
compare_means(root_length_day5~bacteria, data = weight_NaCl, ref.group ="neg. control", method = "t.test")
```

```
## # A tibble: 10 x 8
##    .y.              group1       group2         p p.adj p.format p.signif method
##    <chr>            <chr>        <chr>      <dbl> <dbl> <chr>    <chr>    <chr>
##  1 root_length_day5 neg. control heat con~ 0.211   0.84 0.211    ns       T-test
##  2 root_length_day5 neg. control F4        0.0557  0.45 0.056    ns       T-test
##  3 root_length_day5 neg. control G9        0.355   0.84 0.355    ns       T-test
##  4 root_length_day5 neg. control G6        0.134   0.8  0.134    ns       T-test
##  5 root_length_day5 neg. control G4        0.0969  0.68 0.097    ns       T-test
##  6 root_length_day5 neg. control E4        0.820   0.84 0.820    ns       T-test
##  7 root_length_day5 neg. control F3        0.0135  0.14 0.014    *        T-test
##  8 root_length_day5 neg. control G3        0.0390  0.35 0.039    *        T-test
##  9 root_length_day5 neg. control F8        0.268   0.84 0.268    ns       T-test
## 10 root_length_day5 neg. control F7        0.148   0.8  0.148    ns       T-test
```

Figure 152: Pairwise comparison of root length after 5 days in the mono-association assay with NaCl against the neg. control