Fungicide, nanoparticles, and their combined effect on ectomycorrhizal fungi as non-target organisms

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1 Summary

Sorption can alter the availability of compounds and could explain different findings on pesticide action in laboratory and field conditions. In this thesis, the influence of sorption on the toxicity on non-target organisms was studied. Tailor-made nanoparticles (NPs) and propiconazole were used as model compounds. Propiconazole is a widely used fungicide that inhibits the growth of pathogenic fungi and non-target organisms like ectomycorrhizal fungi. As test organisms, three ectomycorrhizal fungi were utilized that originate from distinct fungal phyla.

The effect of sorption on toxicity was studied in liquid and solid experimental systems. In submerged cultures, three major findings were obtained. First, all three tested fungi responded less sensitively to propiconazole in liquid than in solid cultivation. Second, in the presence of fungi, the amount of freely available propiconazole declined rapidly. In addition, different degradation metabolites were found in varying quantities in the filtrate of each species, which indicates a species-specific fungicide degradation. Third, a combined supply of propiconazole and high NP amounts resulted in slightly increased dry weight levels of the fungi and in greatly increased propiconazole levels in the mycelium. It is assumed that NPs sorbed on the fungal surface and contributed to the dry weight although growth inhibition itself was low. Further, the presence of NPs might trigger fungal uptake of propiconazole or its sorption on the fungal cell. The fungal cell wall might possess a higher sorption affinity for propiconazole than NPs. Due to strive for equilibrium, propiconazole might be continuously released from NPs and cause an elevated attachment on the mycelium. Another possible explanation is that NPs could elicit ROS formation, membrane damage and increased propiconazole permeability.

Fungal cultivation on agar medium and continuous growth monitoring allowed the study of differentiated sorption conditions under sterile conditions. Here, NPs significantly reduced the toxicity of propiconazole in all tested fungi. The observed growth parameters imply reduced fungicide availability in the presence of NPs due to sorption. This was corroborated by HPLC analyses. Propiconazole toxicity occurred in a dose-dependent manner. The higher the applied NP dosage, the lower was the freely available fungicide fraction, and the higher was the fungal growth. Unlike in Ascomycetes, gene expression following an azole treatment has rarely been studied in Basidiomycetes. Existing data on *erg6* and *erg11* gene expression is severely limited and contradicting (Stammler *et al.* 2009, Lee *et al.* 2010). In this respect, the present thesis is the first study to expose a Basidiomycete fungus to propiconazole. This thesis was able to verify that upon exposure to propiconazole, transcription levels of both *erg6* and *erg11* were significantly increased in *L. bicolor*. The levels of upregulation correlated very closely. Therefore, the usage

of *erg6* and *erg11* as marker genes can be recommended. Moreover, NPs mitigated the detrimental effect of propiconazole on the fungal ability to form ectomycorrhizas with poplar.

2 Zusammenfassung

Sorption kann die Verfügbarkeit von Substanzen ändern und könnte die unterschiedlichen Erkenntnisse zur Pestizidwirkung unter Labor- und Feldbedingungen erklären. In dieser Arbeit wurde der Einfluss der Sorption auf die Toxizität von Nichtzielorganismen untersucht. Maßgeschneiderte Nanopartikel (NP) und Propiconazol wurden als Modellverbindungen verwendet. Propiconazol ist ein weit verbreitetes Fungizid, das das Wachstum von pathogenen Pilzen und Nicht-Zielorganismen wie Ektomykorrhizapilzen hemmt. Als Testorganismen wurden drei Ektomykorrhizapilze verwendet, die aus verschiedenen Pilzphyla (Abteilungen) stammen

Die Wirkung der Sorption auf die Toxizität wurde in flüssigen und festen experimentellen Systemen untersucht. Anhand von Flüssigkulturen wurden drei wichtige Erkenntnisse gewonnen. Erstens reagierten in Flüssigkultur alle drei getesteten Pilze weniger empfindlich auf Propiconazol als in fester Kultivierung auf Agarmedium. Zweitens nahm die Menge an frei verfügbarem Propiconazol in Gegenwart von Pilzen rasch ab. Darüber hinaus wurden im Filtrat jeder Art unterschiedliche Abbaumetabolite in unterschiedlichen Mengen gefunden, was auf einen artspezifischen Fungizidabbau hindeutet. Drittens führte eine kombinierte Zufuhr von Propiconazol und hohen NP-Mengen zu leicht erhöhten Trockengewichtsniveaus der Pilze und zu stark erhöhten Propiconazolspiegeln im Myzel. Es wird angenommen, dass NP an der Pilzoberfläche sorbieren und zum Trockengewicht beitragen, obwohl die Wachstumshemmung selbst gering ist. Darüber hinaus könnte das Vorhandensein von NP die pilzliche Aufnahme von Propiconazol oder seine Sorption an der Pilzzelle auslösen. Die Pilzzellwand könnte eine höhere Sorptionsaffinität für Propiconazol als NP besitzen. Aufgrund des Strebens nach einem Gleichgewicht könnte Propiconazol kontinuierlich aus NP freigesetzt werden und eine erhöhte Anhaftung an das Myzel verursachen. Eine andere mögliche Erklärung ist, dass NP ROS-Produktion auslösen könnten, sowie Membranschäden und eine erhöhte Propiconazol-Permabilität.

Die Kultivierung von Pilzen auf Agarmedium und ein kontinuierliches Monitoring des Pilzwachstums ermöglichten die Untersuchung von differenzierten Sorptionsbedingungen unter sterilen Bedingungen. Hier reduzierten die Nanopartikel signifikant die Toxizität von Propiconazol bei allen getesteten Pilzen. Die beobachteten Wachstumsparameter implizieren eine verminderte Verfügbarkeit von Fungiziden in Gegenwart von NP aufgrund der Sorption. Dies wurde durch HPLC-Analysen bestätigt. Die Propiconazol-Toxizität trat dosisabhängig auf. Je höher die angewandte NP-Dosierung war, desto niedriger war die frei verfügbare Fungizidfraktion und desto höher war das Pilzwachstum. Im Gegensatz zu Ascomyceten wurde die Genexpression nach einer Azol-Behandlung bisher selten in Basidiomyceten untersucht.

Vorhandene Daten zur Genexpression von *erg6* und *erg11* sind stark limitiert und widersprüchlich (Stammler et al. 2009, Lee et al. 2010). In dieser Hinsicht ist die vorliegende Arbeit die erste Studie, bei der ein Basidiomycetenpilz Propiconazol ausgesetzt wurde. Diese Doktorarbeit konnte bestätigen, dass bei der Behandlung mit Propiconazol die Transkriptionsniveaus von *erg6* und *erg11* in *L. bicolor* signifikant erhöht waren. Das Niveau der Hochregulation korrelierte sehr stark. Daher kann die Verwendung von *erg6* und *erg11* als Markergene empfohlen werden. Darüber hinaus milderten NP die schädliche Wirkung von Propiconazol auf die Fähigkeit der Pilze, eine Ektomykorrhiza-Symbiose mit Pappel zu bilden.

3 Introduction

Fungicides are chemical compounds that are able to kill fungi or inhibit their growth. Numerous fungi cause various diseases. Therefore, fungicides possess a wide application area. For instance, they are utilized in forest nurseries and afforestation sites to gain control over pathogenic fungi. In the **rhizosphere**, the close area around plant roots, fungicides may affect **non-target** species like **ectomycorrhizal fungi (EMF)** that are known to support tree seedling growth (Björkman 1970, Burgess *et al.* 1993, Bertaux *et al.* 2003b). These EMF live in soil and form symbiotic relationships with trees. Plant roots are colonized and covered by a mantle of dense fungal structures. Single fungal hyphae penetrate plant roots and grow between epidermal and cortical cells to form the Hartig net (Foster and Marks 1966, Smith and Read 1997). In this interface between plant and fungi, water, nutrients, and photoassimilates are exchanged (Foster and Marks 1966, Smith and Read 1997, Hampp and Schaeffer 1999, Finlay 2004). EMF provide an improved water and nutrient supply to plants (Duddridge *et al.* 1980, Marschner and Dell 1994, Smith and Read 1997). Moreover, EMF-associated plants were found to show higher resistance against drought and pathogen stress (Marx 1972, Parke *et al.* 1983).

One fungicide that is used in a large extent in Finnish forest nurseries is **propiconazole**. This triazole fungicide targets 14α demethylase, a key enzyme in ergosterol biosynthesis (Hancock and Weete 1985). Ergosterol is an integral compound in fungal cell membranes that steers membrane fluidity and stability (Weete *et al.* 1983, Henriksen *et al.* 2004). Due to propiconazole exposure, fungal growth is impaired in both detrimental and beneficial fungi. In several cases, laboratory and field experiments with fungicides exhibited contradicting results (Greaves *et al.* 1976, Trappe *et al.* 1984). **Sorption** on soil particles might influence the **toxicity** of a fungicide by modifying its mobility, bioavailability and degradation (Marín-Benito *et al.* 2012, Gámiz *et al.* 2016). The present thesis addresses the question: Does sorption affect the toxicity of propiconazole on EMF? The underlying research was performed within the framework of a joint project involving chemists, biologists, and geologists. The project **Expand** explored the ecotoxicity of particle-associated toxic compounds. **Nanoparticles (NPs)** were designed, characterized and their sorption behavior with propiconazole was studied by team members. In this thesis, the single and combined effect of propiconazole and NPs on three ectomycorrhizal fungal species is described. Fungal growth was investigated both in liquid and in solid cultivation. In submerged cultures, dry weight measurements were implemented. In cultures on solidified agar medium, colony diameter and area measurements were conducted. Moreover, the single and combined effect on the molecular level was analyzed. Here, gene expression of relevant ergosterol biosynthesis genes was evaluated by quantitative real-time PCR (qPCR). In collaborations, propiconazole content of culture filtrate and fungal mycelium was determined.

3.1 Azole fungicides

3.1.1 Azoles

Fungicides are applied in agriculture, forestry, veterinary, and in human medicine to combat pathogenic fungi. In agriculture, fungicides are used to gain control over phytopathogenic fungi that threaten yield. Typical application areas of antifungal agents are cultivations of cereals, beet, corn, potatoes, legumes, vegetables, trees, fruits, herbs, and ornamentals (Schramm 2016). One particular target of fungicides is ergosterol, a sterol and major compound in fungal plasma membranes. Several classes of components with different chemical structures affect and inhibit ergosterol biosynthesis. These include allylamines, thiocarbamates, azoles and morpholines (White *et al.* 1998). Fungicides that inhibit the synthesis of ergosterol are active against Ascomycetes, Basidiomycetes and Deuteromycetes (Siegel 1981).

The present thesis focused on the fungicide propiconazole that belongs to the class of azoles. This major class of fungicides consists of heterocyclic compounds with a five-membered ring that contains at least one nitrogen atom. Azole fungicides are subdivided into two groups (see Figure 1). Imidazoles possess one additional nitrogen atom, whereas, triazoles show three nitrogen atoms (Joseph-Horne and Hollomon 1997). Propiconazole is classified as belonging to the group of triazoles.

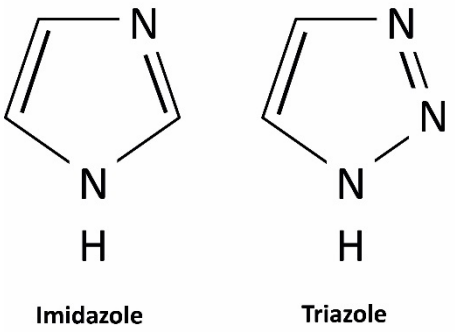

Figure 1: Imidazoles and triazoles are two groups of azole fungicides that contain either two or three nitrogen atoms within the heterocyclic five-membered ring.

It has been possible to purchase azole fungicides since the mid-1960s (Joseph-Horne and Hollomon 1997). The target enzyme of azole fungicides is 14α demethylase, an important enzyme in ergosterol biosynthesis. It is an iron-containing protein of the cytochrome P450 family, whose members are called Cyp proteins (Chambers *et al.* 2014). In the absence of fungicides, 14α demethylase mediates the oxidative removal of the C-14 methyl group from lanosterol or eburicol (Joseph-Horne and Hollomon 1997). In filamentous fungi and yeasts, azoles inhibit the transformation of lanosterol to ergosterol. Likewise, propiconazole impedes the C-14

demethylation during ergosterol or brassicasterol biosynthesis (Weete *et al.* 1983, Weete and Wise 1987). Exposure to azole fungicides results in a shortage of ergosterol and an accumulation of lanosterol and C-14-methylated sterols (Weete and Wise 1987). Consequently, membrane assembly and structure are impaired. Sterols play an important role in the maintenance of membranes, especially their fluidity and stability (Mouritsen and Jørgensen 1994, Joseph-Horne and Hollomon 1997, Rog *et al.* 2008). Furthermore, the allocation and activity of membrane-bound enzymes or ion channels can be affected (Calonne *et al.* 2012). The triazole fungicide propiconazole was observed to induce the chitinase activity of the white rot fungus *Trametes versicolor* at the beginning of wood colonization (Lekounougou *et al.* 2008). Studies revealed that the azole imazalil immediately (within 30 min) prevented the incorporation of [^{14}C] acetate into C-4 desmethyl sterols (ergosterol) in *Aspergillus nidulans* (Siegel and Ragsdale 1978). The effect on mycelial dry weight increase in this fast-growing fungus was visible after 2.5 - 3 h, when growth was severely reduced (Siegel and Ragsdale 1978).

3.1.2 Propiconazole

Propiconazole is widely used for the purpose of plant protection, as it shows a broad antifungal spectrum (Schwinn 1984, Kast-Hutcheson *et al.* 2001, Taxvig *et al.* 2008, Al-Hatmi *et al.* 2016). It is a systemic fungicide that is absorbed by plants and transported upstream in the plant (Syngenta 2005). In the year 2016, the German Federal Office of Consumer Protection and Food Safety (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL) recorded 100 - 250 tons of propiconazole being exchanged in professional domestic sales. Less than 1 ton was exported (BVL 2017a).

In Germany, propiconazole is distributed as a single active ingredient (e.g., Achat, Tilt 250 EC, Bolt XL, Desmel) or in combination with various other fungicides (BVL 2017b). In commercial plant protection products, propiconazole is augmented with azoxystrobin (QUILT XCEL), with difenoconazole (Taspa), with fenpropidin (AGENT), with prochloraz (Cirkon), or with both tebuconazole and fenpropidin (GLADIO) (BVL 2017b). In arable farming, propiconazole is applied against mildew, rust and other diseases in wheat, barley, rye, triticale, winter oats, corn, grasses, and sugar beet (BVL 2017b). In addition, propiconazole is used with ornamentals (BVL 2017b). In countries outside Germany, propiconazole is used to protect the production of legumes, cereals, fruit, rice, sugar beet, ornamentals, and flowers. In the United States, almost 90 % of the fungicide use in wheat cultivation involves propiconazole (Garry *et al.* 1996). Cereals are treated with propiconazole at a concentration of 125 g/ha to control foliar and seed-borne diseases. Typical target organisms for propiconazole application are *Erysiphe graminis*, *Rhynchosporium secalis, Pyrenophora teres, Setosphaeria turcica*, *Drechslera teres*, *Drechslera tritici-repentis*, *Helminthosporium sativum*, *Cercospora arachidicola*, *Cercosporidium personatum*, *Puccinia*

spp., *Cladosporium* spp., and *Septoria* spp. (Schwinn 1984, Hancock and Weete 1985, BVL 2017b). A further example is *Fusarium oxysporum* f. sp. *cubense* that endangers yield in banana plantations (Nel *et al.* 2007). Propiconazole, or its combined use with another fungicide, was reported to restrain the growth of *F. oxysporum* (Nel *et al.* 2007). Additionally, root dipping of plantlets in a suspension of propiconazole significantly reduced disease severity (Nel *et al.* 2007).

In forest nurseries, the spread of fungal infestations is very common due to high humidity and dense cultivation of seedlings in great numbers and confined areas (Stenström and Arvidsson 2001). Fungicides are employed to gain control over fungal infections in tree seedlings (Crous *et al.* 1991, Stenström and Arvidsson 2001, Zambonelli and Iotti 2001, Rai and Mamatha 2005). As wilt diseases are able to spread in the vascular system of tree species, some fungicides are injected directly into the stem of the tree. Elms, oaks or laurel are treated this way with propiconazole (Mayfield III *et al.* 2008). In Finland, propiconazole is one of the most frequently applied fungicides in forest nurseries to prevent the spread of pathogens during seedling growth (Laatikainen and Heinonen-Tanski 2002). Apart from forestry, various fungicides including propiconazole are used as preservatives to reduce wood decay by fungi and other materials (Woo *et al.* 2010, Fernández-Calviño *et al.* 2017).

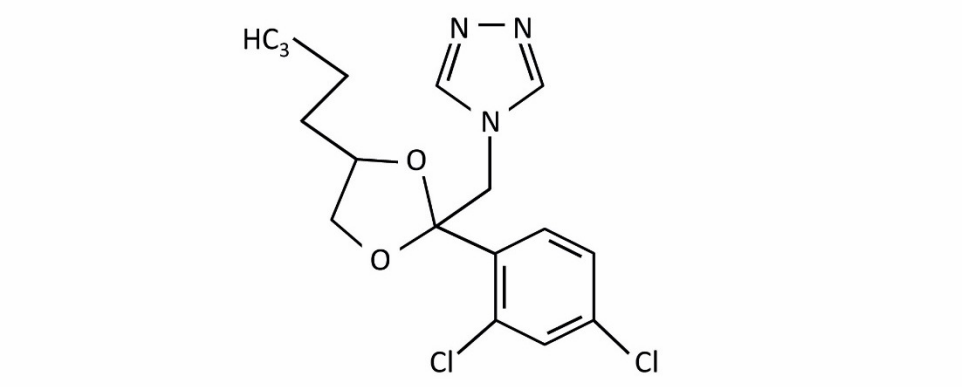

Figure 2: Structure of propiconazole.

Propiconazole isalso referred to as 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole. Propiconazole has the molecular formula $C_{15}H_{17}Cl_2N_3O_2$ and a molecular weight of 342.2 g/mol. Its structure can be seen in Figure 2. This triazole fungicide is active against yeast and most filamentous fungi. It targets the 14α demethylase enzyme and inhibits ergosterol biosynthesis (Hancock and Weete 1985). In the plant pathogenic fungus *Taphrina deformans*, propiconazole was found to inhibit the demethylation of lanosterol and the synthesis of its main sterol, brassicasterol (Weete *et al.* 1983). Brassicasterol, also ergosta-5,22-dienol, is chemically very close to ergosterol or ergosta-5,7,22-trienol (Weete *et al.* 1983). Upon propiconazole treatment, membrane fluidity, structure, and fungal growth are disturbed.

The mobility of propiconazole is categorized into medium to low class. In field soil, propiconazole has a half-life of up to 499 days (Bromilow *et al.* 1999, Yen *et al.* 2009, Calonne *et al.* 2010, Garrison *et al.* 2011, Oliver *et al.* 2012). The lab soil photolysis half-life is 53 - 150 days (Syngenta 2005). The fungicide is considered to be quickly absorbed by soil where it is metabolized by soil microorganisms. The aerobic sediment/water dissipation rate (DT_{50}) is 270 - 427 days (Syngenta 2005). Nevertheless, propiconazole is known to leak or run off from treated areas (Mortensen *et al.* 1998, Egaas *et al.* 1999, Juntunen and Kitunen 2003).

3.1.3 Uptake of propiconazole

Propiconazole is intended for foliar or soil drench applications. It is soluble in water. The fungicide is absorbed by roots or leaves of treated plants. It is transported in the apoplast (Siegel 1981, Stenström and Arvidsson 2001). Within the plant, translocation proceeds through the xylem from lower to upper parts of the plant (Syngenta 2018). It is assumed that the systemic fungicide interacts with sterol carrier proteins (Siegel 1981) and subsequently prevents enzymatic reactions of 14α demethylase.

In *Candida albicans*, the uptake of the antifungal drug ketoconazole was studied (Boiron *et al.* 1987). Low external concentrations led to an active accumulation of ketoconazole within cells that required energy derived from glycolysis. At higher concentrations, ketoconazole was taken up via passive diffusion in *Candida albicans* (Boiron *et al.* 1987). This is probably also the case for propiconazole.

3.1.4 Mode of action of azole fungicides

Azole fungicides are potent sterol biosynthesis-inhibiting agents. They interact with 14α demethylase, a membrane-associated cytochrome P450 protein also referred to as Cyp51 or Erg11. Sterol biosynthesis occurs in all eukaryotes and is mainly absent in prokaryotes. Sterols are crucial for proper membrane function, as they control fluidity and flexibility of plasma membranes (White *et al.* 1998). In animals, the predominant sterol is cholesterol; in fungi, it is ergosterol, and in plants, the main sterol is phytosterol. Furthermore, sterols are precursor molecules for vitamins and steroids, including sex hormones, glucocorticoids, and mineralocorticoids.

The enzyme 14α demethylase is located in the smooth part of the endoplasmic reticulum where sterol biosynthesis occurs, starting from acetyl CoA, HMG CoA, mevalonate, squalene, and lanosterol (in animals and yeast), eburicol (in filamentous fungi, with lanosterol as precursor) or obtusifoliol (in plants) (Podust *et al.* 2004). The latter compounds are substrates for 14α demethylase. This Cyp enzyme catalyzes the oxidative removal of a methyl group of the respective substrate with release of water.

In *Botrytis cinerea*, sterol biosynthesis was studied in the presence of tebuconazole. The triazole fungicide was found to reach its target within 1 to 2 h (Pontzen and Scheinpflug 1989). The Cyp enzyme holds a haem moiety in its active site (White *et al.* 1998). A nitrogen atom of the azole agent binds to the iron atom of the haem. As a consequence, the oxidative demethylation of lanosterol is hindered. A second nitrogen of the azole is assumed to act with the apoprotein and to define the specificity between a certain azole fungicide and the Cyp enzyme (White *et al.* 1998).

The inhibition of the Cyp 14α demethylase results in a shortage of C-4 desmethyl sterols, including the major sterol in the respective organism (Van den Bossche *et al.* 1987). Other sterols pile up, such as C-14 methylsterols (Hancock and Weete 1985, Griffiths *et al.* 2003). Among those C-14 methylsterols are obtusifoliol, eburicol, 24-methylene-dihydrolanosterol and 14α-methylfecosterol in fungi (Hancock and Weete 1985, Joseph-Horne *et al.* 1996). In *Cercospora* and *Cercosporidium*, propiconazole led to an increase of total sterol content (Hancock and Weete 1985). In *Leptosphaeria maculans*, the antifungal flutriafol caused an increase of obtusifoliol and 24-methylene-dihydrolanosterol (Griffiths *et al.* 2003). Besides this, there are reports on the accumulation of toxic sterols like 14α-methyl-3,6-diol that disturb membrane structure as well as membrane function and result in growth inhibition (Kelly *et al.* 1995, Venkateswarlu and Kelly 1996, Abe *et al.* 2009).

The effect of propiconazole is a decrease in ergosterol levels and a reduced fungal growth (Sancholle *et al.* 1984, Hancock and Weete 1985, Fernández-Calviño *et al.* 2017). As the main sterol in fungal plasma membranes, ergosterol is involved in membrane integrity and the function of membrane-bound enzymes (White *et al.* 1998). Moreover, small amounts of ergosterol are essential for a normal cell cycle (White *et al.* 1998). Another effect of propiconazole is an elevated glucosamine content of the fungal cell wall (Hancock and Weete 1985). The content of free fatty acids can decrease due to propiconazole exposure (Hancock and Weete 1985). As composition and structure of the fungal cell wall is altered, the cellular permeability is also affected. Uptake and leakage of nutrients are changed upon propiconazole treatment (Sancholle *et al.* 1984).

3.1.5 Environmental concentrations of propiconazole

Propiconazole gets in contact with soil directly after spraying or indirectly when foliar treated plants are ploughed. Subsequently, the fungicide is released through degradation or is leached into the ground. In soil, propiconazole has a half-life ranging from about 100 to 500 days depending on soil type, temperature, moisture content and microbial activity (Bromilow *et al.* 1999). In soil with low organic carbon content, mobility of this pesticide is considered to be moderate (Singh 2005). Consequently, propiconazole is able to accumulate in the ground, especially in areas of repeated application.

Surface runoff and spray drift are the main entry routes of triazoles to the aquatic environment. Small volumes of fungicides are transported to rivers via wastewater effluents (Chambers *et al.* 2014). Propiconazole is reported to leak out from treated agricultural fields with concentrations of 0.1 - 10 µg/L in runoff waters (Egaas *et al.* 1999). In the surface runoff from an experimental field, 0.56 µg/L propiconazole were detected, whereas the drainage water contained 0.28 µg/L propiconazole (Wu *et al.* 2004). In surface water close to banana cultivations, 24 µg/L propiconazole was found (Mortensen *et al.* 1998). In Sweden, up to 20 µg/L propiconazole were detected in the stream water that was regularly sampled at three sites (Kreuger 1998). In the sediment of river beds in Denmark, up to 130 µg/kg propiconazole were found in the top 1 - 2 cm layer (Kronvang *et al.* 2003). In Norway, streams and rivers revealed an average concentration of 0.28 µg/L propiconazole (Ludvigsen and Lode 2005). The maximum concentration of 1473 analyzed samples was 7.7 µg/L propiconazole (Ludvigsen and Lode 2005).

The octanol-water coefficient (*Kow*) describes the distribution of a chemical in a two-phase system of octanol and water and indicates the water or fat solubility of this compound. Propiconazole has a log *Kow* of 3.7. The fungicide is a lipophilic compound and is assumed to accumulate in the fat tissue of organisms. In fish, propiconazole has a bioaccumulation factor of 130 (Egaas *et al.* 1999). This is equivalent to a concentration of 130 mg propiconazole per kg fish.

3.1.6 Non-target effects and molecular stress marker

Only a small fraction of fungi is considered to be a frequent or severe threat to plants as pathogens, approximately up to 100 species out of estimated 2.5 x 10^5 fungal species (Elmholt 1991). However, as azoles possess a broad activity spectrum, they naturally affect non-target **fungi,** such as saprophytes (Elmholt 1991) or mycorrhizal fungi in terms of growth and mycorrhiza formation (Trappe *et al.* 1984, Marx *et al.* 1986, Von Alten *et al.* 1993). The standard dosage for field application of propiconazole is 0.25 µg/g soil dry weight. At this concentration, mycorrhization of arbuscular mycorrhizal fungi (AMF) was not affected (Kling and Jakobsen 1997). Other studies found a decreased number of arbuscular mycorrhizas with *Glomus etunicatum*, *G. mosseae*, and *G. intraradices* at the recommended application rate of propiconazole (Von Alten *et al.* 1993). Additionally, propiconazole decreased the spore production of *G. mosseae* (Nemec 1985). Changes in sterol content and composition may also occur in response to propiconazole. Further, AMF were reported to have low ergosterol content (Frey *et al.* 1994, Kling and Jakobsen 1997). In *Botrytis cinerea*, *Pyrenophora teres* and *Ustilago maydis*, the ergosterol content decreased upon propiconazole treatment, whereas 14α methyl sterols such as eburicol, obtusifoliol and 14α methylfecosterol accumulated (Loeffler and Hayes 1992). However, these fungal species were also found to respond differently. While the total sterol content decreased in one species, it increased in others (Loeffler and Hayes 1992).

Although the target enzyme is a highly conserved among species, smaller differences in the apoprotein structure regulate the exact extent of the interaction with the fungicide, which in turn is decisive for the effectiveness of the toxic compound (Odds *et al.* 2003).

Due to unspecific activity of some azoles, **soil bacteria and protozoa**, such as amoebas and flagellates can be affected as non-target organisms. Propiconazole is known to manipulate the soil microbial community structure and activity (Yen *et al.* 2009, Fernández-Calviño *et al.* 2017).

Since **plants** and other organisms have a variety of Cyp proteins, these might be impaired during azole treatment. Triazole fungicides may interfere with the isoprenoid pathway in plants. The biosynthesis of gibberellin, for example, comprises several steps that are catalyzed by cytochrome P450 enzymes (Davidson *et al.* 2006). The levels of certain plant hormones may be affected and cause morphological changes like reduced plant growth (Siegel 1981, Steffens 1988, Calonne *et al.* 2012). The triazole fungicide penconazole completely hindered root biomass increase and reduced shoot biomass increase in Norway spruce seedlings (Koele and Hildebrand 2011). Maize seedlings were also reported to show both a decreased stem and root length when exposed to 5 mg/L propiconazole (Taton *et al.* 1988). Moreover, triazoles have been shown to affect plastid development and chlorophyll synthesis (Wolf 1962). Even histidine synthesis is affected by triazoles in plants (Siegel and Gentile 1966). Propiconazole was shown to reduce pollen germination and pollen tube growth of *Tradescantia virginiana* at concentrations of 42.64 - 71.06 mg/L in a strictly dose-dependent way (He *et al.* 1995). In addition, propiconazole was found to have an impact on the distribution of microfilaments and microtubules (He *et al.* 1995). Apart from this, propiconazole caused the swelling of mitochondria and enhanced the senescence of cytoplasm in Scots pine (Manninen *et al.* 1998). In long root tips close to the meristematic area, the fungicide enhanced the formation of vacuoles and the accumulation of tannin therein (Manninen *et al.* 1998). Furthermore, it is known that triazole fungicides may increase ion leakage, such as leakage of K^+ or Cl^- (Radice and Pesci 1991). These fungicides are likely to affect the composition of plant membranes which in turn can affect membrane permeability. In Sudan grass and in orange plants, a correlation was observed between a lower phospholipid content and an increased membrane permeability and an increased root exudation of K^+ ions, amino acids and sugars (Ratnayake *et al.* 1978). These effects may in turn affect soil organisms living in the rhizosphere.

In **humans and animals**, the 14α demethylase enzyme is involved in the synthesis of cholesterol, a constituent of cell membranes. Moreover, cholesterol is a precursor of vitamin D and steroid hormones. Azole fungicides were reported to inhibit the aromatase enzyme and the synthesis of vitellogenin in female zebrafish and female fathead minnow (Chambers *et al.* 2014). Female amphibians were observed to masculinize in the presence of azoles (Chardard and Dournon 1999). Similarly, sex differentiation in reptiles can be influenced by azoles (Chambers *et al.* 2014). In

addition, the embryonic development of *Daphnia magna* was reported to be adversely affected by propiconazole (Kast-Hutcheson *et al.* 2001, Soetaert *et al.* 2006). Abnormal antennae and shell spines developed, as well as dead embryos. Embryo toxicity tests (48 h) with daphnids showed an LC_{50} of 5 mg/L propiconazole, a concentration that is lethal to 50 % of the exposed organisms (Kast-Hutcheson *et al.* 2001). The concentration that was lethal to 10 % of the daphnids (48 h LC_{10}) was 1.2 mg/L propiconazole (Kast-Hutcheson *et al.* 2001). Alongside this, in *Daphnia magna*, propiconazole altered both transcription and translation of genes that are involved in cell processes, embryonic development, energy metabolism, and stress response (Soetaert *et al.* 2006). Furthermore, brown trout displayed an enhanced level for hepatic Cyp1a protein after exposure to 313 µg/L propiconazole (Egaas *et al.* 1999). The authors observed a non-linear dose-response relationship between propiconazole and the induction of the protein synthesis. After 14 days, a significantly elevated Cyp1a activity towards ethoxyresorufin-O-deethylase (EROD) was found at a concentration of 93 µg/L propiconazole or higher (Egaas *et al.* 1999). In addition, the activity of glutathione-S-transferase was increased (Egaas *et al.* 1999), which indicates the induction of defense mechanisms. Glutathione-S-transferase is involved in the binding and transportation of toxins as well as in their detoxification.

Molecular markers indicate various specific reactions. The aim of the present thesis was to test several genes for their suitability as marker genes. Upon exposure to triazole fungicides, the expression of genes involved in the ergosterol biosynthesis is known to be altered. Therefore, this genetic pathway seems promising for the detection of fungicide stress at low concentrations, when there are no or only slight phenotypic reactions. Molecular markers can display changes prior to alterations in macroscopic outward appearance. In fungi, expression of *erg11* and *erg6* genes have been shown to be influenced by azole fungicides (Henry *et al.* 2000, Liu *et al.* 2010). In this thesis, one ectomycorrhizal fungus, *Laccaria bicolor*, was tested for its expression of *erg11* and *erg6* under different propiconazole conditions and the capability of these genes to function as marker genes.

3.2 Rhizosphere

The area of soil in the proximity of living plant roots is referred to as the rhizosphere. Here, fungi exist next to bacteria, archaea, protozoa and animals like nematodes or earthworms. Plants influence the soil surrounding their roots and the organisms that live there by exudation and secretion of so-called rhizodeposits. Plants release 10 to 40 % of photosynthetically fixed carbon into the soil (Grayston *et al.* 1997). Root exudates include water-soluble amino acids, organic acids, sugar, vitamins and phytohormones. These compounds reach the soil without any metabolic energy (Lynch and Whipps 1990). By contrast, secretion requires metabolic energy. Examples for secreted compounds are carbohydrates, enzymes and the root mucilage consisting of mono-

and polysaccharides that protects the very root cap. Via autolysis of root cells, cell ingredients and cell wall compounds are released into the soil. Gases like ethylene and CO_2 are released as well (Lynch and Whipps 1990). Root exudation and secretion can be highly variable depending on plant species, plant age, season and environmental conditions. Roots represent a living source of nutrients for the rhizosphere fauna. Microorganisms are attracted to or deterred by the compounds plants release into the soil. Thus root deposits shape community structure of bacteria or fungi (Philippot *et al.* 2013). Moreover, some rhizodeposits stimulate symbiotic interactions. The organisms in the rhizosphere, in turn, can affect plant nutrition, growth, health and the composition of plant community (Philippot *et al.* 2013). For example, the soil bacterium *Streptomyces* strain AcH 505 is known to excrete auxofuran which promotes the growth of *Amanita muscaria* and supports the formation of ectomycorrhizal structures (Riedlinger *et al.* 2006).

In addition to biotic interactions, the organisms in the rhizosphere are influenced by soil type, agricultural practices, and climatic conditions. The soil type impinges on plant performance and root exudation, which, in turn, affects composition and activity of bacterial and fungal communities. Due to root exudates, the soil in the rhizosphere is carbon-rich and enables high activity of microorganisms (Norton and Firestone 1991). This involves denitrification (Philippot *et al.* 2013), decomposition (Courty *et al.* 2010) or degradation (Cairney and Meharg 2002). Fungal degradation of organic matter depends on the fungal community structure and diversity, which varies with season and soil depths (Courty *et al.* 2010). Some fungal and bacterial species are able to decompose pollutants like persistent aromatic compounds and are, therefore, of certain interest for environmental remediation (Cairney and Meharg 2002).

Several bacteria have a stimulating effect on plants. So-called plant growth-promoting rhizobacteria (PGPR) compete with pathogenic and harmful organisms, crowd them out, and thus have a beneficial effect on plant biomass increase or plant health. PGPR were found to secret siderophores that complex iron and reduce its availability for some organisms (Kloepper *et al.* 1980). Besides this, some PGPR are able to fix free nitrogen or produce phytohormones (Fitter and Garbaye 1994). Bacteria are also found in close association with mycorrhizas, a symbiotic interaction between a fungus and plant roots. These so-called mycorrhiza helper bacteria support the development of mycorrhizas. Some mycorrhiza helper bacteria were reported to increase the formation of lateral roots (Schrey *et al.* 2005). Furthermore, an enhanced dichotomous branching of short roots was observed in the presence of mycorrhiza helper bacteria (Aspray *et al.* 2006).

3.2.1 Mycorrhiza

The four most common classes of mycorrhiza associations are arbuscular mycorrhiza, ectomycorrhiza, ericoid mycorrhiza and orchid mycorrhiza. The focus of the present thesis is exclusively on ectomycorrhiza (ECM). In the beginning of this mutual interaction, fungal hyphae encounter plant root cap cells, attach to the epidermis, and surround the root by forming a mantle structure that contains different layers of fungal hyphae. Some hyphae invade the root, widen the inner space, and form a network of highly ramified hyphae between epidermis and root cortex cells. This structure is called a Hartig net and represents an enormous apoplastic interface between the plant and fungi. Here, nutrients and water are exchanged. ECM is characterized by root tip swelling, the establishment of a Hartig net and root growth arrest (Smith and Read 1997).

Interestingly, only about 3 % of all seed plant species are ectomycorrhizal (Smith and Read 1997). Nonetheless, ectomycorrhizal plant species are of enormous relevance, as they cover a disproportionately large proportion of the earth's surface and represent the main producers of wood (Smith and Read 1997). In temperate and boreal forests, ECM is the most prevalent form of mycorrhiza (Smith and Read 1997). Certain plant families are obligate ectomycorrhizal like *Fagaceae* and *Pinaceae*. There are about 6,000 plant species that form mutual symbioses with more than 20,000 ectomycorrhizal fungal species (Martin *et al.* 2016). All genera of two gymnosperm families are known to form ECMs, namely *Gnetaceae* and *Pinaceae* (Brundrett 2009). Some mycorrhizal fungi are host specific and only form mycorrhizas with one plant species. Other fungi like *L. bicolor* and *A. muscaria* have a broader host range. In forests where spruce trees dominate, about 150 mycorrhizal fungal species exist, whereas oak forests harbor about 100 species, and older forests contain about 50 species (Egli and Brunner 2002).

In the rhizosphere, crosstalk between plant and fungi is used to establish mycorrhizal symbiosis. Plant derived flavonoids, for example, were found to stimulate spore germination of EMF (Lagrange *et al.* 2001, Kikuchi *et al.* 2007). In *L. bicolor*, the plant flavonoids rutin and quercetin induce the biosynthesis of MiSsp7, the mycorrhiza-induced small secreted protein 7, which is essential for symbiosis development with *Populus trichocarpa* (Plett *et al.* 2011, Plett and Martin 2012). In addition, EMF are able to produce auxin and thus alter plant metabolism and root growth. During ECM, the growth of the main root is retarded, whereas lateral root growth is enhanced (Felten *et al.* 2009, Splivallo *et al.* 2009). Often, an increased number of short fine roots occur that apparently lack a hairy root zone, as fungal hyphae form a compact mantle around roots. In addition, hyphae penetrate between epidermal cells to form the Hartig net, which gives the root tips a swollen appearance. In angiosperms, the Hartig net is formed around epidermal root cells, whereas in gymnosperms (*Pinaceae*), the Hartig net is found between epidermal and cortical root cells.

During symbiosis, plant and fungal cell walls are in direct contact and form the apoplastic interface, where signal molecules, carbons, and nutrients are exchanged. Up to 30 % of the carbon assimilated by photosynthesis is present in the plant-fungal interface. In *A. muscaria*, the gene encoding the monosaccharide transporter AmMst1 was found to be four-fold upregulated in mycorrhizas compared to external mycelium (Nehls *et al.* 1998). Higher concentrations of glucose as well as arabinose and ribose enhanced the expression of the gene encoding AmMst1 (Nehls *et al.* 1998). Sucrose can be converted into glucose and fructose by the plant-derived invertase enzyme (Nehls *et al.* 2007). Some fungi like *L. bicolor* are assumed to depend on photoassimilated carbohydrates from plants, as their genome seems to lack the capacity to efficiently degrade lignocellulose (Deveau *et al.* 2008, Martin *et al.* 2016).

By the means of mycorrhizal symbiosis, plants gain better water and nutrient access, as well as an enhanced resistance towards biotic and abiotic stresses, such as pathogens (Sylvia and Sinclair 1983) or drought (Parke *et al.* 1983). Furthermore, ECMs render plants more resistant towards metallic contaminants, as toxic ions are held back by fungal mycelium (Wilkins 1991). Nitrogen and phosphorus are limiting factors for plant growth. To improve P uptake, plants excrete organic acids that chelate Al, Fe and Ca ions from insoluble phosphate compounds and so release P (Dakora and Phillips 2002). Fungal hyphae form a widespread network of fine branches into the soil and enlarge the access to nutrients. Fungi are able to excrete organic acids or siderophores to chelate nutrients and promote the availability of ions that would not be accessible for plants (Szaniszlo *et al.* 1981, Lapeyrie *et al.* 1991, Landeweert *et al.* 2001). Fungi absorb nutrients that eventually may be transported to plant roots. EMF improve the uptake of K, P, NH_4^+, NO_3^- (Marschner and Dell 1994), Mg (Jentschke *et al.* 2000), and Ca (Blum *et al.* 2002). In ectomycorrhizal aspen (*Populus tremuloides*), *L. bicolor* increased P acquisition in the presence of low phosphate contents (Desai *et al.* 2014). To release P from soils or organic complexes, fungi excrete acid or enzymes like acid phosphatase (Marschner and Dell 1994).

3.2.2 Mycorrhizal fungi

Mycorrhizal fungi are found in the bulk soil. However, the majority of fungal mycelia are in the rhizosphere or within plant roots. Fungi interact with plants, bacteria, other fungi, pathogens, protozoa, nematodes, arthropods and animals (Fitter and Garbaye 1994). These interactions are of a stimulating or inhibiting nature. Animals, earthworms or springtails may graze on fungal fruit bodies or spores and thus distribute spores (Fitter and Garbaye 1994). Soil bacteria and fungi may hinder or promote fungal spore germination and hyphal growth (Azcon-Aguilar *et al.* 1986, Wilson *et al.* 1989). Soil organisms compete for nutrients: some are pathogens, while others participate in the decomposition of organic matter, and so alter nutrient conditions in soil. This complex network among organisms is able to influence fungal growth and the formation of mycorrhizas (Fitter and Garbaye 1994). Some bacteria specifically promote mycorrhiza

development and functioning. These mycorrhiza helper bacteria are found closely associated with hyphal mantles that surround root tips. Common mycorrhiza helper bacteria are pseudomonads and bacilli (Duponnois and Garbaye 1991).

A. muscaria is frequently associated with *Picea abies*. The fungal cell wall extract of *A. muscaria* was shown to induce chitinase activity in *Picea abies* cell cultures (Sauter and Hager 1989). Chitinase activity is a part of the plant defense system and is able to degrade chitin, a major compound in fungal cell walls. During mycorrhiza establishment, plant and fungal cell walls are partly degraded to form an apoplastic interface where the transfer of water and nutrients takes place. Fungal hydrolases like pectinase and cellulase are involved in breaking down plant cell walls (Sauter and Hager 1989).

Within 24 h of direct contact between *L. bicolor* and the trembling aspen (*Populus tremuloides*), the fungal gene coding for aquaporin 1, LbAqp1, is 700-fold upregulated (Navarro-Ródenas *et al.* 2015). LbAqp1 was found to be responsible for the correct expression of the gene encoding the MiSsp7 protein and the Hartig net development (Navarro-Ródenas *et al.* 2015). Heterologously expressed in yeast, LbAqp1 was involved in the transport of CO_2, NO and H_2O_2, which might participate in the signaling process for relevant gene expression and ECM development (Navarro-Ródenas *et al.* 2015).

With respect to their genetic background, many mycorrhizal fungi lack the ability to degrade lignocellulose. Especially in ectomycorrhizal Basidiomycetes, there is a deficiency in enzymes necessary for lignin and cellulose degradation (Tedersoo *et al.* 2010). By forming a mantle of hyphae around tree roots, EMF gain carbon-containing photoassimilates and other root exudates. Indeed, during ectomycorrhizal symbiosis with *Populus tremula* × *tremuloides*, an increased hexose uptake capacity was found in *L. bicolor* and *A. muscaria* (Fajardo López *et al.* 2008).

3.3 Nanoparticles and sorption

3.3.1 Categories of nanoparticles

Nanomaterials are usually defined as small particles with a size of 1 - 100 nm in at least one dimension. NPs can be divided into two groups according to their core materials (Ju-Nam and Lead 2008). On the one hand, there are carbon-based NPs. Among them are multiwall and single-wall carbon nanotubes as well as fullerenes. On the other hand, there is the group of inorganic NPs that consist of metal oxides, metals, and quantum dots (Ju-Nam and Lead 2008). Quantum dots are small structures that consist of semiconductor materials and show unique optic and electronic properties. Another way to categorize NPs is based on their origin. There are NPs that have existed naturally since the formation of the earth. Subgroups of these are NPs that are

released unintentionally by human and non-human activity (Handy *et al.* 2008, Navarro *et al.* 2008). In contrast, engineered NPs are produced on purpose (Handy *et al.* 2008). Natural NPs are soil and desert dust, as well as volcanic or star dust particles. Additionally, natural particles include aerosols from pollen, plant fragments, fungal spores, viruses, or forest fires (Navarro *et al.* 2008). Furthermore, soot, black or carbon particles are formed as a result of anthropogenic activity, e.g., the burning of fossil fuels, welding, or the use of vehicles and power plants (Navarro *et al.* 2008). The soot particles that arise from the burning of plant vegetation or fossil fuels have a size ranging from several nm to µm (Nowack and Bucheli 2007). In view of ecotoxicity, not only NPs are of specific interest but also particles with a larger size.

3.3.2 Application area

In recent years, NPs have gained enormous attention. About 1,000 tons of manufactured NPs were produced worldwide in 2004 (Navarro *et al.* 2008). In 2005, about $10 billion was globally invested in nanotechnologies (Navarro *et al.* 2008). There is an expectation that about 60,000 tons of NPs per year will be globally engineered in the year 2020 (Maynard 2006).

There is a wide variety of application areas for NPs: electronics, technical and optical devices, fuel cells, biosensors, biomedicine, pharmaceutics, cosmetics, personal care and household products, food packaging, textiles, material science, environmental analysis, and remediation (Handy *et al.* 2008, Ju-Nam and Lead 2008, Navarro *et al.* 2008). In medicine, engineered NPs are of certain interest given that they can act as carriers for drugs, but NPs can also be used in cancer diagnosis or as magnetic resonance imaging agents.

In the following paragraph, several NPs including their application are exemplarily listed. Solid lipid NPs are employed as carriers for cosmetic ingredients (like coenzyme Q10, retinol and vitamin E) and are able to increase skin elasticity and smoothness (Müller *et al.* 2000). In cosmetics and sunscreens, metal oxide NPs based on TiO_2, Fe_2O_3 and ZnO are used (Nowack and Bucheli 2007, Huang *et al.* 2008, Ju-Nam and Lead 2008). Silver is famous for its antimicrobial effect, and silver-containing NPs (Ag NPs) are used in cosmetics and medicine (as coatings for catheters, for example) (Ju-Nam and Lead 2008). In textiles, Ag NPs are often used to reduce sweat and odor production. Furthermore, NPs can be applied for the purpose of remediation. Zero valent iron NPs gained in importance, as they were found to adsorb arsenic- and chromium-contaminated soils and water (Cundy *et al.* 2008).

3.3.3 Characteristics and NP behavior

The main characteristics of NPs are their small size, relatively high specific surface area, and high surface reactivity. Further features are surface charge and NP composition. It is important to mention that particles with a different size exert a different toxicity even if they consist of the same material. Moreover, shape plays a major role in the activity of NPs. Triangular Ag NPs showed a higher bactericidal effect on *Escherichia coli* than spherical or rod-like Ag NPs (Pal *et al.* 2007). Apart from this, NPs exhibit specific chemical and physical properties like differing electronic states, catalytic reactivity, as well as magnetic and optical properties (Handy *et al.* 2008).

NP surface properties are decisive for NP aggregation, mobility, and interaction with other materials or organisms. The surface charge itself is influenced by temperature, pH, ionic strength, NP size and concentration (Dunphy Guzman *et al.* 2006, Navarro *et al.* 2008). In general, particles in dispersion undergo aggregation and separation processes steered by Brownian motion, shear flow and differential settling (Handy *et al.* 2008). These processes involve the attachment of single particles or clusters of the same and different material. Particle spreading is controlled by Brownian motion, temperature and particle concentration (Handy *et al.* 2008). Attractive and repulsive forces like van der Waals forces, hydrophobic interaction forces or electrostatic diffuse double layer potential contribute as well (Handy *et al.* 2008). Moreover, particle shape, surface quality and surface charge heterogeneity influence particle collision, attachment, aggregation or dispersion (Handy *et al.* 2008).

NPs can be manufactured in such a way that they are soluble by means of surface modification or encapsulation (Ju-Nam and Lead 2008). Often, engineered NPs are coated with organic or inorganic compounds to facilitate good dispersion. To maintain a stable suspension, Ag NPs were reported to be coated with a surfactant (Mafune *et al.* 2000). Metallic-engineered NPs often possess a coating that provides a negatively charged surface in aqueous environments (Navarro *et al.* 2008). Carbon nanotubes and fullerenes are hydrophobic and, therefore, prone to deposition and aggregation as they interact with other hydrophobic or amphiphilic compounds (Navarro *et al.* 2008). With the help of NP functionalization, NP surfaces are equipped with polar groups to acquire NP solubility (Chiang *et al.* 1996, Navarro *et al.* 2008). In sewage and water treatment plants, multi-charged polymers are implemented to bind and remove particles (Handy *et al.* 2008).

In the natural environment, NPs are likely to interact with natural particles or natural organic matter (NOM). The latter consists of polysaccharides, proteins, nucleic acids, humic acids, or lipids and is often released from algae, roots, bacteria or fungi (Navarro *et al.* 2008). NPs can adsorb on any surface like sediments, soils, biofilms, plants, microbes, or animals (Handy *et al.* 2008). Mineral NPs are often positively charged and interact with negatively charged natural acids like humic or fulvic acids (Handy *et al.* 2008).

Toxic effects are influenced by NP size and shape. Moreover, salinity, pH, temperature, and the availability of NOM have an impact on the ecotoxicity of particles as well as NPs. In proximity to cell membranes, NPs are expected to promote the release of reactive oxygen species (ROS) (Handy *et al.* 2008). Furthermore, the adsorption of proteins, polysaccharides, lipids and other compounds may alter NP surface and impact NP solubility and toxicity (Magdolenova *et al.* 2014). Further, the surface charge of NPs can induce the accumulation of toxic compounds like metals or pesticides. In the presence of TiO_2 NPs, an increased adsorption of cadmium in viscera and gills of carp was observed (Zhang *et al.* 2007b).

3.3.4 Environmental behavior of nanoparticles

3.3.4.1 Input of nanoparticles

It is inevitable that engineered NPs are released into the environment. The entry of NPs into terrestrial or aquatic environments is predictable, when NPs are used in agriculture or for the purpose of soil and water remediation. NPs are also released during production, transportation, or usage. For example, NPs are washed out when incorporated in cosmetics, sunscreen, detergents, paintings, tires, or fabrics. After usage, NP-containing products need to be disposed of. Apart from this, unintentional NP production occurs when motor vehicles emit fine and ultrafine particles.

The concentration of natural NPs in the environment is on the mg/L scale, whereas the concentration of engineered NPs is on the µg/L scale (Handy *et al.* 2008, Ju-Nam and Lead 2008). NPs come into contact with fungi, algae, plants, and animals. Inside cells, NPs can interact with membranes and provoke changes in molecules and cellular structures (Navarro *et al.* 2008). Carbon nanotubes were observed to enter thick seed coats of tomato plants (Khodakovskaya *et al.* 2009). Subsequently, a stimulated seed germination and biomass accumulation occurred, which is probably correlated with improved water uptake (Khodakovskaya *et al.* 2009). Via food webs, NP toxicity is able to influence whole communities and ecosystems (Navarro *et al.* 2008).

NPs may move in the water phase within soil pores (Navarro *et al.* 2008). They might interact with natural organic matter (NOM), which is present in natural waters in the range from 1 - 100 mg/L (Lin *et al.* 2009). These interactions are implemented via electrostatic, hydrogen

binding or hydrophobic reactions, and may result in surface changes and enhanced NP aggregation (Navarro *et al.* 2008). NOM with high molecular weight is supposed to foster NP aggregation and deposition into sediments, which consequently reduces NP bioavailability, whereas NOM with lower molecular weight is thought to promote NP bioavailability (Navarro *et al.* 2008). Moreover, NPs have an impact on the fate of chemicals like pesticides. Adsorption on NPs will affect their mobility, availability and toxicity.

3.3.4.2 Uptake of nanoparticles

Internalization of NPs was recorded in bacterial (Kumar *et al.* 2011), plant (Liu *et al.* 2009), animal (Liu *et al.* 2008), and cancer cells (Kam *et al.* 2005). NPs that are exposed to bacteria, plants, and fungi reach the cell wall in the first instance. Cell walls consist of a network of carbohydrates and proteins. In filamentous fungi, the main components of the cell wall are chitin (10 - 20 %), glucans (50 - 60 %), as well as mannans and glycoproteins (20 -30 %) (Bowman and Free 2006). NPs may interact with different functional groups, e.g., carboxylate, phosphate, hydroxyl, amine, sulfhydryl, and imidazole (Navarro *et al.* 2008). Via pores, NPs are able to cross the cell wall and encounter the plasma membrane. NPs are capable to pass this membrane via endocytosis, ion channels or transport carrier proteins (Navarro *et al.* 2008). During endocytosis, a particle is enclosed by membrane components and is finally incorporated into the cell. Inside the cell, NPs may be transported by the means of vesicles to endosomes and lysosomes (Moore 2006) or stored in vesicles or mitochondria (Nowack and Bucheli 2007). NPs are capable of triggering the production of ROS, and interfere with different cell compartments and cell functions (Navarro *et al.* 2008).

In rice plants, fullerene C_{70} NPs were transported from the roots to the stem and then into the leaves (Lin *et al.* 2009). As NPs were mainly detected in or in close proximity to the vascular system in leaves, the authors assumed that the NPs entered root cells via pores and plasmodesmata. Plant cell wall pores have a diameter of 3.5 - 5 nm (Carpita *et al.* 1979). Plasmodesmata have a diameter of 50 - 60 nm at midpoint (Lin *et al.* 2009). However, these numbers have to be treated with care, as pore and plasmodesmata diameters vary highly among species and even within one organism depending on tissue and developmental stage. In the seaweed *Chara zeylanica*, for example, the observed diameter of plasmodesmata ranged from 20 - 400 nm or more, including branched plasmodesmata (Cook *et al.* 1997). For a long time, plasmodesmata were considered to limit the exchange of molecules with a size below 1 kDa (a diameter of 2 nm) (Bresinsky *et al.* 2008). However, plasmodesmata were found to allow the passage of macromolecules up to 70 kDa depending on plant tissue. Furthermore, plasmodesmata may function as regulable pores that allow the passage of distinct macromolecules (Bresinsky *et al.* 2008).

In animals and humans, NPs may be incorporated via nutrition and transition from the gut epithelium. Some NPs have the ability to cross the blood-brain barrier. In rats, the entry and allocation of silica NPs in the brain was proven after intranasal instillation (Wu *et al.* 2011). Those silica NPs cause cell toxicity and oxidative stress which implies their potential neurotoxicity (Wu *et al.* 2011). Apart from these effects, NPs possess the risk of accumulating via the food web in single organisms and to subsequently affect communities and ecosystems.

3.3.4.3 Effect of nanoparticles on organisms

In organisms, NPs may trigger the production of ROS as well as toxic ions that are released from NPs, e.g., Fe^{2+}, Ag^{+}, Cu^{+}, Mn^{2+}, $Cr^{5+}$and Ni^{2+} (Magdolenova *et al.* 2014). As a consequence, lipid peroxidation and membrane damage are likely to occur. Further, ROS might lead to DNA and protein damage (Moore 2006). NPs have direct access to DNA after entering the cell nucleus through pores (diameter of 8 - 10 nm). NPs may be genotoxic when they directly interact with DNA or interfere with DNA replication, transcription and translation. Free ROS radicals cause DNA damage through base lesions, mutations and DNA strand breaks. Larger particles might interact with DNA during mitosis, when the nuclear membrane breaks up (Magdolenova *et al.* 2014). In addition, NPs are able to directly disturb mitosis or intervene in the regulation of the cell cycle (Magdolenova *et al.* 2014). Furthermore, NPs may adversely affect the antioxidant system by reducing glutathione levels and impeding glutathione reductase and superoxide dismutase (SOD) (Magdolenova *et al.* 2014).

In soil, NPs were found to be bactericidal (Fu *et al.* 2005) and able to affect the soil bacterial community structure (Shah *et al.* 2014). Zinc oxide NPs were reported to exert detrimental effects on the membrane of *Escherichia coli* (Zhang *et al.* 2007a).

There is only a limited number of studies that deal with the effect of NPs on fungi. For a long time, publications were confined to the use of fungi in the ecofriendly production of NPs. When subjected to $AgNO_3$, intra- or extracellular formation of Ag NPs was reported for *Verticillium* sp. (Mukherjee *et al.* 2001), *Fusarium oxysporum* (Ahmad *et al.* 2003), and *Aspergillus clavatus* (Verma *et al.* 2010). Besides these, *F. oxysporum* was observed to produce gold NPs in the presence of $AuCl_4^-$ (Mukherjee *et al.* 2002). Recently, the effects of NPs were investigated on soil microbial communities that also include fungi (Simonin and Richaume 2015). For example, the effect of metal oxide NPs was studied on soil microbial biomass and respiration (Antisari *et al.* 2013). In general, metal and metal oxide NPs are considered to be more toxic to soil organisms than organic NPs (Simonin and Richaume 2015). Detailed information is still lacking to distinguish between the effects on fungi, bacteria, algae or protozoa. The fact that little is known about how NPs affect fungi stresses the need for further research in this respect. Several NPs, however, were reported to lead to the inhibition of fungal growth (Kasemets *et al.* 2009, Galindo *et al.* 2013, Kanhed *et al.* 2014) or to changes in the fungal production of lignocellulose degrading

enzymes (Shah *et al.* 2010). The toxicity of certain NPs may stem from the detachment of toxic ions as observed in the case of ZnO NPs (Kasemets *et al.* 2009) or Ag NPs (Ivask *et al.* 2014). Beyond that, Ag NPs were found to increase the infection rate of clover by arbuscular mycorrhizal fungi (Feng *et al.* 2013).

Nevertheless, NPs may directly act on plants. Some studies showed positive, others negative or no effects. Particle composition, size, shape and concentration are crucial for NP impacts. Cerium oxide NPs at concentrations of 500 mg/L or higher led to swollen root tips, retarded root growth, reduced SOD activities, and enhanced lipid peroxidase activities (Cui *et al.* 2014). On the other hand, lipid peroxidation might be reduced due to the presence of NPs. In addition, NPs may influence photosynthetic and respiratory processes as well as seed germination and plant resistance. Additionally, gene expression may be altered, as documented for tomato exposed to carbon nanotubes (Khodakovskaya *et al.* 2011).

If NPs are dispersed in the air, they may have detrimental effects on lung tissue or the immune system. In alveolar macrophages, exposure to NPs resulted in nucleus degeneration and an increased endoplasmic reticulum (Jia *et al.* 2005). Aside from the aerial environment, aquatic environments have been exposed to NPs. In *Daphnia magna*, for example, sublethal NP concentrations were shown to affect heart rate and swimming behavior (Lovern *et al.* 2007). Further, fish exhibited an elevated lipid peroxidation in brains after exposure to fullerene C_{70} (Oberdörster 2004).

3.3.4.4 Challenges in risk assessment

For fish and invertebrates, toxic effects of NPs are often observed in the low mg/L range (Handy *et al.* 2008). In terms of environmental research, it has to be considered that NPs might interact with organic material like polysaccharides and exudates. These interactions themselves are influenced by the dispersion of both materials, by temperature, salinity, or pH (Handy *et al.* 2008). In turn, NP toxicity might be altered. Moreover, colloids may interfere with NP toxicity. Colloids consist of inorganic compounds, humic substances and biopolymers like polysaccharides and peptidoglycans. Their size is defined to be within 1 nm and 1 µm (Lead and Wilkinson 2006, Nowack and Bucheli 2007). The porous structure as well as the charged groups on the outside and inside of organic colloids enable the sorption of nutrients and toxic compounds which, in turn, has an influence on the availability, transport and uptake of these compounds (Nowack and Bucheli 2007).

Apart from this, NPs themselves may serve as sorbents for toxic compounds and can affect the bioavailability and toxicity of pollutants (Navarro *et al.* 2008). These NPs may act as vehicles for the uptake of pesticides in cells. A certain percentage of NP toxicity may also derive from the detachment of toxic ions from specific NPs. It was reported that Ag ions or other metals can be

released from NPs and possess their own toxicity, which might differ from that of the pure NP (Griffitt *et al.* 2007, Ju-Nam and Lead 2008, Kasemets *et al.* 2009, Ivask *et al.* 2014). Ag ions, for example, interfere with the transcription of DNA, the production of ATP, or the respiration process in bacteria (Ju-Nam and Lead 2008).

The next section deals with periodic mesoporous organosilica nanoparticles. In the present thesis, engineered NPs were used as a model to analyze the effect of sorption on the ecotoxicity of propiconazole.

3.3.5 Periodic mesoporous organosilica nanoparticles

In 1999, the generation of periodic mesoporous organosilicas was reported by three groups (Asefa *et al.* 1999, Inagaki *et al.* 1999, Melde *et al.* 1999). Over the last number of years, periodic mesoporous organosilica nanoparticles (PMONPs) have aroused scientific interest. PMONPs are synthesized via hydrolysis and condensation reactions using bridged organosilica precursors and an agent that allows self-assembly and controls the PMONP framework (Park *et al.* 2014). Uniform and periodically ordered pores are the hallmark of PMONPs. These silica materials are stable and possess larger pores than zeolites. As nanocomposites, PMONPs combine both organic and inorganic features. Diameter, shape, core size, shell thickness, host compatibility, as well as outward and inward properties of PMONPs, can be tailored precisely by choosing the proper concentration of precursor or surfactant and by adapting the ratio of ethanol and water during synthesis (Teng *et al.* 2014b). PMONPs can be produced in the shape of particles, rods, films, or yolk-shell structures (Teng *et al.* 2014b). Yolk-shell structured NPs possess radially oriented mesochannels with large pore volumes and are generated via surfactant-templated synthesis (Teng *et al.* 2014a, Teng *et al.* 2014b). The template is crucial for the formation of two shells around the NP core. Through targeted dissolution or calcination of the inner shell, an inner void is generated, which allows the binding of host molecules.

The PMONPs utilized in this thesis are of uniform size, shape and ordered structure; they were synthesized by Leilei Luo, University of Tübingen. Cetyltrimethylammonium bromide (CTAB) was used as a structure directing agent and bis(triethoxysilyl)benzene (BTEE) as a silicon precursor. By acidic ethanol extraction, the template was removed, which resulted in void structured yolk-shell PMONPs (Teng *et al.* 2014a, Teng *et al.* 2014b). Moreover, the binding of propiconazole on these engineered PMONPs was verified in close cooperation with Leyla Guluzada, University of Tübingen.

As their binding characteristics are modifiable, PMONPs possess a broad variety of applications and have gained attention in recent years. PMONPs may be employed for sensing, optics, chromatography, separation, catalysis, microelectronics, light harvesting, energy storage, drug

delivery, and environmental applications (Hatton *et al.* 2005, Park *et al.* 2014, Teng *et al.* 2014a, Teng *et al.* 2014b). Sulfide groups of the PMONPs may be used for selected adsorption of pollutants (Park *et al.* 2014). A promising approach is to utilize PMONPs in water treatment facilities in order to remove heavy metals and other pollutants (Chandra *et al.* 2010). Additionally, volatiles or metallic cations may be detected by mesoporous materials (Melde and Johnson 2010). As PMONPs were shown to be hemocompatible, these particles may be intravenously used for drug delivery (Teng *et al.* 2014a). Moreover, PMONPs have the potential to be applied as vehicles for controlled drug release (Hatton *et al.* 2005, Vathyam *et al.* 2011), which is highly promising in the field of cancer therapy (Wu *et al.* 2013). Biomedical applications like cell or tumor imaging were demonstrated using PMONPs loaded with gold or near-infrared dyes (Teng *et al.* 2014b).

3.3.6 Sorption

Sorption describes the overall process of adsorption and absorption between the interfaces of two phases of either liquid, solid, or gaseous origin (Blume *et al.* 2016). While adsorption refers to the accumulation of a compound directly on the phase interface, absorption represents the accumulation within a phase. Sorption is based on physical and chemical interactions and depends on temperature and concentration. Pollutants may attach to colloids, particles, sediment etc. and, in turn, their availability might be changed. In addition, the adsorbed particle could act as a vehicle for contaminant transportation. In rivers and lakes, pollutants may bind to particles. The size of soil particles is defined as smaller than 2 µm, whereas colloids are smaller than 1 µm (Agriinfo 2015).

At equilibrium in liquid medium, an organic compound is considered to be distributed according to the following equation (Schwarzenbach *et al.* 2003, Ra *et al.* 2008):

$$Kd = \frac{Cs}{Cw} = focKoc$$

Where

$$Kd = distribution\ coefficient$$

$$Cs = concentration\ of\ the\ compound\ in\ the\ particles\ or\ sediment$$

$$Cw = concentration\ of\ the\ compound\ in\ water$$

$$foc = mass\ fraction\ of\ organic\ carbon\ in\ the\ particles\ or\ sediment$$

$$Koc = distribution\ coefficient\ based\ on\ the\ organic\ carbon\ content$$

Sorption depends on pH and ionic strength of the liquid medium, as well as on the structure and the chemical properties of the compound. Molecules or ions that adsorb or desorb from solid surfaces are called sorbates, whereas the solid compound itself is referred to as a sorbent. Different forces contribute to sorption, such as van der Waals interactions, polar interactions, dipole-dipole interactions, or hydrogen bonding. In addition, electrostatic forces occur between charged ions and oppositely charged sorbents (Blume *et al.* 2016). These interactions are weak and ions are easily replaced by others (Blume *et al.* 2016). Covalent bonds may be formed between reactive functional groups of sorbent and sorbate, a process called chemisorption (Blume *et al.* 2016). Adsorption may also take place through hydrogen bonds or hydrophobic interactions. In certain cases, sorption may cause the accumulated adsorption of other sorbates, which is termed surface precipitation or cluster formation (Blume *et al.* 2016). Small particles are assumed to attach and detach immediately on a microscale. Larger particles may have several points of interaction that impede a complete adsorption or desorption.

Motion of a sorbate occurs by diffusion. The compound migrates along a concentration gradient through the surrounding bulk medium and passes the film that covers the solid sorbent through so-called film diffusion (Pignatello and Xing 1995). Subsequently, the compound is able to move via diffusion into particle pores and even within the particle matrix (Pignatello and Xing 1995). The two last-mentioned processes are comparatively slow. The sorption of polycyclic aromatic hydrocarbons on soil material was documented as a two-phase process beginning with a rather quick phase and followed by a slower phase (Weissenfels *et al.* 1992). The slower phase may be explained by the fact that easily accessible sorption sites are already occupied and polycyclic aromatic hydrocarbons have to traverse larger distances to reach further, less accessible sites (Weissenfels *et al.* 1992).

In water treatment plants, activated carbon is used to remove pollutants by sorption. Activated carbon is generated from coal, peat, or coconut shell and has a complex structure consisting of macro and micro pores as well as different surface groups that foster its sorption properties (Srivastava *et al.* 2008). Activated carbon was shown to bind toxic metallic ions like cadmium, nickel, and zinc, where smaller metallic ions had easier access to the sorption sites on the surface and in the pores (Srivastava *et al.* 2008). If activated carbon is treated with NaOH, a complete and better sorption of propiconazole was found compared to commercial activated carbon (Adam *et al.* 2005). Due to their adsorption capacity, nanomaterials are also able to serve as a device for separation and remediation, such as the removal of organic and inorganic pollutants (Walcarius and Mercier 2010). Mesoporous silica materials with a large pore diameter and pore volume are suitable for the removal of DDT (Tian *et al.* 2009). The adsorption is assumed to occur via hydrogen bonding between hydrophilic areas of the DDT molecule and OH groups on the NP surface (Tian *et al.* 2009).

3.4 Expand-Project

Toxic compounds are usually well studied in their dissolved state. However, in nature, compounds often interact with particles or soil colloids. Naturally occurring particles may influence the bioavailability, transportation, degradation and activity of compounds applied in the environment, including chemicals. Particles treated with humic acid as a source of organic matter were shown to reduce the toxicity of various phenolic compounds (Ra *et al.* 2008). Consequently, the influence of toxic compounds on organisms and ecosystems might vary depending on whether or not the compounds are in a sorbed state. Since the effects of these interactions are not yet well understood, a project among different research groups of the University of Tübingen was founded.

Under the leadership of Prof. Dr. Stefan Haderlein and Prof. Dr. Heinz-R. Köhler, a network was established to explore the **e**coto**x**icity of **pa**rticle-associated compou**nd**s (see Figure 3). Derived from that goal, the initiative was named the EXPAND-Project, which in turn forms the context of the work of this thesis.

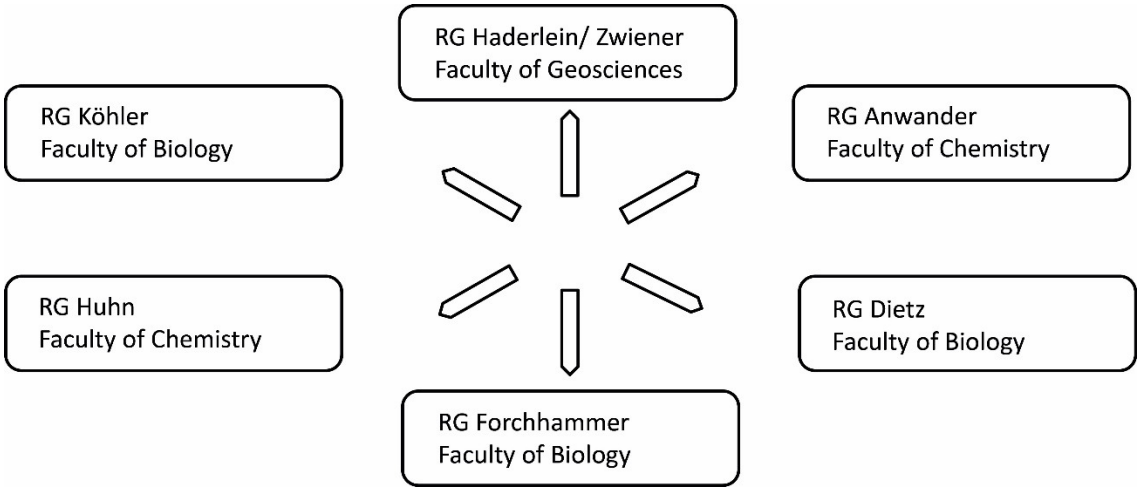

Figure 3: Overview of the research groups (RG) that are involved in the EXPAND-Project (Ecotoxicity of particle-associated compounds).

Several research groups (RG) were involved in this project, and the team benefitted from a variety of different scientific backgrounds. In close cooperation, chemists, geoscientists and biologists studied the impact of NPs and NP-associated toxic compounds. The project focused on commonly applied pesticides that are known to affect non-target organisms and ecosystems. The aim was to investigate whether sorption has an impact on pesticide toxicity, not only on the organism and ecosystem level but also on the molecular and cellular level.

The fundamental tools of this study were designed NPs with known surface characteristics and known physical and toxic parameters. In an interdisciplinary approach, Leilei Luo (RG Anwander, Institute of Inorganic Chemistry, University of Tübingen) engineered particles that showed good sorption capability for pesticides like propiconazole and thiacloprid. Leyla Guluzada (RG Haderlein, Environmental Mineralogy and Environmental Chemistry, Center of Applied Geosciences, University of Tübingen) examined the sorption of these compounds in water and experimental growth media. Carla Lorenz (RG Köhler, Institute of Evolution and Ecology, University of Tübingen) analyzed the impact of aluminum oxide NPs and the insecticide thiacloprid on larvae of the non-biting midge *Chironomus riparius*. The envisaged objective of the present thesis was to observe the effect of NPs and propiconazole on several EMF species and the formation of mycorrhizas in poplar seedlings. Finally, Anna-Jorina Wicht (RG Huhn, Institute of Physical and Theoretical Chemistry, University of Tübingen) evaluated the uptake of toxic compounds in larvae or fungal mycelium.

3.5 Study context and objectives of this thesis

3.5.1 Study context

Toxic compounds like pesticides are widely applied, especially in agriculture but also in forest nurseries to gain control over pathogens. If a toxin does not specifically target a certain group of organisms, other species are adversely affected as well. Fungicides, for example, may not only address pathogenic fungi, but also neutral or beneficial fungi. In order to perform reasonable pesticide management, it is crucial to obtain profound background knowledge. The mode of action of a toxin and side effects on non-target organisms must be known prior to pesticide usage.

Non-target organisms may be directly or indirectly affected by toxic compounds. Within a community, organisms are connected in a functional or nutritional way. If a toxin compromises one organism or a group, others, in turn, may be influenced. Especially in communities with plant-microbial interactions, pesticides with deleterious effects on fungi may indirectly affect associated plants (Yang *et al.* 2011).

Although there are lots of studies on herbicides, insecticides and fungicides, the obtained results are not seldom contradicting and confounding (Greaves *et al.* 1976, Trappe *et al.* 1984). Some fungicides are known to have detrimental effects on certain mycorrhizal fungal species, whereas others are promoted or not affected at all by their application (Laatikainen and Heinonen-Tanski 2002). A single fungicide is able to provoke different reactions in different species. Even isolates of the same species may respond differently. Moreover, studies performed under axenic conditions may not correlate with those performed in the greenhouse or field (Greaves *et al.* 1976, Trappe *et al.* 1984). Different factors influence pesticide toxicity in the field. The fate of utilized

toxic agents is complex: a plant protecting agent, for example, is either translocated within the plant or it runs off to the soil surface. Incorporated in plant material, the pesticide may be ploughed into the soil. Furthermore, the toxin is subjected to degradation by photochemical, biological or chemical processes. It may adsorb on soil colloids or it might be absorbed by plant roots (Greaves *et al.* 1976). All these events influence pesticide half-life, availability, and toxicity.

In this thesis, an investigation into the toxicity level of a given toxin upon sorption was intended. Often, pesticides are studied under aqueous conditions either in submerged cultures or in an agar medium. In nature, however, it is very likely that pesticides are adsorbed on particles or colloids which, in turn, may influence the effect of a toxic compound.

3.5.2 Applied test system

3.5.2.1 Test system

In order to investigate the ecotoxicity of a toxic compound under sorbed conditions, a common fungicide was used that is known to affect mycorrhizal fungi as non-target organisms. These fungi play a crucial role in forest ecosystems, as they interact with the plant root system and support plant growth. Furthermore, NPs that function as sorbents for the toxic compound were inserted into the test system. In what follows, the employed toxin-NP-fungi test system is described in more detail.

Propiconazole was chosen as a model compound. This fungicide is widely applied in agricultural sites and in forest nurseries. Its mode of action is well described. It is known to adversely affect single non-target organisms as well as fungal and bacterial communities (Elmholt 1991, Yen *et al.* 2009, Fernández-Calviño *et al.* 2017). As propiconazole is employed as a pest control agent of pathogenic fungi, it also impairs mycorrhizal fungi. This is an undesirable effect when propiconazole is applied in forest nurseries.

To study the effect of sorption, NPs were selected that exhibited good sorption properties for propiconazole. The PMONPs designed by Leilei Luo have been proven to meet this requirement. Sorption isotherms of propiconazole and PMONPs were analyzed by Leyla Guluzada. Sorption followed the Langmuir model (Figure 4) both in pure water and in fungal growth medium containing either 5 or 10 g/L glucose. Fungi were grown on solidified agar medium and in liquid culture. While fungal cultures performed well on agar medium containing 5 g/L glucose, fungal growth was retarded in liquid medium over time, probably due to the shortage of glucose. Therefore, submerged culture experiments were performed in growth medium containing 10 g/L glucose.

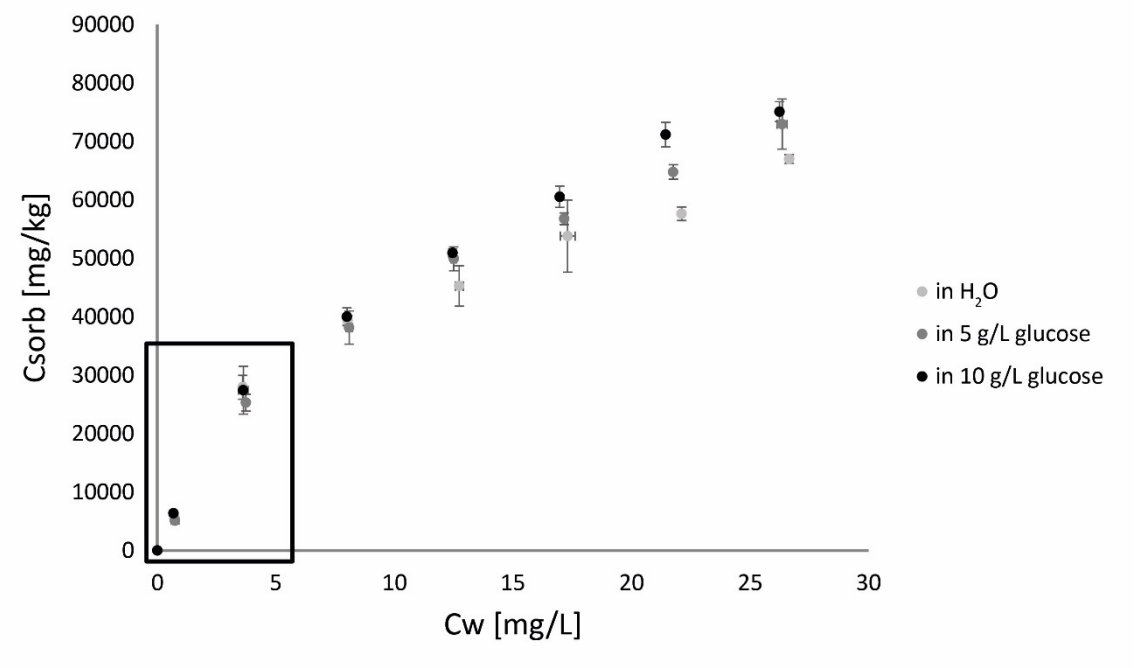

Figure 4: Sorption isotherms of propiconazole and PMONPs in three different liquid media: H_2O and fungal growth medium containing either 5 or 10 g/L glucose. Cw: concentration of dissolved propiconazole in the liquid medium; Csorb: concentration of propiconazole sorbed on PMONPs (unpublished data from Leyla Guluzada).

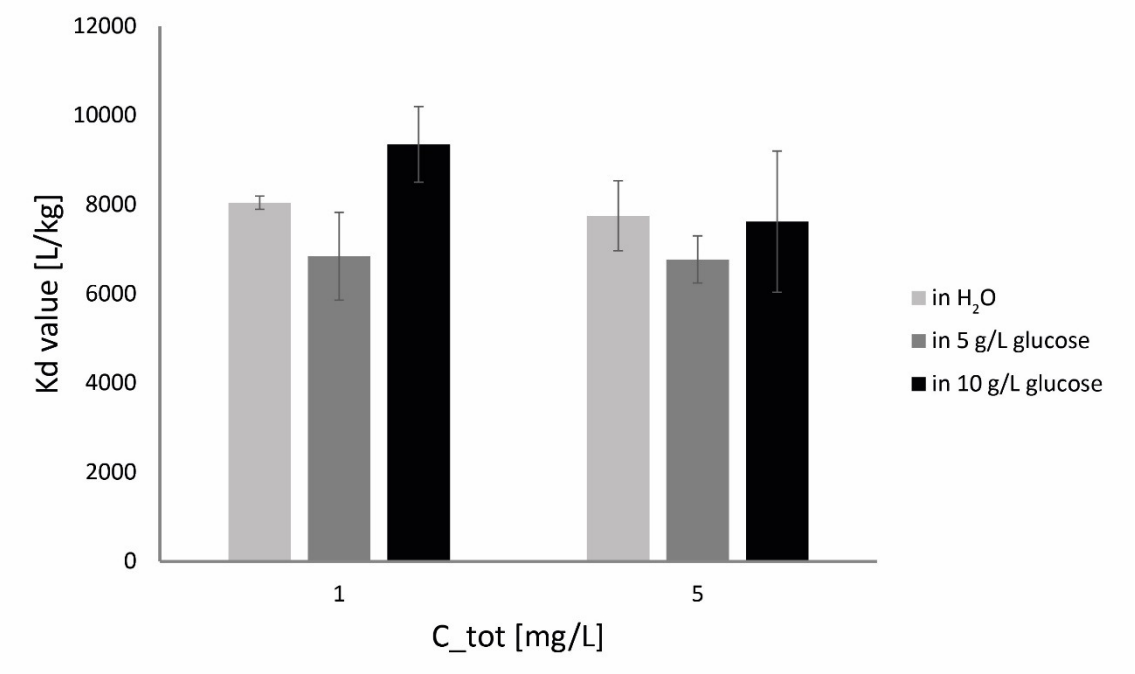

Figure 5: Distribution coefficients (Kd values) of propiconazole sorption on PMONPs in three different liquid media: H_2O and fungal growth medium containing either 5 or 10 g/L glucose. C_tot: total concentration of propiconazole in the liquid medium (unpublished data from Leyla Guluzada).

The distribution coefficients (Kd values) in all liquid media were about the same order of magnitude around 8,000 L/kg (see Figure 5). The mean Kd value in all three media was 7,735.56 L/kg.

It was the aim of the project to study the effect of sorption on toxicity. To create several distinct sorption conditions each with a known percentage of sorbed propiconazole, the following two equations and the above-mentioned Kd value (7,735.56 L/kg) were used to calculate the required amount of NPs (Schwarzenbach *et al.* 2005):

$$fw + fs = 1$$

and

$$r\,sw = \frac{\frac{1}{fw} - 1}{Kd}$$

Where

$$fw = \ fungicide\ fraction\ disolved\ in\ water$$

$$fs = \ fungicide\ fraction\ sorbed\ on\ particles$$

$$r\,sw = amount\ of\ particles\ needed\ for\ a\ given\ fw$$

$$Kd = distribution\ coefficient\ [L/kg].$$

It was intended that experimental systems with a constant propiconazole input would be created, where the percentage of PMONP-sorbed propiconazole was regulated to be 20, 50, and 80 %.

As test organisms, three ectomycorrhizal species from two distinct fungal phyla were selected. *Laccaria bicolor* and *Amanita muscaria* are Basidiomycetes and both are well-established model organisms in the field of mycorrhizal research. *Cenococcum geophilum* is an Ascomycete fungus.

3.5.2.2 Test organisms

3.5.2.2.1 Amanita muscaria

A. muscaria is a common and well-known mycorrhizal fungus in forests of the northern temperate zone and thus of environmental relevance. It belongs to the phylum Basidiomycota in the kingdom Fungi. It is able to establish mycorrhizal structures with poplar and spruce under laboratory conditions and is, therefore, of particular interest in this study.

3.5.2.2.2 Cenococcum geophilum

The studied *Cenococcum geophilum* isolate (*C. g.* K IV 1991) is likewise capable of forming mycorrhizas in vitro. *C. geophilum* is an Ascomycete and associates with a broad variety of tree species (Trappe 1962b). Its distribution ranges from arctic to temperate, tropic and subtropical forests (Trappe 1962a, Fernández-Toirán and Águeda 2007). This fungus may be found in extreme habitats, such as dry environments (Trappe 1962b). With the help of sclerotia, *C. geophilum* is able to endure harsh conditions. It is also considered to be resistant to various stresses. In 2007, the fruiting bodies of *C. geophilum* were described (Fernández-Toirán and Águeda 2007). Previously, its sexual form of reproduction had not been observed and, therefore, the fungus was classed in the former group of imperfect fungi. The oval or spherical fruiting body is a completely closed cleistothecium with three asci (spore bearing cells) containing three ascospores (Fernández-Toirán and Águeda 2007). *C. geophilum* forms typical black-colored ECMs. Its hyphae possess a prevalent diameter of 4 - 5 µm (Fernández-Toirán and Águeda 2007) without clamp connections, as this is the unique feature for most species of the phylum Basidiomycota. The hyphal diameter may vary according to its location in ECMs. In proximity to the mantle surface, the diameter is 5 - 10 µm, whereas in proximity to root cells, the diameter is around 2.5 - 4 µm (Fernández-Toirán and Águeda 2007). *C. geophilum* is a common and widespread fungus that colonizes a high percentage of roots (Heinonsalo *et al.* 2004, Kaldorf *et al.* 2004, Heinonsalo *et al.* 2015). As *C. geophilum* is capable of forming mycorrhizas in vitro, this fungus is of certain relevance in this thesis.

3.5.2.2.3 Laccaria bicolor

The Basidiomycete *L. bicolor* is a highly used model organism to study the development and function of ectomycorrhizal symbiosis in poplar. Next to *C. geophilum*, *Laccaria* sp. are common, widespread species. They were among the six most common species found to colonize aspen clones (*Populus tremula* x *P. tremuloides* Michx.) in an experimental field (Kaldorf *et al.* 2004). The studied *L. bicolor* strain S238N-4 (Maire) P. D. Orton, however, is not able to establish mycorrhizal structures under laboratory conditions. It was selected because a variant of the same strain, S238N-H82, was sequenced in 2008, and relevant information on genetic expression and protein function is available (Martin *et al.* 2008). Apart from this, the tested *L. bicolor* strain shows a fast growth rate under axenic conditions, which is advantageous for laboratory testing.

The genome of *L. bicolor* consists of 65 megabase pairs (Martin *et al.* 2008). Up to 2391 proteins are predicted to be secreted, whereby 82 % of the proteins are species-specific for *L. bicolor* (Martin *et al.* 2008). Compared to other Basidiomycetes, *L. bicolor* exhibits a larger number of ammonium transporters (Martin *et al.* 2008). The acquired nitrogen is probably derived from bacterial, plant, or animal proteins (Martin *et al.* 2008). Genomic analyses revealed that *L. bicolor* lacks the ability to degrade oligosaccharides and polysaccharides originating from plant cell walls (Martin *et al.* 2008).

In ectomycorrhizal symbiosis between *Pinus sylvestris* and *L. bicolor*, plant shoot biomass was found to be significantly increased by 73 % (Christophe *et al.* 2010). Ectomycorrhizal plants showed an enhanced K and Mg acquisition (Christophe *et al.* 2010). The beneficial aspect of mycorrhizal symbioses on plant performance is exploited in forest management. Strains such as *L. bicolor* S238N are commercially produced and applied in forest nurseries to promote tree growth (Bertaux *et al.* 2003a).

3.5.3 Objectives of the thesis

Sorption might alter the bioavailability of pesticides. In addition, their uptake, toxicity and degradation might also change (Navarro *et al.* 2008). This could be one explanation for the often confusing and contradictory results of field and laboratory studies on pesticide efficacy (Greaves *et al.* 1976, Trappe *et al.* 1984). In nature, a compound may interact with materials present in water, air, or soil. In order to be able to predict the toxicity of toxic compounds in an environmental context, it is therefore necessary to gain more information on the effect of sorption processes and their impact on toxicity. The single effect of each sorbent and sorbate needs to be evaluated as well as their combined effect. These effects may be additive, synergistic, or antagonistic.

In this thesis, tailor-made NPs (PMONPs) were used as model compounds that serve as sorbents for propiconazole. The aim of this thesis was to investigate the ecotoxicity of NPs, propiconazole, and particle-associated propiconazole. The association of NPs with toxic compounds could affect NP uptake into cells (Nowack and Bucheli 2007). Further, NPs may act as vehicles for long distance transportation and the import of pesticides. Therefore, NP sorption could intensify the toxicity of propiconazole. Alternatively, NP sorption could reduce the toxicity of propiconazole, as organisms might be protected against the fungicide. Apart from this, there is very limited knowledge available on the effects of NPs on fungi, which underlines the importance of this research (Kasemets *et al.* 2009, Shah *et al.* 2010, Feng *et al.* 2013, Galindo *et al.* 2013, Kanhed *et al.* 2014).

The triazole fungicide propiconazole is widely applied (Schwinn 1984, Taxvig *et al.* 2008, Al-Hatmi *et al.* 2016, Fernández-Calviño *et al.* 2017) and targets ergosterol biosynthesis (Hancock and Weete 1985). In response to propiconazole treatment, growth inhibition occurs in pathogenic fungi as well as in non-target organisms that are often neglected in research (Sancholle *et al.* 1984, Hancock and Weete 1985, Fernández-Calviño *et al.* 2017). Propiconazole is known to affect EMF in forest nurseries and afforestation sites (Laatikainen and Heinonen-Tanski 2002). EMF are of enormous environmental relevance, as they are responsible for the function and the state of present forest ecosystems (Smith and Read 1997). In this thesis, the effect of propiconazole and its sorption on NPs was profoundly studied on the molecular, cellular, and ecosystem level. On the molecular level, we aimed to investigate the effect of propiconazole on gene expression. Several genes should be checked for their utility as marker genes upon propiconazole exposure and various sorption conditions. To date, fungal gene expression upon treatment with azole fungicides has been intensively studied in Ascomycetes (Henry *et al.* 2000, De Backer *et al.* 2001, Agarwal *et al.* 2003, Song *et al.* 2004, Liu *et al.* 2010, Sun *et al.* 2011, Fernandes *et al.* 2016) but only rarely in Basidiomycetes (Stammler *et al.* 2009, Lee *et al.* 2010), where data are conflicting. With the help of this thesis, some light was shed on this knowledge gap. Further, growth performance should be studied in submerged and solid cultivations to assess the toxicity of propiconazole as well as its association with NPs. For this purpose, three EMF species were selected that descend from two distinct phyla to cover a broad range of investigated fungal groups. To gain a better insight into the effect of propiconazole on the ecosystem level, mycorrhization should be studied in the presence and absence of NPs. As plant partner, poplar was chosen, as this fast-growing tree is prevalent in Europe, North Africa and Northeast Asia, and is a mycorrhiza model organism. Additionally, mycorrhization of poplar can be studied under axenic conditions. As fungal partner for these symbioses, one representative each of Ascomycetes and Basidiomycetes should be investigated.

In the present thesis, the following three hypotheses are assessed:

1) PMONPs affect fungal growth in both liquid and solid cultivations.

2) The adsorption of propiconazole on PMONPs alters the bioavailability and therefore the toxicity of propiconazole towards EMF.

3) The adsorption of propiconazole on PMONPs has an impact on the establishment of ECMs.

4 Materials and Methods

4.1 Culture media, solutions and buffers

The composition of culture media, solutions and buffers is presented in Appendix 8.1.

4.2 Chemicals

Utilized chemicals are listed in Appendix 8.2, Table 15.

4.3 Labware, lab equipment and hardware

Lists of utilized labware, lab equipment and hardware can be found in Appendix 8.3 to 8.4, Table 16 and Table 17.

4.4 Organisms and cultivation

4.4.1 Ectomycorrhizal fungi: Laccaria bicolor, Amanita muscaria and Cenococcum geophilum

Three different ectomycorrhizal fungal species were chosen as test organisms. The studied *L. bicolor* strain S238N-4 (Maire) P. D. Orton was generously provided by Prof. Dr. Uwe Nehls, originally provided by Prof. Dr. F. Martin (INRA Nancy). The *A. muscaria* strain referred to as AmRH12 was kindly made available by Prof. Dr. Rüdiger Hampp. The isolate originated from fruiting bodies of *A. muscaria* that were collected from a fir stand in Bad Blankenburg in 2012. *C. geophilum* (*C. g*. K IV 1991) was generously provided by Prof. Dr. Ingrid Kottke.

4.4.1.1 Cultivation of ectomycorrhizal fungi on agar medium

Cultivation of ectomycorrhizal fungi was performed under sterile conditions. Culture media were autoclaved (for 20 min at 121 °C and 2 bar); glucose and MES buffer, being heat labile, were sterile-filtered using either 0.22 µm pore size filters (Carl Roth GmbH + Co. KG, Karlsruhe) or bottle top filters with 0.2 µm pore size (Carl Roth GmbH + Co. KG, Karlsruhe). Fungicide and particles were suspended in 100 % methanol (MeOH, HPLC grade).

EMF were grown on modified Melin-Norkrans medium (Marx 1969, Kottke *et al.* 1987) – here referred to as MMN medium 0.5 % glucose – containing 5 g/L glucose and 1.8 % agar. The fungi were allowed to grow on agar for a certain timespan depending on species-specific growth rate. *L. bicolor* was cultivated for 4 weeks, *A. muscaria* for 12 weeks, and *C. geophilum* for at least 4 months. After this period, fungal material was transferred to solid or liquid growth medium to start new cultures, or stored at 4 °C.

Certain experiments required an end-point removal of fungal material from the agar medium to perform follow-up analyses. In this case, cellophane (Einmach Fix, Folia® Paper, Max Bringmann KG, Wendelstein, Germany) was cut in circles slightly smaller than 9 cm in diameter. Cellophane circles and paper filters were placed in alternate layers in a glass Petri dish, boiled three times in bidistilled water and subsequently autoclaved. One cellophane layer was placed on the surface of an agar plate and a fungal inoculum was placed on top with sterile scalpels.

To start new agar cultures of *L. bicolor*, small pieces of mycelium and agar were cut from the edge of actively growing cultures using the upper end of a sterile glass pasteur pipette (diameter 0.6 cm) and placed in the center of new agar plates by means of sterile scalpels. In the case of the slow-growing fungi, *A. muscaria* and *C. geophilum,* the upper end of a plastic tube with a diameter of 0.9 cm was used to obtain a starting inoculum of equal size for each replicate. All fungal cultures were cultivated at 18 °C in darkness.

4.4.1.2 Cultivation of ectomycorrhizal fungi in liquid medium

For liquid cultivation, agar plugs with fungal material were transferred to flasks with or without baffles with one fifth of their volume filled with liquid MMN medium containing 10 g/L glucose – termed MMN medium 1 % glucose: 100 mL flasks were filled with 20 mL medium; 300 mL flasks were filled with 60 mL medium. Fungi grew for two weeks before the fungal material was homogenized using an Ultra Turrax, a dispersing instrument, for 1 min at 8,000 U/min. Each week, the growth medium was replaced by fresh growth medium; fungal material was homogenized biweekly. To start new liquid cultures, a fungal inoculum of approx. 2 mL of fungal culture that had been rinsed with growth medium was transferred to each new flask containing fresh growth medium. Fungi were cultivated at 18 - 20 °C in darkness under continuous shaking conditions (120 rpm).

4.4.2 Plants: Populus tremula L. x P. tremuloides Michx., clone T89

The plant species *Populus tremula* L. x *P. tremuloides* was used as model plant, as it forms symbiotic relationships under sterile, laboratory conditions. Next to a germination of seeds, *Populus* species are propagated by cuttings. Ectomycorrhizal structures from *Populus tremula* L. x *P. tremuloides* can be studied after one month of cultivation in the presence of EMF.

The hybrid *Populus tremula* L. *x P. tremuloides* Michx., line T89 (Tuominen *et al.* 1995) was generously provided by Dr. H. Tuominen, Umeå Plant Science Center, Umeå, Sweden.

4.4.2.1 Cultivation of *Populus tremula L.* x *P. tremuloides* Michx., clone T89

Poplar cuttings were cultivated on MS6 poplar growth medium in 580 mL glass jars. 2.2 g Murashige & Skoog medium (M0222, Duchefa Biochemie B.V., Haarlem, The Netherlands), 10 g sucrose and 1 % agar (Fluka: 05040, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were used to obtain 1 L MS6 medium. The pH was adjusted to pH 5.8 prior to autoclaving. 200 mL medium were poured into each jar.

Tips of poplar shoots with a size of about 2 cm were separated from established poplar plants in axenic culture. The bases of these cuttings were defoliated and planted into MS6 medium. The jars were covered with glass lids and a layer of plastic wrap. Plant cuttings were cultivated under long day conditions for 4 weeks at 18 - 24 °C and a photon flux density of approx. 50 µmol m^{-2} s^{-1}.

4.4.3 Establishing plant-fungus interactions: mycorrhizas

Interactions between plants and EMF were settled under sterile conditions, while host plant cultivation occurred under semi-sterile conditions. While roots and colonizing fungi were kept in a closed, sterile system, the upper part of the plant was exposed to greenhouse conditions. Petri dishes were filled with 50 mL MMN medium without glucose, a reduced amount of nitrogen (1/10 N), and 2 % agar. When hardened, medium was covered with a sterile cellophane membrane (Einmach Fix, Folia® Paper, Max Bringmann KG, Wendelstein, Germany). In the rim of the Petri dish a small opening was made. Poplar seedlings were positioned in such a way that roots were allowed to grow inside the Petri dish, and the stem was directed through the opening so that leaves were positioned outside the dish (see Figure 6). A fungal inoculum of 1 mL derived from recently dispersed liquid culture was poured onto the roots. A paper filter covered fungi and plant material, and two dental rolls helped to stabilize the arrangement before the lid was closed with parafilm (Parafilm M, Bemis® Bemis Company, Neenah, Wisconsin, USA). The gap around the stem was closed with sterile silicone (GE Bayer Silicones GmbH Co. KG, Erkrath, Germany) to provide a sterile atmosphere inside the Petri dish.

Plants were vertically positioned in mini-greenhouses whose bottoms were covered with a suspension of 0.5 % copper sulfate to prevent contamination. The plants were incubated at 18 - 22 °C and long day conditions with a photon flux density of approx. 50 µmol m^{-2} s^{-1} until mycorrhizal structures were visible.

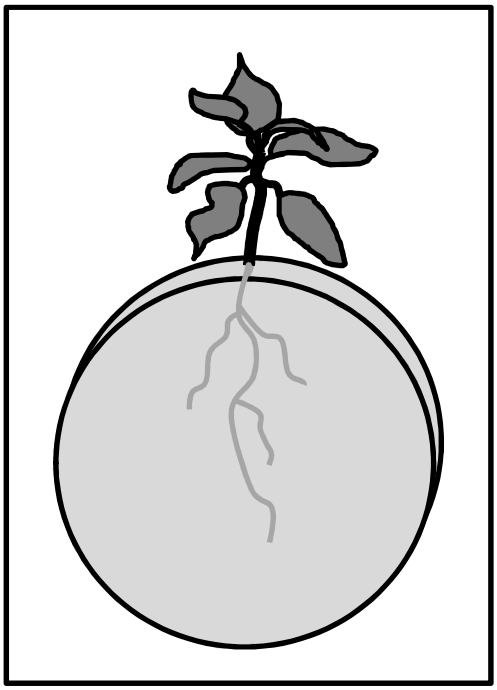

Figure 6: Horizontal cultivation of poplar seedlings for mycorrhiza experiments.

4.5 Propiconazole

The fungicide propiconazole was obtained from Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany (45642, analytical standard, product line PESTANAL®), containing a mixture of stereoisomers.

4.6 Periodic mesoporous organosilica nanoparticles

PMONPs were generously provided by Leilei Luo. These particles had an average size of 360 nm and a specific BET surface area of 1050 m^2/g. The Brunauer, Emmett, and Teller (BET) method takes advantage of nitrogen adsorption at -196 °C to determine the specific surface area including the pore size distribution. The amount of nitrogen is measured that is physically adsorbed on the surface of solid particles and set in correlation with the surface area by assuming a monolayer adsorption. The pores of the NPs showed a size of 1.9 nm with a pore volume of 0.54 cm^3/g. For NP analysis, see Figure 7.

PMONPs were synthesized by Leilei Luo following a surfactant-assembly sol-gel process (Teng *et al.* 2014b). For synthesis, 0.32 g cetyltrimethylammonium bromide (CTAB) were dissolved in a mixture of 150 mL water, 60 mL ethanol, and 2 mL 25 wt % ammonia solution by stirring at 35 °C for 1 h. Subsequently, 2 mL bis(triethoxysilyl)ethane (BTEE) were added. The solution was kept stirring for 24 h for complete hydrolysis of BTEE. The produced PMONPs were centrifuged and washed twice with absolute ethanol. The CTAB template was removed by refluxing 0.75 g of the NPs in 160 mL ethanol supplemented with 4 mL 32 % hydrochloric acid solution at 75 °C for 3 h. This process was repeated twice to ensure complete template removal.

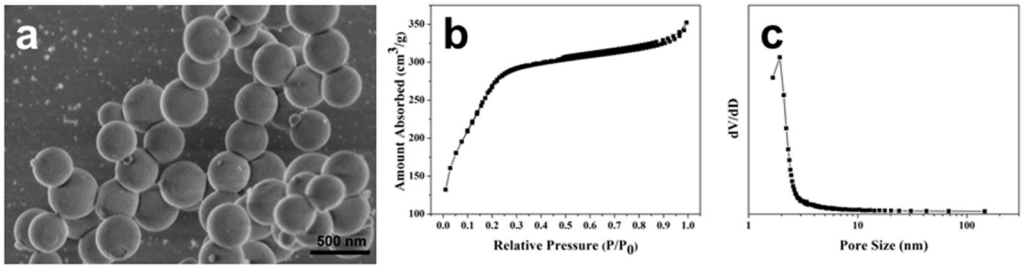

Figure 7: Characterization of periodic mesoporous organosilica nanoparticles (PMONPs). (a) Scanning electron microscopy (SEM) image, (b) nitrogen physisorption isotherm and (c) pore size distribution of PMONPs. Data provided by Leilei Luo.

4.7 Preparation of nanoparticle-containing culture medium

The preparation of NP-containing agar medium was optimized. To keep contamination as low as possible, both NPs and propiconazole were suspended in 100 % MeOH (HPLC gradient grade). Stock solutions of different concentrations were prepared. Before NP usage and before the medium was poured into the Petri dishes, the solutions were treated with ultrasound in a tempered water bath (15 min, 60 °C). This procedure promotes consistent NP dispersion and avoids agglomerations. To prepare 500 mL growth medium, two bottles were required (see Figure 8). One bottle was filled with 200 mL sterile bidistilled water. The other bottle contained the necessary ingredient for 500 mL growth medium but suspended in a volume of only 200 mL. This concentrated medium was supplemented with agar and autoclaved. The aqueous fraction was supplemented with 250 µL of a propiconazole-stock solution or MeOH and 25 mL of a NP-stock solution (0.25 mL NP in MeOH plus 24.75 mL bidistilled water) or an equivalent water-MeOH solution. To enable sorption interactions between NPs and propiconazole, the water fraction was kept stirring for a period of 1 h with a magnetic stirring bar. Then, the aqueous fraction was heated to 60 °C within 2 min by incubating the bottle in a boiling water bath, and immediately combined with the medium fraction under sterile conditions. Prior to combination, the autoclaved agar medium fraction, continuously kept at 60 °C to avoid solidification, was supplemented with

50 mL 0.5 M MES and 25 mL 10 % glucose-stock solution. Media for mycorrhiza experiments were prepared without glucose. After a short stirring step with the magnetic bar, the combined and now complete medium was treated with ultrasound (15 min, 60 °C) and poured into pre-cooled Petri dishes placed on -20 °C cooling racks in order to prevent PMONPs from aggregation and precipitation (Baek and An 2011, Cui *et al.* 2014).

In this medium preparation procedure, the steps were reduced that are usually performed at higher temperatures, as this would affect sorption. In addition, the amounts of liquids in each bottle were adapted to ideal feasibility. The least volume of concentrated growth medium was used that still facilitated complete dissolution after autoclaving and merging with the water fraction. HPLC analysis verified that propiconazole is stable throughout all conditions applied during preparation, e.g., heating, ultrasound treatment, etc. For all treatment groups, the total MeOH content was identical and did not exceed 0.1 %.

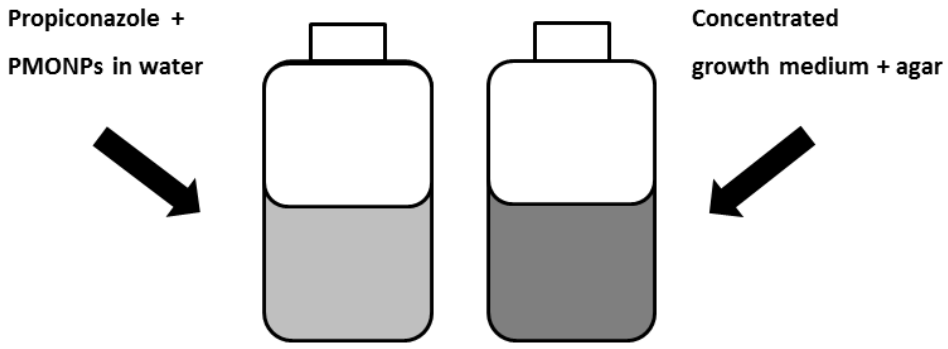

Figure 8: Preparation of agar medium with nanoparticles. Propiconazole and periodic mesoporous organosilica nanoparticles (PMONPs) were suspended in water, stirred for 1 h, incubated in a 60 °C tempered ultrasonic bath for 15 min, and added to concentrated growth medium containing agar. For consistent NP dispersion, the medium was treated once again with ultrasound (15 min, 60 °C) and an equal volume of medium was poured into Petri dishes.

4.8 Study design – effect of nanoparticles and particle-associated propiconazole

Fungal growth should be studied on medium supplemented with propiconazole and different concentrations of PMONPs. Sorption isotherms with propiconazole and PMONPs were performed in different liquids, such as water and growth medium with 5 or 10 g/L glucose (in cooperation with Leyla Guluzada). Derived from the mean Kd value, three concentrations of PMONPs were chosen, so that 20, 50, and 80 % of the initial propiconazole concentration would be adsorbed on the NPs. A fungicide concentration that shows a 70 % inhibition concerning the colony diameter for each of the above-mentioned fungi was applied.

For each of the studied fungal species, the experimental set-up consisted of a full factorial combination of the two predictor variables, NP-concentration (negative control plus the three mentioned concentrations) and fungicide presence. For each of the resulting eight treatment groups (see Figure 9) 19 replicate fungal colonies were measured. As all fungal material descended from one original colony, propagated for years, independency could not be ensured. To establish a lower level of independency, all fungal inocula originating from one Petri dish were homogeneously assigned to all treatment groups. Spatial distribution of all treatment groups was done by a full randomization of replicates and treatment groups.

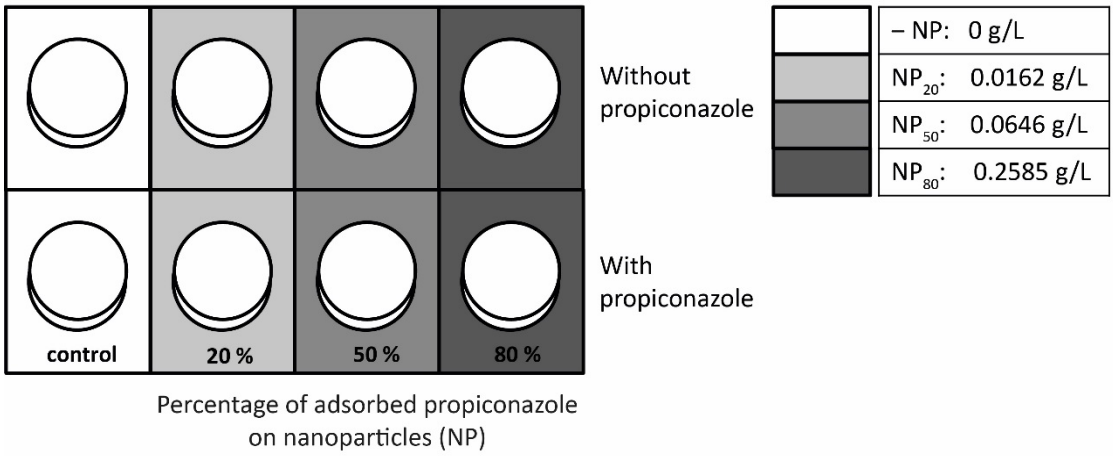

Figure 9: Scheme of the experimental set-up consisting of eight treatment groups with or without addition of propiconazole and nanoparticles (NPs) to fungal growth medium. The amount of NPs added was equivalent to a certain percentage of propiconazole adsorption (20, 50, and 80 %). Depending on fungal species 0.1 mg/L, 2 mg/L, or 5 mg/L propiconazole were introduced for *L. bicolor*, *C. geophilum*, or *A. muscaria*, respectively.

As key response variable, fungal colony growth was measured in each treatment replicate. Over a period of 14 - 70 days (depending on fungal species), the diameter of each fungal colony was recorded weekly along two predefined orthogonal axes, which were drawn on the bottom of the Petri dish prior to growth. Fast-growing fungi were evaluated every 3rd or 4th day. In addition, the total area covered by the fungal mycelium after this period was determined using the software ImageJ2x (Schindelin *et al.* 2015, Rueden *et al.* 2017). Area measurements provide further meaningful information, as colony growth is not strictly circular. Toxicity was assessed as the percentage of growth inhibition (Zambonelli and Iotti 2001).

Furthermore, the effect of NPs, propiconazole, and NPs loaded with propiconazole on the plant-fungus-interaction was studied. Poplar seedlings were used as plant hosts to form separate symbioses with two fungal species, *A. muscaria* and *C. geophilum*. For each species, the experimental set-up consisted of a full factorial combination of the two predictor variables, NP concentration (negative control plus one single NP concentration, resulting in 80 % of propiconazole adsorption) and fungicide presence. For each of the resulting four treatment groups

17 replicate plant-fungus set-ups were measured. Petri dishes were assigned randomly in mini-greenhouses. The establishment of ectomycorrhizal structures was determined using the gridline intersection method (Giovannetti and Mosse 1980, Brundrett *et al.* 1996). This destructive method facilitates measurements of the total length of a plant root and those parts of the root that are colonized with fungal mycelium (see outline point 4.10.5 Quantification of ectomycorrhizal associations using the gridline intersection method).

In addition, the expression of genes related to ergosterol biosynthesis was studied. Some fungi exposed to azole fungicides are known to show elevated levels of mRNA involved in the sterol synthesis pathway. In *Leptosphaeria maculans*, for example, the application of flutriafol resulted in an increased 14α demethylase-encoding mRNA level (Griffiths and Howlett 2002). In *Fusarium graminearum*, the azole fungicide tebuconazole led to an enhanced expression of the gene encoding the C-24 sterol methyltransferase (Liu *et al.* 2010). Therefore, the genes *erg6* and *erg11* were chosen to evaluate their expression in *L. bicolor* grown in the presence of propiconazole. *Erg11* encodes for 14α demethylase, the direct target of propiconazole. *Erg6* encodes for the C-24 sterol methyltransferase, an enzyme that is involved in a later transformation step of zymosterol to fecosterol (see Table 1).

Table 1: Ergosterol biosynthesis from squalene to ergosterol with relevant genes, corresponding enzymes, emerging intermediates, and inhibitors that hinder the biosynthesis at certain steps (modified according to White *et al.* 1998). The names of genes and enzymes that are of major interest in this thesis are marked in bold.

Sterol intermediate	Gene name	Enzyme name	Inhibitor
Squalene			
↓	*erg1*	Squalene epoxidase	Allylamines Thiocarbamates
2,3 - oxidosqualene			
↓	*erg7*	Lanosterol synthase	
Lanosterol			
↓	***erg11***	**Lanosterol (C-14) demethylase**	Azoles
↓	*erg24*	C-14 sterol reductase	Morpholines
↓	*erg25* and others	C-4 sterol demethylase enzymes	
Zymosterol			
↓	***erg6***	**C-24 sterol methyltransferase**	
Fecosterol			
↓	*erg2*	C-8 sterol isomerase	Morpholines
Episterol			
↓	*erg3*	C-5 sterol desaturase	
↓	*erg5*	C-22 sterol desaturase	Azoles (?)
↓	*erg4*	C-24 sterol desaturase	
Ergosterol			

4.9 Molecular biological methods

For the composition of solutions and buffers required for molecular biological methods, please see Appendix 8.1.

4.9.1 Isolation of DNA

DNA from frozen fungal mycelium was isolated using the DNeasy® Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Fungal tissue was ground to fine powder under liquid nitrogen using a mortar and pestle. DNA was eluted in 50 µL Buffer AE rather than 100 µL in two consecutive steps. DNA was immediately stored at -80 °C.

4.9.2 Quantification of nucleic acids

Concentration of isolated DNA and RNA was determined photometrically (Biophotometer Eppendorf, Hamburg, Germany). As a blank, 70 µL bidistilled water was used, and 1 µL DNA or RNA was used for measurements (70 µL plus 1 µL). Additionally, absorbance at 280 nm, 260 nm, and 230 nm was measured, and the ratios A260/A230 and A260/A280 were recorded to assess quality and purity of nucleic acids.

4.9.3 Gel electrophoresis of DNA

DNA fragments were separated via gel electrophoresis for approx. 20 min at 135 V using 2 % agarose gels and 0.5 % TAE-Buffer. 5 µL DNA-Marker was applied and 1 - 5 µL isolated DNA or PCR-product. Gels were stained in 0.0001 % ethidium bromide under agitation (50 rpm, Mini-Shaker Modell Kühner, B. Braun Melsungen AG, Melsungen, Germany). Visualization was performed via UV-light ("Gel Doc 2000", Bio-Rad Laboratories GmbH, München, Germany).

4.9.4 Isolation of RNA

Total RNA of fungal mycelium was isolated using the RNeasy® Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. All steps were performed as quickly as possible. Samples were eluted after an incubation time of 5 min in two consecutive steps using 45 µL and 30 µL RNase-free water each. RNA was immediately preserved in liquid nitrogen and stored at -80 °C.

4.9.5 Gel electrophoresis of RNA

For quantitative real-time PCR (qPCR), integrity of RNA was assessed using gel electrophoresis. 3 µL RNA sample (or 1500 ng RNA) was supplemented with 5 µL loading buffer. RNA and 3 - 5 µL 100 bp marker were separated in a 1 % agarose gel in 1x TBE buffer (see recipe in Appendix 8.1) for 20 min at 120 V. Staining was performed in Midori Green (250 mL water complemented with 40 µL Midori Green Advance, Nippon Genetics Europe GmbH). Only RNA that clearly showed fractions in the gel corresponding to *18S*, *28S*, and *5S rRNA* was further processed and quantified.

4.9.6 DNase treatment

DNA was removed from all RNA samples using the kit "DNase I" (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) according to the manufacturer's instruction. 100 ng RNA were inserted per reaction.

4.9.7 Synthesis of cDNA

DNase-treated RNA (5 µL per reaction, equal to 45.45 ng RNA) was transcribed into complementary DNA (cDNA) using the "iScript™ Advanced cDNA Synthesis Kit for RT-qPCR" (Bio-Rad Laboratories GmbH, München, Germany) according to the manufacturer's instructions.

4.9.8 Polymerase Chain Reaction (PCR)

DNA was amplified using the Taq PCR Core Kit (Qiagen GmbH, Hilden, Germany). Reactions were prepared according to Table 2. PCR reactions were performed in a thermal cycler under the conditions listed in Table 3. By applying a gradient of 13 °C at step 4 (see Table 3) following annealing temperatures were tested for each primer pair: 48.8, 52.1, 55.0, and 57.7 °C.

Table 2: Reaction set-up using the Taq PCR Core Kit (Qiagen GmbH, Hilden, Germany).

20 µL PCR reaction	
2 µL	10x Buffer
4 µL	5x Q-Solution
0.4 µL	Q-dNTPs
0.3 µL	10 µM Primer (fw)
0.3 µL	10 µM Primer (rev)
0.06 µL	Taq-Polymerase (5 U/µL)
1 µL	DNA
11.94 µL	Sterile, bidistilled water

Table 3: Cycling conditions for PCR.

Step	**Temperature**	**Time**	
Lid temperature	105 °C	Preheat	
1.) Start	23 °C	2 min	
2.) Initial denaturation	95 °C	5 min	
3.) Denaturation	95 °C	0.5 min	
4.) Annealing	46 °C	1 min	
5.) Extension	72 °C	0.5 min	Cycle from step 3 - 5, repeated 34x
6.) Final extension	72 °C	1 min	

4.9.9 Quantitative real-time PCR (qPCR)

In order to perform qPCR, the generated cDNA (20µl) was diluted with 80 µL sterile, bidistilled water. To obtain negative controls, 0.5 µL DNase-treated RNA was diluted with 9.5 µL bidistilled water.

Quantitative real-time PCR was performed using "SsoAdvanced™ Universal SYBR® Green Supermix" (Bio-Rad Laboratories GmbH, München, Germany) in line with the manufacturer's instructions. For a 20 µL total reaction mix volume, 10 µL SsoAdvanced™ Universal SYBR® Green Supermix, 7 µL nuclease-free water, 2 µL template (in total 0.909 ng cDNA or RNA), and 1 µL primer pair (forward and reverse primers, 5 µM each) were utilized. Template intended to be amplified with *18S* primer pair was diluted 1:1,000 (equal to 0.000909 ng cDNA).

Several potential reference genes were tested for their suitability, each using various primer pairs. Similarly, different primer pairs for potential target genes were assessed. The sequences of the employed primers are listed in Table 4 and Table 5. The real-Time PCR System Bio-Rad® iQ™ 5 (Bio-Rad Laboratories GmbH, München, Germany) was applied in the standard setting. Cycling conditions are listed in Table 6.

Table 4: Sequence of forward (fw) and reverse (rev) primers of potential reference genes for *L. bicolor* in 5'-3' direction. Protein IDs and coded targets are stated according to the *Laccaria bicolor* database (Joint Genome Institute). Primer sequences originating from publications are indicated by subscript numbers: 1: *18S rRNA* (Fajardo López *et al.* 2008); 2: elongation factor 2 (Xu *et al.* 2015); 3: elongation factor 3 and metalloprotease (Navarro-Ródenas *et al.* 2015).

Protein ID	Coded target	Primer	Sequence 5'-3'
Lacbi2|601976	*18S rRNA*	*18S*_Lbi_fw	cag-agc-cag-cga-gtt-ttt-tc [1]
		*18S*_Lbi_rev	gtt-tcc-ggc-tcc-cca-aag-c [1]
Lacbi2|332611	Elongation factor 2	EF2_1_Lbi_fw	ggc-atg-gga-gaa-ctt-caa-tca [2]
		EF2_1_Lbi_rev	gcc-aga-gac-gca-atc-agt-gtt [2]
Lacbi2|332611	Elongation factor 2	EF2_2_Lbi_fw	gtc-ccg-cct-tct-tat-caa-cg
		EF2_2_Lbi_rev	gcc-aga-gac-gca-atc-agt-gt
Lacbi2|659644	Elongation factor 3	EF3_1_Lbi_fw	caa-gga-gaa-gag-tct-gct-taa-gat-gc [3]
		EF3_1_Lbi_rev	cgt-tgg-gac-cga-gaa-cgg-caa-cac [3]
Lacbi2|659644	Elongation factor 3	EF3_2_Lbi_fw	cct-gtc-tcg-tgg-tct-ctc
		EF3_2_Lbi_rev	ggc-tgg-gaa-gga-aag-gtc-gg
Lacbi2|473056	Metalloprotease	Metallo_1_Lbi_fw	gca-aac-ttg-atg-atc-caa-cga-cc [3]
		Metallo_1_Lbi_rev	gca-aca-tca-cgg-tat-gtt-ctc-agc [3]
Lacbi2|473056	Metalloprotease	Metallo_2_Lbi_fw	acg-cac-atc-acc-tcc-gcc
		Metallo_2_Lbi_rev	agc-ctc-cgt-caa-gag-ttt-cg

Table 5: Sequence of forward (fw) and reverse (rev) primers of potential target genes for *L. bicolor* in 5'-3' direction. Protein IDs and coded targets are stated according to the *Laccaria bicolor* database (Joint Genome Institute).

Protein ID	Coded target	Primer	Sequence 5'-3'
Lacbi2|184995	Sterol 24-C-methyltransferase	Erg6_1_Lbi_fw	gcc-gtc-tgg-gtt-atg-gag
		Erg6_1_Lbi_rev	ggg-gtg-aat-agt-ttt-gtg-cg
Lacbi2|184995	Sterol 24-C-methyltransferase	Erg6_2_Lbi_fw	gag-tgg-gca-atg-act-gac-ga
		Erg6_2_Lbi_rev	aat-cca-acg-gtc-ttg-agg-gc
Lacbi2|184995	Sterol 24-C-methyltransferase	Erg6_3_Lbi_fw	att-ccc-ccg-tca-ctc-gtt-tc
		Erg6_3_Lbi_rev	cat-ctc-gct-gtg-tag-cca-tct
Lacbi2|646218	Sterol 14α demethylase	Erg11_1_Lbi_fw	acc-atc-att-gcc-acc-gtc-at
		Erg11_1_Lbi_rev	tca-gtc-aaa-ctt-tct-cct-ccg
Lacbi2|646218	Sterol 14α demethylase	Erg11_2_Lbi_fw	cgg-tcg-ggc-aaa-ctc-ctc-aa
		Erg11_2_Lbi_rev	tga-tac-agg-gcg-tcg-gca-acc

Table 6: Cycling conditions for qPCR.

Step	Temperature	Time	
1.) Polymerase activation and DNA denaturation	95 °C	30 sec	
2.) Denaturation	95 °C	10 sec	
3.) Annealing/extension and plate read	60 °C	30 sec	Cycle from step 2 - 3, repeated 40x
4.) Final denaturation	95 °C	1 min	
5.) Final extension	60 °C	1 min	
6.) Melt curve analysis	60 - 95 °C	6 sec/step	0.5 °C increment

Primers were designed using the *L. bicolor* genome data base (Martin *et al.* 2008) at the genome portal of the Joint Genome Institute (JGI) (Grigoriev *et al.* 2011, Nordberg *et al.* 2013). The Primer-BLAST tool of the National Center for Biotechnology Information (NCBI) was used to design specific primers (Ye *et al.* 2012). The software Primer Premier 5 (Premier Biosoft) was applied to check for possible secondary structures among potential primer sequences, in order to avoid the formation of hairpins, self-dimers and cross dimers (Lalitha 2000). Primer specificity

was examined by JGI BLAST analysis (Table 7). Only specific primers without secondary structures were utilized. PCR annealing conditions were optimized by performing a gradient PCR with an annealing temperature ranging from 53 to 72 °C. Subsequently, PCR products were separated via gel electrophoresis. The size of the amplicons in the gel coincided with the expected length (see Table 7). In qPCR analysis, the quantification cycle (C_q) is a relevant parameter. It names the PCR cycle, in which fluorescence signals that are correlated to DNA amplification cross a threshold line of fluorescence. Comparing C_q -values, melting curves and bands in the gel, the optimum annealing temperature for all primer pairs was found to be 60 °C. The experiments performed in the work contributing to this thesis focused on the primer pairs Erg6_1, Erg11_2, Metallo_1, EF3_1, and *18S*, as they performed well in qPCR. All EF2 primer pairs resulted in higher C_q -values ranging from 32 to 35 and were, therefore, excluded. All selected primer pairs except *18S* primers consisted of one exon-spanning primer each (see Table 7).

Table 7: Different primer features, primer specificity, and amplicon length of genomic DNA (gDNA) as well as complementary DNA (cDNA). Protein IDs are stated according to the *Laccaria bicolor* database (Joint Genome Institute, JGI). a: Primer nucleotide sequence was compared with the *L. bicolor* genome by JGI BLAST analysis using the database *Laccaria bicolor* v2.0 filtered model transcripts. For each forward (fw) and reverse (rev) primer, the total number of hits is stated and the number of matching nucleotides is shown in brackets.

Protein ID	Primer	Primer feature	Primer specificity [a]	Desired gDNA amplicon [bp]	Desired cDNA amplicon [bp]
Lacbi2|601976	*18S*_Lbi_fw		3 hits (3x 20 bp)	Not possible	231
	*18S*_Lbi_rev		4 hits (1x 14 bp, 3x 19 bp)		
Lacbi2|332611	EF2_1_Lbi_fw		6 hits (21 bp, 2x 17 bp, 3x 15 bp)	67	67
	EF2_1_Lbi_rev		2 hits (21 bp, 16 bp)		
Lacbi2|332611	EF2_2_Lbi_fw		1 hit	126	126
	EF2_2_Lbi_rev		2 hits: (20 bp,16 bp)		
Lacbi2|659644	EF3_1_Lbi_fw	Exon-spanning	1 hit	Not possible	128
	EF3_1_Lbi_rev		5 hits (1x 24, 4x 15 bp)		
Lacbi2|659644	EF3_2_Lbi_fw		2 hits (18 bp, 14 bp)	169	119
	EF3_2_Lbi_rev		2 hits (20 bp, 15 bp)		
Lacbi2|473056	Metallo_1_Lbi_fw	Exon-spanning	3 hits (23 bp,16 bp,15 bp)	Not possible	124
	Metallo_1_Lbi_rev		2 hits (24 bp, 15 bp)		
Lacbi2|473056	Metallo_2_Lbi_fw		3 hits (18 bp, 2x 14 bp)	148	148
	Metallo_2_Lbi_rev		2 hits (20 bp, 16 bp)		
Lacbi2|184995	Erg6_1_Lbi_fw		1 hit	Not possible	119
	Erg6_1_Lbi_rev	Exon-spanning	1 hit		
Lacbi2|184995	Erg6_2_Lbi_fw		1 hit	137	137
	Erg6_2_Lbi_rev		2 hits (20 bp, 15 bp)		

Protein ID	Primer	Primer feature	Primer specificity [a]	Desired gDNA amplicon [bp]	Desired cDNA amplicon [bp]
Lacbi2|184995	Erg6_3_Lbi_fw	Completely within 3' UTR region	1 hit	137	128
	Erg6_3_Lbi_rev		1 hit		
Lacbi2|646218	Erg11_1_Lbi_fw		1 hit	188	132
	Erg11_1_Lbi_rev		1 hit		
Lacbi2|646218	Erg11_2_Lbi_fw		1 hit	Not possible	146
	Erg11_2_Lbi_rev	Exon-spanning	1 hit		

To determine the performance characteristics of the qPCR, a calibration curve was produced. DNase treatment and cDNA synthesis were performed with 1,000 ng RNA input. Obtained cDNA was 10-fold serial diluted to gain 9.09, 0.909, 0.0909, 0.00909, 0.000909, and 0.0000909 ng/µL cDNA. In the case of *18S* amplification, cDNA was further diluted to cover 7 logs of starting quantity. For any other qPCR reaction, template cDNA covered 5 logs. The cDNA of each sample was subjected to qPCRs using all primer pairs in triplicates. PCR efficiency, linearity, dynamic range, sensitivity and specificity were analyzed. For standard curve analysis, C_q -values were plotted against log target concentrations, and slope (m) and R^2 values (coefficient of determination values) were determined. PCR efficiency (E) was calculated by the following equation (Bio-Rad 2013):

$$E = 10^{[-1/m]} - 1$$

The qPCR performed with *18S* primers showed an efficiency of 95.9 %, a slope value of -3.424 Δ C_q/log ng of DNA and 7 logs of dynamic range from C_q 8.83 to 32.85. The R^2 value was 1.000 (see Figure 10). For analysis of qPCRs with *18S* primers, a threshold value of 106.73 RFU (relative fluorescence units) was applied.

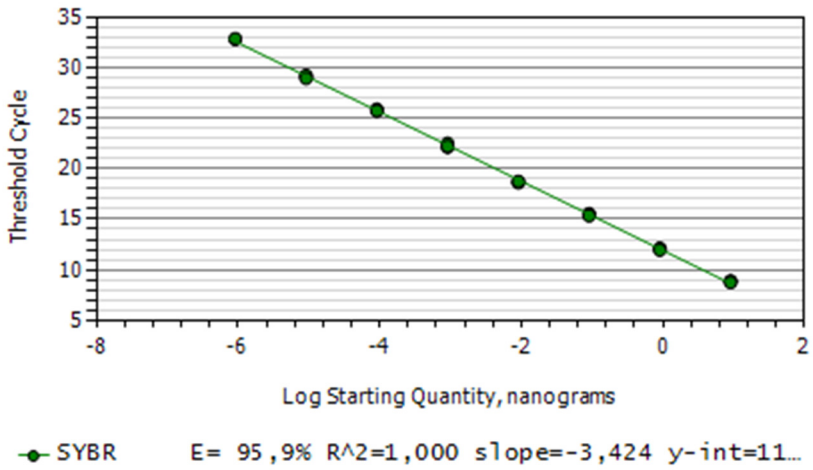

Figure 10: Standard curve of *18S* qPCR reaction.

Amplification reactions with Erg6_1 primers exhibited a qPCR efficiency of 98.9 %, a slope value of -3.3491 Δ C_q/log ng of DNA, and a R^2 value of 0.998. The dynamic range of qPCR reactions was between C_q 20.85 and 30.85, and covered 3 logs of dynamic range (see Figure 11). For analysis of qPCRs with *erg6* primers, a threshold value of 129.56 RFU was applied.

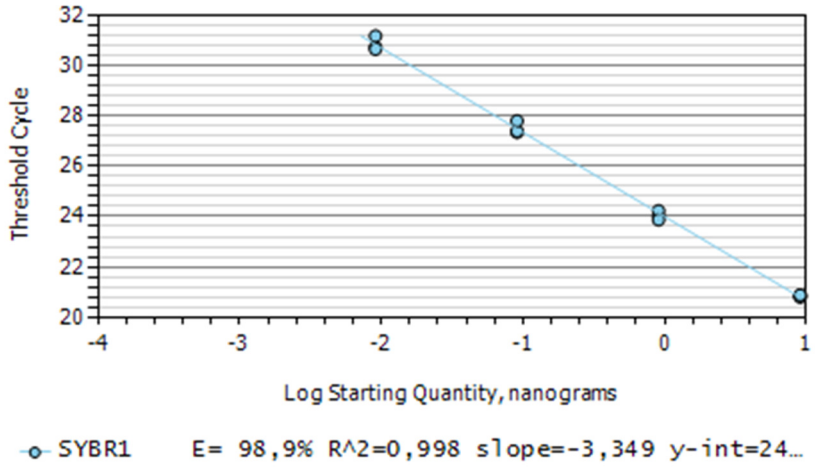

Figure 11: Standard curve of Erg6_1 qPCR reaction.

The qPCR performed with the Erg11_2 primer pair showed a PCR efficiency of 101.6 %, a slope value of -3.284 Δ C_q log ng of DNA, and a R^2 value of 0.999 (see Figure 12). The dynamic range of qPCR reactions reached from C_q 22.77 to 35.89 and herewith covered 4 logs of dynamic range. For analysis of qPCRs with *erg11* primers, a threshold value of 131.73 RFU was applied.

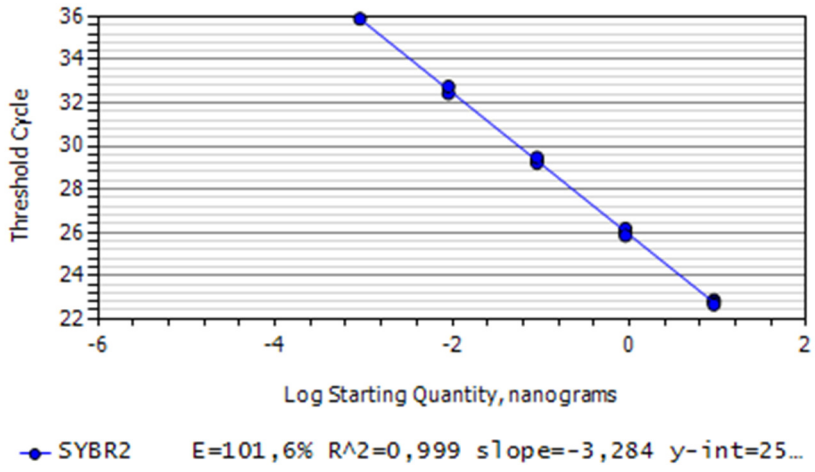

Figure 12: Standard curve of Erg11_2 qPCR reaction.

The qPCR conducted with the EF3_1 primer pair revealed a PCR efficiency of 92.8 %, a slope value of -3.507 Δ C_q log ng of DNA, and a R^2 value of 0.999 (see Figure 13). The qPCR using these primers resulted in 5 logs of dynamic range, whereby the corresponding C_q -values ranged from C_q 18.05 to 35.83.

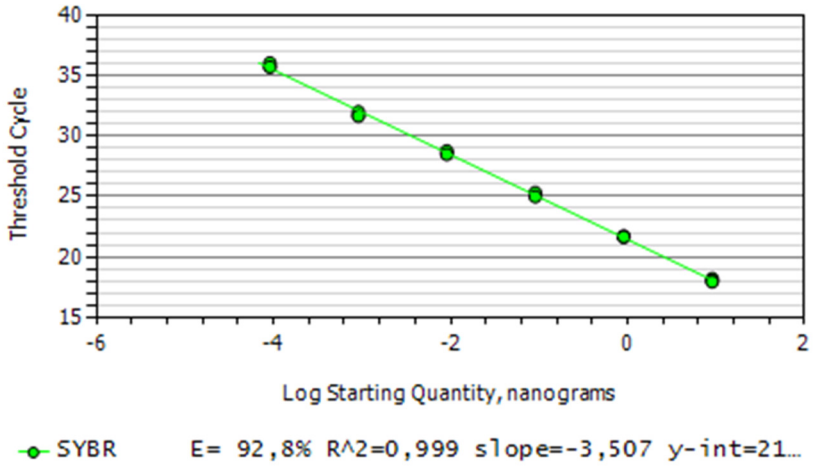

Figure 13: Standard curve of EF3_1 qPCR reaction.

The qPCR performed with the Metallo_1 primer pair showed an efficiency of 92.1 %, a slope value of -3.528 Δ C_q/log ng of DNA, and 4 logs of dynamic range from C_q 16.91 to 30.94. The associated R^2 value was 0.999 (see Figure 14).

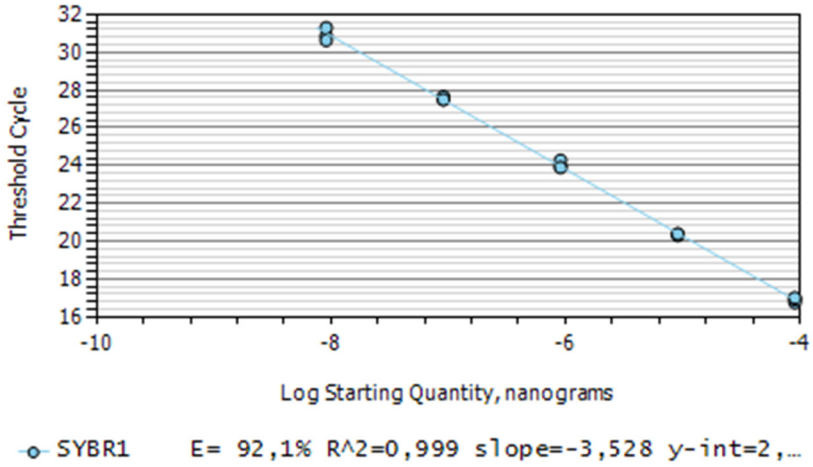

Figure 14: Standard curve of Metallo_1 qPCR reaction.

For all qPCR reactions, melt curve profiles were controlled. No primer dimers or additional PCR products could be detected, and therefore, PCR reactions proved to be highly specific. Moreover, all qPCR parameters were within the accepted range: PCR efficiency was between 90 - 110 %, slope values ranged from -3.6 to -3.1 ΔC_q/log ng of DNA, and R^2 values were ≥ 0.980 (Bio-Rad 2013).

In order to determine an optimal reference gene, cDNA of all sample groups was amplified in technical duplicates with primers of potential reference genes. In total, 4 potential reference genes were tested: *18S rRNA*, elongation factor 1, elongation factor 2 and metalloprotease (see Table 4). For each reference gene, the standard deviation of all C_q -values of all treatment samples was calculated. The qPCRs performed with *18S* primers revealed the lowest standard deviation of C_q -values with a value of 0.65.

All cDNA samples were run in technical duplicates and obtained C_q -values were checked for their reliability. A standard deviation of 0.5 was considered to be in the range of acceptable variance. Samples with a higher variation were rejected or repeated. For each sample, the process of amplification was monitored, and the melt curve of each qPCR reaction was examined for additional peaks. In addition, C_q -values were found to be within the linear dynamic range of the qPCR reaction. Furthermore, non-template controls (NTCs) were run on each 96-well-PCR plate to check for DNA contamination in the master mix of each utilized primer pair. Negative controls of each sample (DNase treatment of RNA not being subscribed into cDNA) were subjected to qPCR reactions and checked for genomic DNA contamination. The C_q -value of each cDNA sample was subtracted from the C_q -value of the corresponding NTC. If the resulting ΔC_q was 7 or higher, gDNA contamination was considered to be irrelevant. A ΔC_q of 7 is equal to a gDNA contribution of 0.78125 % to the overall amount of DNA present in the sample (Bio-Rad 2013).

For analysis, the respective gene-specific threshold value was applied. All samples of a given gene were applied in one plate (sample maximization strategy), which results in the most exact values (D'haene 2013). Reference gene and target genes do not need to be run on the same PCR-well plate. By analyzing all samples of a certain gene in one qPCR run, run-to run variation was avoided, which would be a second type of induced technical variation (D'haene 2013).

PCR characteristics are crucial to define the appropriate applicable math model for qPCR analysis. If the subtraction of the slope of the reference gene from the slope of the target gene results in a value of ≥ 0.1 ΔC_q/log ng of DNA, the $\Delta\Delta C_q$ math model has to be rejected and the Pfaffl method must be applied (Bio-Rad 2013). This was the case for qPCR analysis related to *erg11* expression. For all expression analyses, the Relative Expression Software Tool REST 2009 V2.013 was used (Pfaffl *et al.* 2002). This software considers the different PCR efficiencies of the tested genes and applies randomization and bootstrapping methods to test the statistical

significance of the results. Expression levels of *erg6* and *erg11* were normalized to the expression of the reference gene *18S* in the control and treatment group, and up- or down-regulation concerning the treatment was estimated.

4.10 Analyses

4.10.1 Determination of growth parameters

4.10.1.1 Colony diameter

Fungal growth was determined by measuring the colony diameter along two orthogonal axes placed on the bottom of the Petri dish prior to growth. Growth was recorded on a regular basis depending on fungal specific rate of growth.

4.10.1.2 Area of fungal colony

Fungal growth was regularly recorded by marking the outermost extent the fungal mycelial front reached after a certain time along the two marked axes. At the end of a growth experiment, the complete rim of each fungal colony was marked at the bottom of the Petri dish. The area of fungal growth was calculated using scans of all Petri dishes and the software ImageJ2x (Schindelin *et al.* 2015, Rueden *et al.* 2017). The spatial increase of fungal growth was encircled with software tool devices, and the enclosed area was calculated on the basis of pixel-to-cm-ratio.

4.10.1.3 Dry weight measurements

Besides colony diameter and colony area, fungal growth can be determined by fresh and dry weight measurements. For submerged cultures, vacuum filtration is required to determine the fresh weight of fungal biomass. However, this method is prone to error, as it is difficult to guarantee the same moisture content in all samples. Therefore, dry weight measurements were chosen to compare fungal growth in liquid cultures. Fungal material was collected by means of a büchner funnel lined with filter paper and vacuum. Filter papers were dried before use and their weight was recorded. Cultivation flasks were rinsed with water to gather all fungal material. After drying for 3 days at 60 °C, fungal material adhering to the filter paper was weighed and the dry weight of fungal material alone was calculated.

4.10.2 Growth inhibition – comparing different parameters

Fungi exposed to toxic compounds or harsh environmental conditions show a reduced growth. Inhibition of growth was used as a comparative value to evaluate the detrimental impact of propiconazole and particles. To express percentage inhibition of fungal growth concerning colony diameter, the following modified formula was applied (Pandey *et al.* 1982, Zambonelli and Iotti 2001):

$$Inhibition\ [\%] = \frac{\Delta\ Control - \ \Delta\ Treatment}{\Delta\ Control} * 100$$

Whereas

$$\Delta\ Control =$$

$$\text{Final colony diameter of control group} - \text{Initial colony diameter of control group}$$

and

$$\Delta\ Treatment =$$

$$\text{Final colony diameter of treatment group} - \text{Initial colony diameter of treatment group}$$

In terms of fungal spatial increase, the formula mentioned above was used, replacing diameter data with data derived from area measurements. The area of the fungal inoculum (agar plug) was subtracted from the area that was covered by fungal mycelium at the end of the experiment. To express inhibition with regard to dry weight increase, the relevant data were conformed to the formula in the same vein.

4.10.3 Quantification of propiconazole concentration in liquid growth medium

Samples of liquid growth medium were stored at 4 °C and analyzed via HPLC by Leyla Guluzada to quantify their propiconazole content. Liquid samples were injected into the column (Eclipse Plus C18, 3.5 µm, 4.6*100 nm, Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany) of an HPLC system (Shimadzu Deutschland GmbH, Neufahrn/Freising, Germany) and eluted with a liquid phase consisting of 80 % MeOH (Chromasolv®, gradient grade for HPLC, ≥ 99.9 %, Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) and 20 % Milli-Q water (Milli-Q academic, Filter: Millipak 40 gamma gold 0.22 um). One run lasted for 20 min (pumping rate: 0.4 mL/min). Prior to analysis, propiconazole was applied as a mixture of stereoisomers (analytical standard, PESTANAL®, Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany), which resulted in two peaks with retention times of 16.29 and 16.79 min. The area of the propiconazole peak in the chromatogram is proportional to its concentration and was used to quantify the fungicide concentration.

4.10.4 Quantification of propiconazole concentration in fungal material

Fungal dry material was analyzed for its propiconazole content. Until analysis, samples were stored at -20 °C. Anna-Jorina Wicht performed further sample processing and fungicide determination. The determination of propiconazole concentration via HPLC followed a modified version of the so-called QuEChERS method (Quick, Easy, Cheap, Effective, Rugged und Safe) (Anastassiades *et al.* 2003). Samples were amended with 20 mg NaCl (Merck KGaA, Darmstadt, Germany), ground to fine powder and extracted with water/acetonitrile (1/1, hypergrade, LiChrosolv®, Merck KGaA, Darmstadt, Germany). For liquid-liquid partitioning, samples were mixed with NaCl and $MgSO_4$ (Merck KGaA, Darmstadt, Germany). The acetonitrile phase was evaporated under a nitrogen stream to dryness, and resuspended in 0.5 mL MeOH (hypergrade, LiChrosolv®, Merck KGaA, Darmstadt, Germany). After dilution (1:10 or 1:100), samples were injected into a C18 column of a HPLC-ESI-Q-TOF system (Q-TOF 6550, Agilent Technologies, Santa Clara, California, USA), and analyzed in the positive ionization mode. For elution, a MeOH/water gradient (hypergrade, LiChrosolv®, Merck KGaA, Darmstadt, Germany) containing 0.1 % formic acid (eluent additive for LC-MS, Fluka, Merck KGaA, Darmstadt, Germany) was used ranging from 5 - 95 % MeOH. To perform quantification, samples were spiked with the deuterated analytical standard propiconazole-(phenyl-d3) (Merck KGaA, Darmstadt, Germany), and derived signals were analyzed with the MassHunter Workstation software (Agilent Technologies, Santa Clara, California, USA). The analysis was performed with five biological replicates that were measured in technical duplicates. Using this method, the amount of propiconazole that accumulated in or adhered to the fungus could be analyzed.

4.10.5 Quantification of ectomycorrhizal associations using the gridline intersection method

One goal of this thesis was to analyze whether NP-associated fungicides affect the fungal establishment of mycorrhizas. The symbiotic relationship begins with fungal colonization of plant roots. Outer signs for successful establishment in poplar species are swollen root tips. Dissection microscopes would reveal modifications within roots. As it is not feasible to check every root tip for Hartig net occurrence, those root parts colonized by fungal mycelium were determined as “mycorrhizal”.

These ectomycorrhizal associations were assessed according to the gridline intersection method (Giovannetti and Mosse 1980, Brundrett *et al.* 1996). The root was separated from the stem, the length of the main root was measured using a graph paper, and the whole root system was cut into 1 cm pieces. The root fragments were equally distributed in a round 9 cm Petri dish containing 8 mL water. The Petri dish was positioned on a square Petri dish that contained rectangular gridlines with a distance of 0.5 inch or 1.27 cm between each (see Figure 15).

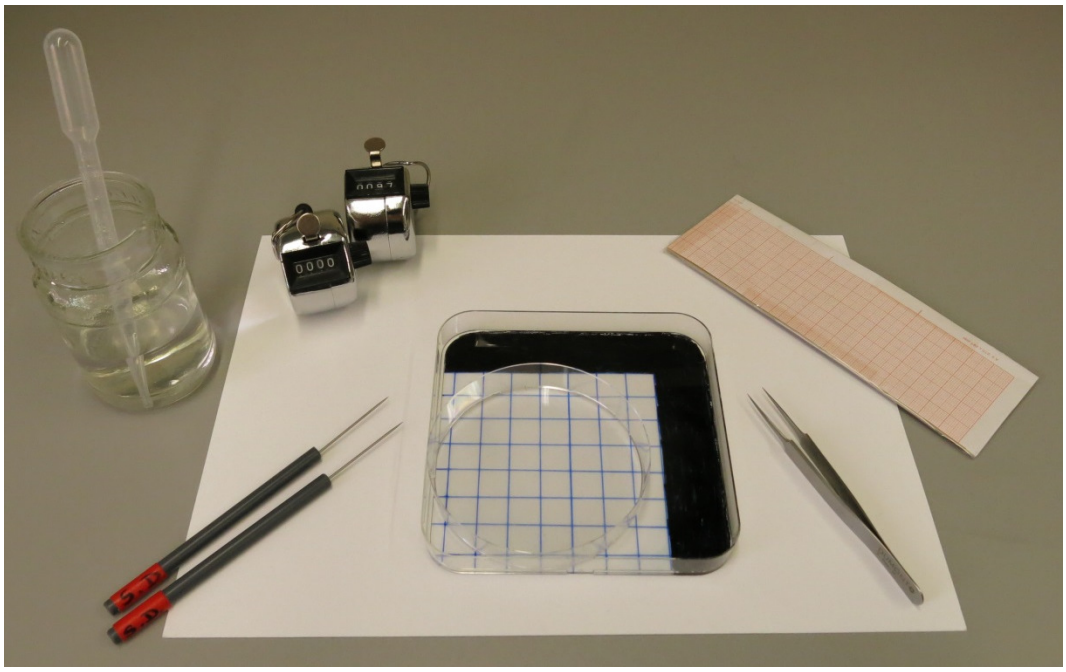

Figure 15: Materials needed for gridline intersection method: water, dissecting needles, two counting devices, forceps, graph paper, and a Petri dish positioned on grid lines with a distance of 0.5 inch.

Using a binocular microscope, all colonized root tips were examined under 90x magnification, and counted if designated mycorrhizal root tips. With the help of a counting device, all crossings of root segments on all horizontal and vertical lines of the gridline system were counted, which results in the total root length [cm] according to Marsh (1971) and Giovannetti and Mosse (1980). In addition, the length of colonized parts of the root was determined by counting all intersections between mycorrhizal root parts and horizontal and vertical gridlines. To compare different treatments, the two parameters "mycorrhizal tips per root length" and "percentage of colonized root length" were used.

As evaluation is prone to observer subjectivity and might vary within a given day as well as from day to day, all treatment groups were homogenously distributed for evaluation issues and assessed by one single, well-trained person. In this way, observer bias was reduced and the same evaluation standard (even when varying) was applied to all plant-fungus set-ups.

4.10.6 Statistical analysis

All data relating to fungal growth, ectomycorrhizal success, and propiconazole concentration in medium or fungal material were statistically analyzed using the software PAST, version 3.0 (Hammer *et al.* 2001). All datasets were tested for normality using Shapiro-Wilk or Jarque-Bera tests. Homogeneity of variance was assessed using Levene's test based on medians. Data sets that did not meet the parametric assumptions were log transformed by applying the functions $y = \log_{10}(x)$ or $y = \log_{10}(x+1)$. Differences among treatment groups were assessed using one-way

analysis of variance (ANOVA) and the Tukey's test for "post hoc" pairwise comparisons (parametric assumptions assumed) or non-parametric Kruskal-Wallis test and Mann-Whitney pairwise post-hoc tests. Parameters and differences among different treatments were considered significant when $p < 0.05$. When there were only two treatments, two-sample *t*-tests in combination with an F-test for the equality of variance were used for comparison. In the case of unequal variance, the unequal variance *t*-statistic was used.

Data derived from qPCR studies were analyzed using the Relative Expression Software Tool, REST 2009 V2.013 (Pfaffl *et al.* 2002). Two biological replicates descending from two independent fungal growth experiments were analyzed. One test sample was obtained by pooling the fungal material cultivated in three Petri dishes. A qPCR was performed using two technical replicates and cDNA templates from 2 - 4 cDNA syntheses of one sample. The percentage coefficient of variation of C_q -values was for all treatment groups under 2.4 % and considered to be reproducible. The software REST takes primer-specific PCR efficiencies into account and determines differential gene expression by randomization and bootstrapping methods. Expression levels of *erg6* and *erg11* were normalized to *18S rRNA* gene expression levels and evaluated in relation to a control treatment.

5 Results

5.1 Effect of nanoparticles, propiconazole, and their combination on fungi in liquid cultivation

The effect of PMONPs and the fungicide propiconazole on fungi was investigated. PMONPs were synthesized by Leilei Luo. According to strict definitions, NPs are compounds with a diameter of 1 - 100 nm in at least one dimension. Having an average size of 360 nm, these PMONPs neglect the stringent criteria for NPs but are nevertheless in the nano range. For the sake of convenience and simplicity, the PMONPs will be stated here as NPs. Sorption behavior of propiconazole and NPs in liquid media was studied by Leyla Guluzada. Previous experiments have revealed that the fungal species *L. bicolor*, *C. geophilum* and *A. muscaria* were more sensitive to propiconazole when cultivated on solid rather than in liquid growth medium. For liquid culture experiments, 5 mg/L propiconazole was chosen. This is the highest fungicide concentration that still enables sorption within the linear range of the Langmuir model, as assessed in isotherm studies by Leyla Guluzada, and thus enables a mathematical determination of the required NP amount to gain a certain percentage of sorbed propiconazole.

5.1.1 Effect of propiconazole on three fungal species

The sole effect of propiconazole on fungi was studied in submerged cultures. All three fungal species were exposed to either 5 mg/L propiconazole or the equivalent solvent control MeOH. The total MeOH content was 0.1 % for each treatment group. The growth media of this experimental set-up were analyzed via HPLC by Leyla Guluzada to quantify the freely available propiconazole concentration present in these conditions before and after the experiment.

At the beginning of an experiment and before fungal material was added, the medium contained a mean concentration of 4.57 mg/L propiconazole, as revealed by an analysis of three biological replicates (see day 0, Figure 16). One equivalent mock culture without fungal inoculation was kept for 7 days under shaking conditions. The concentration of this sample was 4.63 mg/L propiconazole, which is within the standard deviation of the measurements at the starting conditions. After 7 days of cultivation, culture filtrates of *A. muscaria* and *C. geophilum* did not show any propiconazole content. Here, the level of propiconazole dropped below the detectable limit. In contrast, the culture filtrate of *L. bicolor* exhibited a mean concentration of 1.37 mg/L propiconazole (Figure 16).

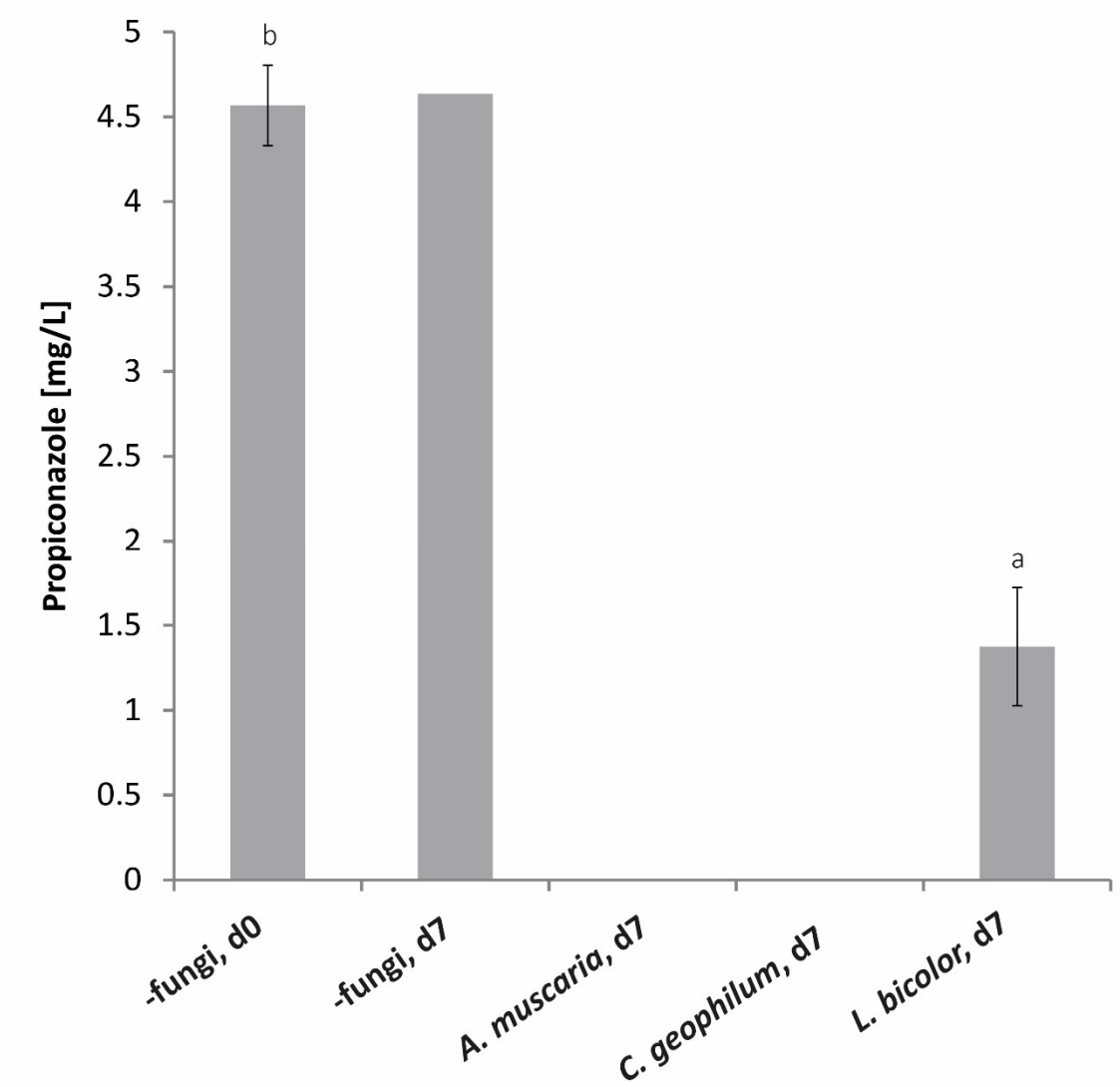

Figure 16: Propiconazole content in cell-free culture media of mock or fungal cultures of *A. muscaria*, *C. geophilum* and *L. bicolor*. All treatment groups were initially supplemented with **5 mg/L propiconazole** and sampled at day 0 or day 7. Analysis was performed via HPLC by Leyla Guluzada. Bars represent mean ± SD, n = 3 with following exception: n = 1 for "-fungi, d7". The two treatment groups "-fungi, d0" and "*L. bicolor*, d7" were solely subjected to statistical analysis as the residual treatment groups either consist of only a single value or have no variance. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to a two-sample *t*-test ($t = 13.147$; $p < 0.001$).

Each fungal culture was started with an inoculum of 0.5 g fresh mycelium. After an incubation of 7 days, fungal biomass was collected, thoroughly rinsed with desalinated water and dried to evaluate its dry weight increase. After one week of cultivation, *C. geophilum* showed the highest biomass increase from 0.057 to 0.138 g dry weight (see Figure 17). Maximum inhibition of fungal growth was observed in *A. muscaria.* In this species, 5 mg/L propiconazole significantly inhibited additional fungal growth by 70 %. In *C. geophilum* and *L. bicolor*, dry weight increase was also significantly reduced by 12 and 25 %, respectively.

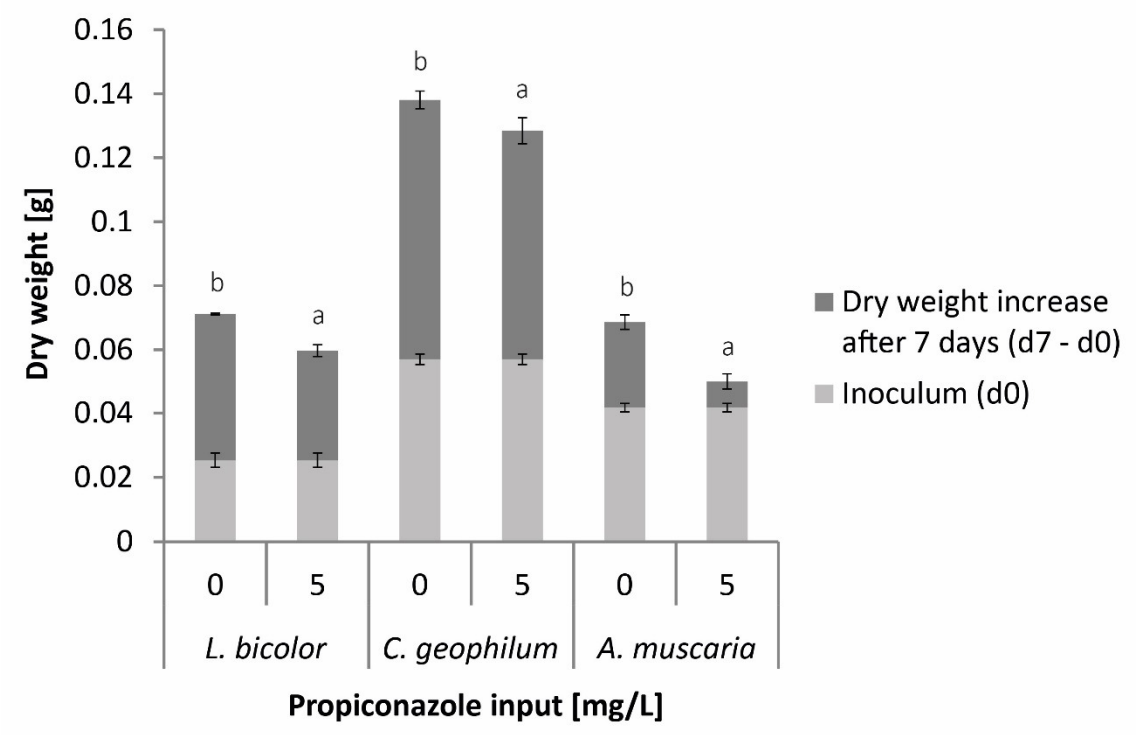

Figure 17: Dry weight of fungal biomass derived from liquid cultures in the presence and absence of 5 mg/L propiconazole. Cultures were started with an inoculum of 0.5 g fungal fresh weight. After 7 days of cultivation, all fungal material was collected and dried. Representing starting amounts were dried (bright grey parts of the column, d0) and subtracted from the final dry weight obtained after cultivation. Dark grey parts of the column (d7 minus d0) represent dry weight increase within 7 days. Bars represent mean ± SD, n = 5. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to a two-sample *t*-test (*L. bicolor*: unequal variance $t = 13.747$; $p < 0.001$; *C. geophilum*: $t = 4.3681$; $p = 0.0023$; and *A. muscaria*: $t = 12.869$; $p < 0.001$).

Fungal dry material was analyzed for its propiconazole content by Anna-Jorina Wicht. In *A. muscaria* a mean content of 33.18 µg propiconazole / g fungal mass was detected after 7 days of cultivation (Figure 18). The highest concentration was observed in *C. geophilum*, which is 784.99 µg/g propiconazole. *L. bicolor* showed a mean concentration of 280.34 µg/g propiconazole.

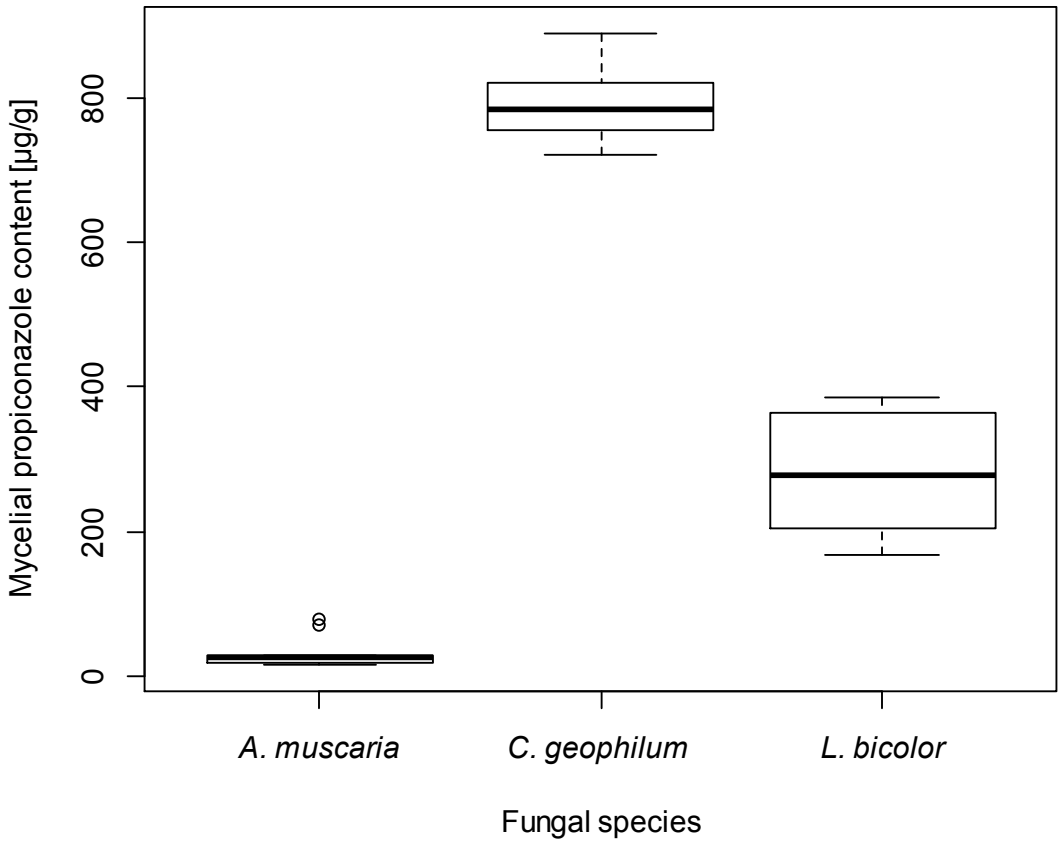

Figure 18: Propiconazole content of fungal mycelium cultivated for 7 days in liquid MMN medium, 1 % glucose supplemented with 5 mg/L propiconazole. Mycelia of *A. muscaria*, *C. geophilum*, and *L. bicolor* were dried and stored at -20 °C until the samples were extracted and analyzed via HPLC by Anna-Jorina Wicht. Propiconazole concentrations are stated in µg propiconazole per g fungal material; n = 5, each analyzed in technical duplicates.

5.1.2 Effect of nanoparticle-sorbed propiconazole on A. muscaria

To determine the effect of sorption on the toxicity of propiconazole, an experimental set-up was chosen consisting of eight treatment groups (see Figure 9). Three different concentrations of NPs were added to the liquid growth medium that establish hypothetical sorptions of 20, 50, and 80 % of the available propiconazole. These NP concentrations are in increasing order: 0.032 g/L (NP_{20}, which sorbs 20 % of the present propiconazole concentration), 0.129 g/L (NP_{50}, which sorbs 50 % of the present propiconazole concentration), and 0.517 g/L (NP_{80}, which sorbs 80 % of the present propiconazole concentration). All three NP concentrations were applied either individually or in combination with 5 mg/L propiconazole. In addition, two control groups were tested: one group without the amendment of NPs and fungicide and one group with the sole input of propiconazole. Since the highest applicable fungicide concentration (5 mg/L propiconazole) showed a strong negative impact on *A. muscaria* in submerged culture after seven days (70 % inhibition of fungal growth) but did not affect *L. bicolor* and *C. geophilum*, *A. muscaria* was exclusively used as test organism in this experimental set-up.

In order to verify propiconazole stability and sorption on NPs during the time of cultivation, growth media derived from mock cultures without fungal inoculum were sampled on day 0 and day 7. The respective propiconazole content of each treatment group was analyzed via HPLC. Media without NP amendment displayed a mean concentration of 5.77 mg/L propiconazole (Figure 19). The higher the amount of NPs added to fungal growth media, the lower was the amount of free propiconazole in media. Within 7 days, the propiconazole content in liquid media was relatively constant across all treatment groups; each were sampled initially and after 7 days. Nonetheless, statistically significant differences in the culture media were observed within the treatment groups that contained either no NPs or NP_{50} (0.129 g/L). However, these differences are regarded as neglectable variances inherent to the experimental or analytical system.

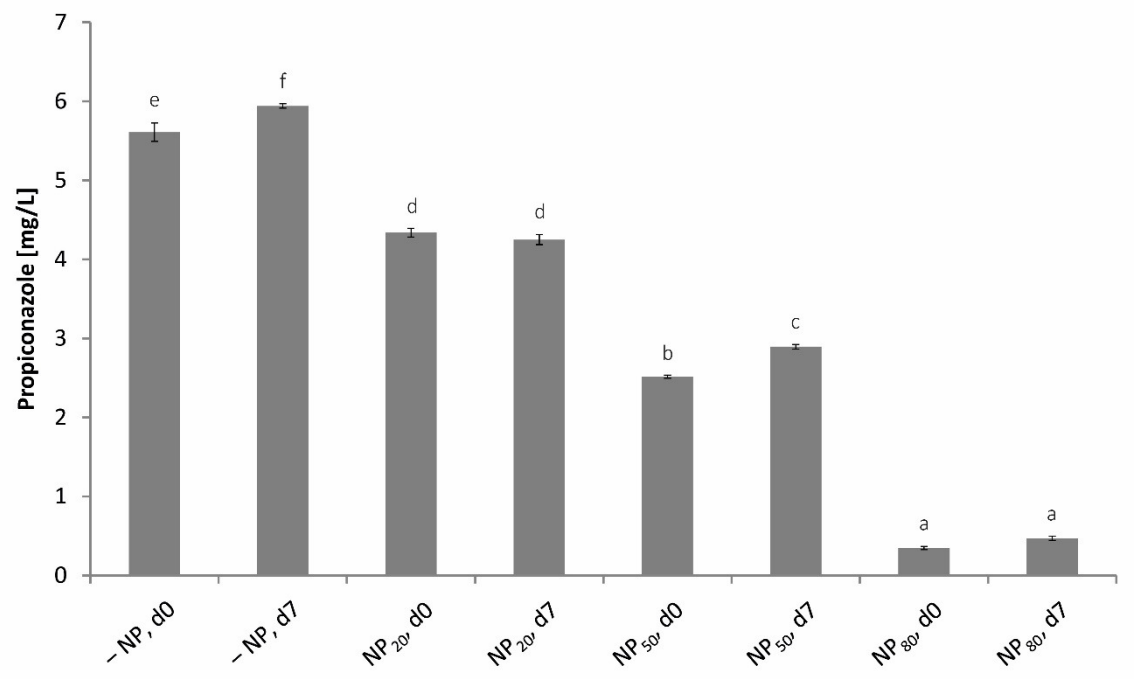

Figure 19: Propiconazole content in culture medium without fungi. Liquid growth medium was supplemented with **5 mg/L propiconazole** (prop) and nanoparticles (NP) or an appropriate solvent control. The propiconazole content was measured at day 0 or day 7 via HPLC analysis. The medium was not inoculated but kept under culture conditions. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. HPLC analysis was performed by Leyla Guluzada. Bars represent mean ± SD, n = 3 technical replicates. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 4618$; $p < 0.001$) and Tukey's post hoc test.

Overall, the samples covered a wide range of freely available propiconazole concentrations. It was intended to achieve sorptions of 20, 50, and 80 % of propiconazole on NPs, which is equivalent to 80, 50, and 20 % of freely available propiconazole in the water fraction. The medium that contained 0.032 g NP/L (NP_{20}) exhibited a content of 4.29 mg/L propiconazole (Table 8). In that medium, about 25 % of the initial propiconazole input was sorbed on NPs according to HPLC analysis. The addition of the middle NP concentration, NP_{50}, led to a sorption of about 50 % of propiconazole, exactly as intended. In the presence of the highest NP concentration, around 90 % of the original propiconazole input sorbed on the NPs.

Table 8: Survey of propiconazole content and sorption of propiconazole (prop) on nanoparticles (NP) in liquid growth media containing different NP levels, as assessed by HPLC analysis. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Mean values of three technical replicates are shown. HPLC analysis was performed by Leyla Guluzada.

Treatment	Free propiconazole [mg/L]	Sorption of propiconazole on NP [%]	Intended sorption [%]
– NP, prop	5.77	0.00	0
NP_{20}, prop	4.29	25.60	20
NP_{50}, prop	2.71	53.12	50
NP_{80}, prop	0.41	92.92	80

In submerged cultures of *A. muscaria*, the propiconazole content in growth medium declined over time. Already thirty minutes after inoculation with *A. muscaria*, the level of propiconazole in liquid medium was 3.78 mg/L. After 7 days of cultivation, the propiconazole content dropped below the detection limit.

Table 9: Dry weight increase and percentage inhibition of liquid cultures of *A. muscaria* in the presence of 5 mg/L propiconazole (prop) and nanoparticles (NP), or appropriate solvent controls. Periodic mesoporous organosilica were added to fungal growth medium in different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs, respectively. After 7 days, fungal material was collected and dried. Dry weight increase within 7 days and percentage inhibition of each treatment in relation to *A. muscaria* grown in the absence of fungicide and NPs are stated, n = 5. If fungal growth was not inhibited but promoted, the percentage promotion is stated in parentheses and marked with an asterisk *.

Treatment	Dry weight increase [g]	Inhibition / promotion [%]
– NP, – prop	0.042	
– NP, prop	0.039	7.19
NP_{20}	0.041	2.13
NP_{50}	0.044	(3.50)*
NP_{80}	0.051	(21.05)*
NP_{20}, prop	0.039	7.47
NP_{50}, prop	0.041	2.89
NP_{80}, prop	0.046	(7.76)*

Next to the analysis of liquid medium, fungal growth of *A. muscaria* was assessed after a cultivation of 7 days by dry weight measurements. Exposure to 5 mg/L propiconazole had only minor inhibitory activity on fungal mycelium (Table 9 and in Appendix 8.5.1, Figure 35). In the absence of the fungicide, a higher NP content resulted in an enhanced fungal dry weight. In the presence of propiconazole, a similar trend was observed. The higher the NP concentration loaded with propiconazole, the higher was the fungal biomass. If growth medium was supplemented with both NPs and propiconazole, fungal dry weight was slightly reduced, but not to a statistically significant degree compared to growth under fungicide-free conditions. This experiment was repeated twice. Similar results were obtained (see Appendix 8.6).

A. muscaria exposed to 5 mg/L propiconazole showed an inhibition of 7 % concerning dry weight increase within 7 days (Table 9). In the absence of propiconazole, growth of *A. muscaria* was promoted by higher NP concentrations. In response to NP_{50} and NP_{80} exposure, fungal dry weight increased by 3.5 and 21 %, respectively. When fungi were exposed to propiconazole that partly sorbed on NPs, their dry weight increase was slightly lower than that of the fungi solely exposed to NPs. An identical experiment was independently performed. For data concerning the propiconazole concentration in growth medium, the evaluated sorption of propiconazole on NPs, dry weight measurements, and respective growth inhibition see Figure 36, Table 21, Figure 37, and Table 22 in Appendix 8.6.

The propiconazole content was not only analyzed in liquid growth medium but also within the fungal biomass of *A. muscaria* after 7 days of cultivation. In the absence of NPs, a propiconazole concentration of 1.43 µg/g dry fungal material was detected (see Figure 20). Fungi exposed to different portions of particle-associated propiconazole were observed to have significantly higher fungicide contents than fungi solely exposed to propiconazole. In the presence of both NP_{50} and propiconazole, a fungicide concentration of 9.46 µg/g dry fungal mycelium was found, which is the highest concentration observed in this test system.

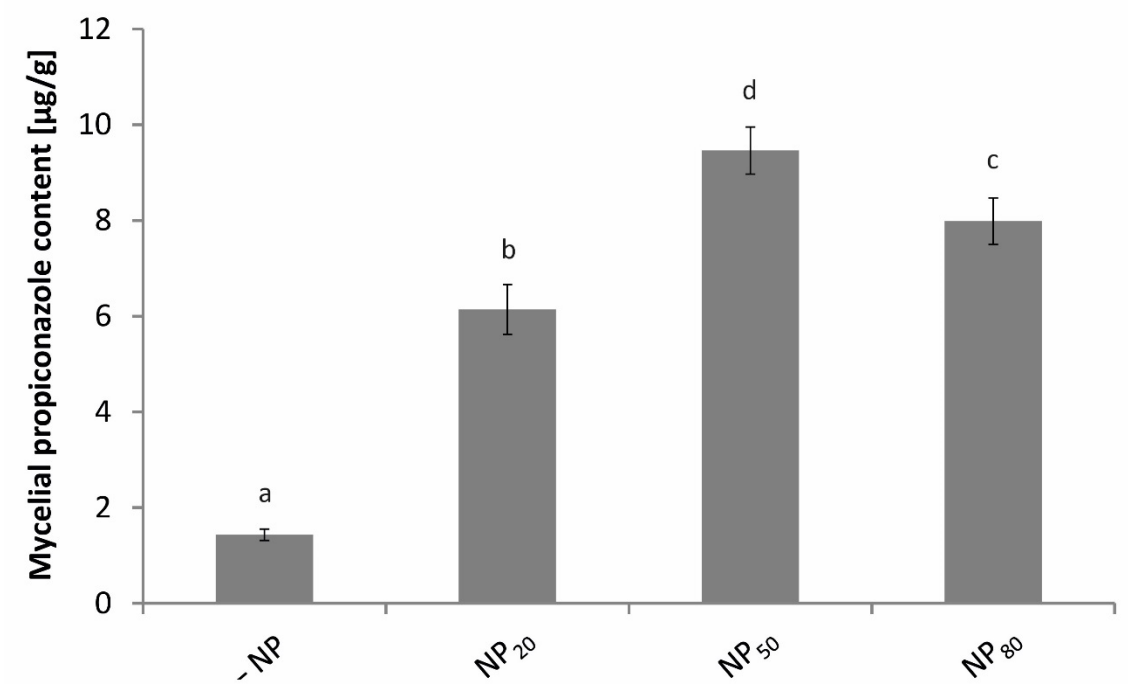

Figure 20: Propiconazole content of dry fungal material derived from liquid cultures of *A. muscaria* started with 5 mg/L propiconazole in the presence of different nanoparticle (NP) concentrations. Periodic mesoporous organosilica nanoparticles were added to fungal growth medium in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs, respectively. Cultures were started with an inoculum of 0.5 g fungal fresh weight. After 7 days, all fungal material was collected and dried. HPLC analysis was performed by Anna-Jorina Wicht. Bars represent mean ± SD, n = 5 with two technical replicates each. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 339.4$; $p < 0.001$) and Tukey's post hoc test.

The combined supply of 5 mg/L propiconazole and either NP_{80}, NP_{50}, or NP_{20} to liquid growth medium hypothetically results in three concentrations of freely available propiconazole, namely 1, 2.5, or 4 mg/L propiconazole. In order to study propiconazole uptake into fungal biomass and propiconazole degradation under these nominal input conditions, a further liquid growth experiment was performed in the sole presence of propiconazole. As a comparative group, the combined application of NP_{50} and 5 mg/L propiconazole was chosen, that generates 2.5 mg/L freely available propiconazole. After 7 days, all treatment groups showed a fungal dry weight of approx. 0.04 g (Figure 21). A one-way ANOVA revealed that there are no statistically significant differences between group means ($F = 1.959$; $p = 0.122$). For exact data on dry weight increase and the respective percentage inhibition see Table 23 in Appendix 8.7.

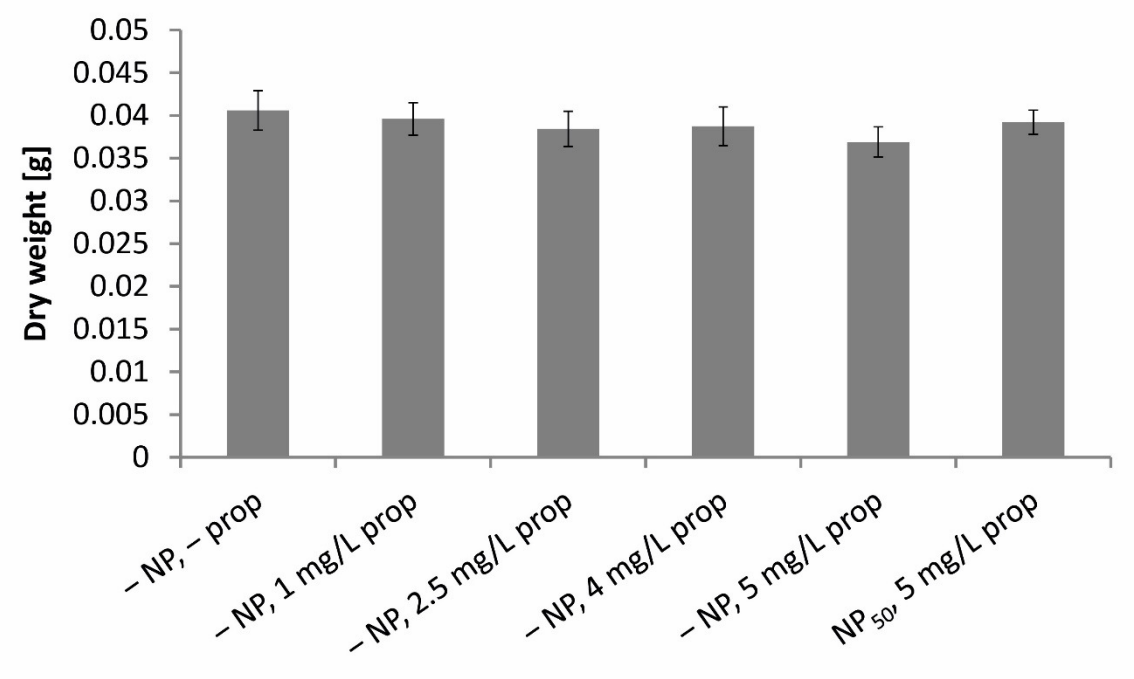

Figure 21: Dry weight of liquid cultures of *A. muscaria* in the presence of various concentrations of propiconazole (prop) and nanoparticles (NP), or appropriate solvent controls after 7 days of cultivation. Periodic mesoporous organosilica nanoparticles were added in a concentration of 0.129 g/L (NP_{50}) that sorb 50 % of the added propiconazole concentration. Cultures were started with an inoculum of 0.5 g fungal fresh weight. After 7 days, all fungal material was collected and dried. Bars represent mean ± SD, n = 5. According to one-way ANOVA there are no statistically significant differences ($p < 0.05$) among treatments groups ($F = 1.959$; $p = 0.122$).

Although the dry weight increase was not statistically significantly different in these treatment groups, the mycelial propiconazole content varied greatly, as analyzed by HPLC (Figure 22). In the absence of NPs and with increasing propiconazole input, the fungicide content of the mycelium of *A. muscaria* increased as well. When cultivated in the presence of 1 mg/L propiconazole, a content of 1.15 µg/g propiconazole was found in the mycelium. Exposure to 5 mg/L propiconazole resulted in a concentration of 4.97 µg/g propiconazole in fungal material. When *A. muscaria* was cultivated in the presence of NP_{50} and 5 mg/L propiconazole, the fungal material contained 23.82 µg/g propiconazole, which is a content twelve times higher than that of the control group cultivated at 2.5 mg/L propiconazole and statistically significantly different.

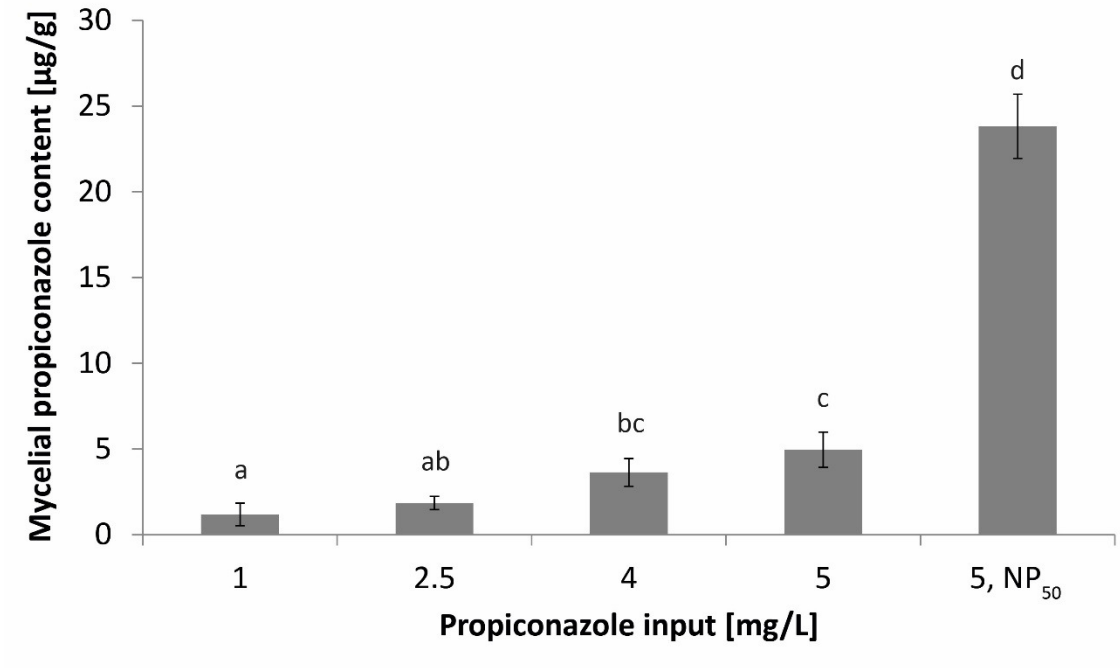

Figure 22: The propiconazole content of fungal material derived from liquid cultures of *A. muscaria* in the presence of different propiconazole concentrations without nanoparticles (NP) and in combination with NP_{50}. Periodic mesoporous organosilica nanoparticles were added to fungal growth medium in a concentration of 0.129 g/L (NP_{50}) representing a sorption of 50 % of the added propiconazole on NPs. Cultures were started with an inoculum of 0.5 g fungal fresh weight. After 7 days, all fungal material was collected and dried. HPLC analysis was performed by Anna-Jorina Wicht. Bars represent mean ± SD, n = 5 with two technical replicates each. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 388$; $p < 0.001$) and Tukey's post hoc test.

5.2 Effect of nanoparticles, propiconazole and their combination in solid cultivation

Furthermore, an aim of the work was to assess the effect of NPs and propiconazole both separately and in association on fungi cultivated on solid agar medium. A method was developed to supplement the fungal growth medium with these compounds. Several obstacles had to be overcome. Agar medium, for example, is sterilized by autoclaving, however, it solidifies when its temperature declines below 50 °C. In addition, neither NPs nor propiconazole are stable under the conditions of autoclaving. Therefore, both compounds were sterilized in MeOH and added in a later step to the autoclaved medium. Further, a certain time span is necessary for sorption processes between NPs and propiconazole. For preparation, the growth medium was thus split into a water and a medium fraction. Based on previous experiments, one hour was reserved for sorption interactions in sterile water. Subsequently, the water fraction was mixed with the concentrated, sterile agar medium. Moreover, ultrasonication of liquids was required to prevent NP agglomeration and to achieve homogenous NP dispersion. In addition, agar medium should quickly harden to minimize NP sedimentation. Hence, the agar medium was poured in pre-cooled Petri dishes placed on -20 °C cooling racks. The detailed procedure on the preparation of NP-containing growth medium is listed in Chapter 4.7.

A fungicide concentration was employed that was supposed to inhibit fungal growth at least by 70 %. For sorption purposes, three different NP concentrations were chosen that hypothetically sorb 20, 50, and 80 % of the present fungicide. For each of the three investigated fungi, namely *A. muscaria*, *C. geophilum* and *L. bicolor*, a species-specific fungicide concentration was applied. Each Petri dish was filled with agar medium and the center was inoculated with an agar plug from the rim of an actively growing fungal colony. The radial fungal growth descending from this agar plug was regularly recorded. Depending on the species-specific growth rate, fungi were cultivated until those colonies grown medium free of both propiconazole and NPs reached a diameter of about 4.2 cm. Accordingly, the experiment lasted 42, 70, and 14 days for *A. muscaria*, *C. geophilum*, and *L. bicolor*, respectively.

5.2.1 Single and combined effect on A. muscaria

After 42 days, *A. muscaria* showed a colony diameter increase of 4 cm, whereas 5 mg/L propiconazole reduced the diameter increase to 1.3 cm (see control groups, Figure 23). All three NP amounts did not affect colony diameter. For all three amounts, the presence of propiconazole partly associated with NPs resulted in each case in growth that was greater than that of the fungicide-containing control group and also lower than that of the fungicide-free control group. The lowest NP concentration combined with propiconazole led to a fungal growth similar to the

growth in the sole presence of the fungicide. There was no statistically significant difference between the two groups that were exposed to propiconazole either in the absence of NPs or in the presence of the lowest NP concentration. The means of the groups containing NP_{50} and NP_{80}, each associated with the fungicide, were statistically significantly different from each other and also different from each control group. The higher the concentration of propiconazole-associated NPs, the higher was the colony diameter increase.

As *A. muscaria* showed an asymmetrical growth behavior on agar medium, the area covered by the fungal mycelium after a growth period of 42 days was additionally investigated. On agar medium free of both propiconazole and NPs, *A. muscaria* increased its average area by 18.14 cm^2 (see Figure 24 and in Appendix 8.5.2, Table 18). In the presence of propiconazole, fungal growth was reduced to 2.98 cm^2 in terms of colony area increase, which is equal to 84 % inhibition. All three NP concentration levels resulted in an area increase that is similar to that of the control group free of both NPs and propiconazole. When NPs were applied in combination with propiconazole, area increase was inhibited by 83, 79, and 56 %. The higher the added NP concentration, the more propiconazole was hypothetically sorbed on the particles and the higher the observed area increase of fungal colonies. Statistical analysis of colony area increase revealed only marginal differences compared to the analysis of colony diameter increase (see Figure 23 and Figure 24). In addition, an overview of the obtained data is listed in Table 18, Appendix 8.5.2.

The described fungal growth experiment using *A. muscaria* as test species was repeated (for results see outline point 8.8.1). The same trend was observed: The addition of NPs did not affect growth. However, the highest NP concentration inhibited colony diameter increase by 26 %, which represents a statistically significant difference compared to the groups containing lower NP amounts (see Figure 38 in Appendix 8.8.1). The repeated experiment confirmed that the higher the applied NP concentration in the presence of propiconazole, the higher was the fungal growth. The combined supply of NP_{80} and propiconazole had the least inhibitory effect when comparing all propiconazole-containing treatment groups. In fact, NP_{20} and NP_{50} each associated with propiconazole led to a fungal area and diameter increase that is not statistically significantly different from that of the control group solely treated with propiconazole (Figure 38 and Figure 39 in Appendix 8.8.1). For exact values concerning colony and area increase as well as respective growth inhibition values see Table 24 in Appendix 8.8.1.

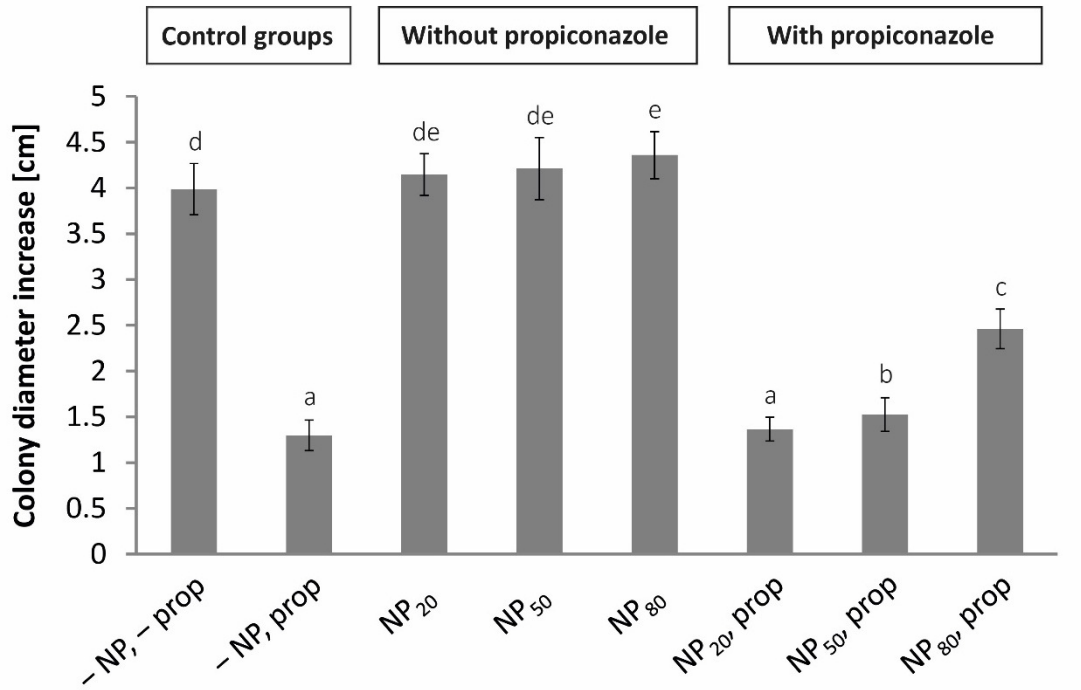

Figure 23: Colony diameter increase of *A. muscaria* cultivated on MMN agar medium supplemented with 0.5 % glucose for 42 days. Growth medium was amended with nanoparticles (NP) and **5 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony diameter was measured and the diameter of the starting inoculum (0.9 cm) was subtracted. Bars represent mean ± SD, n = 19. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 771.8$; $p < 0.001$) and Tukey's post hoc test.

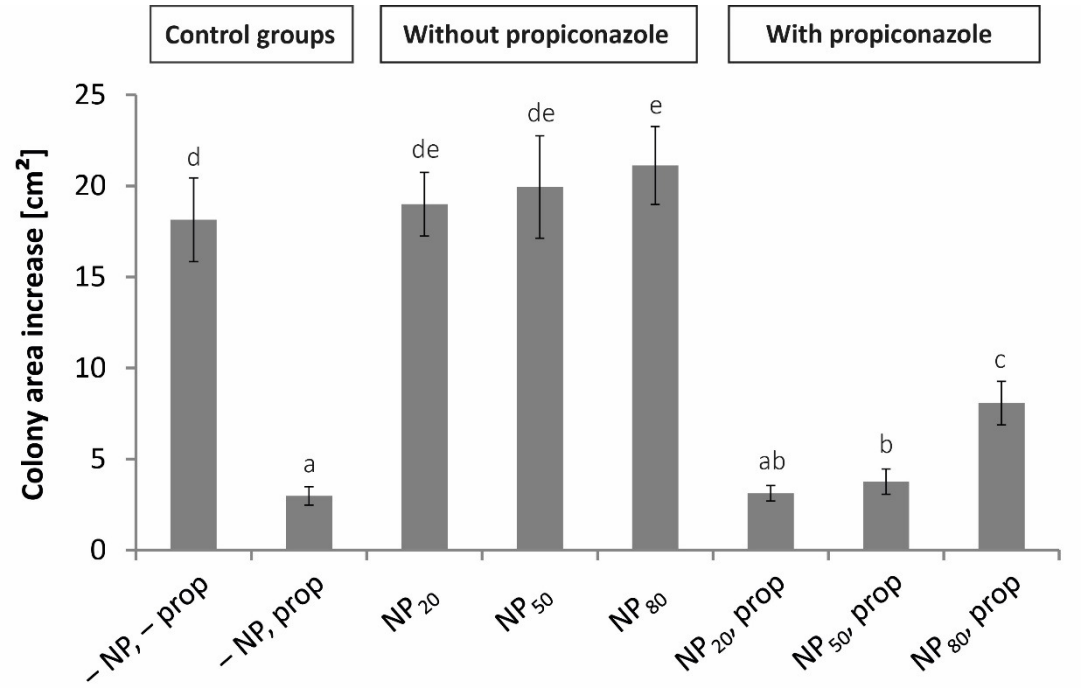

Figure 24: Colony area increase of *A. muscaria* cultivated on MMN agar medium supplemented with 0.5 % glucose for 42 days. Growth medium was amended with nanoparticles (NP) and **5 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony area was measured and the area of the starting inoculum (0.64 cm^2) was subtracted. Bars represent mean ± SD, n = 19. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to Kruskal-Wallis test (Hc (corrected) = 129.8; $p < 0.001$) and Mann-Whitney post hoc with Bonferroni corrected pairwise comparisons.

5.2.2 Single and combined effect on C. geophilum

As a representative of the Ascomycetes, *C. geophilum* was used in the test system to investigate the individual effect of NPs and propiconazole and their combination. As the growth speed of *C. geophilum* is relatively low, the diameter was recorded over a period of 70 days. In this time frame, *C. geophilum* increased its fungal diameter by 4.51 cm (Table 19, in Appendix 8.5.3). Based on previous lab experiments, a fungicide concentration of 2 mg/L propiconazole was chosen, which inhibited diameter increase by 94 %.

In the absence of propiconazole, *C. geophilum* showed a colony diameter increase of 4.5 to 5 cm in the treatment groups holding either no NPs or NPs at various concentrations (Figure 25). In fact, there was no statistically significant difference between these groups according to a Kruskal-Wallis test and Mann-Whitney post hoc test with Bonferroni corrected pairwise comparisons. In the presence of 2 mg/L propiconazole, *C. geophilum* showed an average diameter increase of 0.26 cm, which represents an inhibition of 94 % (Table 19, in Appendix 8.5.3). The exposure to NP_{80} associated with propiconazole resulted in a significantly higher fungal growth than the sole exposure to propiconazole. In addition, the observed growth was significantly lower than that of the control group free of propiconazole. When fungi were cultivated on medium that was amended with propiconazole and one of the two lowest NP concentrations, their growth was not statistically different to fungi that were cultivated in the sole presence of propiconazole (Figure 25).

Moreover, the area increase of these cultures was determined after 70 days. The data of the increase of both colony diameter and colony area are very much alike. The colony area increase of *C. geophilum* exposed to propiconazole was very low (Figure 26 and in Appendix 8.5.3, Table 19). Fungal growth was inhibited by 99 % regarding area increase. Similar to diameter measurements, the colony areas of fungi that were grown at various NP conditions free of propiconazole were not statistically significantly different. Fungal growth was very low in all treatment groups exposed to propiconazole. However, the combined supply of NP_{80} and propiconazole inhibited the area increase of *C. geophilum* by 80 % and resulted in a significantly greater growth than exposure to the fungicide alone. Fungi grown in the absence of both propiconazole and NPs showed a significantly greater growth than all fungi exposed to NP-associated propiconazole.

The described growth experiment testing *C. geophilum* was repeated. The above presented results were confirmed. Only marginal differences were observed (see Figure 40 and Figure 41 as well as Table 25, in Appendix 8.8.2, for an overview).

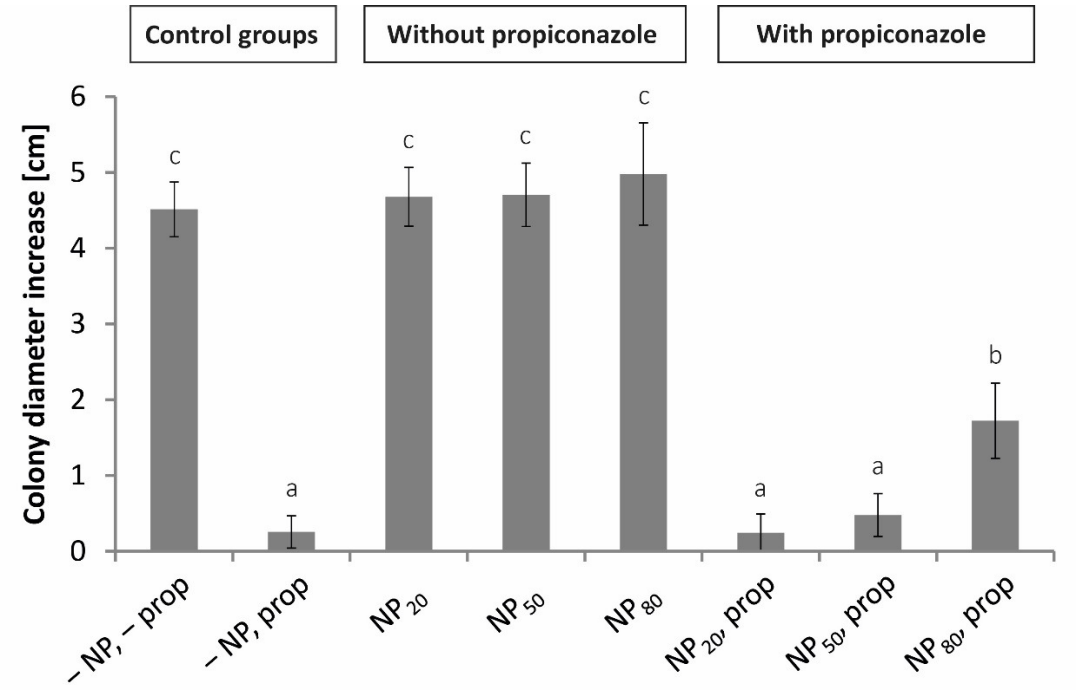

Figure 25: Colony diameter increase of *C. geophilum* cultivated on MMN agar medium supplemented with 0.5 % glucose for 70 days. Growth medium was amended with nanoparticles (NP) and **2 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony diameter was measured and the diameter of the starting inoculum (0.6 cm) was subtracted. Bars represent mean ± SD, n = 19. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to Kruskal-Wallis test (Hc (corrected) = 126.7; $p < 0.001$) and Mann-Whitney post hoc with Bonferroni corrected pairwise comparisons.

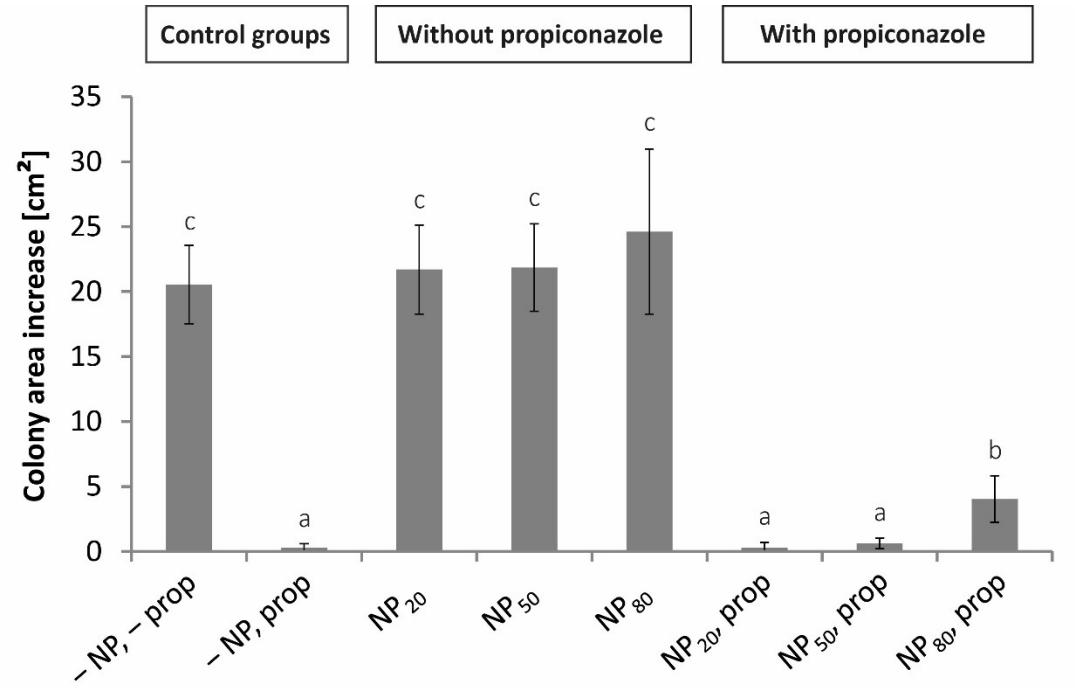

Figure 26: Colony area increase of *C. geophilum* cultivated on MMN agar medium supplemented with 0.5 % glucose for 70 days. Growth medium was amended with nanoparticles (NP) and **2 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony area was measured and the area of the starting inoculum (0.28 cm^2) was subtracted. Bars represent mean ± SD, n = 19. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to Kruskal-Wallis test (Hc (corrected) = 126.2; $p < 0.001$) and Mann-Whitney post hoc with Bonferroni corrected pairwise comparisons.

5.2.3 Single and combined effect on L. bicolor

5.2.3.1 Effects on the morphological growth level

Compared to *A. muscaria* and *C. geophilum*, *L. bicolor* is a fast-growing fungus under laboratory conditions. After only 14 days, the growth experiment was terminated. In the absence of propiconazole, *L. bicolor* showed an average diameter increase of 3.1 to 3.4 cm (Figure 27 and in Appendix 8.5.4, Table 20). Exposure to 0.1 mg/L propiconazole inhibited the diameter increase of *L. bicolor* by 70 %. The sole presence of NPs did not affect the growth of *L. bicolor*. The exposure to each of the three applied NP concentrations led to a slightly higher diameter increase but was not statistically significant except for NP_{50}. In the presence of NPs and propiconazole, growth was less inhibited. The higher the NP concentration in the medium, the higher was the observed fungal growth. NPs sorb freely available propiconazole and the inhibitory effect of propiconazole was reduced. Statistical analysis according to one-way ANOVA ($F = 679.8$; $p < 0.001$) and Tukey's post hoc test verified these findings. NP-associated propiconazole resulted in a colony diameter increase that was significantly different for each applied NP dosage ($p < 0.05$). The lowest NP concentration associated with the fungicide showed a diameter increase that was statistically significantly higher compared to the diameter increase achieved in the propiconazole-containing control group. The highest NP concentration loaded with the fungicide, however, showed a diameter increase that was not statistically different from the diameter increase obtained in the control group free of propiconazole.

The data concerning the area increase of *L. bicolor* correlate very well with the data derived from diameter measurements of the same cultures (Figure 27 and Figure 28). In the absence of propiconazole, the fungus reached area increases of around 10.6 to 11.8 cm^2 and according to one-way ANOVA ($F = 888.6$; $p < 0.001$) and Tukey's post hoc test, the means of area increase under conditions free of fungicide were not significantly different ($p < 0.05$) from one another. The sole presence of NPs in three varying concentrations had no statistically significant effect on the colony area increase. Fungi solely exposed to propiconazole showed an area increase of 1.6 cm^2, which is equivalent to an inhibition of 85 % (Table 20, in Appendix 8.5.4). In the case of NP-associated propiconazole, higher means of area increase were observed for those treatment groups containing higher NP amounts. When the fungicide was associated with the lowest NP concentration, the colony area increase was inhibited by 81 %. The middle NP concentration associated with propiconazole led to an inhibition of 56 %, while the highest NP concentration loaded with the fungicide resulted in a mere inhibition of 8 %.

For *L. bicolor*, fresh weight increase was recorded. Fungi were cultivated on cellophane discs placed on agar medium in order to perform further sample processing and qPCR analysis. Exposure to 0.1 mg/L propiconazole inhibited fungal fresh weight increase by 81 %. The presence

of NPs slightly promoted fungal growth with respect to its fresh weight. NP-associated propiconazole increased fungal fresh weight compared to exposure to propiconazole alone. NP_{20} loaded with propiconazole inhibited fresh weight increase by 74 %, while NP_{80} loaded with propiconazole inhibited fungal fresh weight increase by only 8 %.

An identical experiment was independently performed. For data concerning the colony diameter and area increase as well as respective growth inhibition levels of *L. bicolor* see Figure 42, Figure 43, and Table 26, in Appendix 8.8.3.

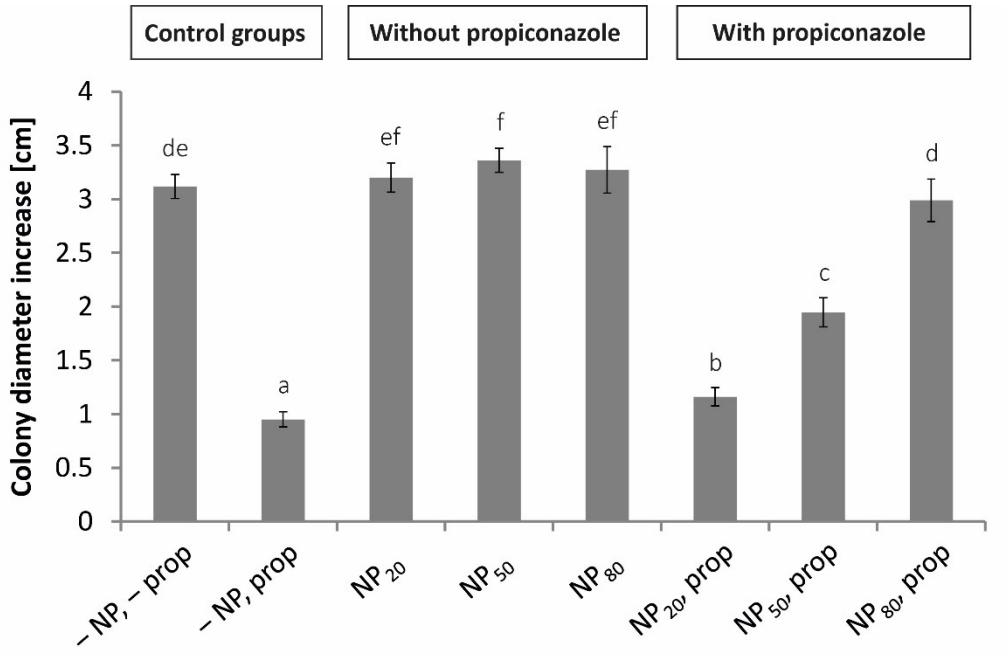

Figure 27: Colony diameter increase of *L. bicolor* cultivated on MMN agar medium supplemented with 0.5 % glucose for 14 days. Growth medium was amended with nanoparticles (NP) and **0.1 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony diameter of 9 replicates was measured and the diameter of the starting inoculum (0.6 cm) was subtracted. Treatment groups containing propiconazole but no NP, NP_{20} or NP_{50} were assessed with 15 replicates each. Bars represent mean ± SD. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 679.8$; $p < 0.001$) and Tukey's post hoc test.

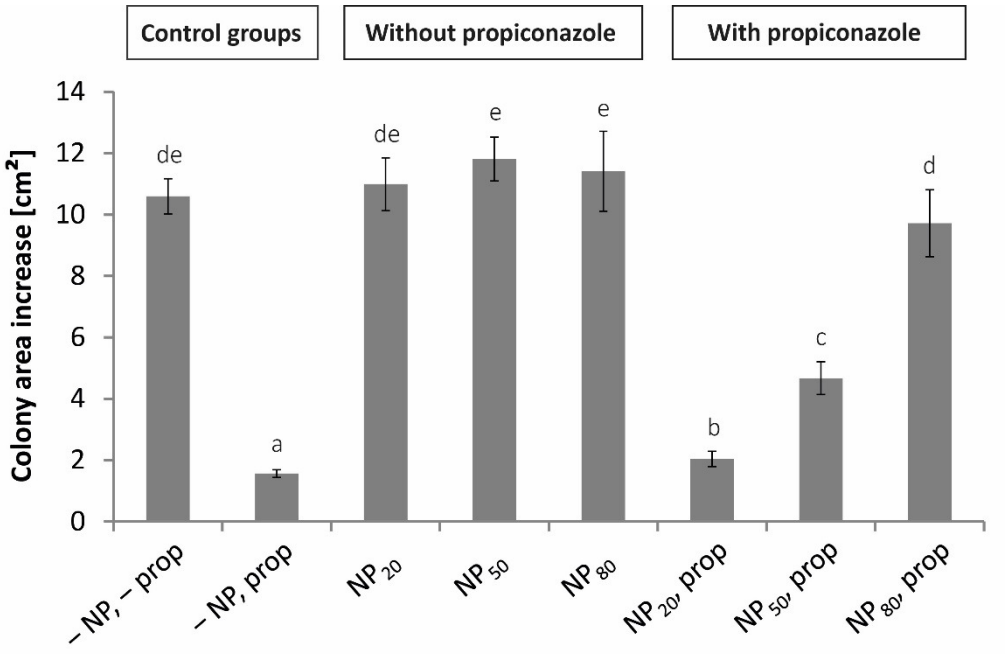

Figure 28: Colony area increase of *L. bicolor* cultivated on MMN agar medium supplemented with 0.5 % glucose for 14 days. Growth medium was amended with nanoparticles (NP) and **0.1 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony area of 9 replicates was measured and the area of the starting inoculum (0.28 cm^2) was subtracted. Treatment groups containing propiconazole but no NP, NP_{20}, or NP_{50} were assessed with 15 replicates each. Bars represent mean ± SD. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 888.6$; $p < 0.001$) and Tukey's post hoc test.

5.2.3.2 Effects on the molecular level

Apart from growth experiments, the single and combined effect of NPs and propiconazole was studied on the molecular level in *L. bicolor*. Fungal material grown on solid agar medium was analyzed for its differential gene expression. Two genes were selected that have been reported to respond transcriptionally to azole fungicides. The two chosen genes, *erg6* and *erg11*, encode the enzymes C-24 sterol methyltransferase and 14α demethylase, respectively. Both enzymes are involved in the ergosterol biosynthesis, which is blocked by propiconazole.

Prior to cultivation of *L. bicolor*, the agar medium was covered with cellophane to enable a subsequent removal of the fungal mycelium. Following a growth period of 14 days, the fungal material out of three Petri dishes was pooled and immediately stored at -80 °C. After RNA isolation, DNase treatment, and cDNA synthesis, qPCR analysis was performed. The expression of *erg6* and *erg11* was normalized by the expression of the reference gene *18S rRNA*, and evaluated in relation to the respective expression under control conditions using the software REST.

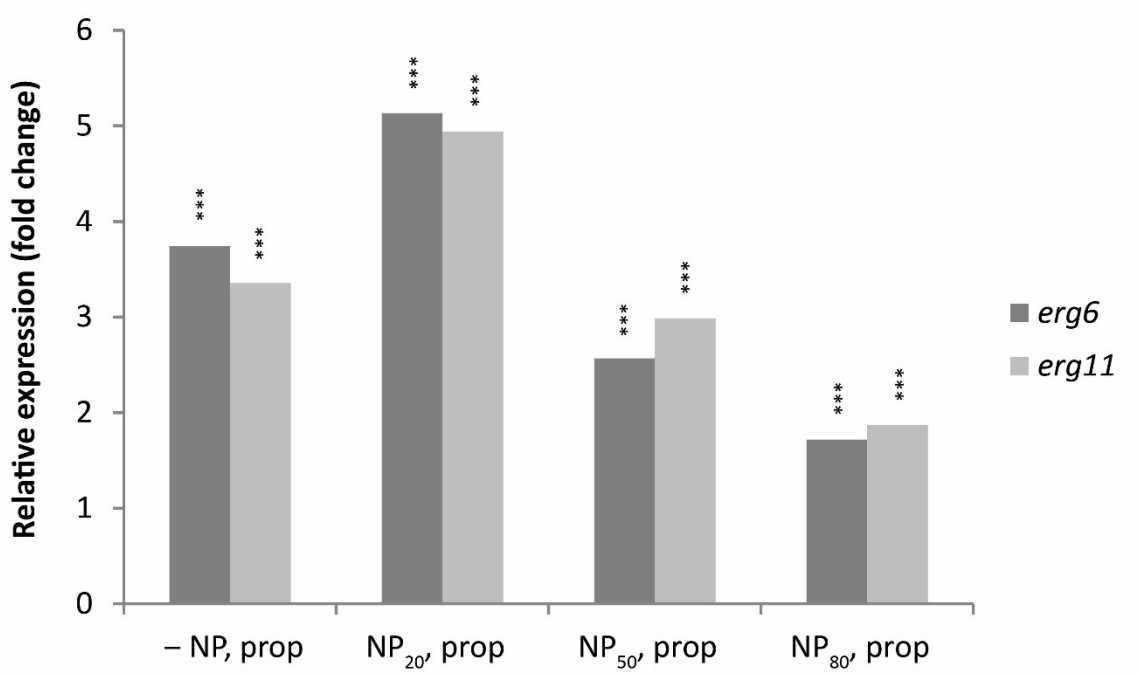

Figure 29: Relative expression of e*rg6* and *erg11* genes in *L. bicolor* cultivated on propiconazole-containing MMN agar medium supplemented with 0.5 % glucose for 14 days. Bars represent mean factors of the upregulation of *erg6* and *erg11* transcript levels, normalized with respect to *18S rRNA* levels, **in relation to the respective gene expression under conditions free of propiconazole but containing identical NP concentrations**. Growth medium was amended with nanoparticles (NP) and **0.1 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. The presented data originate from two independent experiments with two biological replicates, each consisting of three pooled agar plates. The Relative Expression Software Tool (Pfaffl *et al.* 2002) was used to evaluate quantitative real-time PCR reactions with templates descending from two to four cDNA syntheses per replicate and two technical replicates. Coefficient of variation was < 2.4 %. Stars indicate statistically significant differences compared to the respective control group that is free of propiconazole but contains the same amount of NPs: *** for $p < 0.001$.

In a first approach, the gene expression of e*rg6* and *erg11* in *L. bicolor* was evaluated in groups that were exposed to exactly the same NP amount. The gene expression in the presence of propiconazole was set in each case in relation to the gene expression in the absence of propiconazole (Figure 29). In NP-free medium, the presence of 0.1 mg/L propiconazole resulted in a statistically highly significant upregulation of the *erg6* and *erg11* expression by a mean factor of 3.7 and 3.4, related to fungi grown on medium without propiconazole ($p < 0.001$, Figure 29). In the presence of 0.032 g/L NPs (NP_{20}) and 0.1 mg/L propiconazole, *L. bicolor* revealed a statistically highly significant upregulation of the *erg6* and *erg11* expression by a mean factor of 5.1 and 4.9 related to the expression in fungi grown in the presence of the same NP concentration but without fungicide ($p < 0.001$). The presence of 0.129 g/L NPs (NP_{50}) associated with propiconazole resulted in a statistically highly significant upregulation of the *erg6* and *erg11* expression by a mean factor of 2.6 and 3.0 related to the expression in fungi grown in the presence of the same NP concentration but without fungicide ($p < 0.001$). The highest NP concentration loaded with propiconazole led to an upregulation of the *erg6* and *erg11* expression by a mean factor of 1.7 and 1.9 compared to the expression in fungi cultivated in the sole presence of the same NP concentration ($p < 0.001$). In general, the factors of up- and downregulation concerning the expression of *erg6* and *erg11* were similar. The present results corroborate the fact, that both *erg6* and *erg11* are solid marker genes to study the effect of the azole fungicide propiconazole in the Basidiomycete *L. bicolor*.

Another way to analyze the obtained qPCR data is to compare the fold change in expression for each propiconazole-containing treatment group in relation to the respective gene expression under conditions free of both NPs and propiconazole (Figure 30). In *L. bicolor*, the cultivation in the presence of 0.1 mg/L propiconazole led to a statistically highly significant upregulation of the *erg6* and *erg11* expression by a mean factor of 3.7 and 3.4, respectively ($p < 0.001$). The sole application of NP_{20} reduced the *erg11* expression but not statistically significantly. In the presence of higher NP amounts, only a minor upregulation of the *erg6* and *erg11* transcript levels occurred compared to the related control group free of both NPs and propiconazole. Overall, the sole presence of NPs in all three concentrations showed no statistically significant effect on the gene expression of *erg6* and *erg11*.

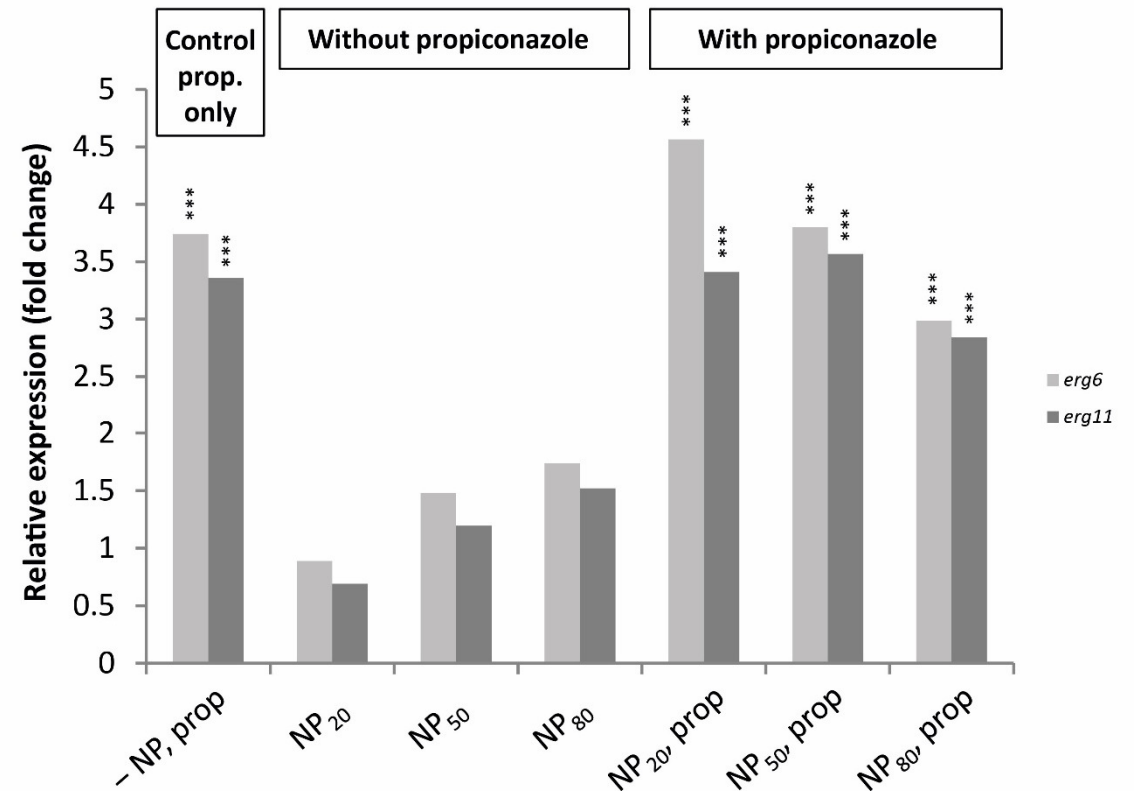

Figure 30: Relative expression of e*rg6* and *erg11* genes in *L. bicolor* cultivated on MMN agar medium supplemented with 0.5 % glucose for 14 days. Bars represent mean factors of up- and downregulation of *erg6* and *erg11* transcript levels, normalized with respect to *18S rRNA* levels, **in relation to the respective gene expression under conditions free of both nanoparticles (NP) and propiconazole**. Growth medium was amended with NPs and **0.1 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Data originate from two independent experiments with two biological replicates, each consisting of three pooled agar plates. The Relative Expression Software Tool (Pfaffl *et al.* 2002) was used to evaluate quantitative real-time PCR reactions with templates descending from two to four cDNA syntheses per replicate and two technical replicates. Coefficient of variation was < 2.4 %. Stars indicate statistically significant differences with at least two-fold changes compared to the control group: *** for $p < 0.001$.

The transcript levels of *erg6* and *erg11* were significantly increased when *L. bicolor* was exposed to NP-associated propiconazole. In the following section, the *erg6* and *erg11* expression is described separately. According to REST analysis, NP_{20} associated with propiconazole led to a statistically highly significant elevation of the *erg6* transcription by a mean factor of 4.6 relative to the RNA levels under conditions free of both NPs and propiconazole ($p < 0.001$). In the presence of higher amounts of NPs associated with propiconazole, the *erg6* expression was 3.8- or 3.0-fold upregulated for NP_{50} or NP_{80}, respectively. In general, the factors of up- and downregulation in the *erg6* and *erg11* expression were similar. In all analyzed treatment groups, the *erg11* expression was slightly lower than the expression of *erg6*. When *L. bicolor* was cultivated in the presence of propiconazole, a highly significant upregulation of the *erg11* expression ($p < 0.001$) was observed. Upon exposure to 0.1 mg/L propiconazole, *erg11* was highly significantly upregulated by a mean factor of 3.4 in relation to the expression under conditions free of both NPs and propiconazole. The presence of NPs associated with propiconazole led to a highly significant upregulation of the *erg11* transcript levels by a mean factor of 3.4, 3.5, and 2.8 for NP_{20}, NP_{50}, and NP_{80}, respectively.

Furthermore, comparing the different factors of fold change, one may notice: The lowest NP concentration associated with the fungicide caused a higher upregulation of the *erg6* expression compared to the upregulation in fungi grown in the sole presence of propiconazole (Figure 30). The highest NP concentration associated with propiconazole, however, resulted in a highly significant upregulation of the *erg6* expression (3.0-fold), which was lower than the upregulation in fungi grown on medium containing only propiconazole (3.7-fold).

5.3 Effect of nanoparticles, propiconazole and their combination on mycorrhiza formation

5.3.1 Single and combined effect on the establishment of mycorrhizas in Poplar – A. muscaria – interactions

To study the effect of fungicide sorption on the formation of mycorrhizas, poplar seedlings were subjected to various culture media and inoculated with *A. muscaria*. The growth medium was amended with either propiconazole-loaded NPs (Figure 31 b) or unloaded NPs (Figure 31 d). The NPs were applied in amounts that hypothetically sorb 80 % of the initial propiconazole concentration (NP_{80}). Emerging mycorrhizal structures were quantified and compared to those occurring in control groups containing no NPs but either propiconazole (Figure 31 c) or the respective solvent control (Figure 31 e).

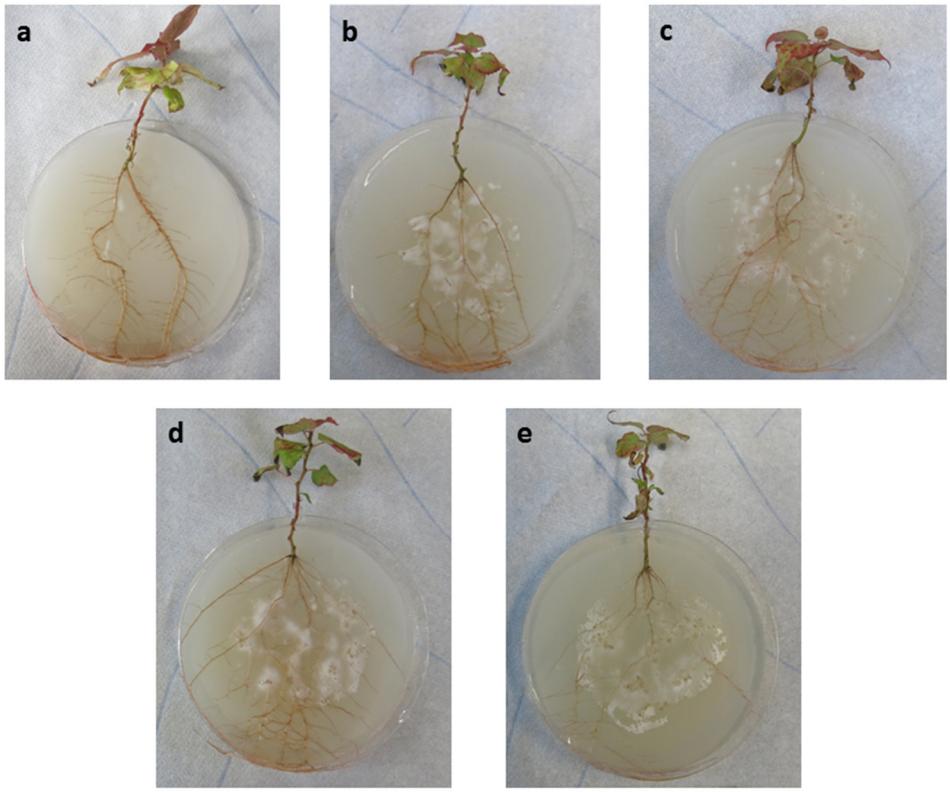

Figure 31: Interaction between *Populus tremula* × *Populus tremuloides* and *A. muscaria.* Plant and fungi were grown over a period of 7 weeks. In this study, four treatment groups were investigated. The growth medium contained 0.517 g/L nanoparticles (NP_{80}) and **5 mg/L propiconazole** (prop), or appropriate solvent controls. Each treatment group was set up with three controls without fungal inoculum (a). The medium was supplemented with NP_{80} and prop (b), without NPs and with prop (c), with NP_{80} and without prop (d), and without NP and without prop (e).

Mycorrhizal structures (Table 12) were quantified according to the gridline intersection method (Giovannetti and Mosse 1980, Brundrett *et al.* 1996). The lowest mycorrhization rate was found in poplar seedlings cultivated in the pure presence of propiconazole determined both as a percentage of mycorrhizal root length and in terms of the number of mycorrhizal tips per one cm root length (Table 10). Statistical analysis using a Kruskal-Wallis test (Hc (corrected) = 42.26; $p < 0.001$) and Mann-Whitney post hoc with Bonferroni corrected pairwise comparisons revealed that the mycorrhization rate was significantly higher when poplar seedlings were cultivated in the presence of NP-associated propiconazole instead in the sole presence of propiconazole. The highest mycorrhization rates were detected under fungicide-free conditions. When the tested organisms were exposed to propiconazole, the presence of NPs had a supporting effect on the establishment of mycorrhizas. In the presence of NP_{80} and propiconazole, plants had a 6.5 times higher percentage of the mycorrhizal root length compared to plants grown in the sole presence of the fungicide. Under fungicide-free conditions, the applied NPs had no effect on the formation of mycorrhizas in poplar.

Table 10: Effects of nanoparticles and propiconazole on total root length and mycorrhiza formation of *Populus tremula* × *Populus tremuloides* inoculated with *A. muscaria*. Growth medium was supplemented with 0.517 g/L nanoparticles (NP_{80}) and **5 mg/L propiconazole** (prop), or appropriate solvent controls. *A. muscaria* was cultivated with poplar seedlings for 7 weeks; mean values of 17 replicates are given. Exceptions are marked with an asterisk *: due to contaminations n = 16. Standard deviation is listed in parentheses below. Different letters (a, b, c) indicate statistically significant differences ($p < 0.05$). The percentage of mycorrhizal root length was analyzed using a one-way ANOVA ($F = 39.59$; $p < 0.001$) and Tukey's post hoc test. The number of mycorrhizal tips per one cm root length was analyzed using a Kruskal-Wallis test (Hc (corrected) = 42.26; $p < 0.001$) and Mann-Whitney post hoc with Bonferroni corrected pairwise comparisons.

Treatment	Total root length [cm]	Mycorrhizal root length [cm]	Number of mycorrhizal root tips	Percentage of myc. root length [%]	Number of myc. tips per one cm root length
– NP, – prop	425.88 (116.88)	13.59 (7.68)	51.41 (27.49)	3.19 c (1.50)	0.12 c (0.06)
– NP, prop*	304.94 (75.82)	0.75 (0.77)	3.13 (3.14)	0.24 a (0.24)	0.01 a (0.01)
NP_{80}	400.53 (92.49)	13.00 (5.76)	62.76 (38.92)	3.43 c (1.64)	0.16 c (0.10)
NP_{80}, prop*	277.31 (73.95)	4.25 (2.4)	18.38 (7.11)	1.56 b (0.91)	0.07 b (0.03)

5.3.2 Single and combined effect on the establishment of mycorrhizas in Poplar-C. geophilum-interactions

To gain a broader spectrum on the establishment of mycorrhizal structures not only in Basidiomycetes but also in Ascomycetes, another test organism was used and the interaction between poplar plant seedlings and *C. geophilum* was studied. Here again, growth medium was supplemented with NPs either loaded with the fungicide propiconazole or without (Figure 32 b and d). The formation of mycorrhizas was analyzed and compared with the mycorrhization rate under conditions free of NPs but in the presence of either propiconazole or the respective solvent control (Figure 32 c and e).

Mycelial growth was strongly reduced due to propiconazole. The least fungal growth occurred on medium solely containing propiconazole (Figure 32 c). In the presence of NP-associated propiconazole, a slightly greater growth was observed (Figure 32 b). Maximum fungal growth occurred on medium free of propiconazole (Figure 32 d and e).

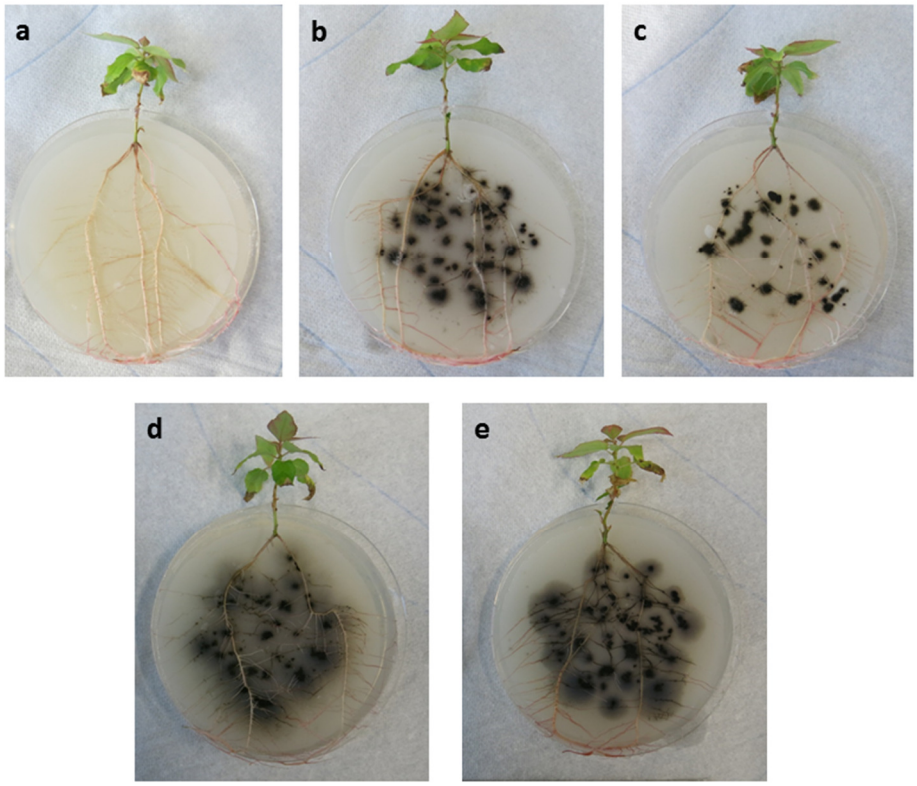

Figure 32: Interaction between *Populus tremula* × *Populus tremuloides* and *C. geophilum*. Plant and fungi were grown over a period of 5 weeks. In this study, four treatment groups were investigated. The growth medium contained 0.517 g/L nanoparticles (NP_{80}) and **2 mg/L propiconazole** (prop), or appropriate solvent controls. Each treatment group was set up with three controls without fungal inoculum (a). The medium was supplemented with NP_{80} and prop (b), without NP and with prop (c), with NP_{80} and without prop (d), and without NP and without prop (e).

The lowest mycorrhization rate was detected in poplar seedlings cultivated in the sole presence of propiconazole. Here, 1.5 % of the root length consisted of mycorrhizal structures (Table 11). In every other treatment group, the mycorrhization rates in terms of percentage of mycorrhizal root and number of mycorrhizal tips per root length were statistically significantly higher according to one-way ANOVA analyses and Tukey's post hoc tests (see legend of Table 11). When cultivated in the presence of NPs, poplar seedlings grown in fungicide-free conditions showed a slightly higher, but not significantly different mycorrhization rate than plants exposed to propiconazole.

Table 11: Effects of nanoparticles and propiconazole on total root length and mycorrhiza formation of *Populus tremula* × *Populus tremuloides* inoculated with *C. geophilum*. Growth medium was supplemented with 0.517 g/L nanoparticles (NP_{80}) and **2 mg/L propiconazole** (prop), or appropriate solvent controls. *C. geophilum* was cultivated with poplar seedlings for 5 weeks. Mean values of 17 replicates are given. Standard deviation is listed in parentheses below. Different letters indicate statistically significant differences ($p < 0.05$). The percentage of mycorrhizal root length was analyzed using a one-way ANOVA ($F = 13.14$; $p < 0.001$) and Tukey's post hoc test. The number of mycorrhizal tips per one cm root length was analyzed using a one-way ANOVA ($F = 53.36$; $p < 0.001$) and Tukey's post hoc test.

Treatment	Total root length [cm]	Mycorrhizal root length [cm]	Number of mycorrhizal root tips	Percentage of myc. root length [%]	Number of myc. tips per one cm root length
– NP, – prop	412.41 (99.88)	25.18 (11.66)	184.29 (63.17)	6.24 b (2.79)	0.46 b (0.17)
– NP, prop	300.00 (73.45)	4.47 (3.02)	24.18 (13.26)	1.53 a (1.03)	0.08 a (0.05)
NP_{80}	419.06 (120.80)	22.94 (12.50)	187.12 (84.91)	5.56 b (2.55)	0.44 b (0.17)
NP_{80}, prop	314.88 (71.3)	12.71 (6.44)	94.00 (31.46)	4.29 b (2.65)	0.31 b (0.13)

Table 12: Mycorrhizal structures between *C. geophilum* and *Populus tremula* × *Populus tremuloides*.

6 Discussion

6.1 Environmental sorption of propiconazole in soils

Propiconazole is predominantly employed in agriculture but also in forest nurseries and forest sites to combat the spread of fungal diseases. In European forest soils, the most common fungal pathogens are *Phytophthora*, *Pythium*, *Fusarium* and *Cylindrocarpon* (Okorski *et al.* 2015). Apart from these, airborne pathogens infest stems and leaves of tree species. After usage, fungicides are expected to accumulate in soils, especially if the pesticide management schedules various applications. Next to fungicide dosage, soil parameters like bulk densities and organic matter content play decisive roles for the fungicide retention in different soil layers. When applied at a rate of 100 g/ha, one estimates that the fungicide concentration in soil is 0.1 mg/dm^3, which is roughly equivalent to 0.1 mg fungicide per Liter substrate (Gisi 2014). In the upper soil layer, however, where EMF are found, fungicide concentration might be even higher. The top 1 cm layer is assumed to contain 1 mg fungicide /L (Gisi 2014). As a lot of fungicides bind to organic matter, the fungicide concentration is presumably higher in organic soils. Propiconazole was shown to be predominantly associated with soil aggregates that were rich in organic matter (Wu *et al.* 2003). Moreover, it was found that the majority of the applied propiconazole was bound in upper 10 cm soil layer of two analyzed soil types with high organic content (Kim *et al.* 2002). Clay and compact soils exhibit high adsorption coefficients and hold fungicides even in the upper 2 cm soil layer, while soils with a higher sand content would allow fungicides to reach lower soil levels (Dodd and Jeffries 1989). Another calculation expects that in mineral soils with bulk densities of 0.5 g/cm^3, a single application of 0.125 kg/ha propiconazole results in a concentration of 0.5 mg/L in the topsoil spanning 0 to 5 cm (Laatikainen and Heinonen-Tanski 2002). In organic soils with bulk densities of 0.05 g/cm^3, the propiconazole concentration is estimated to be 5 mg/L (Laatikainen and Heinonen-Tanski 2002).

Propiconazole is a weakly basic fungicide with a low pk_a value varying between 0.8 and 1.09 (Riise *et al.* 2001, Wu *et al.* 2003, Adam *et al.* 2005). In soil at pH > 1.09, propiconazole is predominantly present in its non-ionic form. Propiconazole has a low mobility in soil and a water solubility of 100 mg/L. Further, it is expected that hydrophobic sorption occurs between propiconazole and the soil organic carbon (Riise *et al.* 2001). As adsorption mainly emerges by adhesion of compounds to the organic matter of the soil, the K_{oc} value is used when comparing different soils. Like Kd value, the K_{oc} value expresses the ratio of a chemical's adsorbed concentration to the dissolved chemical concentration at equilibrium, but it takes the percentage of the total organic carbon content of a given soil type into account. The K_{oc} value of propiconazole ranges from 1086 to around 1800 (Riise *et al.* 2004, Oliver *et al.* 2012).

A published study explored the individual sorption behavior of different size fractions of a soil in Southeast Norway (< 2 µm, < 20 µm or < 2 mm) towards radioactive [^{14}C] propiconazole (Riise *et al.* 2001). Sorption was found to follow the Freundlich isotherm model. The authors observed that particle size, organic carbon content as well as surface reactivity are involved in steering sorption affinity for propiconazole (Riise *et al.* 2001). In addition, humic acids or natural organic matter may attach to particles and influence sorption as well as pesticide toxicity (Ra *et al.* 2008). The present thesis aimed to investigate the effect of sorption on the toxicity of propiconazole.

6.2 The application of NPs and propiconazole in this thesis

Due to their high specific surface area and wide area of application, NPs gained huge interest. Moreover, NPs may be designed in various sizes and with different features concerning pore size and pore distribution. Further, the surface of these engineered NPs can be functionalized to modulate their adsorption capacity. In this thesis, PMONPs were used as model compounds to investigate the impact of sorption on fungicide toxicity. These NPs consist of a macromolecular network that is characterized by a periodic and regular arrangement of pores. In the following, these PMONPs will be referred to as NPs for the sake of simplicity. A comparative study of the effect of sorbed and non-sorbed propiconazole was performed. Sorption of propiconazole on the utilized NPs is assumed to occur in a monolayer way and to follow the Langmuir model (analyses performed by Leyla Guluzada). Propiconazole molecules adsorb on NPs due to their high active surface area and the large pore volume. Compared to mesoporous silica, the employed tailored NPs have a more hydrophobic framework. Therefore, adsorption between propiconazole and NPs is largely implemented by hydrophobic-hydrophobic interactions (personal information by Leilei Luo, 2017).

Propiconazole holds two chiral centers and is present as four stereoisomers. In this thesis, the fungicide PESTANAL® was used, which contains a mixture of different isomers to investigate the overall environmental relevance of propiconazole on fungi. The two 2S isomers are known to be more biological active than the two 2R isomers (Garrison *et al.* 2011). The toxicity of propiconazole was investigated using three EMF cultivated either in liquid medium or on solidified agar medium. The tested EMF descend from two distinct fungal phyla within the kingdom Fungi. *A. muscaria* and *L. bicolor* are Basidiomycetes, while *C. geophilum* belongs to Ascomycetes. Triazole fungicides exert their activity on the target within one to two hours (Pontzen and Scheinpflug 1989). Propiconazole binds to the 14α demethylase enzyme and impedes the synthesis of ergosterol, an integral part of fungal membranes. As a consequence, the fungicide is able to inhibit fungal growth. By adding a constant fungicide amount together with one out of three different NP amounts to the fungal growth medium, the hypothetical concentration of sorbed propiconazole was steered and vice versa the concentration of freely

available propiconazole. The effect of the fungicide on fungal growth was studied as well as the effect on the cellular level, *i. e.* the transcription of genes involved in the biosynthetic pathway of ergosterol.

Propiconazole is known to modify the expression of genes involved in ergosterol biosynthesis (Henry *et al.* 2000, De Backer *et al.* 2001, Agarwal *et al.* 2003, Song *et al.* 2004, Liu *et al.* 2010, Sun *et al.* 2011, Fernandes *et al.* 2016). Therefore, the impact of different proportions of sorbed and freely available propiconazole on the expression of *erg6* and *erg11* was analyzed and the suitability of *erg6* and *erg11* as marker genes was evaluated. Furthermore, the toxicity of particle-associated propiconazole on the level of ecosystem function was assessed. The fungal ability to form ectomycorrhizal structures was surveyed using poplar cuttings. Additionally, microbial degradation of propiconazole was investigated using two different types of cultivation.

6.3 Types of fungal cultivation

In the following, the advantages and disadvantages of liquid and agar cultivations of fungi in the presence of particle-associated toxic compounds are contrasted. The application of submerged cultures has several benefits. In liquid medium, sorption is easily facilitated. Fungal growth medium is simply supplemented with NPs and propiconazole. Since cultivation flasks are kept on a rotating platform, the liquids are in a constant movement, which enables uniform distribution and adsorptive interaction of both components. In liquid cultures, the distribution of nutrients is assumed to be equal, while in agar cultures, radial gradients of nutrients are formed. Besides this, in liquid cultures, fungal mycelium is in direct contact with the medium and what is more, growth rate was found to be accelerated for all tested species. Seven days after inoculation, a considerable biomass increase appeared, whereas on solidified agar medium, cultivation periods of two up to seven weeks were required. However, fungal liquid cultures have to be inoculated with a larger amount of mycelium or propagules than is the case with agar cultures. This fact precludes a comparison of the fungal growth development between different cultivation types. Further, submerged cultures are known to imply irregularities. Inconsistent growth occurs that affects gene expression and sensitive analyses like qPCR (Wösten *et al.* 2013, Lange *et al.* 2014). To evaluate fungal growth in submerged cultures, dry weight measurements were performed. Fresh weight measurements were considered inaccurate, since vacuum conditions could not be controlled during filtration and varied from sample to sample. A disadvantage of dry weight measurements is that, due to the destructive nature of heat, down-stream analysis of fungal material is impaired.

Agar experiments, on the contrary, allow the determination of various fungal features, such as colony diameter and area. Provided that fungi were grown on a cellophane layer directly placed on agar medium, fungal material can be easily removed to perform fresh weight measurements as

well as gene expression analysis. However, the preparation of NP-containing solid growth medium involved several challenges. NPs and propiconazole had to be transferred to the medium in an aseptic state. The lack of thermostability of both components had to be considered. Moreover, agar solidifies at reduced temperatures. In addition, sorption between sorbent and sorbate had to be established in concentrations as close as possible to the final ones. Finally, the sedimentation of NPs should be reduced as much as possible. These difficulties could be overcome by modifying the original process of media preparation. For further details, see 4.7.

6.4 Propiconazole stability in liquid and solid fungal growth medium

This thesis verified that propiconazole is stable over a period of 7 days under submerged culture conditions. In terms of solid growth medium, uniform growth inhibition over a period of 70 days was repeatedly observed. This implies propiconazole stability in agar medium. Apart from this, newly performed analyses revealed that propiconazole could be extracted from agar plates on which *L. bicolor* was cultivated. In agar extracts, no degradation metabolites could be found; these were only found in the dried fungal mycelium (unpublished data in cooperation with Anna-Jorina Wicht). Moreover, a decline in propiconazole content as well as an emergence of propiconazole degradation metabolites were detected in the liquid culture filtrates of *L. bicolor*, *C. geophilum*, and *A. muscaria*. These results show that all three tested species are able to degrade propiconazole. According to our knowledge, propiconazole degradation has been rarely observed in fungi. To date, *Trametes versicolor* is the only fungal species reported to be able to degrade propiconazole (Woo *et al.* 2010). In total, 12 fungal species were assessed the majority being Basidiomycetes (Woo *et al.* 2010). In addition, a low number of bacteria was found to degrade propiconazole (Sarkar *et al.* 2009, Satapute and Kaliwal 2016a, Satapute and Kaliwal 2016b). Therefore, the presented findings are highly interesting. Apart from a decline in the propiconazole content, we were also able to detect different transformation metabolites. Next to *Trametes versicolor*, now two more representatives of the phylum Basidiomycota are shown to be able to degrade propiconazole and *C. geophilum* is the first described Ascomycete. Further, the results of the present thesis imply that fungicide degradation by EMF might differ depending on type of cultivation. In liquid cultures, exoenzymes might be involved, as degradation metabolites were found in the filtrates, but not in the extracts of comparable agar cultures, where diffusion might be hindered. Alternatively, it is also conceivable, that propiconazole is degraded internally and emerging metabolites are excreted into the liquid medium.

6.5 Fungal growth studies

Laboratory growth studies are performed to gain differentiated knowledge of the fungicide effect on individual species. It is known that in vitro growth performance may vary considerably among different species and even among isolates of the same species. *A. muscaria*, for example, was reported to have a colony area of around 10 cm^2 after 8 weeks (Schrey *et al.* 2005), whereas the isolate investigated in the present thesis revealed a higher growth rate. After 6 weeks, an average colony area of 18.8 cm^2 was observed. In another study, the growth rate of three tested *L. bicolor* isolates varied from 125 to 464 µm/day, whereas the growth rate of nine tested *C. geophilum* isolates varied from 208 to 875 µm/day (Coleman *et al.* 1989). Another study also examined nine strains of *C. geophilum* and found growth rates ranging from 7.6 to 15.8 mm/day (Laatikainen and Heinonen-Tanski 2002). Next to isolate specific performance, growth rate may differ depending on growth medium and size of the starting inoculum. On modified MMN agar and on modified Hagem's agar, for example, it took 2.4 to 9 weeks until one *A. muscaria* strain showed a diameter of 1 cm (Laatikainen and Heinonen-Tanski 2002). These researchers utilized three 1 - 3 mm cubic fungal inocula. To compare, in the present thesis, a 9 mm inoculum was used for *A. muscaria* and after 3 weeks, the fungus reached an average diameter of 2.9 cm.

6.5.1 Fungal growth studies in the presence of non-sorbed propiconazole

6.5.1.1 Basidiomycota

On solid agar medium, exposure to propiconazole repeatedly inhibited the growth of the Basidiomycetes *A. muscaria* and *L. bicolor*. To date, inhibition by propiconazole was reported only for a confined number of Basidiomycetes (Table 13). Among those, most species are pathogens. *Armillaria mellea*, for example, causes root rot that is lethal to oak and fruit trees (Adaskaveg *et al.* 1999). In laboratory experiments, 0.15 mg/L propiconazole reduced the growth of *A. mellea* on potato dextrose agar (PDA) by 50 % after 4 weeks of cultivation. The sensitivity of this fungus is roughly in the same range as that of the edible ectomycorrhizal mushroom *L. bicolor* tested in the present thesis. Exposure to 0.1 mg/L propiconazole resulted in a 70 % inhibition of the diameter increase on MMN agar medium, whereas the corresponding area increase was inhibited by 85 or 86 % (data from two independent experiments). Two cacao pathogens *Moniliophthora perniciosa* and *Moniliophthora roreri* revealed a similar sensitivity to propiconazole. In the presence of 0.34 mg/L propiconazole, the in vitro growth on PDA agar was inhibited by 72 and 75 %, respectively (Aneja *et al.* 2005). The present thesis showed that *A. muscaria* is more tolerant. The exposure of *A. muscaria* to 5 mg/L propiconazole inhibited the diameter increase by 67 or 70 %, respectively in two identical but independently performed experiments. The corresponding area increase of *A. muscaria* was inhibited by 84 or 85 %, respectively.

Ustilago maydis is reported to be even more tolerant. This corn pathogen was found to be inhibited by 50 % at a more than 600 times higher propiconazole concentration (Table 13, Loeffler and Hayes (1992)).

Moreover, a Finnish study investigated the response of 15 described ectomycorrhizal species to propiconazole, the majority being Basidiomycetes. More than 60 % of the 45 tested strains were strongly inhibited by 1 mg/L propiconazole when grown on modified MMN medium (Laatikainen and Heinonen-Tanski 2002). The authors observed that the inhibiting effect of propiconazole was more pronounced on modified Hagem's medium. The growth of some mycorrhizal strains, including *Suillus bovinus* and *Suillus variegatus*, was even promoted at 0.1 or 1 mg/L propiconazole (Laatikainen and Heinonen-Tanski 2002). Other strains, like *Amanita muscaria umbria*, were reported to be not affected by propiconazole, even at a concentration of 10 mg/L (Laatikainen and Heinonen-Tanski 2002). This contradicts the results of the present thesis. In the presence of 5 mg/L propiconazole, the diameter increase of *Amanita muscaria* (tested in this thesis) was already inhibited by 70 %.

The causal agent of southern blight, *Sclerotium rolfsii*, was observed to be completely inhibited by 16 mg/L propiconazole when cultivated on agar medium (Waterfield and Sisler 1989). In submerged cultures, this fungus responded more sensitively to propiconazole compared to solid agar cultures. On solid medium, 8 mg/L propiconazole reduced the fungal growth by 84 %, whereas in liquid medium, only 1 mg/L propiconazole was required to achieve an equivalent growth reduction (Waterfield and Sisler 1989). In the present thesis, the opposite phenomenon was noticed. *A. muscaria* exhibited a higher sensitivity to propiconazole when cultivated on solidified agar medium. In comparison to cultivation on agar medium, submerged cultivation provides a complete and permanent contact between fungicide and fungal mycelium. On solid agar medium, aerial mycelium has no direct contact to propiconazole. In addition, the fungicide concentration is assumed to decline within the active growth zone of the fungus and possibly cause depletion. Therefore, fungi in liquid cultures were expected to be more severely affected by propiconazole than in agar cultures. However, this was not the case in the performed experiments.

Table 13: Reported growth performance of different Basidiomycetes upon exposure to propiconazole. The name of the investigated species is given and its environmental relevance. Furthermore, the applied propiconazole concentration, the type of cultivation and the observed growth inhibition are stated.

Species	Environmental relevance	Propiconazole concentration [mg/L]	Cultivation	Growth inhibition [%]	Reference
Armillaria mellea	oak and fruit tree pathogen	0.15 and 10	PDA agar (potato dextrose agar)	50 and 100	Adaskaveg *et al.* (1999)
Hebeloma sinapizans	poisonous fungus	10 and 100	PDA agar	100	Zambonelli and Iotti (2001)
Moniliophthora perniciosa	cacao pathogen	0.34	PDA agar	72	Aneja *et al.* (2005)
Moniliophthora roreri	cacao pathogen	0.34	PDA agar	75	Aneja *et al.* (2005)
Sclerotium rolfsii	crop pathogen	1	liquid modified Coursen and Sisler medium	84	Waterfield and Sisler (1989)
Sclerotium rolfsii	crop pathogen	8 and 16	modified Coursen and Sisler agar medium	84 and 100	Waterfield and Sisler (1989)
Ustilago maydis	corn pathogen	3,079	liquid glucose/yeast medium	50	Loeffler and Hayes (1992)
Amanita muscaria	EMF	5	modified MMN agar medium	70	Results of the present thesis
Laccaria bicolor	EMF	0.1	modified MMN agar medium	70	Results of the present thesis

In liquid cultures containing 5 mg/L propiconazole, the dry weight increase of the two Basidiomycetes *L. bicolor* and *A. muscaria* was inhibited by 25 % and only marginally (2 - 18 %), respectively. On solid agar cultures, the fresh weight increase of *L. bicolor* was strongly inhibited at a much lower fungicide concentration. Exposed to 0.1 mg/L propiconazole on agar medium, the fresh weight increase of *L. bicolor* was inhibited by 81 %. These results indicate a higher resistance of *L. bicolor* to propiconazole in submerged cultures. HPLC analyses of culture filtrates and agar extracts corroborate this conclusion. Liquid culture filtrates contained propiconazole as well as degradation metabolites. In contrast, propiconazole but no degradation metabolites could be detected in agar extracts. It is deduced that propiconazole resistance in *L. bicolor* is caused by a stronger fungicide degradation in liquid medium compared to agar medium.

For *A. muscaria*, four experiments were performed in liquid cultures. While the dry weight increase was once statistically significantly inhibited by 70 % upon fungal exposure to 5 mg/L propiconazole, three independently repeated experiments revealed only minor, but not statistically significantly different inhibitions by 2, 7, or 18 %. Growth performance in liquid cultures is considered as inconsistent (Droce *et al.* 2013, Wösten *et al.* 2013, Lange *et al.* 2014), which is confirmed by this thesis. On solid agar medium, instead, the results obtained for the fungal growth of *A. muscaria* are very close in both independently repeated experiments. The fungal exposure to 5 mg/L propiconazole led to an inhibition of the diameter increase by 67 or 70 %, respectively. The data on the inhibition of the corresponding area increase were even closer, namely 84 or 85 %. Although the growth of *A. muscaria* on agar medium fluctuated among replicates, the obtained results appear to be very stable and reliable. This corroborates the fact that fungal growth on solid agar medium is very regular for *A. muscaria* compared to submerged cultures. Homogenous growth inhibition in terms of colony diameter and colony area was not only observed for *A. muscaria* but also for *L. bicolor* and the Ascomycete *C. geophilum*. Furthermore, in the test system applied in the work contributing to this thesis, up to 5 mg/L propiconazole was utilized, which are realistic and relevant concentrations according to environmental forecasts (Laatikainen and Heinonen-Tanski 2002).

6.5.1.2 Ascomycota

Numerous representatives of the fungal phylum Ascomycota were described to vary considerably concerning their sensitivity to propiconazole (Table 14). For *Mycosphaerella graminicola*, a fungal pathogen on winter wheat, an EC_{50} value of 0.012 mg/L propiconazole in liquid medium was reported (Cools *et al.* 2011). EC_{50} is the effective concentration that caused the half maximal effect. Here, it is a 50 % inhibition of the fungal growth. Other species are far more tolerant. *Pyrenophora teres* and *Botrytis cinerea*, for example, were observed to have an EC_{50} value of 154 and 924 mg/L propiconazole in liquid cultures, respectively (Loeffler and Hayes 1992). It was also reported that sensitivity to propiconazole varied among isolates of the same species (Table 14, Cox *et al.* (2007), Nel *et al.* (2007)). Laboratory studies revealed that the growth of one isolate of *Fusarium oxysporum* f.sp. *cubense* was completely inhibited at 5 mg/L propiconazole, while other isolates were less sensitive and were completely inhibited only at 100 mg/L propiconazole (Nel *et al.* 2007). Furthermore, 0.3 mg/L propiconazole was used to discriminate between sensitive and resistant *Monilinia fructicola* strains (Cox *et al.* 2007).

This thesis showed that 2 mg/L propiconazole inhibited the fungal diameter increase of the Ascomycete *C. geophilum* by 94 or 100 % in two identical but independently performed experiments. The corresponding area increase was inhibited by 99 or 100 %, respectively. Consequently, the investigated *C. geophilum* isolate can be classified as sensitive as *Fusarium graminearum* (see Table 14, Ramirez *et al.* (2004)). One published study found *C. geophilum* to be one of the most sensitive species to numerous pesticides, including propiconazole (Laatikainen and Heinonen-Tanski 2002). In that study, 15 fungal species and 9 strains of *C. geophilum* were assessed (Laatikainen and Heinonen-Tanski 2002). Although some *C. geophilum* strains were found to tolerate propiconazole or to be promoted, *C. geophilum* was categorized as a very sensitive species (Laatikainen and Heinonen-Tanski 2002). However, this finding contradicts the investigations of the present thesis. *L. bicolor* was shown to be even more sensitive to propiconazole than *C. geophilum*. Further, in the present thesis, *C. geophilum* performed more tolerant than *Monilinia fructicola* and *Raffaelea* sp. (Cox *et al.* 2007, Mayfield III *et al.* 2008). While *C. geophilum* was almost completely inhibited at 2 mg/L propiconazole, some isolates of *Monilinia fructicola* were described to be completely inhibited at 0.3 mg/L propiconazole (Cox *et al.* 2007). The in vitro growth of *Raffaelea* sp. was reported to be inhibited by 84 % in the presence of 0.01 mg/L propiconazole (Mayfield III *et al.* 2008). At 0.1 mg/L or higher propiconazole concentrations, no growth of *Raffaelea* sp. occurred. The biocontrol fungus *Trichoderma harzianum* is more tolerant than *C. geophilum* tested in this thesis. Its growth was observed to be inhibited by 56 and 82 % at concentrations of 5 and 10 mg/L propiconazole, respectively (Sarkar *et al.* 2010).

Table 14: Reported growth performance of different Ascomycetes upon exposure to propiconazole. The name of the investigated species is given and its environmental relevance. Furthermore, the applied propiconazole concentration, the type of cultivation, and the observed effect are stated. MIC: minimal inhibitory concentration

Species	Environmental relevance	Propiconazole concentration [mg/L]	Cultivation	Observed effect/ growth inhibition [%]	Reference
Botrytis cinerea	plant pathogen	924	liquid glucose/yeast medium	50	Loeffler and Hayes (1992)
Candida albicans	human opportunistic pathogen	0.1	malt agar	MIC	Rahalison *et al.* (1994)
Cenococcum geophilum	EMF	0.1 - 10	modified MMN agar and modified Hagem's agar medium	various, including tolerance and stimulation	Laatikainen and Heinonen-Tanski (2002)
Cladosporium cucumerinum	cucumber pathogen	1	Sabouraud dextrose agar	MIC	Rahalison *et al.* (1994)
Claviceps purpurea	cereal pathogen	100	filter disks on PDA agar	MIC	Gladders *et al.* (2001)
Fusarium graminearum	wheat pathogen	0.5 and 5	wheat based agar	14 – 90	Ramirez *et al.* (2004)
Fusarium oxysporum f.sp. *cubense*	banana pathogen	5 - 100	PDA agar	100, depending on isolate	Nel *et al.* (2007)
Fusarium oxysporum f.sp. *cubense*	banana pathogen	25	root dipping of plantlets	reduction of disease severity by 75 %	Nel *et al.* (2007)

Species	Environmental relevance	Propiconazole concentration [mg/L]	Cultivation	Growth inhibition [%]	Reference
Monilinia fructicola	stone fruit pathogen	0.3	PDA agar	50 – 100	Cox *et al.* (2007)
Mycosphaerella graminicola	winter wheat pathogen	0.012	liquid Sabouraud dextrose medium	50	Cools *et al.* (2011)
Pyrenophora teres	plant pathogen	154	liquid glucose/yeast medium	50	Loeffler and Hayes (1992)
Raffaelea spp.	tree pathogen	0.01	acidified PDA agar	84	Mayfield III *et al.* (2008)
Trichoderma harzianum	biocontrol fungus against pathogenic fungi	5, 10, and 25	PDA agar	56, 82, and 100	Sarkar *et al.* (2010)
Tuber borchii	edible fungus	10 and 100	PDA agar	100	Zambonelli and Iotti (2001)
Cenococcum geophilum	EMF	2	modified MMN agar medium	94	Results of the present thesis

6.5.1.3 Glomeromycota

All members of the phylum Glomeromycota are obligate symbionts; the majority of these establish arbuscular mycorrhizas. About 80 % of all land plant species live in symbiosis with arbuscular mycorrhiza forming Glomeromycetes. However, for a long time, this group of fungal species was neglected in non-target screenings and is only recently included in ecotoxicity studies. To date, the growth of only one Glomeromycete was reported to be investigated and affected by propiconazole. The extraradical hyphal length of *Glomus irregulare* was found to be significantly reduced at concentrations of 0.02 to 0.2 mg/L propiconazole (Calonne *et al.* 2010, Calonne *et al.* 2012). In contrast, the extraradical hyphal length of *Glomus caledonium* was found not to be impaired by propiconazole, even at the 100-fold dosage of the recommended field rate (Kjøller and Rosendahl 2000).

6.5.2 Fungal growth studies in the presence of NPs and sorbed propiconazole

On solidified agar medium, the sole presence of NPs did not affect the growth of *A. muscaria*. Only the exposure to NP_{80} resulted in a slightly greater growth compared to the growth under NP-free conditions and in the repeated experiment, in a slightly lower growth. Therefore, its impact was considered neglectable. Similarly, minor differences were observed in the two performed experiments using *L. bicolor*. In one experiment, exposure to the middle NP concentration, NP_{50}, led to a slightly lower growth, whereas in the second experiment, the lower and the higher NP concentration (NP_{20} and NP_{80}) led to slightly lower growth compared to the respective other applied NP concentration. This discrepancy was therefore regarded as negligible. In addition, the present thesis clearly revealed that the growth of *C. geophilum* was not affected by all three NP concentrations in both performed experiments. NPs were hypothesized to affect fungal growth. However, this is in contrast to the obtained results. The presented results showed that NPs did not or only marginally affect the growth of all three tested fungi. In submerged cultures of *A. muscaria*, fungal growth was only marginally affected by the employed NP concentrations. Similar to the performed experiment on agar medium, the exposure of *A. muscaria* to NP_{80} resulted in slightly increased growth compared to the growth under NP-free conditions. However, the attachment of NPs on fungal mycelium in submerged cultures may contribute to the observed dry weight increase. NPs were added in high amounts to the culture medium (0.032 to 0.519 g/L) and the surface of the fungal material is inherently large due to the high amount of fine and widespread hyphal branches. Therefore, NPs might be trapped by fungal organisms that act as biosorbents. NPs are able to sorb on any surface including fungi, bacteria, plants, and animals as reviewed by Handy *et al.* (2008). Similarly, heavy metals and other environmental pollutants are known to sorb on dead or living biomass (Sağ 2001, Gadd 2009).

On solidified agar medium, fungi simultaneously exposed to propiconazole and high NP dosages generally showed a greater growth than those fungi that were simultaneously exposed to propiconazole and low NP dosages. In the presence of NP_{80} and propiconazole, *A. muscaria* showed in both of the performed experiments statistically significantly higher diameter and area increase levels compared to the growth in the presence of non-sorbed propiconazole. Additionally, the growth was statistically significantly lower than that of the fungi grown in the absence of propiconazole but in the presence of NP_{80}. A similar observation was made in *C. geophilum*. In the presence of NP_{80} and propiconazole, *C. geophilum* revealed a greater growth in terms of area and diameter increase compared to the sole presence of propiconazole. Furthermore, the observed greater growth was still lower than the growth in the sole presence of NP_{80}. The combined supply of propiconazole and either NP_{20} or NP_{50} to the growth medium considerably impaired fungal growth of *C. geophilum*. In these treatment groups, the colony diameter increase and the area increase was in each case close to the fungal growth obtained after the sole exposure to the fungicide. It is assumed that the utilized fungicide dosage was so high, that only the sorption of propiconazole on NP_{80} alleviated the detrimental effect of the fungicide. Furthermore, *L. bicolor* showed in the presence of NP_{50} and propiconazole both a statistically significantly greater diameter and area increase compared to the sole exposure to the fungicide. In addition, fungal growth was statistically significantly lower than the growth in the sole presence of NP_{50}.

These observations imply that propiconazole had sorbed successfully and dose-dependently on NPs and that consequently less fungicide was available to compromise fungal growth. It was hypothesized that the adsorption of propiconazole on NPs alters the bioavailability and therefore the toxicity of propiconazole. The obtained results demonstrate that the hypothesis can be verified. As shown by analyses of the propiconazole content in agar medium, the sorption of propiconazole on NPs reduced the availability of propiconazole in a NP dose-dependent way (unpublished data in cooperation with Anna-Jorina Wicht). The higher the present NP amount, the smaller was the fraction of the freely available propiconazole. The same was found in liquid medium. The higher the NP concentration, the smaller was the fraction of detectable propiconazole. On agar medium, NPs influenced the toxicity of propiconazole in a dose-dependent manner. The higher the present amount of the NPs, the lower was the growth inhibiting effect of propiconazole. The adsorptive association of NPs with propiconazole obviously removed portions of toxic and freely accessible propiconazole. As a result, fungal growth was less affected. In contrast, liquid cultures of *A. muscaria*, *L. bicolor* and *C. geophilum* were only slightly affected by the employed propiconazole concentration. When *A. muscaria* was exposed to increasing NP amounts, fungal dry weight was found to increase. It is assumed that this increase is due to the attachment of NPs on fungal mycelium. Overall, the presented results are in good agreement with the results gained in gene expression studies, which are discussed below.

6.6 Gene expression studies

6.6.1 Effect of propiconazole and other azole fungicides on the expression of potential marker genes

Apart from fungal growth, the effect of propiconazole was investigated on the cellular level by means of expression analysis of genes involved in the biosynthetic pathway of ergosterol, as propiconazole is known to block its key enzyme, the 14α demethylase (see Figure 33). The present thesis was aimed at evaluating the *erg6* and *erg11* expression in *L. bicolor* and at testing their use as marker genes even if certain percentages of propiconazole are sorbed on NPs.

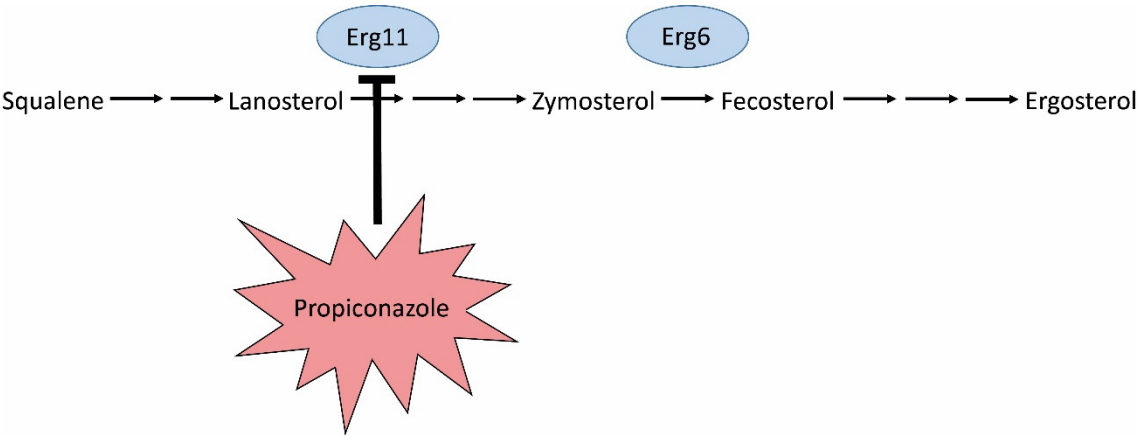

Figure 33: Ergosterol biosynthesis with relevant enzymes and emerging intermediates (modified according to White *et al.* (1998)). The enzyme Erg11 (14α demethylase) is inhibited by the azole fungicide propiconazole. Erg6 (C-24 sterol methyltransferase) catalyzes the transformation of zymosterol to fecosterol.

The analysis of transcript levels as initial step of the gene expression represents an early response to fungicide treatment. The significance of the performed RNA studies is reinforced by several published studies that link transcription and translation in fungi (Liu *et al.* 2005, Hoehamer *et al.* 2010). In the presence of ketoconazole, *C. albicans* showed an enhanced transcription of *erg6* that was accompanied by an increased occurrence of the corresponding protein (Hoehamer *et al.* 2010). In *C. albicans*, the level of the protein Erg6 was 4.3-fold increased in response to an exposure to 19.13 mg/L ketoconazole that is its 50 % inhibitory concentration (Hoehamer *et al.* 2010). Furthermore, it was verified in *F. oxysporum* that increased transcript levels of *erg6* and *erg11* correlated very well with increased amounts of the proteins Erg6 and Erg11 (Deng *et al.* 2015). The study of the present thesis was able to demonstrate that the exposure to propiconazole led to significantly enhanced expression levels of *erg6* and *erg11* in the Basidiomycete *L. bicolor*. Based on the above-mentioned studies, it may be assumed that also in *L. bicolor*, enhanced transcript values of *erg6* and *erg11* are followed by an increase of the respective protein contents.

The study of the present thesis is very important, as according to our knowledge only a very limited number of studies exist that focus on the gene expression in Basidiomycetes following an exposure to azole fungicides (Stammler *et al.* 2009, Lee *et al.* 2010). Plant and human pathogenic Ascomycetes have been studied to a greater extent. In particular, opportunistic pathogens that are a threat for immunocompromised people have been frequently examined. Propiconazole has not been implemented against any of these. Moreover, existing knowledge on the transcriptional impact of azole fungicide treatment is conflicting in Basidiomycetes. While epoxiconazole was reported not to induce the expression of *cyp51* (*erg11*) in *Puccinia triticina* (Stammler *et al.* 2009), itraconazole exposure caused an overexpression of *cyp51* in *Antrodia cinnamomea* (Lee *et al.* 2010). These two are the only available studies focusing on this phylum. The limited and contradicting observations demonstrate the need for further detailed research in Basidiomycetes. The results of the present thesis shed more light on this matter. In the following sections, the expression of *erg11* and *erg6* will be discussed separately.

The observed increase of the *erg11* expression in *L. bicolor* in response to an azole fungicide in this thesis is in agreement with investigations carried out in various Ascomycetes, especially in *Candida* species (Henry *et al.* 2000, De Backer *et al.* 2001, Song *et al.* 2004, Fernandes *et al.* 2016), in *Penicillium digitatum* (Sun *et al.* 2011), in *Saccharomyces cerevisiae* (Agarwal *et al.* 2003), and in *Fusarium graminearum* (Liu *et al.* 2010). Upon exposure to 0.1 mg/L propiconazole, *erg11* expression increased by a factor of 3.4 in *L. bicolor*. Similar upregulation levels were observed in *C. albicans* in response to 9 mg/L fluconazole. As assessed via RNA slot plot hybridization and via reverse transcription PCR analyses, *erg11* expression was 2.6-fold increased (Henry *et al.* 2000). In addition, *C. albicans* showed a 4.5-fold higher level of *erg11* transcripts following an exposure to 10 mg/L miconazole as assayed by northern blot analysis (Song *et al.* 2004). Furthermore, in *S. cerevisiae*, the exposure to 56 mg/L ketoconazole resulted in a 2.2-fold increased expression level of *erg11*, as determined by a microarray experiment (Agarwal *et al.* 2003). According to qPCR analysis of the same samples, *erg11* showed a 7-fold change in expression (Agarwal *et al.* 2003). Overall, the newly obtained data concerning *L. bicolor* coincide very well with the existing data on *erg11* expression in Ascomycetes. Depending on the applied test method, minor or major differences may occur concerning the extent of upregulation. In *F. graminearum*, for example, a deep serial analysis of gene expression (DeepSAGE) showed a 53- or 57-fold increased expression of two out of three genes homologous to *erg11* in the presence of tebuconazole. A qPCR analysis, however, found a 75- and 9-fold increased upregulation of the two *erg11* genes (Liu *et al.* 2010). To the best of our knowledge, the present thesis is one out of only three studies on the effect of azole fungicides on the gene expression in Basidiomycetes (Stammler *et al.* 2009, Lee *et al.* 2010). Moreover, the present thesis is the first study focusing on propiconazole.

With regard to *erg6*, exposure to propiconazole resulted in a 3.7-fold enhanced expression in *L. bicolor*. An increased transcription of *erg6* was also observed in *C. tropicalis* upon treatment with voriconazole (Fernandes *et al.* 2016), in *C. albicans* upon treatment with itraconazole (De Backer *et al.* 2001), in *S. cerevisiae* upon treatment with ketoconazole (Agarwal *et al.* 2003) and in *F. graminearum* upon treatment with tebuconazole (Liu *et al.* 2010). *S. cerevisiae* showed a 2.4-fold change in expression of the *erg6* gene (Agarwal *et al.* 2003), which is about the same extent as observed in *L. bicolor*. Higher upregulations were found in *F. graminearum*. Here, the transcript levels of two genes homologous to *erg6* were 77- and 272-fold increased (Liu *et al.* 2010). In that referred study, the *erg6* gene was among the most upregulated genes following tebuconazole exposure. In *C. albicans*, the transcript level of *erg6* showed a 50.3-fold increase after exposure to 10 µM itraconazole (De Backer *et al.* 2001).

In response to voriconazole, a resistant *C. tropicalis* strain showed a more than 250-fold increased expression level for *erg6* (Fernandes *et al.* 2016). This observation together with an about 200-fold increased expression level of *erg11* indicates that the overexpression of relevant genes is a successful defense mechanism. Overexpression might occur as a consequence of mutations within the *erg6* and *erg11* promoters. A consistent overexpression of the target enzyme is frequently observed in resistant strains (Flowers *et al.* 2012, Lendenmann *et al.* 2015). Moreover, in response to azoles, resistant isolates of several *Candida* species showed overexpression levels of *erg11* (Ribeiro and Paula 2007, Lotfali *et al.* 2017) and *erg6* (Lotfali et al. 2017) compared to sensitive wild type strains. However, general statements should be made with caution. An overexpression of the target enzyme is not always involved in resistance development (Vermitsky and Edlind 2004, Fang *et al.* 2009). Azole resistance might also be induced by enhanced efflux, multiple target-site encoding genes (Cools *et al.* 2013) or an altered amino acid sequence of Erg11 triggered by mutations within the encoding gene (Cools *et al.* 2007, Fang *et al.* 2009).

In the present study, the level of upregulation was marginally higher for *erg6* than for *erg11* when transcript levels were compared in relation to the control group free of both NPs and propiconazole. This is in agreement with several other studies in Ascomycetes. A higher *erg6* expression compared to *erg11* was also revealed in *C. albicans*, *S. cerevisiae*, *F. graminearum*, and *C. tropicalis* (De Backer *et al.* 2001, Agarwal *et al.* 2003, Liu *et al.* 2010, Fernandes *et al.* 2016). Nevertheless, in the present thesis, the transcript levels of *erg6* and *erg11* correlated very closely. Following propiconazole exposure, the levels of the *erg6* and the *erg11* upregulation were about the same. These results confirm that the obtained data are very reliable and that *erg6* and *erg11* are suitable marker genes for the study on the effect of propiconazole on the cellular level of *L. bicolor*. Moreover, the obtained findings corroborate the observations made on the morphological growth level. Exposure to 0.1 mg/L propiconazole on agar medium impaired the growth of *L. bicolor*. After 14 days of cultivation, the colony diameter increase was inhibited by

70 %. The corresponding area increase was inhibited by 85 %. Even on the cellular level, the effect of propiconazole was clearly visible. Propiconazole is known to interact with 14α demethylase (Erg11) and impede the synthesis of ergosterol, the main sterol in fungal membranes. Upon exposure to propiconazole, the transcription of *erg11* was significantly increased, which is coding for the target enzyme Erg11. Similarly, the transcription of *erg6* was significantly elevated. The gene *erg6* is encoding C-24 sterol methyltransferase, an enzyme that catalyzes a later transformation step in the ergosterol synthesis. Therefore, both *erg11* and *erg6* can be used as valid marker genes that indicate cellular stress and possible effects on the fungal growth level. As the presence of propiconazole blocks Erg11, its corresponding gene expression is triggered as well as that of other involved proteins. Despite the increased expression of *erg11* and *erg6*, *L. bicolor* was not able to maintain its ergosterol synthesis and as consequence, fungal growth was reduced. In conclusion, *L. bicolor* is one of the first representatives of Basidiomycetes for which increased *erg6* and *erg11* expression was shown after treatment with azole fungicides. In addition, the present thesis is the only study investigating the effect of propiconazole on the gene expression in a Basidiomycete.

6.6.2 Effect of NPs and sorbed propiconazole on the expression of potential marker genes

In the applied test presented in this thesis, NPs had no statistically significant effect on the investigated gene expression in *L. bicolor*. The NPs caused no change in the expression of *erg6* and *erg11* at low, medium, or high concentrations. In all treatment groups exposed to propiconazole at various sorption conditions, the expression levels of *erg6* and *erg11* were statistically significantly enhanced. The higher the present amount of NPs, the higher is the expected fraction of NP-sorbed propiconazole, and the lower was the observed level of *erg6* and *erg11* upregulation. In general, the transcript levels of *erg6* and *erg11* were very close to each other in the applied test system. Compared to the respective control group, changes in *erg6* and *erg11* expression were approximately of the same size in all cases. This finding reinforces the reliability of the obtained data as well as the reliability of the utilized test system. Furthermore, exposure to propiconazole resulted in statistically significant upregulations of both *erg6* and *erg11* even in the simultaneous presence of NPs. This, in turn, corroborates the suitability of *erg6* and *erg11* as valuable marker genes in *L. bicolor* for the exposure to propiconazole under various sorption conditions. Even low amounts of available propiconazole trigger changes in the respective gene expression of both *erg6* and *erg11*. Moreover, these marker genes show, even on a molecular level, that growth will be affected on the organismic level. The presence of NPs caused a clear downgrading effect of propiconazole on both fungal growth and gene expression. Depending on fungicide availability, colony diameter as well as *erg6* and *erg11* transcription were

affected. Based on previous observations in Ascomycetes, the hypothesis is proposed that *erg6* and *erg11* may be employed as marker genes not only in Ascomycetes but also in Basidiomycetes.

6.7 Mycorrhizal studies

6.7.1 Effect of propiconazole and other azole fungicides on mycorrhization

Fungicides are expected to have a negative impact *per se* on fungal growth and thus on the formation of mycorrhizas. The triazole penconazole, for example, was found to completely eliminate the ectomycorrhizal colonization on Norway spruce seedlings when applied at recommended dosage (Koele and Hildebrand 2011). Even for propiconazole an undesired effect on non-target organisms and the formation of ECMs was reported (Manninen *et al.* 1998, Manninen *et al.* 2000, Teste *et al.* 2006, Nerg *et al.* 2008, Pickles *et al.* 2015). It has to be considered that individual fungal species and strains may respond differently (Carrillo *et al.* 2011). The mycorrhization rate of downy oak plants by *Hebeloma sinapizans*, for instance, was 6.6-fold reduced upon exposure to the fungicide oxycarboxin (100 mg/L), while *Tuber borchii* remained unaffected (Zambonelli and Iotti 2001). In a similar way, the simultaneous treatment with six fungicides did not compromise the mycorrhization of pine seedlings by *Lactarius deliciosus* (Carrillo *et al.* 2011). However, *Pisolithus tinctorius* strain 3SR colonized significantly less seedlings, while *Pisolithus tinctorius* strain Mx could even colonize more plants (Carrillo *et al.* 2011).

In the present thesis, the mycorrhizal length of the root was evaluated according to the well-established gridline intersection method (Giovannetti and Mosse 1980, Brundrett *et al.* 1996). Propiconazole significantly reduced the mycorrhization rate of *A. muscaria*. While 3.2 % of the total root length was colonized by *A. muscaria* under fungicide-free conditions, only 0.2 % of the root was mycorrhized in the presence of 5 mg/L propiconazole. Mycorrhization rate was 16-fold reduced upon fungicide treatment. A consistent higher mycorrhization rate was observed in the *C. geophilum*-poplar set-up. When cultivated without fungicide, *C. geophilum* colonized 6.2 % of the total root length. This mycorrhization level is comparable with an in vitro study using Scots pine seedlings. There, *C. geophilum* was found to colonize 6 % of the fine roots (Heinonsalo *et al.* 2015). The exposure to 2 mg/L propiconazole in the present thesis reduced mycorrhization rate by 76 %.

In Scots pine, propiconazole was described to significantly reduce the root colonization of mycorrhizal fungi by about 35 % when applied at an amount of 0.15 g/m^2, which is a two-fold higher concentration then what is annually used in Finnish nurseries and which is roughly equivalent to an exposure of 19 mg/L (Manninen *et al.* 1998, Manninen *et al.* 2000). In silver birch, an even lower application of 0.025 g/m^2 propiconazole (roughly equivalent to 3 mg/L),

significantly reduced the natural ectomycorrhizal colonization of the lateral short roots by 62.5 % (Nerg *et al.* 2008). This finding is in accordance with the present thesis. Exposure to 2 mg/L propiconazole reduced the mycorrhization by *C. geophilum* by 75 %. Higher fungicidal treatments were reported in greenhouse studies. Upon treatment using Topas® 250E, the ectomycorrhizal colonization level of Rocky Mountain Douglas-fir seedlings was reduced by 53 % (Pickles *et al.* 2015). The seedlings were watered biweekly with a fungicide concentration that resembled exposure to 57 mg/L propiconazole. In the set-up of the present thesis, a maximum exposure of only 5 mg/L propiconazole was utilized, which almost completely eliminated the colonization by *A. muscaria*. The mycorrhization rate was lowered by 94 %.

Another greenhouse experiment found no effect on mycorrhizal colonization of Douglas-fir seedlings by *C. geophilum* when watered with Topas® at 0.5 g/L, which is presumably roughly equivalent to an exposure of 70 to 280 mg/L (Teste *et al.* 2006). However, total ectomycorrhization by natural occurring fungi was reduced by 56 %. Even an enhanced frequency of application (3 and 5 applications) did not reduce the abundance of *C. geophilum* mycorrhizas (Teste *et al.* 2006). This outcome is surprising, since in the experiment of the present thesis, the colonization of poplar roots by *C. geophilum* was already reduced by 75 % at a much lower concentration, namely in the presence of 2 mg/L propiconazole. Further, *C. geophilum* had turned out to be one of the most sensitive fungi in a previous study (Laatikainen and Heinonen-Tanski 2002).

In addition, one study observed that, upon propiconazole treatment, ectomycorrhizal colonization was more reduced in Ascomycetes than in Basidiomycetes (Manninen *et al.* 1998). In terms of growth however, the present thesis found the Basidiomycete *L. bicolor* to be more sensitive than the Ascomycete *C. geophilum*. While 0.1 mg/L propiconazole inhibited the colony diameter increase of *L. bicolor* by 70 %, a 10-fold higher propiconazole concentration was required to observe the same effect in *C. geophilum*.

Next to EMF, AMF and their respective mycorrhization are also affected by sterol biosynthesis inhibitors. Similarly, propiconazole and its commercial mixture with other fungicides were reported to lower the mycorrhization rate of various *Glomus* species (Dodd and Jeffries 1989, Plenchette and Perrin 1992, Calonne *et al.* 2010). In chicory roots, for example, exposure to 0.2 and 2 mg/L propiconazole reduced the mycorrhization rate of *G. irregulare* by 21 and 47 %, respectively (Calonne *et al.* 2010). Further, no effect in terms of root colonization and extraradical hyphal length was observed after the application of propiconazole or its combination with other fungicides at rates up to 100-fold higher than the recommended field rate (Hetrick *et al.* 1988, Kjøller and Rosendahl 2000, Schweiger *et al.* 2001).

6.7.2 Effect of NPs and sorbed propiconazole on mycorrhization

In the applied test system of the present thesis, the presence of NPs did not affect the mycorrhization rate of *C. geophilum* and *A. muscaria* statistically significantly. In the absence of NPs, 3.2 % of poplar root length was colonized by *A. muscaria*, while in the sole presence of NP_{80}, 3.4 % of root length was mycorrhized. In the *C. geophilum*-poplar set-up, a general higher mycorrhization rate was observed compared to the *A. muscaria*-poplar set-up. When cultivated in the presence or absence of NP_{80}, 5.6 or 6.2 % of the total poplar root length was colonized by *C. geophilum*.

The combined supply of propiconazole and NP_{80} to the growth medium led to a mitigated impact of propiconazole. While exposure to propiconazole significantly reduced mycorrhization rate from 6.2 to 1.5 % in *C. geophilum*, exposure to NP-associated propiconazole resulted in a significantly enhanced mycorrhization level of 4.3 %. This level is not significantly different from the level observed under fungicide-free conditions. In the presence of NP_{80}, a larger portion of propiconazole is expected to sorb on the NP surface and the negative overall effect on fungal growth should be less. Consequently, the potential of the fungus to colonize poplar roots and to establish mycorrhizal structures was hypothesized to decrease. Derived from colony and area measurements, a lower mycorrhization of *C. geophilum* was expected to occur in the presence of sorbed propiconazole compared to the sole presence of NP_{80}. However, the mitigating effect of the NPs was less than expected as no statistically significant difference occurred. On the other hand, quantification of mycorrhizal structures is accompanied by a high standard deviation (Koele and Hildebrand 2011), which in turn may complicate unambiguous investigations. Overall, the present thesis pursued to achieve data that depict reality in the best possible way. Experimental as well as evaluation procedures were aimed to reduce observer bias and within-group variation. Therefore, all steps were clearly defined. For the period of cultivation, all treatment groups were randomly assigned in mini-greenhouses. The quantification of ectomycorrhizal associations was assessed by one single, well-trained person, as evaluation is prone to observer subjectivity. Moreover, the treatment groups were evaluated alternately to minimize evaluation differences that might occur within a given day.

In *A. muscaria*, the presence of NPs alleviated the negative effect of propiconazole on fungal growth and on the formation of mycorrhizas. Exposure to propiconazole significantly decreased mycorrhization from 3.2 to 0.2 %. In the presence of propiconazole combined with NP_{80}, the mycorrhization rate was significantly enhanced. It amounted to 1.6 %, which is still significantly different from the control group that was cultivated in the sole presence of particles. Sorption of propiconazole on NP_{80} lowers its availability and thus the inhibitory effect on fungal growth was expected to be reduced. In vitro growth studies performed in the work contributing to this thesis displayed that *A. muscaria* showed increased growth in the presence of sorbed propiconazole

compared to non-sorbed propiconazole. Accordingly, this fungus was also expected to have a higher potential to form mycorrhizas when exposed to sorbed propiconazole. These presumptions were confirmed by the assessed mycorrhization rates. Finally, to our knowledge, the present thesis is the first study that investigates the effect of particle-sorbed fungicide on mycorrhization.

6.8 Further effects of propiconazole on non-target organisms

Triazoles decrease the soil ergosterol content (Hart and Brookes 1996) and alter the soil community structure of both fungi and bacteria (Elmholt 1991, Yen *et al.* 2009, Fernández-Calviño *et al.* 2017). When propiconazole was applied on winter wheat in two stages either at field application rate or its 10-fold concentration (two times 0.61 or 6.13 µg/cm^2), a delayed, significant negative effect on fungi was observed, but not on Ascomycetes, such as yeast and *Penicillium* species (Elmholt 1991). Further, no dose-dependent effect was found. While this study used a soil dilution plate method to assess the soil spore and propagule content, the present thesis investigated fungal biomass increase and discovered contradictory results. First, the fungal growth of the Ascomycete *C. geophilum* was statistically significantly inhibited by 2 mg/L propiconazole. Second, a clear dose-dependent effect of propiconazole was visible when both Ascomycetes and Basidiomycetes were exposed to a constant fungicide concentration but different NP amounts. Sorption on NPs reduced parts of the freely available propiconazole and thereupon, the growth inhibiting effect of propiconazole declined in a dose-dependent manner. In field, multiple factors may interfere and complicate the investigation of dose-response relationships. Our approach was designed to model the effect of particle-bound pesticide, as might occur in natural conditions. In sterile and semi-sterile experimental set-ups, we were therefore able to investigate the effect of particle sorption without further environmental effects. The authors of the aforementioned study, however, reported indeed a high seasonal variation of the fungal soil community structure, both in the presence and absence of the fungicide (Elmholt 1991). As soil organisms are involved in carbon cycling, nitrogen fixing and other ecosystem services, these may be affected as well. Applied at a rate of 0.15 g/m^2, propiconazole was shown to significantly reduce soil respiration, which is carried out by bacteria, fungi, algae, and protozoans (Manninen *et al.* 1998).

Furthermore, ecto- and endomycorrhization is compromised by propiconazole as outlined above. Spore germination was found to be inhibited in the AMF species *G. irregulare*, *G. mosseae* and *G. geosporum* by propiconazole or its commercial mixture with another fungicide (Dodd and Jeffries 1989, Calonne *et al.* 2010). However, different species may respond differently. For instance, the germination of *G. monosporum* was found not to be impeded (Dodd and Jeffries 1989). Propiconazole retarded germ tube length in *G. irregulare* at 0.02 mg/L propiconazole (Calonne *et al.* 2010) and reduced sporulation at 0.2 mg/L propiconazole (Calonne *et al.* 2010,

Calonne *et al.* 2012). Moreover, upon exposure to propiconazole, *G. irregulare* showed a disturbed composition of unsaturated fatty acids and an increased malondialdehyde (MDA) content (Calonne *et al.* 2010). Both observations might be correlated with oxidative stress caused by the fungicide. Under stress conditions, reactive oxygen species interact with unsaturated phospholipids of cell membranes and lipid peroxidation is initiated. In the course of this process, the composition of membrane phospholipids is changed, the level of polyunsaturated fatty acids is disturbed, and MDA is formed (Calonne *et al.* 2010). In our research group, the MDA content has been investigated both in *L. bicolor* and *A. muscaria* cultivated in liquid and in agar cultures (Usenbenz 2016). No reliable effect of propiconazole on MDA formation was observed. In the referred study, *L. bicolor* was exposed to 0.02 mg/L propiconazole and *A. muscaria* was exposed to 0.5 mg/L propiconazole (Usenbenz 2016). Probably, the selected fungicide concentrations were too low and therefore did not trigger an effect on the fungal MDA content. Similarly, catalase activity was not affected at these concentrations, which is another possible indicator for oxidative stress (Usenbenz 2016).

6.9 Environmental fate of propiconazole and the need for water protection efforts

Fungicide application in agriculture and forestry risks leakage to surrounding environments. When applied on Scots pine seedlings, propiconazole was found to leak in high amounts from culture containers, namely 4 to 29 % (Juntunen and Kitunen 2003). Furthermore, following an application at recommended field rate on three agricultural fields in Norway, a high affinity of propiconazole for the solid phase was discovered. At two sites, less than 0.5 % of the applied fungicide was detected in the surface runoff or the drainage water (Riise *et al.* 2004). At the third site, the soil was sensitive to erosion and 36 % of the applied propiconazole was transported in the surface runoff, probably sorbed on soil particles (Riise *et al.* 2004). In the runoff from an apple and an cherry orchard, propiconazole was found to be predominantly transported in association with particles (70 - 90 %) with a size larger than 1.2 µm (Oliver *et al.* 2012). Another research group examined the soil particles collected from the runoff from an agricultural field. Their study revealed that soil material itself, was found to be predominantly transported via surface runoff, namely 84 %, while 16 % of the soil material was transported in the drainage water (Wu *et al.* 2004). Additionally, soil particles with a size smaller than 2 µm showed a high sorption potential towards propiconazole (Wu *et al.* 2003), which again underlines the importance of our study. We used NPs as model compounds to investigate the effect of propiconazole in the presence of sorbents.

To retain pesticides, nutrients, and sediment, as well as to limit the pollution in surface waters, so-called vegetative buffer zones are implemented between agricultural land and streams. The retention ability of a 5 m wide buffer zone was investigated by a direct application of suspensions with known compound and particle concentrations on the upper boarder of the vegetative buffer zone (Syversen 2005). The distribution coefficient (Kd value) of pesticide sorption on soil as well as sediment concentrations are decisive whether a pesticide is dissolved or attached to sediment. The Kd values of propiconazole sorption on a silty clay loam soil were 11 - 22 mL/g soil, which is classified as a moderate adsorption to soil (Syversen 2005). The experimental soil showed a retention efficiency of 63 % for propiconazole and 62 % for soil particles (Syversen and Bechmann 2004). Another investigation of the buffer zone revealed that 85 % of the applied propiconazole was retained, whereas 74 % of the soil particles were retarded (Syversen 2005). Despite the high retention capacity for propiconazole, soil particles may function as vehicles for pesticide transportation to the aquatic environment (Wu *et al.* 2003, Riise *et al.* 2004, Garrison *et al.* 2011, Oliver *et al.* 2012). The organic matter content may be involved in both sorption and release of pesticides, for example, when organic matter is degraded (Wu *et al.* 2003). Particle size, in turn, is decisive for the mobility in water and sedimentation. In the background of this, the necessity of further research on the impact of propiconazole on non-target organisms becomes apparent as well as the need for effective water protection measures.

6.10 Propiconazole degradation and internalization

Propiconazole is considered a persistent pesticide in soils (Riise *et al.* 2004). As fungicide degradation depends on light, oxygen content, as well as soil type, its microbial community, humidity, and temperature, the reported half-life of propiconazole ranges widely from 30 to 499 days (Bromilow *et al.* 1999, Kim *et al.* 2003, Yen *et al.* 2009, Calonne *et al.* 2010, Garrison *et al.* 2011, Oliver *et al.* 2012). Propiconazole is assumed to be degraded by soil microbes (Yen *et al.* 2009). In the following bacteria species, propiconazole degradation was recorded: *Burkholderia* sp., *Pseudomonas aeruginosa*, and *Pseudomonas putida* (Sarkar *et al.* 2009, Satapute and Kaliwal 2016a, Satapute and Kaliwal 2016b). To date, only one fungal species was reported to be able to degrade propiconazole, namely the Basidiomycete *Trametes versicolor* (Woo *et al.* 2010).

The experiments of this thesis showed that EMF, such as *A. muscaria*, *C. geophilum*, and *L. bicolor*, are involved in the degradation of propiconazole. In the presence of each of these EMF, the propiconazole content in liquid culture medium declined sharply, which was initially supplemented with 5 mg/L propiconazole. In the culture filtrates of *A. muscaria*, a fast decrease was observed. Already 30 min after fungal inoculation, propiconazole concentration was reduced by 34 %. After 3 days, no propiconazole could be detected. Even for *C. geophilum* and *L. bicolor*, the propiconazole content in the culture filtrates declined after 7 days of inoculation by 100 and 70 %, respectively. These findings indicate either a quick uptake of the fungicide or its internal or external degradation.

Further analyses showed that propiconazole degradation metabolites could be detected in the culture filtrates of all tested EMF (unpublished data in cooperation with Anna-Jorina Wicht and Leyla Guluzada). 1-(2,4-dichlorophenyl)-2-(1,2,4-triazole-1-yl)ethanol (Figure 34, BTP 4) occurred in high amounts in the culture filtrates of *C. geophilum* and in much lower amounts in the culture filtrates of *L. bicolor*. Further biotransformation products (BTP 2.1, 2.2, 2.3 and 3) were detected in very low amounts. While the overall content of the three isomer BTP compounds 2.1, 2.2 and 2.3 was highest in the culture filtrates of *L. bicolor*, about the same level of BTP 3 (1-(2,4-Dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethanone) was present in the culture filtrates of *C. geophilum* and *L. bicolor*. Additionally, the analysis of dried fungal mycelia revealed that propiconazole and BTP 4 could be detected in *A. muscaria* mycelium. Moreover, propiconazole and all analyzed BTPs could be found in the mycelium of *L. bicolor* that was cultivated on solidified agar medium (unpublished data in cooperation with Anna-Jorina Wicht). The detection of propiconazole in agar medium itself revealed to be more difficult. However, Anna-Jorina Wicht could develop a method to extract and quantify propiconazole in agar medium.

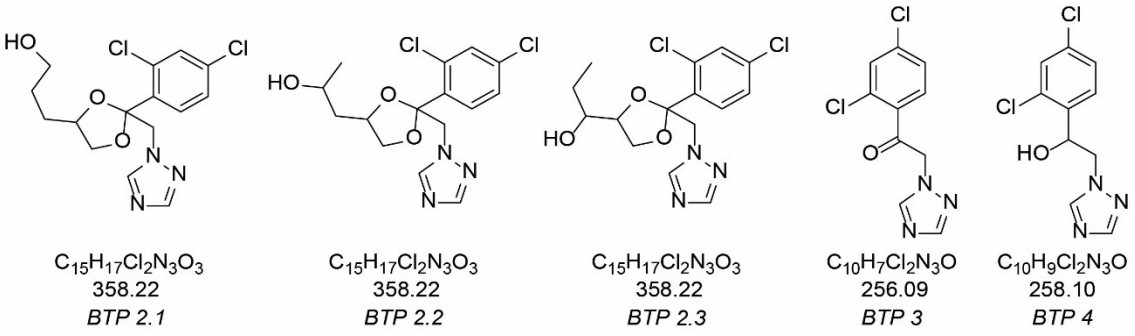

Figure 34: Detected biotransformation products (BTPs) of propiconazole (Kim *et al.* 2003, Konwick *et al.* 2006, Rösch *et al.* 2016) in the culture filtrates and in the dried fungal mycelium of ectomycorrhizal fungi (analyses performed by Anna-Jorina Wicht).

Further sorption studies showed that sorption of propiconazole on NPs protected organisms from toxic compounds on the one hand and on the other hand prevented fungicide degradation. Propiconazole was under the limit of detection within a short period of time in the filtrates of those cultures solely exposed to propiconazole, whereas propiconazole was still present in the culture filtrates that had been additionally amended with NPs. Similarly, the propiconazole content of mycelium was higher in fungi subjected to both NPs and propiconazole than in fungi solely subjected to propiconazole. Therefore, we conclude that propiconazole degradation was slowed down when sorbents were present. Furthermore, single BTPs had their maximum level either in the presence of NP_{80}, NP_{50}, or NP_{20} (unpublished data in cooperation with Anna-Jorina Wicht). This finding suggests that propiconazole degradation even differs depending on fungicide availability. The observation, that propiconazole was shielded from degradation due to sorption on NPs, indicates a longer fungicide persistence in soils treated with this fungicide. This is a drawback, even though pesticide sorption on NPs shields living organisms from negative pesticide effects.

After *A. muscaria* was cultivated in the presence of different propiconazole concentrations ranging from 1 to 5 mg/L propiconazole in liquid growth medium, an increasing propiconazole content could be detected in the mycelium. However, exposed to NP_{50} and 5 mg/L propiconazole, a disproportionate large amount of propiconazole was extracted from the fungal mycelium. The fungicide concentration was about 12-fold higher than that of the control group cultivated in the presence of 2.5 mg/L propiconazole. Initially, we assumed that NPs loaded with propiconazole adhered to the fungal mycelium and that previously sorbed propiconazole was released during extraction and sample preparation for HPLC analysis. However, further experiments showed that the extracting agents do not provoke propiconazole detachment from NPs (unpublished data from Anna-Jorina Wicht). We now suppose that in the presence of NPs, freely available propiconazole may be internalized by fungi or it may attach on the mycelium. Further, we speculate that continuously small portions of propiconazole desorbed from NPs to maintain the equilibrium. As soon as these portions were freely available, they again were internalized or sorbed on the fungal cell wall. This way, an accumulation of propiconazole in or on the fungal biomass could have occurred. Furthermore, propiconazole seems to be degraded only in a non-sorbed state. The reduced bioavailability of propiconazole most probably leads to a lowered biodegradation.

Interestingly, mycelial propiconazole content reached its peak level when fungi were cultivated in the presence of NP_{50}. Perhaps the presence of NP_{50} represents the optimum condition for propiconazole availability triggering the maximum accumulation of propiconazole.

In summary, the present thesis verified that the sorption of propiconazole on NPs and the retarded propiconazole degradation resulted in elevated fungicide levels both in the fungal mycelium and the surrounding medium, as investigated by liquid culture experiments. Transferred to the

ecotoxicological context, the results of the present thesis imply that the sorption of fungicides on soil particles or organic matter favors elevated fungicide concentrations in soils. Furthermore, this thesis showed that sorption caused a reduced biodegradability, as determined by the presence of propiconazole in the filtrates of fungal cultures that contained sorbents, in contrast to its absence in the filtrates of cultures solely exposed to the fungicide. Decreased decomposition in turn contributes to fungicide accumulation in soils. In conclusion, the obtained data imply that apart from repeated pesticide application and a low pesticide mobility, sorption and a resulting reduced biodegradability cause a rise of xenochemical concentrations in soils and sediment. Therefore, the observed effects have an impact on terrestrial but also aquatic environments. Moreover, the studies of this thesis showed that high propiconazole amounts impaired the growth of EMF and their contribution to ecosystem functioning. Hence, the results presented in this thesis are in agreement with a previous study whose results indicate that elevated propiconazole concentrations (100 mg/kg propiconazole) adversely affect soil microbial community structure and its degradation ability (Yen *et al.* 2009).

6.11 Conclusion

The experiments showed that the utilized NPs served successfully as sorbents for propiconazole. Depending on the NP concentration, different fractions of propiconazole sorbed on NP surface and vice versa less propiconazole was accessible for fungi. The fraction of freely available propiconazole revealed to be decisive for the extent of its toxicity. As observed in qPCR studies, the fungicide was able to trigger changes on the cellular level already at conditions with low propiconazole availability. Furthermore, the sorption of propiconazole on the utilized PMONPs altered the toxicity of the fungicide on fungal growth and the formation of ectomycorrhizas. This conclusion is in agreement with a few previous examinations. It was shown that sorption of octylphenol and pentachlorophenol on particles significantly reduced their toxicity on *Daphnia magna* as well as *Vibrio fischeri* (Ra *et al.* 2008). In terms of propiconazole, sorption on nano-zinc oxide (nano-ZnO) reduced the growth inhibiting effect of the fungicide on mouse embryonic fibroblast cells in a dose-dependent way (Li *et al.* 2013). Only one more study has been published, which focused on the combined toxicity of propiconazole and particles. Here, toxicity of propiconazole on the tropical aquatic moss *Vesicularia dubyana* was not reduced (Wu *et al.* 2005). However, only a low sorption of propiconazole (1 - 2 %) was investigated, as the particles themselves caused a strong turbidity and affected the inspected rate of photosynthesis. In contrast, the test system of the present thesis enabled a proper examination. The employed NPs had no or only minor effects on EMF at the utilized concentrations. In addition, the applied fungicide concentration was high enough to achieve different levels of sorbed propiconazole. Moreover, this is the first time that the effect of sorbed propiconazole on EMF was examined.

Sorption affected not only the bioavailability and toxicity of propiconazole but also the bioaccumulation in EMF. Internalization of propiconazole was expected to be reduced in the presence of sorbents whose size prevent a fungal uptake. The tailor-made NPs showed an average size of 360 nm and according to this, fungal uptake is unlikely. In the presence of NPs in liquid growth medium, however, fungal bioaccumulation of propiconazole was significantly increased by a factor of up to 12. It is improbable that extracting agents caused desorption of propiconazole from NPs. Instead, this finding indicates that the presence of sorbents either triggers the fungal uptake of propiconazole or it fosters an increased fungicide attachment on the fungal mycelium. It may be assumed that during the experiment, propiconazole desorbed from NPs and adhered on the fungal cell wall. Further, it may be speculated that propiconazole was continuously released from NPs to achieve the previous solubility and sorption equilibrium. Finally, increasing amounts of the fungicide could have sorbed on the mycelium. It is therefore hypothesized that chemical groups on the fungal surface possess a higher sorption potential than NPs. Another assumption is that NPs elicited the formation of ROS, which are known to mediate membrane damage by lipid peroxidation (Dutta *et al.* 2012). In turn, propiconazole permeability might be increased. However, we have not enough evidence to prove these assumptions.

Apart from particles, bioaccumulation is also influenced by natural organic matter (NOM), such as polysaccharides and humic compounds. To give some examples, cadmium uptake in mussels was increased in the presence of humic acids (Kożuch and Pempkowiak 1996), whereas the uptake of the heavy metal lead by the green algae *Chlorella kessleri* was reduced in the presence of humic, alginic, and polygalacturonic acids (Lamelas *et al.* 2005).

In summary, NPs, soil particles, and organic matter are capable to affect the availability of toxic elements or compounds. In the present study, sorbents revealed a protective role in fungicide bioavailability, toxicity and degradation. Sorption shielded test organisms from fungicide activity and also shielded fungicides from microbial degradation. The reduced degradation of bound pesticides in soils contributes to soil pollution, as toxic compounds may persist and accumulate, which is of great environmental concern. In turn, this shows the environmental relevance and importance of the obtained results.

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8 Appendix

8.1 Composition of culture media, solutions and buffers

All instructions for culture media are related to 1 Liter bidistilled water unless otherwise stated. The pH of the medium was adjusted using HCl or NaOH, and the required amount of agar was added prior to autoclaving.

Trace element stock solution for MMN medium (250 mL)

KCl	7.46 g
H_3BO_3	3.09 g
$MnSO_4*H_2O$	1.69 g
$ZnSO_4*7\ H_2O$	1.15 g
$CuSO_4*5\ H_2O$	0.25 g
$(NH_4)_2Mo_7O_{24}*4\ H_2O$	0.036 g
Fill up to 250 mL with bidistilled water	

MMN medium, 0.5 % glucose – culture medium for ectomycorrhizal fungi

800 mL H_2O	**in total:**
100 µL NaCl stock solution (25 g/100 mL)	0.025 g NaCl
5 mL KH_2PO_4 stock solution (10 g/100 mL)	0.5 g KH_2PO_4
0.25 g $(NH_4)_2HPO_4$ (Merck)	0.25 g $(NH_4)_2HPO_4$
1 mL $CaCl_2$ stock solution (5 g/100 mL)	0.05 g $CaCl_2$
1 mL $MgSO_4$ x 7 H_2O stock solution (15 g/100 mL)	0.15 g $MgSO_4$ x 7 H_2O
100 µL $FeCl_3$ x 6 H_2O stock solution (1 %)	1 mg $FeCl_3$ x 6 H_2O
1 mL thiamin	1 mg Thiamin hypochlorite
2.5 mL trace element stock solution	
Fill up to 1 L with bidistilled water	
Set pH to pH 6.6	
Add 18 g Agar (Merck 1614)	1.8 % agar
Autoclaving	
Add sterile filtered glucose (25 mL 20 % glucose for 1 L)	0.5 % glucose
Pour 25 mL in one Petri dish	

Poplar Medium MS6

2.2 g MS basal medium Sigma M5519
10 g sucrose
Fill up to 1 L with bidistilled water
Set pH to pH 5.8
1 % Agar Fluka

Mycorrhiza-medium: MMN without glucose and with 1/10 N

800 mL H_2O	**in total:**
100 µL NaCl stock solution (25 g/100 mL)	0.025 g NaCl
5 mL KH_2PO_4 stock solution (10 g/100 mL)	0.5 g KH_2PO_4
0.025 g $(NH_4)_2HPO_4$	0.025 g $(NH_4)_2HPO_4$
1 mL $CaCl_2$ stock solution (5 g/100 mL)	0.05 g $CaCl_2$
1 mL $MgSO_4$ x 7 H_2O stock solution (15 g/100 mL)	0.15 g $MgSO_4$ x 7 H_2O
100 µL $FeCl_3$ x 6 H_2O stock solution (1 %)	1 mg $FeCl_3$ x 6 H_2O
1 mL thiamine	1 mg thiamine hypochlorite
2.5 mL trace element stock solution	
Fill up to 1 L with bidistilled water	
Set pH to pH 6.6	
Add 20 g Agar (Merck 1614)	2 % agar
Autoclaving	
Pour 50 mL in one Petri dish	

Solutions and buffers for molecular biological methods are listed below:

10x TBE-buffer

113 g TRIS
55 g boric acid
7.4 g EDTA
Fill up to 1 Liter bidistilled water

1 % agarose gel (1x TBE)

1 g agarose
100 mL 1x TBE-buffer
Boil the solution several times using a microwave oven

0.5x TAE-buffer

0.04 M TRIS
0.019 M acetic acid
0.001 M EDTA

1 % agarose gel (0.5x TAE)

2 g agarose
200 mL 0.5x TAE-buffer
Boil the solution several times using a microwave oven; gel electrophoresis was performed using 0.5x TAE as running buffer.

Ethidium bromide solution (0.0001 %)

30 µL ethidium bromide 1 % (10 mg/mL)
300 mL bidistilled H_2O

8.2 List of chemicals

All utilized chemicals were obtained from a German company or its German branch. If chemicals were bought from abroad, the country of the delivering company is stated as well.

Table 15: List of chemicals.

Chemical compound	**Company, registered office**
$(NH_4)_2HPO_4$	Merck KGaA, Darmstadt
100 bp DNA ladder	New England BioLabs GmbH, Frankfurt am Main
acetic acid	Carl Roth GmbH + Co. KG, Karlsruhe
agar (Fluka 05040)	Sigma-Aldrich Chemie GmbH, Steinheim
agar Merck 1614	Merck KGaA, Darmstadt
agarose Ultra pure^{Tm}	Invitrogen GmbH, Karlsruhe
boric acid ≥ 99,8 % *p. a*. ACS, ISO	Carl Roth GmbH Co. KG, Karlsruhe
buffer WTW trace PL4 pH 4.006	WTW Wissenschaftlich-Technische Werkstätten, Weilheim
buffer WTW trace PL7 pH 6.865	WTW Wissenschaftlich-Technische Werkstätten, Weilheim
$CaCl_2$ *2 H_2O ≥ 99 % *p. a.* ACS	Carl Roth GmbH Co. KG, Karlsruhe
copper sulfate pentahydrate	Carl Roth GmbH Co. KG, Karlsruhe
$CuSO_4$*5 H_2O *p. a.*	Merck KGaA, Darmstadt
DNase I Kit	Sigma-Aldrich Chemie GmbH, Steinheim
DNeasy Plant Mini Kit	Qiagen GmbH, Hilden
EDTA, ethylenediaminetetraacetic acid	Carl Roth GmbH + Co. KG, Karlsruhe
ethanol (absolute, *puriss. p. a.)*	Sigma-Aldrich Chemie GmbH, Steinheim
ethidium bromide (1 %) 10 mg/mL	Carl Roth GmbH + Co. KG, Karlsruhe
$FeCl_3$ x 6 H_2O	Merck KGaA, Darmstadt
formic acid	Merck KGaA, Darmstadt
gel loading dye	New England BioLabs GmbH, Frankfurt am Main
glucose	Carl Roth GmbH + Co. KG, Karlsruhe
H_3BO_3 *p. a.*	Merck KGaA, Darmstadt
hydrogen peroxide (30 %)	Carl Roth GmbH + Co. KG, Karlsruhe
iScript^{TM} Advanced cDNA Synthesis Kit for RT-qPCR	Bio-Rad Laboratories GmbH, München
KCl *p. a.*	Merck KGaA, Darmstadt

KH_2PO_4	Merck KGaA, Darmstadt
MES Pufferan® ≥ 99 %	Carl Roth GmbH Co. KG, Karlsruhe
methanol (HPLC grade)	Thermo Fisher Scientific GmbH, Dreieich
methanol (HPLC grade)	VWR Prolabo, Wien, Österreich
$MgSO_4$ x 7 H_2O	Merck KGaA, Darmstadt
Midori Green Advance	Nippon Genetics Europe GmbH, Düren
$MnSO_4$*H_2O *p. a.*	Merck KGaA, Darmstadt
Murashige & Skoog basal medium: MS salts (5519)	Sigma-Aldrich Chemie GmbH, Steinheim
Murashige & Skoog medium: M0222	Duchefa Biochemie B.V., Haarlem, The Netherlands
NaCl	Carl Roth GmbH + Co. KG, Karlsruhe
NaCl	Merck KGaA, Darmstadt
$NH_4)_2Mo_7O_{24}$*4 H_2O	Merck KGaA, Darmstadt
pH electrolyte KCl 3 mol/L	Mettler Toledo GmbH, Steinbach
propiconazole-(phenyl-d3)	Sigma Aldrich Laborchemikalien GmbH, Seelze
propiconazole, mixture of stereoisomers, PESTANAL®	Sigma-Aldrich Laborchemikalien GmbH, Seelze
RNeasy® Plant Mini Kit	Qiagen GmbH, Hilden
ß-mercaptoethanol	Carl Roth GmbH + Co. KG, Karlsruhe
SsoAdvanced™ Universal SYBR® Green Supermix	Bio-Rad Laboratories GmbH, München
sucrose ≥ 99,9 % *p. a.*	Carl Roth GmbH + Co. KG, Karlsruhe
Taq PCR Core Kit	Qiagen GmbH, Hilden
thiamine hydrochloride	Sigma-Aldrich Chemie GmbH, Steinheim
TRIS ultra quality Pufferan® ≥ 99.9 %	Carl Roth GmbH Co. KG, Karlsruhe
$ZnSO_4$*7 H_2O *p. a.*	Merck KGaA, Darmstadt

8.3 List of labware

All employed labware was obtained from a German company or its German branch. If labware was purchased from abroad, the country of the delivering company is stated as well.

Table 16: List of labware.

Labware	**Company, office**
aluminum foil	Carl Roth GmbH + Co. KG, Karlsruhe
baffled flasks: 100, 300, and 500 mL	Schott AG, Mainz
baffled flasks: 100, 300, and 500 mL	Thermo Fisher Scientific GmbH, Dreieich
bottle top filters (PES membrane, 0.2 µm pore size)	Carl Roth GmbH + Co. KG, Karlsruhe
büchner flask	Schott AG, Mainz
büchner funnel	Morgan Advanced Materials Haldenwanger GmbH, Waldkraiburg
cellophane membrane, Einmach Fix, Folia® Paper	Max Bringmann KG, Wendelstein
centrifuge tubes, eco-friendly, 50 mL, 15 mL	Carl Roth GmbH + Co. KG, Karlsruhe
dental rolls, Celluron®, Ø 10 mm	Paul Hartmann AG, Heidenheim
dewar vessel, Nalgene	Thermo Fisher Scientific GmbH, Dreieich
disposable gloves SafeGrip®	Süd-Laborbedarf GmbH, Gauting
filter paper, type 11A, Ø 70 mm	Carl Roth GmbH + Co. KG, Karlsruhe
filter paper, type 601A, Ø 90 mm	Carl Roth GmbH + Co. KG, Karlsruhe
forceps	Carl Roth GmbH + Co. KG, Karlsruhe
freezer packs	Liebherr, Bulle, Switzerland
glass bottles: 100, 250, 500, and 1,000 mL	Schott AG, Mainz
glass bottles: 100, 250, 500, and 1,000 mL	Thermo Fisher Scientific GmbH, Dreieich
glass funnel	Carl Roth GmbH + Co. KG, Karlsruhe
glass jar, 580 mL	Weck GmbH u. Co. KG, Wehr-Öflingen
glass pasteur pipette	Brand GmbH + Co. KG, Wertheim
hand tally counters	Carl Roth GmbH + Co. KG, Karlsruhe
magnetic stirring bars	Carl Roth GmbH + Co. KG, Karlsruhe
mortar	Morgan Advanced Materials Haldenwanger GmbH, Waldkraiburg
nitex membrane: 50, 60, and 100 µm	Züricher Tuchfabrik, Zürich, Switzerland
nitrile gloves	Halyard health, Weinheim
nitrile gloves	Süd-Laborbedarf GmbH, Gauting

paper filters, Whatman, Ø 90 mm	Whatman GmbH, Dassel
parafilm M, Bemis®	Bemis Company, Neenah, Wisconsin, USA
PCR reaction vessels, 8-stripes	Brand GmbH + Co. KG, Wertheim
pestle	Morgan Advanced Materials Haldenwanger GmbH, Waldkraiburg
Petri dishes (round)	Greiner Bio-One GmbH, Frickenhausen
Petri dishes (square)	Greiner Bio-One GmbH, Frickenhausen
pH-indicator stripes (2 - 9; 3.6 - 6.1)	Carl Roth GmbH + Co. KG, Karlsruhe
pipette 0.5 - 10, 2 - 20, 10 - 100, 20 – 200, and 100 – 1,000 µL	neoLab Migge GmbH, Heidelberg
pipette tips, Ultratip, 0.1 – 1,000 µL	Greiner Bio-One GmbH, Frickenhausen
plastic wrap, alio	Wentus Kunststoff GmbH, Höxter
reaction vessels: 1.5, and 2 mL	Carl Roth GmbH + Co. KG, Karlsruhe
scalpel	Thermo Fisher Scientific GmbH, Dreieich
silicone baysilone paste	GE Bayer Silicones GmbH Co. KG, Erkrath
spring steel forceps	Thermo Fisher Scientific GmbH, Dreieich
syringe filters, Rotilabo: 0.22, and 0.45 µm	Carl Roth GmbH + Co. KG, Karlsruhe
syringe, 3 mL Luer lock	B. Braun Melsungen AG, Melsungen
UV cuvettes, micro, z = 8.5 mm	Brand GmbH + Co. KG, Wertheim
water jet pump	Brand GmbH + Co. KG, Wertheim

8.4 List of lab equipment and hardware

All used lab equipment and technical hardware was obtained from a German company or its German branch. If obtained from abroad, the country of the delivering company is stated as well.

Table 17: List of lab equipment and hardware.

Lab equipment	**Manufacturer**
-20 °C freezer	Liebherr, Bulle, Switzerland
-80 °C freezer, C660 premium	New Brunswick Scientific GmbH, Nürtingen
ABI PRISM® 3130*xl* Genetic Analyzer	Applied Biosystems, Foster City, CA, USA
balance sartorius 2004 MP	Sartorius GmbH, Göttingen
balance sartorius portable	Sartorius GmbH, Göttingen
binocular Leica S8 APO	Leica Microsystems GmbH, Wetzlar
binocular Stemi SV6	Carl Zeiss AG, Oberkochen
binocular WILD M7A	WILD, Heerbrugg, Switzerland
biophotometer	Eppendorf, Hamburg
centrifuge Hermle z 513	Hermle Labortechnik GmbH; Wehingen
clean bench Msc Advantage™	Thermo Fisher Scientific GmbH, Dreieich
electrophoresis chamber	Biometra, Göttingen
electrophoresis chamber "i-Mupid-mini gel migration trough"	Cosmo Bio Co. Ltd., Tokyo, Japan
gel Doc 2000	Bio-Rad Laboratories GmbH, München
gooseneck lamp Schott KL 1500	Schott AG , Mainz
heating cabinet	Memmert GmbH & Co. KG, Schwabach
ice system Scotsman MF26	Sctosman Ice Systems, Milan, Italy
magnetic stirrer Heidolph MR 2000	Heidolph Instruments GmbH & Co.KG, Schwabach
magnetic stirrer Heidolph MR 3001 K	Heidolph Instruments GmbH & Co.KG, Schwabach
microscope camera Axio Cam ICc1	Carl Zeiss AG, Oberkochen
microscope camera Leica MC190 HD	Leica Microsystems GmbH, Wetzlar
microwave MICROMAT	AEG, Frankfurt am Main
mini-shaker model Kühner	B. Braun Melsungen AG, Melsungen
pH measuring instrument "Microprocessor pH-Meter"	WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim
pocket thermometer Checktemp® 1	Hanna instruments, Inc. Woonsocket, USA

pressure cooker WMF perfect	Württembergische Metallwarenfabrik WMF AG, Geislingen/ Steige
quantum/radiometer/photometer, LI-250A light meter	LI-COR Biosciences, Lincoln, Nebraska USA
real-time PCR system Bio-Rad® iQ™ 5	Bio-Rad Laboratories GmbH, München
scanSpeed mini	Labogene, Lynge, Denmark
shaker GFL 3017	Gesellschaft für Labortechnik mbH, Burgwedel, Deutschland
shaker Infors AG	Infors AG, Bottmingen, Switzerland
shaker KS-15	Edmund Bühler GmbH, Tübingen
thermocycler FlexCycler	Analytic Jena AG, Jena
ultra Turrax T25	Janke & Kunkel IKA®-Labortechnik, Staufen im Breisgau
ultrasonic cleaner Branson 2200 E1	Branson, Soest, The Netherlands
vortex Genie 2	Bender & Hobein AG, Bruchsal
vortex Mixer neoLab 7-2020	neoLab Migge GmbH, Heidelberg
vortex Reax 2000	Bender & Hobein AG, Bruchsal

8.5 Supplementary data on liquid and solid experiments

8.5.1 Liquid culture experiment with A. muscaria

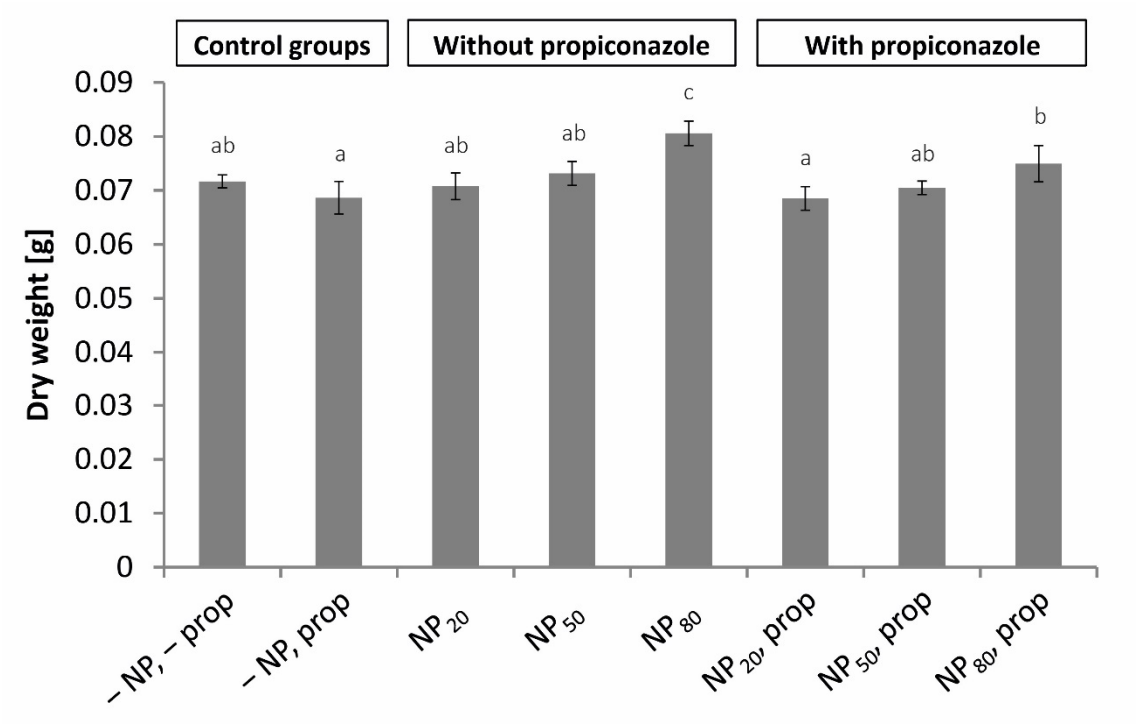

Figure 35: Dry weight of liquid cultures of *A. muscaria* in the presence of 5 mg/L propiconazole (prop) and nanoparticles (NP), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added to fungal growth medium in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs, respectively. Cultures were started with an inoculum of 0.5 g fungal fresh weight. After 7 days, all fungal material was collected and dried. Bars represent mean ± SD, n = 5. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 14.2$; $p < 0.001$) and Tukey's post hoc test.

8.5.2 Solid agar experiment with A. muscaria

Table 18: Increase of colony diameter and area of *A. muscaria* cultivated on MMN agar medium supplemented with 0.5 % glucose for 42 days and respective inhibition related to *A. muscaria* grown in the absence of both nanoparticles and propiconazole. Growth medium was amended with nanoparticles (NP) and **5 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs; n = 19. If fungal growth was not inhibited but promoted, the percentage promotion is stated in parentheses and marked with an asterisk *.

Treatment	Diameter increase [cm]	Area increase [cm^2]	Inhibition / promotion [%] of diameter increase	Inhibition / promotion [%] of area increase
– NP, – prop	3.97	18.14		
– NP, prop	1.30	2.98	67.46	83.57
NP_{20}	4.18	19.00	(4.03)*	(4.72)*
NP_{50}	4.21	19.94	(5.61)*	(9.93)*
NP_{80}	4.36	21.13	(9.31)*	(16.46)*
NP_{20}, prop	1.37	3.12	65.74	82.78
NP_{50}, prop	1.53	3.76	61.72	79.27
NP_{80}, prop	2.46	8.07	38.28	55.52

8.5.3 Solid agar experiment with C. geophilum

Table 19: Increase of colony diameter and area of *C. geophilum* cultivated on MMN agar medium supplemented with 0.5 % glucose for 70 days and respective inhibition related to *C. geophilum* grown in the absence of both nanoparticles and propiconazole. Growth medium was amended with nanoparticles (NP) and **2 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs; n = 19. If fungal growth was not inhibited but promoted, the percentage promotion is stated in parentheses and marked with an asterisk *.

Treatment	Diameter increase [cm]	Area increase [cm^2]	Inhibition [%] of diameter increase	Inhibition [%] of area increase
– NP, – prop	4.51	20.54		
– NP, prop	0.26	0.28	94.34	98.64
NP_{20}	4.68	21.69	(3.67)*	(5.62)*
NP_{50}	4.71	21.85	(4.26)*	(6.40)*
NP_{80}	4.98	24.61	(10.38)*	(19.84)*
NP_{20}, prop	0.24	0.31	94.58	98.51
NP_{50}, prop	0.48	0.62	89.39	97.00
NP_{80}, prop	1.72	4.03	61.81	80.36

8.5.4 Solid agar experiment with L. bicolor

Table 20: Increase of colony diameter, area, and fresh weight of *L. bicolor* cultivated on cellophane on MMN agar medium supplemented with 0.5 % glucose for 14 days and respective inhibition related to *L. bicolor* grown in the absence of both nanoparticles and propiconazole. Growth medium was amended with nanoparticles (NP) and **0.1 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony diameter and area of 9 replicates were measured. Treatment groups containing propiconazole but no NP, NP_{20}, or NP_{50} were assessed with 15 replicates each. Total fresh weight of three colonies was determined, n = 2 with following exceptions: n = 3 for the group "NP_{20}, prop", and n = 4 for the groups "– NP, prop" and "NP_{50}, prop". If fungal growth was not inhibited but promoted, the percentage promotion is stated in parentheses and marked with an asterisk *.

Treatment	Diameter increase [cm]	Area increase [cm^2]	Fresh weight increase [g]	Inhibition [%] of diameter increase	Inhibition [%] of area increase	Inhibition [%] of fresh weight increase
– NP, – prop	3.12	10.59	0.52			
– NP, prop	0.95	1.56	0.10	69.52	85.25	81.49
NP_{20}	3.20	10.99	0.55	(2.67)*	(3.74)*	(5.83)*
NP_{50}	3.36	11.81	0.60	(7.84)*	(11.50)*	(14.37)*
NP_{80}	3.27	11.41	0.59	(4.99)*	(7.71)*	(11.95)*
NP_{20}, prop	1.16	2.04	0.14	62.78	80.70	74.01
NP_{50}, prop	1.95	4.67	0.26	37.54	55.90	49.40
NP_{80}, prop	2.99	9.72	0.48	4.10	8.26	7.63

8.6 Independently repeated experiments in liquid medium

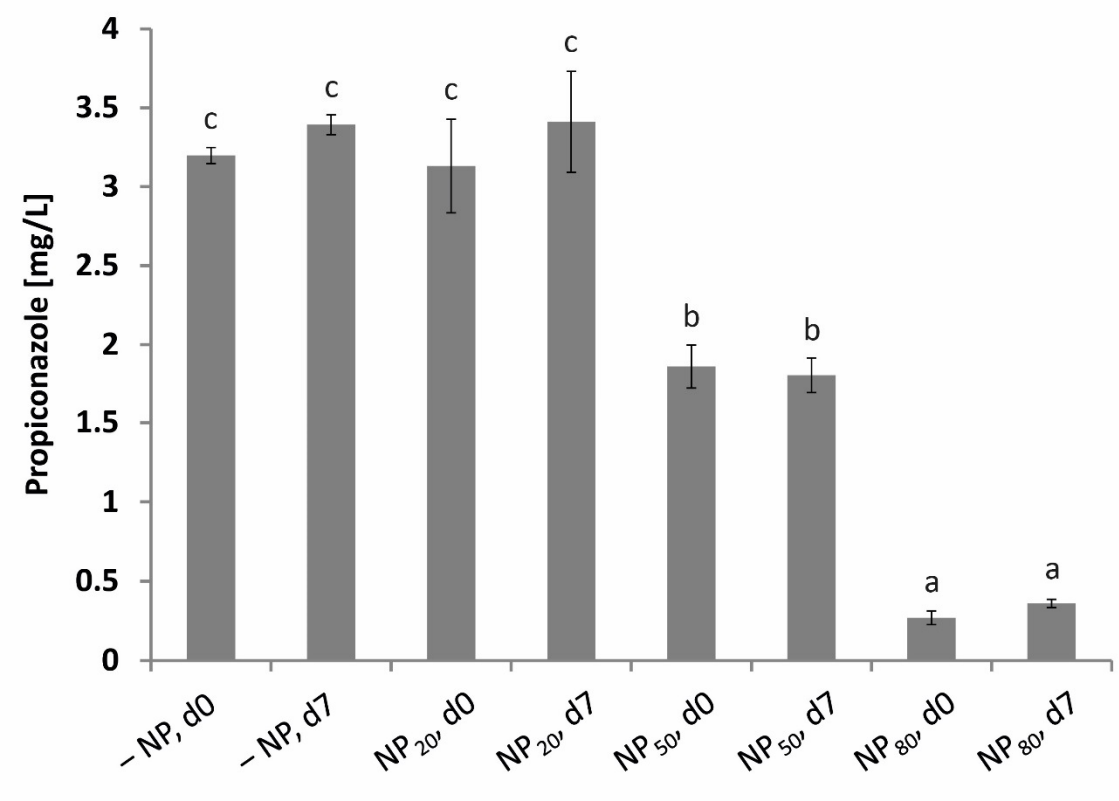

Figure 36: Propiconazole content in medium supplemented with 5 mg/L propiconazole and different concentrations of nanoparticles or an appropriate solvent control at day 0 (d0) and day 7 (d7) under cultivation conditions. All samples contained no fungal inoculum and were analyzed via HPLC. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs, respectively. Bars represent mean ± SD, number of technical replicates = 3. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 181.1$; $p < 0.001$) and Tukey's post hoc test (HPLC analysis performed by Leyla Guluzada).

Table 21: Survey of propiconazole content and sorption of propiconazole (prop) on nanoparticles in different growth media containing different levels of nanoparticles. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs, respectively. Sample analysis was performed using HPLC. Mean values of three technical replicates are shown (HPLC analysis performed by Leyla Guluzada).

Treatment	Free propiconazole [mg/L]	Sorption of propiconazole on NP [%]	Intended sorption [%]
– NP, prop	3.30	100.00	0
NP_{20}, prop	3.27	0.72	20
NP_{50}, prop	1.83	44.37	50
NP_{80}, prop	0.31	90.46	80

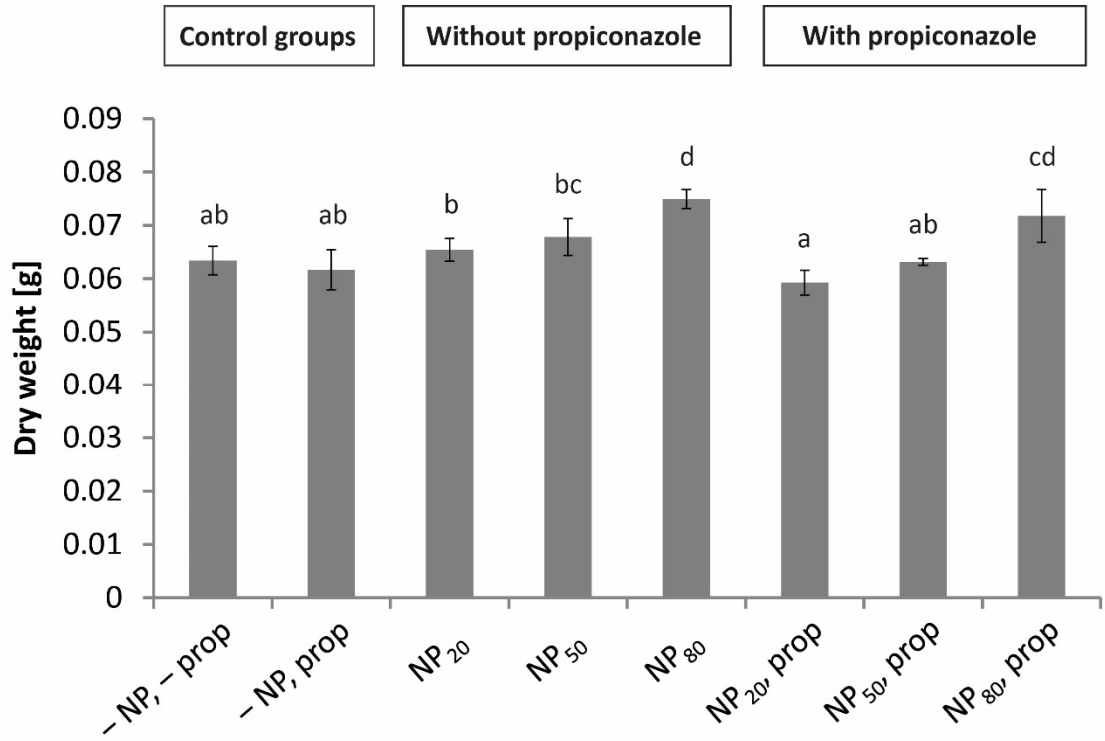

Figure 37: Dry weight of fungal cultures of *A. muscaria* in the presence of 5 mg/L propiconazole (prop) and nanoparticles (NP), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs, respectively. Cultures were started with an inoculum of 0.5 g fungal fresh weight. After 7 days, all fungal material was collected and dried. Bars represent mean ± SD, n = 5. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 15.69$; $p < 0.001$) and Tukey's post hoc test.

Table 22: Liquid cultivation of *A. muscaria* in the presence of 5 mg/L propiconazole (prop) and nanoparticles (NP), or appropriate solvent controls. Periodic mesoporous organosilica were added to fungal growth medium in different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs, respectively. After 7 days, fungal material was collected and dried. Mean dry weight increase within 7 days and inhibition of each treatment in relation to *A. muscaria* grown in the absence of both fungicide and NPs are stated; n = 5. If fungal growth was not inhibited but promoted, the percentage promotion is stated in parentheses and marked with an asterisk *.

Treatment	Dry weight increase [g]	Inhibition / promotion [%]
– NP, – prop	0.063	
– NP, prop	0.061	2.72
NP_{20}	0.065	(3.26)*
NP_{50}	0.067	(7.06)*
NP_{80}	0.074	(18.47)*
NP_{20}, prop	0.058	6.62
NP_{50}, prop	0.062	0.38
NP_{80}, prop	0.071	(13.39)*

8.7 Concentration series of propiconazole in liquid medium

Table 23: Dry weight increase and percentage inhibition of liquid cultures of *A. muscaria* in the presence of various concentrations of propiconazole (prop) and nanoparticles (NP), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added to fungal growth medium in a concentration of 0.129 g/L (NP_{50}) representing a sorption of 50 % of the added propiconazole on NPs. After 7 days, fungal material was collected and dried. Dry weight increase within 7 days and percentage inhibition of each treatment in relation to *A. muscaria* grown in the absence of fungicide and NPs are stated, n = 5. According to one-way ANOVA there are no statistically significant differences ($p < 0.05$) among treatments groups ($F = 1.959$; $p = 0.122$).

Treatment	Dry weight increase [mg]	Inhibition [%]
– NP, – prop	20.10	
– NP, 1 mg/L prop	19.10	4.98
– NP, 2.5 mg/L prop	17.94	10.75
– NP, 4 mg/L prop	18.22	9.35
– NP, 5 mg/L prop	16.40	18.41
NP_{50}, 5 mg/L prop	18.70	6.97

8.8 Independently repeated experiments on solid agar medium

8.8.1 A. muscaria as test organism

Table 24: Increase of colony diameter and area of *A. muscaria* cultivated on MMN agar medium supplemented with 0.5 % glucose for 42 days and respective inhibition related to *A. muscaria* grown in the absence of both nanoparticles and propiconazole. Growth medium was amended with nanoparticles (NP) and **5 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs; n = 19.

Treatment	Diameter increase [cm]	Area increase [cm^2]	Inhibition [%] of diameter increase	Inhibition [%] of area increase
–NP, – prop	4.48	21.61		
–NP, prop	1.36	3.23	69.74	85.06
NP_{20}	4.27	19.93	4.58	7.82
NP_{50}	4.33	20.42	3.41	5.54
NP_{80}	3.33	13.02	25.56	39.76
NP_{20}, prop	1.33	3.10	70.21	85.67
NP_{50}, prop	1.50	3.65	66.57	83.10
NP_{80}, prop	2.07	5.99	53.76	72.31

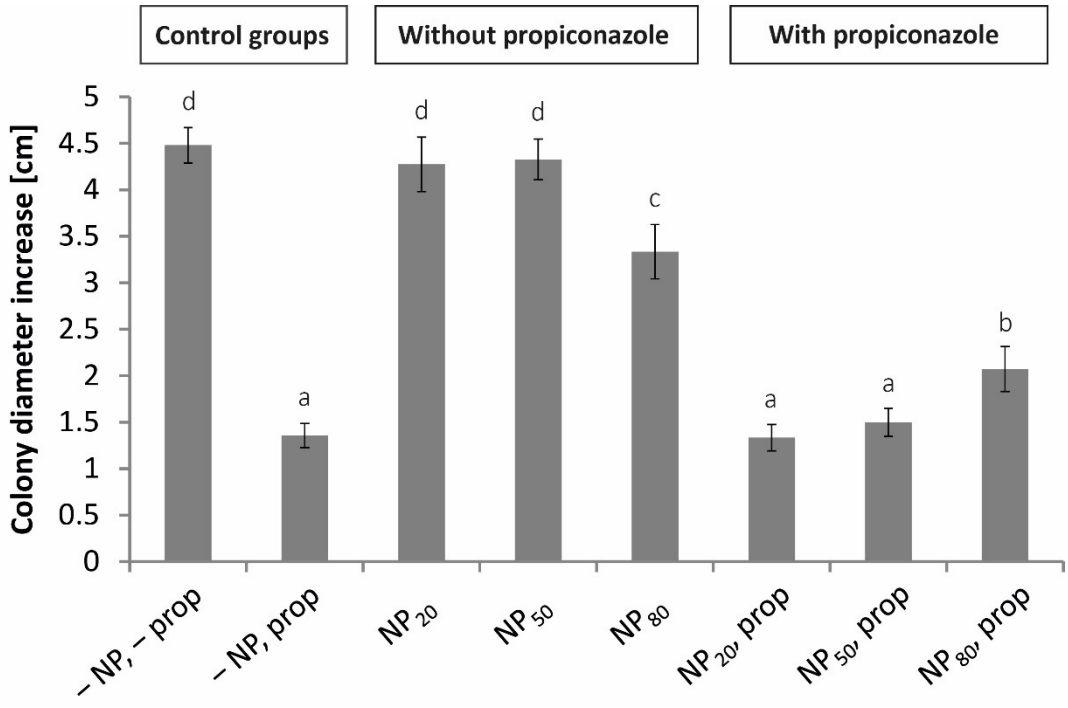

Figure 38: Colony diameter increase of *A. muscaria* cultivated on MMN agar medium supplemented with 0.5 % glucose for 42 days. Growth medium was amended with nanoparticles (NP) and **5 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony diameter was measured and the diameter of the starting inoculum (0.9 cm) was subtracted. Bars represent mean ± SD, n = 19. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to Kruskal-Wallis test (Hc (corrected) = 136.7; $p < 0.001$) and Mann-Whitney post hoc with Bonferroni corrected pairwise comparisons.

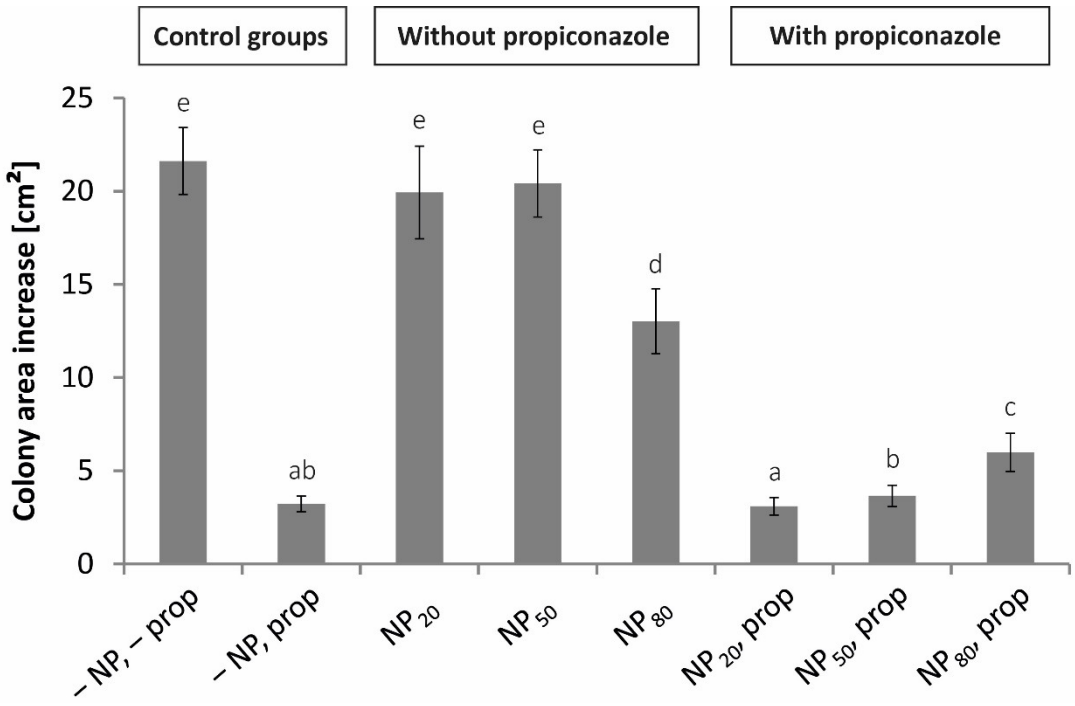

Figure 39: Colony area increase of *A. muscaria* cultivated on MMN agar medium supplemented with 0.5 % glucose for 42 days. Growth medium was amended with nanoparticles (NP) and **5 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony area was measured and the area of the starting inoculum (0.64 cm^2) was subtracted. Bars represent mean ± SD, n = 19. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 765.3$; $p < 0.001$) and Tukey's post hoc test.

8.8.2 C. geophilum as test organism

Table 25: Increase of colony diameter and area of *C. geophilum* cultivated on MMN agar medium supplemented with 0.5 % glucose for 70 days and respective inhibition related to *C. geophilum* grown in the absence of both nanoparticles and propiconazole. Growth medium was amended with nanoparticles (NP) and **2 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs; n = 19. If fungal growth was not inhibited but promoted, the percentage promotion is stated in parentheses and marked with an asterisk *.

Treatment	Diameter increase [cm]	Area increase [cm^2]	Inhibition / promotion [%] of diameter increase	Inhibition / promotion [%] of area increase
– NP, – prop	4.48	20.38		
– NP, prop	0.00	0.03	100.00	99.87
NP_{20}	4.43	20.21	1.12	0.83
NP_{50}	4.37	19.52	2.58	4.21
NP_{80}	5.24	27.11	(16.90)*	(33.04)*
NP_{20}, prop	0.04	0.04	99.06	99.82
NP_{50}, prop	0.07	0.06	98.47	99.72
NP_{80}, prop	0.69	1.16	84.68	94.31

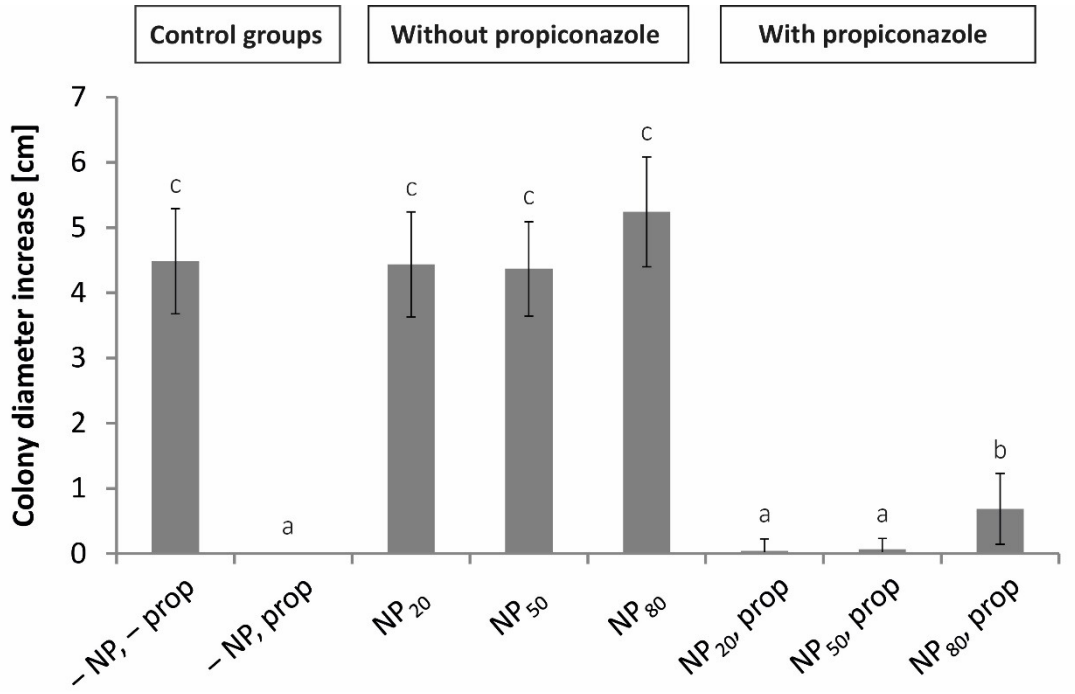

Figure 40: Colony diameter increase of *C. geophilum* cultivated on MMN agar medium supplemented with 0.5 % glucose for 70 days. Growth medium was amended with nanoparticles (NP) and **2 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony diameter was measured and the diameter of the starting inoculum (0.6 cm) was subtracted. Bars represent mean ± SD, n = 19. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to Kruskal-Wallis test (Hc (corrected) = 129.2; $p < 0.001$) and Mann-Whitney post hoc with Bonferroni corrected pairwise comparisons.

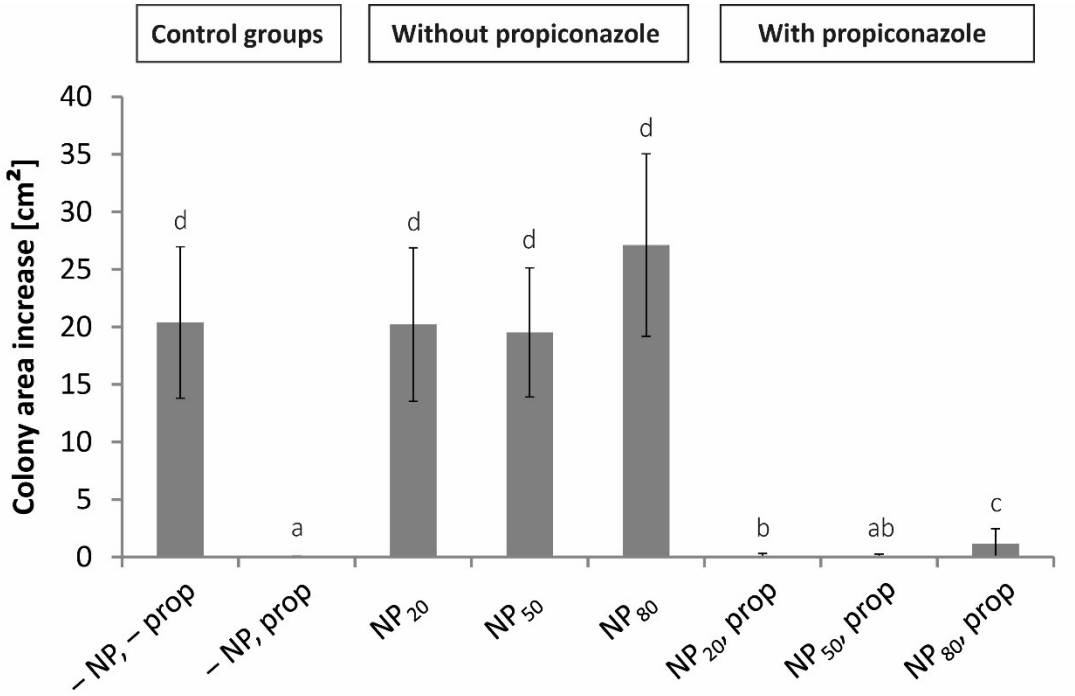

Figure 41: Colony area increase of *C. geophilum* cultivated on MMN agar medium supplemented with 0.5 % glucose for 70 days. Growth medium was amended with nanoparticles (NP) and **2 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony area was measured and the area of the starting inoculum (0.28 cm^2) was subtracted. Bars represent mean ± SD, n = 19. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to Kruskal-Wallis test (Hc (corrected) = 125.7; $p \leq 0.001$) and Mann-Whitney post hoc with Bonferroni corrected pairwise comparisons.

8.8.3 L. bicolor as test organism

Table 26: Increase of colony diameter, area, and fresh weight of *L. bicolor* cultivated on cellophane on MMN agar medium supplemented with 0.5 % glucose for 14 days and respective inhibition related to *L. bicolor* grown in the absence of both nanoparticles and propiconazole. Growth medium was amended with nanoparticles (NP) and **0.1 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs; n = 9. If fungal growth was not inhibited but promoted, the percentage promotion is stated in parentheses and marked with an asterisk *.

Treatment	Diameter increase [cm]	Area increase [cm^2]	Fresh weight increase [g]	Inhibition / promotion [%] of diameter increase	Inhibition/ promotion [%] of area increase	Inhibition / promotion [%] of fresh weight increase
– NP, – prop	3.11	10.43	0.16			
– NP, prop	0.93	1.46	0.03	69.95	86.05	83.69
NP_{20}	3.25	11.16	0.17	(4.65)*	(6.99)*	(8.06)*
NP_{50}	2.92	9.43	0.17	6.08	9.63	(7.88)*
NP_{80}	3.28	11.45	0.18	(5.55)*	(9.77)*	(14.39)*
NP_{20}, prop	1.03	1.71	0.04	66.73	83.61	75.61
NP_{50}, prop	1.51	3.08	0.05	51.34	70.45	66.03
NP_{80}, prop	2.83	8.65	0.13	8.94	17.09	17.62

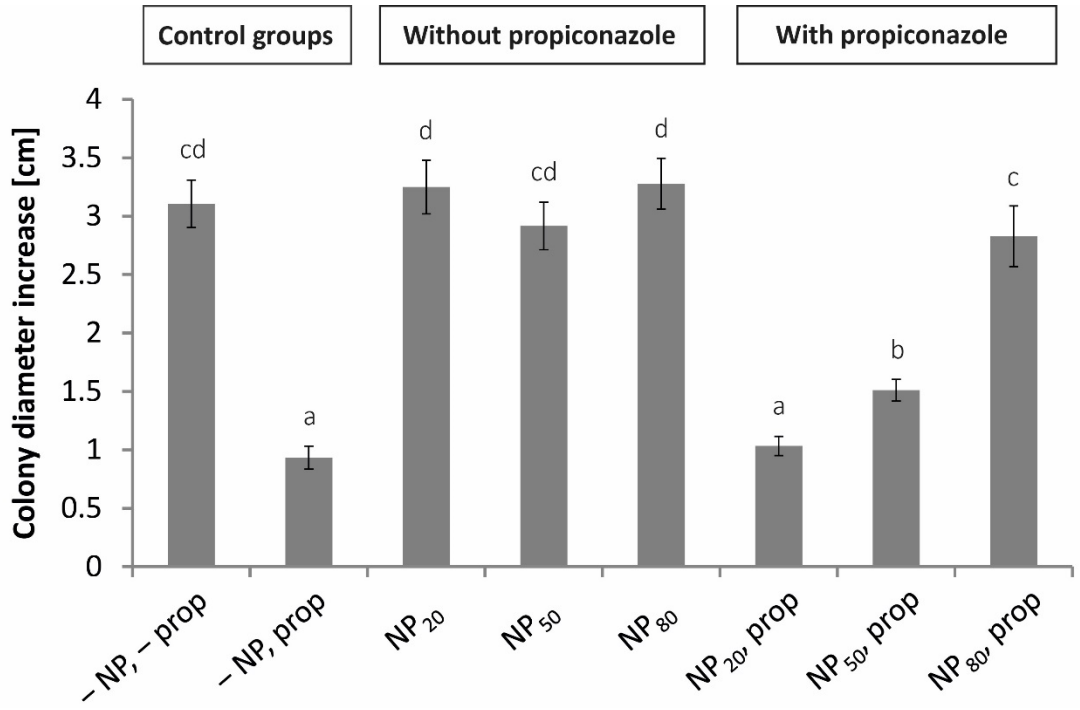

Figure 42: Colony diameter increase of *L. bicolor* cultivated on MMN agar medium supplemented with 0.5 % glucose for 14 days. Growth medium was amended with nanoparticles (NP) and **0.1 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony diameter was measured and the diameter of the starting inoculum (0.6 cm) was subtracted. Bars represent mean ± SD, n = 9. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 411.3$; $p < 0.001$) and Tukey's post hoc test.

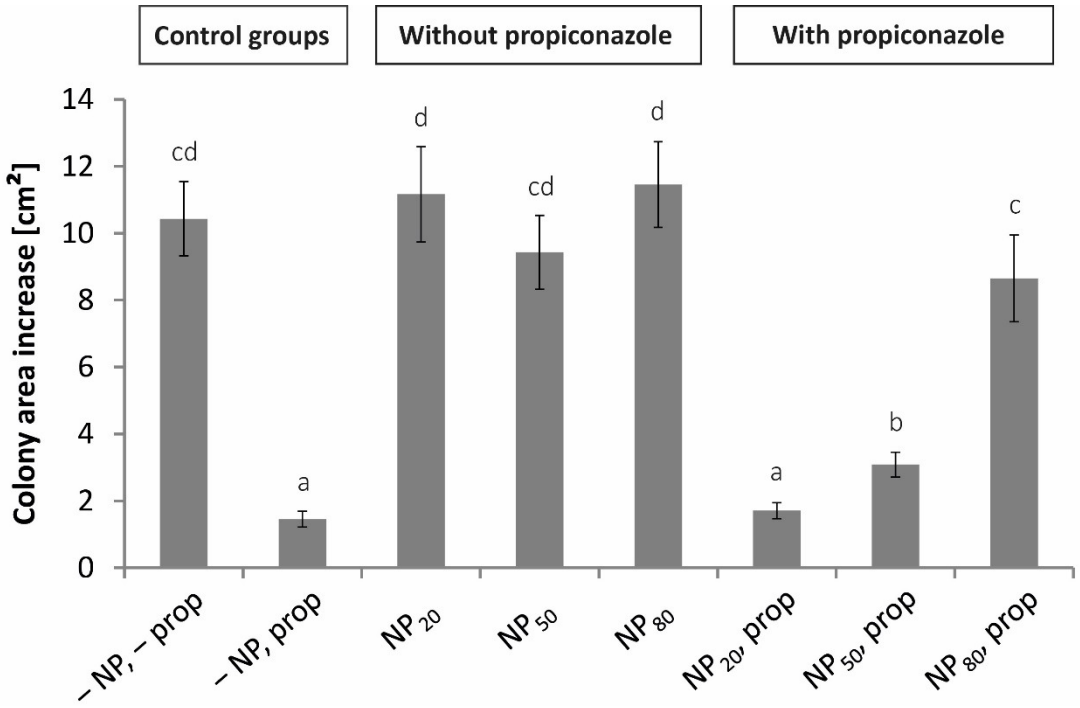

Figure 43: Colony area increase of *L. bicolor* cultivated on MMN agar medium supplemented with 0.5 % glucose for 14 days. Growth medium was amended with nanoparticles (NP) and **0.1 mg/L propiconazole** (prop), or appropriate solvent controls. Periodic mesoporous organosilica nanoparticles were added in three different concentrations: NP_{20} (0.032 g/L), NP_{50} (0.129 g/L), and NP_{80} (0.519 g/L) representing hypothetical sorptions of 20, 50, and 80 % of the added propiconazole on NPs. Colony area was measured and the area of the starting inoculum (0.28 cm^2) was subtracted. Bars represent mean ± SD, n = 9. Different letters above each bar indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA ($F = 392.2$; $p < 0.001$) and Tukey's post hoc test.

8.9 List of abbreviations and symbols

Table 27: List of utilized abbreviations and symbols.

$	dollar
%	percent
$(NH_4)_2HPO_4$	diammonium phosphate
$(NH_4)_2Mo_7O_{24}$*4 H_2O	ammonium molybdate tetrahydrate
Δ	difference
ΔC_q	difference between two C_q -values
®	registered trademark
°C	degree Celsius
µL	microliter
µm	micrometer
µmol m^{-2} s^{-1}	micromole per square meter and second
2-D PAGE	two-dimensional polyacrylamide gel electrophoresis
A	absorbance
A. muscaria	*Amanita muscaria*
ACS	American Chemical Society
Ag	silver
$AgNO_3$	silver nitrate
Al	aluminum
AMF	arbuscular mycorrhizal fungi
ANOVA	analysis of variance
approx.	approximately
ATP	adenosine triphosphate
$AuCl_4^-$	tetrachloroaurate
BET	Brunauer, Emmett and Teller, a method to determine the specific surface area
bp	base pairs
BTEE	bis(triethoxysilyl)ethane
BTP	biotransformation product
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
C	carbon
C. geophilum	*Cenococcum geophilum*
Ca	calcium
$CaCl_2$	calcium chloride

cDNA	copy desoxynucleic acid
cm	centimeter
Co. KG	Compagnie Kommanditgesellschaft (limited commercial partnership)
CO_2	carbon dioxide
CoA	coenzyme A
C_q	quantification cycle
Cr	chromium
CTAB	cetyltrimethylammonium bromide
Cu	copper
$CuSO_4$*5 H_2O	copper sulfate pentahydrate
Cyp	cytochrome P450
Da	Dalton
DDT	dichlorodiphenyltrichloroethane
deepSAGE	deep serial analysis of gene expression
DNA	desoxynucleic acid
dNTP	deoxynucleotide triphosphates
DT	dissipation time, disappearence time
e.g.	example given, or *exempli gratia*
EC_{50}	the effective concentration that caused the half maximal effect
ECM	ectomycorrhiza
EMF	ectomycorrhizal fungi
Erg	ergosterol
EROD	ethoxyresorufin-O-deethylase
ESI	electrospray ionisation
et al.	*et alii* (and others)
etc.	*et cetera*
EU	European Union
F	Test statistic named after Sir Ronald Fisher
Fe	iron
Fe_2O_3	ferric oxide, or iron(III) oxide
$FeCl_3$ x 6 H_2O	iron(III) chloride hexahydrate
fw	forward
g	gram
gDNA	genomic desoxynucleic acid
GmbH	Gesellschaft mit beschränkter Haftung (limited liability company)

h	hour
H_2O	water
H_2O_2	hydrogen peroxide
H_3BO_3	boric acid
ha	hectare
HCl	hydrogen chloride
HIV	human immunodeficiency virus
HMG CoA	3-hydroxy-3-methylglutaryl-CoA
HNO_3	nitric acid
HPLC	high-performance liquid chromatography
HSA	human serum albumin
i. e.	*id est*, that is
ID	identifier
ISO	International organization for standardization
JGI	Joint genome institute
K	potassium
KCl	potassium chloride
Kd	distribution coefficient
KGaA	Kommanditgesellschaft auf Aktien (partnership limited by shares)
KH_2PO_4	potassium dihydrogen phosphate
Koc	organic carbon partition coefficient
Kow	octanol-water coefficient
L	liter
L. bicolor	*Laccaria bicolor*
LC	lethal concentration
log	logarithm
M	Molarity [moles/Liter]
m^2	square meter
MALDI-TOF MS	matrix-assisted laser desorption ionization-time of flight mass spectroscopy
MDA	malondialdehyde
MeOH	methanol
MES	2-(N-morpholino)ethanesulfonic acid
Mg	magnesium
$MgSO_4$ x 7 H_2O	magnesium sulfate heptahydrate

MIC	minimal inhibitory concentration
min	minutes
MiSsp7	mycorrhiza-induced small secreted protein 7
mL	milliliter
MMN medium	modified Melin-Norkrans medium
Mn	manganese
$MnSO_4*H_2O$	manganese(II) sulfate monohydrate
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog
Mst	monosaccharide transporter
myc.	mycorrhizal
N	nitrogen
n	number of replicates
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National center for biotechnology information
ng	nanogram
NH_4^+	ammonium
Ni	nickel
nm	nanometer
NO_3^-	nitrate
NOM	natural organic matter
NP	nanoparticle
NPs	nanoparticles
NTC	no template control
Ø	diameter
OH	hydroxy group
P	phosphorus
p or *p* value	probability value
p. a.	*pro analysi*
PCR	polymerase chain reaction
PDA	potato dextrose agar
PGPR	plant growth-promoting rhizobacteria
pH	potential hydrogen (*pondus Hydrogenii* or *potentia Hydrogenii*)
pk_a	logarithmic acid dissociation constant
PMONP	periodic mesoporous organosilica nanoparticle

PMONPs	periodic mesoporous organosilica nanoparticles
puriss.	*purissimum*
qPCR	quantitative real-time PCR
Q-TOF	quadrupole time-of-flight
R	*rectus*, right, clockwise (configuration of an enantiomer)
R^2	coefficient of determination
rev	reverse
RFU	relative fluorescence units
RG	research group
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
S	*sinister*, left, counterclockwise (configuration of an enantiomer)
SD	standard deviation
sec	seconds
SEM	scanning electron microscopy
SOD	superoxide dismutase
sp.	species
spp.	*species pluralis*
TAE	tris-acetate-EDTA
Taq	*Thermus aquaticus*
TBE	TRIS, boric acid, EDTA
TiO_2	titanium dioxide
™	unregistered trademark
TRIS	tris(hydroxymethyl)-aminomethane
U/µL	units/microliter
U/min	rotations per minute
USA	United States of America
UV-light	ultraviolet light
V	volts
wt	weight
wt %	percent by weight
ZnO	zinc oxide
$ZnSO_4$*7 H_2O	zinc sulfate heptahydrate