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Daniela Vogl, BSc

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CONTENT

GLOSSARY

C	Control
Cd	Cadmium
CLSM	Confocal Laser Scanning Microscope
Co	Cobalt
Cu	Copper
EPS	extracellular polysaccharides
IUPAC	International Union of Pure and Applied Chemistry
MS	Murashige and Skoog Medium without major salts
Ni	Nickel
NPG	Newport Green™
RH	*Rhodotorula mucilaginosa*
RH dead	*Rhodotorula mucilaginosa* dead
ROS	Recative Oxygen Species
Pb	Lead
PGPR	Mycorrhiza & plant growth promoting bacteria
SD	Standard deviation
SP	*Sporobolomyces sp.*
SP dead	*Sporobolomyces sp.* dead
Zn	Zinc

ABSTRACT

Menschliche Aktivitäten führen zunehmend zu erhöhten Mengen an toxischen Metallen in Böden. Diese haben zahlreiche Effekte auf Pflanzen, die auf diesen Standorten wachsen, wie reduziertes Pflanzenwachstum und Metall-Akkumulation. Schlussendlich könnten diese Metalle dadurch in den Nahrungsketten enden und weiteren Schaden anrichten. In den letzten Jahren wurden zahlreiche Strategien entwickelt, um diesen Agglomerationen entgegenzuwirken und die Standorte zu restaurieren, beispielsweise mit der Hilfe von Pflanzen, so genannter Phytoremediation. Bestimmte, spezialisierte Arten sind an das Leben mit erhöhten Schwermetallkonzentrationen angepasst und daher vielversprechende Partner für Restaurationsversuche. Als zusätzliche Helfer rücken vermehrt endophytische Pilze in den wissenschaftlichen Fokus, da diese Möglichkeiten bieten, diese Schwermetall-belasteten Böden zu restaurieren, indem sie die Pflanzen auf diesen Standorten unterstützen.

Nickel und Zink wurden als Kontaminanten gewählt. Landwirtschaftlich bedeutsame Arten, Amarant und Weizen, und schwermetall-adaptierte Arten, wie *Noccaea caerulescens*, wurden verwendet, um die Interaktion und die Effekte zweier Hefepilze, *Sporobolomyces sp.* und *Rhodotorula mucilaginosa*, und eines filamentösen Pilzes, *Diaporthe columnaris*, auf die Pflanzen zu untersuchen. Alle drei Pilzarten sind als pflanzenbewohnende Endophyten bekannt und zeigten in früheren Studien positiven Einfluss auf das Pflanzenwachstum.

In vitro Experimente wurden durchgeführt, ebenso wie Topfexperimente, um den Einfluss der Pilze, unter dem Einfluss von Nickel und Zink, auf die Pflanzenentwicklung zu untersuchen. Co-Habitationsexperimente in Agarplatten zeigten großen Einfluss der Metalle auf die Pflanzen, und weisen auf mögliche Co-Abhängigkeiten mit den Pilzen hin. Die Pilze hatten nur punktuellen Effekt auf die Pflanzen, kein generelles Muster konnte festgestellt werden. Langfristig hatte die Co-Habitation mit Pilzen negative Effekte auf die Pflanzen.

Ein zusätzliches Studienziel war, die Interaktion von Pilz und Pflanze zu beobachten. Verschiedene Färbemethoden wurden dafür getestet. Anilinblau und Sudan IV erwiesen sich dabei als hilfreich, um die Penetration der Pilze in die pflanzlichen Zellen abzubilden, limitiert von starken Ablagerungen auf den Wurzeloberflächen. Interne Strukturen wurden mit diesen Methoden nicht dargestellt. Newport Green ™ Färbung wurde verwendet um Nickel- und Zinkaufnahme in den Pflanzen darzustellen. Experimente zu Wachstum und Entwicklung der Pilze gemeinsam mit Pflanzen zeigten kein direktionales Wachstum der Pilze zu den Pflanzen.

Topfexperimente in Erde wurden durchgeführt um den Einfluss der Pilze auf die Pflanzenentwicklung unter natürlicheren Umständen zu untersuchen. Verschiedene Wachstumsentwicklungsparameter wurden ermittelt, ebenso wie osmotische Toleranz und photosynthetische Aktivität. Dieser Teil der Experimente zeigte erneut den starken Einfluss der verwendeten Metalle, speziell von Nickel, und nur punktuellen Einfluss der Hefen. Dieser resultierte meist in negativen Effekten für die Pflanzen.

Pilze können Pflanzen bei der Entwicklung auf schwierigen Böden unterstützen, und stellen damit ein vielversprechendes Werkzeug dar, um diese Böden zu restaurieren. Dennoch legen die Ergebnisse nahe, dass diese punktuellen Verbesserungen ein präzises Wissen und damit intensive Studien voraussetzen, bezüglich der Interaktion beider Partner und ihrer Umgebung.

ABSTRACT

Human activities increasingly lead to elevated levels of toxic metals in soils. These have numerous effects on plants growing on these sites, like reduced plant growth and metal accumulation. Ultimately, these metals might therefore end up in food chains and cause further damage. In recent years numerous strategies have been developed to counteract these agglomerations as well as to restore these sites, eg. by the aid of plants, so called phytoremediation. Certain species specialized to sites with elevated concentrations of metals and are therefore promising partners for remediation attempts. As additional helpers, endophytic fungi are increasingly gaining scientific attention, as they might offer possibilities to restore heavy metal polluted soils by supporting plants inhabiting these sites.

Concentrating on nickel and zinc as contaminants, species of agricultural interest were used, amaranth and wheat, as well as species adapted to heavy metal polluted sites like *Noccaea caerulescens*, to investigate the interaction and effects of two yeast species, *Sporobolomyces sp.* and *Rhodotorula mucilaginosa* and a filamentous fungus, *Diaporthe columnaris*, on the plants. All three fungal species are known endophytes and have been reported to positively influence plant growth.

In vitro experiments were performed, as well as soil pot experiments, to investigate the fungal influence on plant development under the influence of nickel and zinc stress. Co-habitation experiments in agar dishes revealed strong metal impact on plants, and suggest a possible co-dependence with fungi. Fungi had only punctual effects on plants, no general pattern was observed. On a long term, co-habitation with fungi was affecting the plants in a negative way.

An additional aim of the work was to observe how fungi and plants interact. By testing various staining methods, we found Aniline blue and Sudan IV to be helpful tools to reveal fungal entrance points to the roots, yet limited by strong exudates on root surfaces. Internal structures were not depicted by these methods. Newport Green™ staining was performed to depict nickel and zinc uptake to plants. Furthermore growth development experiments with fungi and plants revealed non-directional fungal distribution, which did not approach the plants directly.

Soil pot experiments were conducted to evaluate the fungal influence on plant development under more natural conditions. Several growth development parameters were estimated, accompanied by osmotic tolerance tests and photosynthetic activity tests. This part of the experiments revealed a strong metal impact, especially by nickel, and only punctual influence by yeasts, mostly resulting in adverse effects on the plants.

Fungal partners may aid plants to develop on demanding sites and might therefore provide a promising aid in restoring these sites. Nevertheless, the results suggest that these punctual improvements would recommend a precise knowledge and therefore intense studies of the interaction of both partners and their environment.

INTRODUCTION

Pollution by toxic metals is a well known and highly discussed problem. All kind of toxic metals are of environmental concern, including nickel and zinc. Contaminations with heavy metals root in diverse anthropogenic activities and natural sources. Numerous attempts to counteract this pollution have been developed and explored, like the remediation of polluted soils using plants with the aid of fungi and bacteria. In this study we focus on the interaction of plants living on nickel and zinc contaminated substrates under the influence of three different endophytic fungi to get insights into the causes, the effects and the possible solutions offered by these liaisons.

HEAVY METALS, TOXIC METALS

The term "heavy metals" is widely discussed, with varying criteria depending on context and author. A commonly used criterion includes metals and metalloids with relatively high atomic weights, or atomic numbers, with densities of more than 5 g/cm^3. (Koduru et al., 2017, Abbaszadeh-Dahaji et al., 2016)

NICKEL

Nickel (Ni), atomic number 28, exists in five oxidation states (−1 to +4), the most common is 2+. The silverish-white metal is deformable and widely used, for example in steel and alloy production, electroplating, nickel-cadmium battery production, in electronic components, coins, household items and jewelery, to name just a few. (Klein & Costa, 2015)

Occurrence

Nickel exists in sulphide or oxide ores as a natural earth element, getting mined underground or in open pits. Natural emissions of nickel into the atmosphere are volcanic activities and rock shear processes. Anthropogenic sources include metal mining, smelting, vehicle emissions, fossil fuel burning, industrial processes, fertilizer application, and organic manures. In natural waters concentrations from 2 to 10 µg/L have been estimated for tap water, and 0.2 to 0.7 µg/L in marine waters. In soils, nickel occurs with broad variations, ranging from 3 to 1,000 ppm. In total, the Earth's crust contains about 84 mg/kg, primarily as divalent cations. Levels rise dramatically in areas of nickel refinery or in dried sludge, where concentrations between 24,000 and 53,000 ppm have been reported. (Klein & Costa, 2015; Chen et al., 2009)

Biological importance

It is still under question if nickel is essential to animals. Rats suffer from reduced growth when fed with low nickel diets, but a proof for a necessity in humans is still lacking. Nickel seems to have limited function in higher eukaryotes. Nevertheless, nickel is essential for plants and bacteria. In bacteria nickel serves as cofactor in nine enzymes. As a consequence, nickel might be necessary as well for humans regarding interactions in the human biome. (Klein & Costa, 2015)

Biological toxicity in animals and humans

Excess uptake of nickel causes headache, vertigo, nausea, vomiting, nephrotoxic effects and pneumonia. Inhalation can lead to chronic rhinitis, sinusitis, nasal septum perforations, and asthma. Skin absorption might induce contact dermatitis and nickel allergy. Nickel is toxic to reproductive systems, reduced number of sperm is observed under the influence of nickel, as well as deformed uteri and accumulation of abortions in fetuses of rats. In humans, reductions in birth rates and increased

abortion rates as well as structural malformations have been reported in a study of nickel refinery workers. In Syrian hamsters, nickel ions induced cytotoxicity, apoptosis, chromosomal aberrations, and morphological transformation. Nickel is teratogenic and has carcinogenic potential. (Klein & Costa, 2015; Denkhaus & Salnikow, 2002)

Biological importance and toxicity in plants

In plants nickel serves as micro-nutrient in very low concentrations between 0.05 and 10 mg/kg dry weight (Chen et al., 2009; Seregin et al., 2006). Nickel deficits result in reduced urease activity in some legumes and other dicots. As a consequence urea accumulates to toxic levels preferably in the leaf tips, which finally get necrotic. Furthermore, synthesis of anthocyanins is affected. Soy shows reduced nodulation and seed yield. In grasses plants under nickel deficiency suffer from intervenal chlorosis and young leaves show necrosis spots. (López et al., 2011)

Excess nickel leads to symptoms similar to iron deficiency. Reduced growth of the root system and leaf bud distortions may be the result, as well as reductions in fresh weight. In wheat, fresh weight of shoots is reduced. (Chen et al., 2009)

ZINC

The "Handbook on the Toxicology of Metals" describes zinc as ubiquitious, essential for life, and toxic in excess. Zinc (Zn) is the 25th most abundant element, with atomic number 30. In crystalline form, it is a blueish-white metal. Zinc is used to coat other metals, in construction, noncorrosive alloys and in brass. As oxide it is used in rubber production and for white pigment. Furthermore, it is used as fungicide, topical antibiotics, lubricants, dental, medical and household applications, to name a few. (Sandstead, 2015)

Occurrence

On average, 1 kg of rock from the Earth's crust contains 78 mg of zinc. Aerial concentrations are usually ranging between 10 and 300 ng/m^3, the highest concentrations are found in urban and industrial areas, where values of up to 2,400 ng/m^3 are reported. In water, zinc is occurring in microgram concentrations, ranging from below 10 µg/L in natural surface waters, and 10 to 40 µg/L in groundwater. In soils, the concentrations vary with ranges reported from 1 to 2,000 mg/kg. Areas impacted by anthropogenic activities, like industrial sources, might cause higher values, sewage sludge for example might contain up to 49,000 mg/kg dry weight. Soils along motorways show elevated zinc contents in topsoils up to 1,500 mg/kg dry weight in various sites in the EU. (Sandstead, 2015; WHO, 2003; Bodar et al., 2005)

Biological importance

Zinc serves as micronutrient in plants and is involved in numerous processes, like enzyme activation, gene expression, phytohormone activity and protein synthesis. It is also essential for animals and humans, serving as cofactor or structural component with involvements in numerous biochemical pathways and functions. (Sadeghzadeh & Rengel, 2011)

Biological importance and toxicity in humans

In uncontaminated soils, zinc levels are mostly below 125 ppm (Tsonev & Lidon, 2012). Half of the world's croplands show low zinc levels, resulting in low zinc levels in yields and ultimately in zinc deficits in about two to three billion people (Sadeghzadeh & Rengel, 2011). Zinc deficiency in humans causes growth failure during growth periods and affects numerous systems like the epidermal, gastrointestinal, central nervous or the immune system, to name just a few (Roohani et al., 2013).

Zinc is relatively non-toxic to humans if ingested orally. Uptakes of about 100 to 300 mg zinc per day, well above the recommended dietary allowance of 15 mg of zinc per day, might interfere with utilization of copper and iron and affect HDL cholesterol concentrations. An excess uptake of zinc may cause nausea, vomiting, epigastric pain, lethargy and fatigue. (Fosmire, 1990)

Biological importance and toxicity in plants

In plants, elevated zinc levels cause phytotoxicity in leaves. An excess of zinc reduces growth, photosynthetic rates decrease, photochemical reactions are affected, as well as carbonic anhydrase activity, chlorophyll synthesis and cell wall integrity. Wilting and necrosis of older leaves, decline in biomass and inhibition of cell elongation and devision are also observed. (Tsonev & Lidon, 2012)

SOIL POLLUTION & REMEDIATION

Contaminations with heavy metals root in anthropogenic activities like mining activities, traffic, agricultural, military and industrial operations and natural sources like pedogenesis of soils derived from ultramafic rocks (Ma, 2016; Abbaszadeh-Dahaji et al., 2016). Polluted soils and waters pose substantial risks to human and environmental health, as these toxic metals are non-biodegradable and accumulate in the environment. Plants are affected by decreased photosynthesis rates, decreased chlorophyll content, reduced stomatal conductance and so on. Various physiological and biochemical processes are limited, plant growth is inhibited. Heavy metals reduce the activity of soil microorganisms and crop production; and may ultimately end up in the food chain by accumulating in edible plant parts. (Salt et al., 1998; Abbaszadeh-Dahaji et al., 2016; Sarwar et al., 2017)

Remediating these sites bases on two basic principles: removing the pollutants from the contaminated site or transforming them to non-harming forms. The latter includes various methods and technologies like excavation, separation, extraction, electrokinesis, washing, oxidation, reduction, phytoextraction, phytovolatilization, solidification, or vitrification. (Li, 2013)

PHYTOREMEDIATION

A sustainable and eco-friendly solution to remove metal pollutants from contaminated soil and water may be found in a plant-based remediation approach—phytoremediation—which uses green plants to remove pollutants from the environment, or in rendering these pollutants harmless. For example, by the use of specific shrubs, trees or grasses that immobilize, degrade and remove contaminants from the soils, in a secure, cost effective, large scale and non-invasive way. (Ma, 2016; Salt et al., 1998; Raskin et al., 1997; Rajkumar et al. 2012; Abbaszadeh-Dahaji et al., 2016)

Different approaches to phytoremediation

Plants can be used in various ways to approach heavy metal contaminated sites, like phytovolatilization, phytofiltration, or phytoextraction and phytostabilisation. (Raskin et al., 1997; Ma 2016; Sarwar et al., 2017)

In phytovolatilization, biomethylated, volatile molecules are converted out of hazardous metals like selenium, arsenic or mercury, which are then released to the atmosphere eg. through stomata. (Raskin et al., 1997; Sarwar et al., 2017)

Phytofiltration uses plants to purify water. Aquatic and semi-aquatic plants are used as well as plants cultivated as hydroponics. The latter are used for rhizofiltration by using plants with rapidly growing roots that allow to remove toxic heavy metals over longer timespans. (Raskin et al., 1997)

Phytostabilisation uses plants to reduce the bioavailability and mobility of metals, by absorbing them into the roots, precipitation and complexation in the root zone. This approach is not permanent, as heavy metals remain in the soil. (Sarwar et al., 2017)

Phytoextraction, the most important approach to phytoremediation (Sarwar et al., 2017), is a concept reaching back to the 1980s, bases on the discovery that numerous wild plants contain high amounts of metals, like nickel, zinc, or copper. They often inhabit sites with naturally mineralized soils, where the essential and non-essential heavy metals are incorporated and stored in eg. leaves. Accumulation often reaches 1 to 5 % of dryweight, exceeding the concentrations of these metals to be found in non-accumulating plants by orders of a magnitude. Possible reasons for this hyper-accumulation might be eg. protection from herbivores. (Raskin et al., 1997)

Soil properties, metal speciation and plant characteristics affect the efficiency of phytoextraction. The ideal phytoextractor plant should grow fast, produce a lot of biomass while accumulating high amounts of heavy metals. It should be an ubiquitous species, store the desired metals in the shoots, be tolerant to heavy metals, resistant to diseases and unattractive to herbivores to avoid entrance to the food chain. (Sarwar et al., 2017)

Ultimately, these hyperaccumulating plants offer the possibility to harvest the metal enriched leaves and use them as bioore, so called “metal phytominig”, to recover valuable metals (Thij, 2017).

Phytoremediation and the underlying plant strategies

Plants growing in substrates with elevated metal concentrations take up metal ions at varying degrees. Three strategies have been described as response by the plants to elevated levels of heavy metals in soils, metal excluders, metal indicators and metal hyperaccumulators. (Ma, 2016)

Metal excluders do not accumulate high concentrations of metals in their aboveground tissues, the contaminants are preferably stored in roots. (Ma, 2016)

Metal indicators store metals in aerial organs with a linear correlation between metal concentration in soil and plant tissue, they indicate pollution. (Ma, 2016)

Hyperaccumulating plants accumulate high levels of metals, up to two or three orders of magnitude higher compared to common plants, without showing signs of toxicity. A species is defined as nickel or zinc hyperaccumulator when it is accumulating more than 1,000 µg g^{-1} dry weight nickel, or 10,000 µg g^{-1} dry weight zinc. (Ma, 2016; Kazakou, et al., 2010; Thij, 2017)

To date, approximately 450 species, that is about 0.2 % of all angiosperms, are known hyperaccumulators (Verbuggen et al., 2013). A hyperaccumulator needs to have a factor of bioconcentration bigger than 1.0 (the ratio of metal concentration in the plant to the metal concentration in the soil), and a translocation factor greater than 1.0 (the ratio of metal concentration in the shoots to the metal concentration in the roots). (Ma, 2016; Sarwar et al., 2017; Baker et al., 1994)

Hyperaccumulators tend to develop quite low biomass. Slow growth and variable uptake of metals additionally limit the phytoextraction and mining potential of hyperaccumulating plants. Therefore remediation attempts soon started to focus on plants with lower accumulation rates, but higher growth-yields that are easy to cultivate. (Raskin et al., 1997; Thij, 2017)

Nowadays, the scientific focus is concentrated on the additional use of chemical-assisted phytoremediation, on the use of biochar, on the use of transgenic plants and on microbial-assisted phytoremediation. (Sarwar et al., 2017)

Phytoremediation assisted by bacteria and fungi

This approach to restore contaminated soils uses soil living microorganisms of the rhizosphere to increase the efficiency of phytoremediaton by the use of bacteria or fungi, so called "bio-remediation" or "microbial-assisted phytoremediation" (Abbaszadeh-Dahaji et al., 2016; Cicatelli et al., 2019; Sarwar et al., 2017). Until recently, plants and microorganisms have been used separately for remediation attempts. In recent years, the focus started to shift towards the interaction of both, they can act synergistically to degrade, transform and accumulate contaminants (Cicatelli et al., 2019).

Hyperaccumulators carry a high diversity of bacteria and fungi in their rhizosphere and their endosphere (Thijs et al., 2017). Rhizosphere describes the densely populated microsystem of the root consisting of microorganisms, plants and surrounding soil (1 to 2 mm), where roots compete and collaborate with invading root systems, with bacteria, fungi and insects (Walker et al. 2003; Cicatelli et al., 2019; Thijs et al., 2017). Endosphere describes the habitat provided by the plant-roots themselves. Various bacteria and fungi enter the roots and colonize the interior of the roots. Both, rhizosphere and endosphere inhabiting microorganisms can assist plants to grow in contaminated soils and help them with taking up the metals. (Thijs et al., 2017)

Environmental stresses, like contamination with toxic metals of the soil, can be buffered by mycorrhiza and also by endophytic associations (Kothe & Turnau, 2018). Plants exude up to 30 % of photosynthetis byproducts into the soil, which get utilized by rhizosphere microorganisms. Microorganisms improve cation exchange capacity, alter the soil water pH, release numerous substances like phosphatases that are beneficial for plant nutrition and exude plant stress reducing enzymes. (Cicatelli et al., 2019)

Mycorrhiza & plant growth promoting bacteria (PGPR)

Mycorrhizal fungi can build associations with most higher plants, they are a well studied part of the rhizosphere. Different forms of mycorrhiza are known, like ectomycorrhizas, arbuscular mycorrhizas, orchid mycorrhizas and ericaceous mycorrhizas. Plant growth promoting bacteria (PGPR) form another important community influencing metal uptake. Two groups are known, symbiotic bacteria and free living rhizobacteria. Both may have positive impacts on the plants' performance. (Sarwar et al., 2017)

Some fungi have formed intense symbioses with plants for more than 400 million years: endo- and ecto-mycorrhizal symbioses. Other fungi and bacteria live, at least parts of their lifespan, inside plant tissue and are therefore called "endophytes". (Cicatelli et al., 2019)

ENDOPHYTES

The term "endophyte" describes organisms that live and develop within healthy plant tissue. These infections of plants with bacteria, fungi, other plants or even insects stay mostly symptomless, non-pathogenic or latent and are omnipresent in all kinds of species and climatic regions. Any organ of the host can be infected. (Carroll, 1988; Larran, S. et al., 2007; Isaeva et al., 2010; Schulz & Boyle, 2005)

In this plant-microbe interaction, plants provide nutrients and a stable "habitat", where external stresses and competitions are excluded (Schulz & Boyle, 2005). The invaders on the other hand follow diverse strategies, ranging from mutualistic interactions, to exploitive, to parasitic and facultatively saprobic (Isaeva et al., 2010; Schulz & Boyle, 2005). Endophytes may enhance plant stress tolerance, they promote growth and affect the accumulation of elements, which may be useful regarding phytoremediation and tolerance to environmental stresses, by turning inorganic forms of toxic metals to non-toxic or even beneficial, organic forms (Lindblom, 2018; Domka et al., 2019)

Fungal endophytes

The term "endophyte" covers a broad variance of possible intruders, therefore it seems necessary to describe the interaction more precisely. From a mycological perspective, "fungal endophytes" sums up fungi that spend at least some time of their lifespan inside of plants, without harming their hosts (Schulz & Boyle, 2005). Fungi inhabit almost all plant species studied so far, at all stages of plant life, from seed to grown up plant to fruit. Numerous cryptic taxa are involved in this taxonomically diverse group. (Isaeva et al., 2010; Schulz & Boyle, 2005; Domka et al., 2019)

All niches provided by the plants are used by fungi, they grow inter- and intracellularly, some are found specifically on distinct organs, some live above ground as well as inside root tissue, some are specified on certain hosts, others are ubiquiteous (Schulz & Boyle, 2005). In return for getting provided with nutrients and shelter, plants may receive secondary metabolites, endophytes can support plants in reducing disease and in tolerating unfavorable conditions (Isaeva et al., 2010). Plants inhabited with fungal endophytes show better growth, perform better, and show higher resistance to nematodes, fungi, pathogenic bacteria and fungi (Domka et al., 2019). Plants are positively affected by fungi. On one hand directly by optimizing the plants adaptation to the soil it grows on, on the other indirectly by soil formation. These positive impacts for plants growing in areas with metal pollution, offer promising possibilities regarding phytoremediation. (Domka et al., 2019)

Fossil records suggest that fungal endophytes have been living with plants for more than 400 Myr, played an important role when plants started to colonize land and are important drivers for the evolution of plants on terrestric environments. (Rodriguez et al., 2009)

Classification of fungal endophytes

Two groups with four classes of endophytic fungi can be distinguished (Domka et al., 2019; White et al., 2017; Rodriguez et al., 2009):

Group one (Class 1) consists of clavicipitaceous endophytes, with related species that live in cold- and warm-season grasses (Domka et al., 2019). Endophytes belonging to this group are systemic in leaves, stems and seeds, they are obligately endophytic, transmitted per seed and enter the embryo within in the host plants' seeds (White et al., 2017). Class 1 endophytes frequently offer diverse benefits to plants, like increasing biomass, confer drought tolerance, and the production of chemicals that decrease herbivory and are toxic to animals. The benefits provided by class 1 endophytes depend on the host plant species, its genotype and environmental conditions. (Rodriguez et al., 2009)

The second group, non-clavicipitaceous endophytes, can be further divided to three classes:
- Class 2 fungi inhabit systemically host tissues.
- Class 3 fungi grow only in plant material above ground.
- Class 4 fungi live in roots, a group including dark septate endophytes that belong to Ascomycota and non-mycorrhiza of Sebacinales and Basidiomycota. (Domka et al., 2019)

Class 2 to 4 endophytes are not obligately endophytic, they do not show coevolution with particular hosts and are rather transmitted by surface than within embryos. They may also become pathogenic. (White et al., 2017)

Differentiation of endophytes from mycorrhiza

Unlike mycorrhiza, endophytic fungi are not synchronized regarding their development with their hosts, they are facultative symbionts (Rodriguez et al., 2009; Domka et al., 2019).

Class 1 to 3 endophytic fungi are not restricted to the plants' roots. Unlike mycorrhiza, they colonize leaves, stems, flowers and seeds as well. Furthermore, endophytic fungi do not cause morphological changes in the host, no specific interface like an arbuscule is developed. Endophytic fungi do not provide specific hyphae for communication with the host. They may finish their live cycles outside of plant cells, and therefore can be cultivated under artificial conditions, a circumstance making them attractive for phytoremediation and production of inoculum. (Domka et al., 2019)

Fungal endophytes, metallophytes and hyperaccumulators

Non-clavicipitaceous endophytes (Class 2 to 4) include Ascomycota, Basidiomycota and Mucoromycotina and inhabit bryophytes, ferns, gymnosperms, angiosperms including non-mycorrhizal Brassicaceae, which are often metallophytes or hyperaccumulating species. Like mycorrhizal fungi, these endophytic fungi promote plant growth under nutrient limitation one may find for example in soils contaminated with toxic metals. (Domka et al., 2019)

Some endophytic fungi are resistant to high concentrations of metals. They are able to tolerate concentrations up to mM ranges. Fungal species that showed tolerance to toxic metals have been also isolated from plants growing on soils with high concentrations of toxic metals. These fungi show great potential for microbially assisted phytoremediation as they show high tolerances to pollution. (Domka et al., 2019)

Mechanisms of metal uptake into fungal endophytes

Generally, uptake mechanisms of toxic metals into fungi are not well understood today. After uptake into the hyphae, toxic metals are complexated in the cytosol and translocated into vacuoles to neutralize them. Inside vacuoles, compartimentalization of metal chelates helps to separate toxic metals from sensitive cellular compartments. Chelating molecules are for example thiol-containing substances GSH (glutathiones). These are found in all cell compartments eg. mycorrhizal fungi, and act as antioxidant that reduces oxidative stress. (Domka et al., 2019)

Additionally, endophytic fungi also secret chelating substances, such as oxalic axids or citrates. This mechanism prevents uptake by plant roots as toxic metals are stabilized in the soil. Some reports suggest that fungal endophytes bind ions of toxic metals to cell wall components. (Domka et al., 2019)

Endophytic fungi can form nanoparticles, which reduce the exposure to toxic metals of the host plants and the fungi. These ions can be precipitated as nanoparticles intra- or extracellularly. On the other hand, nanoparticles are taken up by plant cells. Therefore, these nanoparticles might be used as fertilizer by facilitating uptake of micronutrients. (Domka et al., 2019)

Effect of fungal endophytes on plants

Endophytic fungi offer a broad range of action to plants regarding metal uptake and distribution. Some species decrease metal uptake, others increase uptake and translocation from root to shoot. Some endophytic fungi, like the yeast *Rhodotorula sp.*, are known to induce root hair elongation and alter root architecture, which leads to an increase in root surface in unpolluted soils as well as soils contaminated with toxic metals. Most studies showed an increase of plant biomass after inoculation with endophytic fungi compared to not-inoculated control groups. Furthermore, plants infected with endophytes take up more water and nutrients. The rhizophagy theory suggests that endophytic yeasts and bacteria may be also used by plants as nutrient source when the surroundings are limited in nutrients, serving as prey for the plant root cells. (Domka et al., 2019)

AIM OF THE WORK

The study wants to increase knowledge of a) how plants react to toxic metals, b) how they interact with endophytic fungi, and c) to observe where the fungi are actually entering the plants' roots and how they develop inside the plants. Ultimately the goal was d) to develop possibilities to strengthen plants by the aid of endophytic fungi to improve their performance and quality when growing on contaminated soil, so they might be used in sustainable agriculture or in remediating polluted sites. Two different approaches were conducted to reach these goals. We used *in vitro* cultures growing on agar dishes one hand and soil experiments on the other.

In vitro experiments

In vitro experiments allowed us to directly observe the effects of heavy metals on plants and fungi, as well as the interaction of the organisms. We used a combination of plants of agricultural interest, wheat and amaranth, and plants adapted to increased amounts of heavy metals, *Noccaea caerulescens* and *Noccaea goesingensis*. The plants were infected with endophytic fungi, *Rhodotorula mucilaginosa*, *Sporobolomyces sp.* and *Diaporthe columnaris*, that had been isolated from heavy metal adapted plants.

In a first step we analysed the effect toxic metals have on plants, and on fungi. In a second step we looked at the reaction of both when growing in co-habitation. Plant growth parameters were analysed, like root length and the length of root hair. The analysis of fungal development was based on macroscopic observation.

The goal was to see if the interaction with fungi allows plants to tolerate high amounts of heavy metals. Analysing these interactions, we additionally evaluated the usability of bright field- and fluorescence microscopy aided by different staining methods. Plant-fungi interactions were depicted, focusing on fungal entrance points into the roots and its dispersal within the plants.

Different fungal partners might also affect where and how much heavy metals are stored, inside the fungi themselves as well as within plant roots. Using a small experimental dataset, we estimated the usability of Newport Green to locate the heavy metals in plant and fungal material with fluorescence microscopy and scanning laser microscopy. The interaction of fungus and plant was finally studied by time laps movies, that focused on fungal growth and dispersal, and its interaction with the roots.

Soil experiments

Nevertheless, *in vitro* experiments remain highly artificial and reflect natural systems only in a very simplified way. To address point d), strengthening the plants with the aid of fungi, we decided to perform soil experiments to investigate the interactions under more natural conditions. This time we focused on the effect of heavy metals and yeasts on wheat. After one month of growing in soil we analysed the plants' germination rates, photosynthetic activity, cellular tolerance, and growth parameters like root and shoot length.

plants and fungal

MATERIAL

PLANT MATERIAL

The following species were chosen as plant material to work with, representing a selection of metal accumulating plants and important crop species.

AMARANTHUS CRUENTUS & AMARANTHUS CAUDATUS

Amaranthus species belong to the *Caryophyllales* order, *Amaranthaceae* family, *Amaranthoideae* subfamily, and *Amaranthus* genus. The cosmopolitan plants are widely distributed in temperate and tropical regions, often pioneering on open habitats. They are annual or short lived. They have a herbaceous habit and a prostrate to erect stem and alternating, ovate to linear leaves with terminate or axillary inflorescence. Their tiny lenticular seeds are dispersed by wind, water or birds. The genus has C_4-photosynthesis. (Assad, 2017; Sauer, 1967)

Use

Among the 60 to 70 species of *Amaranthaceae*, about 17 produce edible leaves, only three are grain-producing species, *Amaranthus caudatus*, *Amaranthus cruentus* and *Amaranthus hypochondriacus* (Wolosik & Markowska, 2019; Sauer, 1967; Achigan-Dako et al., 2014). Amaranth grains are used in America and Asia as pseudo-cereals (Zehring, 2015), a term describing dicotyledonous plants like amaranth, quinoa or buckwheat, which produce seeds rich in starch, but are not monocotyledonous cereal grain grasses (Schönlechner et al., 2008). Amaranth leaves are consumed as leafy vegetable in African countries (Zehring, 2015). The plant is used for medicinal purposes, as dye plant, forage, fuel and as an ornamental (Assad, 2017).

Phytoremediation potential

Amaranth species show potential for phytoremediation, like *Amaranthus hypochondriacus* which is capable of accumulating toxic metals like cadmium; and other species containing higher concentrations of a broad range of heavy metals like Cd, Cu, Cr, Fe, Hg, Mn, Ni, Pb and Zn if grown on the respective contaminated sites (Assad, 2017; Li et al., 2013).

In our experiments we focused on *Amaranthus caudatus* and *Amaranthus cruentus*:

Amaranthus caudatus L.

Amaranthus caudatus is used as pseudo-cereal, as leafy vegetable and for ornamental purposes. Inflorescences are dense spikes hanging from their bases. *Amaranthus caudatus* tolerates cool and dry highland conditions, eg. elevations of 1,000 to 3,200 m in South America. (Achigan-Dako et al., 2014)

Amaranthus cruentus L.

Amaranthus cruentus is used for its leaves and grains, as medicine and for ornamental purposes. Its large inflorescences are complex, the terminal spike can reach a length of up to 45 cm. (Achigan-Dako et al., 2014)

Amaranthus cruentus is an annual herbaceous plant, which reproduces only by seeds during a relatively short growing period of four to six weeks. The pseudocereal has a particularly highly regarded nutritional value (Wolosik & Markowska, 2019), but also shows the capability of hyperaccumulating heavy metals like cadmium and zinc, and accumulating lead when growing in polluted areas. Toxic thresholds in the plants leaves were reached for cadmium and lead posing a health risk to humans if used for dietary purposes (Ogunkunle, 2015).

NOCCAEA CAERULESCENS

Noccaea caerulescens (J. Presl & C. Presl) F. K. Mey., the alpine pennycress, formerly *Thlaspi caerulescens*, is a *Brassicaceae* belonging to genus *Noccaea* (Koch, 2013) and is hyperaccumulating zinc, nickel and cadmium (Sterckeman et al., 2015). It is endemic to various European calamine soil types, which are enriched with zinc, lead and often cadmium, including mine and smelter wastes. It is also endemic to serpentine soils, enriched with nickel, chromium and eventually cobalt. (Reeves et al., 2001; Baker et al., 1994)

Noccaea caerulescens is capable of accumulating zinc in its shoots to more than 10,000 µg g^{-1} foliar dry weight, and can therefore be classified as zinc hyperaccumulating plant. It is hyperaccumulating zinc regardless if growing on soils without contamination, with moderate or with high contamination of zinc. (Zhao et al., 2003; Whiting et al., 2001; Baker et al., 1994; Reeves et al., 2001)

Phytoremediation potential
Hyperaccumulators like *Noccaea caerulescens* have received increased interest due to their potential use in phytoremediation, phytomining and biofortification (Verbruggen et al., 2013).

NOCCAEA GOESINGENSIS

Noccaea goesingensis (Halácsy) F. K. Mey. (*Brassicaceae*), formerly *Thlaspi goesingensis*, is a hyperaccumulator of nickel when growing on ultramafic soils (Idris et al., 2004). It is native to serpentine regions of Eastern Austria (Rosenkranz et al., 2019).

Phytoremediation potential
The potential for phytomining using *Noccaea goesingensis* was evaluated by Rosenkranz et al. (2019). The plants reached a maximum nickel yield of 36 kg per hectare, but were outreached by the second species tested, *Odontarrhena chalcidica* (syn. *Alyssum murale*). (Rosenkranz et al., 2019)

TRITICUM AESTIVUM

Common wheat, *Triticum aestivum* L., family *Poaceae*, is one of the most important crops globally and fundamental for feeding the human civilization (Shiferaw et al., 2013). In 2018, the European Union (EU27 plus United Kingdom) produced 138.3 million tons of wheat, making the EU the biggest producer of the 732.4 million tons that were harvested globally (FAO, 2020). 20 per cent of the calories we consume are produced by *Triticum aestivum* (Brenchley et al., 2012; Shiferaw et al., 2013).

Its origins date back to an hybridization event taking place 8,000 years ago, when tetraploid emmer wheat (AABB, *Triticum dicoccoides*) hybridized with diploid goat grass (DD, *Aegilops tauschii*), resulting in an hexaploid genome containing around 17-gigabase-pairs. To date, about 95% of all wheat grown is hexaploid wheat. (Nesbitt, 1996; Brenchley et al., 2012; Shewry, 2009)

The cultivation and domestication of wheat is directly linked to the spread of agriculture and settled societies. Today, wheat is widely cultivated, offering high yields, as well as nutritional and processing qualities. (Brenchley et al., 2012)

FUNGAL MATERIAL

DIAPORTHE COLUMNARIS

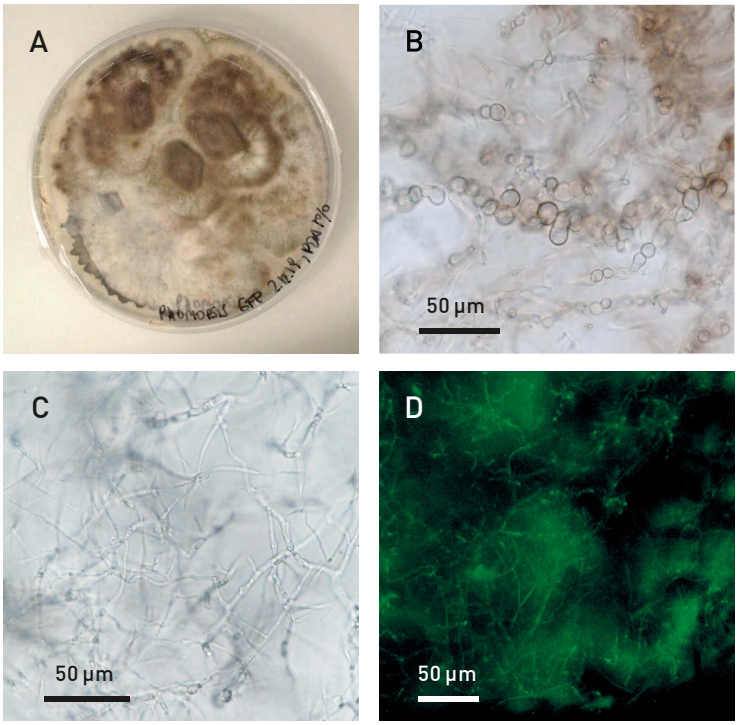
Diaporthe columnaris (D.F. Farr & Castl.) Udayanga & Castl., formerly known as *Phomopsis columnaris*, is belonging to the phylum of Ascomycota, class Sordariomycetes, order Diaporthales, genus *Diaporthe* (Kirk, 2020).

Diaporthe species have been isolated from numerous hosts. They are described as plant pathogens, non-pathogenic endophytes or saprobes. Some species can be both, endophytes to some species and pathogens to others, depending on the hosts and the plant's condition. They can cause fruit rot, die-back, cankers, leaf spots, blights, decay and wilt. *Diaporthe* spp. are frequent endophytic fungi, that produce enzymes and secondary metabolites showing anticancer and antibiotic activity, they deter herbivory and have been applied as bioherbicides. (Gomes et al., 2013)

Diaporthe species also include its asexual states, *Phomopsis*. Since the deletion of Article 59 in the International Code of Nomenclature for algae, fungi and plants, both states, sexual and asexual, are listed as equal status, in this case *Diaporthe* which has been described before *Phomopsis*. (Gomes et al., 2013)

Diaporthe columnaris has been isolated and described by Farr et al. (2002) from a dying lingonberry stem (*Vaccinium vitis-idaea*, *Ericaceae*). It has typical conidiophores, made of cells that are arranged vertically in lines at the side and base of the conidiomata, and that allow differentiation from other *Diaporthe* species. (Farr et al., 2002)

We used GFP-transformed specimen (Figure 1, D) as well as non-GFP-ones, generously provided by Agnieszka Domka from the plant-microbial interactions team, JU, Krakow, for our experiments which have been isolated from various hyperaccumulating plants on zinc and nickel contaminated sites. In Ramingstein, a zinc contaminated site in Austria, *Diaporthe columnaris* was isolated from *Noccaea caerulescens*. It was furthermore isolated from three nickel sites: from *Noccaea goesingensis* in Redlschlag, Austria; from *Noccaea caerulescens* and from *Alyssum serpyphyllum*, both in Spain.



DIAPORTHE COLUMNARIS

Figure 1:
Diaporthe columnaris,
GFP transformed;
A) as macroscopic culture, growing on PDA in 1 % agar on 9 cm diameter petri dishes. (Bar = 3 cm)
B), C) Hyphae under bright field;
D) Hyphae under fluorescence light.

RHODOTORULA MUCILAGINOSA

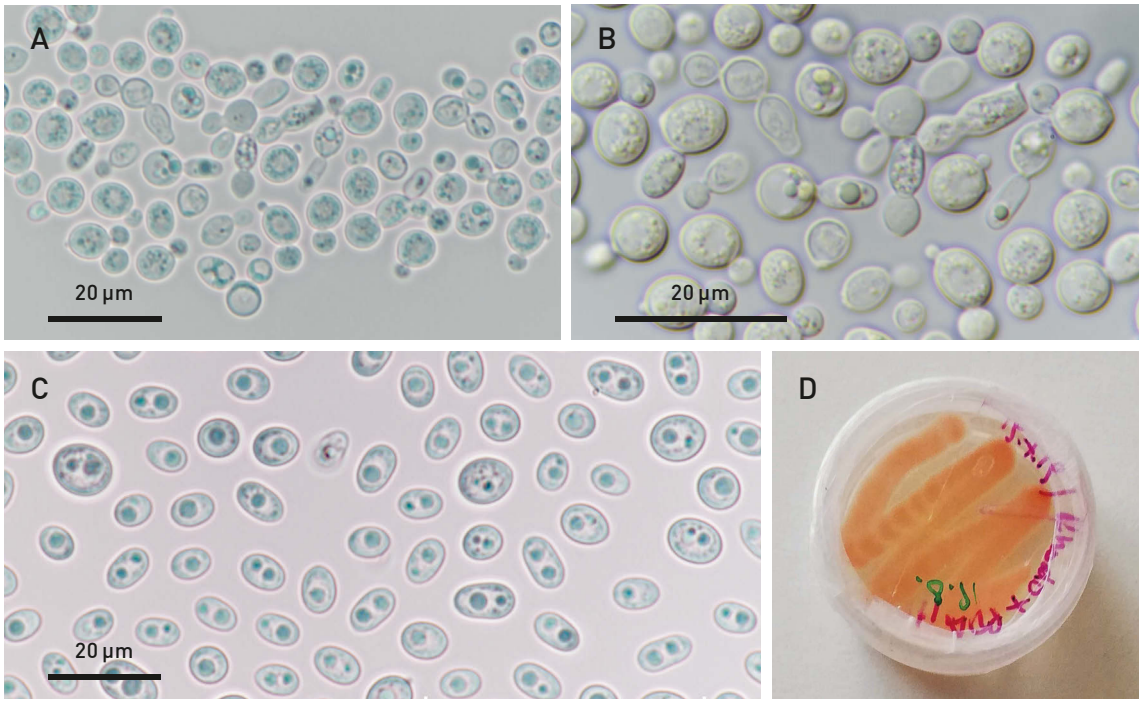


Figure 2: Rhodotorula mucilaginosa: *A) under bright field; B) and C) differential interference contrast; D) macroscopic culture, growing on PDA in 1 % agar on 3 cm diameter petri dishes.*

SPOROBOLOMYCES

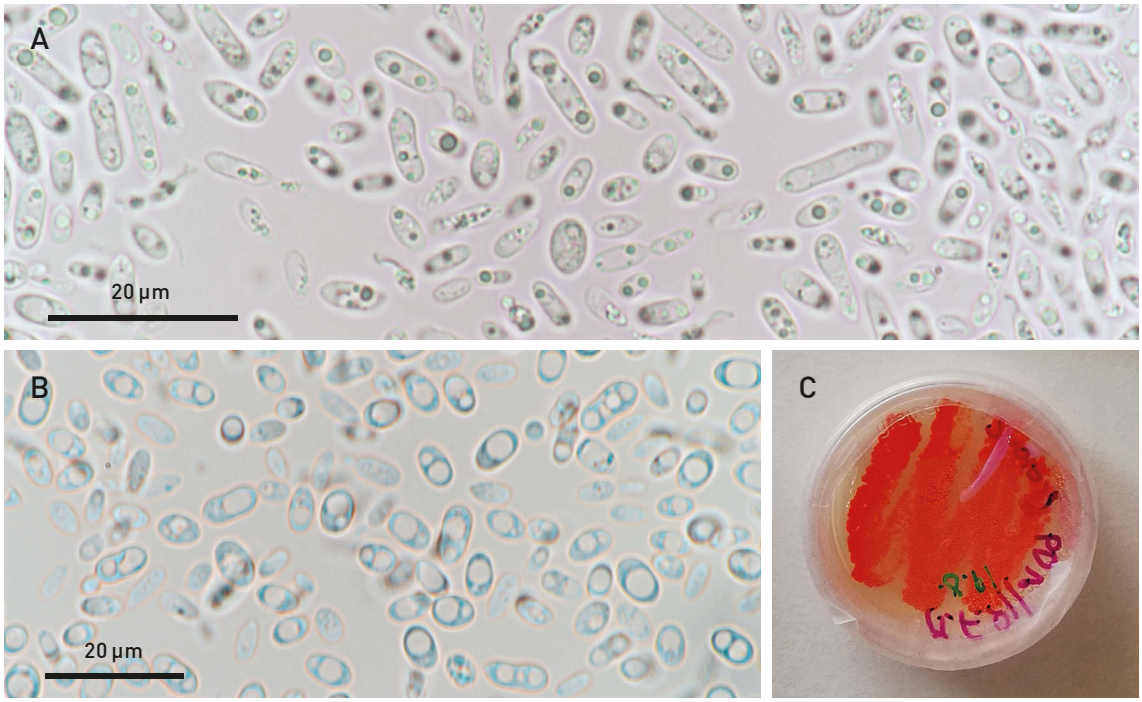


Figure 3: *A)* Sporobolomyces ruberrimus *under bright field, B)* Sporobolomyces sp. *under bright field; Bars in A) and B) equal 20 µm; C) macroscopic culture, growing on PDA in 1 % agar on 3 cm diameter petri dishes.*

RHODOTORULA MUCILAGINOSA

Yeasts of the genus *Rhodotorula* belong to the phylum Basidiomycota, class Microbotryomycetes, order Sporidiobolales (Sen et al., 2019, Roskov et al., 2020). *Rhodotorula* species inhabit a wide range of environments and are found in deep-sea vents, hypersaline environments, in deserts, in soil and air, and in other organisms (Sen et al., 2019). They show strong affinity for plastic and are the most common microorganisms that have been isolated from employees and patients in hospitals (Wirth & Goldani, 2012).

Rhodotorula mucilaginosa (A. Jörg.) F.C. Harrison has been isolated from common food like peanuts, sausages and crustaceans (Sen et al., 2019; Wirth & Goldani, 2012). The yeast has even made its way to the international space station, ISS, where it has been detected onboard in 2017 by Reidt et al. (Reidt et al., 2017). In a clinical environment *Rhodotorula mucilaginosa* might cause pathogenesis in patients that are immune-suppressed (Sen et al., 2019). Nevertheless, the yeast may be present on skin, in sputum, faeces and urine, and is seen as opportunistic pathogen causing meningitis, endocarditis, skin infections, etc. (Falkes-Romero et al., 2017).

Plants on the other hand seem to profit from the interaction with *Rhodotorula mucilaginosa*. Endophytic yeasts of the genus *Rhodotorula* can improve plant growth (Sen et al., 2019). *Rhodotorula mucilaginosa* is a class 2 endophyte that inhabits host tissues systemically (Domka et al., 2019). It promoted growth in *Typha angustifolia*, where Sen et al. (2009) had isolated it from.

Macroscopic Features on (malt) agar (Figure 2, D)
Rhodotorula mucilaginosa produces pinkish to reddish colonies, due to production of carotenoids, with a glistening and usually smooth surface, with smooth to mucous texture. (Wirth & Goldani, 2012; Nunes et al., 2013; Sampaio, 2011)

Microscopic Features (Figure 2, A to C)
The genus *Rhodotorula* is described as monomorphic, no transition between yeast and filamentous from is known (Sen et al., 2019). Balistoconidia are unicellar and lack pseudohyphae and hyphae (Wirth & Goldani, 2012), the ability for fermentation is lacking (Sampaio, 2011).

Rhodotorula mucilaginosa is an unicellular, pigmented yeast. Cells are described as subglobose to ellipsoid or ovoid, with sizes ranging from 1.5 to 4 × 4 to 12 µm (Sampaio, 2011). Budding cells are described as spherical to oval (Nunes et al., 2013). Budding is predominantly polar (Sampaio, 2011).

Like other basidiomycetes, *Rhodotorula* yeasts produce natural pigments like carotenoids. Carotenoids can function as antioxidants, they are helping by protecting from photodamage, by peroxy radical scavenging and by singlet oxygen quenching. *Rhodotorula yeasts* produce beta- and gamma carotene, torulene and tolarhodin, a specific xanthophyll. *Rhodotorula mucilaginosa* can produce high amounts of lipids, up to 70 % of its biomass. (Cobban et al., 2016; Pacia et al., 2016)

Rhodotorula species have shown great potential in removing heavy metals, growing as biofilm it was able to remove copper, mercury and lead ions as shown by Grujić (2018). Furthermore, their work demonstrated a nickel and zinc impact on *Rhodotorula mucilaginosa* living planktonic and as biofilm. Both showed high tolerances to elevated cadmium, nickel and zinc levels, the biofilm-yeasts survived all offered concentrations of metals, due to extracellular polymeric substances that absorb pollutants (Domka et al., 2019). The biofilm gave promising results regarding the removal of metals. (Grujić et al., 2018)

SPOROBOLOMYCES

Genus *Sporobolomyces* is polyphyletic and currently holding 50 accepted species in four lineages. It can be easily discerned from many other species of yeast by the formation of reproductive ballistoconidia. Ballistoconida are mitotically produced cells, capable of forced ejection from the parent fungus. Many *Sporobolomyces* species where isolated from dead leaves. Glueing them to one side of a petri dish might form mirror images on the other half of the dish, due to ballistoconidia ejection, a "shadow" yeast is emerging. Furthermore, genus *Sporobolomyces* is not capable of fermenting sugars. (Cobban et al., 2016)

Cobban et al. (2016) isolated various *Sporobolomyces* species from different leaves in Vermont and Washington. All of the isolates were showing red pigmented, smooth colonies grown on solid media, all formed ballistoconidia. The red color is caused by natural pigments, like carotenoids, torularrhodin, beta-carotene and torulene. All isolates were tolerant to low temperatures but sensitive to desiccation and incubation temperatures of 37 °C. All isolates could grow on solid and liquid media and needed aerobic conditions for growth. Heating for 15 minutes at 70 °C, followed by an ice bath inhibited any further growth after transferring the yeasts to new growing media. (Cobban et al., 2016)

Sporobolomyces ruberrimus

Sporobolomyces ruberrimus Yamasaki & H. Fujii ex Fell, Pinel, Scorzetti, Statzell & Yarrow, belongs to the phylum of Basidiomycota, genus *Sporobolomyces* (Kirk, 2020). The isolate used in our experiments originates from Kraubath, Austria and is resistant to cadmium, nickel and zinc.

Macroscopic Features on (malt) agar (Figure 3, D)

Sporobolomyces ruberrimus grows in whitish to orange colonies that are flat and dull (Hamamoto, 2011).

Microscopic Features (Figure 3, A to C)

Single yeast cells are ovoid or elongate, measuring 3 to 5 × 2 to 3 µm. They occur in small clusters of yeast cells, or as single cells. They reproduce by blastoconidia, which are forming directly on the parental cell. Short conidiophores have been observed as well. Buds appear ovoid to reniform. (Hamamoto, 2011)

The

METHODS

EFFECTS OF HEAVY METALS ON PLANTS

A set of different plant species, hyperaccumulating plants as well as species more sensitive to toxic metals but with economic value, was used in *in vitro* cultures to test their suitability for further experiments.

Seeds of *Amaranthus cruentus*, *Noccaea caerulescens*, *Noccaea goesingensis* and *Triticum aestivum* were surface sterilized by immersing them in 70 % ethanol for two to five minutes, followed by ten minutes in 50 % hypochlorid with a few drops of triton (serving as detergent), and rinsing them in sterilized water for five times. The seeds were placed on agar dishes under sterile conditions, which contained 1% agar, ½ Murashige and Skoog Medium without minor salts (MS), and either 1 mM nickel, 1 mM zinc or no heavy metals. The number of seeds per plate was estimated according to their size: five seeds of *Triticum aestivum*, the biggest seeds used, were put on the plates. Approximately 30 seeds of the smaller *Amaranthus cruentus*, *Noccaea caerulescens*, and *Noccaea goesingensis* seeds were sown. Five repeats per species were prepared, for *Triticum aestivum* twelve repeats were prepared for each treatment, respectively. The plates were sealed with PARAFILM® and rested in the dark at 4 °C for 48 hours for stratification. Plants were allowed to germinate and grow under artificial light, at 22 °C, at a diagonal position to direct the growth of the roots downwards. Only the zinc treatment plates rested at horizontal orientation, as the respective agar did not solidify completely.

HEAVY METAL EFFECT ON GERMINATION RATE AND DEVELOPMENT OF THE PLANTS

After one week, germination rates were estimated. Macroscopic pictures were taken to document the development of the plants. Root hair development was documented using a stereomicroscope. We decided to continue working with *Amaranthus cruentus* and *Noccaea caerulescens*.

EFFECTS OF HEAVY METALS ON FUNGI

Amaranthus cruentus and *Noccaea caerulescens* were inoculated with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* respectively after 18 days of growing under heavy metal influence. Yeasts were added to the plates under sterile conditions. Fresh cultures of yeast were transferred from cultures growing on agar, using disinfected, flame-clean inoculation-loops. They were applied next to the roots, with a distance of approximately 0.5 cm and no direct contact to the plants. Plates were sealed using PARAFILM® to minimize contaminations. After six days of growth, macroscopic pictures were used to compare fungal development under heavy metal influence.

In a second approach, *Sporobolomyces sp.* was co-cultured with *Amaranthus cruentus*. *Sporobolomyces sp.* was applied to agar dishes with 1 month old seedlings of *Amaranthus cruentus*. Agar dishes contained ½ MS to provide the major nutrients, 1 mM nickel, 1 mM zinc, or no metals. Nickel and zinc dishes were prepared with 1 % agar, zinc agar with 2 % to achieve a solid medium. The yeast was applied under sterile conditions in three ways: With great distance to the plants; close to the plants, but without direct contact; and directly onto the plants. Plates were sealed using PARAFILM®. After three days the development of the yeasts was analysed.

FUNGAL INFLUENCE ON *TRITICUM AESTIVUM*

In a second step, we wanted to get a closer look into the performance of *Triticum aestivum* growing by its own, or with *Diaporthe columnaris*, or *Rhodotorula mucilaginosa*. Plants and fungi were grown either as control without heavy metal influence or with 100 µM nickel on 0.6 % agar in twelve cm wide, quadratic petri dishes. No additional nutrients were added. (Figure 4)

The concentration of the heavy metal was chosen based on preliminary experiments resulting in sufficient germination and growth of *Triticum aestivum*, while the plants were still clearly affected by the heavy metal. 0.6 % agar was prepared to offer the plants a stable medium, yet easy to enter for the roots.

Seeds were soaking in double distilled water for twelve hours and were surface sterilized afterwards (three minutes in 70 % ethanol, ten minutes in 50 % hypochlorid with a few drops of triton, rinsing them in sterilized water for five minutes four times). Five seeds were placed per petri dish under sterile conditions with the furrow facing downside. The plates were sealed with PARAFILM® and rested in the dark at 4 °C for 24 hours for stratification. Plants were allowed to germinate and grow under artificial light, at 22 °C, at a diagonal position to direct the growth of the roots downwards into the agar.

After four to six days fresh cultures of *Diaporthe columnaris* or *Rhodotorula mucilaginosa* were added to the plates under sterile conditions. The goal was to add the fungi to seedling that had developed roots with an approximate length of 1 cm. After seven days of incubation, root lengths and the length of the longest root hairs were estimated with NIS software or FIJI/Image J (Version:2.1.0/1.53c), additionally germination rate was estimated. Stereoscopy was used to document the experiment.

Figure 4: *Quadratic petri dish with 0.6 % agar used to grow* Triticum aestivum *with* Rhodotorula mucilaginosa, *or* Diaporthe columnaris.

EFFECTS OF HEAVY METALS ON PLANTS AND YEASTS

Effects on root length, root hair length and the distance between the first root hair and the root tip in *Amaranthus cruentus* and *Noccaea caerulescens* were estimated after three days of growing with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* respectively. For documentation and measurements we used a long distance lens with a Nikon Eclipse microscope, as well as stereomicroscopy, both equipped with NIS software (Nikon Corporation).

HEAVY METAL LOCALIZATION, STAINING USING NEWPORT GREEN™

One of the aims of the study was to estimate the usability of Newport Green™ to locate nickel and zinc in plant and fungal material with fluorescence microscopy and scanning laser microscopy. We approached this aim with a small, experimental dataset as follows:

Heavy metal localization using Newport Green™ with fluorescence microscopy to detect nickel and zinc in roots of *Amaranthus cruentus* and *Noccaea caerulescens*

Nickel and zinc deposits in root cells and root hairs of *Amaranthus cruentus* and *Noccaea caerulescens* seedlings were detected using Newport Green™ DCF indicator (NPG), which complexes with nickel and zinc. The cell-permeant has its excitation/emission maxima at 505/535 nm. It moderately binds to zinc (Kd for Zn ~1 µM), but is quite insensitive to calcium. Furthermore, NPG is an exceptionally sensitive probe for Ni^{2+} in solution. (Thermo Fisher, 2010).

We wanted to see if the different treatments, seedlings growing in agar plates without heavy metal contamination or with 1 mM nickel or 1 mM zinc, show different contents of heavy metals in roots and root hairs, as well as different intracellular heavy metal storage, visualised by the use of fluorescence microscopy. Additionally we wanted to investigate if growing with *Rhodotorula mucilaginosa* or *Sporobolomyces sp*. affected the incorporation of heavy metals into roots and root hairs.

Seedlings of *Amaranthus cruentus* and *Noccaea caerulescens*, grown under the conditions above-mentioned, were placed on microscope slides, 125 µl of 10 µM NPG were added. The plants were analysed using a fluorescence microscope (Nikon ECLIPSE Ni set up with a Nikon DS-Ri2 camera and Nikon intenselight C-HGFI Fluorescence equipment) after two hours of incubation time. Pictures were taken under blue excitation, without additional filters, at various magnifications (10 × 1; 20 × 1; 20 × 2; 40 × 1; 40 × 2). Using the NIS-elements software (Nikon Corporation), thresholds were estimated at which the samples barely showed any fluorescence. The slides were stored overnight in moist chambers at 4 °C. After 20 hours they were re-examined to see if the increased incubation time affected the fluorescence by giving in- or decreased signals.

Heavy metal localization using NPG, bright field and confocal microscopy to detect nickel and zinc in *Sporobolomyces* Sp.

In addition, we were interested in the heavy metal uptake and its storage into the yeast cells. Slides with yeasts that had been growing with *Amaranthus cruentus* and *Noccaea caerulescens* were prepared and analysed after 48 h of infiltration with NPG. Analysation was performed using bright field and fluorescence microscopy as well as confocal laser scanning microscopy (CLSM).

To get a closer look into the storage of nickel and zinc inside the yeast cells, we decided to use confocal microscopy. Six microscopy slides were prepared in total, with *Sporobolomyces sp.* grown on agar plates together with *Amaranthus cruentus*. Pinhead-sized pieces of agar and yeast were cut out and placed on the slides. One set contained pieces of agar with yeast from the control group, one from the nickel treatment and one from the zinc treatment. A second, identical set was prepared and additionally stained with 10 µM NPG for 3 hours to compare the autofluorescence to the actual dye. Slices were kept at 4 °C in moist chambers after the preparation. Analysation was performed using CLSM.

INTERACTION OF PLANTS AND FUNGI

Interactions of plants and fungi might include cellular penetration of the plants by the fungi. To estimate the amount of infection in the cortex of the root, different staining methods were exercised. (Philipps and Hayman, 1970)

If not mentioned otherwise further down in the text, plants were grown as follows: Seedlings of *Triticum aestivum* were grown in 12 cm wide, quadratic petri dishes with 0.6 % agar and 100 µM of nickel. After immersing in water overnight at 4 °C, seeds were surface sterilized by soaking in 50 % bleach (DanKlorix Hygienereiniger, 2.8 g sodium hypochlorite per 100 g fluid) for 5 minutes, soaking in 70 % ethanol for 5 minutes, and rinsing thrice in sterile water. Five seeds were placed on the agar under sterile conditions, plates were sealed with PARAFILM® and rested in the dark at 4 °C for 24 hours for stratification. After germination and roots having reached lengths of approximately 1 cm, *Diaporthe columnaris*, *Rhodotorula mucilaginosa* or *Sporobolomyces ruberrimus* were applied to the petri dishes, respectively. After one week, roots were cut out with the surrounding agar. To examine the interactions various rapid conventional staining methods were applied, as described hereinafter.

STAINING USING CALCOFLUOR-WHITE

Calcofluor-White stain is a fluorescent brightener used to detect yeasts, fungi and parasitic organisms. It is binding to beta 1-3 and beta 1-4 polysaccharids, compounds found in cellulose and chitin, and fluoresces in blueish and white under UV radiation. Absorption is shown over a range of 300 to 412 nm, peaking at 347 nm. (Harrington & Hageage, 2003)

One to two drops of 0.1 % Calcofluor-White were added to the samples, and examined under the microscope after one minute of incubation time.

STAINING USING SUDAN IV

Sudan IV stains oils, fat, shoe polish, floor polish and pomade in red. In biology it is used to detect protein-bound lipids and triacylglycerols in tissues. (Welsch, 2018)

The following staining protocol (Mulisch, 2015) was applied: 50 mg Sudan IV were solved in 50 ml ethanol. The mixture was filtered and 50 ml glycerol were added. Plant tissue was allowed to immerse in the liquid and was carefully heated in dyeing glasses. As the protocol was lacking the duration time of immersion, we started from 1 min and increased immersion- and heating-times.

Bright field microscopy: Sudan IV immersion time estimation

Diaporthe columnaris had been grown on agar with 1 mM nickel. Small pieces of agar containing the fungi were cut out, and put into moist chambers with 1 mM nickel solution. A seed of *Triticum aestivum* was soaked overnight in distilled water at 4 °C and placed on top the agar piece. Seedlings were allowed to germinate for 6 days in the lab. Samples were stained by immersing and heating in Sudan IV solution for 1 minute, with increasing time intervals up to 15 minutes. Samples were observed under the microscope under bright field.

Sporobolomyces sp. was chosen to estimate the immersion time necessary to stain yeast cells. The yeast was stained by cutting out a piece of growing medium including the culture and heating it in the staining solution for 5 up to 15 minutes.

STAINING WITH ANILINE BLUE

Aniline blue, also referred to as methyl blue, water blue, Poirrier's blue, cotton blue, etc., is widely used to stain plant-fungal interactions. Its application area includes fungal infection and colonization of plants, as well as visualizing the presence of hyphae in tissue. Using fluorescence microscopy, it is widely used to stain callose depositions, these "callose plugs" are formed by plants as response to biotic and abiotic stress. (Hood & Shew, 1996; Liu et al., 2018)

The following, slightly adapted, protocol was used to stain fungi, based on the suggestions of Hood and Shew (1996): 0.05 % Aniline blue dye were mixed with 0.067 M K_2HPO_4 (Merck) at least two hours prior to use. Samples embedded in agar were treated at room temperature in 10 M KOH for 4 hours, and rinsed three times in deionized water. The staining solution was added directly dropwise onto the specimen on microscopy slides. Samples were examined under bright field as well as under fluorescence light using the 420 nm filter. Additionally a shorter immersion time was used for samples not embedded in agar. Samples were stained with 0.05 % or 0.1 % Aniline blue after heating for 15 or 30 minutes in 1 M KOH.

GROWTH DYNAMICS OF THE FUNGI

To analyse the development of the fungus and the first contact with the plants timelapse was used. We used direct examination of the plants growing in petri dishes, eg. by using stereomicroscopy. Alternatively microchambers were prepared, which allowed a closer look:

Growth development in microchambers

Microchambers were built to study the interaction of plant and fungi over time. Figure 5 illustrates the chamber-setup, where seedlings of *Triticum aestivum* were placed with *Diaporthe columnaris*. Root and fungus were placed as close as possible to each other, without direct contact. Samples were prepared with and without nutrient containing agar, to compare the effect on the fungus. (Figure 5)

The aim was to see, if the fungus is attracted by the plants and shows directional growth towards the plant. Pictures were taken every 1 or 5 minutes to examine where and how the interaction between both takes place.

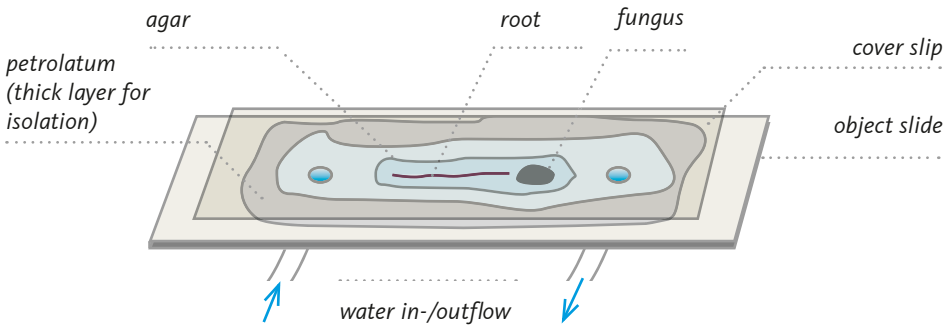


Figure 5: *Microchambers were used to examine the fungus-wheat interaction over time. A petrolatum "wall" was applied to an object slide to seal the chamber later on. Fluid agar was applied to the slice and allowed to solidify. A root piece of* Triticum aestivum *was placed on the agar next to* Rhodotorula mucilaginosa. *To avoid air bubbles, fluid agar was injected using a syringe after applying the cover slip. Samples were kept moist by pumping fresh water into the chamber continuously.*

INTERACTIONS OF PLANTS, HEAVY METALS AND YEAST

To get further insight into the interactions of yeast and plants under more natural conditions, we decided to perform pot experiments with a growing time of one month, using *Triticum aestivum* as model plant. In prior experiments, *Noccaea caerulescens* had shown poor, slow growth after a growing period of one month, *Amaranthus caudatus* had given quite divergent results. *Triticum aestivum* had grown fast and quite homogenous within the respective treatments and therefore was chosen as model plant for the experiment. Different concentrations of heavy metal contaminated soil were used in combination with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* as yeast-partner to see, if the yeasts have an effect on the plants' performance under heavy metal stress.

The soil used for the experiment consisted of 70 % potting soil (Franz Kranzinger GmbH), 20 % perlite (GramoFlor) and 10 % quartz (Min2C, 0.5 mm to 2 mm grain size), a mixture commonly used by the gardeners of the University of Vienna. The soil mixture was filled into labelled, sterile 60 ml plastic pots.

Soil experiment 1

In the first set-up a liquid culture of *Rhodotorula mucilaginosa* was used to infect seedlings of *Triticum aestivum* growing in heavy metal contaminated soil. Nickel or zinc contaminated soil was prepared in two concentrations, 1 mM and 0.1 mM, respectively. To prepare the zinc-contaminated soil, zinc sulfate heptahydrate (EMSURE®, Merck Chemicals) was used. Nickel-contamination was prepared using nickel(II) sulfate hexa-/heptahydrate (Sigma-Aldrich). Heavy metal solutions were added to the soil by watering each pot three times using 30 ml of a heavy metal solution, a total volume of 90 ml was added per pot. Non-contaminated control pots were treated likewise, using 90 ml double-distilled water instead. Each treatment was prepared in triplicates.

Biological controlled seeds of *Triticum aestivum* (Nestelberger) were soaked overnight at 4 °C in double-distilled water. Five seeds were added per pot. Seeds were allowed to germinate under lab conditions. After three days, when the first green tips of the germ buds became visible, the seedlings were infected with *Rhodotorula mucilaginosa.* To infect the plants with yeast, a liquid yeast culture was prepared by diluting a colony of *Rhodotorula mucilaginosa*, growing on an agar plate, in a falcon tube with 25 ml sterile, autoclaved water. After vortexing the solution, 5 µl were added to an Erlenmeyer flask with 50 ml of liquid WYEM growing medium (100 ml H_2O, 0.3 g yeast extract, 0.3 g maltose, 0.5 g peptone, 1 g glucose). The liquid cultures were put on a shaker with 70 rpm, at approximately 28 °C. After having reached sufficient cell-density, the yeast cells were separated from the growing medium by centrifuging them twice. The supernatant growing medium was replaced with autoclaved water. The remaining yeast-pellet was resuspended by vortexing. 60 µl of the liquid yeast cultures were added per plant (3 ml per pot in total).

Additionally, unpolluted control groups were prepared without any heavy metal pollution. To see if the yeast itself has nourishing effects to the plants—without any active interaction of the yeast with the plants—a control group with dead yeast cells was prepared as well. One liquid culture was heated to kill the yeast cells within and added to the plants after cooling down to room temperature.

The procedure was repeated after four days, to further increase the yeast density in the pots. Compare with figure 6 for the whole set-up.

After the first inoculation with *Rhodotorula mucilaginosa*, the plants were moved to the glass house of the University of Vienna, Althanstr. 14, to grow at a temperature range between 20 °C and 28 °C, at 40 % to 65 % humidity. Plants were watered as needed every two to three days with 30 ml of deionized water. To limit nutritional deficiency of the plants, WUXAL® Super (8 % N, 8 % P_2O_5, 6 % K_2O) was added twice during the growing period of 30 days. Additionally, 30 ml of heavy metals in the respective concentrations were added twice to limit leaching of the heavy metals from the pots.

Photosynthetic activity

Chlorophyll fluorescence was measured as a proxy for stress, using a Handy PEA+ meter (Hansatech Instruments Ltd.) after 29 days of growth. Three measurements were taken per treatment, after darkening the measurement spot for 20 min with leafclips provided.

Harvesting the plants

The plants were harvested after a total growing period of 30 days by carefully taking them out of the pots. Roots were cleaned from soil particles by washing them using deionized water and by carefully detangling them. Root length, shoot length and the number of leaves were estimated.

Analysis & Statistics

For the statistical analysis Microsoft Excel, Version 15.33, and Past software, version 3.14 were used. Data from the Handy PEA+ meter was exported using the PEA+ software provided by Hansatech Instruments Ltd. and further processed using Microsoft Excel. Graphs were edited with Adobe Illustrator CS6 software.

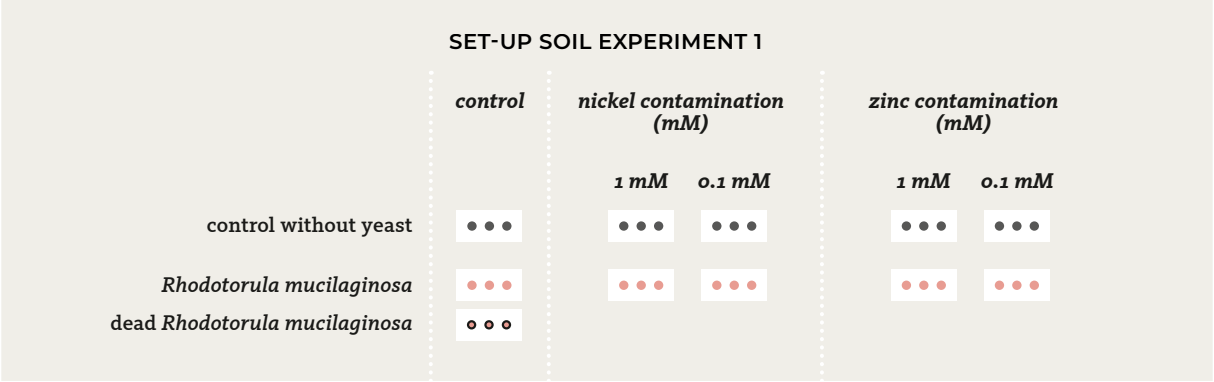


Figure 6: *Experimental setup of soil experiment 1.*

Soil Experiment 2

For the second soil experiment we used a slightly adapted experimental design: To reduce the number of naturally occurring fungal soil-contaminants, soil and pots were autoclaved at 121 °C for 20 minutes. The pots were prepared using the same composition of soil as mentioned above.

90 ml of zinc-solution (Zinc sulfate heptahydrate; EMSURE®, Merck Chemicals), nickel-solution (Nickel(II) sulfate hexa-/heptahydrate; Sigma-Aldrich) or double-distilled water were added to the pots, by watering each pot three times with 30 ml of the respective solutions. Three different concentrations of heavy metals were used, respectively 10 mM, 5 mM or 1 mM of nickel, and 50 mM, 10 mM or 1 mM of zinc. Additionally control pots without any heavy metal treatment were prepared. All treatments were prepared in triplicates. Compare to figure 7 for the experimental setup.

Triticum aestivum seeds (Nestelberger, AT-BIO-301) soaked overnight at 4 °C in double-distilled water. Prior to sowing, the seeds were infected with liquid cultures of *Rhodotorula mucilaginosa* or *Sporobolomyces sp*. The liquid yeast cultures were prepared by diluting colonies of *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* from fresh cultures on agar plates in falcon tubes with 25 ml sterile, autoclaved water. The solution was vortexed to reach an even distribution of yeast cells. 5 µl of the water-yeast suspension were added to an Erlenmeyer flask with 50 ml of WYEM growing medium. The liquid cultures were put on a shaker with 70 rpm, at approximately 28 °C for growth. To separate yeast and growth medium, the suspensions were centrifuged twice, with 4,000 rpm, at 25 °C, for 5 min each. The supernatant liquid was discarded, the pellet was resuspended in an equal amount of sterile water. To inoculate the seeds with *Rhodotorula mucilaginosa*, one third of the seeds were allowed to immerse in a liquid yeast culture for 1 hour. The second third of *Triticum aestivum* seeds immersed in a liquid culture of *Sporobolomyces sp.* for 1 hour. The remaining plants served as non-infected control seeds and were immersed in sterile water. Additionally, yeast viability was checked by striking on agar plates. Five seeds were planted per pot. After 3 days of germination in the lab, yeast solutions were added a second time. 60 µl of the liquid yeast cultures were added per plant (3 ml per pot in total).

The final setup consisted of triplicates of *Rhodotorula mucilaginosa*, *Sporobolomyces sp*. and a control set containing no additional fungi, in heavy metal contaminated pots, with 10 mM, 5 mM or 1 mM of nickel-contamination, or 50 mM, 10 mM or 1 mM of zinc-contamination. Additionally, a blank set containing no heavy metals was prepared. This control set contained either *Rhodotorula mucilaginosa*, *Sporobolomyces sp.*, no yeasts at all, or dead yeast cells. The dead yeast cells were added to the pots to see if the yeast cells themselves have nutritional value for the plants. This control group was prepared by sterilizing liquid yeast cultures by boiling for 20 minutes. Inoculation followed the procedure mentioned above for living cultures.

Right after the second inoculation, plants were moved to the glass house of the University of Vienna, Althanstr. 14. They grew for four weeks, at a temperature range between 20°C and 28 °C, and a humidity range of 40 % to 65 %. Plants were watered every second day with 30 ml of deionized water. After the first week of growth, 0.1 % WUXAL® Super (8 % N, 8 % P_2O_5, 6 % K_2O) was added to allow sufficient plant nutrition and healthy growth. The concentration of WUXAL® Super was increased to 0.2 % in the following weeks, added once a week. Additionally 30 ml of heavy metals in the respective concentrations were added once a week to limit leaching of the heavy metals from the pots.

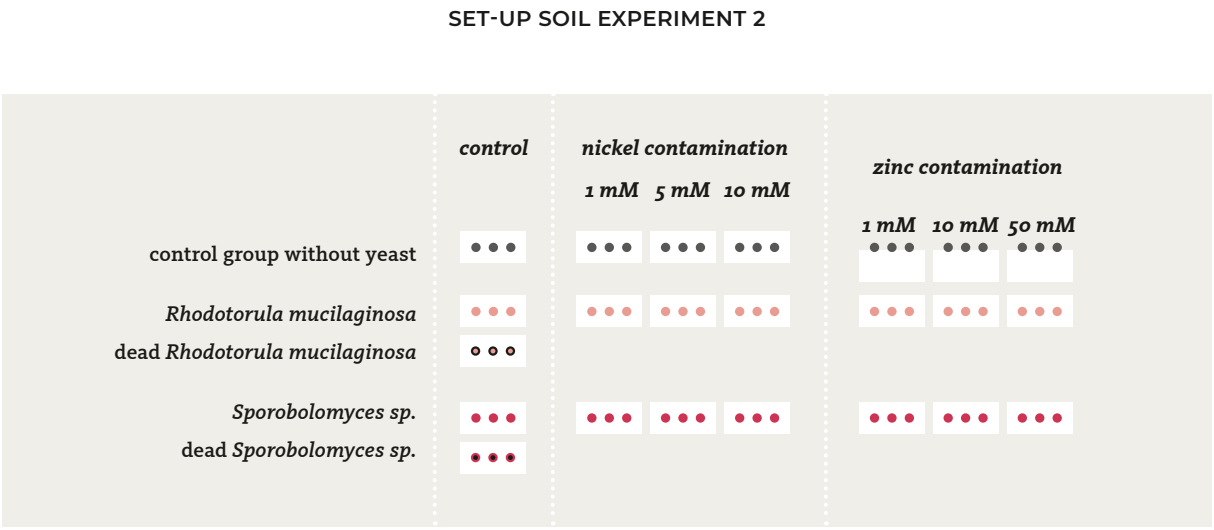


Figure 7: *Experimental setup of soil experiment 2.*

Photosynthetic activity

To estimate photosynthetic activity of the plants chlorophyll fluorescence was measured after 19 days of growth using a Handy PEA+ meter (Hansatech Instruments Ltd.). The measurement spot was darkened for 20 min by leafclips prior to measures. Three measurements were taken per treatment.

Cellular tolerances

Heavy metal treatment in our experimental design had strong macroscopic impact on our plants. In this step we wanted to get an insight into the cellular effect the combined treatments of heavy metal contamination and inoculation with yeast might have, and decided to perform cellular tolerance tests. The basic idea is to find the lethal threshold of heavy metal solution on a cellular level, which might differ if growing under different conditions. Surface cuts were immersed in concentration rows of heavy metal solutions, in our case from 1 M to 1 µM of nickel sulfate and zinc sulfate solutions. After resting in these solutions for 48 h, a plasmolyte was added, like mannitol, forcing surviving cells to adapt to the new osmolytic situation by showing a shrinking protoplast, a reversible process if the cells are still alive.

We chose plants that were heavily affected by the heavy metal treatments and showed reduced growth compared to the control group, yet leaving enough plant material for further analysis. For the nickel treatment plants from the 5 mM group were chosen. The zinc group was represented by plants treated with 10 mM zinc. Additionally plants from the control group were selected. Each soil treatment group, control, nickel or zinc, consisted of a plant inoculated with *Rhodotorula mucilaginosa*, *Sporobolomyces sp.*, and a plant without yeasts serving as control.

Leaf-surface cuts were prepared and allowed to immerse for 48 hours in nickel sulfate or zinc sulfate solutions with concentrations of 1 M to 1 µM and an additional tap water control containing no heavy metals. To avoid any photosynthesis, the samples were kept in the dark during immersion. Cuts were put on slides, drops of 0.8 M mannitol were added, and after ten minutes cuts were analysed under the microscope.

Cells were counted “alive” if they reacted to the 48 hours heavy metal solutions treatment with plasmolysis, showing a shrunken protoplast, if they showed healthy, clearly defined chloroplasts with a “healthy” green colour and if they deplasmolysed again. If the cells did not show any signs of plasmolysis, with crushed, brownish or yellowish chloroplasts or if they did not react with deplasmolysis, they were counted as being “dead”.

Harvesting the plants

The plants were harvested after four weeks of growth. Plants were carefully taken out of the pots and washed using deionized water to remove soil particles and to clean the roots. After estimating length of roots, length of leaves and the number of leaves, the plants were oven-dried at 45 °C. Finally, dry weight of roots and shoots was estimated.

Statistics

Data was analysed using Microsoft Excel, Version 15.33, and Past software, version 3.14 for Mac. Analysis of Variance (ANOVA) was used followed by Tukey's test ($p = .05$) to test for statistical significance. Kruskal-Wallis tests were used if normality of the data or homogeneity of variances were violated. Graphs were edited with Adobe Illustrator CS6.

RESULTS

EFFECTS OF HEAVY METALS ON PLANTS

A mixture of plant species, from accumulators and excluders to heavy metal sensitive but economically important plant, was tested for their suitability for further experiments in *in vitro* cultures. Surface sterilized seeds of *Amaranthus cruentus*, *Noccaea caerulescens*, *Noccaea goesingensis* and *Triticum aestivum* were allowed to germinate on petri dishes under sterile conditions and either 1 mM nickel, 1 mM zinc, or no heavy metals.

EFFECT OF HEAVY METALS ON GERMINATION AND GENERAL DEVELOPMENT OF THE PLANTS

After one week of growth, germination rates were estimated. Additionally, macroscopic pictures were taken to get an impression of the heavy metal effects on the plants. One-way ANOVA was conducted to assess the effects of the heavy metals on the plants. (Figure 8).

Amaranthus cruentus

Amaranthus cruentus had the highest germination rate in zinc (84 %) with more seeds germinating in comparison to the control (81 %). Nickel had a negative impact (68 %) compared to the control and led to reduced germination as well as poor general development. There was a significant difference between the factors nickel and zinc treatment. One-way ANOVA, $F(2, 13) = 3.858$, $p = .0484$, omega$^2 = .263$. Tukey post-hoc analysis revealed a significant difference ($p = .0362$) between 0.1 mM nickel and 0.1 mM zinc. (Figure 8, A)

Noccaea caerulescens

Noccaea caerulescens had the highest germination rates in the whole experiment, showing the best results under nickel (92 %) and zinc treatment (91 %) with higher germination rates as compared to the control group (85 %). Yet, no statistically significant differences between the respective heavy metal concentrations were found. (Figure 8, B)

Noccaea goesingensis

Noccaea goesingensis showed low germination rates, ranging from 8 % in the control, to 5 % in the nickel treatment, and 5 % in the zinc treatment. After 18 days of growth, plants growing under the influence of zinc seemed best-developed, but were quite rare. No statistically significant differences between the respective heavy metal concentrations were found. Due to low germination rates and slow development *Noccaea goesingensis* was excluded from further experiments. (Figure 8, C)

Triticum aestivum

Triticum aestivum seeds germinated best in the control group (87 %), showing reduced rates in nickel (72 %) and zinc (77 %). After 18 days of growing, plants growing in the control group seemed most vital. Nickel treatment had negative effects resulting in limited growth. No statistically significant differences between the respective heavy metal concentrations were found. (Figure 8, D)

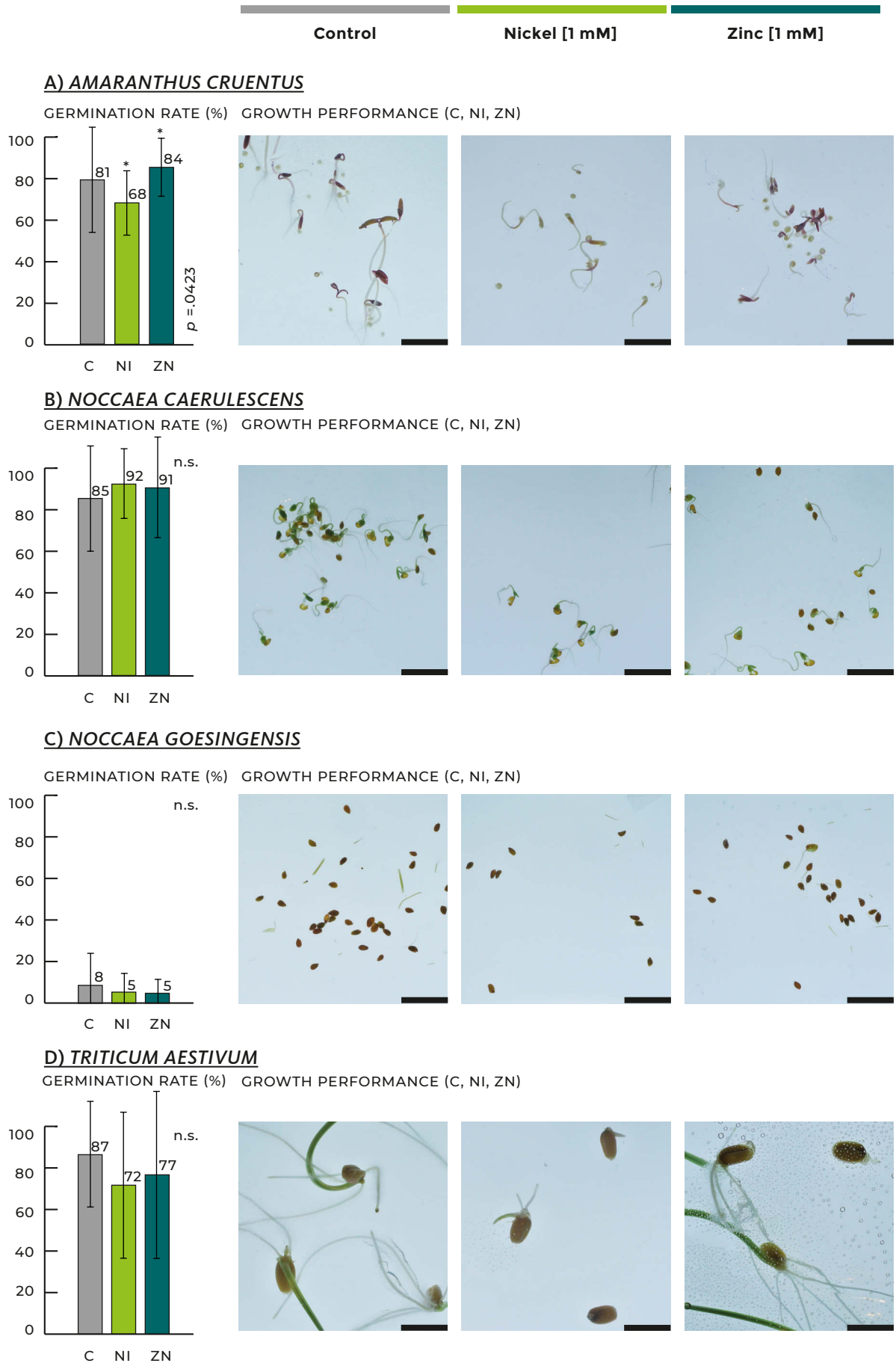


Figure 8: *Basic effect of the heavy metal treatment (1 mM nickel, 1 mM zinc) on* Amaranthus cruentus *(A),* Noccaea caerulescens *(B),* Noccaea goesingensis *(C), and* Triticum aestivum *(D). The first column shows the germination rate (%) of the respective treatments (C = control, NI = 1 mM nickel, ZN = 1 mM zinc).* Vertical lines *on each bar show ±SD, 95 % confidence interval. Bars for control are grey, nickel is green, zinc is petrol. "n. s." indicates that differences were non significant after ANOVA (p < .05). asterisk indicate two groups with significant differences. Columns two to four show overviews over the growth performance in the respective treatment 18 days after sowing (column 2: control group; column 3: 1 mM nickel treatment; column 4: 1 mM inc treatment). Bars equal 1 cm*

HEAVY METAL EFFECT ON ROOT LENGTH AND ROOT HAIR DEVELOPMENT

The effect of heavy metal treatment on the roots and root hairs was documented using stereomicroscopy after seven days of growth.

Amaranthus cruentus

Root length and root hairs in *Amaranthus cruentus* developed best in the control, as shown in Figure 9, 1 mM nickel treated plants did not produce visible root hairs and only short roots. 1 mM zinc reduced length of root hairs compared to the control group. (Figure 9)

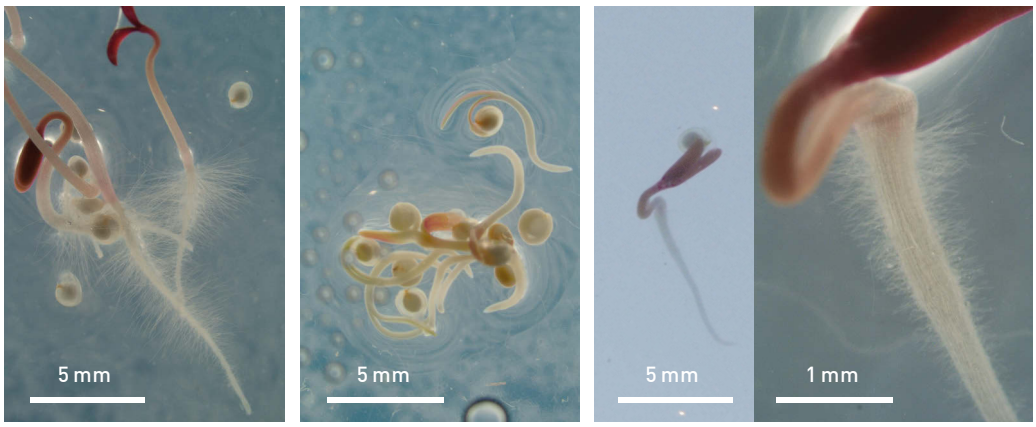


Figure 9: *Development of* Amaranthus cruentus *in the control and affected by heavy metals (1 mM nickel, 1 mM zinc). Pictures were taken seven days after sewing using stereomicroscopy. Different magnifications used.*

Noccaea caerulescens

Noccaea caerulescens root lengths did not show any clear tendency regarding the influence of heavy metals based on macroscopic observation. Root hairs were longer when growing in the control group or with nickel treatment. Zinc treated plants produced root hairs reduced in length. (Figure 10)

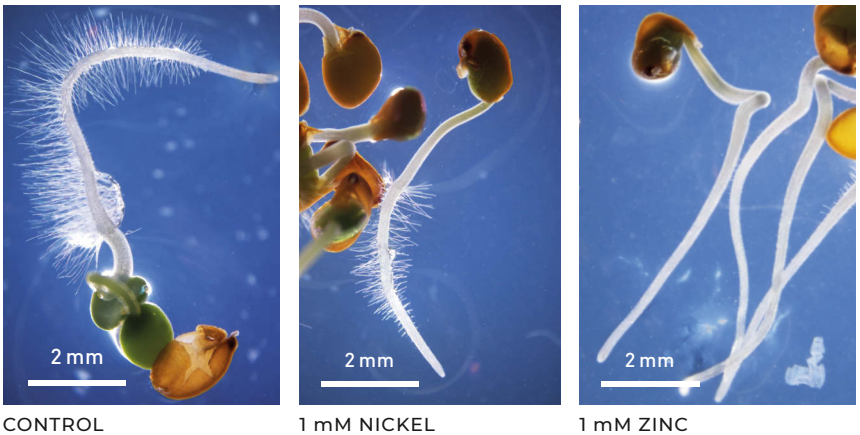


Figure 10: *Development of* Noccaea caerulescens *in control agar and affected by heavy metals (1 mM nickel, 1 mM zinc). Pictures were taken seven days after sewing using stereomicroscopy.*

Noccaea goesingensis

Noccaea goesingensis root lengths did not show any clear tendency regarding the influence of heavy metals. Root hairs grew longer in the control group or with nickel treatment. Zinc treated plants had reduced root hairs. Due to generally slow development and insufficient germination rates we excluded *Noccaea goesingensis* from further experiments. (Figure 11)

Figure 11: *Development of* Noccaea goesingensis *in the control and affected by heavy metals (1 mM nickel, 1 mM zinc). Pictures were taken seven days after sewing using stereomicroscopy.*

Triticum aestivum

Triticum aestivum was severely affected by nickel treatment resulting in reduction of length. Plants developed well in the control and the zinc treatment, respectively. (Figure 12)

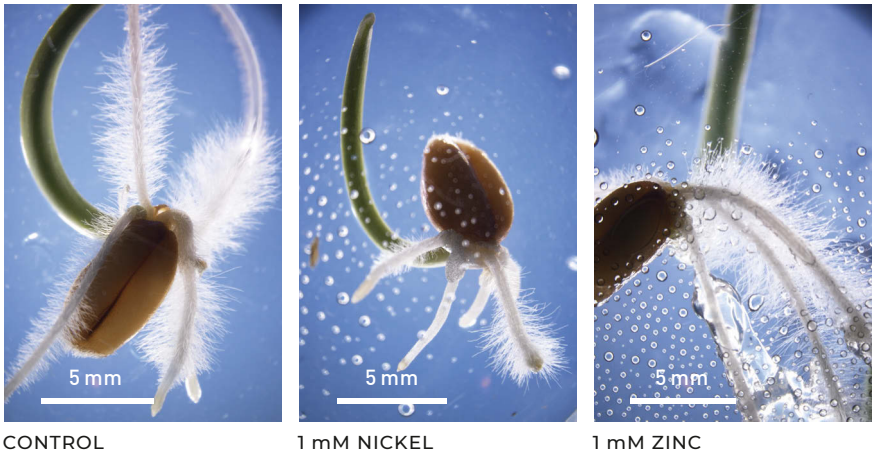
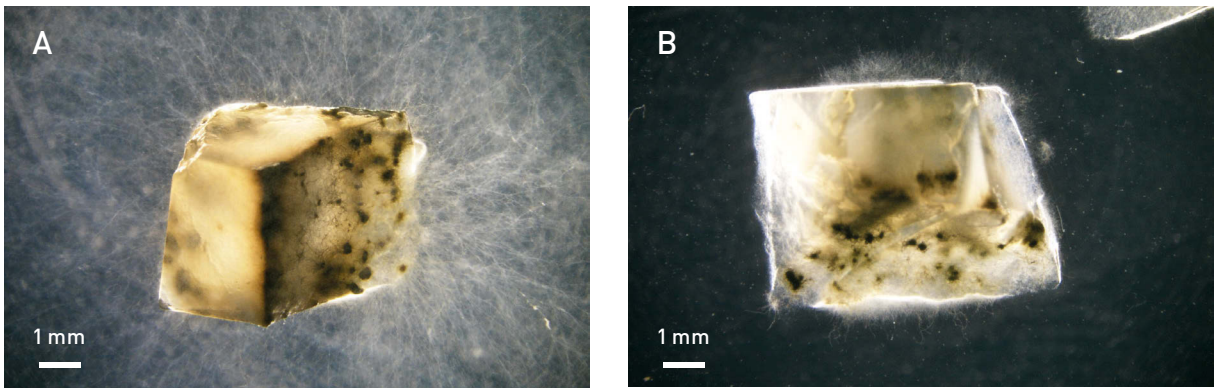


Figure 12: *Development of* Triticum aestivum *in the control and affected by heavy metals (1 mM nickel, 1 mM zinc). Pictures were taken seven days after sewing using stereomicroscopy.*

EFFECTS OF HEAVY METALS ON FUNGI

HEAVY METAL EFFECT ON *DIAPORTHE COLUMNARIS*

Diaporthe columnaris was grown in petri dishes without nutrients on pure agar. *Diaporthe columnaris* was placed on pure agar with 100 µM nickel and a control without metals. After seven days the control group had been growing throughout the plate, while the nickel treated plate showed only reduced growth. (Figure 13; A & B)



CONTROL

1 mM NICKEL

Figure 13: Diaporthe columnaris *growing on control media (A) and 100 µM nickel (B) after seven days.*

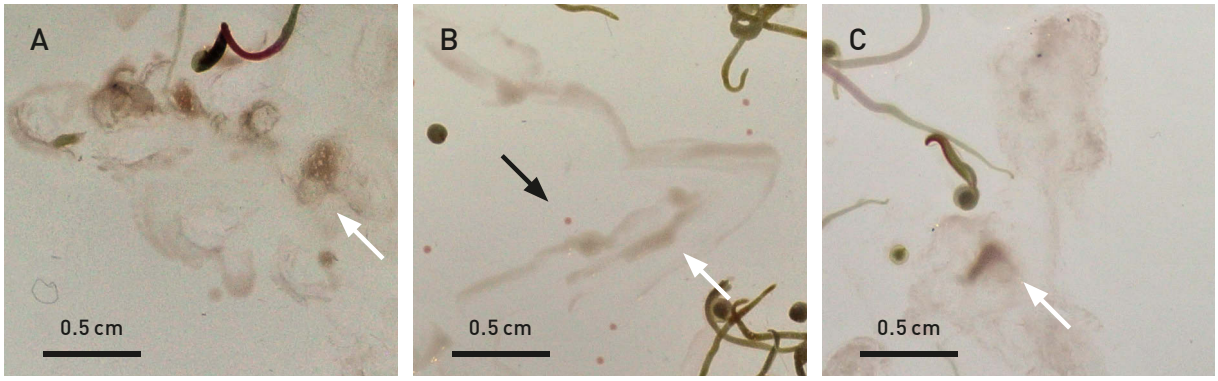
HEAVY METAL EFFECT ON YEAST SPECIES

In an experimental setup yeasts were co-cultured with *Amaranthus cruentus* or *Noccaea caerulescens* on agar plates with ½ MS as control, with 1 mM nickel or with 1 mM zinc. The aim was to observe the growth response of the yeast species to heavy metals macroscopically to evaluate the usability of the yeasts.

1. Heavy metal effect on *Rhodotorula mucilaginosa*

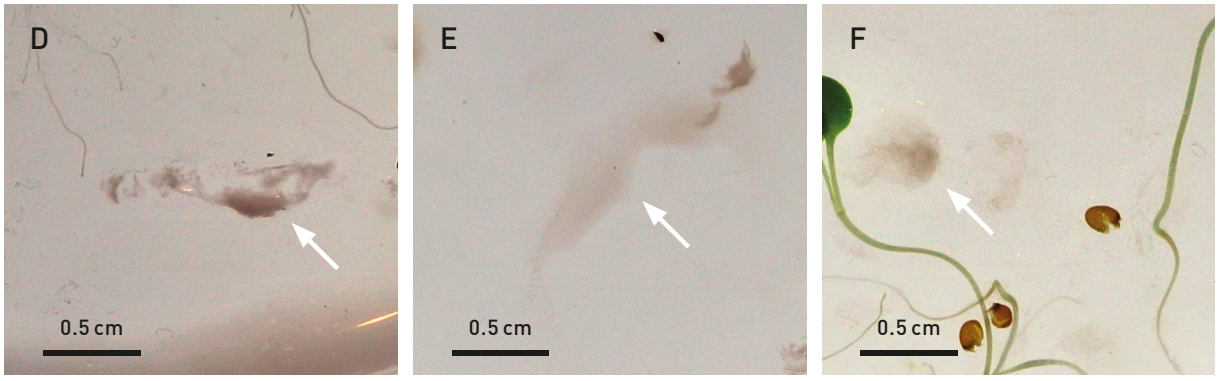
Rhodotorula mucilaginosa was co-cultured with *Amaranthus cruentus* Figure 14; A, B, C) or *Noccaea caerulescens* (Figure 14; D, E, F). *Rhodotorula mucilaginosa* seemed capable of growing on all offered conditions, no obvious reduction in macroscopic growth was estimated after six days of fungal growth in either treatment. Bacterial colonies were growing especially on nickel treatments.

***RHODOTORULA MUCILAGINOSA* (Growing with *Amaranthus cruentus*)**



CONTROL | 1 mM NICKEL | 1 mM ZINC

***RHODOTORULA MUCILAGINOSA* (Growing with *Noccaea caerulescens*)**



CONTROL | 1 mM NICKEL | 1 mM ZINC

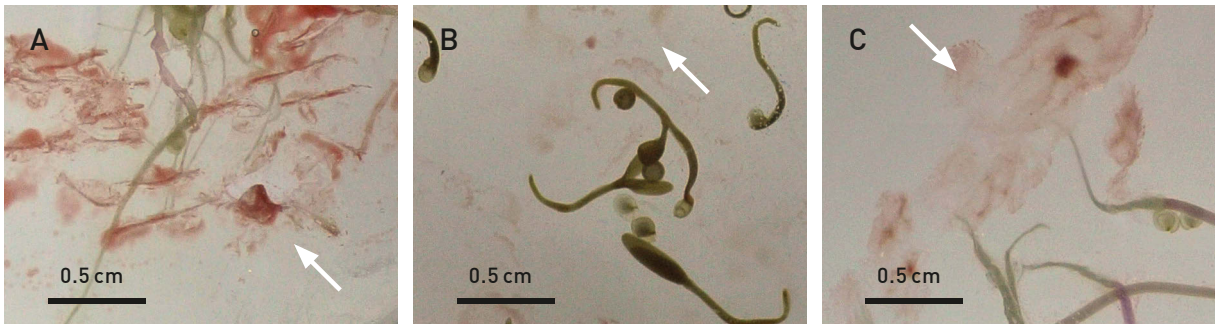
Figure 14: Rhodotorula mucilaginosa *after 6 days of growing on petri dishes with ½ MS and no additional heavy metals (A, D), 1 mM of nickel (B, E), and 1 mM of zinc (C, F). Yeasts were co-cultured with* Amaranthus cruentus (*Pictures A to C), or with* Noccaea caerulescens (*Pictures D to F). White arrows mark examples of yeast colonies. Black arrows mark bacterial colonies.*

2. Heavy metal effect on *Sporobolomyces sp.*

In an experimental dataset, *Sporobolomyces sp.* was co-cultured with *Amaranthus cruentus* or *Noccaea caerulescens* as control, with nickel and with zinc exposition. Yeasts grew well in the control treatment with both plant partners (Figure 15; A, D). Nickel treatment (Figure 15; B, E) resulted in reduced yeast growth compared to the control group (Figure 15; A, D). Zinc treated yeasts (Figure 15; C, F) appeared to be depressed in development compared to the control group (Figure 15; A, D). *Sporobolomyces sp.* was negatively affected by exposition to 1 mM nickel and zinc after six days of fungal growth.

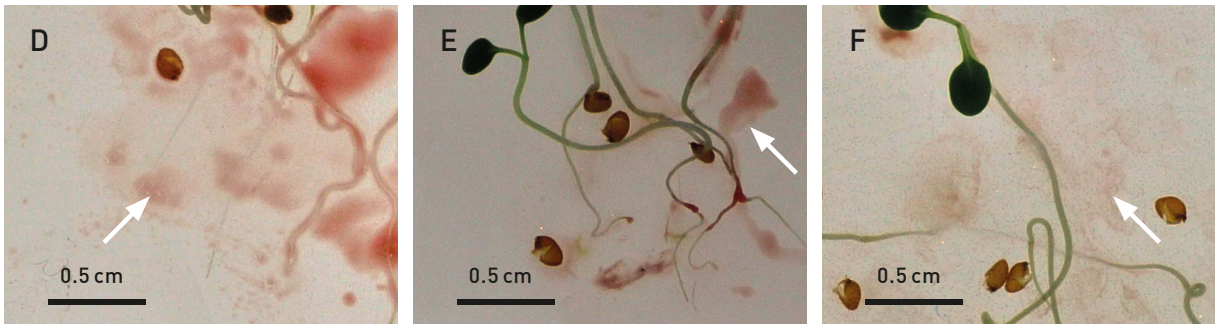
Comparing the co-habitation with the two different plant species, a positive effect in the nickel treated plants could be observed when growing with *Noccaea caerulescens*. In this case, with yeast in co-habitation with plants that were tolerant to the increased heavy metal levels, yeasts seemed more capable to develop, as compared to co-cultures with *Amaranthus cruentus*, where both, plant and yeast, seemed to suffer from the metal treatment.

***SPOROBOLOMYCES SP.* (Growing with *Amaranthus cruentus*)**



CONTROL | 1 mM NICKEL | 1 mM ZINC

***SPOROBOLOMYCES SP.* (Growing with *Noccaea caerulescens*)**

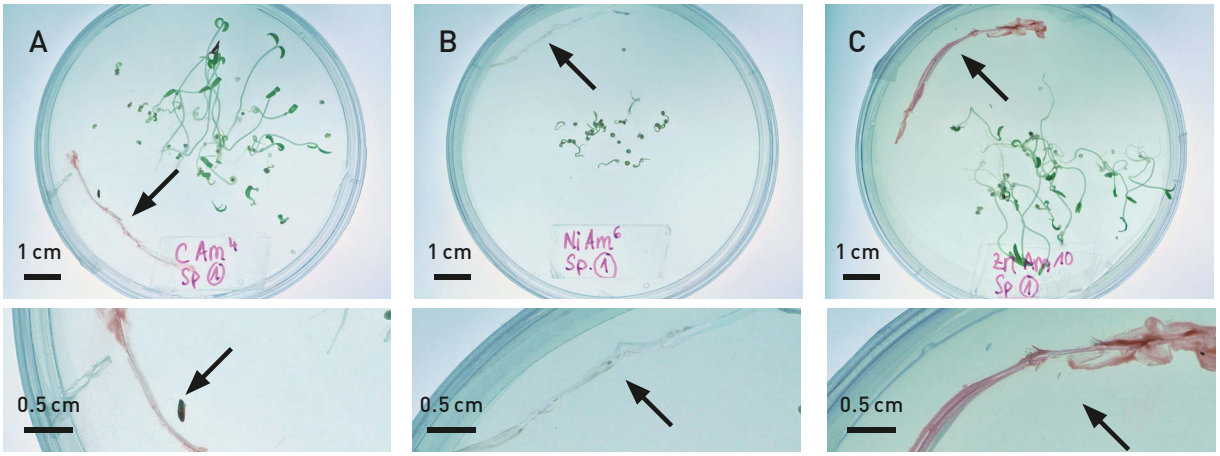


CONTROL | 1 mM NICKEL | 1 mM ZINC

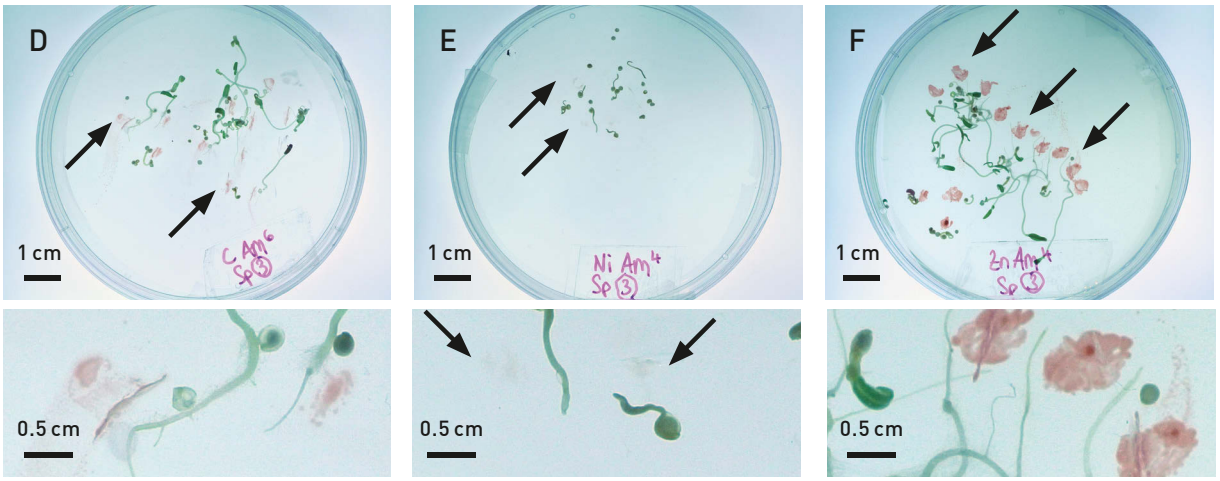
Figure 15: Sporobolomyces sp. *after 6 days of growing on petri dishes with ½ MS and no additional heavy metals (A, D), 1 mM of nickel (B, E), and 1 mM of zinc (C, F). Yeasts were co-cultured with* Amaranthus cruentus (*Pictures A to C*), *or with* Noccaea caerulescens (*Pictures D to F*). White arrows mark examples of colonies.

The development of *Sporobolomyces sp.* was compared in a second approach using the same heavy metal concentrations as mentioned above and three different application methods. Higher concentrated agar in the zinc contaminated dishes ensured a solid surface. After three days of growth yeasts grew well on the control, while suffering from nickel treatment (compare figure 16 A, D, G to B, E, H). Zinc treatment boosted yeast growth. Yeasts grew better, even compared to the control (compare figure 16 A, D, G to C, F, I). Application without contact to the plants did not affect growth. Yeast with direct contact to the plant roots seemed to use the plants for distribution.

Sporobolomyces sp.* applied with great distance to *Amaranthus cruentus



Sporobolomyces sp.* next to *Amaranthus cruentus



Sporobolomyces sp.* on *Amaranthus cruentus

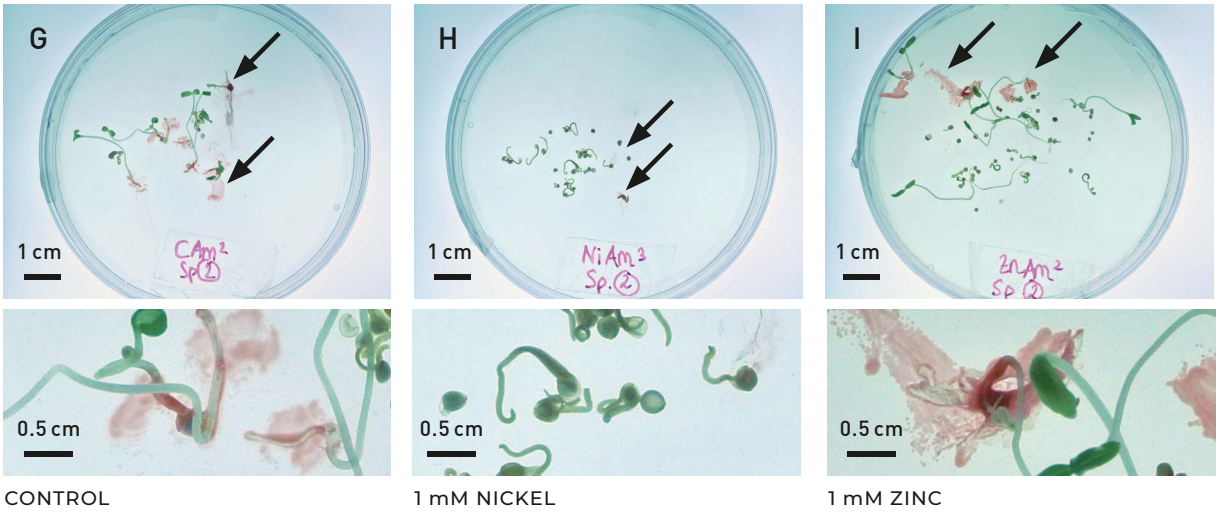


Figure 16: Sporobolomyces sp. *after 3 days of growing on petri dishes with ½ MS and no additional heavy metals (A, D, G), 1 mM of nickel (B, E, H), and 1 mM of zinc (C, F, I). Yeasts were co-cultured with* Amaranthus cruentus. *Arrows mark examples of colonies. Each treatment is shown as overview and as detail.*

EFFECTS OF HEAVY METALS ON PLANTS AND YEASTS

FUNGAL INFLUENCE ON *AMARANTHUS CRUENTUS*

After 18 days of growing under heavy metal influence, 1 mM nickel or 1 mM of zinc, respectively, we inoculated *Amaranthus cruentus* with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* in a small and experimental dataset. Effects on root length, root hair length of the longest root hairs and the distance between the first root hair and the root tip were estimated, if possible, after three days of growing with yeast. Additionally, control groups were prepared.

Root lengths

In the control treatments without yeasts, root lengths were significantly reduced by toxic metals (Figure 17, A). *Amaranthus cruentus* developed the longest roots in the control group without heavy metal influence. Nickel caused the shortest roots. Zinc had disadvantageous effects too, but plants performed better compared to the nickel treatment. One-way ANOVA was performed, $F(2, 14) = 85.76$, $p = 1.394E\text{-}08$, omega² = .909. Tukey post-hoc analysis revealed a significant difference between the control group and the nickel group ($p = 1.194E\text{-}08$), between the control group and the zinc group ($p = .1.601E\text{-}06$) and between nickel and zinc treatments ($p = .008$).

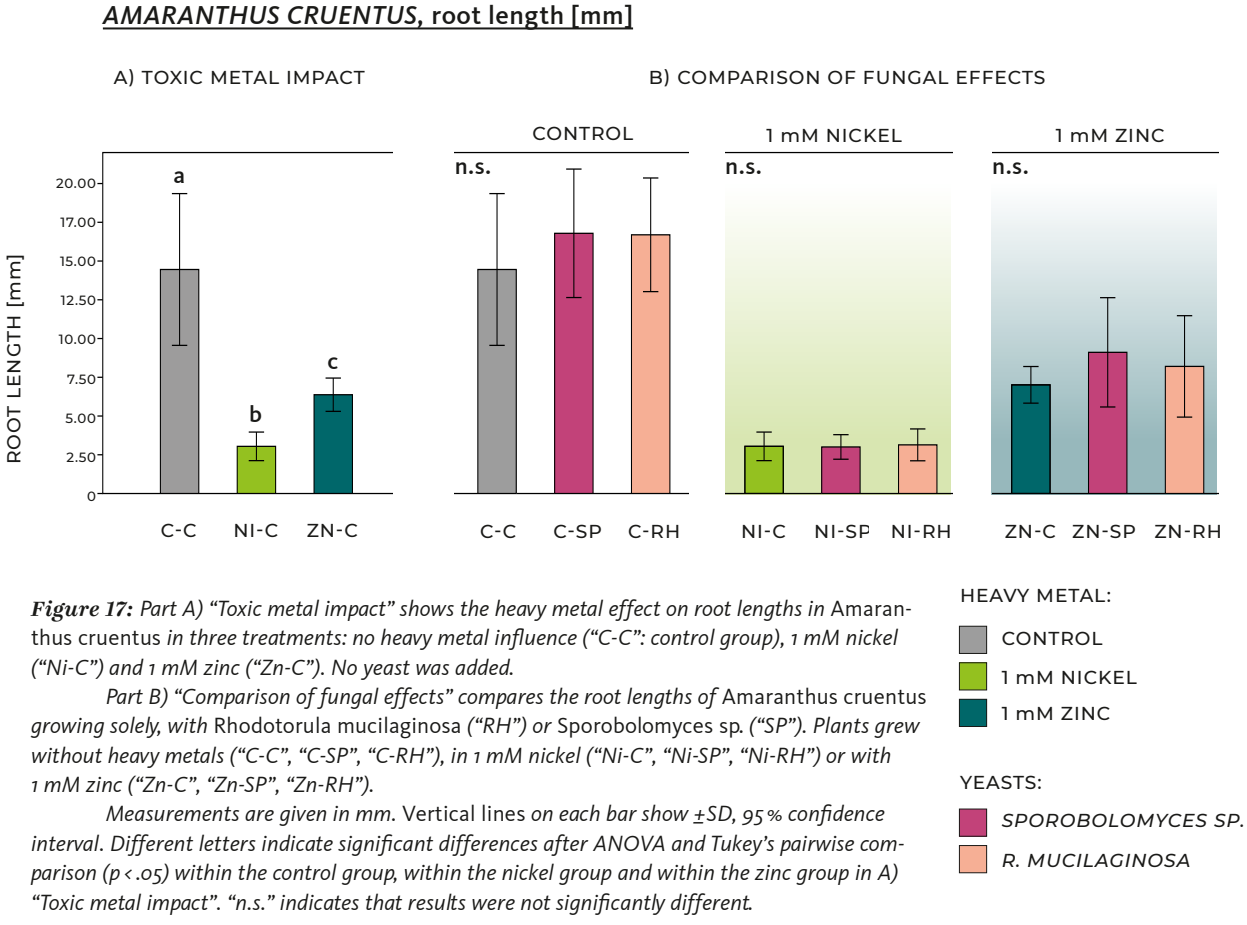


Figure 17: *Part A) "Toxic metal impact" shows the heavy metal effect on root lengths in* Amaranthus cruentus *in three treatments: no heavy metal influence ("C-C": control group), 1 mM nickel ("Ni-C") and 1 mM zinc ("Zn-C"). No yeast was added.*

Part B) "Comparison of fungal effects" compares the root lengths of Amaranthus cruentus *growing solely, with* Rhodotorula mucilaginosa *("RH") or* Sporobolomyces sp. *("SP"). Plants grew without heavy metals ("C-C", "C-SP", "C-RH"), in 1 mM nickel ("Ni-C", "Ni-SP", "Ni-RH") or with 1 mM zinc ("Zn-C", "Zn-SP", "Zn-RH").*

Measurements are given in mm. Vertical lines *on each bar show ±SD, 95 % confidence interval. Different letters indicate significant differences after ANOVA and Tukey's pairwise comparison ($p < .05$) within the control group, within the nickel group and within the zinc group in A) "Toxic metal impact". "n.s." indicates that results were not significantly different.*

Addition of *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* had no significant effects on root length in the control group, one-way ANOVA, $F(2, 14) = 2.128$, $p = .156$, omega² = .117 (Figure 17, B "Control"). In the nickel treated plants, yeasts had no significant effect on root lengths (One-way ANOVA, $F(2, 15) = .131$, $p = .878$, omega² = 0; Figure 18, B "1 mM nickel"). Zinc treated plants also showed no difference in root length when growing with fungal partners (One-way ANOVA, $F(2, 14) = 2.662$, $p = .105$, omega² = 0.164; figure 17, B "1 mM zinc").

Root hair length

In the control group, without influence of yeasts, root hairs grew longest in the control and the zinc treatment. Addition of nickel led to almost complete reduction of root hairs. Root hair length differed significantly between the control and nickel treated plants. (One-way ANOVA, $F(2, 13) = 6.201$, $p = .0129$, omega² = .394, Tukey Pairwise, $p = .01$). (Figure 18 A "Toxic metal impact".)

Adding *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* had no significant effect on root hair length in the control group (One-way ANOVA, $F(2, 13) = .996$, $p = .396$, omega² = 0), shown in figure 18 B, "Control". There was also no significant difference in the nickel group (Kruskal-Wallis, Chi square = .0117, $p = .986$, $df = 2$) and in the zinc treated group (One-way ANOVA, $F(2, 8) = 3.09$, $p = .101$, omega² = .275). (Figure 18 B, "1 mM nickel" and "1 mM zinc").

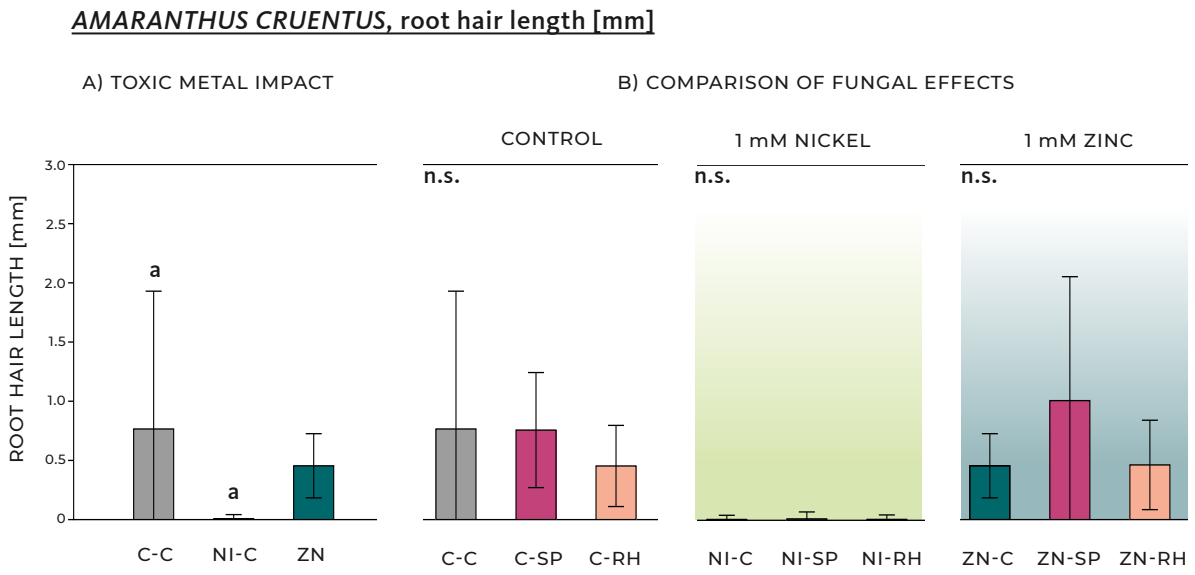


Figure 18: *Part A) "Toxic metal impact" shows the effect of heavy metal treatment on root hair length in* Amaranthus cruentus. *Three treatments are compared: no heavy metal influence ("C-C"), 1 mM nickel ("Ni-C") and 1 mM zinc ("Zn-C"). No yeast was added.*

Part B) "Comparison of fungal effects" compares the influence of Rhodotorula mucilaginosa *("RH") or* Sporobolomyces sp. *("SP"). Plants grew without heavy metals ("C-C", "C-SP", "C-RH"), in 1 mM nickel ("Ni-C", "Ni-SP", "Ni-RH") or with 1 mM zinc ("Zn-C", "Zn-SP", "Zn-RH").*

Measurements are given in mm. Vertical lines *on each bar show ±SD, 95 % confidence interval. Different letters indicate significant differences after One-way ANOVA and Tukey's pairwise comparison ($p < .05$) within the control group and the nickel group in A) "Toxic metal impact". "n.s." indicates that results were not significantly different.*

Distance of the root tip to the first visible root hair

In the control group with plants growing without additional fungal influence, no statistically significant difference emerged after One-way ANOVA regarding the distances between root tip and the first root hair in the different heavy metal treatments. ($F(2, 13) = 3.065$, $p = .081$, omega² = .205), Compare to figure 19 A, “toxic metal impact”.

Comparing the influence of yeast no significant differences were found in the control group in the distance from the tip of the root to the first root hair, as shown in figure 19, B. (One-way ANOVA, $F(2, 14) = .343$, $p = .716$, omega² = 0). Compare to figure 19 B, “control”.

Nickel treated plants did not produce sufficient amounts of root hairs and therefore had to be excluded from this analysis.

Zinc treated plants showed significant differences in the root tip to root hair distance, (One-way ANOVA, $F(2, 8) = 10.82$, $p = .005$, omega² = .641). Tukey post hoc test revealed that plants treated with *Sporobolomyces sp.* had shorter root tip to first root hair distances compared to the control group ($p = .007$). Plants treated with *Rhodotorula mucilaginosa* showed the same reduced distance compared to the control group ($p = .015$). Compare to figure 19 B, “1 mM zinc”.

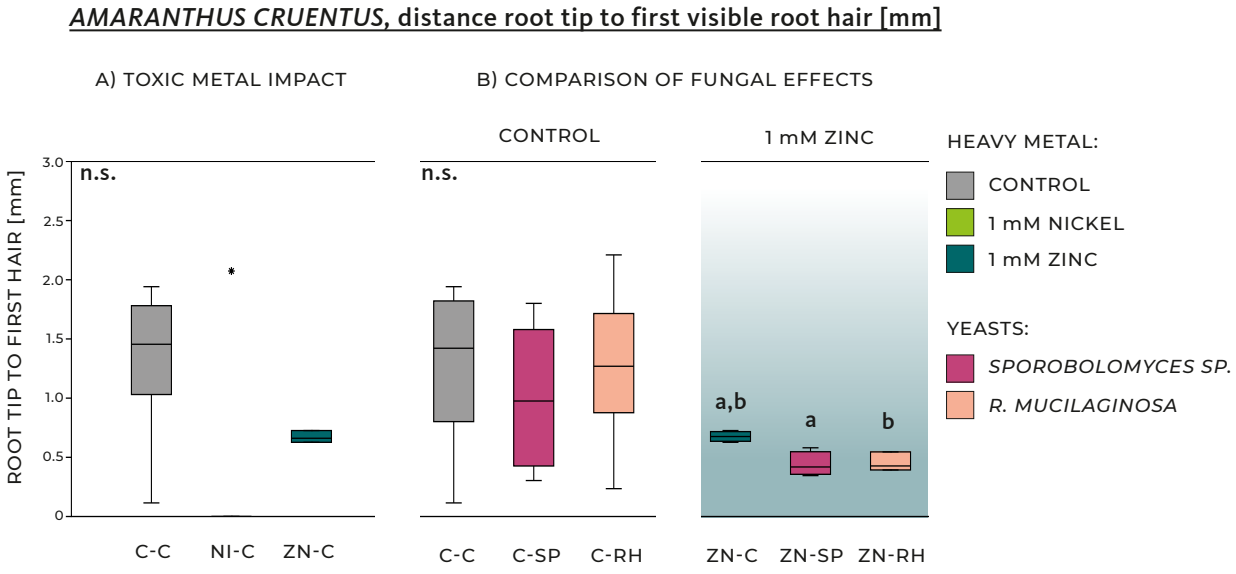


Figure 19: *Part A) “Toxic metal impact” shows the effect of heavy metal treatment on the distance between the root tip and the first root hair in* Amaranthus cruentus. *Three treatments are compared: no heavy metal influence (“C-C”), 1 mM nickel (“Ni-C”) and 1 mM zinc (“Zn-C”). No yeast was added.*

Part B) “Comparison of fungal effects” compares the influence of Rhodotorula mucilaginosa *(“RH”) or* Sporobolomyces sp. *(“SP”). Plants grew without heavy metals (“C-C”, “C-SP”, “C-RH”), or with 1 mM zinc (“Zn-C”, “Zn-SP”, “Zn-RH”).*

Measurements are given in mm. Vertical lines *on each bar show ±SD, 95 % confidence interval. Different letters indicate significant differences after one-way ANOVA and Tukey’s pairwise comparison ($p < .05$) within the control group, within the nickel group and within the zinc group in B) 1 mM zinc. "n.s" indicates that results were not significantly different.*

FUNGAL DEVELOPMENT AND DISTRIBUTION ON *AMARANTHUS CRUENTUS*

Rhodotorula mucilaginosa or *Sporobolomyces sp.* grew on petri dishes with *Amaranthus cruentus* on 0.5 MS agar under three different conditions, as control group with no heavy metal influence, and under the influence of 1 mM nickel or 1 mM zinc, respectively. The petri dishes were analysed without opening them using a long distance objective. Plates were turned 180°, if possible. This restricted us in terms of magnifications, as well as image quality, due to plants growing in and under the agar, misty dishes and thick agar layers.

Sporobolomyces sp.
Yeast cells grew on top of the agar, not within. In the control we could see *Sporobolomyces sp.* partly growing alongside the roots after 18 hours of inoculation. Inhabited roots were densely covered with yeast. Root hairs did not seem to be inhabited. (Figure 20, "control")

Nickel treatment led to huge accumulation of yeast around the root tips. Yet, yeasts did not appear to be healthy. Nickel led to reduced growth of the yeast. (Figure 20, "1 mM nickel")

The sample shows individual yeast cells of *Sporobolomyces sp.*, not huge colonies, growing next to the roots. (Figure 20, "1 mM zinc")

Rhodotorula mucilaginosa
Again, yeast cells grew on top of the agar, not within. In the control *Rhodotorula mucilaginosa* grew as thick coatings alongside the roots on top of the agar after 18 hours of inoculation. Root hairs were covered with yeast (Figure 21, A1, A2, A3).

In nickel treatments we could not detect interaction between yeast and root. The roots remained uninfected, no directional growth towards the roots has happened (Figure 21 B1). Plates showed a lot of pinkish dots, most likely bacterial contaminations as vacuoles, structures in the plasma were lacking. (Figure 21, B2, dot: black arrow, yeast: white arrow)

Zinc treated plates, did not show much interaction between root and yeast. (Figure 21, C)

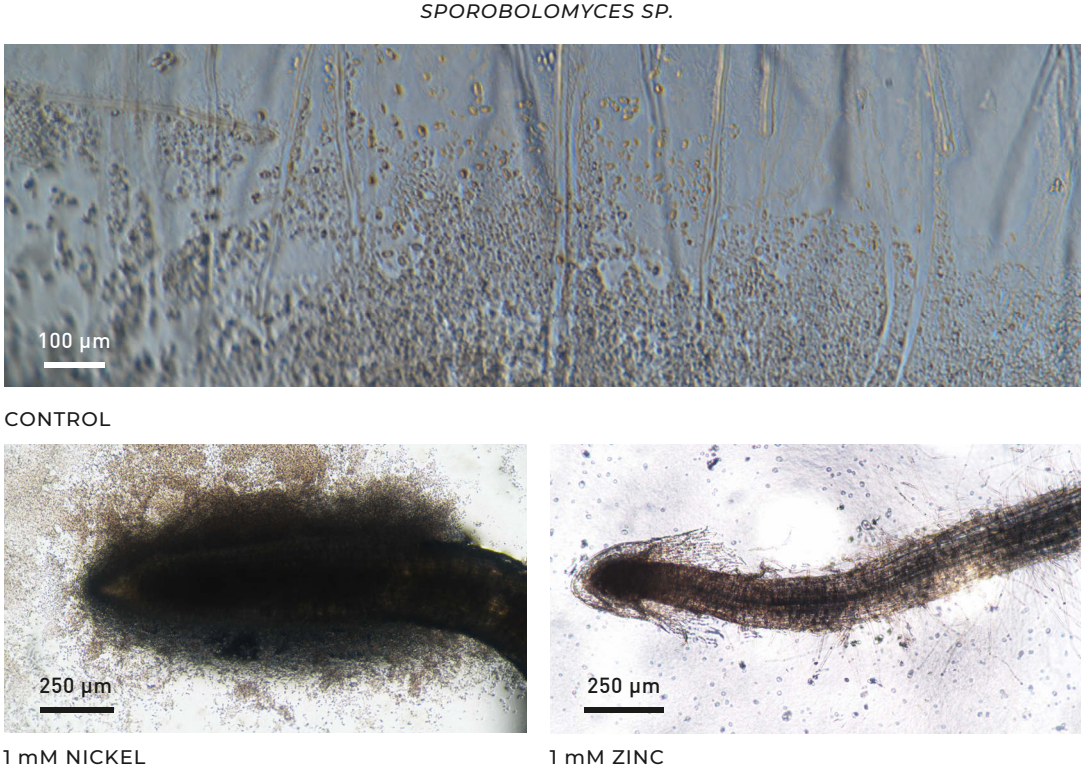
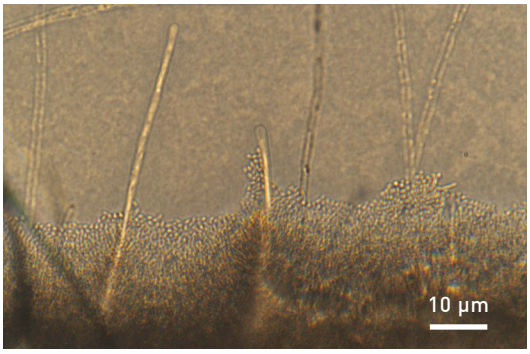
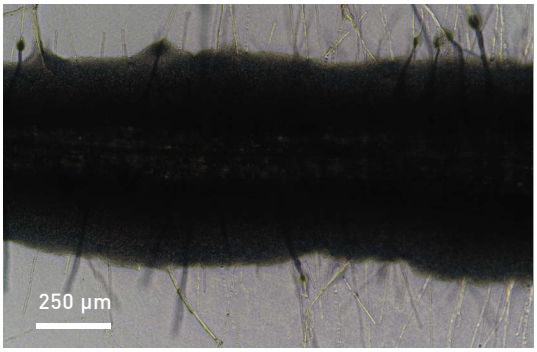


Figure 20: *Seedlings of* Amaranthus cruentus *after 18 hours of inoculation with* Sporobolomyces sp. *in A) the control group without toxic metals, B) 1 mM nickel, C) 1 mM zinc. All pictures were taken after 18 hours of inoculation.*

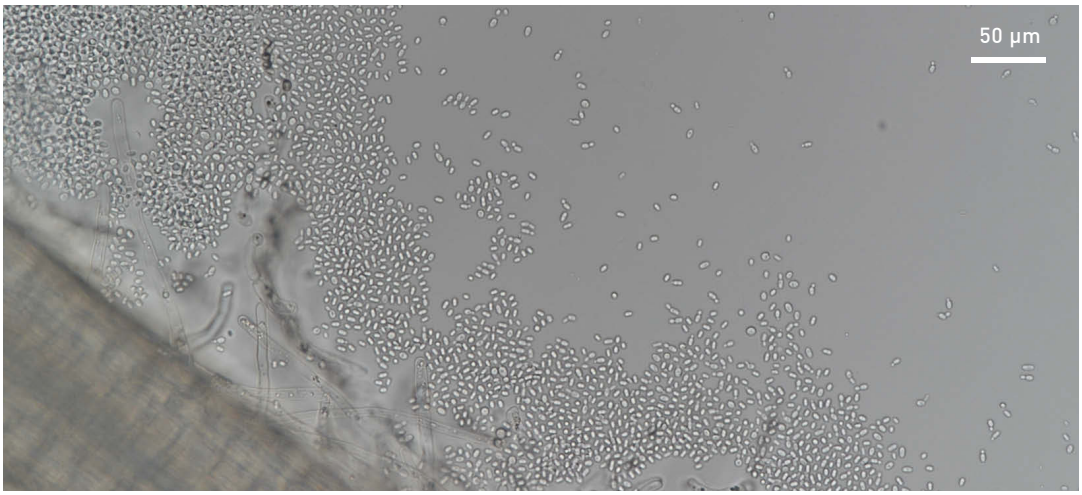
RHODOTORULA MUCILAGINOSA



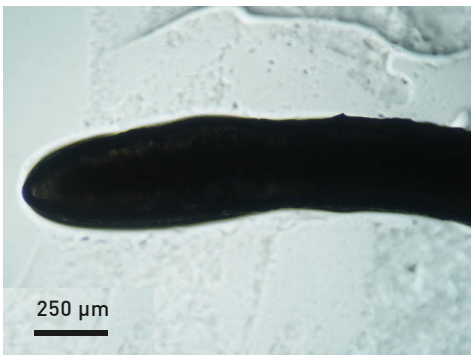
A1) CONTROL (18 hours inoculation)



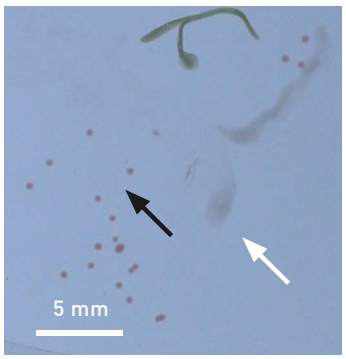
A2) CONTROL (18 hours inoculation)



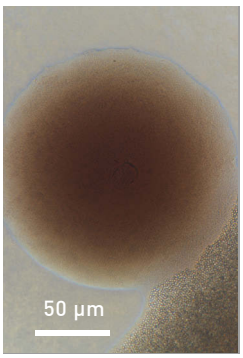
A3) CONTROL (one week inoculation)



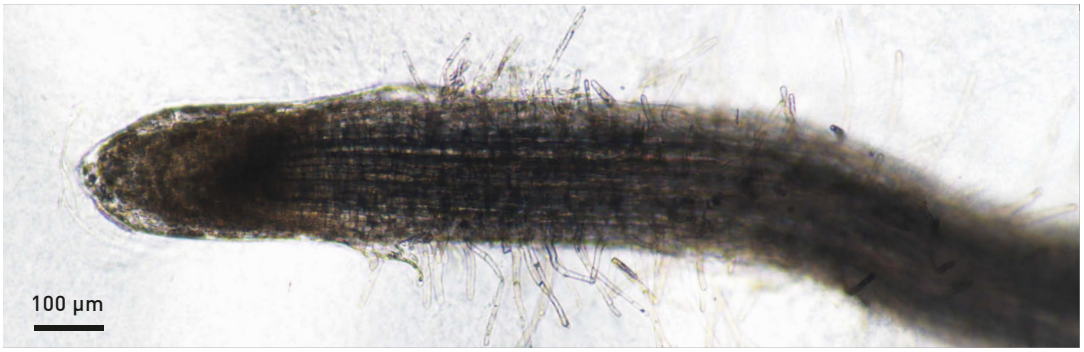
B1) 1 mM NICKEL (18 hours inoculation)



B2) CONTAMINATION



B3) CONTAMINATION



C) 1 mM ZINC (18 hours inoculation)

Figure 21: *Seedlings of* Amaranthus cruentus *after 18 hours of inoculation with* Rhodotorula mucilaginosa *in A) the control group without toxic metals, B) 1 mM nickel, C) 1 mM zinc.*

FUNGAL INFLUENCE ON *NOCCAEA CAERULESCENS*

We inoculated 18 days old *Noccaea caerulescens* seedlings with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* Three days after inoculation with yeast, root length, root hair length and the distance between the first root hair and the root tip were estimated. Control groups were treated likewise.

Root lengths

In the control group, heavy metal treatment had a significant effect on root length (Figure 22 "Root length"). Roots of *Noccaea caerulescens* were significantly longer in the control group compared to the nickel treatment (One-way ANOVA, $F(2, 12) = 6.881$, $p = .0102$, omega² = .44, Tukey Pairwise, $p = .024$) and the zinc treatment ($p = .008$).

Addition of *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* did not affect root lengths in the control group, there was no significant difference between the treatments, (One-way ANOVA, $F(2, 13) = .814$, $p = .464$, omega² = 0; figure 22, B "Control"). In plants growing with nickel no significant difference regarding root lengths could be detected (One-way ANOVA, $F(2, 13) = 2.138$, $p = .152$, omega² = .112; figure 22, B "Nickel"). Zinc treated plants developed the longest roots when growing with *Rhodotorula mucilaginosa*, roots grew significantly longer in the group treated with *Rhodotorula mucilaginosa*, compared to the control group (One-way ANOVA, $F(2, 12) = 5.933$, $p = .0162$, omega² = .397, Tukey Pairwise: $p = .032$), and compared to the plants treated with *Sporobolomyces sp.* (Tukey Pairwise: $p = .047$). Compare to figure 22 B, "Zinc".

Root hairs

Plants growing with 1 mM zinc had the shortest root hairs, that were almost completely reduced. There was a significant difference in root hair length between the nickel and the zinc treatment. (One-way ANOVA, $F(2, 12) = 5.384$, $p = .02143$, omega² = .369, Tukey Pairwise: $p = .044$). Compare to figure 23, A.

Addition of yeast made no significant difference regarding length of root hairs. ("Control": One-way ANOVA, $F(2, 12) = .829$, $p = .462$, omega² = 0; "Nickel": One-way ANOVA, $F(2, 13) = .678$, $p = .525$, omega² = 0; "Zinc": One-way ANOVA, $F(2, 13) = .858$, $p = .447$, omega² = 0). Compare to figure 23, B.

NOCCAEA CAERULESCENS, root length [mm]

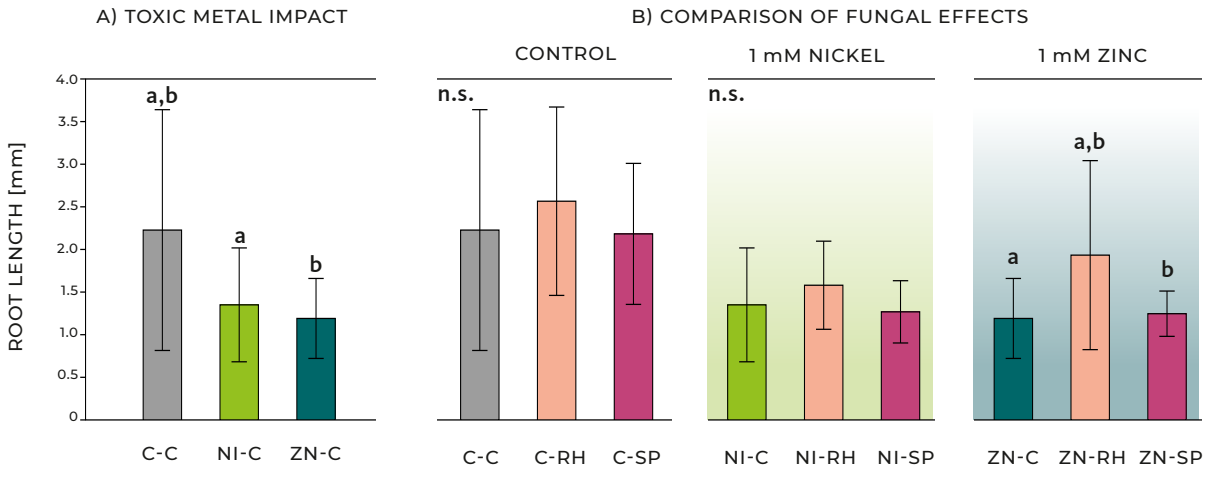


Figure 22: *Part A) "Toxic metal impact" shows the effect of heavy metal treatment on root length (in mm) in seedlings of* Noccaea caerulescens. *Three treatments are compared: no heavy metal influence ("C-C"), 1 mM nickel ("Ni-C") and 1 mM zinc ("Zn-C"). No yeast was added.* Vertical lines *on each bar show ±SD, 95 % confidence interval. Different letters indicate significant differences after ANOVA and Tukey's pairwise comparison ($p < .05$).*

Part B) "Comparison of fungal effects" compares the influence of Rhodotorula mucilaginosa *("RH") and* Sporobolomyces sp. *("SP") on root lengths. Plants grew without heavy metals ("C-C", "C-SP", "C-RH"), in 1 mM nickel ("Ni-C", "Ni-SP", "Ni-RH") or with 1 mM zinc ("Zn-C", "Zn-SP", "Zn-RH").*

Measurements are given in mm. Vertical lines *on each bar show ±SD, 95 % confidence interval. Different letters indicate significant differences after one-way ANOVA and Tukey's pairwise comparison ($p < .05$) within the control group, within the nickel group and within the zinc group. "n.s" indicates that results were not significantly different.*

NOCCAEA CAERULESCENS, root hair length [µm]

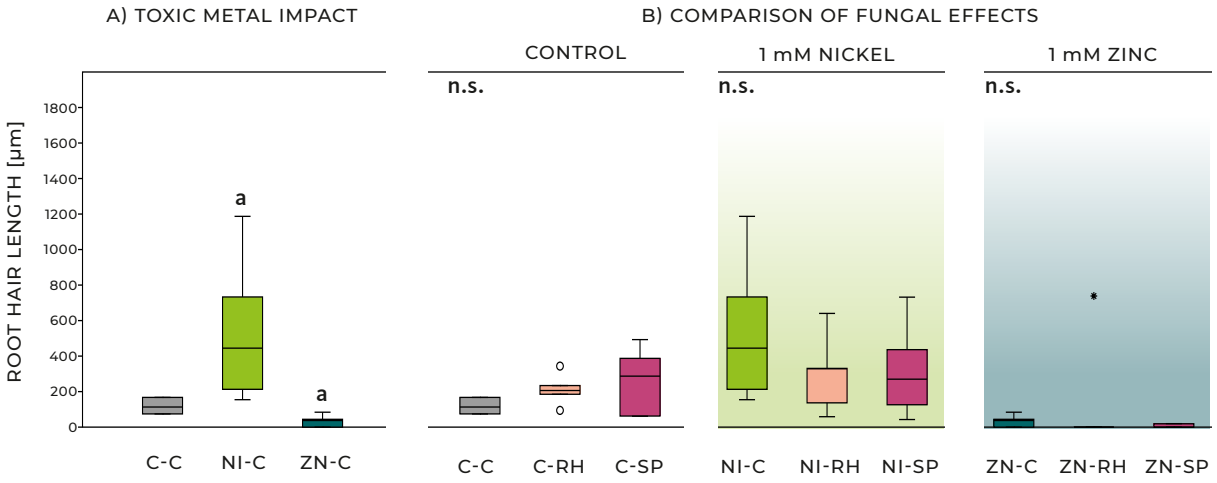


Figure 23: *Part A) "Toxic metal impact" shows the effect of heavy metal treatment on the development of root hair length (in µm) in seedlings of* Noccaea caerulescens. *Three treatments are compared: no heavy metal influence ("C-C"), 1 mM nickel ("Ni-C") and 1 mM zinc ("Zn-C"). No yeast was added.* Vertical lines *on each bar show ±SD, 95 % confidence interval. Different letters indicate significant differences after ANOVA and Tukey's pairwise comparison ($p < .05$).*

Part B) "Comparison of fungal effects" compares the influence of Rhodotorula mucilaginosa *("RH") or* Sporobolomyces sp. *("SP") on root hair length. Plants grew without heavy metals ("C-C", "C-SP", "C-RH"), in 1 mM nickel ("Ni-C", "Ni-SP", "Ni-RH") or with 1 mM zinc ("Zn-C", "Zn-SP", "Zn-RH").*

Measurements are given in µm. Vertical lines *on each bar show ±SD, 95 % confidence interval. Different letters indicate significant differences after one-way ANOVA and Tukey's pairwise comparison ($p < .05$) within the control group, within the nickel group and within the zinc group. "n.s." indicates that results were not significantly different.*

Distance of the root tip to the first visible root hair

The distance of the root tip to the first visible root hair (Figure 24, "Distance root tip to first root hair") had its maximum length in the zinc group. Kruskal Wallis test for equal medians revealed no significant difference between the treatments (Kruskal-Wallis, Chi square = 5.769, p = .056).

The plants growing without heavy metal contamination did not show significant differences regarding the distance of the root tip to the first root hair, regardless if growing with yeast or not, (One-way ANOVA, $F(2, 12)$ = 2.008, p = .1769, omega² = .119; Figure 34, B "Control"). Plants with nickel exposure also showed no significant differences between treatment with or without yeast (One-way ANOVA, $F(2, 13)$ = .0472, p = .634, omega² = 0; Figure 34, B "Nickel"). Zinc treated plants growing with yeast showed strongly reduced development of root hairs, partly heavily overgrown by fungi. Therefore the estimation of root tip to first root hair had to be excluded from the analysis.

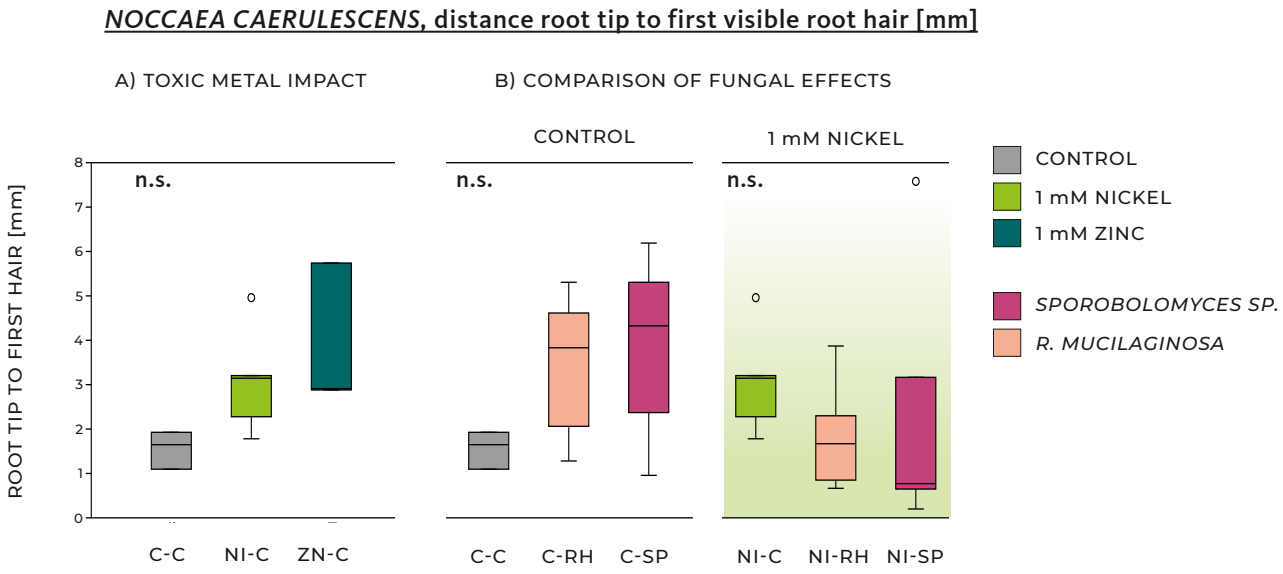


Figure 24: *Part A) "Toxic metal impact" shows the effect of heavy metal treatment on the development of the distance between the root-tip to the first root-hair in seedlings of* Amaranthus cruentus. *Three treatments are shown: no heavy metal influence (Control, "C-C"), 1 mM nickel ("Ni-C") and 1 mM zinc ("Zn-C"). Measurements are given in µm.* Vertical lines *on each bar show ±SD, 95 % confidence interval. Different letters indicate significant differences after ANOVA and Tukey's pairwise comparison ($p < .05$).*

Part B) "Comparison of fungal effects" compares the influence of Rhodotorula mucilaginosa *("RH") and* Sporobolomyces sp. *("SP") in the respective heavy metal treatments (1 mM nickel "Ni"; 1 mM zinc "Zn"; control "C").*

Measurements are given in mm. Vertical lines *on each bar show ±SD, 95 % confidence interval. "n.s." indicates that differences are non significant.*

FUNGAL DEVELOPMENT AND DISTRIBUTION ON *NOCCAEA CAERULESCENS*

Rhodotorula mucilaginosa and *Sporobolomyces sp.* grew on petri dishes with *Noccaea caerulescens* on 0.5 MS agar as control group with no heavy metal influence, and under the influence of 1 mM nickel or 1 mM zinc. The petri dishes were analysed without opening them, using a long distance objective. Plates were turned 180°, if possible.

Rhodotorula mucilaginosa

Yeast cells grew on top of the agar, not within. In the control *Rhodotorula mucilaginosa* grew alongside the roots covering them with thick coats after 18 hours of inoculation. (Figure 25; A1 to A2)

In nickel roots were inhabited too by the yeast. Root tips were used as "hot spot" for accumulation of yeast. (Figure 25; B1 to B3)

Yeasts and plants growing in zinc did not show much interaction between root and yeast. (Figure 25; C1 & C2)

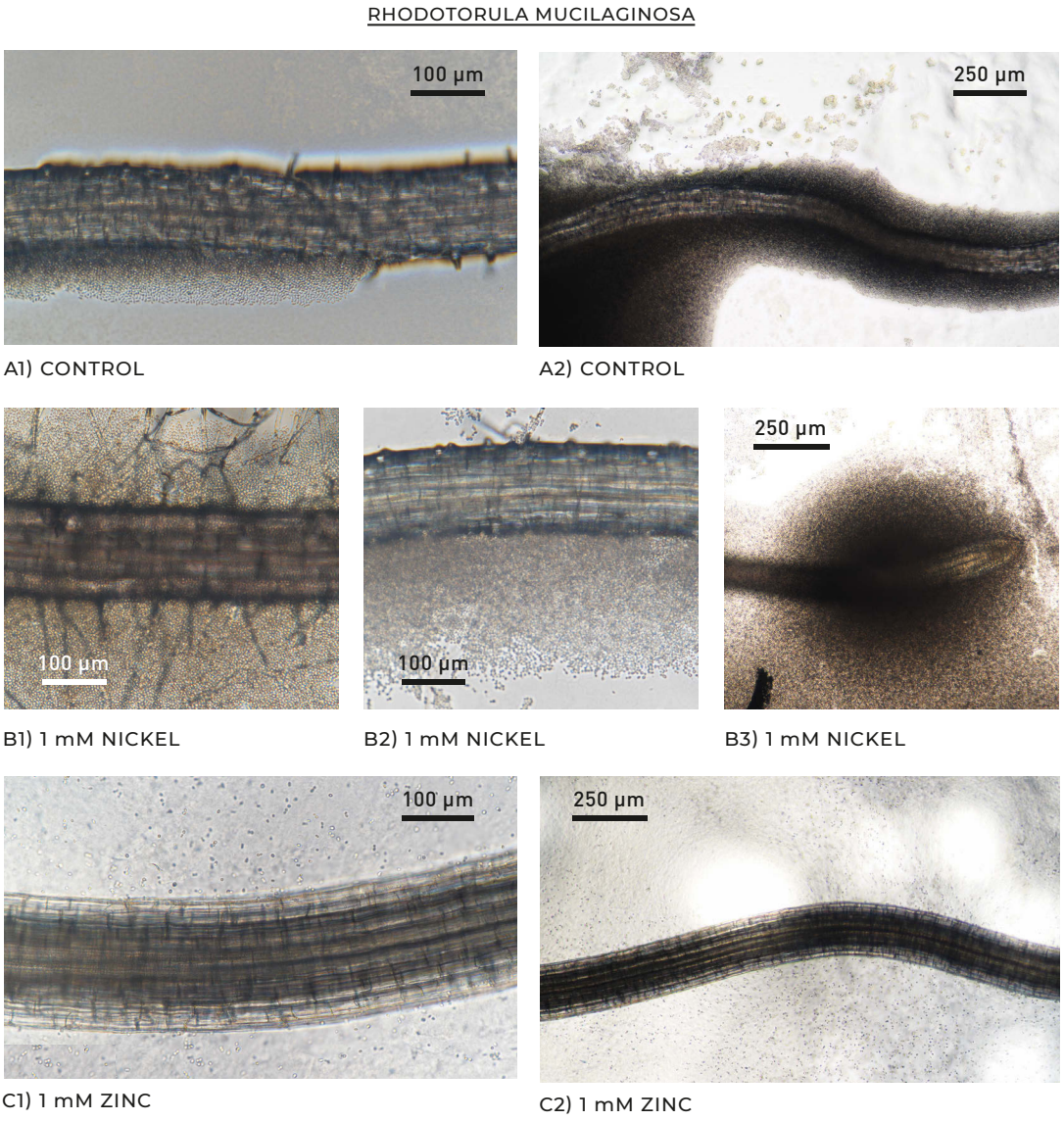


Figure 25: *seedlings of* Noccaea caerulescens *after 18 hours of inoculation with* Rhodotorula mucilaginosa *in A) the control group without toxic metals, B) 1 mM nickel, C) 1 mM zinc. All pictures were taken after 18 hours of inoculation.*

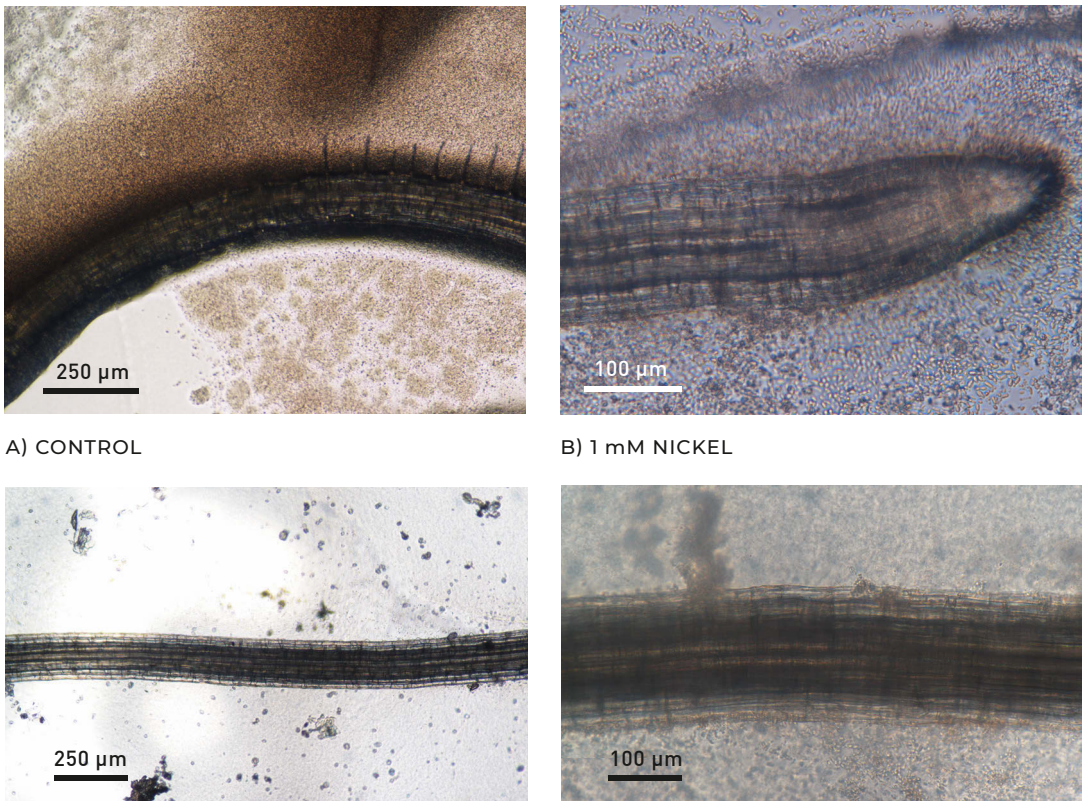
Sporobolomyces sp.
Sporobolomyces sp. developed best on uncontaminated plates and with zinc. Nickel reduced the growth of the yeast on a macroscopic scale (Macroscopic analysis, figures 15 & 16). Yeast cells grew on top of the agar, not within.

Looking closer (Nikon Eclipse), we could see *Sporobolomyces sp.* growing alongside the roots after 18 hours of inoculation in the control group. (Figure 26, "Control")

Nickel treated plates were also showing interactions between roots and yeast. (Figure 26, "1 mM nickel")

Zinc contaminated plates did not show much interaction between roots and yeast. Yeast colonies appeared to be "cloudy". (Figure 16, "1 mM zinc, left and right")

SPOROBOLOMYCES SP.



A) CONTROL
B) 1 mM NICKEL
C1) 1 mM ZINC
C2) 1 mM ZINC

Figure 26: *seedlings of* Noccaea caerulescens *after 18 hours of inoculation with* Sporobolomyces sp. *in A) the control group without toxic metals, B) 1 mM nickel, C) 1 mM zinc. All pictures were taken after 18 hours of inoculation.*

Table 1 gives an overview over the results of the experiment. All significant effects are listed for *Amaranthus cruentus*, *Noccaea caerulescens* and yeasts.

Table 1: *Summarized results of influence by yeasts, significant effects are listed for A)* Amaranthus cruentus *and B)* Noccaea caerulescens*: "+" indicates an increase compared to the other treatments "–" indicates a reduction. C) "Yeasts on ½ MS" summarizes the yeast performances based on observations.*

A) *Amaranthus cruentus*

	Root length	**Root hair length**	**Root tip to 1st hair**
control			
1 mM nickel			
1 mM zinc			RH, SP (–)

B) *Noccaea caerulescens*

	Root length	**Root hair length**	**Root tip to 1st hair**
control			
1 mM nickel			
1 mM zinc	RH (+)		

C) Yeasts on ½ MS

	control	**1 mM nickel**	**1 mM zinc (1 % agar)**	**1 mM zinc (2 % agar)**
R. mucilagonosa				
with *A. cruentus*	+	+	+	
with *N. caerulescens*	+	+	+	
Sporobolomyces sp.				
with *A. cruentus*	+	–	+/–	++
with *N. caerulescens*	+	+	+/–	

FUNGAL INFLUENCE ON *TRITICUM AESTIVUM*

Triticum aestivum was grown on quadratic petri dishes without toxic metals (control) or with 100 µM nickel under lab conditions. After roots had reached 1 cm in length, *Diaporthe columnaris* or *Rhodotorula mucilaginosa* were added to the plates from fresh cultures. No additional nutrients were added. Germination rates were estimated, as well as length of roots and the longest root hair. Furthermore we were interested to determine if the effects were acting locally, just at the site of interaction, or systemically, affecting the whole plant.

Effect of nickel without yeast

Germination rates showed slightly increased rates for the control group, with 88 % (*Mdn* = 100) compared to the nickel treatment, where 83 % germinated (*Mdn* = 80). The difference was not significant after comparing the mean germination rates of several dishes with Mann-Whitney-U-test. $U = 96.5$, $p = 0.4$); compare to figures 27 and 28.

GERMINATION RATE OF *TRITICUM AESTIVUM* ON CONTROL AND NICKEL

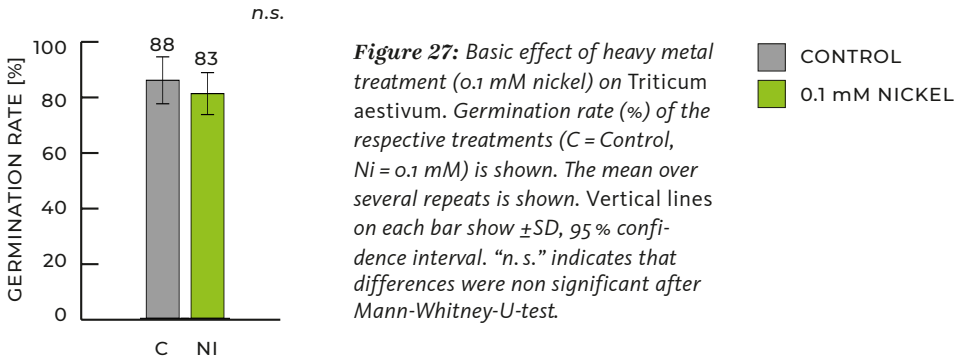


Figure 27: *Basic effect of heavy metal treatment (0.1 mM nickel) on* Triticum aestivum. *Germination rate (%) of the respective treatments (C = Control, Ni = 0.1 mM) is shown. The mean over several repeats is shown.* Vertical lines *on each bar show ±SD, 95 % confidence interval. "n. s." indicates that differences were non significant after Mann-Whitney-U-test.*

HEAVY METAL EFFECT ON *TRITICUM AESTIVUM*

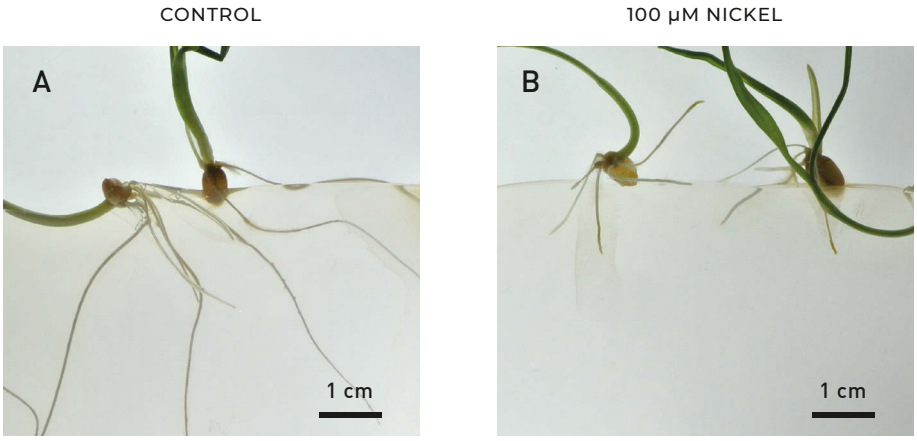


Figure 28: *Comparison of heavy metal effect on* Triticum aestivum *growing A) without heavy metal contamination and B) in 100 µM nickel. Bars equal 1 cm.*

General observations

Two basic root morphology types were noticed: Roots growing outside the agar-medium had dense and long root hair. Roots growing inside the agar, or between the agar and the bottom of the petri dish, had less and shorter root hair, regardless if treated with nickel or not. (Figure 29, A)

The 100 µM nickel treatment resulted in plants that avoided to grow into the agar, most roots were found growing into the air. (Figure 29, B)

Lots of exudates and deposits were found in both treatments (control and nickel treatments), yet nickel treatments caused thicker and denser deposits surrounding roots (Figure 29, C & D).

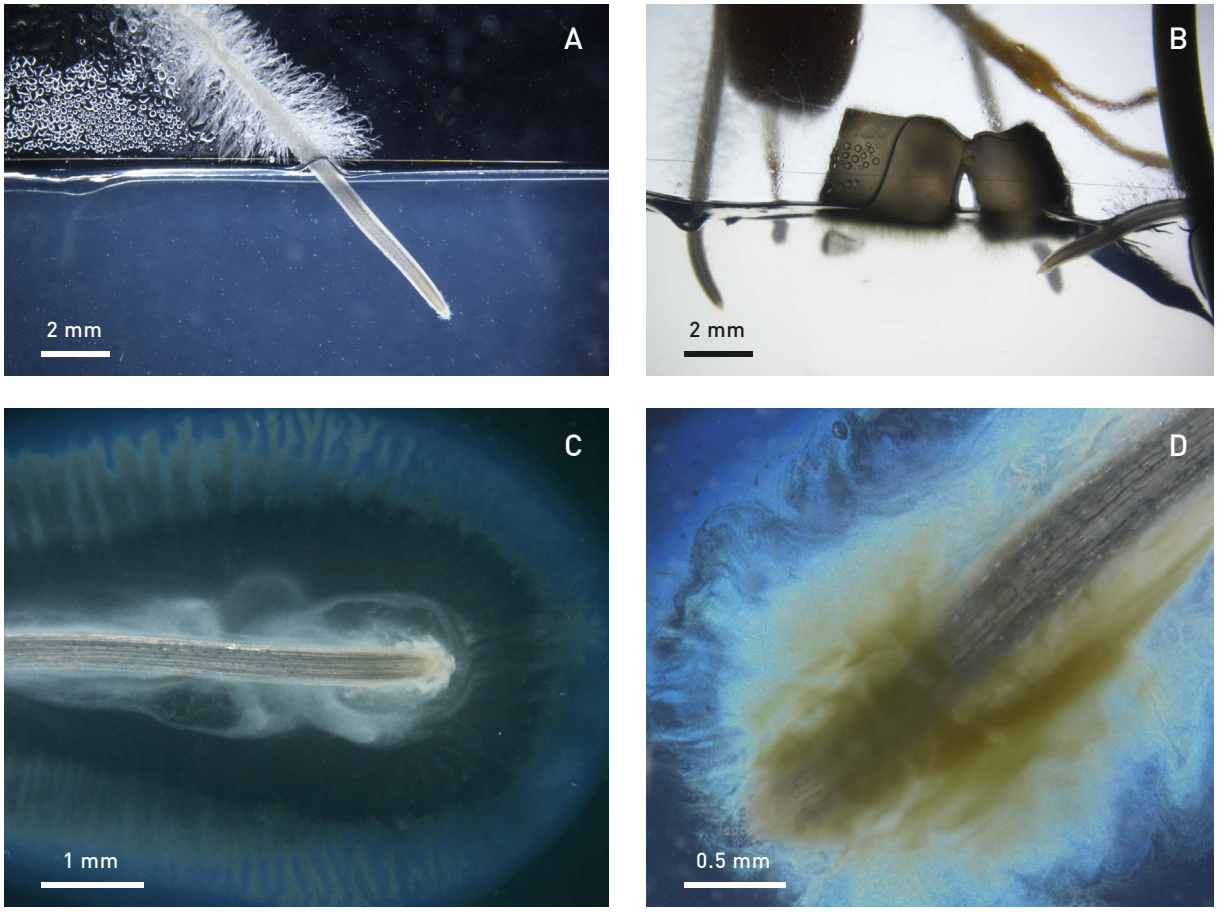


Figure 29: Triticum aestivum *growing on agar, partly with 100 µM nickel. A) Length of root hairs was dependent if the root was growing inside the agar or in air. B) Roots tended to avoid nickel agar, shown samples were growing with* Diaporthe columnaris. *C) Exudates and deposits in a control treatment and D) in nickel agar, sample was growing with* Diaporthe columnaris.

Root lengths

Heavy metal treatment had a significant effect on root length. Roots of *Triticum aestivum* were significantly longer in the control group (M = 51.373, SD = 24.408) compared to the nickel treatment (M = 16.018, SD = 6.649) after a t-test. $t(40) = 4.708$, p = 2.9884E-5). (Figure 30, A)

Addition of *Diaporthe columnaris* in the control treatment reduced root length significantly, after conducting one-way ANOVA, $F(2, 65) = 4.859$, p = .011, $omega^2$ = .1019, compared to the control group (Tukey Pairwise, p = .02778) and treatment with *Rhodotorula mucilaginosa (*Tukey Pairwise, p = .017). (Figure 30, B "Control")

Plants growing with *Diaporthe columnaris* or *Rhodotorula mucilaginosa* in the nickel treatment did not significantly differ regarding root length. (One-way ANOVA, $F(2, 41) = 1.565$, p = .221, $omega^2$ = .025). (Figure 30, B "0.1 mM Nickel")

Root hairs

Nickel treated plants did not produce many roots that were growing into the agar. Therefore the data-set remained quite small, in contrast to the control group.

A Mann-Whitney test indicated that there was no significant difference between the metal treatment (Mdn = 140) and the control group (Mdn = 158,95) U = 1413,5, p = .705. (Figure 31, A)

In the control treatment, there was no significant difference regarding the treatment with different fungi. (Kruskal-Wallis, Chi square = 4.33, p = .1147, df = 2). (Figure 31, B "Control")

Nickel treated plants were not significantly different regarding root hair length. (Kruskal-Wallis, Chi square = .669, p = .716, df = 2). (Figure 31, B "0.1 mM Nickel")

Distance of the root tip to the first visible root hair

Nickel treated plants that were growing into the agar were rare. Root tips growing in the agar, not too overgrown by fungi or deposits were to enable an estimation between the root tip and the first root hair were almost non-existent. Nickel treatment had to be excluded completely from this part of the analysis. In the control group, a comparison between root tips to first hair reveals that *Rhodotorula mucilaginosa* treated plants (Mdn = 2156.685) did not differ significantly fromt the control (Mdn = 1799.615) after conducting a Mann-Whitney test, U = 10, p = .179). (Figure 32)

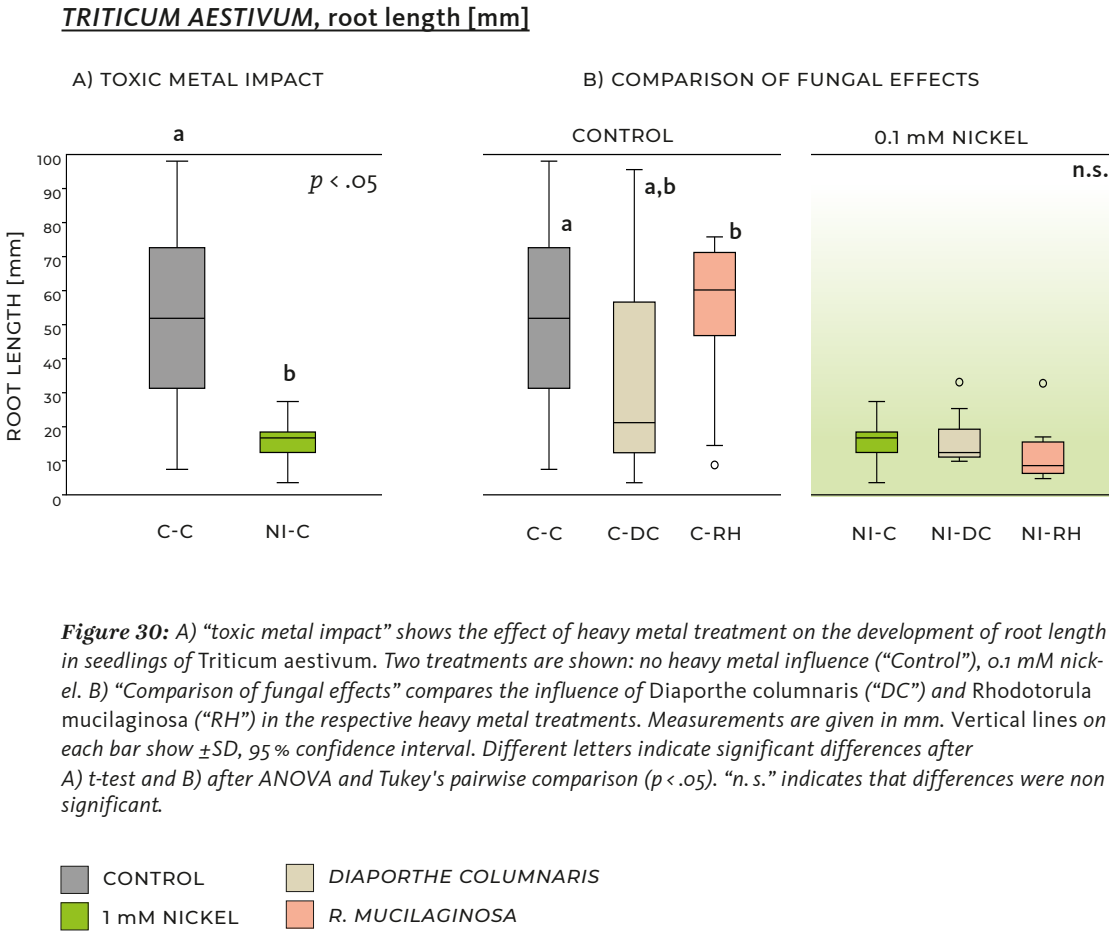


Figure 30: *A) "toxic metal impact" shows the effect of heavy metal treatment on the development of root length in seedlings of* Triticum aestivum. *Two treatments are shown: no heavy metal influence ("Control"), 0.1 mM nickel. B) "Comparison of fungal effects" compares the influence of* Diaporthe columnaris *("DC") and* Rhodotorula mucilaginosa *("RH") in the respective heavy metal treatments. Measurements are given in mm.* Vertical lines *on each bar show ±SD, 95 % confidence interval. Different letters indicate significant differences after A) t-test and B) after ANOVA and Tukey's pairwise comparison (p < .05). "n. s." indicates that differences were non significant.*

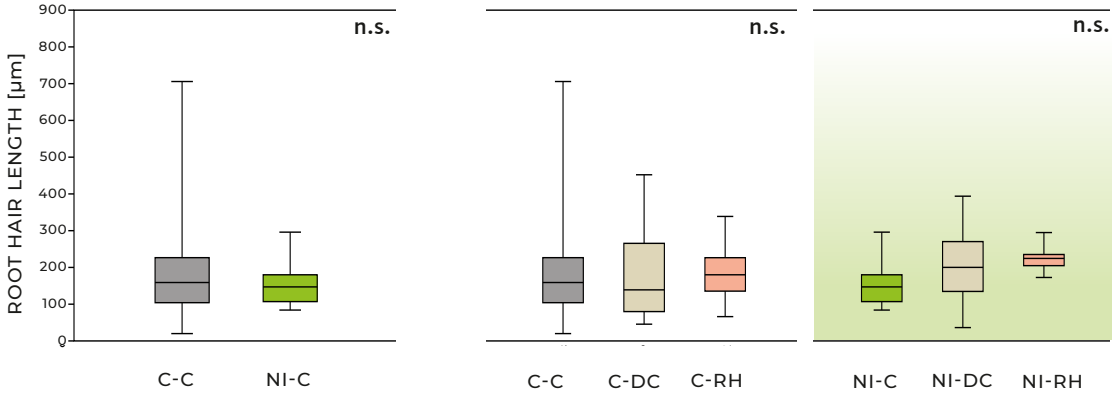


Figure 31: *A) "toxic metal impact" shows the effect of heavy metal treatment on the development of root hair length in seedlings of* Triticum aestivum. *Two treatments are shown: no heavy metal influence ("Control"), 0.1 mM nickel. B) "Comparison of fungal effects" compares the influence of* Diaporthe columnaris *("DC") and* Rhodotorula mucilaginosa *("RH") in the respective heavy metal treatments. Measurements are given in µm.* Vertical lines *on each bar show ±SD, 95 % confidence interval. Different letters indicate significant differences (p < .05). "n. s." indicates that differences were non significant.*

TRITICUM AESTIVUM, distance root tip to hair [mm]

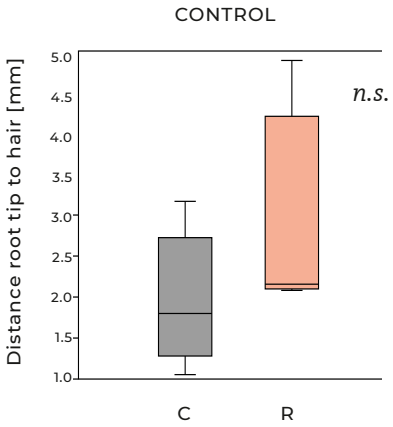


Figure 32: *The distance between the root tip and the first root hair in the control treatment is compared in the control plants ("C") and plants treated* Rhodotorula mucilaginosa *("RH"). Measurements are given in mm.* Vertical lines *on each bar show ±SD, 95 % confidence interval. "n. s." indicates that differences were non significant after conducting a Mann-Whitney test (p < .05).*

CONTROL

R. MUCILAGINOSA

FUNGAL DEVELOPMENT AND DISTRIBUTION ON *TRITICUM AESTIVUM*

Rhodotorula mucilaginosa and *Diaporthe columnaris* grew on petri dishes with *Triticum aestivum* without additional nutrients, as control group with no heavy metal influence, and under the influence of 0.1 mM nickel. The petri dishes were analysed using stereomicroscopy. (Figure 33)

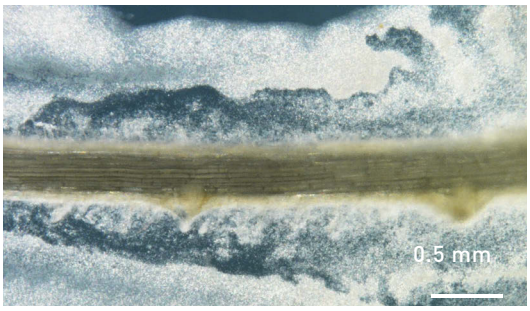
Rhodotorula mucilaginosa

Rhodotorula mucilaginosa seemed to profit from the nickel treatment, and appeared to be cloudy in the control after 7 days of inoculation. No clear distribution pattern was observed, the yeast was flushing over the agar as soon as the plates were moved. Colonies on "air" roots got crusty and did not spread any further. Roots were covered with yeast and exudates which made it difficult to analyse them. (Figure 33; A, B)

Diaporthe columnaris

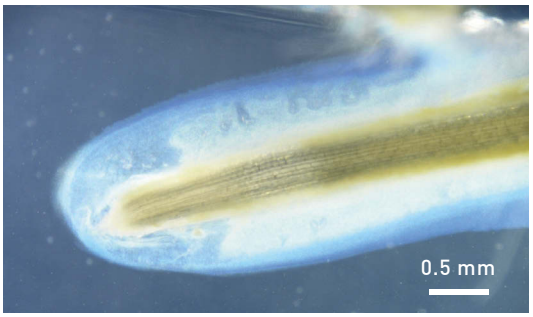
Diaporthe columnaris did not show directional growth toward the roots and spread into all directions, but appeared to be attracted into the deposits, exudates and bacterial colonies on top of the agar (Figure 33, C1). Roots on the other hand seemed to be attracted by the agar block the fungi was transferred with. (Figure 33, D1)

RHODOTORULA MUCILAGINOSA

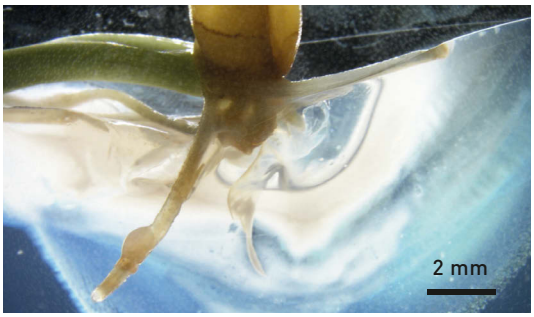


A1) CONTROL

A2) CONTROL

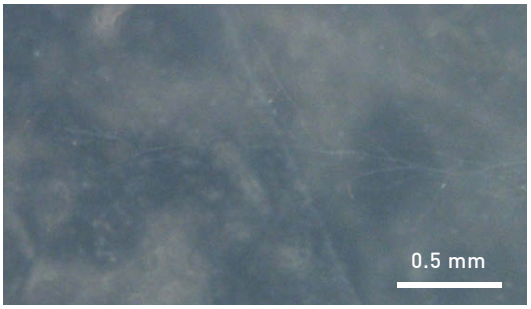


B1) 0.1 mM NICKEL

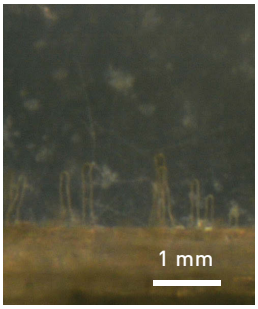


B2) 0.1 mM NICKEL

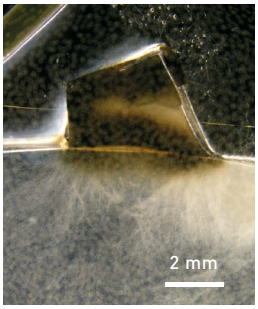
DIAPORTHE COLUMNARIS



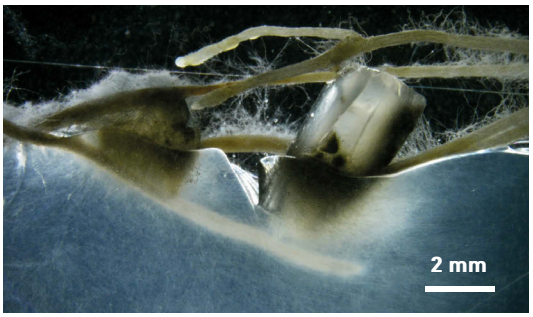
C1) CONTROL



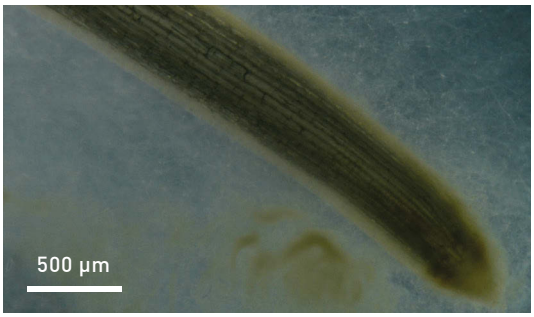
C2) CONTROL



C3) CONTROL



D1) 0.1 mM NICKEL



D2) 0.1 mM NICKEL

Figure 33: *seedlings of* Triticum aestivum *inoculated with* Rhodotorula mucilaginosa *in A) the control group without toxic metals, B) 0.1 mM nickel, or* Diaporthe columnaris *in C) the control group without toxic metals, D) 0.1 mM nickel. All pictures were taken after 7 days of inoculation*

HEAVY METAL LOCALIZATION, STAINING USING NEWPORT GREEN™ (NPG)

Using small, experimental datasets we wanted to depict interactions between plants and fungi. First we wanted to estimate the usability of NPG to detect and display nickel and zinc in plants and in yeast cells, in cohabitation as well as on their own.

Heavy metal localization using NPG with fluorescence microscopy to detect nickel and zinc in roots of *Amaranthus cruentus* and *Noccaea caerulescens*

Seedlings of *A. cruentus* (1) and *N. caerulescens* (2) were co-habited with both yeast each, and were dyed with NPG to visualize nickel and zinc uptake using fluorescence microscopy. The method worked well for the roots in bright field. Yeasts were very small in contrast to the roots, easy to wash off and partly hard to identify, results were better using CLSM (performed only for nickel treatments). Results do not allow quantifications of nickel or zinc uptake or differentiation of yeasts.

1.1) Amaranthus cruentus & Rhodotorula mucilaginosa

Samples of *Amaranthus cruentus*, infected with *R. mucilaginosa*, were infiltrated with 10 µM of NPG and analysed using fluorescence microscopy after two hours and again after 20 hours at various magnifications. *Rhodotorula mucilaginosa* in nickel was additionally analysed after 48 hours using CLSM.

Root tip:

After two hours of drenching in NPG, a clear increase in intensity could be determined in the nickel treated sample compared to the control. Zinc treatment did not affect the signal. Soaking for 24 hours in NPG resulted in an increase in intensity, especially in the nickel treatment, with increased background noise.

In the control group, cell walls got stained. Nickel treatment lacked the central cylinder, cells seemed lysed. Zinc treated plants showed dead cells in the calyptra, the root cap, also the central cylinder showed increased fluorescence. (Table 2, figure 34)

Root hair

Root hairs were poorly dyed after 2 hours in the control. Nickel plants did not produce root hairs, results are therefore missing. Zinc treatment did not affect the signal. In the zinc treatment the dye stained especially the tips of the root hairs which gave brighter results. After 24 hours in NPG, signals were generally increased. (Table 2, figure 35)

Rhodotorula mucilaginosa

The yeast was hard to detect next to the roots, which showed much brighter signals in bright field (data not shown). Using CLSM, after 48 hours drenching time, the yeasts showed stained cell walls. (Table 2, figure 36)

Table 2: *NPG staining of* Amaranthus cruentus *growing with* Rhodotorula mucilaginosa *in A) control, B) 1 mM nickel, C) 1 mM zinc. "0" indicates no change in intensity, "+" to "+++++" increase in intensity of fluorescence. Thresholds of the control after 2 hours and after 20 hours are listed at the end of the table.*

Amaranthus cruentus + Rhodotorula mucilaginosa	**control**	**1 mM nickel**	**1 mM zinc**
root tip (2 hours)	0	+	0
root tip (20 hours)	+++	+	+
root hair (2 hours)	0	missing	0
root hair (20 hours)	+	missing	+
yeast (2 hours)	0	+++	+
yeast (20 hours)	+++	+++++	+++
	root tip	**root hair**	**root & yeast**
threshold (2 hours)	10 ms	100 ms	2000 ms
threshold (20 hours)	2 ms	30 ms	20 ms

AMARANTHUS CRUENTUS WITH *RHODOTORULA MUCILAGINOSA*, ROOT TIP

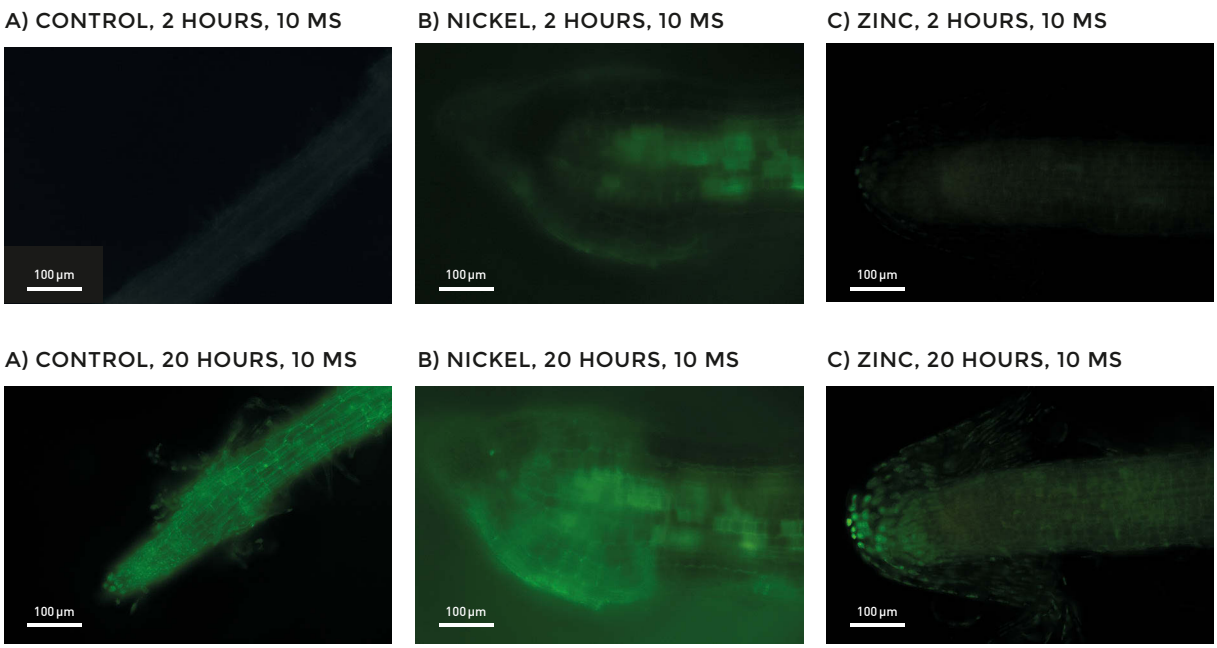


Figure 34: *Root tip of* Amaranthus cruentus *growing with* Rhodotorula mucilaginosa *in A) control, B) 1 mM nickel, C) 1 mM zinc. Pictures were taken with 10 ms exposure after drenching plants and yeast for two and twenty hours in* npg *using fluorescence microscopy.*

AMARANTHUS CRUENTUS WITH *RHODOTORULA MUCILAGINOSA*, ROOT HAIR

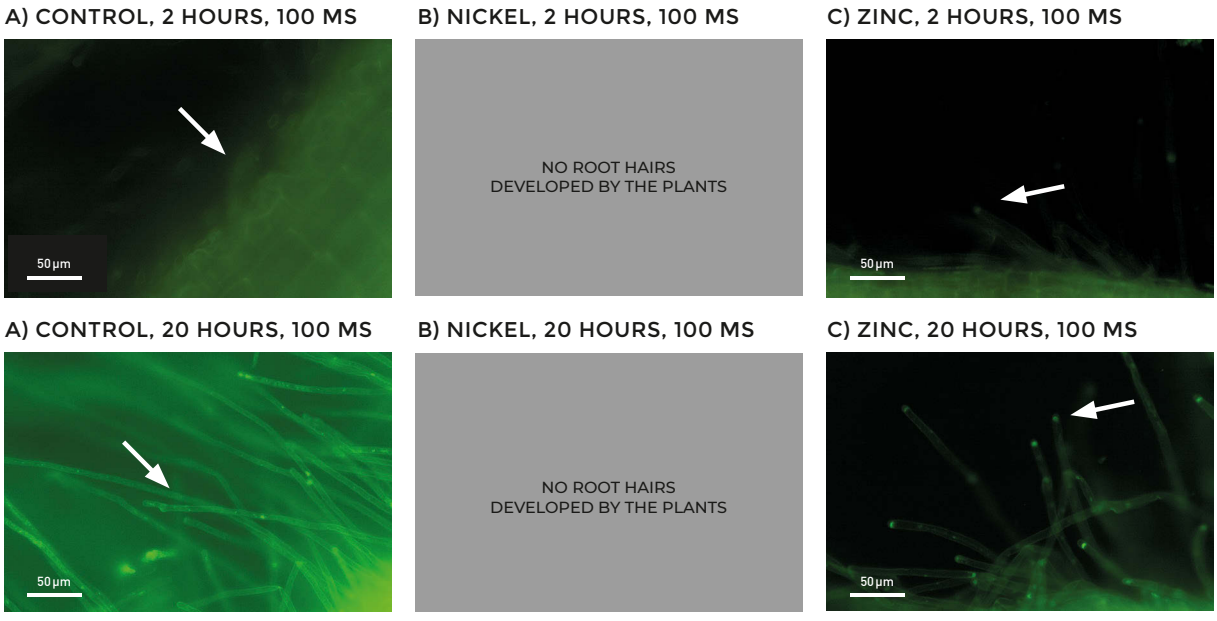


Figure 35: *Root hairs of* Amaranthus cruentus *growing with* Rhodotorula mucilaginosa *in A) control, B) 1 mM nickel, C) 1 m*M *zinc. Pictures were taken with 100 ms exposure after drenching plants and yeast for two and twenty hours in NPG using fluorescence microscopy. Arrows mark exemplary root hairs.*

RHODOTORULA MUCILAGINOSA IN NICKEL

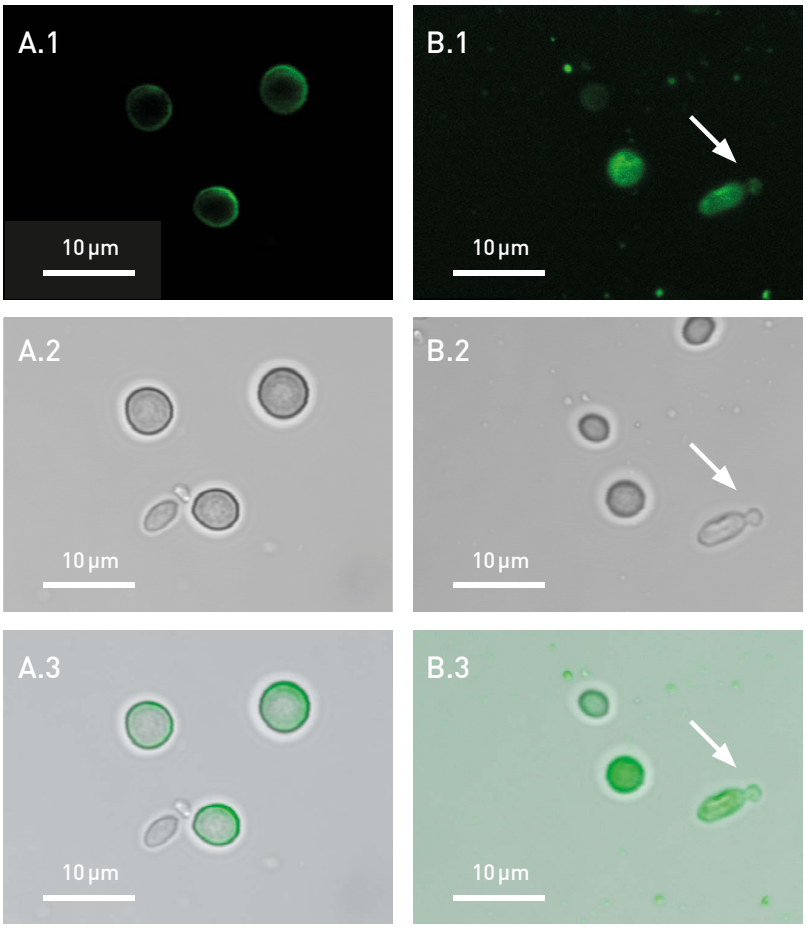


Figure 36: *Two examples for* Rhodotorula mucilaginosa *in 1 mM nickel after drenching in NPG for 48 hours. Pictures were taken using CLSM. The arrow marks a budding yeast cell.*

1.2) Amaranthus cruentus & Sporobolomyces sp.

Seedlings of *Amaranthus cruentus* grew with *Sporobolomyces sp.* and were drenched with 10 µM of NPG for two hours and 20 hours. *Sporobolomyces sp.* in nickel was analysed after 48 hours using CLSM.

Root tip

Nickel treated sample showed increased fluorescence compared to the control, irrespective of the drenching time. Zinc treatment did not increase fluorescence compared to the control. (Table 3, figure 37)

Nickel plants had dead roots, root hairs and hypocytl looked like stained from outside. Zinc treated plants indicate apoplastic transport of zinc after dyeing, the central cylinder did not show much fluorescence.

Root hair

Nickel treated plants did not develop root hairs. Zinc treatment led to well developed root hairs that showed higher fluorescence compared to the control. (Table 3, figure 38)

Sporobolomyces sp.

Yeasts did not show much fluorescence in the control, nickel slightly increased the signal, zinc treated yeasts showed intense fluorescence after 2 hours of drenching in NPC. Yeast did not take up the dye very well. (Table 3, figure 39)

After 48 hours, using CLSM, the yeasts showed stained cell walls, as well as stained internal structures in nickel treatment, like microbodies. Young yeast cells appeared less stained than old ones. (Table 3, figure 40)

Table 3: *NPG staining of* Amaranthus cruentus *growing with* Sporobolomyces sp. *in A) control, B) 1 mM nickel, C) 1 mM zinc. "o" indicates no change in intensity, "+" to "+++++" increase in intensity of fluorescence. Thresholds of the control after 2 hours and after 20 hours are listed at the end of the table.*

Amaranthus cruentus + Rhodotorula mucilaginosa	**control**	**1 mM nickel**	**1 mM zinc**
root tip (2 hours)	o	+	o
root tip (20 hours)	+	++	+
root hair (2 hours)	o	missing	+
root hair (20 hours)	+	missing	++
yeast (2 hours)	o	+	++
yeast (20 hours)	+++	+++	+++
	root tip	**root hair**	**root & yeast**
threshold (2 hours)	7 ms	400 ms	400 ms
threshold (20 hours)	2 ms	100 ms	60 ms

AMARANTHUS CRUENTUS WITH *SPOROBOLOMYCES SP.*, ROOT TIP

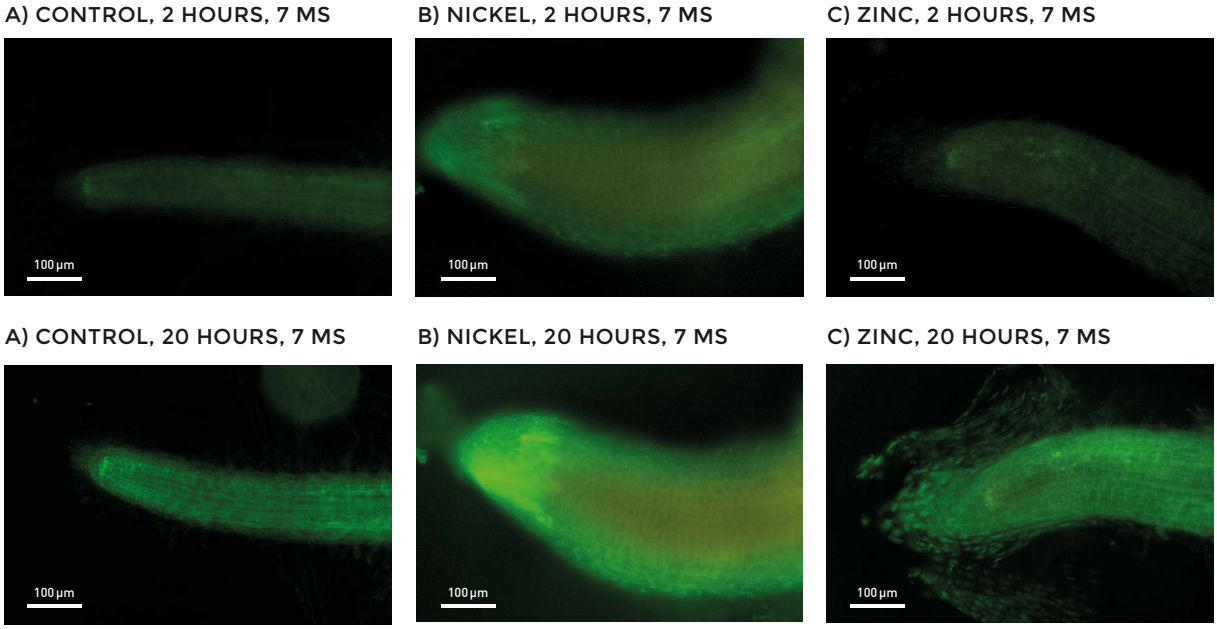


Figure 37: *Root tip of* Amaranthus cruentus *growing with* Sporobolomyces sp. *in A) control, B) 1 mM nickel, C) 1 mM zinc. Pictures were taken with 7 ms exposure after drenching plants and yeast for two and twenty hours in* npg *using fluorescence microscopy.*

AMARANTHUS CRUENTUS WITH *SPOROBOLOMYCES SP.*, ROOT HAIR

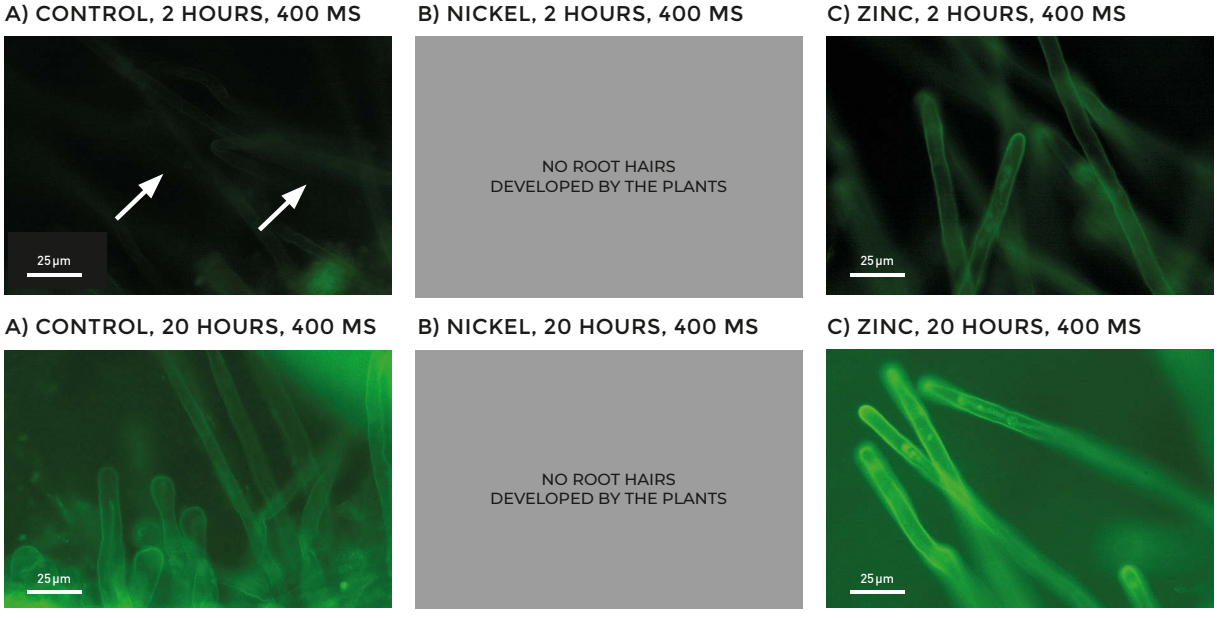


Figure 38: *Root hairs of* Amaranthus cruentus *growing with* Sporobolomyces sp. *in A) control, B) 1 mM nickel, C) 1 m*M *zinc. Pictures were taken with 400 ms exposure after drenching plants and yeast for two and twenty hours in* npg *using fluorescence microscopy. Arrows mark exemplary root hairs.*

AMARANTHUS CRUENTUS WITH *SPOROBOLOMYCES SP.*

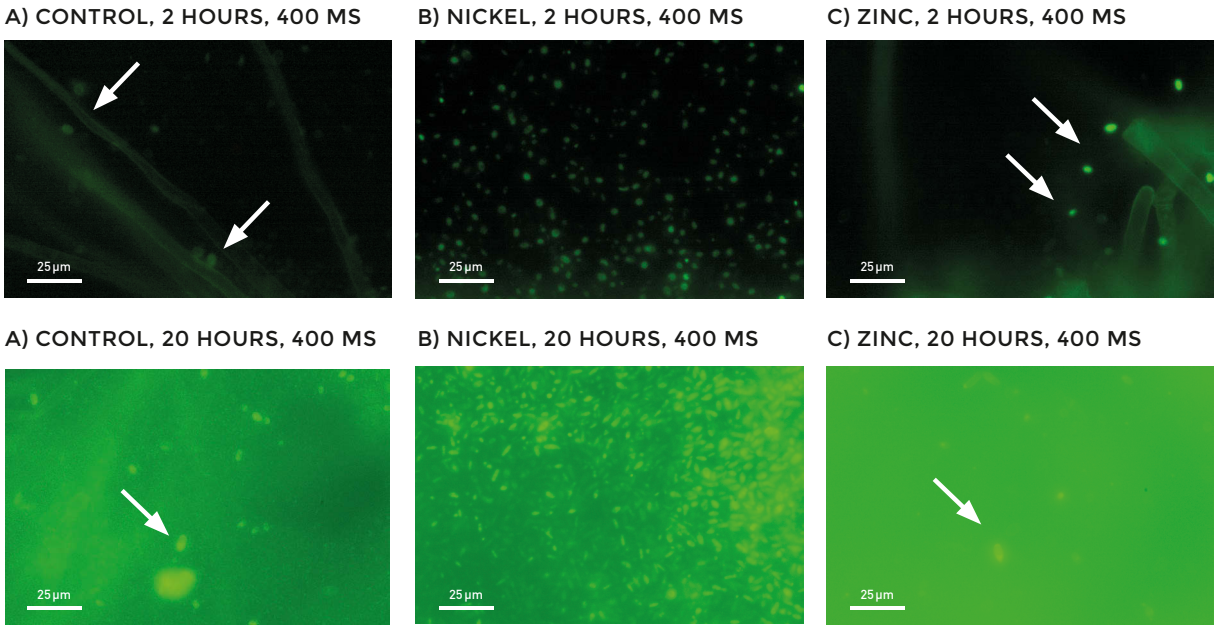


Figure 40: Amaranthus cruentus *growing with* Sporobolomyces sp. *in A) control, B) 1 mM nickel, C) 1 mM zinc. Pictures were taken with 400 ms exposure after drenching plants and yeast for two and twenty hours in* npg *using fluorescence microscopy. Arrows mark exemplary yeast cells.*

SPOROBOLOMYCES SP. IN NICKEL

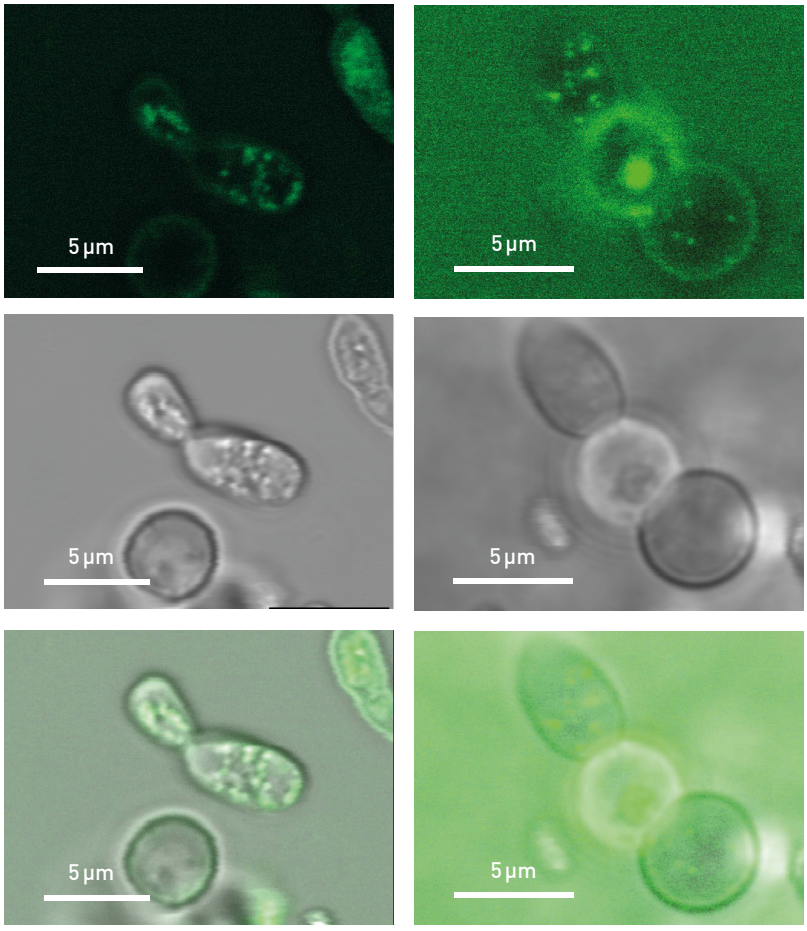


Figure 41:
Two examples for Sporobolomyces sp. *in 1 mM nickel after drenching in* npg *for 48 hours. Pictures were taking using* clsm. *Cell walls show staining.*

2.1) Noccaea caerulescens & Rhodotorula mucilaginosa

Noccaea caerulescens seedlings, infected with *Rhodotorula mucilaginosa*, were infiltrated with 10 µM of NPG and analysed using fluorescence microscopy after two and 20 hours.

Root tip

Metal treatments did not cause increased fluorescence compared to the control after two hours of drenching in NPG. In the nickel treatment, fluorescence did not appear in the central cylinder, but in the endodermis. Zinc treated plants seemed affected by the heavy metal treatment, with non-fluorescent root tips. Tracheids were partly visible. (Table 4, figure 42)

Root hair

NPG fluorescence intensity did not increase in the nickel treated sample compared to the control after two hours. Zinc treated root hairs showed slightly increased fluorescence compared to the control. After 24 hours in NPG, signals were generally increased.

Nickel treated plants did produce root hairs. Zinc treatment caused short root hair. (Table 4, figure 43)

Rhodotorula mucilaginosa

Yeasts did not show high fluorescence after two hours immersion time. After 20 hours, in control, and nickel treated samples, increased values and background staining could be determined, with strongest signals in the nickel treated plants. Yet, only a part of the yeast got stained. Zinc treatment led to slightly increased fluorescence, again, only part of the yeast cells got stained. (Table 4, figure 44)

Table 4: *NPG staining of* Amaranthus cruentus *growing with* Rhodotorula mucilaginosa *in A) control, B) 1 mM nickel, C) 1 mM zinc. "o" indicates no change in intensity, "+" to "+++++" increase in intensity of fluorescence. Thresholds of the control after two hours and after 20 hours are listed at the end of the table.*

Noccaea caerulescens + Rhodotorula mucilaginosa	**control**	**1 mM nickel**	**1 mM zinc**
root tip (2 hours)	o	o	+
root tip (20 hours)	++	+	+
root hair (2 hours)	o	o	+
root hair (20 hours)	+	+	+
yeast (2 hours)	o	o	o
yeast (20 hours)	++	++	+

	root tip	**root hair**	**root & yeast**
threshold (2 hours)	10 ms	150 ms	100 ms
threshold (20 hours)	2 ms	20 ms	20 ms

NOCCAEA CAERULESCENS WITH *RHODOTORULA MUCILAGINOSA*, ROOT TIP

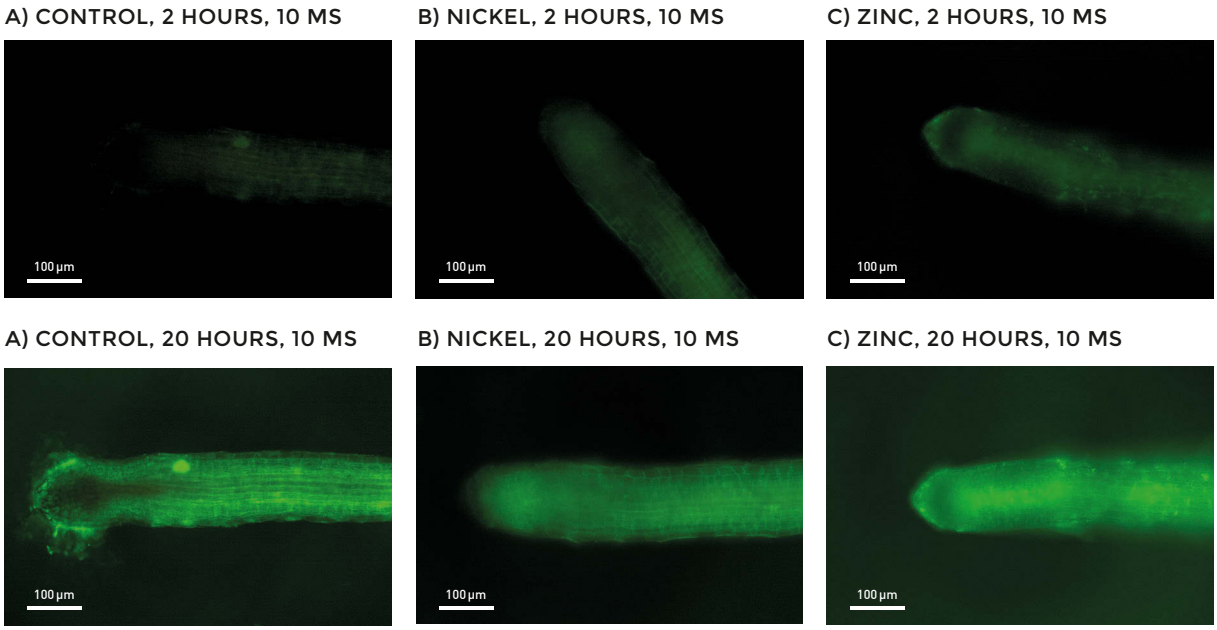


Figure 42: *Root tip of* Noccaea caerulescens *growing with* Rhodotorula mucilaginosa *in A) control, B) 1 mM nickel, C) 1 mM zinc. Pictures were taken with 10 ms exposure after drenching plants and yeast for two and twenty hours in* npg *using fluorescence microscopy.*

NOCCAEA CAERULESCENS WITH *RHODOTORULA MUCILAGINOSA*, ROOT HAIR

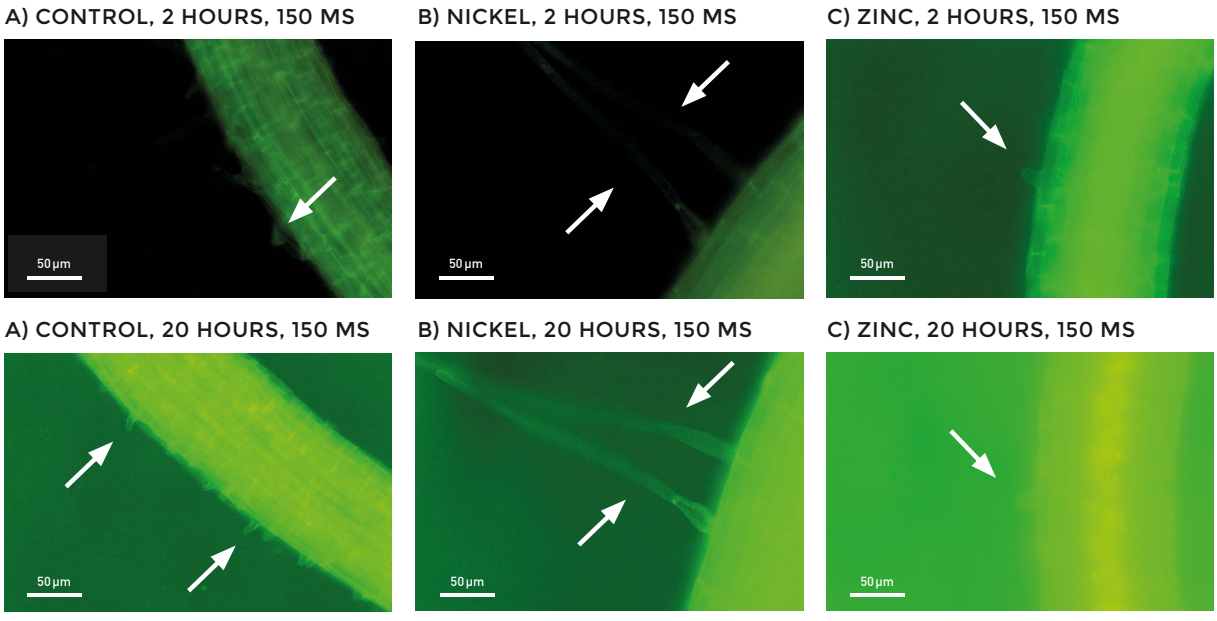


Figure 43: *Root hairs of* Noccaea caerulescens *growing with* Rhodotorula mucilaginosa *in A) control, B) 1 mM nickel, C) 1 m*M *zinc. Pictures were taken with 100 ms exposure after drenching plants and yeast for two and twenty hours in* npg *using fluorescence microscopy. Arrows mark exemplary root hairs.*

NOCCAEA CAERULESCENS WITH *RHODOTORULA MUCILAGINOSA*

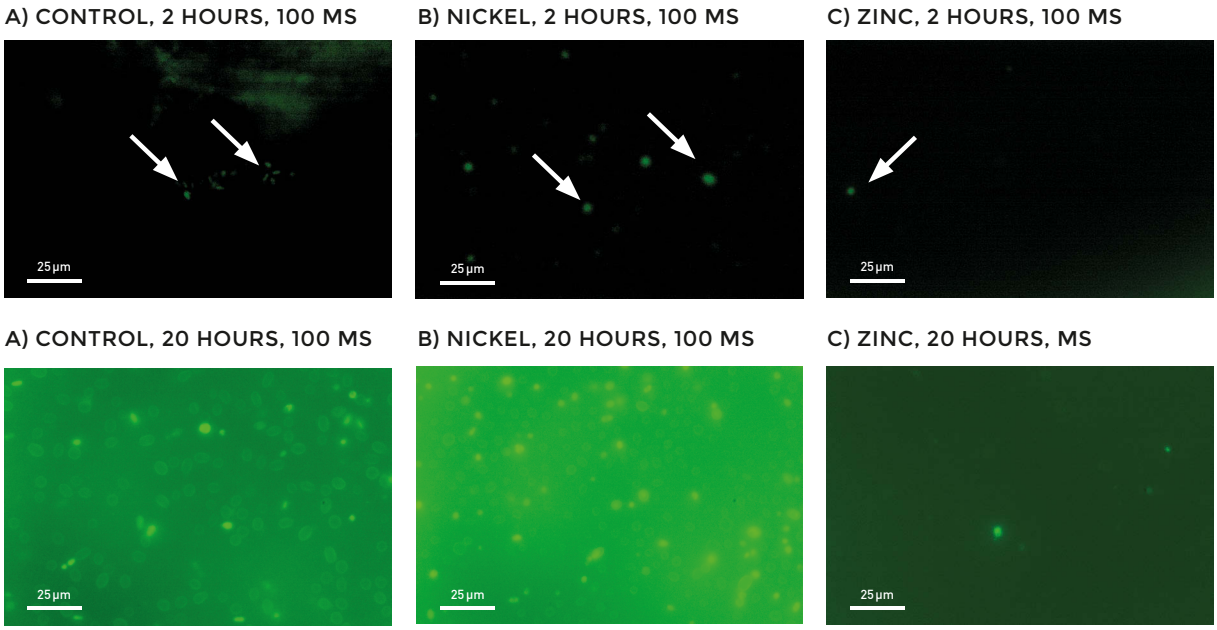


Figure 44: Noccaea caerulescens *growing with* Rhodotorula mucilaginosa *in A) control, B) 1 mM nickel, C) 1 mM zinc. Pictures were taken with 100 ms exposure time after drenching plants and yeast for two and twenty hours in* npg *using fluorescence microscopy. Arrows mark exemplary yeast cells.*

2.2) Noccaea caerulescens & Sporobolomyces sp.

Noccaea caerulescens seedlings, infected with *Sporobolomyces sp.*, were infiltrated with 10 µM of NPG and analysed using fluorescence microscopy after two and 20 hours.

Root tip

The nickel treated sample showed strong fluorescence with strong background noise, while zinc did not affect the signal compared to the control after two hours of drenching in NPG. Zinc plants had fluorescent tips, indicating dead cells there. (Table 5, figure 45)

Root hair

NPG fluorescence intensity strongly increased in the nickel treated sample compared to the control after two hours. Zinc treated root hairs did not show stronger fluorescence after two hours. After 24 hours in NPG, signals were clearly increased in all samples. Nickel treated plants did produce root hairs. Zinc treatment caused short root hair. (Table 5, figure 46)

Sporobolomyces sp.

Yeasts in the nickel treated samples showed intense fluorescence, accompanied by strong background noise after 2h immersion time. Zinc treated plants showed increased fluorescence compared to the control. After 20 hours, in control, and zinc treated samples, increased values and background staining could be determined. Nickel treated samples showed an over-exposure and made analysation quite impossible with the used exposure time of 300 ms. Again, staining seemed to involve only a part of yeast cells. (Table 5, figure 47)

Table 5: *NPG staining of* Amaranthus cruentus *growing with* Sporobolomyces sp. *in A) control, B) 1 mM nickel, C) 1 mM zinc. "o" indicates no change in intensity, "+" to "+++++" increase in intensity of fluorescence. Thresholds of the control after 2 hours and after 20 hours are listed at the end of the table.*

Noccaea caerulescens + Rhodotorula mucilaginosa	**control**	**1 mM nickel**	**1 mM zinc**
root tip (2 hours)	o	++++	o
root tip (20 hours)	+	++++	+
root hair (2 hours)	o	++++	+
root hair (20 hours)	+++	+++++	+++
yeast (2 hours)	o	++++	++
yeast (20 hours)	++	+++++	+

	root tip	**root hair**	**root & yeast**
threshold (2 hours)	30 ms	150 ms	300 ms
threshold (20 hours)	4 ms	20 ms	100 ms

NOCCAEA CAERULESCENS WITH *SPOROBOLOMYCES SP.*, ROOT TIP

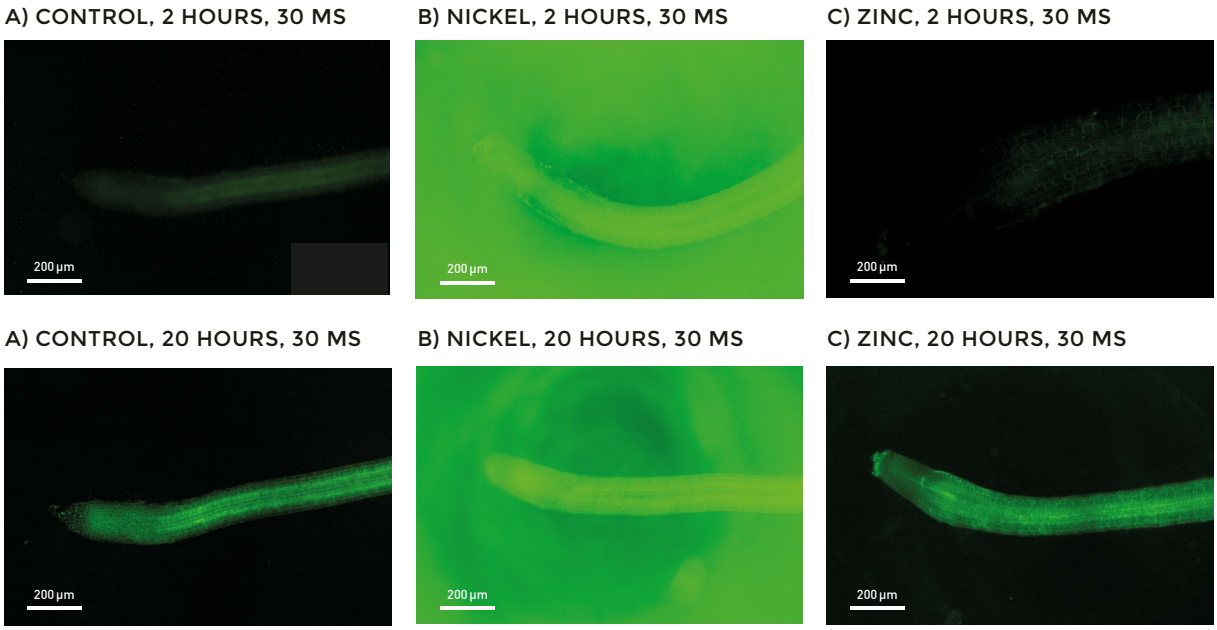


Figure 45: Noccaea caerulescens *growing with* Sporobolomyces sp. *in A) control, B) 1 mM nickel, C) 1 mM zinc. Pictures were taken with 30 ms exposure time after drenching plants and yeast for two and twenty hours in* npg *using fluorescence microscopy.*

NOCCAEA CAERULESCENS WITH *SPOROBOLOMYCES SP.*, ROOT HAIR

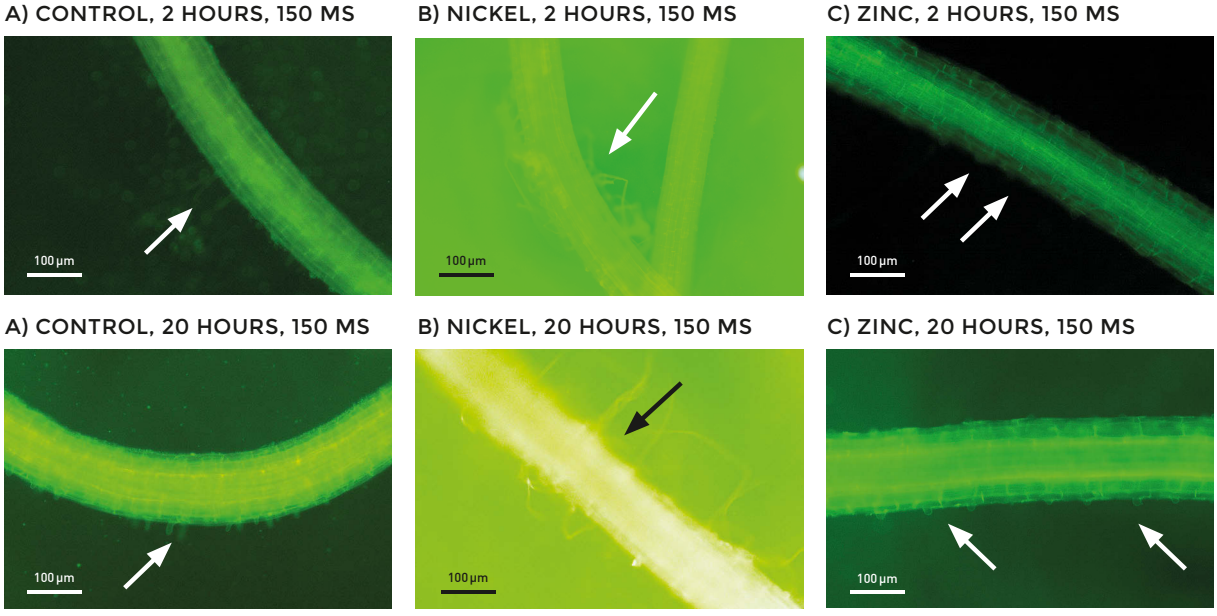


Figure 46: Noccaea caerulescens *growing with* Sporobolomyces sp. *in A) control, B) 1 mM nickel, C) 1 mM zinc. Pictures were taken with 150 ms exposure after drenching plants and yeast for two and twenty hours in* npg *using fluorescence microscopy. Arrows mark exemplary root hairs.*

NOCCAEA CAERULESCENS WITH *SPOROBOLOMYCES SP.*

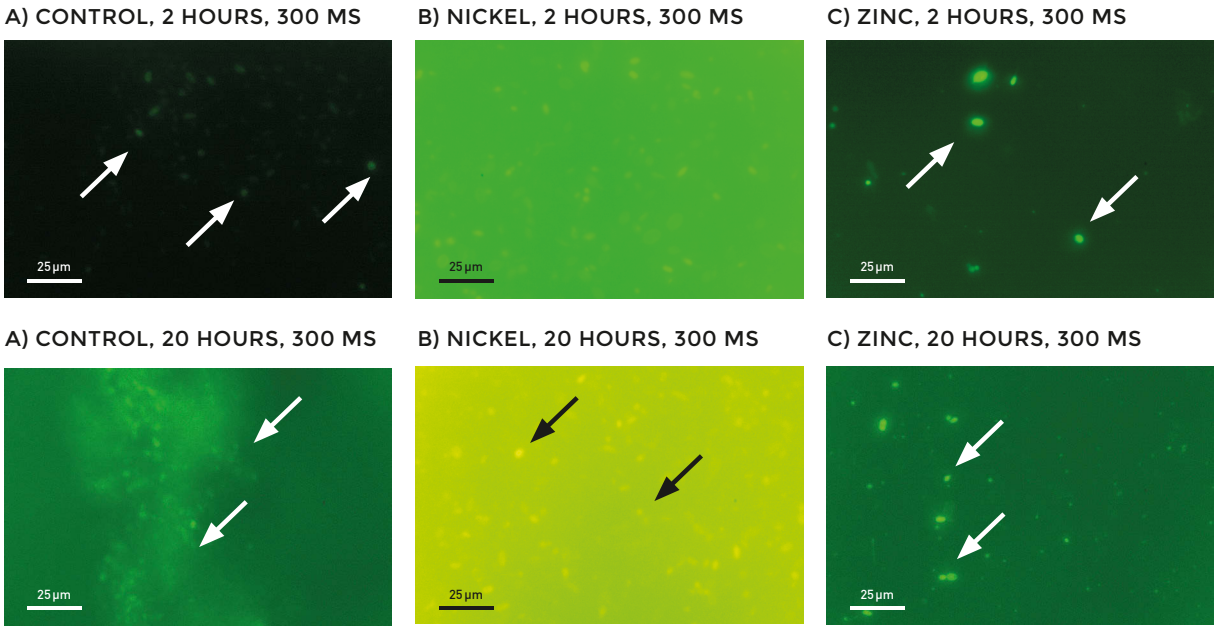


Figure 47: Noccaea caerulescens *growing with* Sporobolomyces sp. *in A) control, B) 1 mM nickel, C) 1 mM zinc. Pictures were taken with 300 ms exposure after drenching plants and yeast for two and twenty hours in* npg *using fluorescence microscopy. Arrows mark exemplary yeast cells.*

Heavy metal localization using NPG with CLSM to detect nickel and zinc in *Sporobolomyces* sp.

We wanted to estimate usability NPG for detecting nickel and zinc uptake in yeasts. CLSM was used to get further insights into the intracellular storage of nickel and zinc in *Sporobolomyces sp. S*ix microscopy slides were prepared with *Sporobolomyces sp.* originally growing with *Amaranthus cruentus*. These were used in two sets. Autofluorescence of unstained samples of control-, nickel- and zinc-treatment, was compared to an identical set that had been stained with 10 µM NPG for 3 hours. Volt-thresholds of both sets, where fluorescence was visible first, were compared.

Generally, cell walls were not fluorescent, but small vacuoles, cell plasm and small grains.

Control, autofluorescence without staining
In the unstained control treatment cells showed autofluorescence. Cells were alive, no big central vacuole was visible. The threshold where the fluorescence of cells was visible for the first time was estimated at 720 V. (Table 6; figure 48, A)

Control, with NPG
NPG stained cells in the control group were dividing and showed partly fluorescence. Dots in the cells interior were visible. The threshold for even the brightly fluorescent cells was estimated at 370 V. (Table 6; figure 48, D)

Nickel, autofluorescence without staining
The sample did not contain enough cell material, but the nickel agar itself showed autofluorescence. The shown picture was taken with 711 V. (Table 6; figure 48, B)

Nickel, with NPG
NPG stained cells in the nickel group were dividing and partly fluorescent. The nickel agar showed increased fluorescence compared to the unstained sample. Yeast cells appeared to be smaller, and in bad condition. Brightly fluorescent dots were visible, coagulated cell plasm was fluorescent. Threshold: 530 V (Table 6; figure 48, E)

Zinc, autofluorescence without staining
In the unstained zinc treatment cells showed partly autofluorescence. Small vacuoles were fluorescent, the cell wall did not emit any signal. Partly plasm and grains were fluorescent. Threshold: 950 V. (Table 6; figure 48, C)

Zinc, with NPG
Zinc treated cells with dye were partly fluorescent. Budding was visible. Cells appeared to be smaller, while more cells were fluorescent. In general, yeast dyed with NPG, showed weaker signals compared to the control group stained with NPG. Threshold: 616 V. (Table 6; figure 48, F)

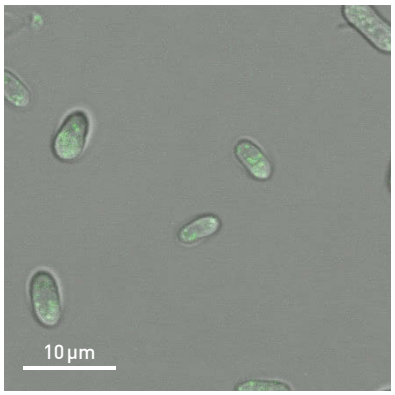
Table 6: *Comparison of autofluorescence of* Sporobolomyces sp. grown *in control, nickel, zinc to fluorescence after staining with NPG. Fluorescence thresholds are shown after three hours of drenching in NPG. Values were estimated using CLSM.*

Sporobolomyces sp.			**Threshold (V)**
	Control	Unstained	720
		Stained	370
	Nickel	Unstained	711
		Stained	530
	Zinc	Unstained	950
		Stained	619

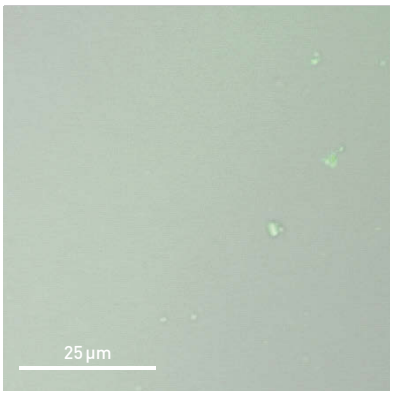
SPOROBOLOMYCES SP. GROWN ON *AMARANTHUS CRUENTUS*

AUTOFLUORESCENCE WITHOUT ADDITIONAL STAINING

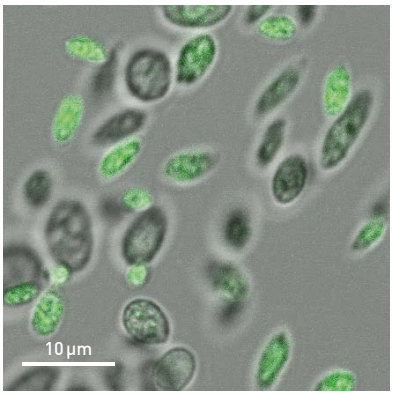
A) CONTROL



B) NICKEL

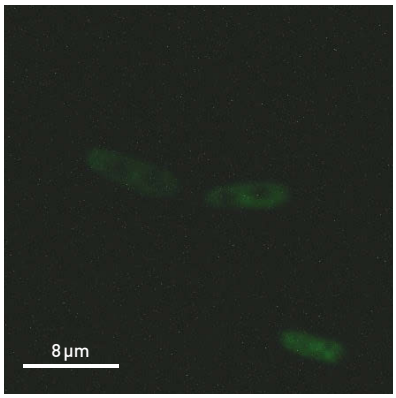


C) ZINC

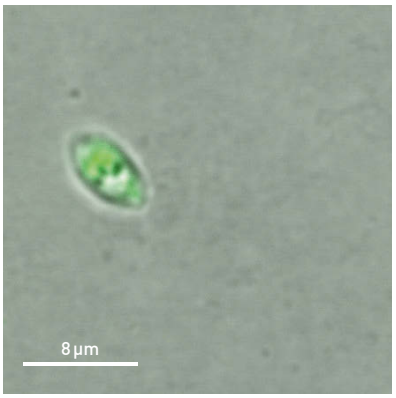


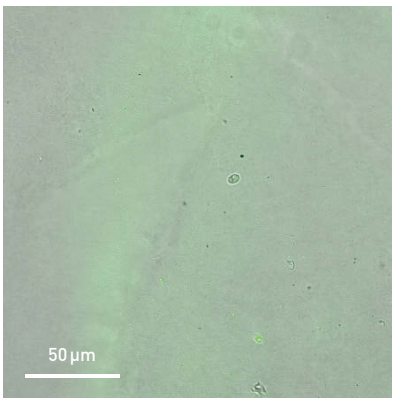
ADDITIONAL STAINING WITH NPG

D) CONTROL



E) NICKEL





F) ZINC

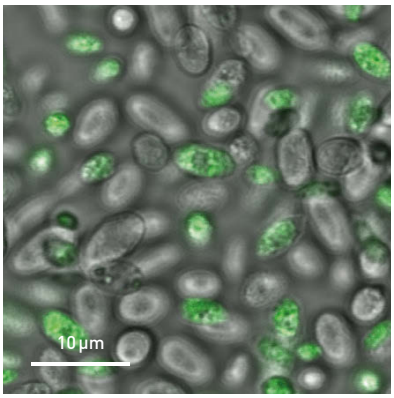


Figure 48: *Pictures A to C show the autofluorescence of* Sporobolomyces sp. *growing as control or with nickel or zinc. Pictures D to F show* Sporobolomyces sp. *after drenching for three hours in* npg. *Pictures were taking using CLSM, in the green channel only (emission filter 500–550 nm).*

INTERACTION OF PLANTS AND FUNGI

Different staining methods were performed to examine the co-habitation between *Triticum aestivum* roots and *Diaporthe columnaris, Rhodotorula mucilaginosa* or *Sporobolomyces sp.*

YEAST AND PLANT INTERACTION: STAINING USING CALCOFLUOR-WHITE

Calcofluor-White stain, a fluorescent brightener, was added in droplets to roots of *Triticum aestivum* growing with *Diaporthe columnaris, Rhodotorula mucilaginosa or Sporobolomyces ruberrimus* on agar with 100 µM nickel and examined immediately under the fluorescence microscope.

Fluorescence microscopy with Calcofluor-White and *Diaporthe columnaris*

The stain binds unspecifically to cellulose as well as chitin. Root cell hairs of *Triticum aestivum* showed bright fluorescence at an exposure time of 50 ms, with slightly brighter root hair-tips, cell epidermal cell walls are stained less, but still visible (Figure 49, A). *Diaporthe columnaris* showed weaker fluorescence, and needed 100 ms exposure time (Figure 49, B), making it difficult to distinguish it in between of the wheat's root hair cells (marked by the arrows in figure 50 and figure 51, where UV excitation and bright field are compared).

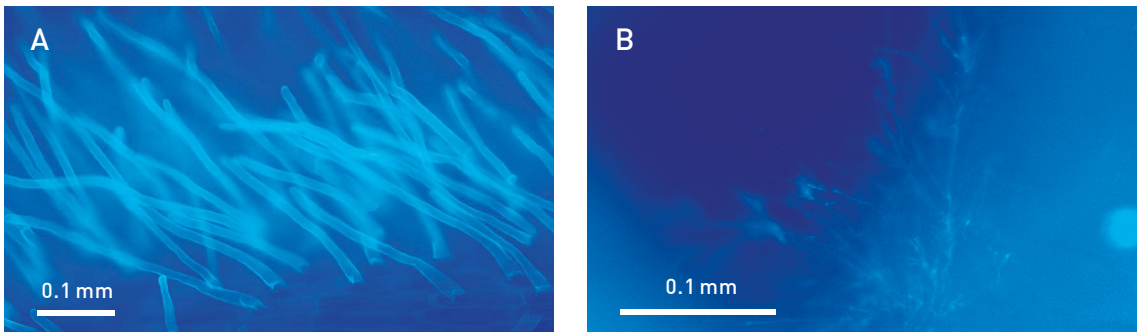


Figure 49: *Calcofluor-White staining of* Triticum aestivum *growing with* Diaporthe columnaris *on 100 µM nickel agar. The left picture A shows an overview of the root hairs of* Triticum aestivum *in UV-excitation at an exposure time of 50 ms. The right picture B shows hyphae of* Diaporthe columnaris *at an exposure time of 100 ms. Root hairs (A) are showing bright fluorescence, hyphae had weaker fluorescence (B).*

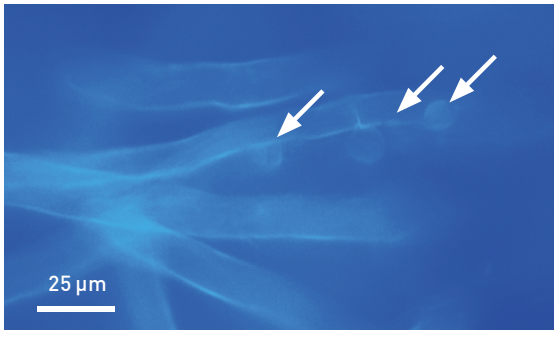


Figure 50: *Calcofluor-White staining of* Triticum aestivum *growing with* Diaporthe columnaris *on 100 µM nickel agar. The picture shows an overview of the root hairs of* Triticum aestivum *in UV-excitation. The arrow marks* Diaporthe columnaris.

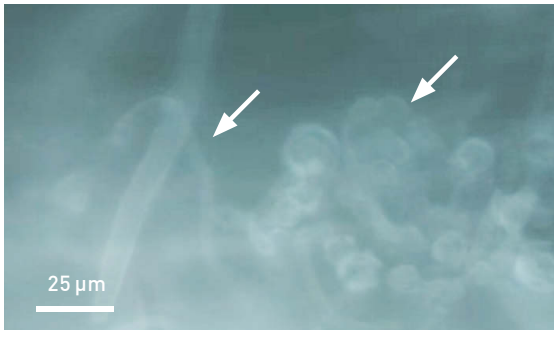


Figure 51: *Calcofluor-White staining of* Triticum aestivum *growing with* Diaporthe columnaris *on 100 µM nickel agar in bright field. The arrows marks* Diaporthe columnaris.

Fluorescence microscopy with Calcofluor-White and *Rhodotorula mucilaginosa*

Calcofluor-White staining of *Triticum aestivum* growing with *Rhodotorula mucilaginosa* showed intense staining of the wheat's epidermal cell walls, making it difficult to differentiate stained *Rhodotorula mucilaginosa* hyphae from the wheat epidermis. Yeast cells were easier to distinguish in bright field. (Figure 52, A and B)

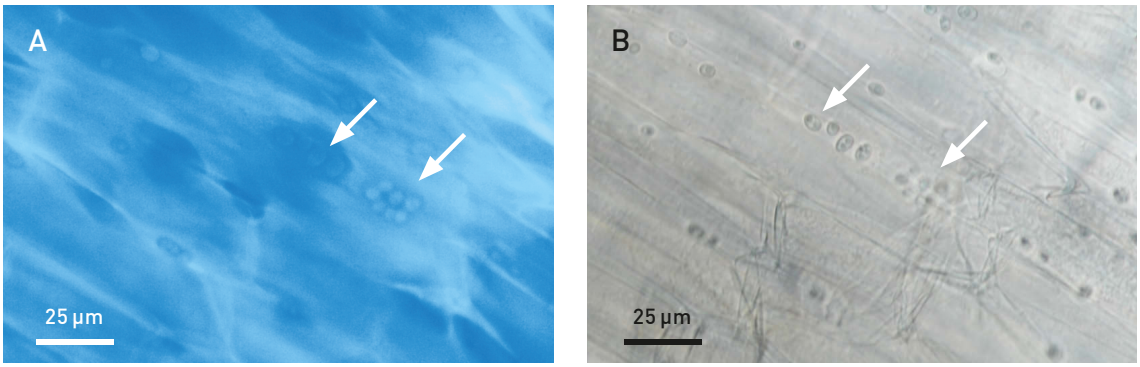


Figure 52: *Calcofluor White staining of* Triticum aestivum *growing with* Rhodotorula mucilaginosa *on 100 µM nickel agar. Picture A shows an overview of the root epidermis of* Triticum aestivum *inhabited by cells of* Rhodotorula mucilaginosa *in UV-excitation at an exposure time of 10 ms. Picture B shows the same situation under bright field. Arrows mark yeast cell examples.*

Fluorescence microscopy with Calcofluor-White and *Sporobolomyces ruberrimus*

Calcofluor-White staining of *Triticum aestivum* growing with *Sporobolomyces rubberimus* showed again an intense staining of the wheat, making it difficult to identify *Sporobolomyces rubberimus* on the surface of the wheat root. Yeast cells were easier to distinguish in bright field. *(Figure 53, A and B)* Yeast cells were covering the root surface, yet no penetration into the roots was visible.

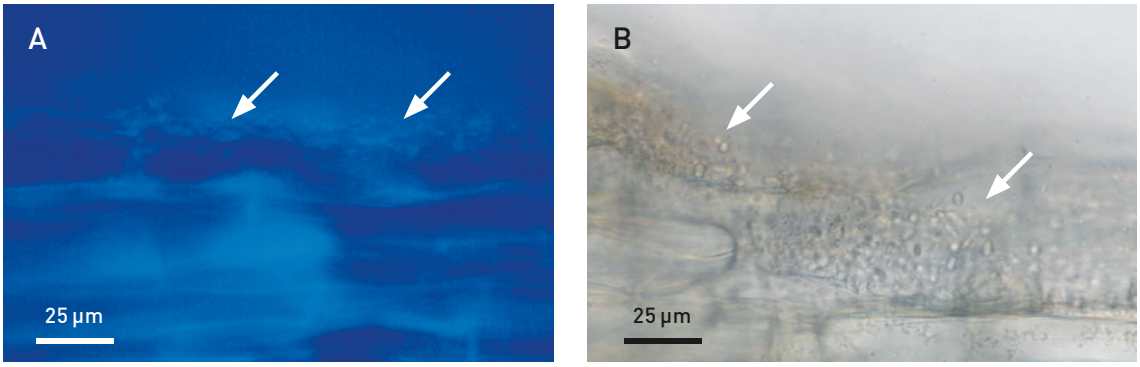


Figure 53: *Calcofluor White staining of* Triticum aestivum *growing with* Sporobolomyces ruberrimus *on 100 µM nickel agar. Picture A shows an overview of the root epidermis of* Triticum aestivum *inhabited by cells of* Sporobolomyces ruberrimus *in UV-excitation at an exposure time of 900 µs. Picture B shows a similar situation under bright field. Arrows mark yeast cell examples.*

CLSM: Calcofluor-White and *Rhodotorula mucilaginosa*

Stainings analysed with CLSM showed clear signals for cell walls of *Triticum aestivum* and *Rhodotorula mucilaginosa*. Internal cell structures of *Rhodotorula mucilaginosa* were not visible in UV-excitation. UV-excitation helped to distinguish the cell material from the cloudy deposits which did not show fluorescence. (Figure 54 and 55)

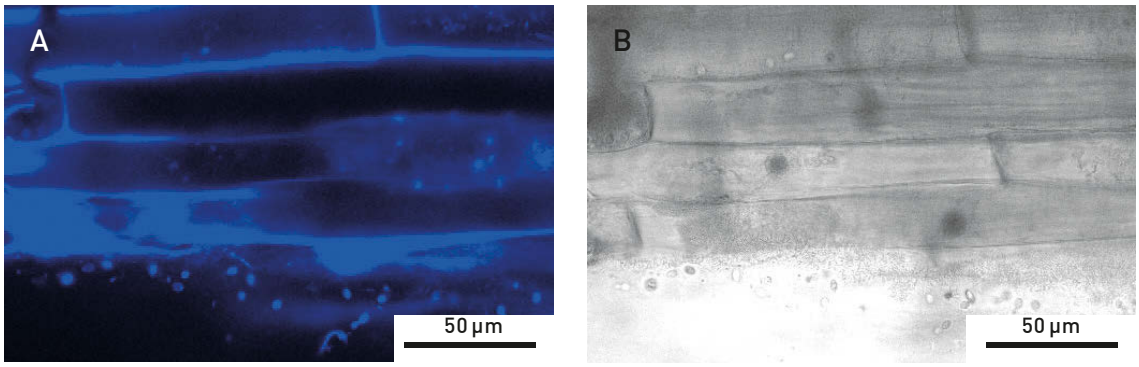


Figure 54: *Calcofluor-White staining of* Triticum aestivum *growing with* Rhodotorula mucilaginosa *on 100 µM nickel agar. Picture A was taken in UV-excitation. Picture B shows the same situation under bright field.*

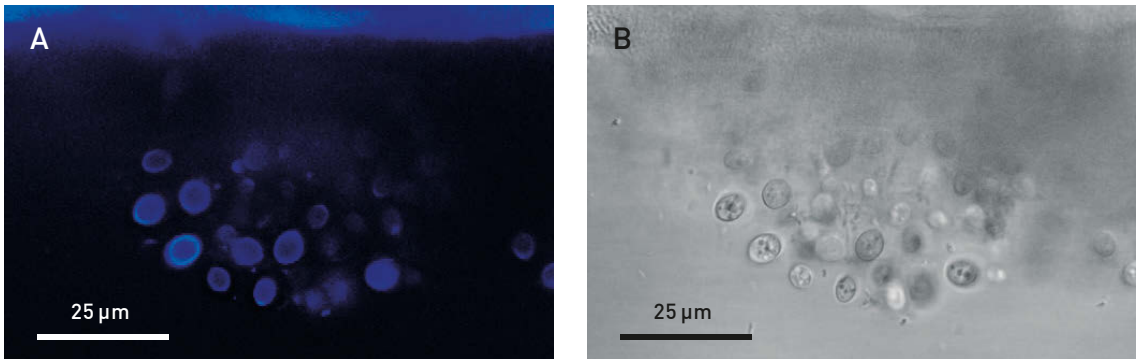


Figure 55: *Calcofluor-White staining of* Triticum aestivum *growing with* Rhodotorula mucilaginosa *on 100 µM nickel agar. Picture A shows a group of yeast cells next to a root* Triticum aestivum *in UV-excitation. Picture B shows the same situation under bright field.*

Figures 56 to 58 show CLSM pictures taken of *Rhodotorula mucilaginosa* stained with Calcofluor-White. In UV-Excitation primarily the yeast cell walls were visible. Internal structures were not visible (Figures 56 to 58: A). Yeast cells were not stained evenly, budding yeast cells showed a higher fluorescence (Figure 57).

Deposits on the agar plates were not stained, therefore a differentiation between *Triticum aestivum* and *Rhodotorula mucilaginosa* was improved with CLSM (Figure 58, A and D). Internal structures got visible. (Figure 56, B and C)

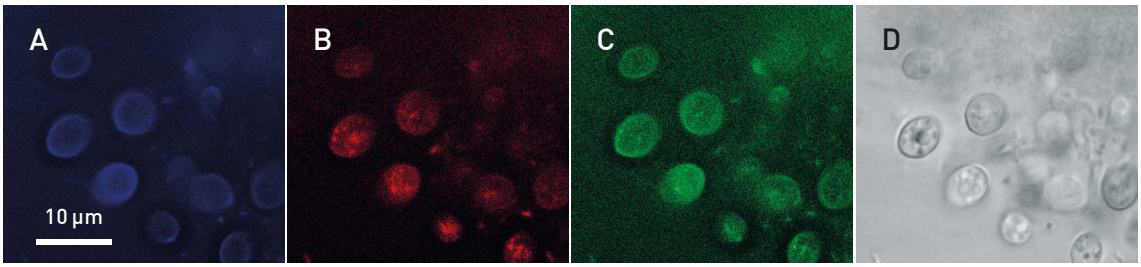


Figure 56: *Calcofluor-White staining of* Rhodotorula mucilaginosa *growing with* Triticum aestivum *on 100 µM nickel agar in different excitations. Scale bar accounts for pictures A to D.*

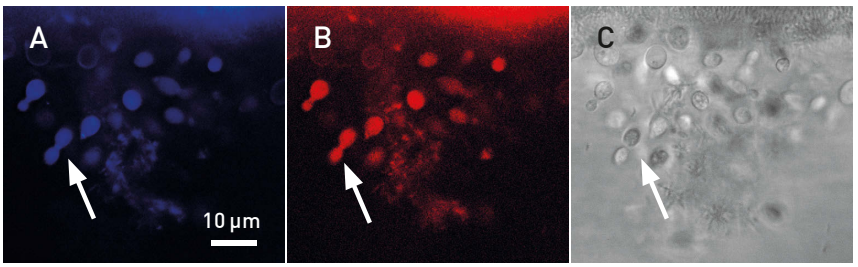


Figure 57: *Calcofluor-White staining of* Rhodotorula mucilaginosa *growing with* Triticum aestivum *on 100 µM nickel agar in different excitations. Examples for budding yeast cells are marked with arrows. Scale bar accounts for pictures A to C.*

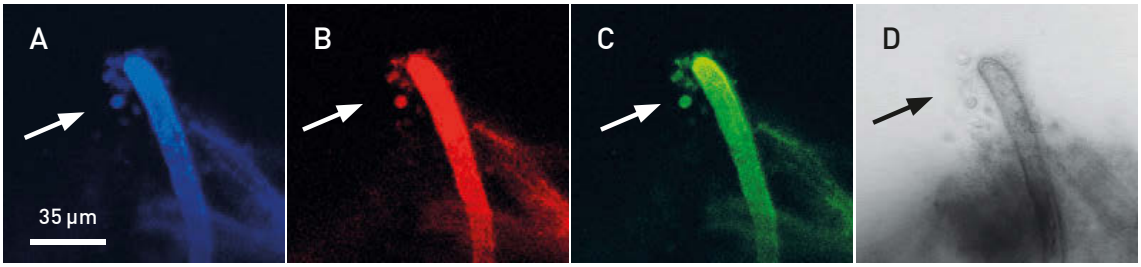


Figure 58: *Calcofluor-White staining of* Triticum aestivum *growing with* Rhodotorula mucilaginosa *on 100 µM nickel agar in different excitations. The arrow marks a root tip and yeast cells surrounded by deposits in the Bright Field. Scale bar accounts for pictures A to D. Arrows mark yeast cells on root hair*

YEAST AND PLANT INTERACTION: STAINING WITH SUDAN RED IV

Sudan IV was used to detect protein-bound lipids. The goal was to depict interaction and possible penetration of yeast into the plant cells.

BRIGHT FIELD MICROSCOPY: Sudan IV immersion time estimation

Figure 59 A to C shows stained *Diaporthe columnaris* and roots of *Triticum aestivum*. The yeast was grown on agar with 1 mM nickel, cut out and put into moist chambers with 1 mM nickel solution. A seedling of *Triticum aestivum* was placed on top the agar piece, and germinated for 6 days in the lab. Samples were stained by immersing and heating in Sudan IV solution starting from 1 minute. Samples were observed under the microscope.

Triticum aestivum showed well stained protoplasts (Figure 59, A and B). In cells of the calyptra, stained lipids became visible surrounding the nucleus and the vacuole.

Diaporthe columnaris was stained well if examined by itself without interaction with wheat. Hyphae showed internally stained lipids (Figure 59, C).

Sporobolomyces sp. was stained by cutting a piece of growing medium including the culture and heating it in the staining solution for 5 up to 15 minutes. Figure 60 A shows the results after 5 minutes immersion time, which gave a slightly uneven staining. After 15 minutes (Figure 60, B) yeasts were stained more evenly. The staining solution contained fibre-like structures, possibly remains from filtering the solution during preparation with filter-paper (arrow-marked).

BRIGHT FIELD MICROSCOPY: Sudan IV and *Diaporthe columnaris*

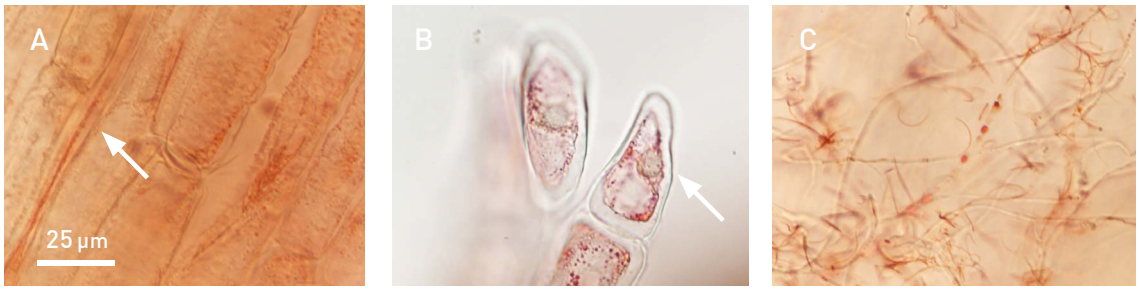


Figure 59: *Optical microscopy images of Sudan IV stainings of* Diaporthe columnaris *and* Triticum aestivum *grown with 1 mM nickel solution for 6 days. All examples were allowed to immerse and heat in Sudan IV for 1 minute. Picture A shows* Diaporthe columnaris *and* Triticum aestivum. *The arrow marks a longitudinally grown hyphae. Picture B shows stained cells of the calyptra. The arrow marks a nucleus. Picture C shows stained* Diaporthe columnaris. *Scale bar accounts for pictures A, B, and C. Scale bar accounts for pictures A to C*

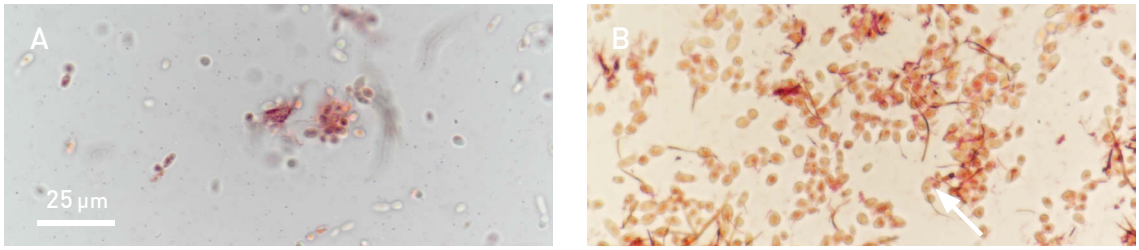


Figure 60: *Optical microscopy images of Sudan IV staining of* Sporobolomyces sp., *pieces of agar with the colonies were cut out and heated for 5 min (A) up to 15 min (B) in the staining solution. The arrow marks particles stained together with the yeast, possibly originating from the staining solution itself. Scale bar accounts for both pictures, A and B.*

Seedlings of *Triticum aestivum* were grown in quadratic petri dishes with 12 cm diameter with 0.6 % agar and 100 µM of nickel under sterile conditions. After roots had reached lengths of approximately 1 cm, *Diaporthe columnaris* was applied. After one week both, roots and fungi, were cut out with the surrounding agar and stained with Sudan IV to examine the interaction.

Figure 61 A shows both root and fungi after 1 min of heating in the staining solution. Both root exudates and/or bacteria surrounding the roots got stained, hindering the exact differentiation between roots and hyphae. Picture B shows the stained fungi by itself after 10 min of heating in Sudan IV solution, resulting in stained internal lipids visible inside the hyphae.

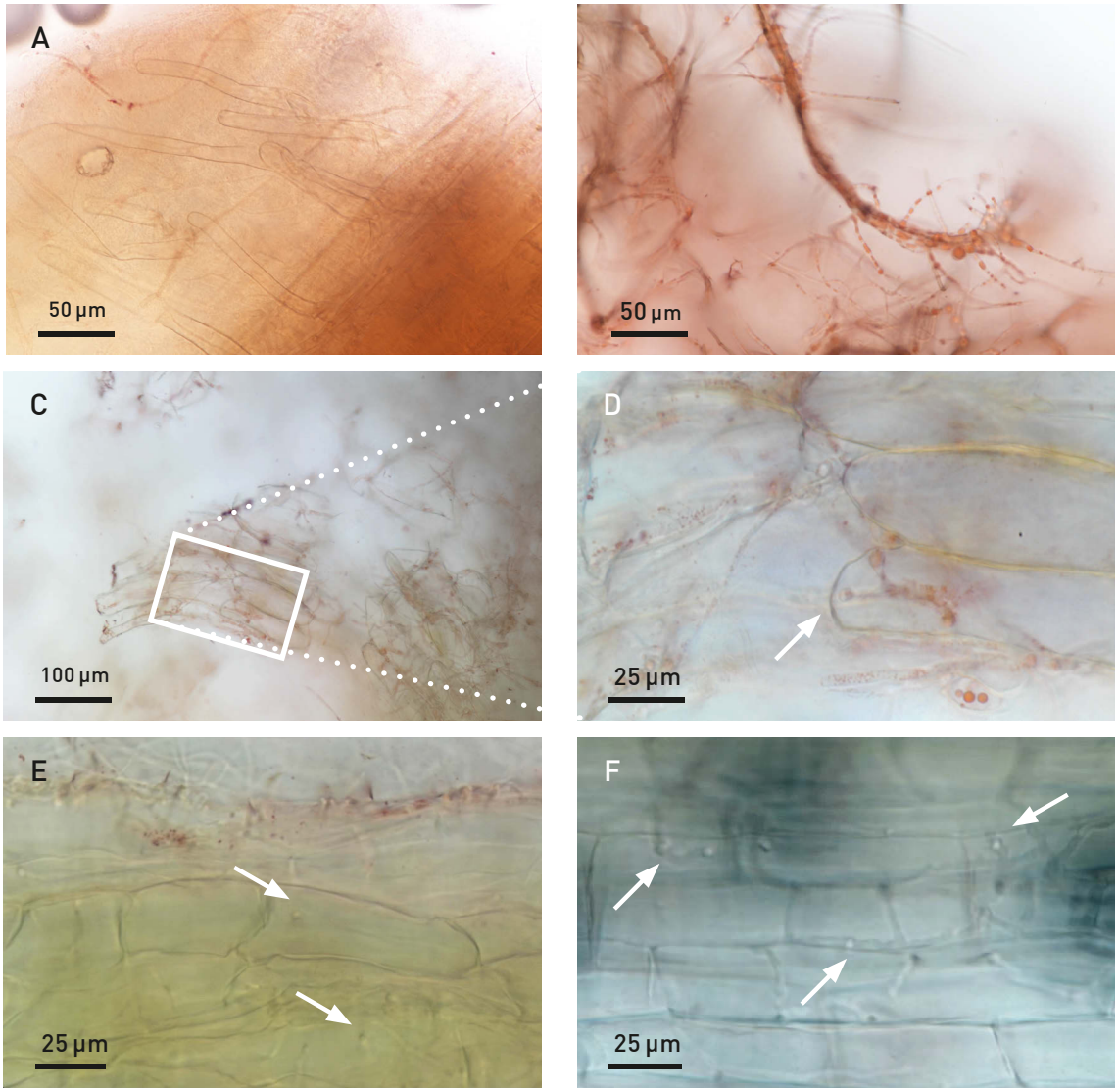


Figure 61: *Optical microscopy images of Sudan IV stainings of* Diaporthe columnaris, *GFP transformed, grown with* Triticum aestivum *on 100 µM nickel agar. Pictures A) shows* Diaporthe columnaris *and* Triticum aestivum *after cutting out pieces of agar and heating for 1 min in the dye. Stained exudates hinder exact differentiation between roots and hyphae. Picture B) shows* Diaporthe columnaris, *immersion time 10 minutes. Pictures C) and D) show* Diaporthe columnaris *after cutting out pieces of agar with contaminated roots and heating for 10 min in the dye. The rectangular in C) is highlighting the surrounding of picture D). The arrow marks possible callose spots in epidermal root cell walls (D to F)a.*

Figure 61 C to F show the interaction of both, wheat root and fungi, after staining by heating in Sudan IV solution for 10 minutes. The arrows mark areas where papillae were detected. Plant cells react to possible invaders with the formation of callose plugs, papillae, to prevent penetration (Flors, 2005). The examined samples showed these structures, although not always stained, the fungi might have penetrated into the root cortex.

BRIGHT FIELD MICROSCOPY, Sudan IV and *Rhodotorula mucilaginosa*

Seedlings of *Triticum aestivum* were grown in petri dishes with 0.6 % agar and 100 µM of nickel under sterile conditions. After germination of the seeds, with roots having reached lengths of approximately 1 cm, *Rhodotorula mucilaginosa* was applied to the seedlings' roots. For analysis, roots were cut out including the surrounding agar, and stained with Sudan IV to examine the interaction.

Roots were densely covered with exudates, bacteria and yeast, making it difficult to identify endophytic yeasts (Figure 62, A). *Rhodotorula mucilaginosa* was not detected inside the root cortex, the cells were preferably found aligning on the root surface using possible slots (Figure 62, B to F).

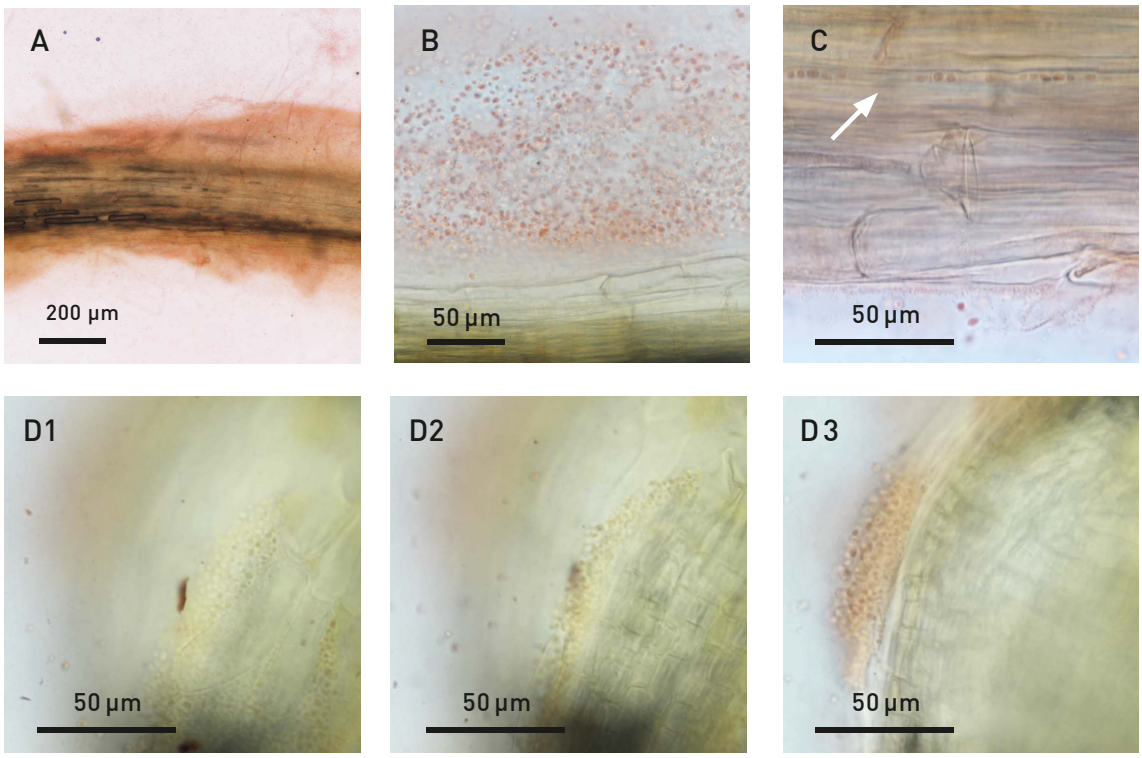


Figure 62: *Optical microscopy images of Sudan IV staining of* Rhodotorula mucilaginosa, *grown with* Triticum aestivum *on 100 µM agar. Pictures A) shows an overview of* Rhodotorula mucilaginosa *and* Triticum. *Picture B) shows* Rhodotorula mucilaginosa, *growing on the roots. Picture C) shows single cells of* Rhodotorula mucilaginosa, *aligned on the root surface, as marked by the arrow. Pictures D1) to D3) show* Rhodotorula mucilaginosa *by focusing along the root surface. Yeast seems to inhabit just the root surface.*

BRIGHT FIELD MICROSCOPY, Sudan IV and *Sporobolomyces ruberrimus.*

Seedlings of *Triticum aestivum* were grown in petri dishes with 0.6 % agar and 100 µM of nickel in a sterile environment. After the roots germinated reaching lengths of approximately 1 cm, *Sporobolomyces ruberrimus* was applied to the seedlings' roots and allowed to grow under a sterile environment. Roots were cut out with the surrounding agar and stained with Sudan IV to examine the interaction.

Especially roots growing in nickel contaminated agar produced lots of exudates, regardless if growing with or without fungal partner. Figure 63, A shows a control without yeast. Root exudates and deposits enveloping the roots got stained too, resulting in blurry effects, which complicated differentiating the fungus inhabiting the root. Additionally, roots growing inside the agar with *Sporobolomyces ruberrimus* were rare and did not show much colonization in general, as plants avoided growing into the nickel contaminated agar. (Figure 63; B, C).

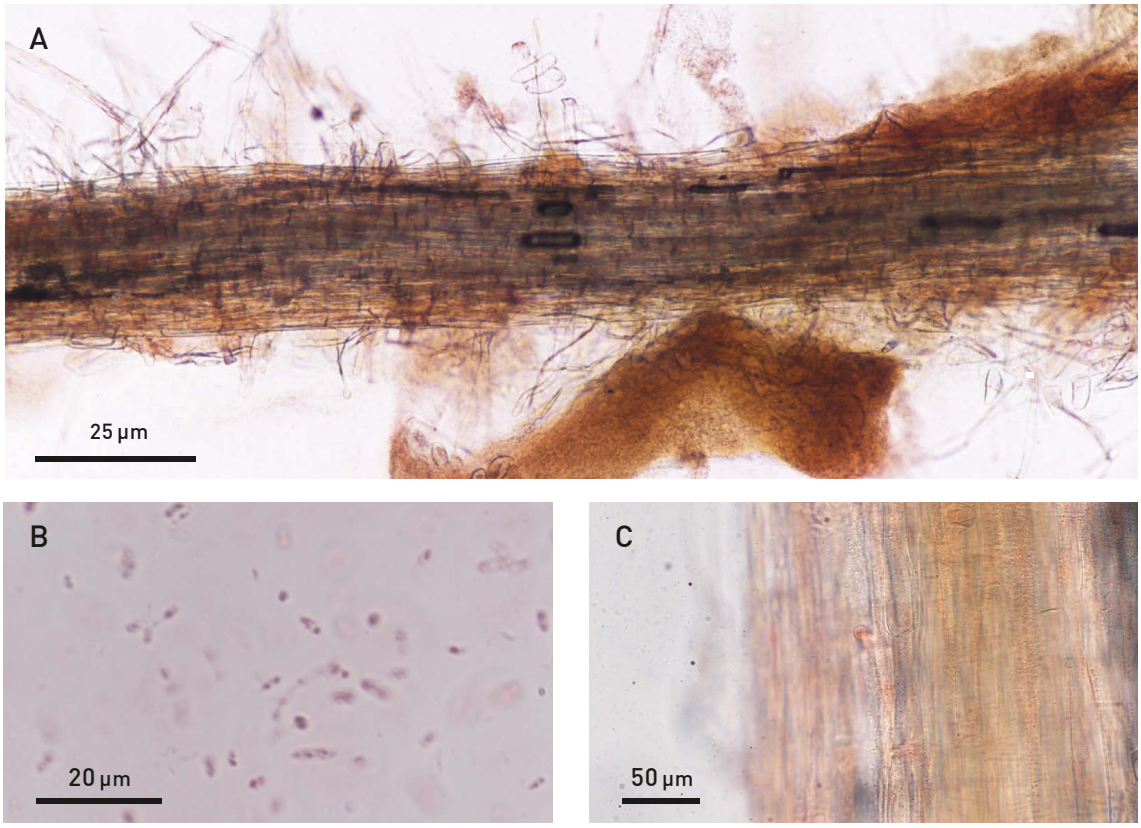


Figure 63: *Optical microscopy images of Sudan IV stainings of* Sporobolomyces ruberrimus *grown with* Triticum aestivum *on 100 µM agar. Picture A) was taken from a control plate and did not contain* Sporobolomyces ruberrimus, *enormous deposits are visible. Pictures B) shows* Sporobolomyces ruberrimus *extracted from a root growing without contact to agar. Picture C) shows* Sporobolomyces ruberrimus *on* Triticum aestivum *extracted from a root growing in agar, unfortunately little interaction was happening.*

CLSM MICROSCOPY, Sudan IV and *Rhodotorula mucilaginosa*

This CLSM picture series (Figure 64, A to F) shows the epidermis of a root of *Triticum aestivum* which has been growing with *Rhodotorula mucilaginosa* on agar with 100 µM nickel. Yeasts seemed to colonize the outside of the epidermis, without penetration into the root. Yeast cells were not detected inside cells of *Triticum aestivum*. *Rhodotorula mucilaginosa* seemed to inhabit interstitial space only on the rough root surface.

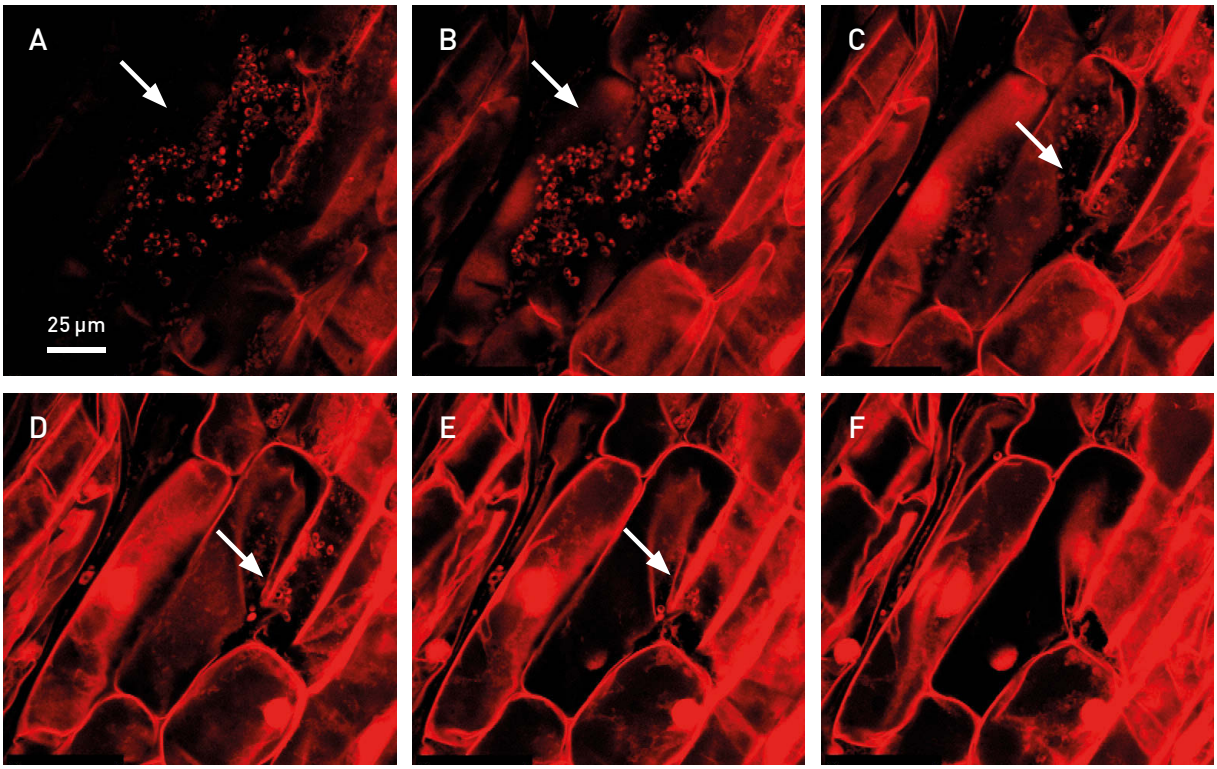


Figure 64: *Series of CLSM pictures "scanning" through a root of* Triticum aestivum *growing with* Rhodotorula mucilaginosa *on 100 µM nickel agar. Samples were stained with Sudan IV. Pictures show yeast cells growing on the outside of the epidermal cells, preferably in the interstices. Penetration into the host cells is not detectable. Arrows mark* Rhodotorula mucilaginosa.

YEAST AND PLANT INTERACTION: STAINING WITH ANILINE BLUE

BRIGHT FIELD MICROSCOPY, Aniline blue and *Diaporthe columnaris*

Seedlings of *Triticum aestivum* were grown with *Diaporthe columnaris* in petri dishes with 0.6 % agar and 100 µM of nickel in a sterile environment. Roots were cut out with the surrounding agar and stained with 0.01 % or 0.05 % Aniline blue after resting for four hours in 10 M or after being heated for 15 to 30 minutes in 1 M KOH, to examine the interaction of yeast and root. Samples were analysed in bright field and under UV-excitation.

Aniline blue and KOH staining resulted in damaged root material that was well destained on one hand, but ended up being very soft on the other. Root hairs were stained as well, as were phloem-, xylem-, and root cap cells in the control. The fungus was stained well in the bright field, UV-excitation showed partly fluorescent hyphae. The fungus had entered into epidermal cells and had grown into the intercellular space. (Figure 65, A to E)

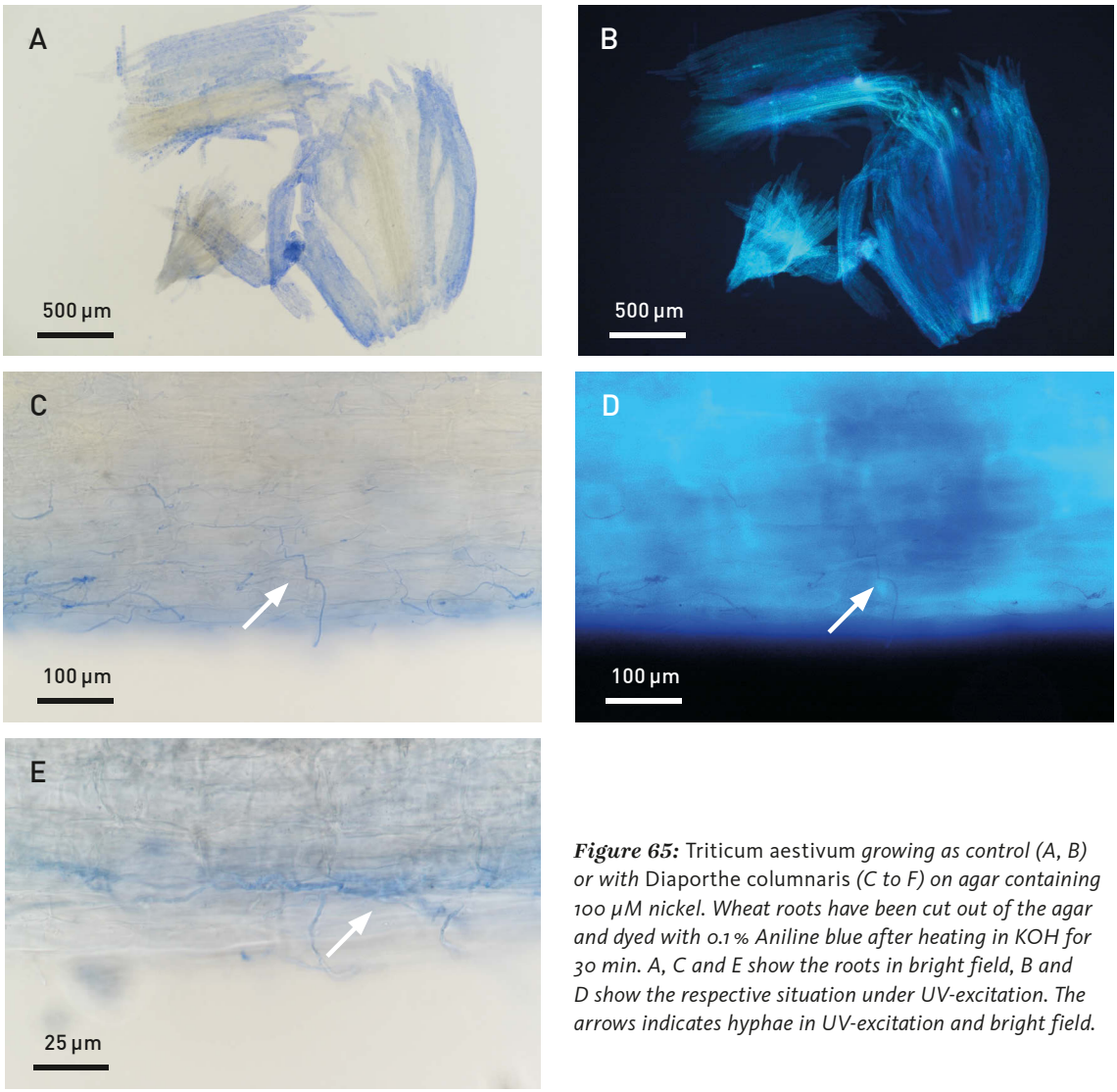


Figure 65: Triticum aestivum *growing as control (A, B) or with* Diaporthe columnaris *(C to F) on agar containing 100 µM nickel. Wheat roots have been cut out of the agar and dyed with 0.1 % Aniline blue after heating in KOH for 30 min. A, C and E show the roots in bright field, B and D show the respective situation under UV-excitation. The arrows indicates hyphae in UV-excitation and bright field.*

The dye entered best into the plant tissue next to the cutting spot, or in plant material that had been deformed by the KOH treatment, therefore hyphae were best stained in these areas. Hyphae showed partly bright fluorescence under UV-excitation. Callose plugs were visible under UV-Excitation. (Figure 66, A to E)

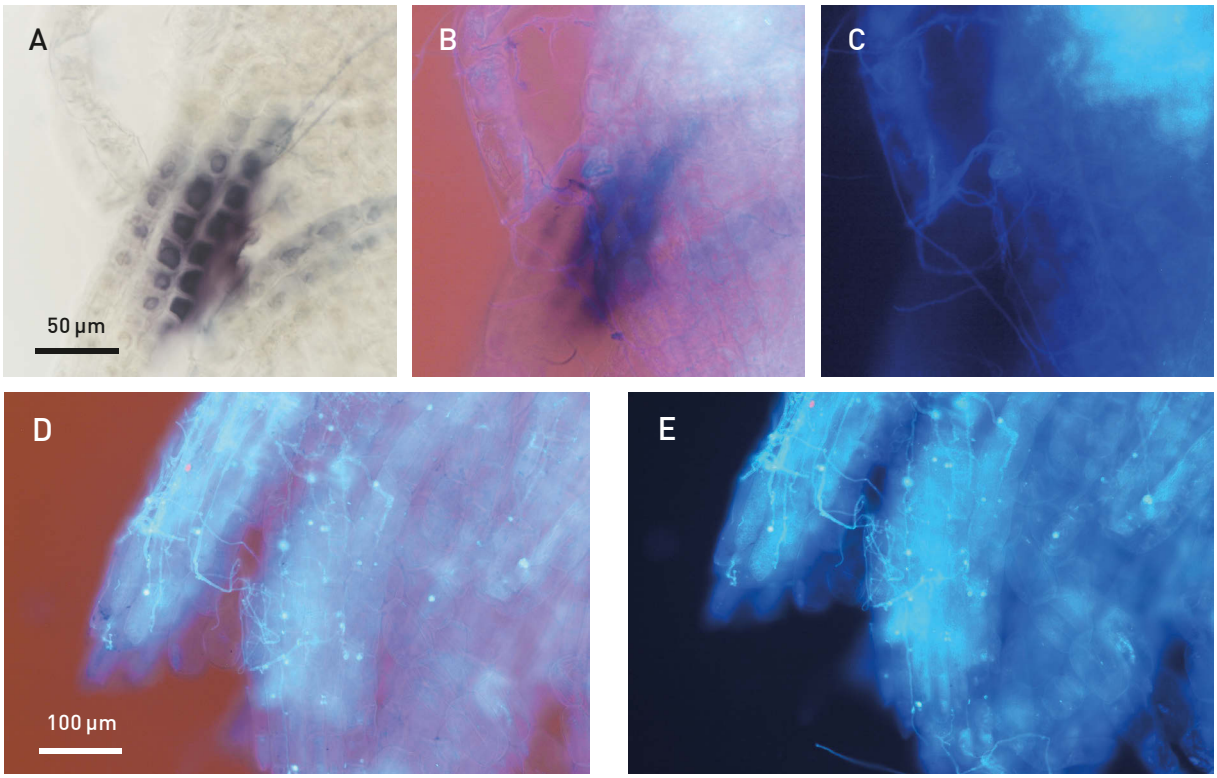


Figure 66: *Aniline blue staining of* Triticum aestivum *growing with* Diaporthe columnaris *on 100 µM nickel agar. The arrow marks yeast cells. Pictures A to C show the staining in bright field, as an overlay with UV-excitation, and as UV-excitation only. Pictures D and E show an overlay of bright field and UV-excitation, and UV-excitation only.*

BRIGHT FIELD MICROSCOPY & CLSM, Aniline blue with *Rhodotorula mucilaginosa & Sporobolomyces ruberrimus*

Seedlings of *Triticum aestivum* were grown with *Rhodotorula mucilaginosa* or *Sporobolomyces ruberrimus* in petri dishes with 0.6 % agar and 100 µM of nickel in a sterile environment. Roots were cut out with the surrounding agar and stained with 0.01 % or 0.05 % Aniline blue after resting for four hours in 10 M or after being heated for 15 to 30 minutes in 1 M KOH, to examine the interaction. Samples were analysed in bright field and under UV-excitation.

In the control group that grew with out fungi, staining with Aniline blue and KOH resulted in slightly stained exudates and deposits on the root surface (Figure 67, A, B). These appeared as minimal background stainings on the surface, if examined under UV-excitation and blue "halos" on the root surface observed under bright field.

Rhodotorula mucilaginosa was hard to be detected in the bright field sample. Few yeast cells were visible, but these had assimilated the dye. Under UV-excitation, epidermal cells of *Rhodotorula mucilaginosa* showed high fluorescence, outshining yeast cells. Again exudates and deposits got stained in bright field. No penetration to the epidermal cells was detected. (Figure 67 A1 to B3)

Sporobolomyces ruberrimus got stained by this method as marked in figure 68 (A to D). Budding yeast cells were visible. Due to fluorescent wheat root cells, it was difficult to identify the yeast cells on the root surface. It was not possible to see if cells entered the roots. Yeast cells did not show any fluorescence when examined using CLSM. (Figure 69)

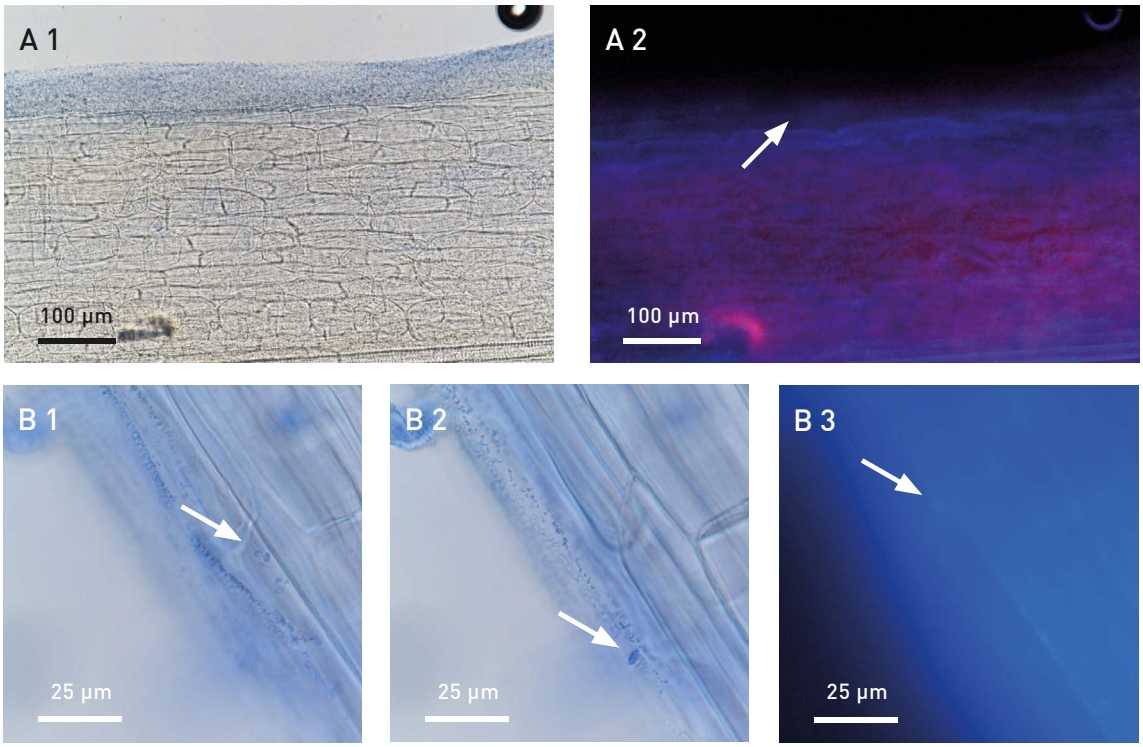


Figure 67: *Aniline blue staining of* Triticum aestivum *on 100 µM nickel agar growing with A) the control group, exudates and deposits are marked by an arrow; or growing with B)* Rhodotorula mucilaginosa. *The arrow marks yeast cells. Bright field pictures are shown together (A1, B1, B2) with the respective situation under UV-excitation, where fluorescence of wheat reduced visibility of yeast cells (A2, B3).*

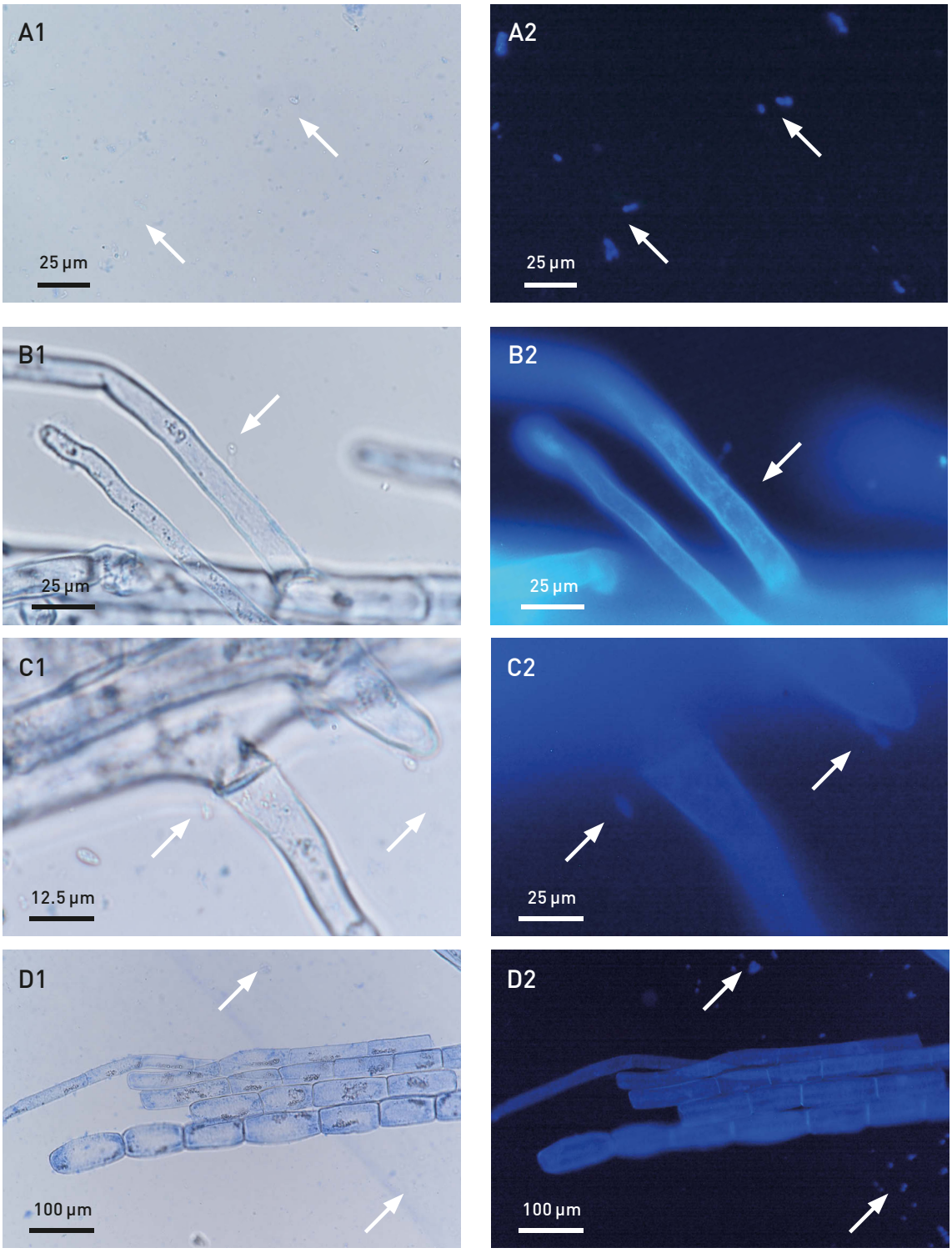


Figure 68: *Aniline blue staining of* Triticum aestivum *growing on 100 µM nickel agar growing with* Sporobolomyces ruberrimus. *The arrow marks yeast cells. Bright field pictures are shown, together with the corresponding UV-excitations.*

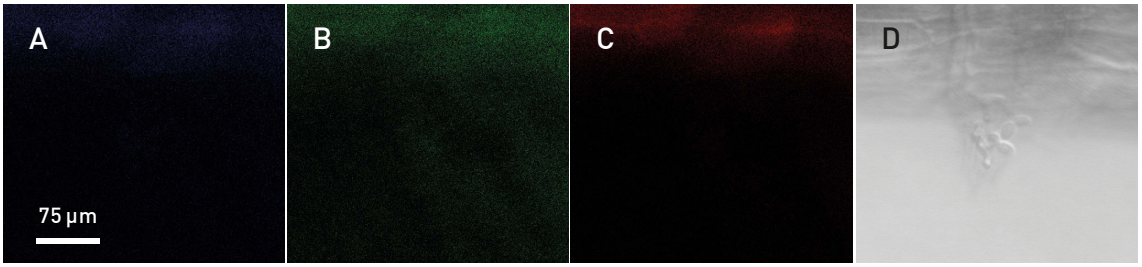


Figure 69: *CLSM pictures of an Aniline blue staining of* Triticum aestivum *on 100 µM nickel agar growing with* Sporobolomyces ruberrimus.

GROWTH DYNAMICS OF *DIAPORTHE COLUMNARIS*

Microchambers were built to examine the interaction of *Triticum aestivum* and *Diaporthe columnaris* over time. Both partners had been positioned close to each other, but without direct contact. They were enclosed in pure agar, or agar with PDA to provide the fungus with nutrients and promote growth. Pictures were taken every minute or in intervals of five minutes.

Results show that no directional growth of the fungus could be observed, hyphae expanded slowly in all directions, regardless if roots were nearby or not. Roots did not seem to have attracting functions. (Figure 70, 71 and 72).

Additionally, we observed that the area where the fungus was cut out of the petri dish it was growing in, as well as the surface, showed decelerated development. New hyphae did not grow in the described area. The fungus developed preferably from uncut areas.

Figure 70: *Series of light field pictures taken over a time span of five hours. A cut off root of* Triticum aestivum *and* Rhodotorula mucilaginosa *were observed on PDA agar using an Olympus BX41 microscope. Picture A) shows the situation after 1 hour, B) after 3 hours and C) after 5 hours.*

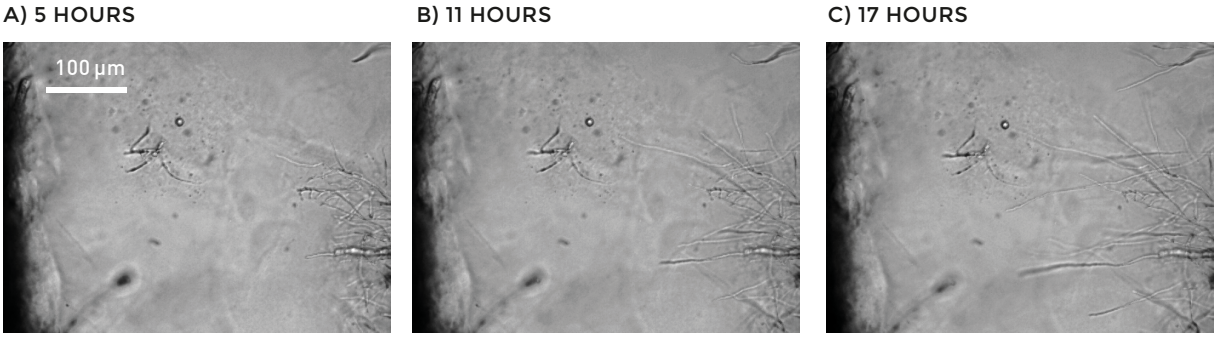


Figure 71: *Series of light field pictures taken over a time span of 17 hours. A cut-off root of* Triticum aestivum *(left part of the pictures) and* Diaporthe columnaris *(right part of the pictures) were observed on agar without nutrients using an Nikon EFD microscope. Picture A) shows the situation after 5 hours, B) after 11 hours and C) after 17 hours.*

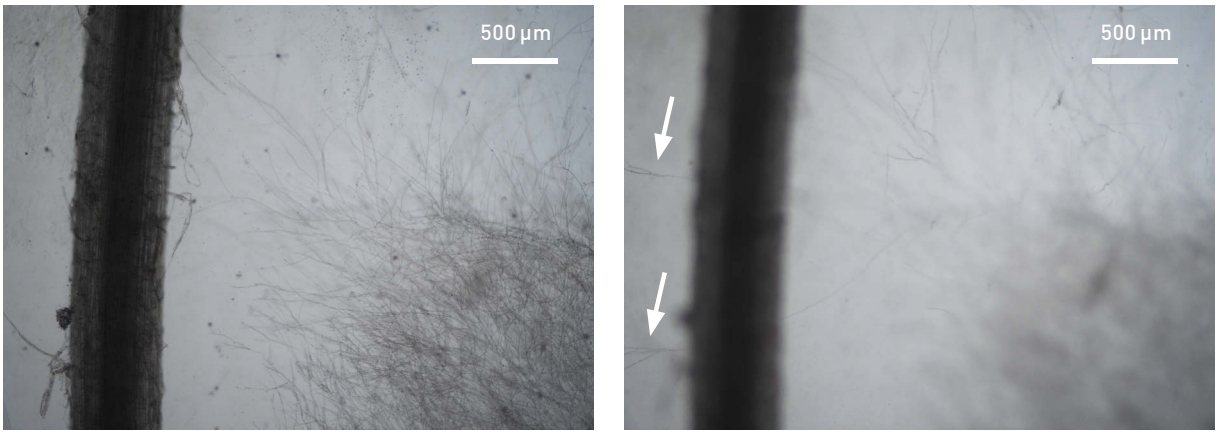


Figure 72: *Light field pictures taken after roots of* Triticum aestivum *were examined with* Rhodotorula mucilaginosa *on PDA agar using an Olympus BX41 microscope. Growth of the fungus was happening in all directions. Arrows mark hyphae growing beneath the root.*

INTERACTIONS OF PLANTS, HEAVY METALS AND YEAST

The goal of the experiments was to get insights into the interactions of *Triticum aestivum* with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* under the influence of nickel and zinc. The soil cultures should provide more realistic conditions compared to *in vitro* cultures. The experiment consisted of two parts:

In the first setup ("Soil experiment 1") *Triticum aestivum* was inoculated after three and again after eight days of germination with *Rhodotorula mucilaginosa* in a control group, and in nickel or zinc contaminated soil (0.1 mM, 1 mM, respectively).

In the second setup ("Soil experiment 2") we inoculated *Triticum aestivum* with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* immediately when sewing the seeds, and again after three days of germination in control, nickel and zinc contaminated soil. Heavy metal concentrations were increased, due to the minor differences resulting from part 1. We were using 1 mM, 5 mM and 10 mM for nickel treated plants, and 1 mM, 10 mM and 50 mM for zinc treated plants.

SOIL EXPERIMENT 1: Germination rate

Triticum aestivum was growing in soil contaminated with no heavy metal exposure, nickel or zinc (1 mM, 0.1 mM, respectively) and with *Rhodotorula mucilaginosa*. Five seeds were planted per pot, treatments were executed in triplicates. Germination rates are given in %. Rates were estimated after harvesting the plants after one month growing time.

Effects of heavy metals (compare to figure 73)

In the control group 87 % of the seeds germinated.

Nickel had a positive effect in both concentrations used. *Triticum aestivum* growing in nickel showed higher germination rates compared to the control with both concentrations. 100 % of the plants in 0.1 mM nickel germinated, they had higher germination rates than plants 1 mM nickel, with a germination rate of 93 %.

Zinc had a positive effect in the lower concentration, and a negative effect in the higher concentration used. Plants growing in zinc showed higher germination rates compared to the control group, 100 % germinated, if grown in 0.1 mM. 1 mM zinc group reduced the germination rate to 73 %.

Effects of *Rhodotorula mucilaginosa* (compare to figure 74)

Rhodotorula mucilaginosa had no effect on germination of *Triticum aestivum* in the control group. They had identical germination rates of 87 %. Plants treated with killed yeast had a slightly reduced germination rate of 80 %.

Rhodotorula mucilaginosa had a positive effect on germination of *Triticum aestivum* in the higher concentration of nickel. 0.1 mM nickel treated plants growing with *Rhodotorula mucilaginosa* had slightly reduced germination rates of 87 % compared to the nickel control group where 100 % of the seed germinated. In 1 mM nickel, *Rhodotorula mucilaginosa* plants showed increased germination rates with 100 % germination compared to the 1 mM nickel control with 93 %.

Rhodotorula mucilaginosa had a negative effect on zinc treated plants growing in 0.1 mM of zinc, germination rates went down to 93 % compared to 100 % of the 0.1 mM zinc control. *Rhodotorula mucilaginosa* treated plants in 1 mM zinc had higher germination rates compared to the 1 mM zinc control group. 93 % germinated with *Rhodotorula mucilaginosa* compared to 73 % without yeast in 1 mM zinc.

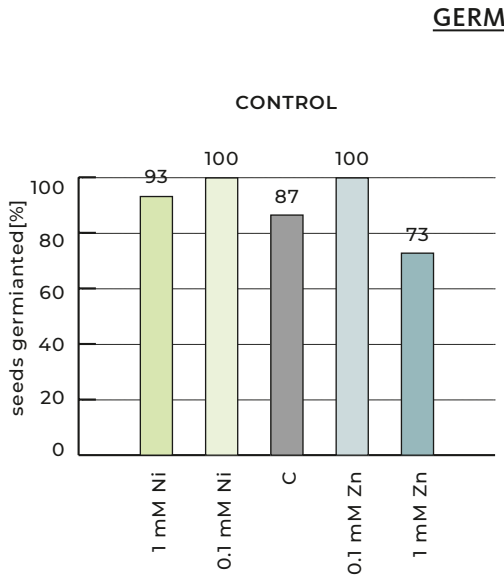


Figure 73: *Germination rates of* Triticum aestivum *grown for 29 days as control, nickel (0.1 mM or 1 mM), and with zinc (0.1 mM or 1 mM) in pots.*

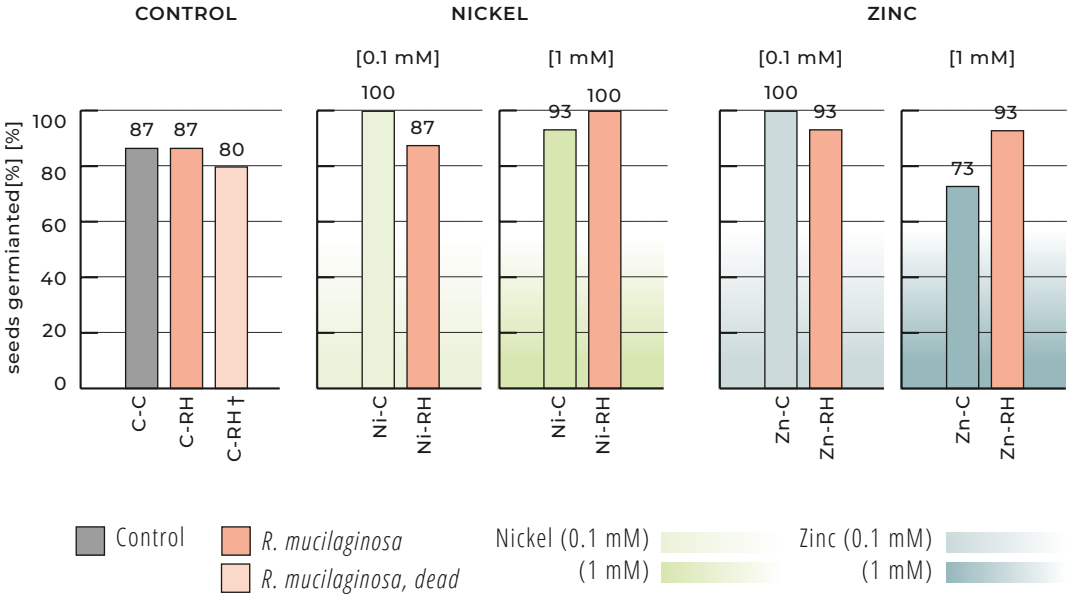


Figure 74: Germination rates *of* Triticum aestivum *grown with/without* Rhodotorula mucilaginosa *and killed* Rhodotorula mucilaginosa *for 29 days as control, nickel (0.1 mM or 1 mM), and with zinc (0.1 mM or 1 mM) in pots.*

SOIL EXPERIMENT 1: Photosynthetic activity

Triticum aestivum was growing in soil contaminated with no heavy metal exposure, nickel or zinc (1 mM, 0.1 mM, respectively) and with *Rhodotorula mucilaginosa* or without yeast. Photosynthetic activity of *Triticum aestivum* was estimated by measuring chlorophyll fluorescence after 29 days of growth, using a Handy PEA+ meter. Measuring spots were darkened with clips for 20 minutes prior to measurements. Fv/fm ratios ranged between a minimum of 0.762 (1 mM nickel with *Rhodotorula mucilaginosa)* and a maximum of 0.83 (control with dead *Rhodotorula mucilaginosa)*.

Effects of heavy metals in general

At first glance the chosen heavy metal concentrations did not have much impact on the plants in general. Fv/fm ratios reflect this impression, regardless if yeast had been added. One-way ANOVA was conducted to assess the effects of the heavy metals on the plants growing with different fungal partners. No statistically significant differences between the different heavy metal concentrations were found in the control group which had been growing without yeast. (Compare to figure 75)

Effects of yeasts in general

The addition of yeast did not make obvious differences on plant development in the respective heavy metal treatments. Again, fv/fm ratios reflect this impression. One-way ANOVA or two-sample t-tests were conducted to assess the effects of the yeasts on the plants growing in different heavy metal concentrations. (Compare to figure 76)

Effects of yeasts on the control group

No statistically significant differences were found in the control group growing with no heavy metal contamination between control and yeast treated plants. (Compare to figure 76, A)

Effects of yeasts on nickel treated plants (0.1 mM, 1 mM)

No significant statistical differences were found in the plants treated with 0.1 mM or 1 mM nickel between control and yeast treated plants. (Compare to figure 76, B and C)

Effects of yeasts on zinc treated plants (0.1 mM, 1 mM)

No significant statistical differences were found in the plants treated with 0.1 mM or 1 mM zinc between control and yeast treated plants. (Compare to figure 76, D and E)

FV/FM RATIOS, EFFECT OF HEAVY METALS

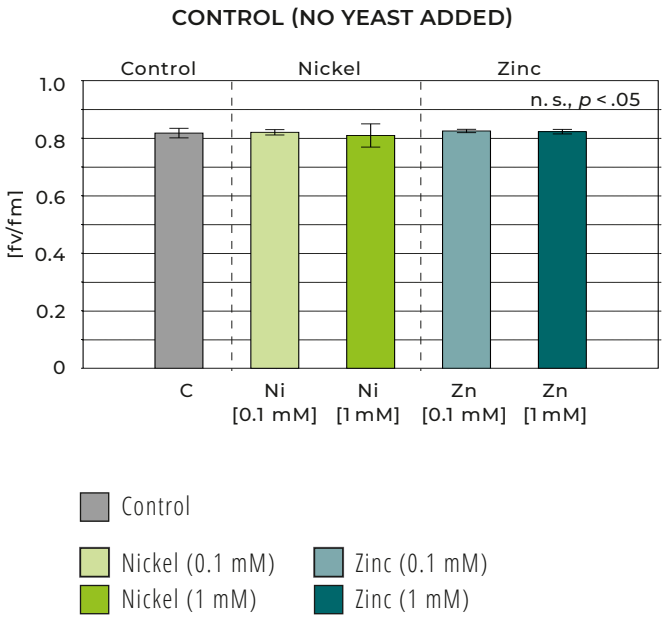


Figure 75: *Influence of heavy metal treatment on the mean fv/fm ratio of* Triticum aestivum *grown for 29 days without yeast.*

Plants were grown without heavy metals as control ("C"), with nickel ("Ni"; 0.1 mM or 1 mM), or with zinc ("Zn"; 0.1 mM or 1 mM).

Vertical lines *on each bar show ±SD, 95 % confidence interval* (n = 3). *Bars for control are grey, nickel is petrol, zinc is green. The dotted lines separate control, nickel and zinc treatments. "n.s." indicates that differences were non significant after one-way ANOVA (p < .05).*

FV/FM RATIOS, EFFECT OF YEASTS

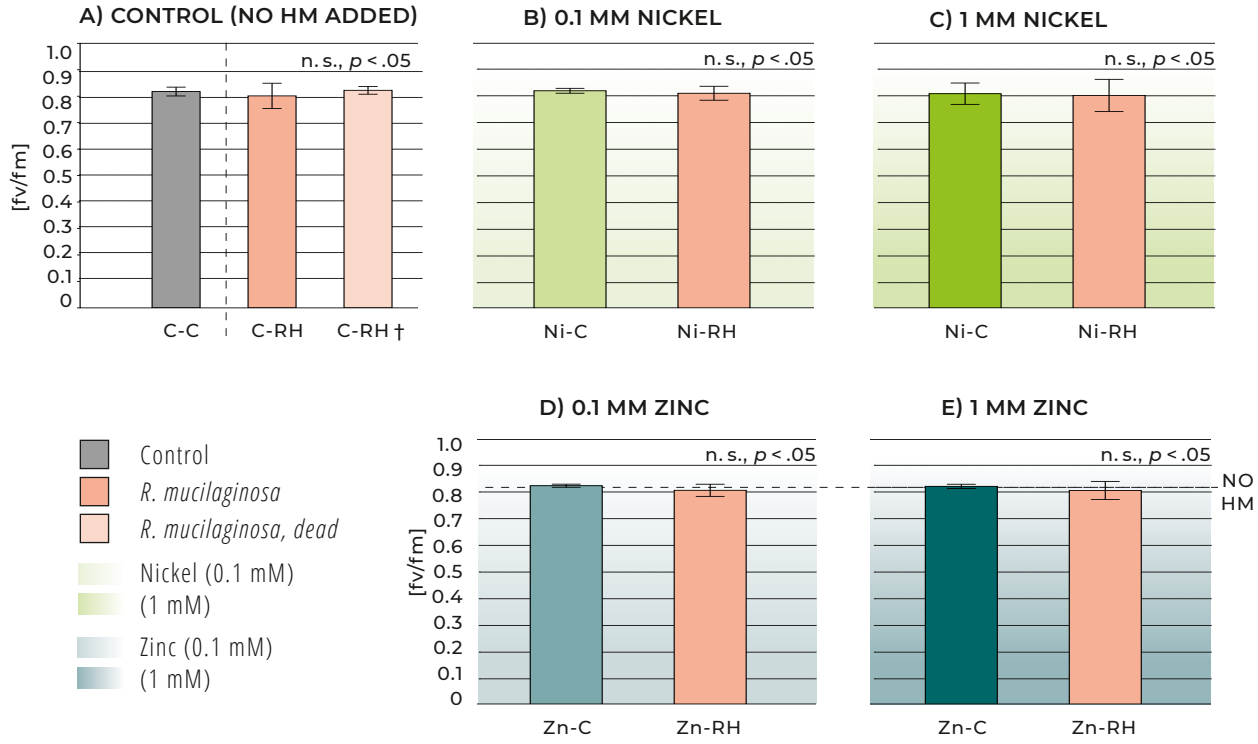


Figure 76:
Mean fv/fm ratios of Triticum aestivum *grown for 29 days without heavy metals (A), with 0.1 mM nickel (B), with 1 mM nickel (C), with 0.1 mM zinc (D) or with 1 mM zinc (E). Additionally, plants were grown either as control ("C", grey bar), with* Rhodotorula mucilaginosa *("RH", purple bar), or with dead* Rhodotorula mucilaginosa *("RH †", lavender bar).* Vertical lines *on each bar show ±SD, 95 % confidence interval* (n = 3). *The horizontal dotted line shows the mean fv/fm ratio without heavy metal treatment ("NO HM" in B, C, D and E). "n.s." indicates that differences were non significant in A) after one-way ANOVA* (p < .05) *and in B) to E) after two sample t-tests.*

SOIL EXPERIMENT 1: Plant growth

After one month of growing time *Triticum aestivum* inoculated with *Rhodotorula mucilaginosa* was harvested. Length of roots and shoots and the number of leaves were estimated, embryonic leaves were not counted. The results are presented in two parts:

1) the effect of heavy metals on the plants
2) the effect of yeasts on the plants

Representing pictures of the results are summarized in figure 78.

1. The effects of heavy metals on the plants

The effects of heavy metals on the control group were analysed using one-way ANOVA and Tukey's pairwise test. (Figure 77: A.1, B.1, C.1)

Effects of heavy metals on root length (Figure 77, A.1)
In the control group, root lengths differed statistically significantly for the different concentrations of heavy metals, $F(4, 67) = 5.419$, $p = .0004096$, omega² = .161. Tukey post-hoc analysis revealed a significant difference ($p < .001$) between 0.1 mM nickel and all other treatments. Roots were significantly longer in 0.1 mM nickel compared to the other treatments. Root lengths ranged from 4 cm in 1 mM zinc to 22.5 cm in 0.1 mM nickel.

Effects of heavy metals on shoot length (Figure 77, B.1)
No statistically significant differences in shoot lengths were found in the control. Minimum and maximum values were found in 1 mM zinc with 15.5 cm length and 41 cm were found in 0.1 mM zinc.

Effects of heavy metals on number of leaves (Figure 77, C.1)
No statistically significant differences were found in the control group in regarding number of leaves The highest number of six leaves in the control group in were found in plants growing with 1 mM nickel, minimum count of leaves was estimated as 3 leaves in 1 mM zinc.

2. The effects of yeasts on the plants

Differences between control groups and treatment with *Rhodotorula mucilaginosa* in the respective heavy metals concentrations were estimated conducting one-way ANOVA and Tukey's post hoc or t-tests. (Figure 77: A.2, B.2, C.2)

Effects of yeasts on root length (Figure 77, A.2)
A statistically significant differences in root length between the control groups and the treatment with *Rhodotorula mucilaginosa* was found in the treatment with 0.1 mM nickel ($p = .009$) and in the treatment with 1 mM zinc ($p = .005$) after conducting t-tests.

Effects of yeasts on shoot length (Figure 77, B.2)
No statistically significant differences in shoot lengths, between the control groups and the treatment with *Rhodotorula mucilaginosa* were found.

Effects of yeasts on number of leaves (Figure 77, C.2)
No statistically significant differences in number of leaves, between the control groups and the treatment with *Rhodotorula mucilaginosa* were found.

PERFORMANCE OF *TRITICUM AESTIVUM*

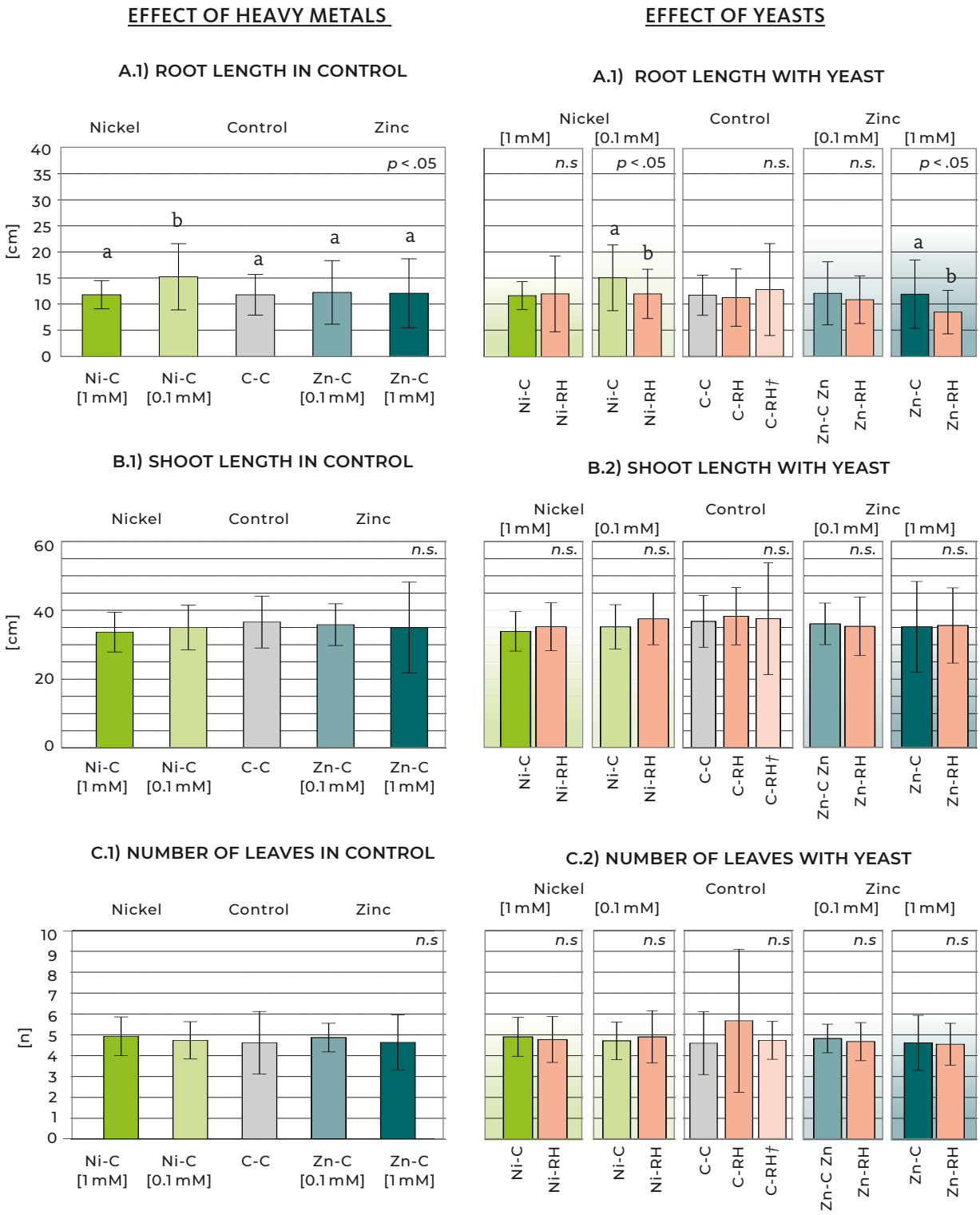


Figure 77: *Performance of* Triticum aestivum *under nickel and zinc stress (0.1 mM, 1 mM, respectively) and the influence of* Rhodotorula mucilaginosa. *A.1 shows root lengths under heavy metal stress; A.2 compares the control group of each heavy metal treatment to treatment with yeast. B.1 and B.2 show the influence of metals and yeast on shoot lengths, C.1 and C.2 on the number of leaves.* Vertical lines *on each bar show ±SD, 95 % confidence interval. "n. s." indicates that developmental differences between the different amounts of applied heavy metals were non significant after ANOVA or t-tests (*p < .05*). Different small letters indicate significant differences between the different heavy metal concentrations applied (Tukey's pairwise in A.1, B.1 and C.1; t-tests in A.2, B.2 and C.2).*

OVERVIEW OF POT EXPERIMENT 1

Figure 78: *Comparison of* Triticum aestivum *grown for 29 days without heavy metals (control), with 1 mM or 0.1 mM nickel or zinc, respectively. Plants were inoculated with* Rhodotorula mucilaginosa *three days after germination. Scale bars equal 10 cm.*

SOIL EXPERIMENT 2: Germination rate

Triticum aestivum was inoculated with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* immediately when sewing the seeds, and again after 3 days of germination. Seeds grew in nickel or zinc contaminated soil. Heavy metal concentrations were increased compared to part 1 of the experiment using 1 mM, 5 mM and 10 mM of nickel, and 1 mM, 10 mM and 50 mM of zinc to increase the heavy metal effect on the plants' development and cause a broader range in plant feedback, compared to part 1.

Effects of heavy metals

In the control group 87 % of the seeds germinated. (compare to Figure 79 A)

Triticum aestivum growing in nickel showed higher germination growing in 1 mM compared to the control group, 93 % of seeds germinated. Higher metal concentrations reduced germination rates down to 60 % in 5 mM and to 53 % in 10 mM. (compare to Figure 79 A and B)

Zinc-treated plants showed germination rates of 87 % in 1 mM and 50 mM, identical to the control group, and increased germination rates of 100 % in 10 mM. (compare to Figure 79 A and C)

Effects of *Rhodotorula mucilaginosa.*

In the control group *Rhodotorula mucilaginosa* treated plants showed higher germination rates (93 %) compared to the control containing no additional heavy metals (87 %). Dead *Rhodotorula mucilaginosa* treated plants showed reduced germination rates (80 %). (compare to Figure 80 A)

In 1 mM nickel, *Rhodotorula mucilaginosa* treated plants had lower germination rates of 87 % compared to the control with 93 % (Figure 81 A and B, "1 mM"). In 5 mM nickel, germination rates where increased from 60 % in the control to 80 % when treated with *Rhodotorula mucilaginosa* (Figure 81 B, "5 mM"). 10 mM of nickel plants treated with yeast had increased germination rates of 67 % compared to the control with 53 % (Figure 80 B, "10 mM").

In 1 mM zinc plants 93 % germinated in the *Rhodotorula mucilaginosa* group, compared to 87 % in the control (Figure 81 A and C, "1 mM"). In 10 mM zinc plants 93 % germinated in the *Rhodotorula mucilaginosa* group, compared to 100 % in the control (Figure 81 C, "10 mM"). 50 mM zinc showed decreased germination rates of 73 % with *Rhodotorula mucilaginosa*, compared to 87 % in the control (Figure 80 C, "50 mM").

Effects of *Sporobolomyces sp.* (Figure 81)

Seedlings of the control treatment showed a lower germination rate of 73 % compared to plants growing without yeast (87%). Higher germination rates of 93 % were observed when the plants grew with dead *Sporobolomyces sp.* (compare to Figure 80 A)

In 1 mM nickel, germination rates were reduced from 93 % in the control to 80 % when treated with *Sporobolomyces sp* (Figure 81 A and B, "1 mM"). In 5 mM nickel, germination rates were increased from 60 % in the control to 87% when treated with *Sporobolomyces sp*. (Figure 81 A and B, "5 mM"). 10 mM of nickel plants had increased germination rates of 73 % compared to the control with 53 % (Figure 80 B, "50 mM").

In 1 mM zinc all seedlings germinated in the *Sporobolomyces sp*. group, compared to 87 % in the control, (Figure 81 A and C, "1 mM"). In 10 mM zinc all seedlings germinated in the *Sporobolomyces sp*. group, compared to 100 % in the control (Figure 81 C, "10 mM"). 50 mM zinc yielded germination rates of 93% with *Sporobolomyces sp*. group, compared to 87 % in the control (Figure 80 C, "50 mM").

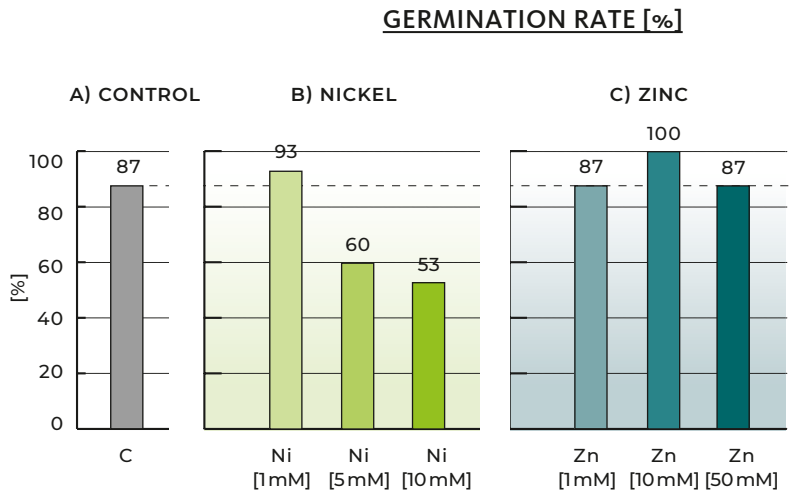


Figure 79: *Germination rates (%) of* Triticum aestivum *grown for 19 days as control ("A"; dotted line), with nickel ("B"; 1 mM, 5 mM or 10 mM), and zinc ("C"; 1 mM, 10 mM or 50 mM) in pots.*

Control (no HM)
Nickel
Zinc

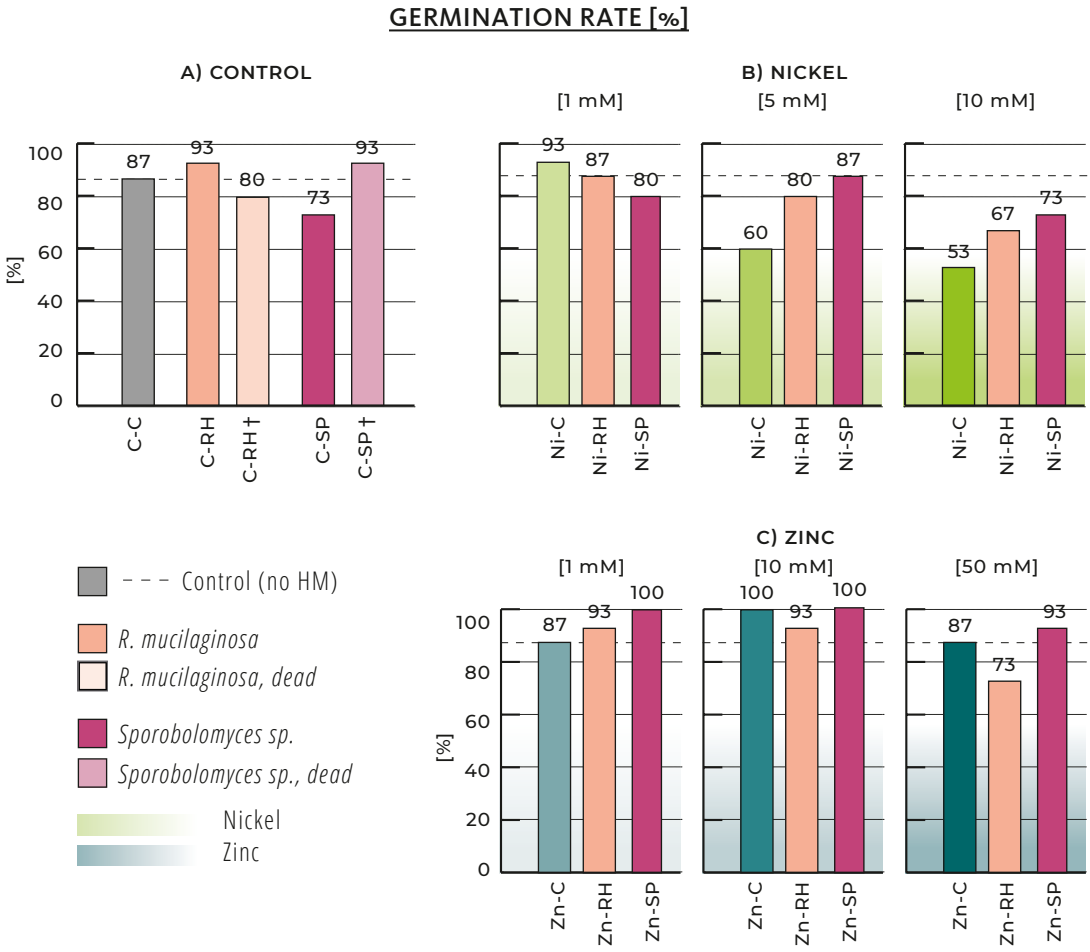


Figure 80: *Germination rates (%) of* Triticum aestivum *grown with/without* Rhodotorula mucilaginosa *(dead and alive) and* Sporobolomyces sp. *(dead and alive) for 19 days as control ("A"; dotted line), with nickel ("B"; 1 mM, 5 mM or 10 mM), or with zinc ("C"; 1 mM, 10 mM or 50 mM) in pots.*

SOIL EXPERIMENT 2: Photosynthetic activity

Photosynthetic activity of *Triticum aestivum* was estimated by measuring chlorophyll fluorescence after 19 days of growth, using a Handy PEA+ meter. Measuring spots were darkened with clips for 20 minutes prior to measurements. Fv/fm ratios ranged between a minimum of 0.686 (50 mM zinc with *Rhodotorula mucilaginosa)* and a maximum of 0.836 (*Sporobolomyces sp.* with 10 mM nickel).

Effects of heavy metals in general

Increasing heavy metal influence led to reduced plant development. Treatment with 10 mM nickel left us with plants which did not offer enough leaf material, hence they had to be excluded from this part of the analysis. One-way ANOVA was conducted to asses the effects of the heavy metals on the plants growing with yeasts.

No statistically significant differences between the mean fv/fm ratios were found in the control group which had been growing without yeast-partner. Plants growing with higher metal concentrations seem to have broader standard deviations. (Figure 81)

FV/FM RATIOS, EFFECTS OF HEAVY METALS IN GENERAL

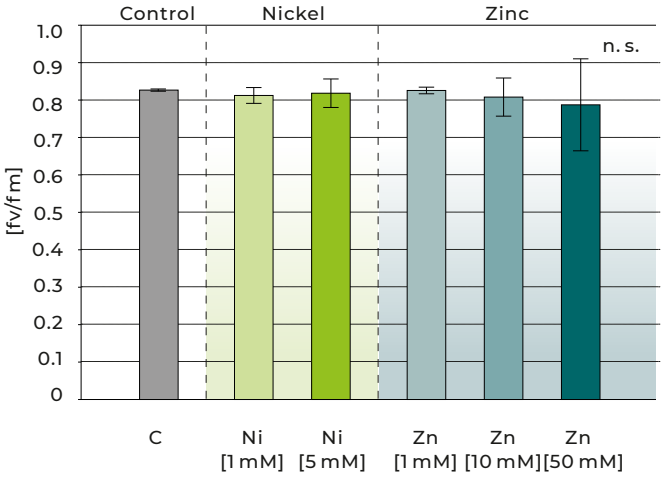


Figure 81: *Mean fv/fm ratio of* Triticum aestivum *grown for 19 days as control, with nickel (1 mM or 5 mM), and with zinc (1 mM, 10 mM or 50 mM).* Vertical lines *on each bar show ±SD, 95 % confidence interval* (n = 3). *"n.s." indicates that differences between the heavy metal treatments were non significant.*

Color code: The control is light grey, bars for nickel are green, bars for zinc are blue.

Effects of yeasts

Effects of *Rhodotorula mucilaginosa* and *Sporobolomyces sp.* in the control group

The control group had significant difference in fv/fm ratios, F (4, 10) = 8.135, p = .003, omega² = .656. Tukey post-hoc analysis revealed a significant difference between dead *Sporobolomyces sp.* and the control (p = .01), the *Rhodotorula mucilaginosa* treatment, (p = .018), the dead *Rhodotorula mucilaginosa* treatment, (p = .04977), and the treatment with *Sporobolomyces sp.* (p = .003). (Figure 82, A)

Effects of *Rhodotorula mucilaginosa* and *Sporobolomyces sp.* in nickel treatments

No significant differences were found regarding the mean fv/fm ratios in 1 mM (Figure 83, B) and 5 mM nickel treatments (Figure 82, B and C).

Effects of *Rhodotorula mucilaginosa* and *Sporobolomyces sp.* in zinc treatments

No significant differences were found regarding the mean fv/fm ratios in 1 mM, 10 mM and 50 mM zinc treatments. (Figure 83, A, B, C)

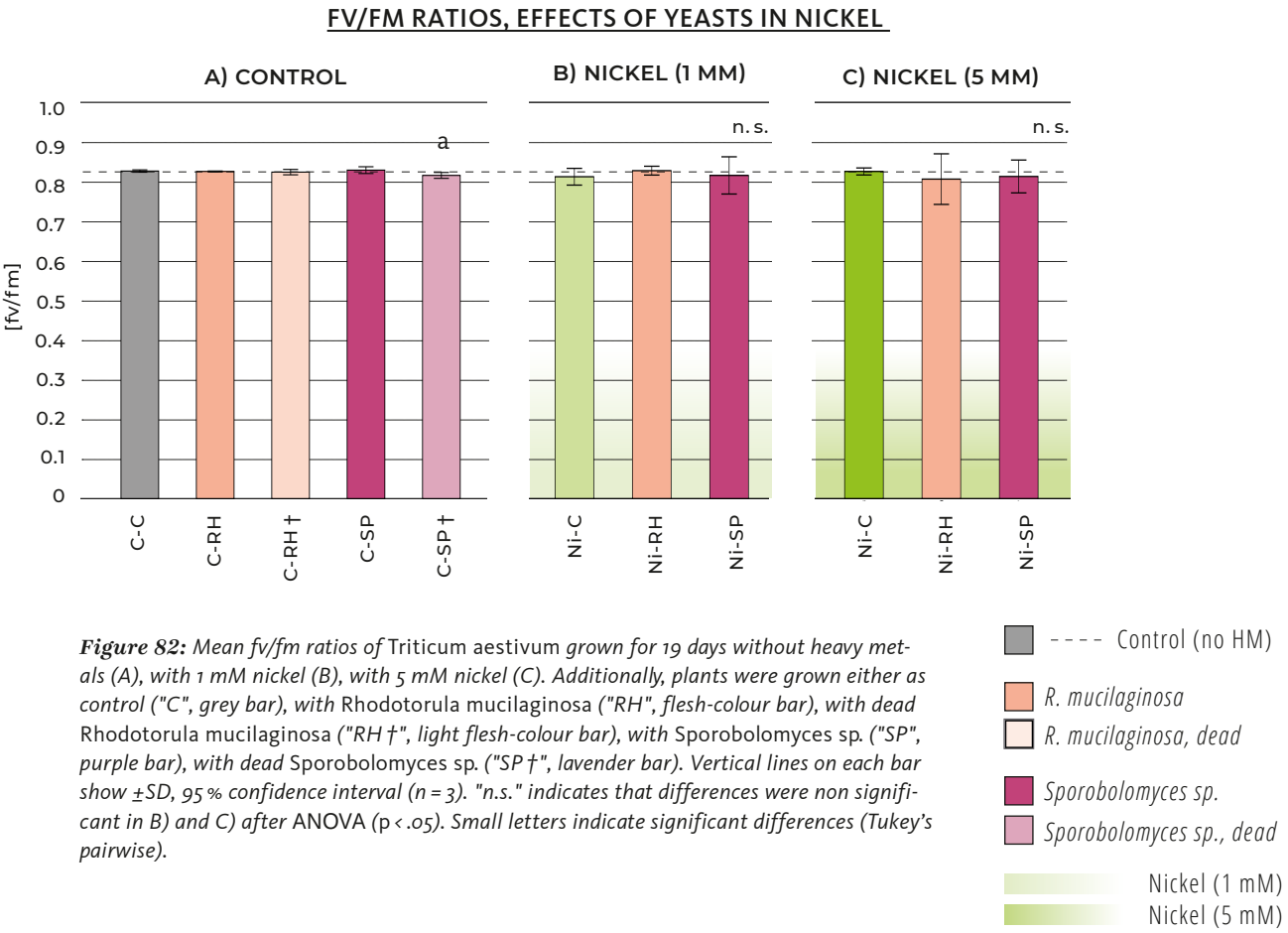


Figure 82: *Mean fv/fm ratios of* Triticum aestivum *grown for 19 days without heavy metals (A), with 1 mM nickel (B), with 5 mM nickel (C). Additionally, plants were grown either as control ("C", grey bar), with* Rhodotorula mucilaginosa *("RH", flesh-colour bar), with dead* Rhodotorula mucilaginosa *("RH †", light flesh-colour bar), with* Sporobolomyces sp. *("SP", purple bar), with dead* Sporobolomyces sp. *("SP †", lavender bar). Vertical lines on each bar show ±SD, 95 % confidence interval (n = 3). "n.s." indicates that differences were non significant in B) and C) after* ANOVA ($p < .05$). *Small letters indicate significant differences (Tukey's pairwise).*

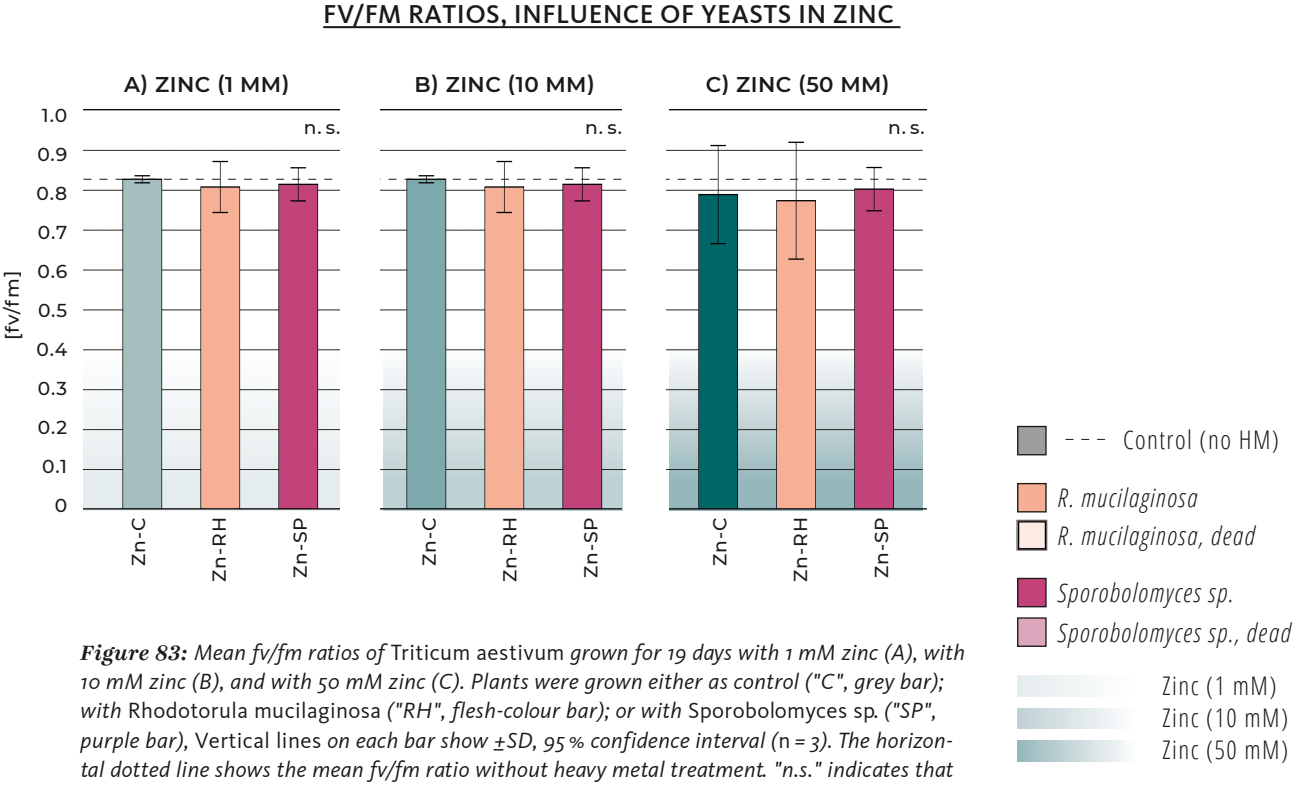


Figure 83: *Mean fv/fm ratios of* Triticum aestivum *grown for 19 days with 1 mM zinc (A), with 10 mM zinc (B), and with 50 mM zinc (C). Plants were grown either as control ("C", grey bar); with* Rhodotorula mucilaginosa *("RH", flesh-colour bar); or with* Sporobolomyces sp. *("SP", purple bar),* Vertical lines *on each bar show ±SD, 95 % confidence interval* (n = 3). *The horizontal dotted line shows the mean fv/fm ratio without heavy metal treatment. "n.s." indicates that differences were non significant after ANOVA* ($p < .05$).

SOIL EXPERIMENT 2: Cellular tolerances

To estimate the effect of the heavy metals and yeasts on a cellular level, a tolerance test using plasmolysis was performed. We chose to work with plants that had been growing either as control with no exposure to heavy metals, growing in soil contaminated with 10 mM zinc, or with 5 mM nickel contamination. Leaf surface cuts that rested for 48 h in concentration rows of nickel or zinc (1 M to no heavy metal) were checked for their cellular tolerance, hence their surviving in these heavy metal solutions. By adding 0.8 M mannitol, healthy cells adapt with plasmolysing to adjust to changes in osmotic conditions. A sample was counted "alive" if cells reacted with plasmolysis, showed healthy chloroplasts and deplasmolysed again. "Dead" samples did not show any signs of plasmolysis, and/or crushed, yellowish or brownish chloroplasts and/or no sign of deplasmolysis. (Compare to figure 84)

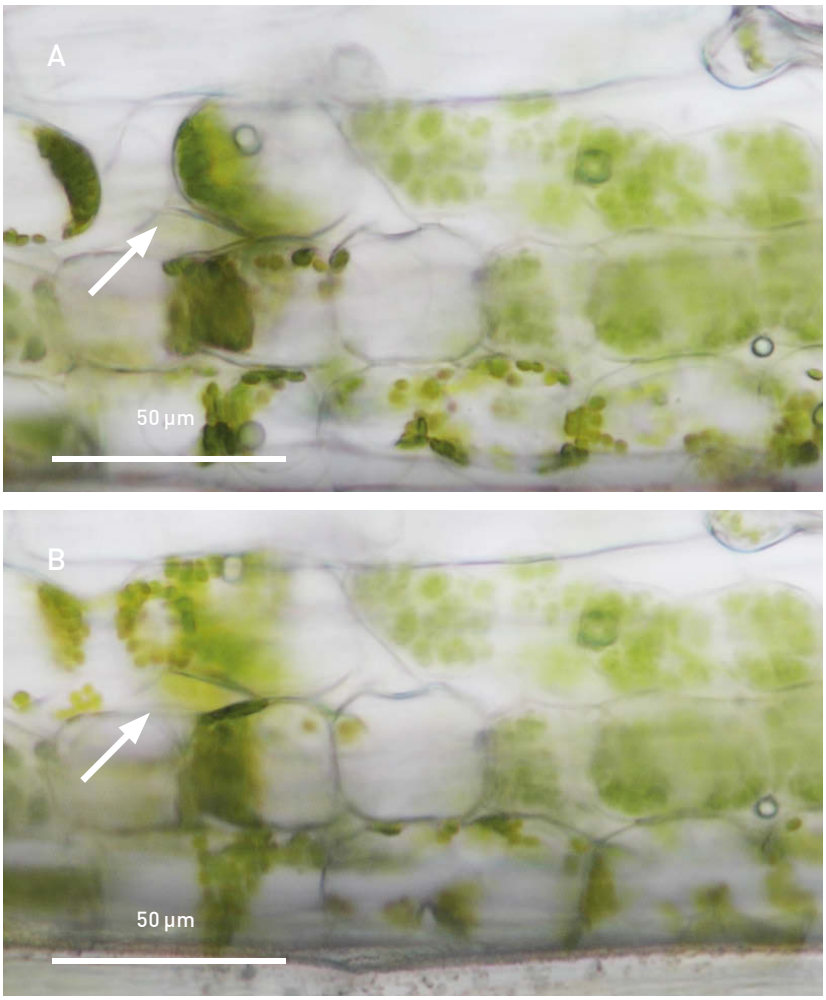


Figure 84: *Cellular tolerance, optical microscopy image of leaf surface cuts of* Triticum aestivum *grown in contaminated soil. Control group: the plant material immersed in water for 48 h and was examined after adding 0.8 M mannitol under the microscope. Picture A shows a sample grown with 10 mM zinc, after plasmolysis; picture B shows the same situation after deplasmolysis (marked by the arrows)*

Heavy metal impact on cellular tolerances

Effects of nickel solutions *versus* zinc solutions

Sections that had been resting in metal solutions for 48 hours were generally stronger affected by the nickel solutions compared to zinc solutions. Nickel solutions were up to two orders of magnitude more toxic compared to zinc solutions: Cells tended to die at lower concentrations of nickel solutions, whereas the same concentration of zinc solution was better tolerated, cells survived zinc solution concentrations of 1 mM up to 10 mM. (Figure 85, comparing the lines "in nickel solution" to "in zinc solution")

Effects between the treatments in nickel solutions

Nickel solutions were more toxic to plants that were growing with heavy metal contaminations compared to the control treatment, especially if plants were growing with zinc. (Figure 86, comparing the different treatments in the line "in nickel solution")

Effects between the treatments in zinc solutions

Zinc solutions were better tolerated by plants growing with heavy metal contaminated soil compared to the control pots, an inverse effect compared to the nickel solutions. (Figure 86, comparing the different treatments in the line "in zinc solution")

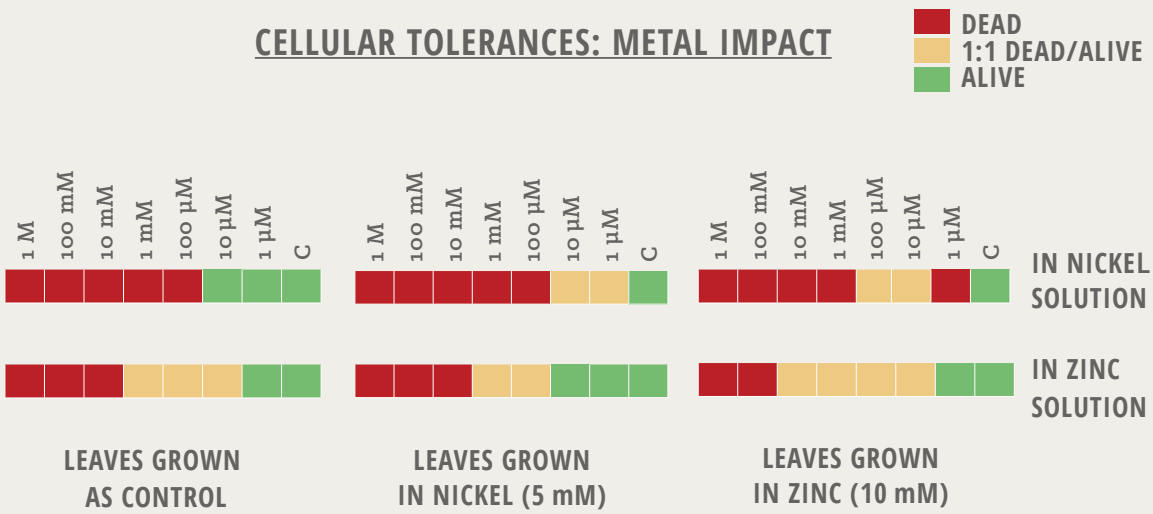
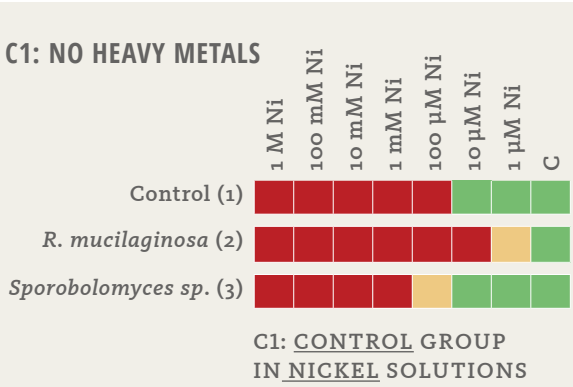


Figure 85: *Cellular tolerance, heavy metal impact:* Triticum aestivum *was grown either as control group with no heavy metal impact, with 5 mM nickel or 10 mM zinc. Leaf surface cuts were prepared and immersed in 1 M to 1 µM of nickel or zinc solutions for 48 hours, cell viability was estimated. Colour code: Red accounts for mostly dead cells, green accounts for mostly surviving cells, yellow was used if both appeared in equal amounts.*

Effect of yeasts on cellular tolerances

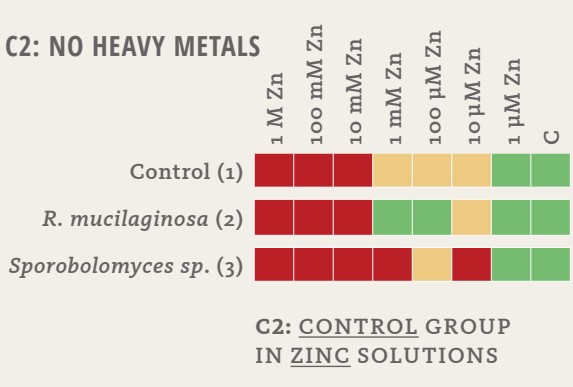
Figure 86 C1, C2, N1, N2, Z1, Z2: *Cellular tolerance, impact of yeasts:*
Triticum aestivum *was grown either as control group with no heavy metal impact, or with 5 mM nickel or 10 mM zinc. Additionally each of the 3 groups was either processed as control group, inoculated with* Rhodotorula mucilaginosa *or* Sporobolomyces sp. *Leaf surface cuts were prepared and immersed in 1 M to 1 µM of nickel or zinc solutions for 48 hours and cell viability was estimated. Colour code: Red accounts for mostly dead cells, green accounts for mostly surviving cells, yellow was used if both were found in equal amounts.*

DEAD
1:1 DEAD/ALIVE
ALIVE

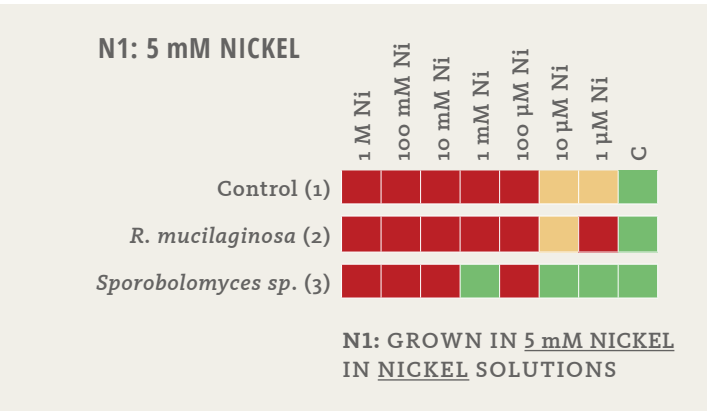


C1: The control group in nickel solutions:
In the control group, that had had no previous contact to elevated nickel or zinc levels, *Rhodotorula mucilaginosa* did not improve the cellular tolerance of the cell, the leaf-surface cuts of the control group resting in nickel solutions had even reduced tolerances if the plants were treated with *Rhodotorula mucilaginosa* (2). *Sporobolomyces sp.* (3) had a slightly positive effect.

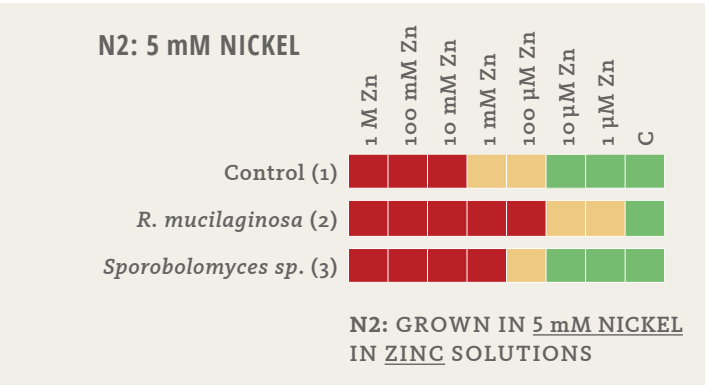
Cells in the control group (1) died when resting in 100 µM nickel solution. Cells from plants infected with *Rhodotorula mucilaginosa* (2) died at lower concentrations of 1 µM nickel solution. Cells from plants infected with *Sporobolomyces sp.* (3) performed better and survived at least partly up to 100 µM nickel contamination. (Compare to figure 86, C1)



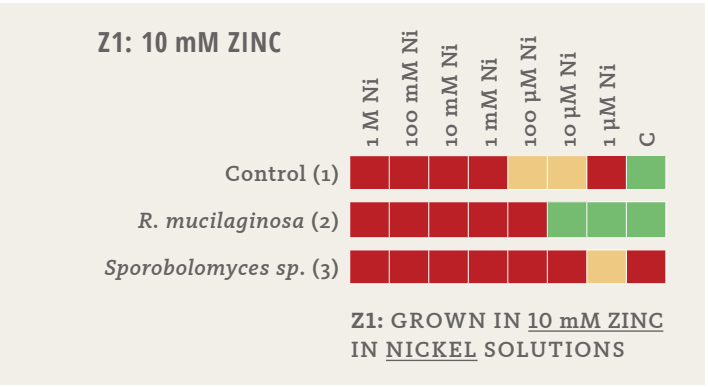
C2: The control group in zinc solutions:
Plants of the control group (1), without previous contact to heavy metal stress, were able to survive up to 1 mM of zinc. *Rhodotorula mucilaginosa* (2) had minor positive effects on cells in 1 mM of zinc solutions, but did not allow the cells to survive higher concentrations. *Sporobolomyces sp.* treated cells (3) survived up to 100 µM. *Sporobolomyces sp.* had negative effects on the surviving of the control group in zinc solutions compared to the control and the *Rhodotorula mucilaginosa* group. (Compare to figure 86, C2)



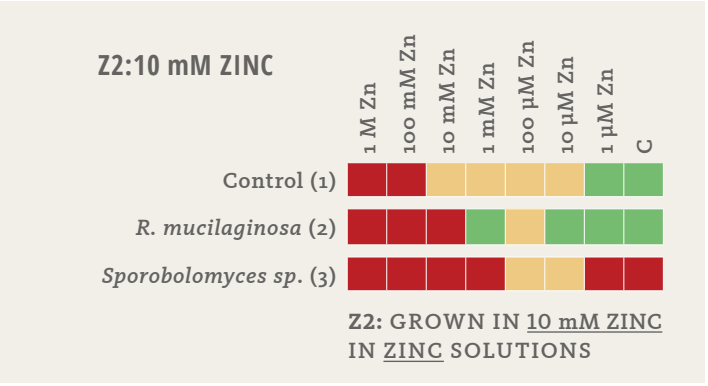
N1: The 5 mM nickel group in nickel solutions:
Plants growing in 5 mM nickel and resting in nickel solution had divers results: 100 µM nickel were lethal for the control group (1). *Rhodotorula mucilaginosa* treated cells (2) were heavily affected by most concentrations, besides the tap water and cells resting in 10 µM nickel solutions. *Sporobolomyces sp.* treated plants (3) performed best, surviving in 1 mM and up to 10 µM of nickel. (Compare to figure 86, N1)



N2: The 5 mM nickel group in zinc solutions:
The control group (1) performed best, surviving up to 1 mM zinc. *Rhodotorula mucilaginosa* treated cells (2) coped with concentrations of zinc up to 10 µM. *Sporobolomyces sp.* treated plants (3) survived up to 100 µM of zinc. (Compare to figure 86, N2)



Z1: The 10 mM zinc group in nickel solutions:
Cells from plants growing in zinc contaminated soil that were treated with nickel solutions survived concentrations up to 100 µM in the control (1) and 10 µM if treated with *Rhodotorula mucilaginosa* (2). Cells with prior contact to *Sporobolomyces sp. (3)* died in all nickel concentrations besides 1 µM. (Compare to figure 86, Z1)



Z2: The 10 mM zinc group in zinc solutions:
Cells from plants of the zinc contaminated soil that were treated with zinc solutions survived concentrations up to 10 mM in the control (1) and 1 mM if treated with *Rhodotorula mucilaginosa* (2). Treatment with *Sporobolomyces sp.* (3) was disadvantageous, cells only survived 100 µM and 10 µM of zinc, dying in all other tested solutions, even tap water. (Compare to figure 86, Z2)

Figure 86 C1, C2, N1, N2, Z1, Z2: *Cellular tolerance, impact of yeasts:* Triticum aestivum *was grown either as control group with no heavy metal impact, or with 5 mM nickel or 10 mM zinc. Additionally each of the 3 groups was either processed as control group, inoculated with* Rhodotorula mucilaginosa *or* Sporobolomyces sp. *Leaf surface cuts were prepared and immersed in 1 M to 1 µM of nickel or zinc solutions for 48 hours and cell viability was estimated. Colour code: Red accounts for mostly dead cells, green accounts for mostly surviving cells, yellow was used if both were found in equal amounts.*

SOIL EXPERIMENT 2: Plant growth

Triticum aestivum inoculated with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* was harvested after one month of growing in soils with different heavy metal concentrations. Concentrations were increased compared to part 1 to intensify the heavy metal effect, and to achieve more diverse results. Length of roots and shoots was estimated as well as the respective dry weight and the number of leaves. Embryonic leaves were not counted. Control groups growing without heavy metal influence were treated likewise. Representational pictures of the results are summarized in figure 93 to 95.

The results are presented in two parts: 1) the effect of heavy metals on the plants
2) the effect of yeasts on the plants

1. The effects of heavy metals on the plants

Effects of heavy metals on root length

In the control group, root lengths differed statistically significantly between the different concentrations of heavy metals, after one-way ANOVA was conducted. There were significant differences found among the heavy metal treatments, $F(6, 94) = 23.79$, $p = 1{,}541E{-}16$, omega^2: .59. To locate the differences, Tukey-Kramers post hoc test was conducted. Results are listed in table 8 and figure 87.

A beneficial effect could be estimated for 1 mM nickel compared to the control treatment. Plants growing with high concentrations of heavy metals had shorter roots, high concentrations of nickel affected the plants more negatively compared to zinc treatment.

Root lengths ranged from 0.1 cm in 50 mM zinc to 25.7 mm to 0.1 mM nickel, both treated with *Rhodotorula mucilaginosa*.

Table 8: *Tukey's pairwise comparison, effects of heavy metals on root length. Tukey's Q below the diagonal, p(same) above the diagonal. Significant comparisons are marked in bold.*

	Ni [10 mM]	**Ni [5 mM]**	**Ni [1 mM]**	**C**	**Zn [1 mM]**	**Zn [10 mM]**	**Zn [50 mM]**
Ni [10 mM]		**0.004958**	**0.0001218**	**0.0001218**	**0.0001218**	**0.000144**	0.9999
Ni [5 mM]	5.367		**0.0001811**	**0.003672**	0.09632	0.8018	**0.001832**
Ni [1 mM]	12.46	7.09		0.9186	0.2779	**0.008796**	**0.0001218**
C	10.86	5.498	1.592		0.9174	0.1757	**0.0001218**
Zn [1 mM]	9.268	3.901	3.189	1.597		0.8208	**0.0001218**
Zn 10 mM]	7.343	1.976	5.113	3.522	1.925		**0.0001275**
Zn [50 mM]	0.4286	5.796	12.89	11.29	9.696	7.772	

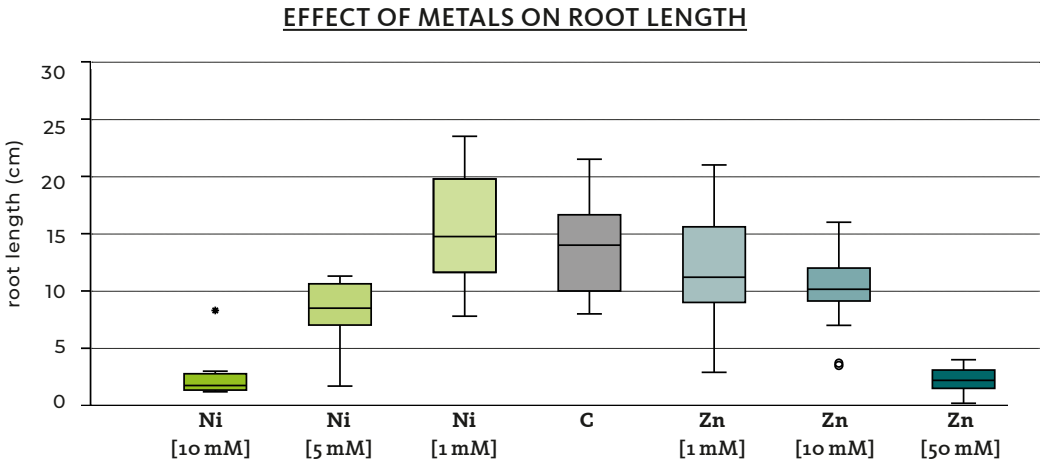


Figure 87: *Effect of heavy metals on root length.* Triticum aestivum *was grown for 29 days without heavy metals (control), with 1 mM, 5 mM or 10 mM nickel, or 1 mM, 10 mM, or 50 mM zinc.*

Effects of heavy metals on shoot length

Shoot lengths differed significantly between the different concentrations of heavy metals, (Kruskal-Wallis, Chi square = 65.1, p = 4.02E-12, df = 6). Table 9 lists the effects located with Mann Whitney pairwise comparison. Results are listed in table 9 and figure 88.

1 mM nickel had a positive effects on shoot length. Heavy metal treatment in higher concentrations had greater impact on shoot lengths, especially regarding nickel treated plants, causing shorter shoots.

The longest leaves were found in the control group treated with *Sporobolomyces sp.* with 46.5 cm length. The shortest leaves with 0.5 cm were found in the plants treated with 10 mM nickel and treated with *Rhodotorula mucilaginosa.*

Table 9: *Mann-Whitney pairwise comparison, effects of heavy metals on shoot length. Significant comparisons are marked in bold.*

	Ni [10 mM]	**Ni [5 mM]**	**Ni [1 mM]**	**C**	**Zn [1 mM]**	**Zn [10 mM]**	**Zn [50 mM]**
Ni [10 mM]		**0.0009139**	**0.0001493**	**0.0002514**	**0.0003358**	**0.0006717**	**0.04628**
Ni [5 mM]	**0.0009139**		**6.453E-06**	**0.0002812**	**0.000694**	0.2761	**0.000284**
Ni [1 mM]	**0.0001493**	**6.453E-06**		**0.03028**	0.6099	**7.055E-05**	**1.111E-05**
C	**0.0002514**	**0.0002812**	**0.03028**		0.2181	**0.004626**	**1.633E-05**
Zn [1 mM]	**0.0003358**	**0.000694**	0.6099	0.2181		**0.004651**	**2.071E-05**
Zn [10 mM]	**0.0006717**	0.2761	**7.055E-05**	**0.004626**	**0.004651**		**4.952E-05**
Zn [50 mM]	**0.04628**	**0.000284**	**1.111E-05**	**1.633E-05**	**2.071E-05**	**4.952E-05**	

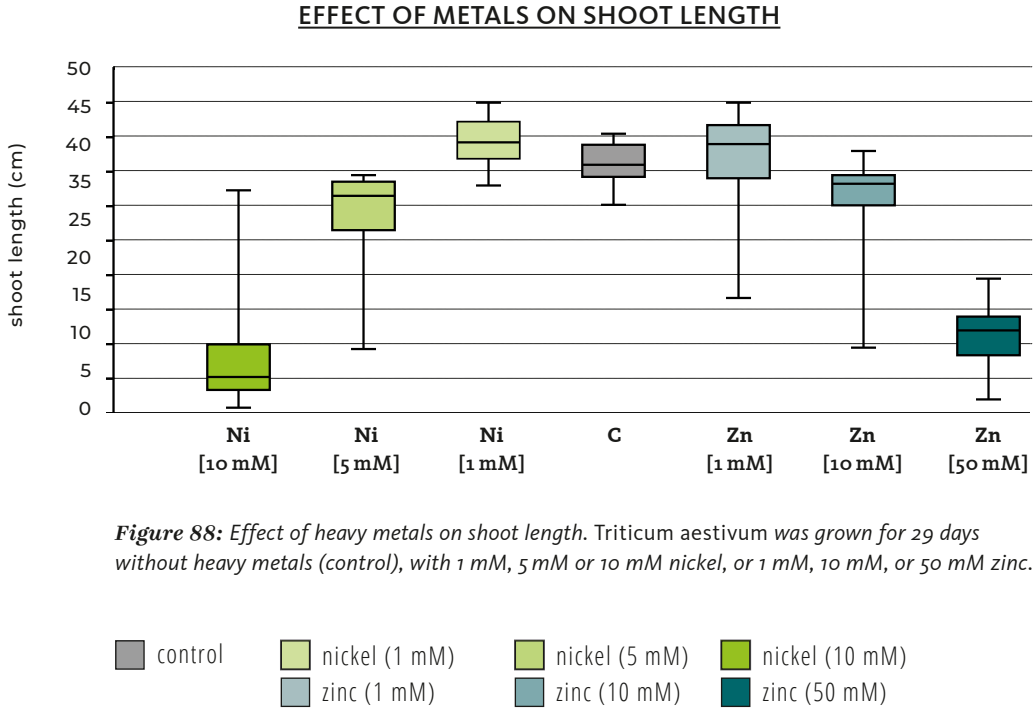


Figure 88: *Effect of heavy metals on shoot length.* Triticum aestivum *was grown for 29 days without heavy metals (control), with 1 mM, 5 mM or 10 mM nickel, or 1 mM, 10 mM, or 50 mM zinc.*

Effects of heavy metals on number of leaves

To examine the differences between the number of leaves under the influence of different heavy metal concentrations a Kruskal-Wallis test was conducted. There were significant differences found among the heavy metal treatments (Chi square = 51.14, p = 8.99E-11, df = 6; table 10, figure 89). To locate the differences, a Mann-Whitney pairwise comparison was conducted.

Again, increased concentrations of heavy metal reduced the number of leaves, while nickel treatment had stronger impact on the number of leaves in the plant, yet 5 mM of nickel caused an increase in the number of leaves.

The highest number of leaves was found in 5 mM nickel treated plants that grew without yeast and developed 11 leaves. Least leaves were estimated in plants treated with 10 mM nickel, no leaves besides the embryonic leave were produced.

Table 10: *Mann-Whitney pairwise comparison, effects of heavy metals on number of leaves. Significant comparisons are marked in bold.*

	Ni [10 mM]	**Ni [5 mM]**	**Ni [1 mM]**	**C**	**Zn [1 mM]**	**Zn [10 mM]**	**Zn [50 mM]**
Ni [10 mM]		**0.0003168**	**2.561E-05**	**6.7E-05**	**6.7E-05**	**0.0001102**	0.1279
Ni [5 mM]	**0.0003168**		**0.005794**	**0.01197**	**0.01197**	**0.003715**	**3.511E-05**
Ni [1 mM]	**2.561E-05**	**0.005794**		0.6247	0.6247	0.1992	**1.931E-06**
C	**6.7E-05**	**0.01197**	0.6247		0.9674	0.1363	**4.523E-06**
Zn [1 mM]	**6.7E-05**	**0.01197**	0.6247	0.9674		0.1363	**4.523E-06**
Zn [10 mM]	**0.0001102**	**0.003715**	0.1992	0.1363	0.1363		**4.03E-06**
Zn [50 mM]	0.1279	**3.511E-05**	**1.931E-06**	**4.523E-06**	**4.523E-06**	**4.03E-06**	

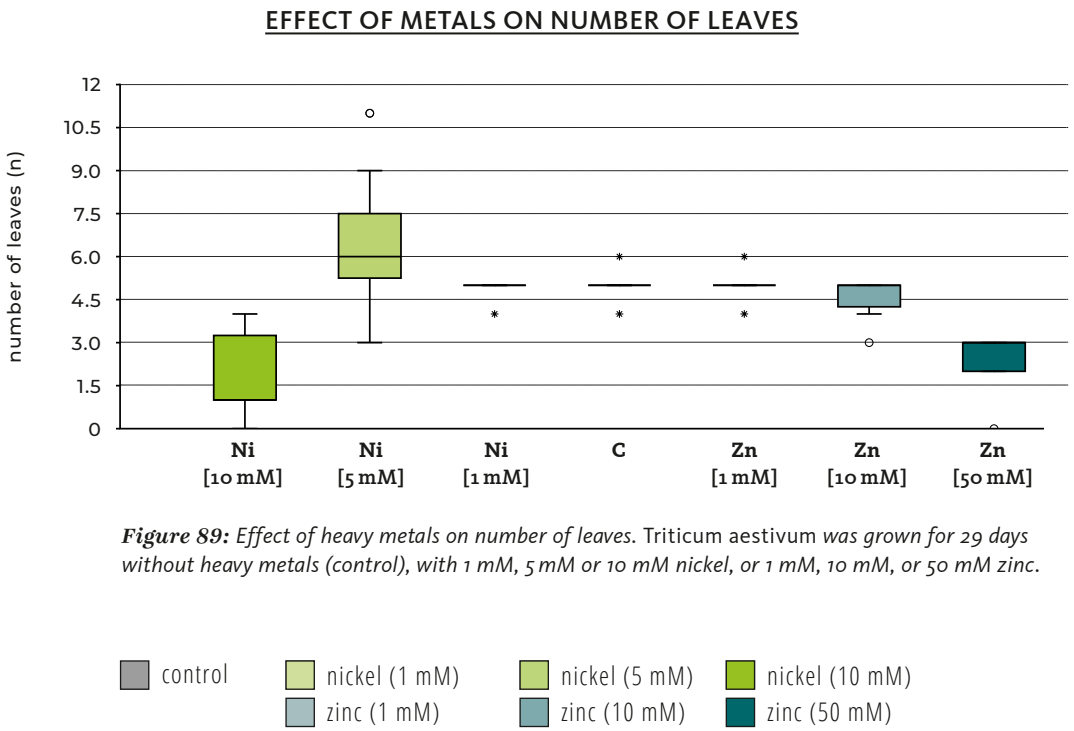


Figure 89: *Effect of heavy metals on number of leaves.* Triticum aestivum *was grown for 29 days without heavy metals (control), with 1 mM, 5 mM or 10 mM nickel, or 1 mM, 10 mM, or 50 mM zinc.*

Effects of heavy metals on root weight

To examine the differences in root weight under the influence of different heavy metal concentrations one-way ANOVA was conducted. Significant differences were found among the heavy metal treatments, $F(6, 94) = 25.02$, $p = 4E{-}17$, omega^2: .603. To locate the differences, Tukey-Kramers post hoc test was conducted. Results are listed in table 11 and figure 90.

1 mM nickel caused slightly heavier roots compared to the control. Increased heavy metal concentration reduced root weight, 10 mM nickel affected the plants negatively more severely compared to 10 mM zinc.

Root weight was lowest with zero gram in 10 mM nickel, treated with *Rhodotorula mucilaginosa;* or in 50 mM zinc, treated with both yeasts. The heaviest roots were found in 1 mM nickel treated plants inoculated with *Sporobolomyces sp.* weighing 0.07 gram.

Table 11: *Tukeys pairwise comparison, effects of heavy metals on root weight. Tukey's Q below the diagonal, p(same) above the diagonal. Significant comparisons are marked in bold.*

	Ni [10 mM]	**Ni [5 mM]**	**Ni [1 mM]**	**C**	**Zn [1 mM]**	**Zn [10 mM]**	**Zn [50 mM]**
Ni [10 mM]		0.9664	**0.0001218**	**0.0001221**	**0.0001218**	**0.02488**	0.9392
Ni [5 mM]	1.318		**0.0001221**	**0.0001467**	**0.0001218**	0.2379	0.4301
Ni [1 mM]	9.959	8.641		0.9641	0.9988	**0.005349**	**0.0001218**
C	8.623	7.305	1.336		0.7725	0.08154	**0.0001218**
Zn [1 mM]	10.68	9.357	0.7163	2.052		**0.001023**	**0.0001218**
Zn [10 mM]	4.625	3.307	5.334	3.998	6.05		**0.0008798**
Zn [50 mM]	1.493	2.81	11.45	10.12	12.17	6.118	

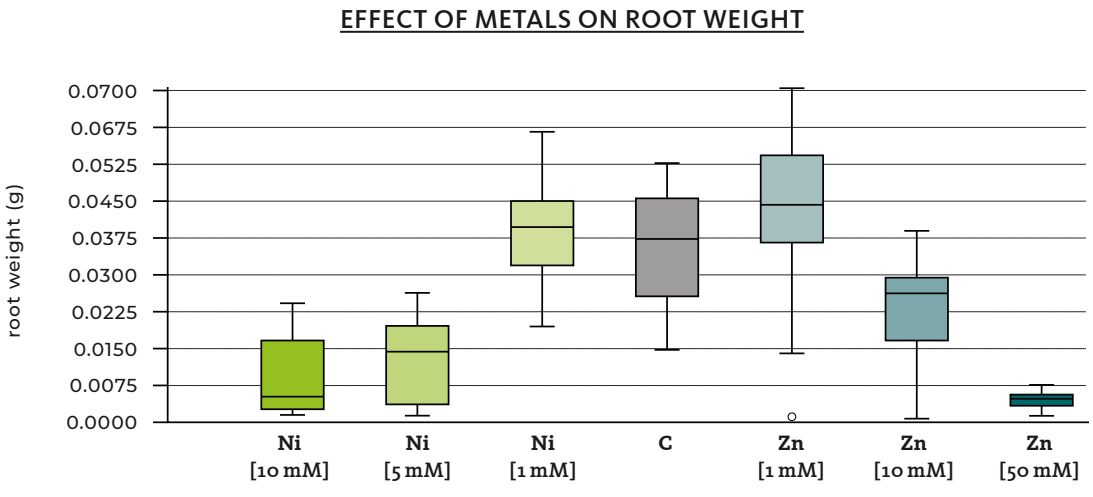


Figure 90: *Effect of heavy metals on root weight.* Triticum aestivum *was grown for 29 days without heavy metals (control), with 1 mM, 5 mM or 10 mM nickel, or 1 mM, 10 mM, or 50 mM zinc.*

Effects of heavy metals on shoot weight

Shoots differed significantly in weight for the different concentrations of heavy metals, after One-way ANOVA was conducted. There were significant differences found among the heavy metal treatments, $F(6, 94) = 16.84$, $p = 7.502E{-}13$, omega^2: .5). To locate the differences, Tukey-Kramers post hoc test was conducted. Results are listed in table 12 and figure 91.

Shoot weights were ranging from zero grams, in cases like 10 mM nickel in the control group, where just the embryonic leave was produced, up to 0.24 gram in 1 mM nickel, in plants treated with *Sporobolomyces sp.*

1 mM zinc treated plants weighed more compared to the control. With increasing metal concentrations of nickel and zinc, shoots weighed less. The effect was stronger in 10 mM nickel treated plants compared to 10 mM zinc treated plants.

Table 12: *Tukeys pairwise comparison, effects of heavy metals on shoot weight. Tukey's Q below the diagonal, p(same) above the diagonal. Significant comparisons are marked in bold.*

	Ni [10 mM]	**Ni [5 mM]**	**Ni [1 mM]**	**C**	**Zn [1 mM]**	**Zn [10 mM]**	**Zn [50 mM]**
Ni [10 mM]		0.1957	**0.0001235**	**0.0001224**	**0.0001243**	0.2251	0.7929
Ni [5 mM]	3.447		**0.02199**	**0.01196**	**0.0275**	1	**0.004127**
Ni [1 mM]	8.133	4.686		1	1	**0.01789**	**0.0001218**
C	8.421	4.974	0.2884		1	**0.009633**	**0.0001218**
Zn [1 mM]	8.023	4.576	0.1096	0.3981		**0.02245**	**0.0001218**
Zn [10 mM]	3.348	0.09964	4.785	5.074	4.676		**0.005184**
Zn [50 mM]	2	5.447	10.13	10.42	10.02	5.347	

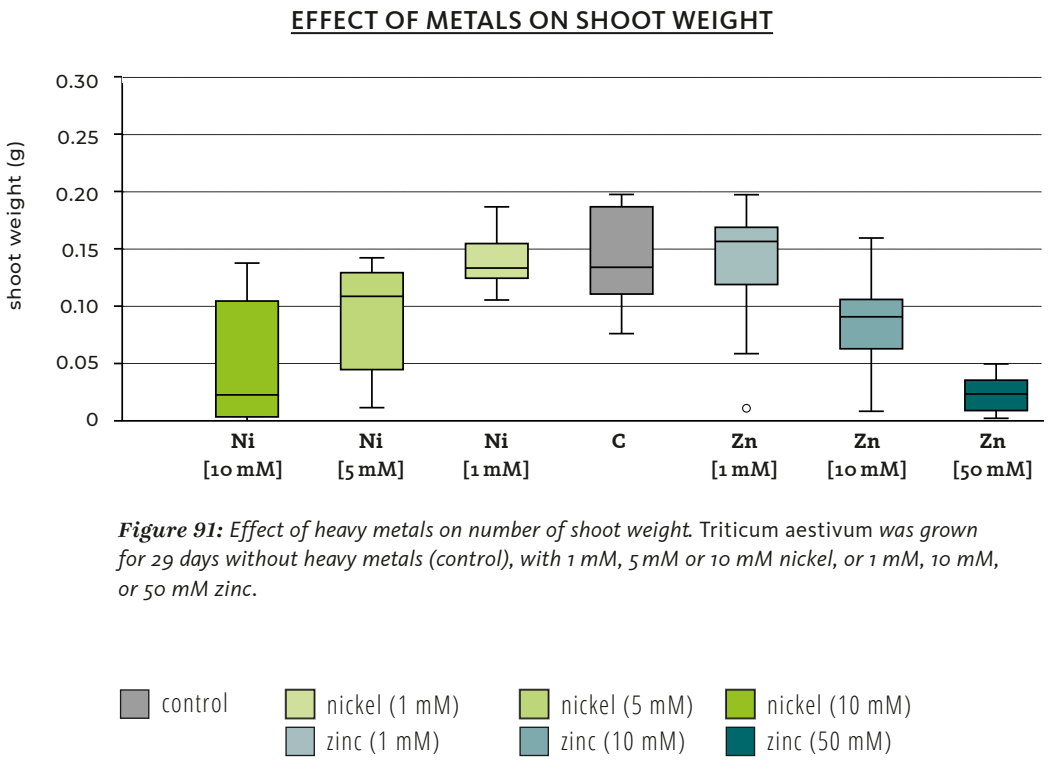


Figure 91: *Effect of heavy metals on number of shoot weight.* Triticum aestivum *was grown for 29 days without heavy metals (control), with 1 mM, 5 mM or 10 mM nickel, or 1 mM, 10 mM, or 50 mM zinc.*

2. The effects of yeasts

Differences between plants growing without heavy metals as control, and plants growing with *Rhodotorula mucilaginosa* or *Sporobolomyces sp. i*n the different heavy metals concentrations, were estimated conducting one-way ANOVA. Effects were located with Tukey's post hoc test. Statistical differences between number of leaves were estimated using a Kruskal Wallis Test. A summary of significant results is provided in table 13 and figure 92. Results are also presented as barcharts in Appendix 1 and 2.

Effects of yeasts on the control group (no heavy metal treatment)
There was no statistically significant difference in root length, shoot length, number of leaves, root weight and shoot weight, between the control and the treatment with *Rhodotorula mucilaginosa* (alive or dead) or *Sporobolomyces sp.* (alive or dead). Yeast treatment did not influence the development of the plants growing without heavy metal exposure. (compare to table 13)

Effects of yeasts on nickel treated plants
In 1 mM nickel only *shoot length* differed statistically significant, one-way ANOVA, $F(2, 38) = 4.968$, $p = .012$, omega^2: .169, between control and *Rhodotorula mucilaginosa* ($p = .011$). Plants treated with *Rhodotorula mucilaginosa* had shorter shoots compared to the control. (compare to table 13 and figure 92)

There was no statistically significant difference in *root length*, *number of leaves*, *root weight* and *shoot weight* between the control and the treatment with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.*

In 5 mM nickel *shoot length* differed statistically significant between control and *Sporobolomyces sp.* ($p = .032$). Further significant differences could be estimated in *root length* (One-way ANOVA, $F(2, 33) = 4.555$, $p = .018$, omega^2: .173), between control and *Sporobolomyces sp.* ($p = .0119$). Also *root weight* differed significantly (One-way ANOVA, $F(2, 33) = 3.633$, $p = .037$, omega^2: .135), between control and *Sporobolomyces sp.* ($p = .026$). Shoots and roots grown in 5 mM nickel and treated with *Sporobolomyces sp.* were longer, and roots were heavier compared to the control group. (compare to table 13 and figure 92)

There were no statistical differences in *number of leaves* and *shoot weight* between the control group and yeast treatments.

In 10 mM nickel a statistical significant difference was found between the control group and *Rhodotorula mucilaginosa* (Kruskal-Wallis, Chi square = 7.019, $p = .03$, $z = 2$) regarding *root weight.* Roots weighed less when growing with *Rhodotorula mucilaginosa.* (compare to table 13 and figure 92)

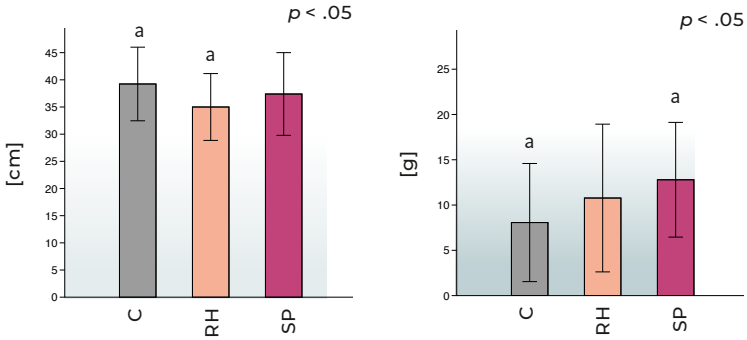
Treatment with yeast did not affect *root length*, *shoot length*, *shoot weight* and *number of leaves* in the 10 mM nickel group.

Effects of yeasts on zinc treated plants
No statistically significant differences were estimated between the control and the treatment with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* in 1 mM, 10 mM or in 50 mM zinc regarding *root length*, *shoot lengths*, *number of leaves*, *root weight and shoot weight.* Growing with yeast therefore did not affect the plants' performances.

Table 13. *Summarised effects of yeasts on the different treatments. " – " marks not significant differences, "(+)" marks an increased value in the respective treatment, "(–)" marks negative effects, "C" stands for the control group, "RH" for Rhodotorula mucilaginosa., "SP" for Sporobolomyces sp.*

	Root length	Shoot length	Root weight	Shoot weight	n leaves
C	–	–	–	–	–
NICKEL					
1 mM	–	(–) C – RH	–	–	–
5 mM	(+) C – SP	(+) C – SP	(+) C – SP	–	–
10 mM	–	–	(–) C – RH	–	–
ZINC					
1 mM	–	–	–	–	–
10 mM	–	–	–	–	–
50 mM	–	–	–	–	–

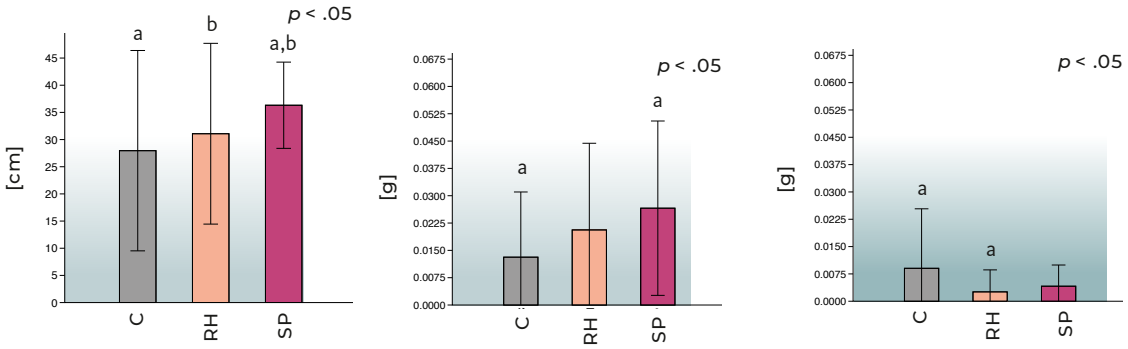
OVERVIEW OVER THE SIGNIFICANT EFFECTS OF YEAST



1 mM nickel
shoot length

5 mM nickel
root length

Figure 92:
Overview over the significant effects of yeast on the development of Triticum aestivum *grown with different toxic metal conditions. Plants grew as control group ("C"; grey bar), with* Rhodotorula mucilaginosa *("RH"; salmon pink bar), or with* Sporobolomyces sp. *("SP"; purple bar). Vertical lines on each bar show ±SD, 95 % confidence interval. Treatments marked with "a" or "b" are significantly different.*



5 mM nickel
shoot length

5 mM nickel
root weight

10 mM nickel
root weight

OVERVIEW OF POT EXPERIMENT 2, HARVESTED PLANTS

CONTROL GROUP:
TRITICUM AESTIVUM WITH *RHODOTORPULA MUCILAGINOSA* OR *SPOROBOLOMYCES SP.*

CONTROL

RHODOTORULA MUCILAGINOSA

SPOROBOLOMYCES SP.

RHODOTORULA MUCILAGINOSA DEAD

SPOROBOLOMYCES SP. DEAD

Figure 93: *Comparison of* Triticum aestivum *grown for 29 days. Plants were inoculated with* Rhodotorula mucilaginosa *or* Sporobolomyces sp., *and alternatively with inactive, dead inoculates of* Rhodotorula mucilaginosa *or* Sporobolomyces sp. *Scale bar equals 10 cm.*

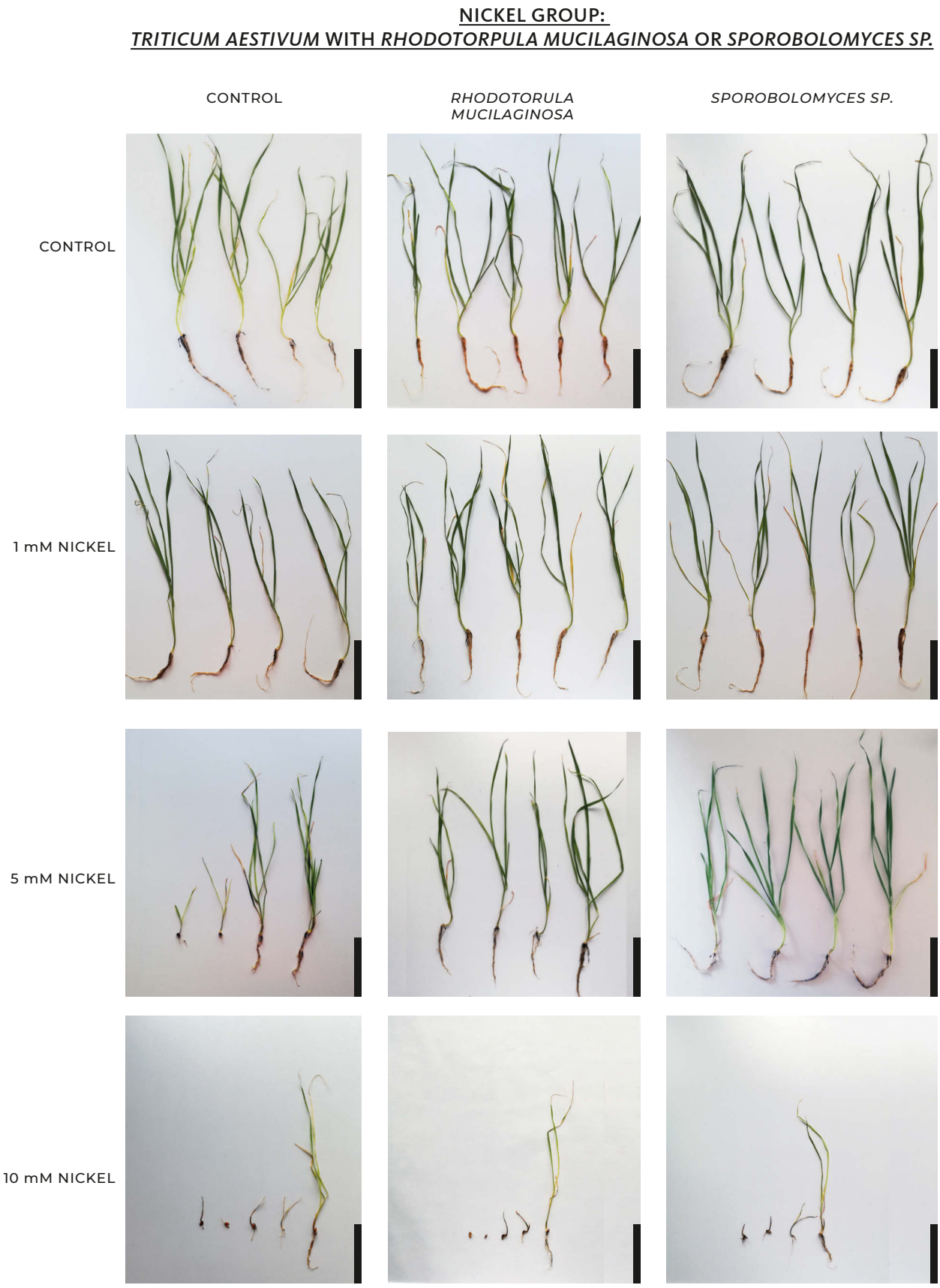


Figure 94: *Comparison of* Triticum aestivum *grown for 29 days without heavy metals (control), with 1 mM nickel, 5 mM nickel or 10 mM nickel. Plants were inoculated with* Rhodotorula mucilaginosa *or* Sporobolomyces sp. *Scale bar equals 10 cm.*

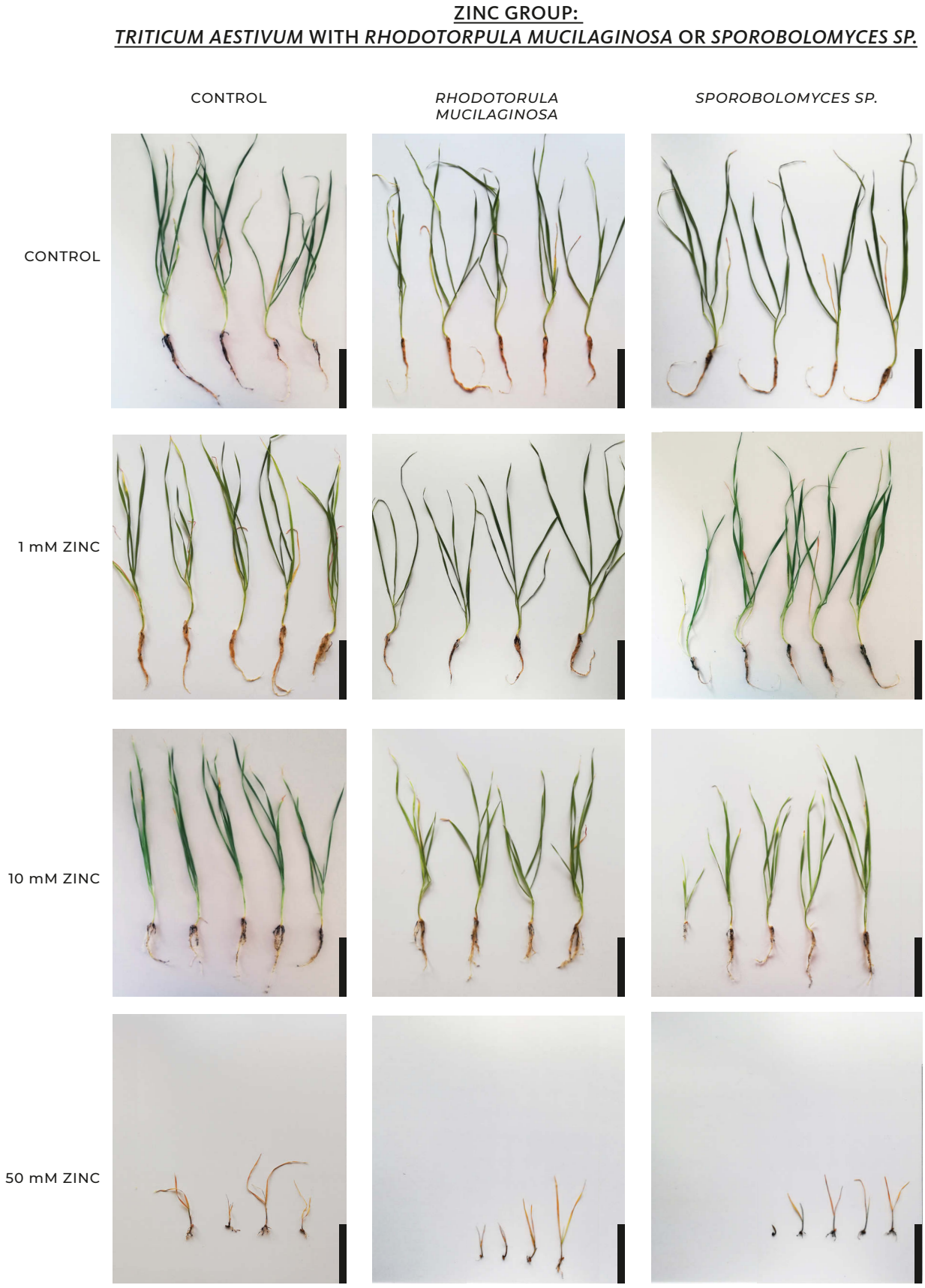


Figure 95: *Comparison of* Triticum aestivum *grown for 29 days without heavy metals (control), with 1 mM zinc, 10 mM zinc or 50 mM nickel. Plants were inoculated with* Rhodotorula mucilaginosa *or* Sporobolomyces sp. *Scale bar equals 10 cm.*

DISCUSSION

The effect of toxic metals on plants is a widely studied and discussed topic. In recent years, the interaction of plants under heavy metal stress with fungi, and their possible positive effects on the development of these plants, received increased attention. The aim of this study was to increase the knowledge on the interaction of plants with endophytic fungi on contaminated sites. Do endophytic fungi affect the plants in a negative or a positive way? Do they improve the plants' performance on these demanding sites? How do they enter the plants? May these endophytes be used on contaminated soils as phytoremediators in future scenarios?

Two general parts of experiments can be distinguished in this work to answer our numerous questions. On one hand *in vitro* experiments were performed using microscopical methods with experiments conducted on agar plates. Agar plates offer the possibility to directly investigate the interaction of metals, plants and yeasts. The effect of heavy metals on numerous plant growth parameters can be distinguished, as well as the interaction with yeast.

Plants of agricultural interest and species adapted to elevated levels of heavy metals, in this case nickel and zinc, were grown in heavy metal contaminated agar dishes. The effect of heavy metals on general plant growth parameters was documented, like germination rate, root and shoot length and weight. Additionally, the interaction with one filamentous fungus, *Diaporthe columnaris*, and two yeast species, *Rhodotorula mucilaginosa or Sporobolomyces sp.*, was observed in co-habitation experiments. Again, growth parameters were determined to estimate the effect the interaction with fungi might have on the plants. The usability of different staining methods in combination with microscopy was analysed. The goal was to visualize the interaction of fungi and plants, the entrance points of the fungi into the plants, as well as the distribution of the fungi inside the plants using different staining methods with bright field- and fluorescence microscopy. Furthermore, to focus on the uptake and the distribution of the heavy metals between plants and fungi, we analysed the potential of Newport Green to answer this question, aided by the use of fluorescence microscopy and CLSM.

Experiments using agar dishes though remain "artificial". They offer possibilities to focus on details, yet they only reflect natural systems in a simplified way. Therefore, for the second part of the experiments, soil pot experiments were used as a more "natural" approach. Metals in soils are less available to the plants, they may be present in a variety of chemical and physical forms, like soil adsorbed species, soluble complexed species and ionic solutes (Olaniran et al., 2013) and interact with numerous organisms inhabiting these habitats. The interaction between yeasts and wheat was observed after a growing period of one month. Again, various growth parameters were estimated, like germination rates, photosynthetic rates, root and shoot lengths, etc. to estimate the effect yeasts had on wheat under nickel and zinc stress.

EFFECTS OF HEAVY METALS ON PLANTS CULTIVATED *IN VITRO*

In this first approach we concentrated on the selection of species to work with in further experiments. The selection was composed of heavy metal sensitive plant species, as well as specialists adapted to elevated levels of heavy metals, and non adapted species. We focused on the effect of nickel and zinc on germination and the development of the seedlings.

SELECTION OF PLANTS ACCORDING TO THEIR REACTION TO HEAVY METALS FOR FURTHER EXPERIMENTS WITH ENDOPHYTES

Amaranthus cruentus

Amaranthus cruentus, an important, worldwide used pseudo-cereal, germinated significantly better on 1 mM zinc plates (84 %) and the control group (81 %), compared to 1 mM nickel treated plates (68 %). After one week of growing, plants appeared to be macroscopically best developed in the control group, regarding development of length of roots and root hairs. Nickel treated plants showed the worst development with short roots compared to the control group and a total lack of root hairs. Zinc treated plants had shorter roots and shorter root hairs compared to the control group.

Amaranth species have been described as zinc accumulators, storing zinc in their aerial parts (Ogunkunle et al., 2013; Assad, 2017). Regarding the remediation potential of *Amaranthus cruentus*, the species might be promising for remediating zinc contaminated sites. Our plants showed slightly reduced performance when growing with 1 mM zinc compared to the control group, which might nevertheless offer sustainable amounts of plant material on contaminated sites for remediation purposes.

Akubugwo et al. (2012) described *Amaranthus hybridus* as accumulator for zinc and nickel, amongst other metals, and suggests this species as phytoremediator, based on shoot to root ratios bigger than 1.0, following Baker's theory (Baker, 1981). In the experiments mentioned, the plants were collected from various polluted dump sites and were analysed using HNO3-HCLO4-HF digestion followed by atomic absorption spectrometry. Additionally, Akubugwo et al. (2012) mention potential to remediate iron, zinc, cadmium, chrome, and lead. *Amaranthus cruentus* did not perform well in our experiments under the influence of nickel. The remediation potential is not given in this high concentration of nickel with agar plates.

Noccaea caerulescens

Noccaea caerulescens is a classical model species for cadmium, nickel- and zinc-hyperaccumulation (Sterckeman et al., 2015) and has received much interest as possible phytoremediation candidate (Verbruggen et al., 2013).

In our experiments, *Noccaea caerulescens* could cope quite well with the offered metal concentrations, the plants had the highest germination rates in the experiment. Yet, no significant difference in the germination rate between the different metals could be determined. After one week plants were best developed in the control and nickel treatment. Zinc led to a reduction in root hairs, indicating suboptimal growing conditions.

Zhao et al. (2003) state that phytoremediation of soils using *Noccaea caerulescens* is feasible only at moderately contaminated sites, slow growth and small biomass are limiting factors for phytoremediation.

Noccaea goesingensis

Noccaea goesingensis, a nickel hyperaccumulator when growing on ultramafic soils (Idris et al., 2004), like serpentine soils, showed poor germination rates in our experiments, with 8 % germinating in the control, and 5 % in nickel and zinc, respectively.

The few germinated plants in the nickel treatment had the longest root hairs, based on macroscopic observation. Zinc treated plants had shorter root hairs compared to the nickel and the control group. *Noccaea goesingensis* was therefore excluded from further experiments due to poor germination rates and slow development. An explanation for the low germination rates might be found in the usage of aged seeds, suboptimal storing or unsufficient germination conditions.

Triticum aestivum

Triticum aestivum represented, additionally to *Amaranthus cruentus*, a species of agricultural value. Its seeds showed generally good germination rates, irrespective the metal treatment. No significant differences between the germination in the different heavy metal treatments could be estimated.

After one week plants from the control group appeared most vital. Nickel treated plants were clearly suffering, growth seemed to be reduced compared to the control and the zinc treatment.

In vitro experiments with *Amaranthus cruentus*, *Noccaea caerulescens*, *Noccaea goesingensis* and *Triticum aestivum* growing on agar revealed strong impacts of contamination with 1 mM nickel or zinc. Based on these first results, *Amaranthus cruentus*, *Noccaea caerulescens* and *Triticum aestivum* were chosen for further experiments.

EFFECTS OF HEAVY METALS ON FUNGI

Endophytic fungi are able to adapt to many different environmental situations and habitats, like sites with elevated metal levels, certain strains show high resistance to toxic metals. They occur in almost every plant in the natural environment and contribute to plant growth and stress tolerance. (Domka et al., 2019)

The effect of exposure of *Diaporthe columnaris*, *Rhodotorula mucilaginosa*, and *Sporobolomyces sp.* to nickel and zinc contaminated media was examined. Co-habitation experiments were used to analyse if the offered metal concentrations were affecting the fungi in a positive or a negative way, and furthermore, as explained in a chapter later on, to investigate the interaction of both.

Heavy metal effect on *Diaporthe Columnaris*

Diaporthe species have been described as plant pathogens, endophytes and saprobes, sometimes both (Gomes et al., 2013). The species used in our experiments, *Diaporthe columnaris*, has been isolated as endophyte from plants growing on various heavy metal contaminated sites, therefore the interaction of plant and fungus was of obvious interest, as it might reveal growth promoting interaction effects between fungus and plants.

Growing on nickel agar without nutrients and no carbon source, resulted in slow development of *Diaporthe columnaris* compared to the control group. Nevertheless, the fungus was able to grow, possibly fuelled by the tiny piece of agar the fungus was transferred with onto the metal media, which contained nutrients and might have enabled a minimum of growth.

Heavy metal effect on *Rhodotorula mucilaginosa*

Rhodotorula species are ubiquitous yeasts isolated from all kinds of environments, like common food (Sen et al., 2019; Wirth & Goldani, 2012), and clinical environments, where they might act as a pathogen to patients (Sen et al., 2019). *Rhodotorula mucilaginosa* has been shown to improve plant growth (Sen et al., 2019), inhabiting the tissue of the host systemically (Domka et al., 2019).

Rhodotorula mucilaginosa showed great potential for phytoremediation in experiments where the fungi were grown as a biofilm in elevated nickel and zinc concentrations (Domka et al., 2019). Grujić et al. (2018) tested the resistance of *Rhodotorula mucilaginosa* to nickel and zinc in biofilms. Nickel was used in concentrations between 1.30 to 20.67 mM, zinc was tested with 0.1, 1, 10 and 100 mM. The yeast was tolerant to all of the used concentrations. (Grujić et al., 2018)

The results of Grujić et al. (2018) correspond well with the result of our *in vitro* experiments. The yeast grew well on agar dishes (½ MS, without minor salts), irrespective if it was exposed to 1 mM nickel or 1 mM zinc. One reason for the tolerance of elevated metal concentrations might have been reported by Domka et al. (2019) and Grujić et al. (2018), stating that extracellular polymeric substances absorb the pollutants and surround the cells in biofilms.

No direct carbon source was offered on the agar dishes to the yeasts. Nevertheless they were able to grow. This circumstance might be explained by interaction with bacteria or plants, both are able to provide the yeast with exudates. Plant root exudates include primary metabolites, like sugars, amino acids, and organic acids (Canarini et al., 2019), and might have been transported to the yeast, even when growing without direct contact to them, possibly by the liquids present in the agar dishes. These might have been flushed all over the surface, eg. when taking the macroscopic pictures, where we had to change the slight angle the plates had been stored with during growth. Alternatively, carbon sources could have also been provided by bacterial communities, e.g. via lysis, a thought which offers possibilities for further studies, as the study did not focus on bacterial communities within the plates. Dennis et al. (2010) state, that root-derived carbon is rapidly assimilated by microorganisms, further on modified, independent of plant influences, and finally released into the rhizosphere by microorganisms themselves, and might finally be available to the fungi as well.

Heavy metal effect on *Sporobolomyces sp.*

Sporobolomyces sp. gave diverse results growing on agar with different heavy metal concentrations. Nevertheless, the yeasts showed reduced macroscopic growth in the 1 mM nickel treatment compared to the other treatments. Zinc treatment appeared to be less harmful to the yeast. These results adapts well to Vadkertiová et al. (2005) who investigated the effect of copper, zinc, nickel and cadmium on seventy stains of yeasts, belonging to 15 species. They found that, in liquid yeast cultures, most species were able to grow at zinc concentrations of 10 mM or higher, while nickel was found to be quite toxic, tolerated up to 5 mM. (Vadkertiová et al., 2005)

However, numerous additional factors might have influenced yeast development in our experiments. One might be found in the consistency of the agar medium. Growing media with 1 mM zinc, 1 % agar and ½ MS did not solidify completely and remained more fluid compared to nickel and control growing media. Therefore humidity conditions within zinc plates might have been very different compared to the control and the nickel treatment. *Sporobolomyces sp.* grew better on denser, solid agar, where the yeast appeared much more vital, based on macroscopic observation. Using 2 % agar resulted in more solid growing media, yeast cells were developing best on zinc treatments compared to nickel and control treatments.

Furthermore, *Sporobolomyces sp.* under nickel stress seemed to profit from growing with well developed nickel hyperaccumulator *Noccaea goesingensis*, in contrast to co-habitation with suffering *Amaranthus cruentus*. Co-habitation with hyperaccumulators might therefore effect yeast viability under metal stress. A reason might be found in a low level of exudates provided by the plants which might result in insufficient carbon supply for the yeast. An explanation might be given by Broeckling et al. (2008), who state that "root exudates regulate soil fungal community composition and diversity". In their study they focused on *Arabidopsis thaliana* and *Medicago truncatula* and showed that both were able to "maintain resident soil fungal populations but were unable to maintain nonresident soil fungal populations", mediated by root exudates that they added to the plants and fungi, root exudates may be used by the plants to regulate the fungal community, (Broeckling et al., 2008) which might also hold true for heavy metal adapted plant species and might be worth further investigations.

EFFECTS OF HEAVY METALS ON PLANTS AND YEASTS

Endophytic fungi offer provision of nutrients to their hosts and buffer external environmental stress factors and microbial competition from the plants. There are diverse possibilities for interaction with plants, mutualistic mycorrhizal fungi colonizing the roots, mutualistic interactions and pathogenic ones. (Schulz et al., 2005)

To compare the effects on development implemented by heavy metals on plants with a fungal partner, experiments with *Amaranthus cruentus* and *Noccaea caerulescens* growing on 1 mM nickel or zinc, or as control in petri dishes on agar containing additional nutrients (½ MS) were conducted. After 18 days of growing they were inoculated with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* Developmental parameters like root length, root hair length and the distance between the first root hair and the root tip were estimated after three days of co-habitation.

Fungal influence on *Amaranthus cruentus*

Amaranthus cruentus showed reduced root lengths when growing with zinc and especially when growing with nickel, the latter causing an almost complete reduction of root hairs. Heavy metals reduce plant growth by interfering with metabolism in roots, eg. with water and mineral uptake, they inhibit cell devision and enzyme activities, affect the functioning of membranes, cause oxidation and cross-linking of proteins, they damage DNA, cause cell death, and cause oxidative stress in roots (Tamás et al., 2008).

Rhodotorula mucilaginosa and *Sporobolomyces sp*. reduced the tip to first root hair distance in zinc treated plants. No further significant effects could be estimated. This might be explained by the effect of yeast exudates interacting with the growth center cells at the root tips and causing a retardation of development, a thought offering possibilities for further studies. Root tip cells are thought to favor diffusion of metabolites from the roots to the soil or growing medium, caused by a lack of cell differentiation (Canarini et al. 2019).

Fungal influence on *Noccaea caerulescens*

Noccaea caerulescens was performing best in the control, while metal treatments were reducing root lengths. Nickel treated plants produced long root hair. The distance between root tip and the appearance of the first root hair was longest in the zinc treated plants.

Zinc treated plants seemed to profit from inoculation with *Rhodotorula mucilaginosa*. Roots were significantly longer compared to the control group and compared to the treatment with *Sporobolomyces sp.*

Fungal development and distribution on *Amaranthus cruentus and Noccaea caerulescens*

Rhodotorula mucilaginosa and *Sporobolomyces sp.* were densely covering the roots growing on top of the agar of the control treatment. Using bright field we could not detect entrance points into root hairs or epidermal cells, plates were too overgrown by the yeasts. (Uni-)directional growth towards the roots was not detectable, yeasts spread into all directions on top of the agar. Yet, having reached a root, they used the plant material to spread alongside the root.

Amaranthus cruentus treated with nickel produced short, dense and reduced roots, that were hard to analyse using long distance objectives in bright field. Bacterial contaminations appeared on the nickel treatments as small dots with increasing incubation time. Roots growing in nickel inoculated with *Rhodotorula mucilaginosa* were partly covered with unhealthy looking yeasts, agglomerating especially around the tip of the roots. As already mentioned, Canarini et al. (2019) state that the flux of primary metabolites is concentrated at the root tip, which might have offered the yeasts a temporary microhabitat with more suitable conditions than the surrounding agar.

Yeasts on zinc plates with 1 % agar did not show much interaction with the plants, which might have been caused by the quite liquid medium. Using more solid media, yeasts seemed to profit from the denser agar, yet no directional growth was observed. This is indicating further effects that important for development and might be worth further observations.

Growing with *Noccaea caerulescens* yeasts were found on top of the agar coating the roots in the control, growing alongside the roots. Nickel treated plants were inhabited by the yeasts too. Yeasts were growing alongside the roots, partly forming hot spots around the root tips found in treatments with *Rhodotorula mucilaginosa*. In the zinc treatments, less interaction was observed with both yeasts.

Nevertheless, co-habitation might also be a factor of adaption. Schulz et al. (2005) state, that endophytes from any particular host usually include one to several taxa that are adapted to that host, an adaption which includes chemotaxic signaling between them. This signaling causes specific stimuli produced by the respective hosts. They refer to Peters et al. (1998) who were using plant calli and their endophytic fungi to study their interactions and found that the growth of the fungi was enhanced when they were hosted. Lu & Clay (1994) suggest, that growth of fungi correlates positively with host compatibility. (Schulz et al., 2005)

Fungal influence on *Triticum aestivum*

In this part of the experiments the interaction of *Triticum aestivum* with *Diaporthe columnaris* or *Rhodotorula mucilaginosa* was of interest. *Triticum aestivum* grew in quadratic petri dishes in 0.1 mM nickel and a control set and was inoculated with *Diaporthe columnaris* or *Rhodotorula mucilaginosa*. This time, no additional nutrients were added.

Heavy metals were affecting the whole plants systemically. Plants growing in agar with nickel avoided to grow into the media, producing lots of roots growing into the air, where root hair grew densely and long, in contrast to the short and rare root hairs growing inside the agar. Exudates and encrustations were found especially on the roots in the nickel treatment, and less in the control treatment.

Germination rates were not different in the control and the nickel treatment. Root lengths were reduced by nickel treatment, interaction with fungi did not change that, so only the metal effect was induced. In the control group, roots were shorter when growing with *Diaporthe columnaris*, a negative effect can be found here. Root hairs were scarce in the nickel treatment, but their length was not affected by metal concentration or the presence of fungi. The distance between the tip of the root and

the first root hair could only be estimated on the control group between *Rhodotorula mucilaginosa* treated plants and the control, which did not show any significant difference.

Triticum was therefore mainly suffering from heavy metal exposure, addition of fungi only affected root length in a negative way. *Diaporthe columnaris* had negative effect on the plants, we could not find positive interactions or promoting effects, it might therefore be described as pathogenic under these circumstances. Endophytic fungi show excellent versatility regarding lifestyle and can act mutualistic, commensalic or as pathogens, depending on the partner, development, nutrition and further environmental factors (Domka et al., 2019; Schulz et al., 2002). The balance between the two partners might be disturbed, which might hold true for our experiments, by either a decrease in plant defence or an increase in fungal virulence, as a consequence, disease develops (Schulz et al., 2002).

Fungal development and distribution on *Triticum aestivum*

Both, *Diaporthe columnaris* and *Rhodotorula mucilaginosa* were able to cope with the offered conditions. *Rhodotorula mucilaginosa* appeared more viable on the nickel treatment, in the control it gave a cloudy impression, macroscopically seen. In both cases roots growing on top of the agar were covered by the yeast. No clear distribution pattern was observed, additionally yeast got flushed over the plates when they were moved for analysis.

Diaporthe columnaris spread into all directions, without addressing roots directly on the first hand. Nevertheless, interactions were observed. Additionally, an interaction between bacterial colonies, exudates and deposits by roots on the agar surface was observed, which seemed to be attracting the fungus and might be interesting for further studies. Domka et al. (2019) state that fungal endophytes can complete their lifecycles outside hosts, their development is not synchronized to the host, in contrast to mycorrhizal fungi.

Roots on the other hand appeared to be attracted by the block the fungus had been transferred to the plate. This might be explained by the nutrients found in the block itself, as the fungus was cultivated on PDA-media, while no further nutrients were provided in the quadratic dishes.

On the long term, an increased mortality was noticed in plants treated with *Diaporthe columnaris*. The partnership did not seem to be advantageous for *Triticum aestivum*, the plants were the first to die compared to the control and yeast treated plants.

HEAVY METAL LOCALIZATION, STAINING USING NEWPORT GREEN ™

Samples of *Amaranthus cruentus* and *Noccaea caerulescens*, infected with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.*, were infiltrated with NPG and analysed using fluorescence microscopy and CLSM. The goal was to localize nickel and zinc uptake into roots and yeasts. If possible, we wanted to determine if toxic metal uptake differed in the respective treatments and when using different yeasts.

NPG enters the cells as an uncharged permeant acetate ester, allowing it to pass the cell wall. After hydrolysis it becomes charged and binds to charged protein metal complexes, fluorescence is induced. (Cadosch et al., 2009)

Two different drenching times were used. Results suggest to use a longer drenching time for roots and root hair, both were depicted clearer after 20 hours of drenching, compared to 2 hours. An interaction between plant and yeast was hard do detect, due to the strong fluorescence of wheat roots and root hair. The dye showed a great affinity for nickel throughout the experiment, partly causing strong background noise when working with plants growing in nickel media.

The dye depicted the status of the plants: *Amaranthus cruentus* suffering from nickel treatment had roots that were most likely dead, plants did not actively take up the dye, which was just staining the outside of the root.

Comparing the uptake between the two plants we would have expected stronger uptake into the roots, staining seemed to happen mostly externally, maybe due to a lack of suction by the plant caused by the humid atmosphere they grew inside the agar dishes, as a consequence of the artificial environment.

Rhodotorula mucilaginosa generally needed long exposure times to get visible. 48 hours of drenching and using CLSM revealed that the dye was only staining cell walls. This might be explained by strong cell walls, which are highly refractive and hard to be dyed, cells that took up the dye easily were most likely dead. Nickel agar showed intense autofluorescence and was outshining the yeasts giving intense background noises. Storage of nickel or zinc appears to be a question of cell age, old cells store lots of nickel, in contrast to young ones.

In *Sporobolomyces sp.* internal storages were detectable with CLSM, in contrast to *Rhodotorula mucilaginosa.* CLSM revealed nickel storage in internal organelles. Old cells seemed to store more nickel. Again, staining seemed to involve only a part of yeast cells.

Duran et al. (2011) state, that a lot of endophytes are able to form nanoparticles, which might reduce the toxicity of metals to the plants, they can be precipitated internally and externally (Domka, 2019), which might further explain the different staining patterns of the yeast species. Yet, further studies might reveal deeper insights.

Ultimately, results do not allow quantifications of nickel or zinc uptake or differentiation of yeasts, but might be a useful tool for further experiments.

Heavy metal localization using NPG with confocal microscopy to detect nickel and zinc in *Sporobolomyces* Sp.

Using CLSM we wanted to further analyse intracellular storage of nickel and zinc in *Sporobolomyces sp.* in a small, experimental dataset. This time autofluorescence of the yeasts was compared to stainings with NPG to in control-, nickel-, and zinc-treatment.

All tested situations showed autofluorescence. Nickel treated samples had strong background noise, most likely caused by the nickel containing growing medium. Zinc treated yeast cells showed partly fluorescence of small vacuoles. Fluorescence of vacuoles, plasm and small grains was detected.

Stainings with NPG revealed coagulated cellplasm in the only rarely appearing nickel treated cells which were in bad condition. Zinc treated yeast cells seemed to be smaller, budding was visible. NPG staining reduced thresholds in all cases tested.

NPG seems useful to mark cells with uptaken nickel and zinc, allowing to work with lower thresholds. Yet quantitative results should be estimated using other methods, accompanied by NPG. A basic problem of the approach, as already mentioned, is that plants were mounted and stained on coverslips in humid chambers, an environment which results in a lack of suction by the plant. This might cause insufficient, mostly external staining, as plants are not taking up the dye properly. Based on this, further experiments were conducted using soil to achieve more natural results. As previous

experiments with *Amaranthus cruentus* and *Noccaea caerulescens* gave quite unsatisfying results regarding homogeneity of growth within one treatment and plant development (data not shown) we decided to work with *Triticum aestivum*, which gave better results.

TESTING POSSIBILITIES TO LOCALIZE FUNGI IN AND ON PLANTS

Using several different staining methods, the interaction between *Triticum aestivum* roots and *Diaporthe columnaris, Rhodotorula mucilaginosa* or *Sporobolomyces sp.* was observed. The goal was to identify interactions, entrance points and internal structures built by endophytic fungi.

Roots exudates and deposits presented a general obstacle in this part of the experiments, due to densely covering the root surfaces which complicated their examination.

YEAST AND PLANT INTERACTION, STAINING USING CALCOFLUOR WHITE

Calcofluor White binds unspecifically to cellulose and chitin and is used to rapidly detect microorganisms and parasites in clinical mycology and parasitology (Harrington et al., 2003). We were interested in the interaction between *Triticum aestivum* roots and *Diaporthe columnaris*, searching for a dye that would easily give separable results.

Calcofluor White is easy and quick to use. Nevertheless, with optical microscopes, *Triticum aestivum* root hairs showed strong autofluorescence under UV-excitation, due to the dye's binding affinity to cellulose, which made it difficult to detect the fine hyphae of *Diaporthe columnaris* in between.

The same problem appeared with the yeasts, *Rhodotorula mucilaginosa* and *Sporobolomyces sp.*, the tiny yeast cells were hard to identify on the root surface under UV-excitation. Pictures were partly even easier to interpret using bright field.

We could not detect any internal hyphae or yeast cells using Calcofluor White in optical microscopy. Fungi were just detected on external structures.

CLSM, Calcofluor White and *Rhodotorula mucilaginosa*
Using Calcofluor White with CLSM, we could clearly determine between deposits on the root surface and root cells. Cell walls of both, *Triticum aestivum* and *Rhodotorula mucilaginosa*, got stained. Especially budding yeast cells seemed to take up the dye. Regarding the size of yeast cells it might be therefore recommendable to use CLSM in these cases. As our experiments were primarily focusing on the use of light microscopy, further experiments using CLSM might reveal more promising results.

YEAST AND PLANT INTERACTION, STAINING WITH SUDAN IV

Using Sudan IV entrance points of yeast to wheat roots could not be detected, nevertheless, using filamentous *Diaporthe columnaris*, callose deposits were observed, that indicated penetration of the hyphae to the epidermal cells. Unfortunately internal structures were not depicted well by the dye. Again, root deposits and exudates were densely covering the roots impeding observation.

YEAST AND PLANT INTERACTION, STAINING WITH ANILINE BLUE

Aniline blue followed the same picture given by the previous dyes, yeasts were only detected on the surface. Yeast were hard to detect in bright field as well as when using fluorescence, due to the intense signals given by roots and root hair in fluorescence and the stained exudates found in bright field. For filamentous fungi Aniline blue offered better results, hyphae were visible, partly also indicating penetration into the host.

Therefore a single staining approach might be improved by adding further dyes, an approach performed by Osuna Avila et al. (2012). They were specially focusing on lipid bodies and the interaction between plants and fungal endophytes in root and leaf material of *Bouteoula eriopoda* and used Trypan blue and Sudan IV with differential interference contrast microscopy. They found hyphae to be sometimes visible, while fruiting bodies and other clear details useful for identification were typically absent. Furthermore, they reported amorphous fungal structures which they thought to be derived from yeasts. (Osuna Avila et al., 2012)

Nevertheless, further studies would improve the depiction of these interaction, ideally accompanied by molecular and genetic analysis.

EXAMINATION OF GROWTH DEVELOPMENT

Using growth chamber experiments no clear directional growth towards wheat roots could be estimated over time, *Diaporthe columnaris* grew into all directions. A result which is reflecting previously mentioned distribution patterns quite well and was found for example in the quadratic petri dishes.

INTERACTIONS OF PLANTS, HEAVY METALS AND YEAST

Two pot experiments were conducted to address the interaction of *Triticum aestivum* with *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* under the influence of nickel and zinc in a "more natural" surrounding, compared to the previously mentioned *in vitro* experiments that were working with agar media only. Both experimental series have been designed as follows:

Soil Experiment 1
Triticum aestivum was inoculated with *Rhodotorula mucilaginosa* after three and again after eight days after sewing, both times by pouring liquid yeast cultures into the pots. Treatments consisted of a control group, and nickel or zinc contaminated soils (0.1 mM, 1 mM, respectively).

Soil Experiment 2
The second version of the experiment was conducted with increased concentrations of toxic metals, due to the similar results obtained in part 1. Nickel was administered in 1 mM, 5 mM and 10 mM, zinc in 1 mM, 10 mM and 50 mM. *Rhodotorula mucilaginosa* or *Sporobolomyces sp.* were applied immediately by allowing the seeds to soak in a liquid yeast culture, and again after three days of germination.

GERMINATION RATE

Germination is one of the most important developmental stages in a plant's life, affecting growth as well as yield. Numerous factors are influencing germination, including availability of light, water, the mineral composition of soil and various stressors, like metals in toxic concentrations. (Ahmad & Ashraf, 2012)

Nickel (without yeast)
The germination rates of our pot experiments were negatively affected with increasing influence of nickel. In the first part of our pot experiments, where low concentrations were used, nickel treatment had a positive effect on germination. More seeds germinated in 0.1 mM nickel treated plants (+ 13 %) and in 1 mM (+ 6%), compared to the control group (87 %). With increasing levels of nickel in the second pot experiment, these values declined. Germination rates were reduced in plants growing with 5 mM to 60 % and down to 53 % in 10 M, compared to the control (87 %). (Table 14)

Nickel had slightly positive effects in lower concentrations, while causing reduced germination rates in higher concentrations, a result also found for germination of rice by Das et al. (1978) and numerous authors like Welch (1981) and Shweti & Verma (2018). This might be explained by the fact that nickel is an essential micronutrient for plants and improves seed germination at low concentration for many species (Ahmad & Ashraf, 2012), yet it becomes toxic in higher concentrations (Shweti & Verma, 2018). Nickel in high concentrations affects ribonuclease-activity, and the activity of proteases and amylases, which is influencing the mobilization and digestion of reserves that are found in germinating seeds, (Shweti & Verma, 2018; Ahmad & Ashraf. 2012). It affects protein synthesis, carbohydrate metabolism, and it severely retards seed germinability of many crops. Nickel is furthermore a known competitor for micro- and macronutrients, reducing the uptake of elements into germinating seeds, which results in low germination rates, increasing germination rates and poor development of the seedlings (Ahmrad & Ashraf, 2012).

Zinc (without yeast)
Zinc treated plants had higher germination rates, regardless the metal concentration, compared to the nickel treated plants. Interestingly, least plants germinated at an intermediate heavy metal concentration: 1 mM zinc reduced germination rates down to 73 % in part 1. Munzuroglu et al. (2002)

found similar patterns in their experiments. They soaked *Triticum* seeds in various metal solutions and observed the germination after 24 h, 48 h and 72 h. Plants treated with zinc showed only slightly reduced germination rates of less than 15 %, down to about 85 %. The results were similar from 0.5 M up to zinc concentrations of 6.5 mM. Higher concentrations decreased germination. The study focused on Cd, Co, Cu, Pb and Zn and found zinc to be least harmful regarding germination in wheat, its root elongation, coleoptile and hypocotyl growth. (Munzuroglu et al., 2002)

Table 14: *Effect of nickel and zinc treatment on germination rates. Values without brackets represent the percentage of seeds that germinated in the respective concentration. Values in brackets stand for in-/decrease compared to the control. Missing values were not conducted in the respective pot experiment. (n = 15 for each concentration)*

Nickel	control	0.1 mM Ni	1 mM Ni	5 mM Ni	10 mM Ni
pot experiment 1	87 %	100 % (+13 %)	93 % (+6 %)		
pot experiment 2	87 %		93 % (+6 %)	(60 % (– 27 %)	53 % (– 34 %)

Zinc	control	0.1 mM Zn	1 mM Zn	10 mM Zn	50 mM Zn
pot experiment 1	87 %	100 % (+13 %)	73 % (– 14 %)		
pot experiment 2	87 %		87 % (+/–0 %)	100 % (+13 %)	87 % (+/–0 %)

Additionally, we wanted to estimate the effect of yeasts on germination rates:

Nickel with *Rhodotorula mucilaginosa*

In the first pot experiment *Rhodotorula mucilaginosa* was added three days after sewing to the pots. *Rhodotorula mucilaginosa* treated plants showed identical germination rates to the control group, which might be explained by fast seed germination and a belated addition of yeast, which could not affect germination anymore. In the lowest concentration of nickel (0.1 mM) germination rates decreased 13 % compared to the control group. In 1 mM nickel, the germination rate increased by 7 % when the yeast was present. (Compare to table 15)

In pot experiment two, where seeds were allowed to soak in a liquid *Rhodotorula mucilaginosa* culture, the presence of yeast increased wheat germination rates compared to the control, as plants were immediately able to profit from co-habitation. Only 1 mM treated plants showed decreased germination rates. Increasing levels of nickel led to increasing germination rates when *Rhodotorula mucilaginosa* was added. (Compare to table 15).

EXPERIMENT 1	without yeast	with yeast	increase/decrease
control	87 %	87 %	+/– 0 %
0.1 mM nickel	100 %	87 %	– 13 %
1 mM nickel	93 %	100 %	+ 7 %
EXPERIMENT 2	without yeast	with yeast	increase/decrease
control	87 %	93 %	+ 6 %
1 mM nickel	93 %	87 %	– 6 %
5 mM nickel	60 %	80 %	+ 20 %
10 mM nickel	53 %	67 %	+ 14 %

Table 15: *Germination rate (%) of the nickel treatments in pot experiment 1 and 2 with* Rhodotorula mucilaginosa

The positive effect of *Rhodotorula mucilaginosa* in higher metal concentrations might be explained by shielding the seeds from toxic metals. The fungus has already shown potential to remove nickel and zinc when growing as a biofilm as well as growing planktonic (Grujić et al., 2018). Possible mechanisms in our experiments might therefore be found in exudation of EPS (Grujić et al., 2018) that protect yeasts from stress factors and are involved in metal sorption (Breierová, 2018) or in uptake, which might facilitate germination of wheat seeds in difficult environments. After uptake, toxic metals are neutralized by complexation in the cytosol and translocated for storage in the vacuole (Domka et al., 2019). Mowll and Gadd (1983) describe zinc uptake as biphasic, with a rapid first phase, where cations bind to cell walls, followed by slower, metabolism-independent intracellular uptake. They further conclude, that zinc surface binding capacities are species dependant, due to different cell wall structures (Mowll & Gadd, 1983), which might explain the different influences by the yeasts.

Differences between experiment one and two, (eg. 1 mm nickel; part 1: +7 %; part 2: –6 %) might be explained by the different application forms. Fedotov et al. (2017) analysed the role of yeasts in germination, they found that different taxa of soil yeasts stimulated germination in wheat, barley and rye and further stated that method of application impacts the yeasts' performance. They compared a soaking method and a semi dry variant (20 l of solutions were spent per 1 t of seeds), and state that the method of seed treatment with yeast preparations greatly affects their action, on one hand they might stimulate surface microorganisms (soaking method), on the other the development of seed and epiphytic microorganisms (semi-dry method). (Fedotov et al., 2017)

Nickel with *Sporobolomyces sp.*

Plants growing with *Sporobolomyces sp.* had 14 % lower germination rates in the control group, and the low concentrated nickel treatment, respectively. The yeast had positive effects in higher metal concentrations (5 mM: + 27 %; 10 mM: + 20 %). These results follow the same trend as the plants treated with *Rhodotorula mucilaginosa*, where positive effects were also especially visible in higher concentrations. As already discussed, possible explanations might be found in shielding effects offered by the yeast cells, uptake or the exudation of EPS. (Compare to table 16)

EXPERIMENT 2	without yeast	with yeast	increase/decrease
control	87 %	73 %	– 14 %
1 mM nickel	93 %	80 %	– 13 %
5 mM nickel	60 %	87 %	+ 27 %
10 mM nickel	53 %	73 %	+ 20 %

Table 16:
Germination rate (%) of the nickel treatments in pot experiment 2 with Sporobolomces sp.

EXPERIMENT 1	without yeast	with yeast	increase/decrease
control	87 %	87 %	+/– 0 %
0.1 mM zinc	100 %	93 %	– 7 %
1 mM zinc	73 %	93 %	+ 20 %
EXPERIMENT 2	without yeast	with yeast	increase/decrease
control	87 %	93 %	+ 6 %
1 mM zinc	87 %	93 %	+ 6 %
10 mM zinc	100 %	93 %	– 7 %
50 mM zinc	87 %	73 %	– 14 %

Table 17:
Germination rate (%) of the zinc treatments in pot experiment 1 and 2 with Rhodotorula mucilaginosa

Zinc with *Rhodotorula mucilaginosa*

Addition of *Rhodotorula mucilaginosa* did not affect germination rates in the control treatment. No clear pattern is visible, results are inconsistent comparing experiment 1 and 2, the different application methods might be the cause. In contrast to nickel treatments (table 17), *Rhodotorula mucilaginosa* had a negative effect in higher concentrations on germination rates. An explanation might be found again in the different application methods used, further studies would possibly reveal deeper insights.

Zinc with *Sporobolomyces sp.*

Addition of *Sporobolomyces sp.* reduced germination rates in the control treatment, in higher concentrations plants had higher germination rates when growing with *Sporobolomyces sp.* This results is the opposite compared to the zinc treatment with *Rhodotorula mucilaginosa*, which might be explained by species' differing affinities for zinc. (Compare to table 18)

EXPERIMENT 2	without yeast	with yeast	increase/decrease
control	87 %	73 %	– 14 %
1 mM zinc	87 %	100 %	+ 13 %
10 mM zinc	100 %	100 %	+/– 0 %
50 mM zinc	87 %	93 %	+ 6 %

Table 18: *Germination rate (%) of the zinc treatments in pot experiment 2 with* Sporobolomces sp.

Nickel and dead *Rhodotorula mucilaginosa* or *Sporobolomyces sp.*

The addition of dead yeasts to one control group of pot experiment one and two was conducted to estimate the nutritional value these dead yeast cells might have for the plants. Plants growing with dead *Rhodotorula mucilaginosa* had reduced germination rates compared to the control of both experiments.

Plants that were growing with dead *Sporobolomyces sp.* (pot experiment 2), had 6 % increased germination rates compared to the control group, while plants growing with living yeast showed reduced germination rates (– 14 %). (Compare to table 19)

Results were contradicting: Dead yeast cells had no ubiquitous nutritional value for the plants. Killed *Rhodotorula mucilaginosa* did not seem to have nutritional value for the plants when applied after three days after sewing and immediately after soaking. In this case, the method of application seemed irrelevant. Killed *Sporobolomyces sp.* increased the plants' germination rate, when applied through soaking before sewing. The two species might therefore offer different nutritional values, which might be explained by difference in cell wall structure affecting the accessibility, a thought offering possibilities for further experiments.

Table 19: *Effect of dead yeasts, increase/decrease compared to control*

	without yeast	with *RH*	with *RH †*	with *SP*	with *SP †*
pot experiment 1	87 %	0	– 3 %		
pot experiment 2	87 %	+ 6 %	– 7 %	– 14 %	+ 6 %

PHOTOSYNTHETIC ACTIVITY

As a proxy for measuring stress in plants and stress tolerance, measurement of fluorescence was conducted. It is a low cost, non-invasive measurement of photosystem II activity, widely used in plant physiology, and often used as indicator of how plants respond to environmental change (Murchie & Lawson, 2013). Chlorophyll fluorescence can give insights into a plants ability to deal with environmental stresses, which are indicating photoinhibition. (Maxwell & Johnson, 2000)

Soil Experiment 1: low concentrations of heavy metals

In pot experiment 1 photosynthetic activity of *Triticum aestivum* did not reveal significant differences between either the different heavy metal treatments or the treatment with *Rhodotorula mucilaginosa*. F_v/F_m values were ranging between 0.762 in 1 mM nickel growing with *Rhodotorula mucilaginosa* and 0.83 in the control group growing with dead *Rhodotorula mucilaginosa*.

The results reflect the appearance given by the harvested plants, all seemed to be quite unaffected by the heavy metal exposure and the interaction with *Rhodotorula mucilaginosa*. This might be explained by metal concentrations that were too small to severely negatively affect the plants and their photosynthetic performance. A result which encouraged us to repeat the experiment using increased concentrations of heavy metals.

Soil Experiment 2: high concentrations of heavy metals

In the second run plants were more affected. Plants growing with 10 mM nickel were severely suffering from the treatment, growth was clearly affected, plants lacked sufficient leaf material. Hence they had to be excluded from the experiment. Values ranged from 0.686 in 50 mM zinc growing with *Rhodotorula mucilaginosa* to 0.836 estimated in plants growing with *Sporobolomyces sp.* with 10 mM nickel. Yet, there were no significant differences in F_v/F_m values comparing the different heavy metals used, without fungal interference.

Treatment with yeast revealed significant differences in the control group. Plants that had been inoculated with dead *Sporobolomyces sp.* had significantly lower F_v/F_m ratios compared to the other treatments in the control group. The estimated values in the control group ranged between 0.813, in a sample treated with dead *Sporobolomyces sp.*, and 0.832, in a sample treated with living *Sporobolomyces sp.*—a quite small effect. Treatment with yeast was not different to any of the other heavy metal treatments.

According to our measurements all of our plants should have been growing well, which might reflect the situation in experiment 1, but simply does not reflect the reality for part 2. *Triticum aestivum* growing with *Sporobolomyces sp.* in 10 mM was severely affected by the treatment, just one measurement could be performed in this treatment as just one plant offered enough material to conduct the measurement. It was excluded from statistical analysis, yet this one plant showed the highest F_v/F_m ratio of the whole experiment.

Nickel disrupts photosynthesis in numerous ways, by a combination of different factors, like affecting chloroplast structures, chlorophyll content and photosynthetic protein complexes. Nickel decreases chlorophyll content, it damages the photosynthetic apparatus by destroying cells of mesophyll and epidermal tissue. It is furthermore competing with other metal ions, which causes deficiencies of other essential metals ions in plants. (Chen et al., 2009)

It indirectly causes increased concentrations of hydroxyl radicals, simultaneously many antioxidant enzymes get reduced, resulting in ROS accumulation and oxidative stress, as stated in a study on wheat leaves by Gajewsak et al. (Gajewska et al., 2007, Chen 2009).

Excess zinc disturbs photosynthetic electron transport processes, resulting in suppression of the efficiency of energy transformation in photosystem II, furthermore it reduces carotenoid and chlorophyll concentration, as stated by Paunov et al. (2018) who studied the effect of cadmium and zinc on photosynthesis in drum wheat. (Paunov, Momchil et al., 2018)

An explanation for the unaffected and homogenous ratios might be given by Murchie and Lawson (2013), who state that one of the possible reasons might be found in the simple fact that fluorescence is not a "generic plant stress detector", stress acting on other plant parts, like the roots, may not simply be manifested in reduced F_v/F_m ratios. Although Chen et al. (2009) state, that over 50 % of nickel absorbed by plants is retained in the roots, and Timberley et al. (1973) found that nickel is stored only in minor percentages inside chloroplast in a study focusing on four tree species, which might reduce the heavy metal effect on the chloroplasts.

PLANT GROWTH

To get further insight into the plants' development we analysed various growth parameters, like length of roots and shoots, root and shoot dryweight, and the number of leaves the plants had produced.

Plant reactions under heavy metal influence, without yeast

The experiments showed a strong reduction of various growth parameters with increasing concentrations of metals used. Root lengths, root weights, shoot lengths, shoot weights and the number of leaves were increasingly diminished. Low concentration treatments stayed mostly unaffected. Yet a positive effect of nickel was found in the low concentrated 0.1 mM nickel treatment, where roots grew longer compared to the control. This result is following the trend given in the germination rates, with more seeds germinating in the lower concentrated treatments. Low concentrated nickel might therefore serve as growth propagating micronutrient as already mentioned.

Interestingly 10 mM of nickel and 50 mM of zinc caused quite similar reductions. Highly concentrated nickel had more impact compared to zinc, which caused similar results in five-fold concentrations. Plants treated with zinc were particularly affected by the highest concentrations used, 50 mM.

Plant reactions under heavy metal influence, with yeast

No general effect of yeasts regarding developmental parameters could be estimated. The addition of *Rhodotorula mucilaginosa* to low concentrations of heavy metals of the experiment had little effect. Plants treated with 0.1 mM nickel and 1 mM zinc had shorter roots when growing with *Rhodotorula mucilaginosa* compared to the control group in part one. In part two shoot lengths were reduced in 1 mM nickel and in 10 mM nickel, in the plants treated with *Rhodotorula mucilaginosa* compared to the control group.

Sporobolomyces sp. had punctual effects in pot experiment two, especially in the 5 mM nickel treatment. Plants growing in 5 mM nickel with *Sporobolomyces sp.* had longer and heavier roots compared to the control group, also shoots were longer with fungal partner compared to the control. Zinc treated plants on the other hand, did not significantly respond to the addition of *Sporobolomyces sp.*, regardless the concentration.

Addition of *Rhodotorula mucilaginosa* caused partly negative effects, while addition of *Sporobolomyces sp.* caused partly positive effects. This contradicts our expectations for *Rhodotorula mucilaginosa* which showed strong growth promoting effects eg. in *Typha*, in a study conducted by Sen et al. (2019), who discovered positive effects on shoot lengths when growing with *Rhodotorula mucilaginosa*. Both, roots and shoots were colonized by the yeast in this study, plants were clearly profiting from the

infection (Sen et al., 2019). Boby et al. (2008) found growth promoting effects of *Rhodotorula mucilaginosa* in cowpeas, when growing with *Glomus mosseae*, a mycorrhizal fungus (Boby et al., 2008). Endophytic yeasts offer various advantages over bacterial and filamentous endophytes, like the possibility of longtime storage, it is easy to culture them, and easy to apply them to crops (Joubert et al., 2018), yet our results show only partly positive effects and only for *Sporobolomyces sp.*

CELLULAR TOLERANCES

Plants from experiment two were observed on a cellular level using tolerance tests. Plants were chosen from the control, from the 5 mM nickel treatment, and from the 10 mM zinc treatment. Leaf surface cuts rested in nickel and zinc concentration rows (0 to 1 M) and were checked for surviving after two days.

Cellular tolerances of plant cells under heavy metal influence, without yeast
Regarding the effect of nickel or zinc on a cellular level in this control treatments, without fungal partner, an impact by nickel was detected, cells were more sensitive to nickel solutions than to the zinc solutions. This result is following the general trend of the study and well in line with the overall outcome of our experiments. Interestingly, it did not matter if the plants had been growing with nickel or without, zinc solutions were up to two magnitudes less harmful compared to nickel solutions, cells were not able to "pre-adapt" to nickel exposure.

Cellular tolerances of plant cells under heavy metal influence, with yeast
Additionally to the control groups each group was represented by a plant growing with *Rhodotorula mucilaginosa*, and by a plant growing with *Sporobolomyces sp.*

In many cases observed, yeast treated cells were more sensitive towards the exposure to nickel or zinc solutions compared to the control group, yeast treatment had mostly negative effects. In four cases yeast treated plants performed equal or better compared to the control:

1+2. Cells from leaf surface cuts of the control and of the 5 mM nickel plant performed better in nickel solutions, when growing with *Sporobolomyces sp.*

3. Cells from leaf surface cuts of the control plant performed slightly better in zinc solutions, when growing with *Rhodotorula mucilaginosa*.

4. *Rhodotorula mucilaginosa* treated plants were performing well in zinc solutions in the plant grown with 10 mM zinc.

Cells from the plant treated with *Sporobolomyces sp.* were sensitive to exposure to zinc solution, in all tested plants: in the control, in the 5 mM nickel plant and the 10 mM zinc plant, respectively. The most harmful combination was found in plants growing with 10 mM zinc and *Sporobolomyces sp.*, with surface cuts emerging in zinc and nickel solutions, even the control group emerging in tap water was rated as dead. This result leaves us quite puzzled and doubtful, up to our expectations at least the cuts emerging in tap water should have survived. Yet the cells in tap water were only partly reacting to the plasmolysis test, chloroplasts were yellow instead of green, and cells did not deplasmolyse again, and were therefore declared as dead. The simplest explanation might be found in methodological flaws, or an error. A repetition might therefore be advisable.

Nevertheless, surface cuts from the 5 mM treatment with nickel were performing better while emerging in nickel solutions if they had been growing with *Sporobolomyces sp.* This result is further supporting the results from germination rates and plant performance, and outlining the positive effect in this concrete concentration of nickel.

CONCLUSION

Endophytic yeasts can contribute to plant growth and health by producing plant hormones and other factors (Doty et al., 2013), even on demanding heavy metal polluted sites. The aim of the work was to determine the effect of two endophytic yeasts, *Rhodotorula mucilaginosa* and *Sporobolomyces sp.*, and a filamentous fungus, *Diaporthe columnaris*, on selected plants species under nickel and zinc stress.

Two general approaches were conducted to investigate the effect on plants of cultural interest (*Amaranthus cruentus*, *Triticum aestivum*) and *Noccaea caerulescens*, which is adapted to sites with increased levels of zinc and nickel. On one hand, *in vitro* experiments revealed insights into the interactions of heavy metals, plants and fungi, on the other hand, pot experiments were conducted to get insights into more natural interactions.

In vitro experiments

In experimental datasets several circumstances were found that made it problematic and difficult to accurately evaluate the effect of heavy metals and fungi on plants:

- The humid atmosphere within the agar dishes prevented water transport in the plants, which might have affected e.g. the efficiency of dyeing methods.
- Also the import of metals into the plants might have been affected by these conditions.
- Root length and especially root hairs developed differently inside agar and in humid atmosphere.
- Roots tended to grow onto the agar and not within, especially with nickel, irrespective if the dishes where kept at slight angles to direct root growth downwards. This results in limited contact with media and therefore metals, so our knowledge about the uptake is limited in these cases.
- Wheat in agar reacted to lower concentrations of heavy metals compared to soil pots. Metal conditions in agar dishes were more toxic compared to wheat growing in soil.
- Plant performance was mainly influenced by increasing heavy metal conditions. *Rhodotorula mucilaginosa*, *Sporobolomyces sp.*, and *Diaporthe columnaris* had only punctual effects: Root tip to first hair distance was reduced in *Amaranthus cruentus* under the influence of *Rhodotorula mucilaginosa*, *Sporobolomyces sp.* when growing with 1 mM zinc, *Noccaea caerulescens* growing with *Rhodotorula mucilaginosa* showed increased root lengths in 1 mM zinc. *Triticum aestivum* in co-habitation with *Diaporthe columnaris* in 1 mM nickel had shorter roots compared to the control group.
- Co-dependency between plants and yeast was indicated in the agar experiments and provides opportunities for further studies.
- NPG staining efficiency might have been reduced by the humid atmosphere in the dishes, resulting in insufficient uptake of the dye into the plants and mainly cell wall staining. Wheat showed strong fluorescence making it hard to detect yeasts in between. Yeast got only partly stained, a circumstance which might reflect the living status of the cells. *Sporobolomyces* seemed to take up the dye internally while *Rhodotorula* showed mainly dyed cell walls, depicted well using CLSM. A quantification of nickel or zinc uptake does not seem appropriate using NPG.
- Staining using Sudan IV, Calcofluor white and Aniline blue revealed no penetration caused by yeast, often disturbed by heavy deposits on root surfaces. The dyes worked better with filamentous fungi, callose spots as well as hyphae were depicted.
- Growth chamber experiments revealed that no directional growth towards the wheat and *Diaporthe columnaris* has happened.

Nevertheless, results indicate a primarily impact by heavy metals, punctually affected by fungi:
› *Rhodotorula mucilaginosa* and *Sporobolomyces sp.* treated *Amaranthus cruentus* had shorter root tip to first visible hair distances in 1 mM zinc.
› *Rhodotorula mucilaginosa* treated *Noccaea caerulescens* had longer roots when growing in 1 mM zinc.
› *Triticum aestivum* treated with *Diaporthe columnaris* had shorter roots in the control.

Pot experiments

The pot experiments revealed overall a clear negative impact on the plants caused by increasing heavy metal concentrations, only germination rates were partly profiting from increased zinc and nickel levels, root lengths were increased by minor levels of nickel. Overall, elevated levels of zinc and especially nickel reduced germination rates, growth parameters like root length and weight and the number of leaves. Nickel was by far more toxic to the plants in higher concentrations. 10 mM nickel affected plants similar to the five fold concentrated 50 mM zinc treatment. Data provided by cellular tolerance test and plant performances support this impression, which already indicated itself when observing the harvested plants. Interestingly this result was not reflected by photosynthetic activity. A repetition of the experiment might therefore advisable.

Application of *Rhodotorula mucilaginosa* had only partly positive effects on germination rates in nickel treatments and low concentrations of zinc. Utilization of dead yeast did not increase the germination rates. *Rhodotorula mucilaginosa* did not affect photosynthetic activity. Overall, *Rhodotorula mucilaginosa* had negative effects on wheat. Plants were growing shorter shoots when growing with *Rhodotorula mucilaginosa* in 1 mM nickel, and lighter roots in 10 mM zinc.

Sporobolomyces sp. had positive effects on germination rates in 0.1 mM nickel or zinc. Furthermore positive effects could be determined in 5 mM nickel, roots and shoots grew longer, roots were heavier when growing with yeast, this result was also reflected by tolerance tests. Photosynthetic activity was not affected. Yet, plants growing with *Sporobolomyces sp.* had higher germination rates in 10 mM zinc.

Outlook

Overall the results suggest that the interaction between plants growing on heavy metal sites, regardless if naturally adapted to these sites as hyperaccumulator or suffering from the exposure to heavy metals, might be aided by the help of endophytic fungi, yet only punctual improvements were found, beneficial only at very distinct concentrations of heavy metals. This punctual improvements would recommend intense further studies and knowledge of the site, the contamination, the plants and the interaction with the respective fungal partner, yet one might find interesting opportunities for sustainable remediation approaches.

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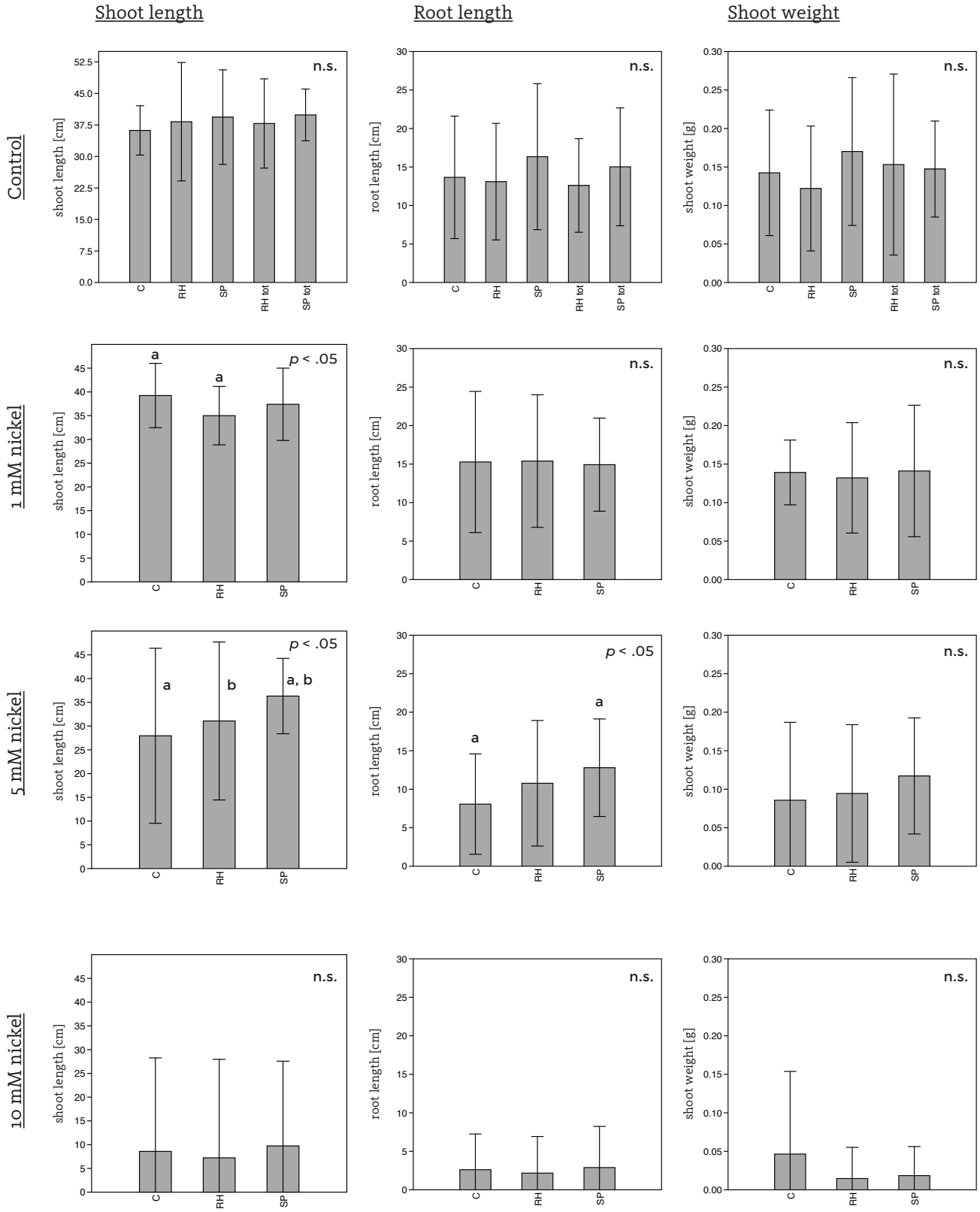
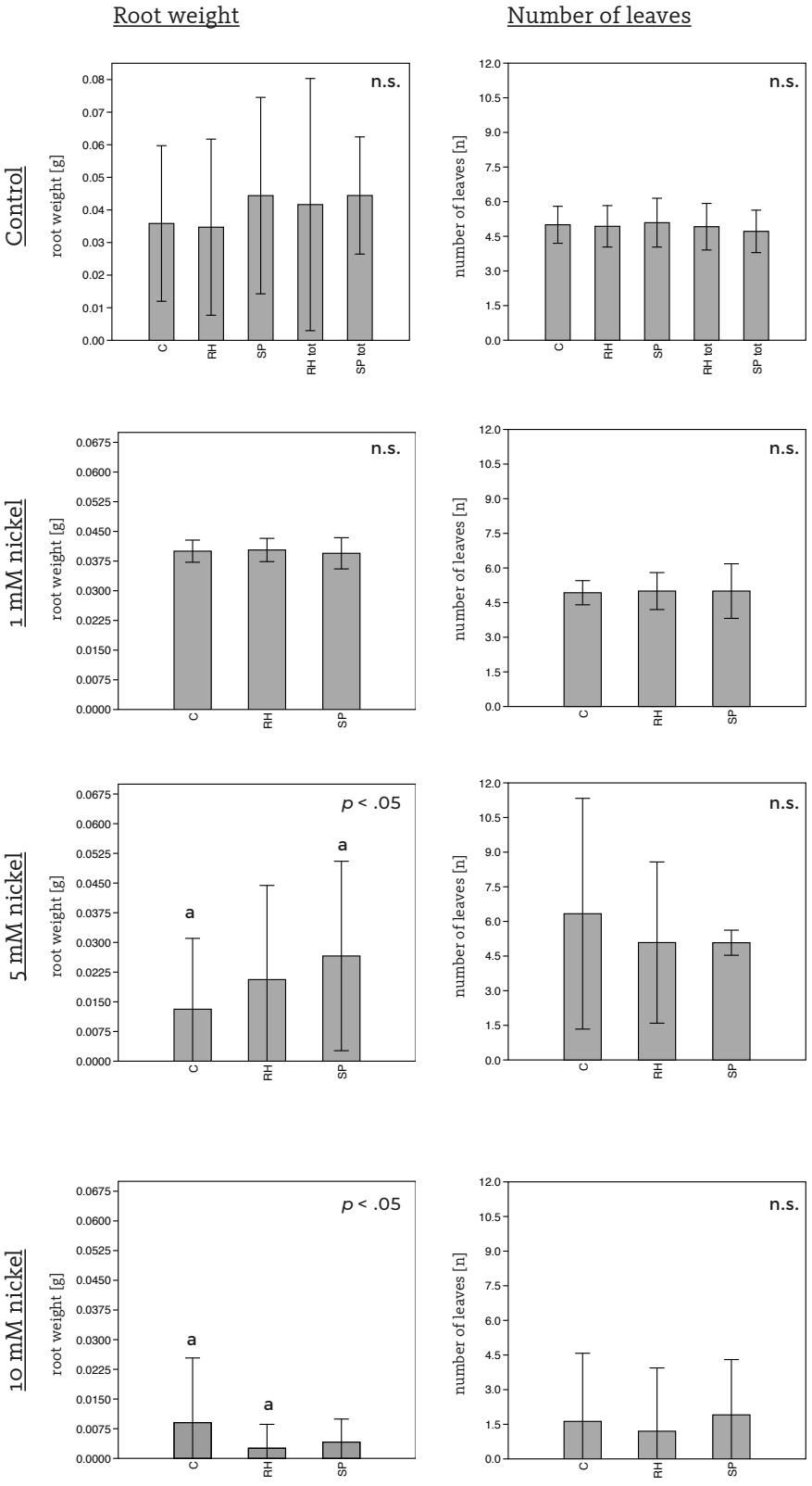
APPENDIX

APPENDIX 1
RESULTS POT EXPERIMENT 2, NICKEL



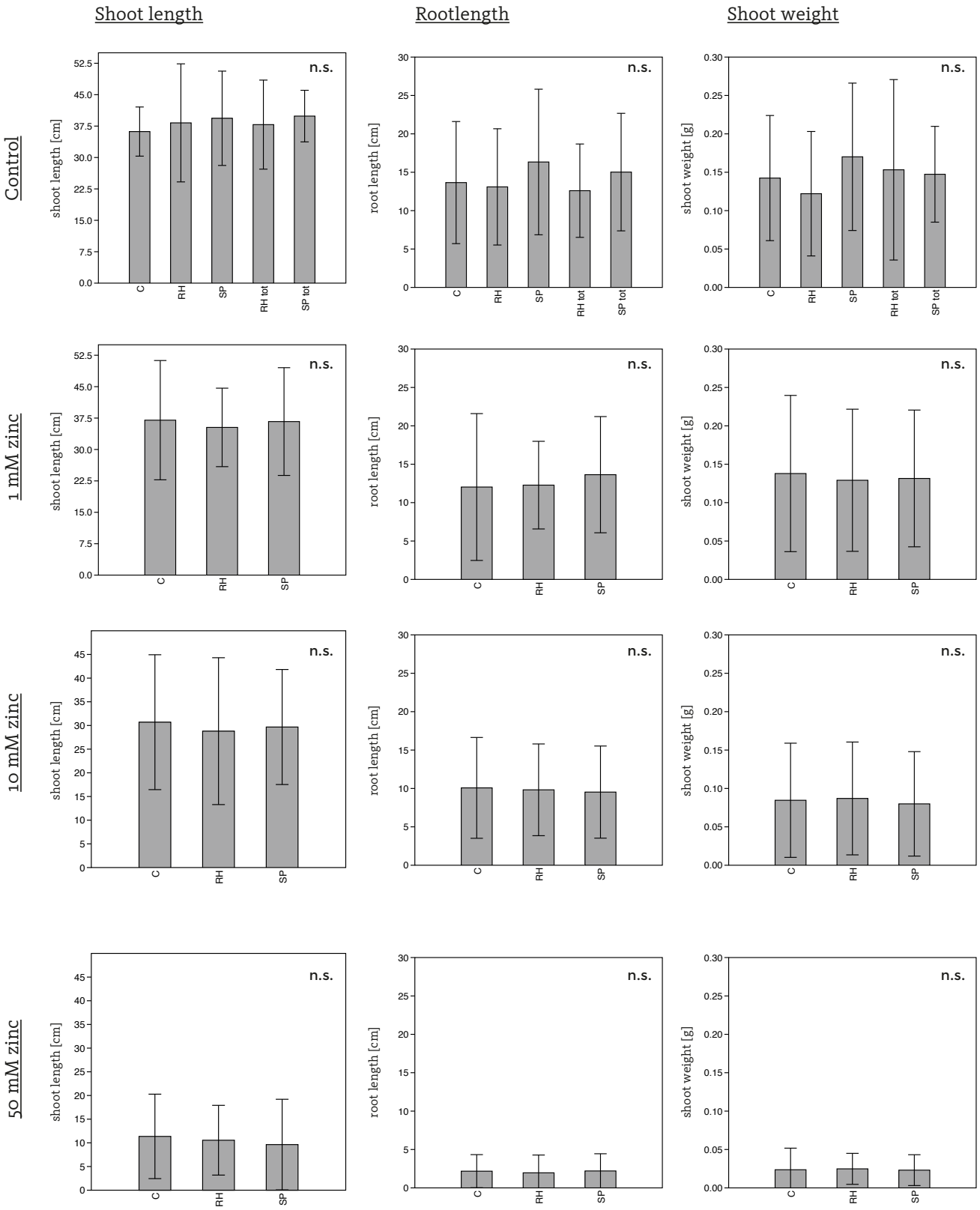


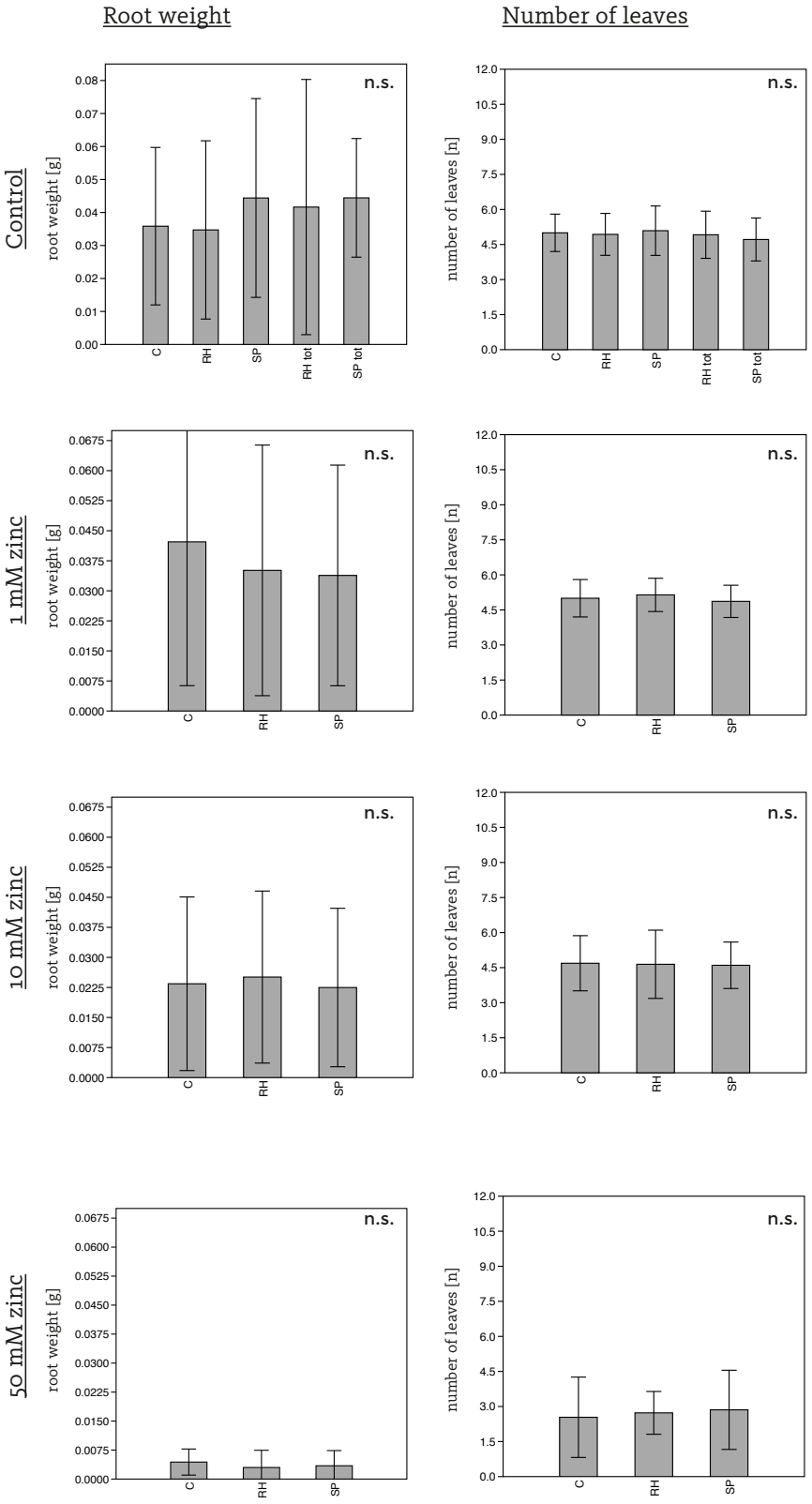
C . . . . . Control
RH . . . . . *Rhodotorula*
. . . . . *mucilaginosa*
RH tot . . *Rh. mucilaginosa*
dead
SP . . . . . *Sporobolomyces sp.*
SP tot . . . *Sporobolomyces sp.*
. . . . . *dead*

Appendix 1:

Triticum aestivum *was grown for 29 days without heavy metals (control), with 1 mM nickel, 5 mM nickel or 10 mM nickel. Plants have been inoculated with* Rhodotorula mucilaginosa *or* Sporobolomyces sp. *In addition, dead yeast cells of both species were added in the control treatment, to evaluate the nutritional affect of dead yeast cells. "n.s." means that differences were not statistically different. Treatments marked with "a" or "b" are significantly different.*

APPENDIX 2
RESULTS POT EXPERIMENT 2, ZINC





C . . . . . Control
RH . . . . . *Rhodotorula*
. . . . . *mucilaginosa*
RH tot . . *Rh. mucilaginosa*
dead
SP . . . . . *Sporobolomyces sp.*
SP tot . . . *Sporobolomyces sp.*
. . . . . *dead*

Appendix 2:

Triticum aestivum *was grown for 29 days without heavy metals (control), with 1 mM zinc, 10 mM zinc or 50 mM zinc. Plants have been inoculated with* Rhodotorula mucilaginosa *or* Sporobolomyces sp. *In addition, dead yeast cells of both species were added in the control treatment, to evaluate the nutritional affect of dead yeast cells. "n.s." means that differences were not statistically different. Treatments marked with "a" or "b" are significantly different.*