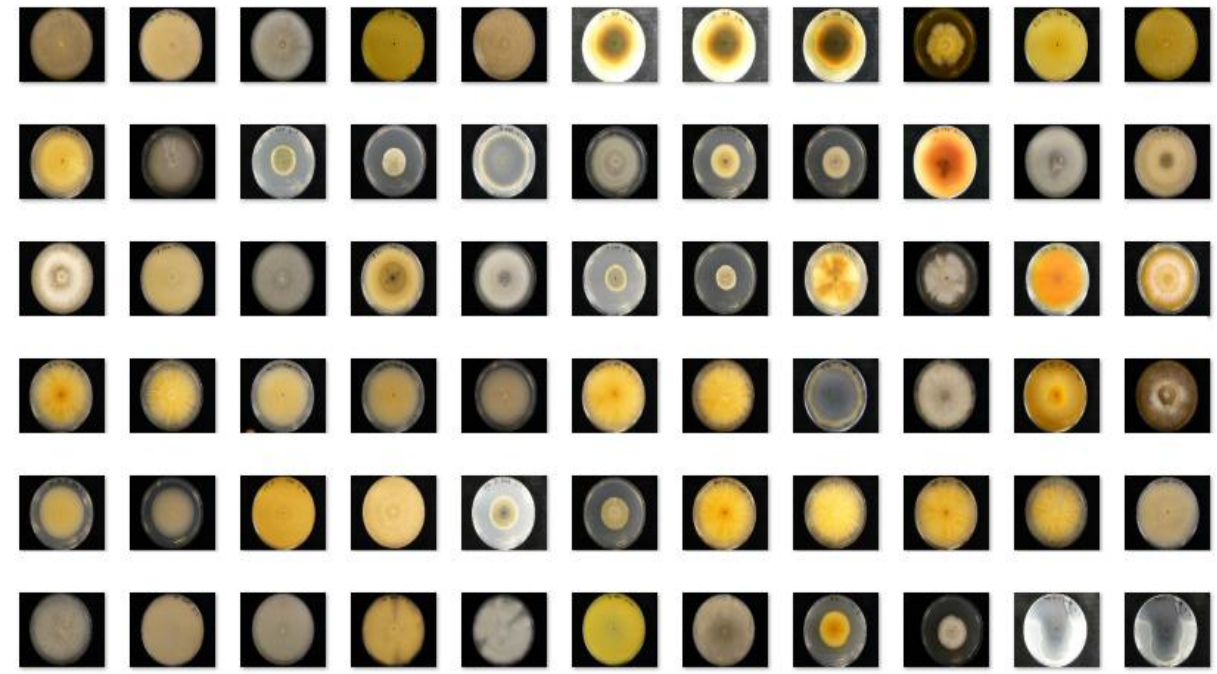
Unravelling plant soil feedback:

Interactions between grassland species and soil-borne fungi



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Abstract

Recent studies have shown that the positive relationship between plant diversity and ecosystem productivity can be explained by plant-soil feedback. An explanation for the increased productivity of plants in mixtures would be the deleterious effects of species-specific pathogens depressing yield in monocultures. Other studies have found positive effects of soil biota on plant productivity, which they believe to be the cause of increased productivity in mixtures. However, the rhizophere contains a high number of micro-organisms making it hard to identify both the pathogens and the mutualists.
In this experiment we tried to unravel the mechanisms of plant-soil feedback by investigating the interactions between two common grassland species: *Anthoxanthum odoratum* and *Leucanthemum vulgare* and several endophytic fungi. We tested both the direct and indirect interactions of these fungi on both plant species.
For both plant species a species-specific fungal pathogen has been identified which could have been responsible for the reduction of biomass in monocultures. Positive interactions between the fungi and plant species have been found as well. These interactions were most profound when the fungi and plant species were grown separately, which raised the idea of volatiles to be responsible for the found enhancement of plant productivity.

Introduction

Anthropogenic exploitation of the environment leads to biodiversity decline and impairment of ecosystem functioning, such as primary productivity, decomposition and trophic interactions (Loreau *et al.* 2001; Hooper *et al.* 2005). Many large scale biodiversity experiments have been performed in order to determine the correlation between the loss of biodiversity and ecosystem productivity. The results of these experiments show that species richness is positively correlated with the total productivity of these grassland ecosystems (Cardinale *et al.* 2007; Tilman *et al.* 2001; Hector *et al.* 1999). The classic explanation for this so-called 'overyielding' has long been niche complementarity and selection effects (Loreau and Hector 2001). Niche complementarity explains overyielding by a greater use of limited resources by the plant species due to spatial niche differentiation like rooting depth (Berendse 1983) or differences in nitrogen preference (von Felten *et al*. 2009). Selection effect explains higher productivity of mixtures by the dominance of individual, highly productive species (Roscher *et al*. 2005). However these hypotheses have been challenged by an 'alternative hypothesis' suggesting that soil biota cause the higher production in mixtures compared to the monoculture yield (van der Putten *et al*. 1993; Bradley *et al*. 2008; Maron *et al.* 2011; de Kroon *et al.* 2012; Wardle *et al*. 2004). This idea is derived from agriculture where higher productivity is realized in intercropping systems than in monocultures (Vandermeer 1989) and the same correlation has been found in recent biodiversity experiments focussing on how soil biota affect ecosystem productivity (Bever *et al.* 2010; Maron *et al*. 2011; Schnitzer *et al.* 2011).

Plant-soil feedback: negative effects studied

Most experiments which studied the effects of soil biota on productivity, found a negative feedback between plants and soil biota (van der Putten. 1993; Klironomos 2002; Petermann *et al*. 2008, Bever 1994). In monocultures, species specific pathogens may accumulate leading to a reduction of biomass compared to the yield found in mixed communities. In agriculture, negative plant-soil feedback is a well-known phenomenon and often referred to as "soil sickness" (Patrick *et al.* 1963; Bonamoni *et al.* 2005). In order to investigate whether the found negative correlation between plant productivity and soil-composition was due to either biotic (soil biota) or abiotic (nutrient availability) factors, several experiments looked

at the differences between conditioned soil (containing species specific pathogens) and sterilized soil and found the negative effects to disappear once the soil biota were eliminated through sterilization (Petermann *et al*. 2008; Hendriks *et al*. in prep, Maron *et al.* 2011; Schnitzer *et al.* 2011).

Plant-soil feedback: positive effects studied.

However in contrast to this negative feedback, soil microbes may increase the biomass productivity of mixed plant communities (Schnitzer *et al*. 2011; Maron *et al*. 2011; Hendriks *et al*. in prep; Bradley *et al*. 2008).

Plant growth-promotion is widely studied for plant mutualists such as arbuscular mycorrhizal fungi (AMF) (van der Heijden *et al.* 2008; Bever *et al.* 2001) and plant growth promoting rhizobacteria (RPGR) (Gray and Smith 2005; Compant *et al*. 2005; Raaijmakers *et al*. 2009). However, several studies concluded that different endophytic fungi, which are not AMF, are able to promote growth as well; either directly by stimulating longer root hairs and enhancing water absorption or indirectly by competing with plant pathogens and herbivores (Carrol 1988; Latch *et al*. 1985; Schulz *et al*. 2002; Schardl *et al*. 2004; Rodriguez *et al*. 2009, Malinowski and Belesky 2000).

Soil-borne fungi

All plants in natural ecosystems host a high number of endophytic fungi which can positively or negatively affect plant productivity (Brundrett *et al.* 2006; Rodriguez *et al*. 2009). One of the main issues concerning endophytic fungi is their high number of species and high variance in functional characteristics, depending on both biotic and abiotic factors like plant composition or nutrient availability. Many endophytic fungi are host specific, thus being beneficial on one species but neutral or even pathogenic on another, but the influence on plant performance differs according to the environment in which the plant is growing (Barrett *et al*. 2009; Hersch *et al*. 201). Furthermore, endophytic fungi are known to switch under certain conditions from beneficial to pathogenic and *vice versa* (Schultz and Boyle 2005; Kogel *et al*. 2006). For example, moisture level can influence the extent to which *Discula quericina* is parasitic or mutualistic on its host *Quercus cerris* (Morrica and Ragazzi 2008). Therefore it is difficult to make a clear distinction between pathogenic and beneficial fungi; the outcome of the interaction depends on a wide range of factors differing from

species-specificity to the composition of soil and plant community and abiotic stress factors (Hersch *et al*. 2012).

In this study we investigated the positive and negative effects of several endophytic fungi on biomass of two common grassland species. Mommer *et al.* (2010) carried out a biodiversity experiment in the Nijmegen Phytotron, which is an outdoor facility to study plant growth under near ambient conditions. In the experiment, four plant species were used; two forbs (*Leucanthemum vulgare* and *Plantago lanceolata)* and two grasses (*Festuca rubra* and *Anthoxanthum odoratum)* and they were grown both in mono- and mixed cultures. The experiment showed a much higher root biomass of the plant community as a whole in the mixture as compared to that in monoculture. In order to further unravel the species contribution to this belowground overyielding, a molecular method developed by Mommer *et al.* (2008) was used to unravel the species abundance in the root mixtures. It appeared that this belowground overyielding was mainly driven by one of the four species used in the experiment: *Anthoxanthum odoratum*, which made 3 times more roots in mixtures compared to monocultures.

In an additional study, addressing the importance of plant soil-feedback in a biodiversity experiment, the same four plant species were used (Hendriks *et al*. in prep). A plant-soil feedback approach makes use of soil conditioned by either a monoculture or a mixture of certain plant species, thus influencing the composition and abundance of soil biota present in the soil and testing these effects on plant biomass. As a control, the conditioned soils were sterilized, thus eliminating the influences of soil biota on plant biomass. In this experiment they performed the plant-soil feedback experiment on both monocultures of the four plant species and on mixtures that were grown in soil conditioned by single plant species (monoculture) or 1:1:1:1 mixtures of those soils (soil mixtures). In general, all species produced less biomass on the non-sterilized soil conditioned by its own species than on the soil conditioned by the other three species, varying on average from 1.6 to 4.5 times less. Especially biomass production of *Leucanthemum vulgare* was strongly inhibited on its 'own' soil type compared to the average biomass on the conditioned soil of the other three species. *Leucanthemum vulgare* produced the most biomass, 2.5 times more than on its 'own' soil type, when it was grown on soil conditioned with *Anthoxanthum odoratum*. Furthermore the experiment showed that *Anthoxanthum odoratum* produced more on the

soil mixture, 1.5 times, and even 3 times as much on the soil conditioned by *Leucanthemum vulgare* compared to the biomass production on its 'own' soil type.
On the sterilized soil, both the production of *L. vulgare* and *A. odoratum* was not affected on their ´own´ soil type and, opposite to the non-sterilized soils, there was no overyielding due to soil mixing. Plant mixtures did not produce more biomass than monocultures. Overall total biomass was significantly higher on sterilized soil compared to the non-sterilized soil types, production of *A. odoratum* was 20 times more on sterilized soil compared to non-sterilized and for *L. vulgare* 12 times as much.
In accordance to both Maron (2011) and Schnitzer (2011), plant-soil feedback either positive or negativ could be the main driver of this belowground overyielding (Mommer *et al*, 2010; Hendriks *et al*, in prep).

To determine the effects of plant-soil feedback, several fungi were isolated from surface sterilized roots of all four plant species grown in monoculture (Kempin and Reijers 2010; Deurhof 2011). In this study presented here seven of the additional 19 fungal isolates were investigated for their effects on plant growth. The following endophytic fungi were used in this research project: *Chaetomium udagawae*, *Paraphoma chrysanthemicola*, *Gaeumannomyces incrustans, Gaeumannomyces cyclindrosporus*, *Fusarium oxysporum* and *Plectosphaerella cucumerina*.
Some of these fungi are known to have negative effects on the growth of some plant species. For example the genus *Gaeumannomyces* is known to be pathogenic on grass species (Landschoot and Jackson 1989, Hornby *et al.* 1977) and *Paraphoma chrysanthemicola* on chrysanths, same family as *L. vulgare.*

The two main hypotheses of this thesis were:
-Negative effects: Plant production is reduced in monocultures compared to mixtures through the accumulation of species-specific pathogens. These species-specific pathogens will reduce growth through direct contact by infecting the host. Therefore we expect that some fungal isolates will negatively but differentially influence the biomass of plant species.

- Positive effects: Plant biomass productivity in mixtures is on average higher than expected from their yield in monocultures. We expect this increase in biomass to be caused by

positive feedback between the plant species and soil biota. We expect that some fungal isolates will be able to positively, but differentially, influence growth of plant species. The positive interactions can be either directly (enhancing nutrient/water uptake) or indirectly by releasing volatiles as has been shown for bacteria (Ryu *et al*. 2003).

Materials and Methods

1. Materials: Plants, fungi and conditioned soils.

1.1 Plant species

In this thesis project, two plant species were used; a grass, *Anthoxanthum odoratum* L. and a forb, *Leucanthemum vulgare* L. These two species appeared important players in the preceding biodiversity studies of Mommer *et al.* (2010); Hendriks *et al.* subm. The seeds were provided by Cruydthoek, The Netherlands (batch November 2011).

Seeds of *Leucanthemum vulgare* were sterilized for 6 hours in an exicator containing two beakers of 50 ml Sodiumhypochlorite and 1.5 ml HCl each. *Anthoxanthum odoratum* seeds were sterilized in eppendorf tubes containing 0.8 ml 96% ethanol and 0.2 ml household chlorine bleach for 15 minutes. After the 15 minutes the solution was removed and the seeds were washed three times with 96% ethanol and six times with sterile H_2O. After sterilization the seeds were kept in a small petridish(ø 60 mm) on a sterile filterpaper with 0.8 ml sterile H_2O in the dark at 4°C for a minimum of four days.

1.2 Fungal species

In earlier student projects (Deurhof 2011; Kempin and Reijers 2010) 17 different soil fungal species were isolated from the surface sterilised roots of four plant species; *Anthoxanthum odoratum* L., *Leucanthemum vulgare L., Plantago lanceolata* L. and *Festuca rubra* L., used in the biodiversity experiment of Mommer *et al* (2010). Based on the pilot studies with these 17 fungal species, seven of these 17 fungal species were selected for further tests on the two plant species (Table 1).

Table 1: Names of the seven species selected for the bioassays. The code responds with all the other figures in this report. The last column shows the host species of which the different fungi were isolated.

Species	**Code**	**Host**
Chaetomium udagawae	F1	*L. vulgare*
Paraphoma chrysanthemicola	F2	*L. vulgare*
Gaeumannomyces incrustans	F9	*F. rubra*

Gaeumannomyces cyclindrosporus	F10	*F. rubra*
Fusarium oxysporum	F13	*F. rubra*
Pyrenochaeta inflorescentiae	F21	*A. odoratum*
Plectosphaerella cucumerina	F26	*P. lanceolata*

1.3 Soil types

In this project, I also used conditioned soil of monocultures of *A. odoratum* and *L. vulgare*. This soil was originally obtained from the phytotron experiment (Mommer *et al.* 2010). Four years after the start of the phytotron experiment several soil cores from the four monocultures were taken. These trained soils were used to inoculate sterile soil on which the plants were planted again. After two months half of this soil was sterilised (25kGray gammaradiation; Isotron, Ede), to remove the soil biota, and half not. In my experiments, I thus used conditioned soils of *L. vulgare* and *A. odoratum* and sterile soil of these two different plant species.

2 Experimental setups: Plate assays, soil assays & ethylene measurements

2.1 Soil assay

The direct effects of the fungi on the growth of *A. odoratum* and *L. vulgare* in the soil were tested at Nijmegen University. The sterilized seeds of both species were placed on 0.5 MS medium without sucrose for germination. Small pots (5.5 cm) were filled with autoclaved riverine sand. On the bottom of each pot filter paper was placed and a small plastic bag served as a “saucer” to prevent any contact between the pots.

The seedlings were planted in the small pots and randomly placed in the climate room (day/night regime: 16 hrs light/8 hrs dark, temp 23/19°C; 210 $\mu mol \cdot m^{-2} \cdot sec^{-1}$).

The first four days a plastic sheet was placed on top of the plants to reduce the evaporation. Watering of the plants occurred every other day. Nutrition was added once a week (day 8, 14 and 20); 10 ml of 0,5 gram L^{-1} Kristalon (Yara) containing: NO_3^-: 11.9%, NH_4^+: 7.1%, P_2O_5: 6%, K_2O: 20%, MgO: 3%, SO_3: 7.5%, B: 0.025%, Cu: 0.01%, Fe: 0.07%, Mn: 0.04%, Mo: 0.004%, Zn: 0.025%.

Two sets of plants were inoculated with the fungi at different times; either one day or seven days after transplantation. We expected the fungal effects to be dependent on seedling stage. Half of the plants were inoculated one day after planting the seedlings (t=1). Soil was removed and a fungal plug (5mm) was placed on the root of the plant, 0.5-1cm below the soil surface. The other half of the plants was inoculated seven days after planting the seedlings (t=7).
The number of replicas per treatment for *A. odoratum* was 10 and for *L. vulgare* 15.
The plants were harvested 23 days after they were transferred to the soil (32 days after germination). The roots were washed from the soil and both the roots en shoots were dried for 48 hours at 70 °C and weighed.

2.2 Agar plate assays

In this project, several different bio assays have been executed with plants growing on agar plates, with or without fungi. Generally, plants were grown on round or square petridishes of ø 145 mm or 100 x 100 respectively, filled with 50 ml agar each. The plant agar consisted of 0.5 MS medium containing 2.2 g L^{-1} Murashige-Skoog salts + vitamins (pH: 5,8) and 12 g L^{-1} Plant Agar. Whenever fungi were supposed to direct interact with the plants, sucrose was added (5 g L^{-1}); otherwise no sucrose was added to the agar medium.

The fungi were kept in stock at 18 °C on 1/5 PDA plates (92 x 16 mm). These plates contained 4.8 g L^{-1} Potato dextrose (pH 6.5) and 15 g L^{-1} 1,5% Technical Agar. Whenever the fungi were grown with plants, they were transferred (plugs of 5 mm) to 0.5 MS with sucrose.

All plate assays were placed in a growth chamber with the following conditions: 21 °C day / 21 °C night temperature, light 200 µmol m^{-2} s^{-1} at plant level, 12h light/ dark, 70% relative humidity. On all plant plates (round and square) seven seeds were placed 3.5 cm from the top of the plate; 1.5 cm apart in a straight line. The number of replicas per treatment was 4-7 in all plate assays. All the plate assays were performed at Wageningen University, department of Phytopathology, Bacterial Ecology & Genomics group.

All plants grown on the 0.5 MS plates were harvested by separating the roots from the shoot, after 21 days of growth. The roots from every plate were kept in an eppendorf tube and the

shoots in a paper bag. Both were kept for 48 hours in a stove (60 °C) and weighted afterwards. The number of plants grown on each individual plate was counted and used to determine the dry weight per plant.

2.3 Ethylene production by fungi

Sterile cap flasks of 10 ml were filled with 3 ml 0.5 MS medium with sucrose. Plugs (5mm) of the fungal species were inserted on the medium in the flask, and incubated for seven days at 25 ℃; five replicates. Controls received a plug of 1/5 PDA plate without fungus. After the incubation, the ethylene concentration was measured at Utrecht University, department of Plant Ecophysiology, with help of dr. R. Pierik using a gas chromatograph (Syntech (2000) GC 955-800; flame ionization detector; column: Haye Sep 80/100). Measurements with the fungi on MS medium without sugar and 1/5 PDA agar were also performed to investigate the effect of the medium on fungal ethylene production.

In order to determine if the micro-organisms of the conditioned soil of *A. odoratum* and *L. vulgare* monocultures produced ethylene as well, 40 ml cap flasks were used and filled with approximately 20 gram of both conditioned soil types and the same sterilized soil. The cap flasks were incubated for seven days at 25 °C, four replicates, and measured at Utrecht University using the gas chromatograph.

2.4 Ethylene dosis response curves on plants

For the ethylene growth curve seeds of both plant species were placed on 0,5 MS without sucrose and placed in the growth chamber. After ten days the plates were transferred to Utrecht University and placed in closed glass containers (35 dm^3) that were flushed continuously (0.5 L min^{-1}) with various concentrations of ethylene (0 ppb, 60 ppb, 100 ppb, 460 ppb, 800 ppb and 200 ppb) in air, which were checked using the gas chromatograph. Each treatment was carried out in four or five replicates. The plants were harvested after ten days.

3. *Details of Plate assays*

3.1 Direct interaction

To test the direct effects of the fungi on the plants, the plant and fungal species were grown together on agar. Sterilized seeds of both plant species were sown on 0.5 MS medium with sucrose (square plate (100 x 100 mm)) and kept in the growth chamber. After a week four plugs (5 mm each) of the different fungi were added per plate; as a control plugs of 1/5 PDA were used; in between two plants, 1,0 cm from the roots of the plant (Fig. 15 & 16). The plant species and fungi were grown together for two weeks and then harvested.

3.2 Separation experiment

In order to determine if the growth of both *A. odoratum* and *L. vulgare* was influenced by volatiles produced by the seven fungal species, plants and fungi were grown on petridishes with a separated compartments for plant and fungus A small petridish (ø 35 mm; i.e. fungal compartment) was placed in a bigger petridish (ø 145 mm; plant compartement) (Fig. 1a). The fungal compartement was filled with 2 ml 0.5 MS medium with sucrose and the plant compartment with 50 ml 0.5 MS medium without sucrose. Sterilized seeds were already germinating on the plates for a week before the fungal plugs were added to the small petridish. As a control a plug of 1/5 PDA was used.

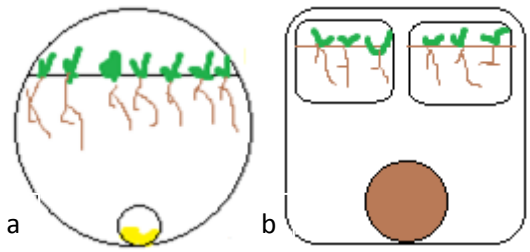


Figure 1: a) design of the separation experiment b) design of the 'big' plate assay

3.3 "Big" plate assay

The indirect effects of the isolated fungi on the growth of the plant species were investigated in the *separation experiment.* However, in the biodiversity experiment carried out by Mommer *et al* (2010) the plants may have been subject to a blend of volatiles produced by many fungal species. Therefore, seedlings on agar were subject to volatiles produced by soils; either sterilized or non-sterilized, of the monocultures.

The test these effects two plates with seedlings on agar and one plate containing soil were inserted in one “big” plate (241 x 241 mm) (Fig. 1b). In this way any volatile compound produced by the soil biota would accumulate and thus influence the growth of the plants. The plant compartements were filled with 40 ml 0.5 MS medium without sucrose; these plates were stuck with agar in the upper half of the big plate (Fig. 1b). On the bottom the lid of a round petridish (ø 92 mm) was inserted, filled with approximately 35 grams of one of the four soil types and covered with foil, in which approximately 30 small holes were punctured. The plates were moved to the growth chamber and after three weeks the plants were harvested.

4. Statistical analysis

Statistical analysis was performed for the soil assay using univariate ANOVAs, with fungal treatment, plant species and timing as fixed factors. Statistical analysis of the plate assays was performed using univariate ANOVAs with plant species and fungal treatment as fixed factors, except for the ‘big’ plate assay which was analysed using univariate ANOVAs with plant species and soil type as fixed factors. For the ethylene production of the fungi, statistical analysis was performed using fungal species as a fixed factor and for the ethylene growth curve univariate ANOVAs were performed with plant species and ethylene concentration as fixed factors. All data were ln- or sqrt-transformed prior to analysis.

Results

1. Soil assay

Species–specific response on fungal treatment

A. odoratum and *L. vulgare* differ significantly (F-value: 38.1 ***) in total root biomass and an overall effect of fungal treatment on total plant biomass was found as well (F-value: 10.7 ***) (Table 2). Furthermore the effects of fungal treatment differ between the two plant species, which means the effects are host-specific (F-value$_{root}$ 10.9 ***, F-value$_{shoot}$ 16.1***) (Table 2). Disentangling these interactions, without distinguishing between the two different times of inoculation, it is shown that both root and shoot biomass of *L. vulgare* plants inoculated with *Paraphoma chrysanthemicola* (F2) was significantly reduce2d compared to control (P-value: 0.000). However there were no significant effects of *P. chrysanthemicola* on the biomass production of *A. odoratum.* Root biomass of *A. odoratum* was significantly reduced for the plants inoculated with *Gaeumannomyces incrustans* (F9) (P-value 0.039), but no effects were found for *L. vulgare.*

Both *A. odoratum* and *L. vulgare* showed only mild effects when inoculated with the other fungi. For example root biomass of *A. odoratum* was enhanced when inoculated with *Plectosphaerella cucumerina* (F26), whereas shoot biomass was reduced. However these effects were not significant.

Effects of plant age on response to fungal treatment

The plants were inoculated on two different times (t=1, 1 day after planting; t=7, 7 days after planting) to test whether plant age would influence the effects of fungal treatment on plant biomass. In overall timing had a significant effect on the total biomass (F-value$_{root}$:12.5 ***, F-value$_{shoot}$ 16.9***) (Table 2). The interaction between the plant species and time of inoculation had no significant effects, which means that the plant species did not differ in their response to the differences in times of inoculation. However the effects of fungal treatments on the total biomass of both plant species were different between the two times of inoculation (F-value$_{root}$: 4.4***, F-value$_{shoot}$: 10.9***).

For *A. odoratum* this difference in response resulted for most fungal treatments in a reduction of biomass on t=1 (Fig. 3) compared to the control. The only significant negative effect on *A. odoratum* was *G. incrustans* (F9). Differentiating between the two times of

inoculation we find this negative effect only on t=1 (P-value$_{root}$:0.041; P-value$_{shoot}$: 0.004). On t=2 there were no significant negative effects found, only mild effects (Fig. 2).
No significant positive effects were found. On t=1 all plants which were inoculated with a fungus had a lower total biomass compared to the control. On t=2 root biomass was enhanced of the plants inoculated with *P. cucumerina* (F26), however shoot biomass was reduced compared to control (Fig. 2). Shoot biomass was enhanced on t=2 for the plants inoculated with both *P. chrysanthemicola* (F2) and *F. oxysporum* (F13) (Fig. 2).

For *L. vulgare* differences in biomass between the two times of inoculation were found as well. On t=1 a clear negative effect was found between the control and the plants inoculated with *P. chrysanthemicola* (F2) (P-value$_{root}$: 0.000; P-value$_{shoot}$: 0.000). This strong negative effect was less on t=2, there was still a significant negative effect on the root biomas (P-value: 0.009) but for the shoot biomass a trend was observed (P-value: 0.053). Pictures were taken of the plants as well (Fig. 14). These pictures show clearly the strong negative effects of *P. chrysanthemicola* on t=1 and the milder negative effects on t=2.
In general most plants show an increase in biomass on t=2, compared to t=1, except for the *L. vulgare* plants inoculated with *C. udugawae* (F1). On t=2 root biomass was significantly reduced compared to the control (P-value: 0.018).
No significant positive effects were found. On t=1 mean total biomass of most plants inoculated with fungi, except for *P. chrysanthemicola*, was the same as the mean biomass of the control plants. Only the biomass of the plants inoculated with *P. cucumerina* (F26) was slightly enhanced compared to the control plants. On t=2 a mild positive effect was found on the root biomass of the plants inoculated with *F. oxysporum* (F13).

*Table 2: full model ANOVA results of the effects of plant species, fungal treatmen and time of inoculation on the total biomass (root + shoot). Analyses are performed on ln-transformed data. P-values are indicated with: * <0.05, **< 0.01, ***< 0.001, $< 0.1, N.S.> 0.1. N=10 except for F13; N=9.*

Source	Df	F-value root	F-value shoot
Plant species	1	38.1 ***	1.1 N.S.
Fungal treatment	7	10.7 ***	10.9 ***
Time of inoculation	1	12.5 ***	16.9 ***

P x F	7	10.9 ***	16.1 ***
P x T	1	1.8 N.S.	0.0 N.S.
F x T	7	4.4 ***	10.9 ***
P x F x T	7	4.7 ***	3.7 ***
Error (MS)	360	0.2	

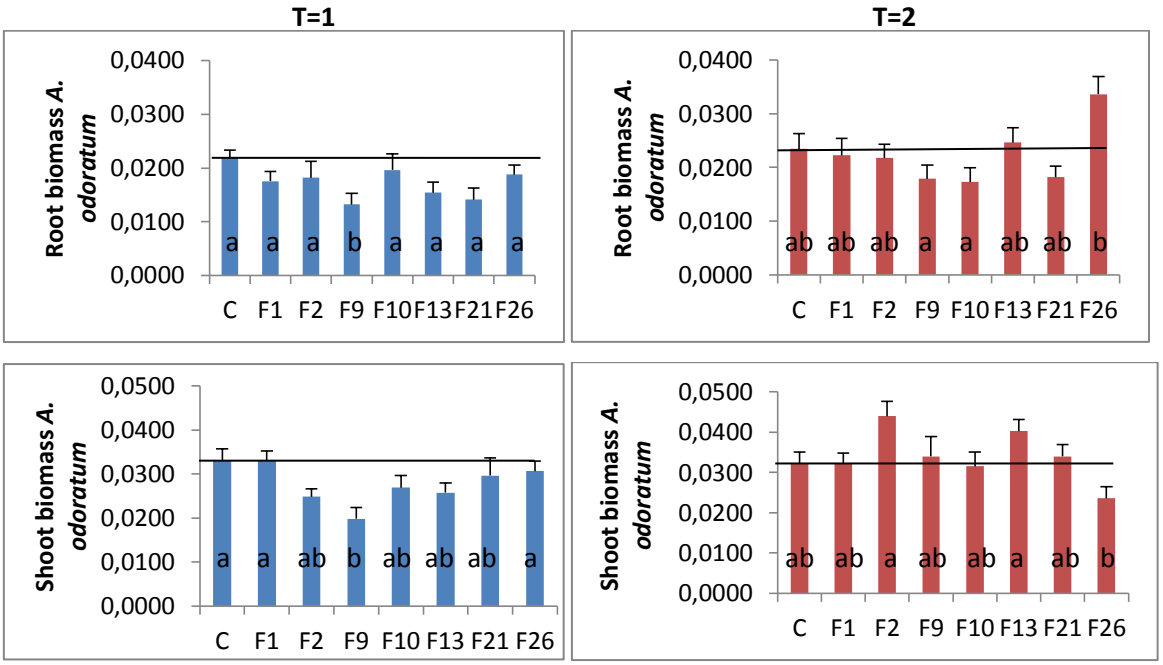
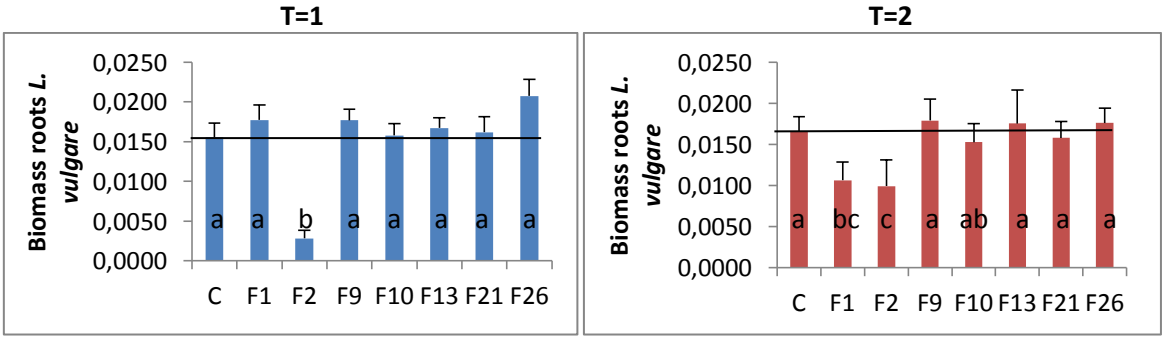


Figure 2: Mean dryweight of the roots (up) and shoots (below) of Anthoxanthum odoratum plants. The different fungi are placed on the roots on two different times (t=1 (left) and t=7 (right)). The line indicates the mean biomass of the plant species grown under the control situation. Data are means + S.E., N=10. Codes are fungal treatment (table 1). Letters indicate statistical differences between the treatments.



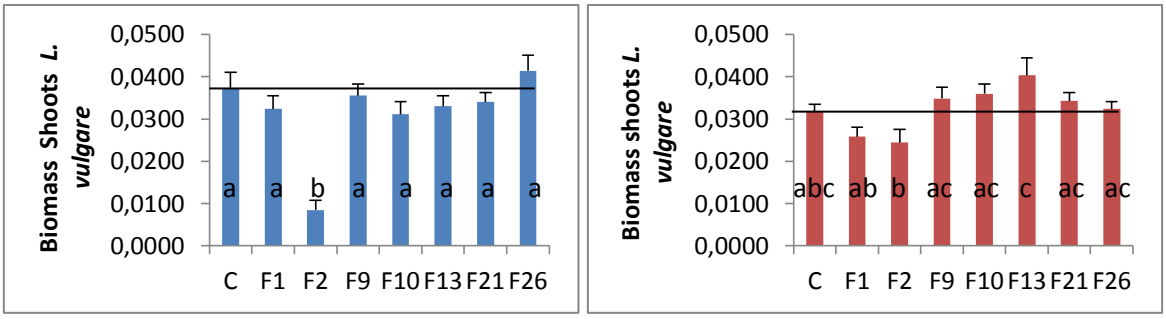


Figure 3: Mean dryweight of the roots (up) and shoots (below) of Leucanthemum vulgare plants. The different fungi are placed on the roots on two different times (t=1 (left); t=7(right)).The line indicates the mean biomass of the plant species grown under the control situation. Data are means ± S.E., N=15. Codes are fungal treatment (table 1). Letters indicate statistical differences

*Table 3: ANOVA results, split per plant species and time of inoculation, of the effects of treatment (fungal inoculation table 1) on the biomass of A. odoratum and L. vulgare. Data have been ln-transformed prior to analysis. Df= degrees of freedom. P-values are indicated with: * <0.05, **< 0.01, ***< 0.001, $< 0.1, N.S.> 0.1. N=10 except for F13; N=9.*

	Df	Ao Root 1 F-value	Ao Shoot 1 F-value	Df	Lv Root 1 F-value	Lv Shoot 1 F-value
Treatment	7	2.2 *	3.3 **	7	13.3***	21.6***
Error	71			106		
	Df	Ao Root 2 F-value	Ao Shoot 2 F-value	Df	Lv Root 2 F-value	Lv Shoot 2 F-value
Treatment	7	2.9 **	3.8 **	7	5.8 ***	4.8 ***
Error	72			112		

2. Plate assays

Direct interaction

The direct effects of the different fungal species on the biomass production of both *A. odoratum* (Fig. 4) and *L. vulgare* (Fig. 5) on 0.5 MS agar + sucrose were tested. Treatment in overall had a significant effect (F-value$_{root}$: 9.1***; F-value$_{shoot}$: 7.4***) (Table 4). The fungal treatment had a significantly positive effect on both root and shoot biomass of both *A. odoratum* and *L. vulgare* (Table 5). However there were no different effects of the fungi on the plant species (F-value$_{root}$: 1.4 N.S.; F-value$_{shoot}$: 1.5 N.S.).

For *A. odoratum* there were significant differences found between the control plants and the ones treated with *P. chrysanthemicola* (F2)*, G. incrustans* (F9) *and G. cylindrisporus* (F10). For *L. vulgare* there were significant differences found between the control plants and all fungal treatments (only a trend (P-value= 0.052) was observed for the shoot biomass of *C. udagawae* (F1). In overall both plants performed better compared to the control when the fungal plugs were present. There were some differences in the effects between the plants. For the *A. odoratum* plants, the ones treated with *G. incrustans* (F9) showed the highest increase (3.07 times for the root biomass). Whereas for *L. vulgare* the treatment with *G. cylindrisporus* (F10) had the highest biomass compared to the control (2.75 times).

Pictures of the assay were taken at the time of harvest and shown in the appendix (Fig.15 & 16). For *A. odoratum* the increase in biomass is seen in the number and length of the roots. For *L. vulgare* the effects are easier to spot since the leaves of the forb are easier to compare between the treatments than the leaves of the grass. However when looking at the *L. vulgare* plants treated with *P. chrysanthemicola* (F2), it is shown that the size of the plants differs a lot within the treatment. Some are a lot bigger than the control plants, whereas others remained small with signs of infection like brown leaves and dark root tips (Fig. 16). The *A. odoratum* plants that were treated with *F. oxysporum* showed signs of infection as well, such as brownish leaves and fragile roots with dark tips.

*Table 4: full model ANOVA results of the effects of plant species (P) and treatment (fungal treatment for direct interaction and separation experiment and soil type for 'big' plate assay) (T) on the total biomass (root + shoot) of all the plate assays. Analyses are performed on ln-transformed data. P-values are indicated with: * <0.05, **< 0.01, ***< 0.001, $< 0.1, N.S.> 0.1.*

Source	*Direct interaction*			*Separation experiment*			*'big' plate assay*		
	df	F-value root	F-value shoot	df	F-value root	F-value shoot	df	F-value root	F-value shoot
P	1	32.3***	20.1***	1	34.3***	91.7***	1	0.8 N.S.	6.5 *
T	7	9.1***	7.4***	7	19.9***	19.7***	4	2.1 N.S.	1.7 N.S.
P x T	7	1.2 N.S.	1.5 N.S.	7	0.9 N.S.	2.6 *	4	5.3 **	4.6 *

Anthoxanthum odoratum

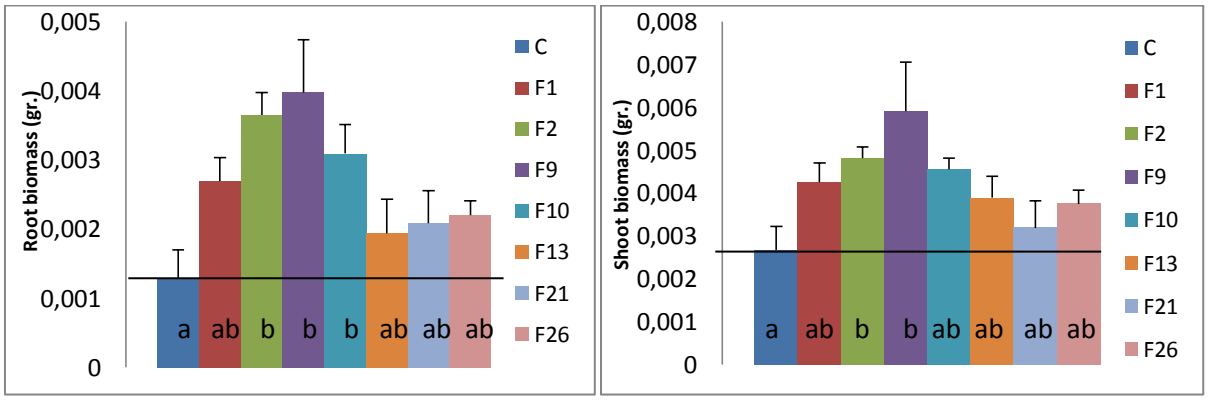


Figure 4: Mean dryweight of the roots (left) and shoots (right) of the Anthoxanthum odoratum plants grown on 0.5 MS agar with the four fungal plugs added after 7 days. The line indicates the mean biomass of the plant species grown under the control situation. Data are means ± S.E., N=6 for C and F13; N= 5 for F1, F2, F9, F10, F26, N=4 for F21, F26 and N=3 for C, F9. Codes are fungal treatment (Table 1).

Leucanthemum vulgare

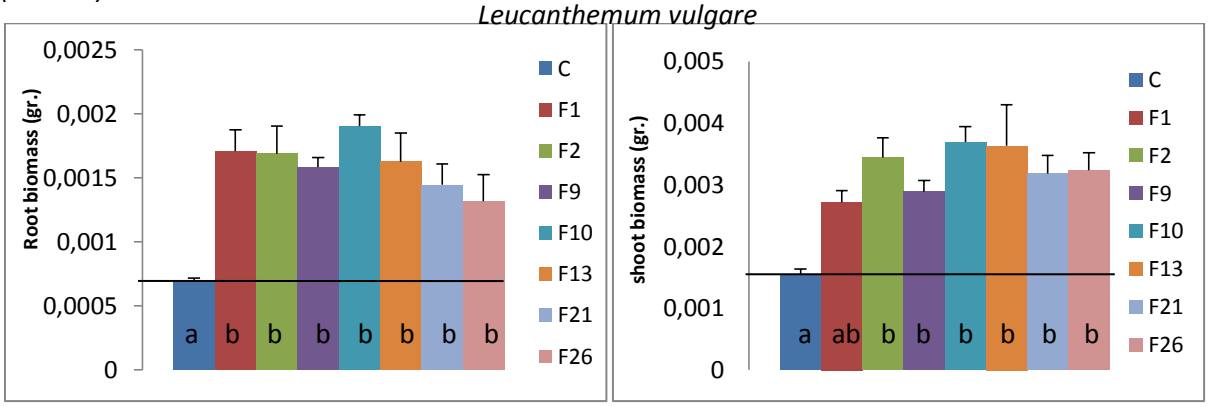


Figure 5: Mean dryweight of the roots (left) and shoots (right) of the Leucanthemum vulgare plants grown on 0.5 MS agar with the four fungal plugs added after 7 days. The line indicates the mean

biomass of the plant species grown under the control situation. Data are means ± S.E N=6. Codes are fungal treatment (Table 1).

*Table 5: ANOVA results of effects of treatment (four fungal plugs) on the biomass of both A. odoratum and L. vulgare. Data have been ln-transformed prior to analysis. Df= degrees of freedom. P-values are indicated with: * <0.05, **< 0.01, ***< 0.001, $< 0.1, N.S.> 0.1.*

	Df	Ao Root F-value	Ao Shoot F-value	Df	Lv Root F-value	Lv Shoot F-value
Treatment	7	4.5 ***	3.6 **	7	6.9 ***	5.4 ***
Error	33			34		

Separation assay

The indirect effects of the different fungal species on the biomass production of both *A. odoratum* (Fig. 6) and *L. vulgare* (Fig. 7) were tested in the separation experiment. Treatment in overall had a significant effect (F-value: 19.9***) (Table 4). In general both plant species produced more biomass when a fungus was present. For *A. odoratum* this resulted in an increase in root biomass of average 2.7 compared to control and in shoot biomass varying on average from 1.5 to 2 compared to the control plants. For *L. vulgare* the increase in root biomass was around 2.7 compared to control and the increase in shoot biomass on average 2.2 compared to the control plants.

Only *C. udugawae* (F1) did not have a significant effect on the biomass of both species and *G. incrustans* (F9) did not have a significant effect on the biomass of *L. vulgare.* So the effect of *G. incrustans* seems to be host-specific.

Pictures were taken of some of the treatments of both plant species (Fig. 17 & 18). These pictures show a clear increase of biomass in all cases. Both root and shoot biomass of both plants were increased when the fungi were present. The treatment effect was significant on both root and shoot biomass of the two plant species (*Table 5).*

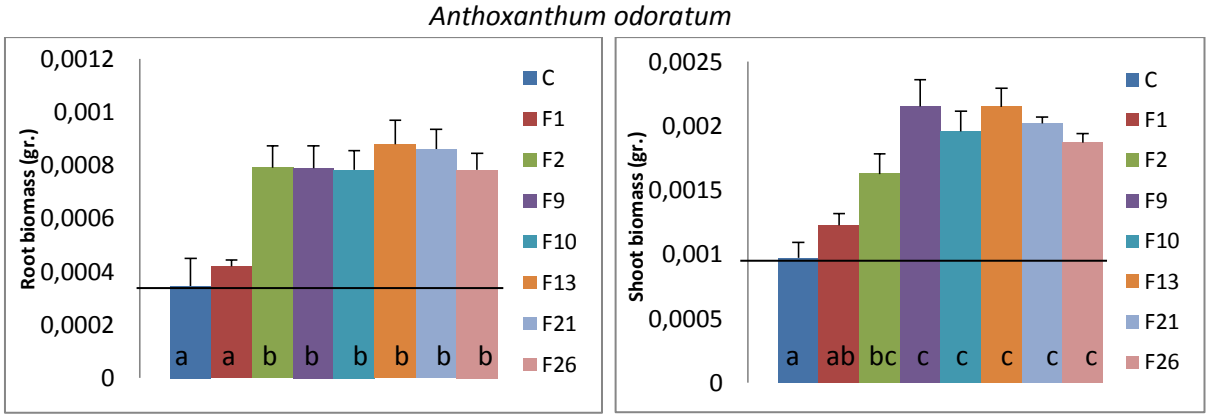


Figure 6: Mean dryweight of the roots (left) and shoots (right) of the Anthoxanthum odoratum plants grown on 0.5 MS agar with the fungi growing separately on 0.5 MS + sucrose. The line indicates the mean biomass of the plant species grown under the control situation. Data are means ± S.E., N=6. Codes are fungal treatment (Table 1).

Leucanthemum vulgare

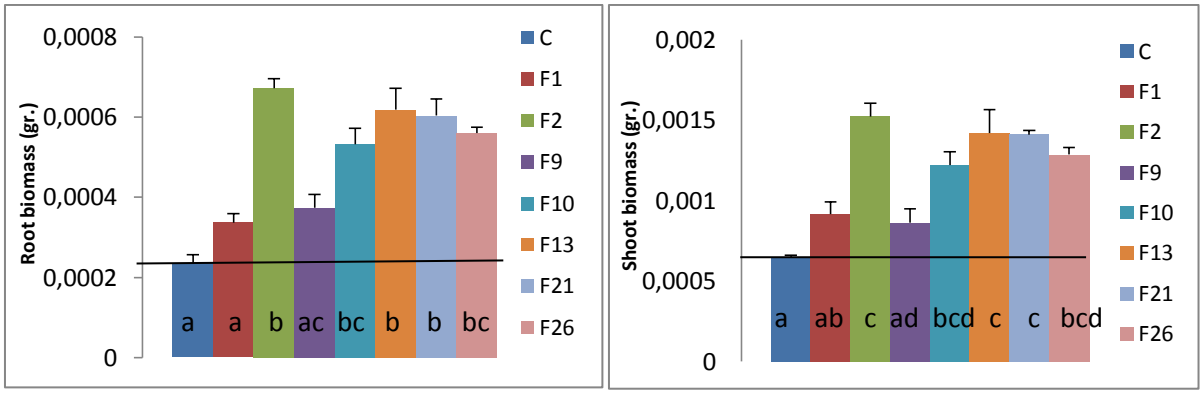


Figure 7 : Mean dryweight of the roots (left) and shoots (right) of the Leucanthemum vulgare plants grown on 0.5 MS agar with the fungi growing separately on 0.5 MS + sucrose. The line indicates the mean biomass of the plant species grown under the control situation. Data are means ± S.E., N=6 for F1, F2, F10 and F13; N=4 for F21, F26 and N=3 for C, F9. Codes are fungal treatment (table 1)

*Table 6: ANOVA results of effects of treatment (fungus grown separately of plant) on the biomass of both A. odoratum and L. vulgare. Data have been ln-transformed for A. odoratum and sqrt-tranformed for L. vulgare prior to analysis. Df= degrees of freedom. P-values are indicated with: * <0.05, **< 0.01, ***< 0.001, $< 0.1, N.S.> 0.1.*

	Df	Ao Root F-value	Ao Shoot F-value	Df	Lv Root F-value	Lv Shoot F-value
Treatment	7	4.5 ***	3.6 **	7	6.9 ***	5.4 ***
Error	33			34		

"Big" plate assay

The indirect effect of the conditioned soil, containing species specific soil biota, on the growth of both plant species was investigated in the "big" plate assay. Neither the biomass of *A. odoratum* nor *L. vulgare* was influenced by the soil types compared to the control. However there was an effect of treatment for the roots of *L.vulgare* (F-value: 4.313; P-value: 0.018) and shoots (F-value: 3.612; P-value: 0.030). This effect was found when comparing the sterilized conditioned soil of *Leucanthemum vulgare (*Lvst) to the non-sterilized conditioned soil (LvNS) (P-value: 0.010).

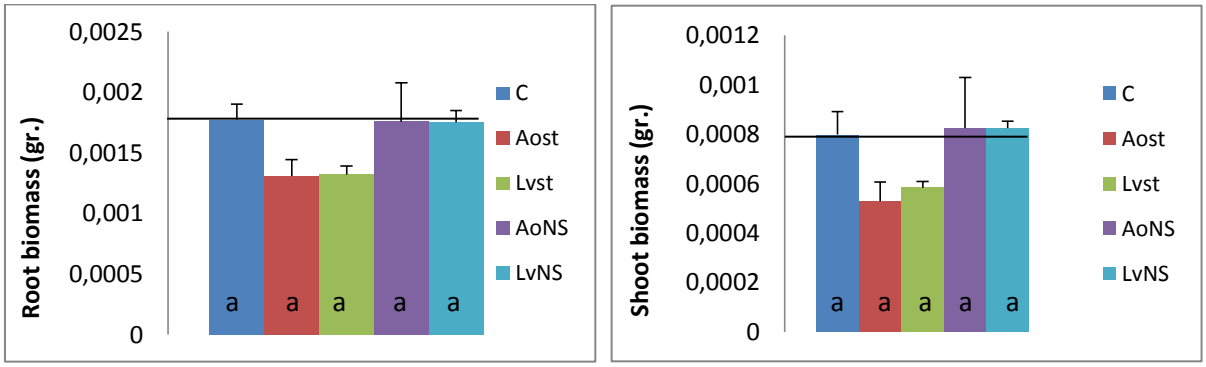


Figure 8: Mean dryweight of Anthoxanhum odoratum plants grown on 0.5 MS agar, separated from a round petridish filled with the different soil types (C: no soil, Aost: conditioned soil of monocultures of A. odoratum, sterilized using gamma radiation, Lvst: conditioned soil of monocultures of L. vulgare, sterilized using gamma radiation, AoNS: conditioned soil of monocultures of A. odoratum, not sterilized and LvNS: conditioned soil of monocultures of L. vulgare, not sterilized).The line indicates the mean biomass of the plant species grown under the control situation.Data are means ± S.E. N=4.

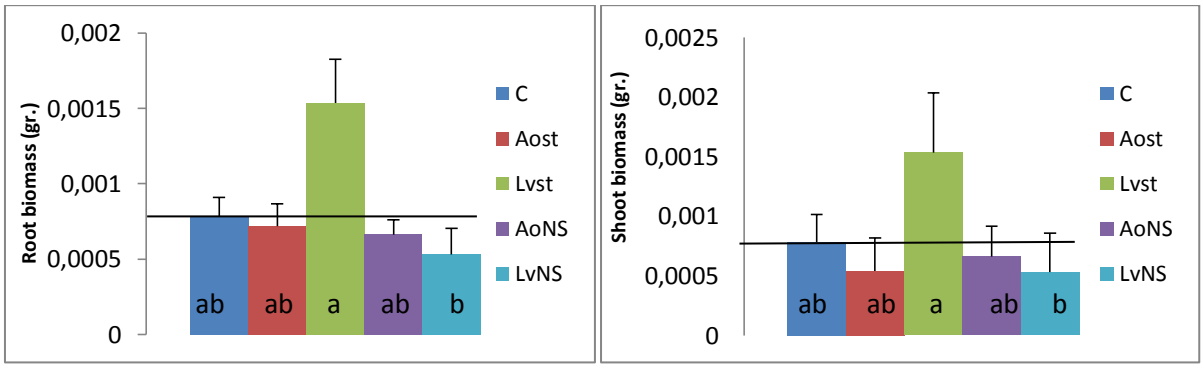


Figure 9: Mean dryweight of roots (a) and shoots (b) of Leucanthemum vulgare plants grown on 0.5 MS agar, separated from a round petridish filled with the different soil types (codes are according to Table 1) The line indicates the mean biomass of the plant species grown under the control situation. Data are means ± S.E. N=4.

*Table 6: ANOVA results of the indirect interactions of treatment (soil type (Figure7) on the biomass of both A. odoratum and L. Vulgare. Data have been ln-transformed prior to analysis. Df= degrees of freedom. P-values are indicated with: * <0.05, **< 0.01, ***< 0.001, $< 0.1, N.S.> 0.1.*

	Df	Ao Root F-value	Ao Shoot F-value	Df	Lv Root F-value	Lv Shoot F-value
Treatment	4	2.0 N.S.	2.2 N.S.	4	4.3 *	3.6 *
Error	15			15		

3. Ethylene measurements

A large variation in ethylene production between the fungi was found, varying between 4.6*10^3 ppb to 2.7 ppb. Most fungi produced large amounts of ethylene. Only *Penicillium ochrochloron* (F6), *Gaeumannomyces cylindrisporus* (F10), *Fusarium oxysporum* (F13), *Mortierella minutissima* (F18), *Chaetomium coarctatum* (F19), *Pyrenochaeta lycopersici* (F24) did not differ significantly from the control concentration . The highest concentration of ethylene (mean: 4.6*10^3 ppb) was found with *C. udugawae* (F1). Of the fungi used in this project only *G. cylindrisporus* (F10) (P-value: 0.955) and *F. oxysporum* (F13) (P-value of 0.196) did not produce ethylene. These measurements were performed for different media (MS-sucrose and 1/5 PDA) as well. However, these measurements were not used since the variation between the measurements was very high and no fungi were found to produce ethylene. According to other studies (Lynch and Harper 1976; Graham and Linderman 1980) fungi need sugar in their medium to produce ethylene. Some of the fungi grew fast on MS-medium whereas others stayed small. However, there was no correlation between the size of the fungus and production of ethylene. The conditioned soil types, both sterilized and non-sterilized, were used to measure the concentrations of ethylene excreted by specific soil biota. However no production of ethylene was found for any of the soil types.

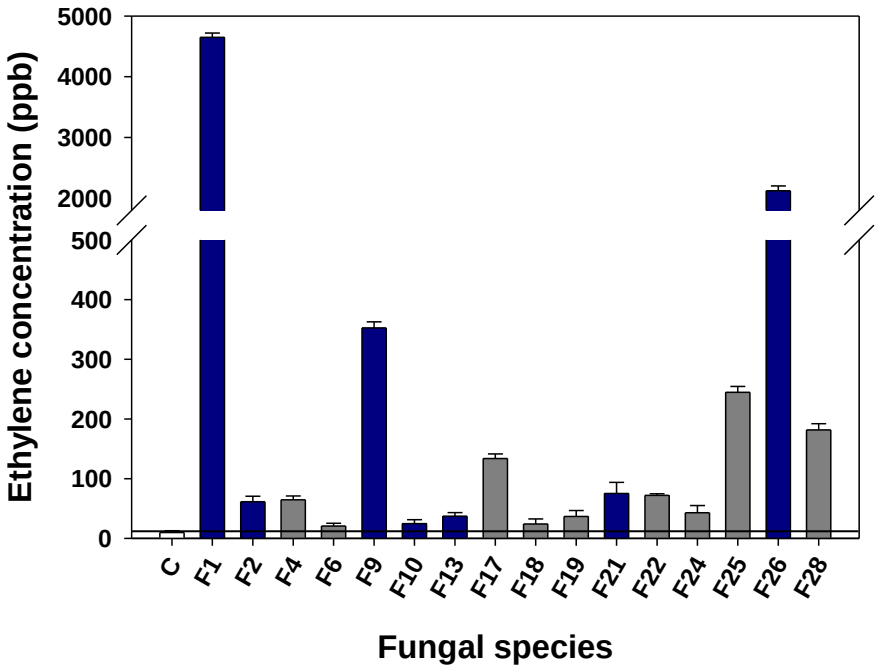


Figure 10: Mean concentration ethylene (ppb) produced by all fungal isolates grown on 0.5MS with sucrose. As a control a plug of 1/5 PDA was used. The blue bars indicate the used species in this report. The line indicates the mean biomass of the plant species grown under the control situation. Data are means + S.E.

In order to test the response of both plant species on ethylene, the plants were grown under exposure of different concentrations of ethylene. Neither *A. odoratum* (Fig. 10) nor *L. vulgare* (Fig. 11) showed any significant effects to the ethylene concentrations (*Table 6)*.

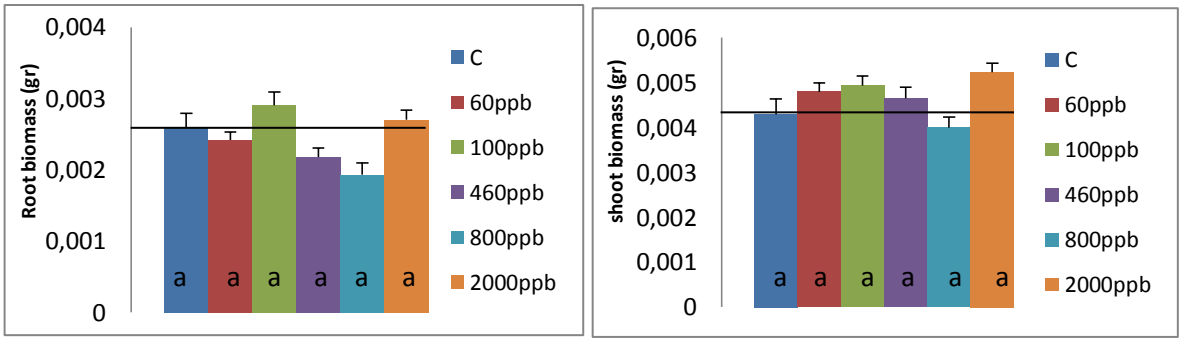


Figure 11 : Mean biomass of roots (left) and shoots (right) of A. odoratum plants, growing under different ethylene concentrations in ppb. The line indicates the mean biomass of the plant species grown under the control situation. Data are means + S.E. N=5 for 100, 800 and 2000 ppb; N=4 for C, 60 and 460 ppb.

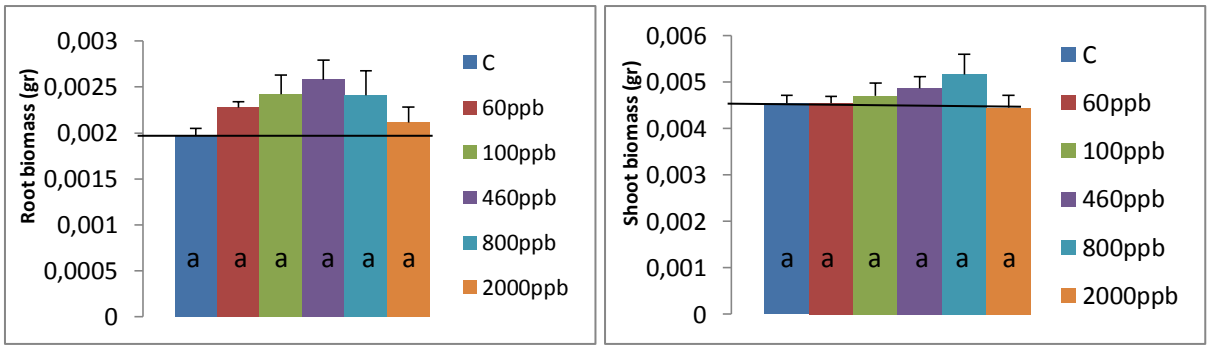


Figure 12: Mean biomass of roots (left) and shoots (right) of L. vulgare plants, growing under different ethylene concentrations in ppb. The line indicates the mean biomass of the plant species grown under the control situation. Data are means + S.E. N=5 for C, 60 and 460 ppb; N=4 for 100, 80 and, 2000 ppb

*Table 6: ANOVA results of both the ethylene concentration measurements and the ethylene growth curve. Data of the ethylene concentrations per fungus have been sqrt-transformed prior to analysis. Data of the ethylene growth curve have been ln-transformed prior to analysis. Df= degrees of freedom. P-values are indicated with: * <0.05, **< 0.01, ***< 0.001, $< 0.1, N.S.> 0.1.*

	Df	Ethylene conc. F-value	Df	Ao Root F-value	Ao Shoot F-value	Df	Lv Root F-value	Lv Shoot F-value
Treatment	16	580.5***	5	1.2 N.S.	0.9 N.S.	5	0.3 N.S.	0.1 N.S.
Error	66		21			21		

Discussion

1. Negative feedback

In both the phytotron experiment of Mommer *et al.* (2010) and the plant-soil feedback experiment of Hendriks *et al.* (in prep) a negative correlation was found between plant productivity and plants growing either in monoculture or on soil conditioned by its own species. These results provide strong support for the pathogen niche hypothesis since Hendriks *et al.* (in prep) showed the deleterious effects disappeared once the soil biota were eliminated through sterilization.

Species–specific response to pathogenic fungi

In the soil assay, seedlings of both plant species were inoculated with each of the seven fungal species to test their direct effects on plant growth. Most of these fungi only induced mild effects, which were not significant compared to the control. However, two fungi infected the plant species and significantly reduced biomass compared to the control. In overall there was an interaction found between the effects of fungal treatment and the plant species, indicating host-specific effects (F-value$_{root}$ 10.9 ***, F-value$_{shoot}$ 16.1***) (Table 2). This interaction has been confirmed for the two pathogens found in this assay. Neither *A. odoratum* plants inoculated with *P. chrysanthemicola* (F2) nor *L. vulgare* plants inoculated with *G. incrustans* (F9) showed any reduction in biomass compared to the control plants. From earlier experiments we know that *L. vulgare* is strongly inhibited when grown in monoculture or on soil conditioned by conspecifics (van Ruijven *et al.* 2003; Mommer *et al*. 2010; Hendriks *et al.* in prep). Based on the results of the soil assay we can confirm the pathogen hypothesis for *L. vulgare.* Half of the plants, which were inoculated with *P. chrysanthemicola* (F2) one day after planting, died. Besides an significant reduction of total biomass of 2.2 was found and differentiating between the two different times of inoculation a reduction of 4.7 was found on t=1 between the plants inoculated with *P. chrysanthemicola* (F2) and the control plants. *P. chrysanthemicola* (F2) was isolated from the root tips of *L. vulgare* and is therefore a prime candidate for the observed negative plant-soil feedback. Biomass of *A. odoratum* was less reduced by negative plant-soil feedback than biomass of *L. vulgare* (Hendriks *et al.* in prep). Biomass of *A. odoratum* was the lowest when grown on soil

of conspecifics or on the soil of *Festuca rubra,* another grass species, suggesting the involvement of grass specific pathogens (Hendriks *et al.* in prep; Petermann *et al*. 2008; Harrison and Bardgett (2010). This hypothesis has been confirmed since biomass of *A. odoratum* was significantly reduced when inoculated with *G. incrustans* (F9)*,* a fungal isolate from *F. rubra.* Total biomass was reduced by 1.3 compared to the control plants..

Influence of plant age on pathogenicity

In the soil assay we differentiated between two different times of inoculation. Pathogens are known to infect plant species at different life stages and the impact of the disease depends on both the life stage of the plant that is attacked and the life history of the pathogen (Gilbert 2002). In this experiment we found the time of inoculation to be important. In overall the biomass of both plants species that were inoculated with fungi was lower on t=1 (inoculated one day after planting) compared to t=2 (inoculated one week after planting). The pathogenicity of both *P. chrysanthemicola* (F2) on *L. vulgare* (Fig. 14) and *G. incrustans* (F9) on *A. odoratum* was reduced as well. On t=1 half of the *L. vulgare* plants inoculated with *P. chrysanthemicola* (F2) died, whereas on t=2 only one of the 15 plants showed clear symptoms of disease. The differences in productivity between the two different times of inoculation can be explained by several factors. The plants that were inoculated at t=2 were seven days older than the first series, and therefore, larger, resulting in increased defence against pathogen attack. Uptake of nutrients will have been easier since their root system had expanded compared to the plants that were inoculated one day after they were transferred to their new environment. Furthermore this transportation could have caused stress for the young seedlings and stress may have reduced their defence mechanisms, causing a high infection and mortality rate. Finally, the plants of the second series were infected one week less, which could have decreased the chance of observing growth reduction.

However in the field it is likely that the infection propagules of the fungi are present in the soil and might already start infecting the plant at seed germination. The highest rates of disease-related mortality of plants in natural ecosystems are usually due to seed and seedling diseases (Gilbert 2002).

Conclusion

The results of the biodiversity experiment of Mommer *et al.* (2010) and the plant-soil feedback experiment of Hendriks *et al.* (in prep) are consistent with the results found in this study. For both plant species, different pathogenic fungi have been identified which could have caused the reduction of biomass found in the respective monocultures. The found pathogenic effects of *P. chrysanthemicola* (F2) on *L. vulgare* were stronger than the effects of *G. incrustans* (F9) on *A.odoratum*. This correlates with the previous studies (Hendriks *et al.* in prep, Mommer *et al.* 2010) where the reduction of biomass of monocultures compared to mixtures was most profound in *L. vulgare.*

However, in this experiment we only looked at the direct interaction between one plant species and one fungal species. In the field or in biodiversity experiments the reduction is caused by negative feedback of the soil, containing a blend of different fungi, bacteria and nematodes. For future work it would therefore be interesting to quantify the abundance of the identified pathogenic fungi in preconditioned soil and roots of the monocultures and mixtures. If the observed negative plant-soil feedback is indeed caused by an accumulation of species-specific pathogens in monocultures it would mean that the abundance of the pathogen should be higher in monocultures compared to the abundance in mixtures.

2. Positive feedback

The phytotron experiment of Mommer *et al.* (2010) showed that overyielding of *A. odoratum* in particular occurred when the four plant species were grown in mixtures. In the plant-soil feedback experiment of Hendriks *et al.* (in prep) *A. odoratum* was found to overproduce the roots when grown on soil conditioned by *L. vulgare* (3 times more than on its 'own' soil). *L. vulgare* was found to produce 2 times more on soil conditioned by *A. odoratum* than on its 'own' soil. This led to the idea that overyielding could partly be caused by positive plant-soil feedback.

Direct interaction

In the soil assay, some positive effects of the fungi on biomass of both plant species were indeed observed (Fig. 2 & 3). Although none of these positive plant-fungal interactions were significant, it is an indication that fungi are able to positively influence productivity of plant species. Furthermore it appeared that these positive interactions are species-specific and

depended of the time of inoculation. Root biomass of *A. odoratum* plants inoculated on t=2 with *P. cucumerina* (F26) was enhanced, although not significantly, while only poor positive effects of *P. cucumerina* (F26) on *L. vulgare* are found with the plants inoculated on t=1 (Fig. 2 & 3).

Shoot biomass of *A. odoratum* on t=2 was highest with the plants inoculated with *P. chrysanthemicola* (F2), a fungus isolated from the monoculture of *L. vulgare.* This positive interaction might be the first clue to understanding the positive influence of the soil biota of conditioned soil of *L. vulgare* on the biomass production of *A. odoratum.*

However, the soil assay was carried out in a short period of time (23 days) and the positive interactions might have become more profound after a longer time period.

The direct interaction assay on agar plates (Fig. 4 & 5) gave completely different results than the direct interaction investigated in the soil assay (Fig. 2 & 3). On the plates, total biomass of both plant species were always significantly increased when the fungi, also the specific pathogens, were present compared to the biomass of the control plants.

The positive effects on agar posed questions on how the fungi enhanced growth of the plant species. Was it through direct or indirect interaction via producing volatiles?

Indirect interaction

When the fungi were in a compartment separate from the plants, growth promotion was observed for the *A. odoratum* plants in combination with all fungi except *C. udugawae* (F1) and for the *L. vulgare* plants with all fungi except both *C. udugawae* (F1) and *G. incrustans* (F9). Therefore we can conclude that the fungi are able to indirectly influence plant growth by excreting certain volatiles, which stimulate the plants to grow better.

A striking observation is the effects of *G. incrustans* (F9) on *A. odoratum*. In the soil assay it was identified to be pathogenic on *A. odoratum* (Fig. 2). However in the separation assay *G. incrustans* (F9) is found to be, especially for the shoot biomass, one of the most growth promoting fungus on *A. odoratum*. Same results were found for *L. vulgare* and *P. chrysanthemicola* (F2).

Volatiles

In the separation assay it was shown that fungi can release volatiles that influence plant growth. Some bacteria (especially *Pseudomonas fluorescens)* are well known to produce

plant growth-promoting volatiles (Ryu *et al.* 2003; Schulz and Dikschat 2007). The effects of these compounds have been tested using compartmental petri dish assays with plants on one side and bacteria on the other side, and found to both promote and reduce the growth of the plants (Kai *et al.*2009; Blom *et al.* 2011). Fungi are known to produce a wide range of volatile compounds as well but their effects on plant growth are still to be further investigated (Wenke *et al.* 2011). Some endophytic fungi were found to promote plant performance indirectly by producing a wide range of antimicrobial volatiles of which many were lethal to both pathogenic bacteria and fungi (Strobel *et al*. 2001; Ezra *et al*. 2004). Other fungi are found to directly influence plant growth by either releasing general compounds as planthormones, such as ethylene (Splivallo *et* al. 2009) or species specific compounds (Menotta *et al.* 2004).

From the results of the separation assay it appeared that most fungi had a positive effect on plant growth; total biomass was increased (on average 2 times) and their phenotype was altered resulting in more lateral roots, more and longer root hairs and bigger, darker green leaves. These observations raised the idea that it could be a general volatile rather than a very specific volatile or different volatiles with the same effect. Based on the phenotypical traits of the plants in the separation assay; ethylene was thought to be a prime candidate. Ethylene is a plant hormone which can stimulate plant growth in low concentration, but is deleterious in higher concentrations (Pierik *et al*. 2006).

However a few results contradict the idea of a general volatile. Firsty, *C. udugawae* (F1) was the only fungus, of the seven used in this thesis, that did not have significant effects on both plant species in the separation assay. If the volatiles the fungi are excreting would be something general, the effects of the fungal treatments would be the same for every fungus on both plant species. A possible explanation for this effect could be that all fungi do not excrete the same volatile so the effects would be fungal specific. Or the quantity of the excreted volatile differs between the fungal species, leading to a different response of the plant species to fungal treatment.

Secondly, the effects of *G. incrustans* (F9) on plant productivity differ between the two plant species. Both root and shoot biomass of the *A. odoratum* plants is significantly increased, compared to the control plants, when *G. incrustans* (F9) is present (P-value: 0.000 for both root and shoot). However, neither root nor shoot biomass of *L. vulgare* was significantly increased when *G. incrustans* (F9) was present compared to the control plants (P-value$_{root}$:

0.140; P-value$_{shoot}$: 0.758). If all the fungi would excrete the same volatile the plant species specific response would be the same for all fungal treatments. However in this case the plant specific response differs for only one of the seven fungi.

Ethylene

Ethylene was a prime candidate for our research. Fungi are known to produce phytohormones like cytokines, indole-3-acetic acid (IAA) (Tan and Zou 2001; Römmert *et al*. 2002). In 1968 Llag and Curtis found a wide range of fungi to be able of producing ethylene as well. An increase in ethylene production is often observed during interaction between a host and a pathogen (Abeles *et al*. 1991). Ethylene is associated with both the induction of the defence system of the plant and in the development of disease symptoms (Boller 1991). However, the highest concentration of ethylene was found for *C. udugawae* (F1) which was the only fungus in the separation assay which had no significant effects on the biomass of both plant species (Fig. 6 & 7).

This falsified the idea that ethylene was responsible for the observed plant growth promotion. An additional experiment was executed in which an ethylene dosis response curve was produced (Fig. 11 & 12).

The 5 different concentrations of ethylene had no effect on the biomass production of both plant species. *A. odoratum* showed no response, but there were some differences observed in the phenotypes of *L. vulgare* (Fig. 20), concerning the angle and length of the leaves and the density and length of root hairs.

Therefore we can conclude that ethylene is not responsible for the observed fungal induced growth promotion.

As another volatile candidate carbon dioxide has been brought up. More carbon dioxide will increase plant productivity, at least in the short term (Rogers *et al.* 1994; Hungate *et al.* 1997). Future work will investigate the role of fungal CO_2 for plant growth.

Conclusion

Our data suggest, that fungi are able to positively influence plant growth, either directly or indirectly. However, in these experiments we looked at the individual responses of plant species to fungal species. In order to further investigate the mechanisms of positive plant-soil feedback, it is important to look at the interactions between the soil biota as well. Both

bacteria and fungi are known to have antagonistic effects against plant pathogens (Campanile *et al.*; Strobel *et al.* 2001; Ezra *et al.* 2004, Suarez-Estrella *et al.* 2007). For futher research it would be interesting to test the antagonistic effects of the soil biota on the plant pathogens. A possible set up to test these effects would be to grow both plant species on the conditioned soil of the other species, and then inoculate both plant species with the species-specific pathogen. For example, *L. vulgare* would be planted on the soil conditioned by *A. odoratum* and inoculated with *P. chrysanthemicola*. If the species-specific soil biota of *A. odoratum* is antagonistic to pathogen attack of *P. chrysanthemicola*, the reduction of biomass would be less on the conditioned soil of *A. odoratum* compared to the biomass of the inoculated plants on sterile soil.

3. Summary

As a final conclusion my thesis results suggest that endophytic fungi can both negatively and positively influence plant productivity. For both *L. vulgare* and *A. odoratum* we identified two species-specific pathogens which may be responsible or at least in part for the reduced plant productivity in different monocultures. The data for enhanced productivity of plants species found in mixtures are less clear, but suggest that fungi are able to promote plant productivity, since this growth promotion was observed when the plant species were grown separately from the fungi on agar. The precise volatiles responsible for this growth promotion have not been identified yet.

We can conclude that the results of this thesis provide more insight in the micro-organisms responsible for plant-soil feedback in biodiversity studies. As a first step, the interactions between the individual plant species and endophytic fungi have been studied here. Future work will scale up the complexity between fungi and plants roots in soil.

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Appendix 1.

1. Soil assay

Figure 13: Pictures taken from the soil assay of A. odoratum; control (left), G. incrustans (middle) and C. cucumerina (right)

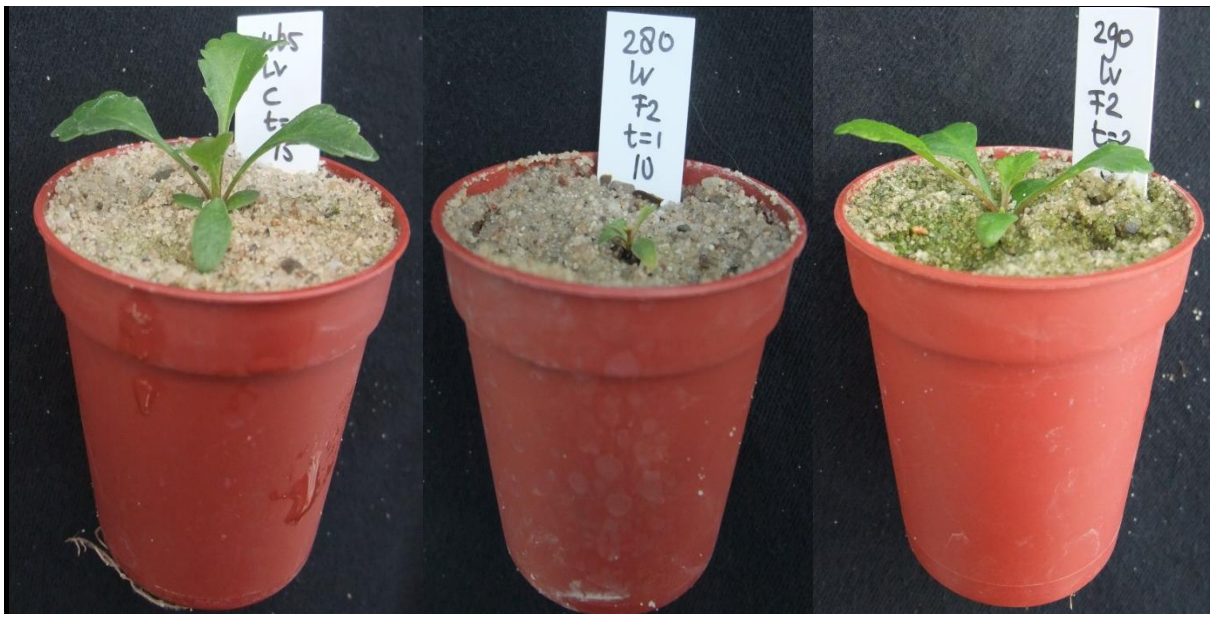

Figure 14: Pictures taken from the soil assay of A. odoratum; control (left), P. chrysanthemicola t=1(middle) and P. chrysanthemicola t=2 (right)

2. Plate assays

Infection assay

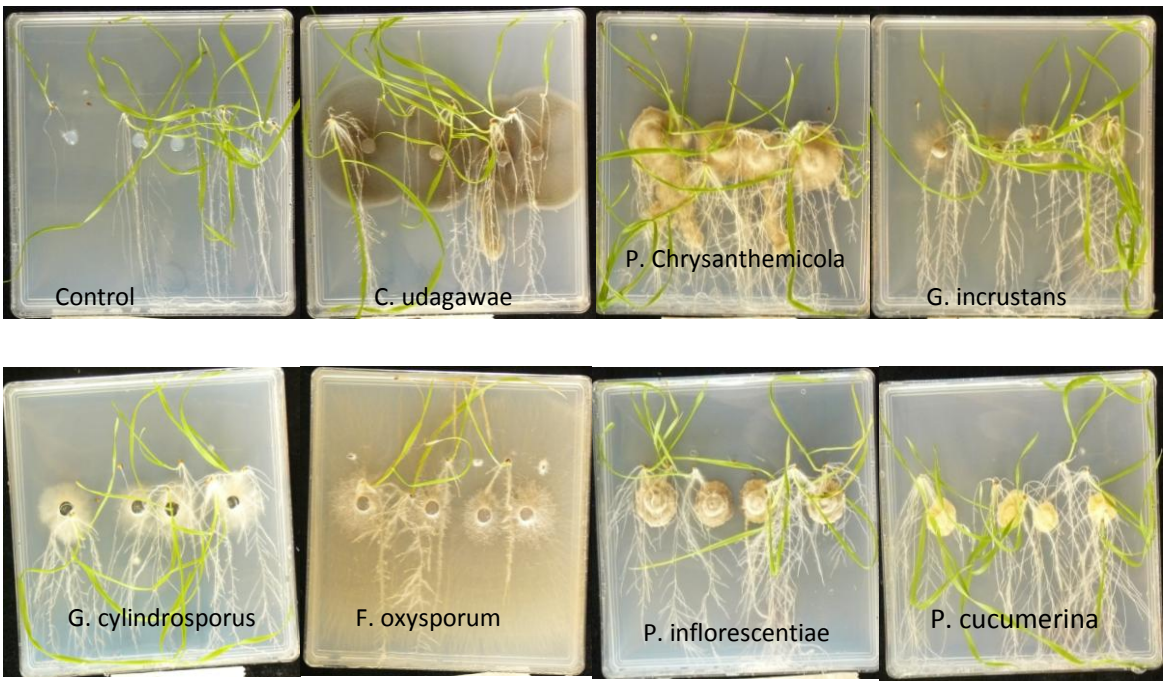

Figure 15: Pictures taken from the infection experiment of the A. odoratum plants treated with different fungal species.

Figure 16:Pictures taken from the infection experiment of the L. vulgare plants treated with different fungal species. Codes are fungal treatment (Table 1).

Seperation assay

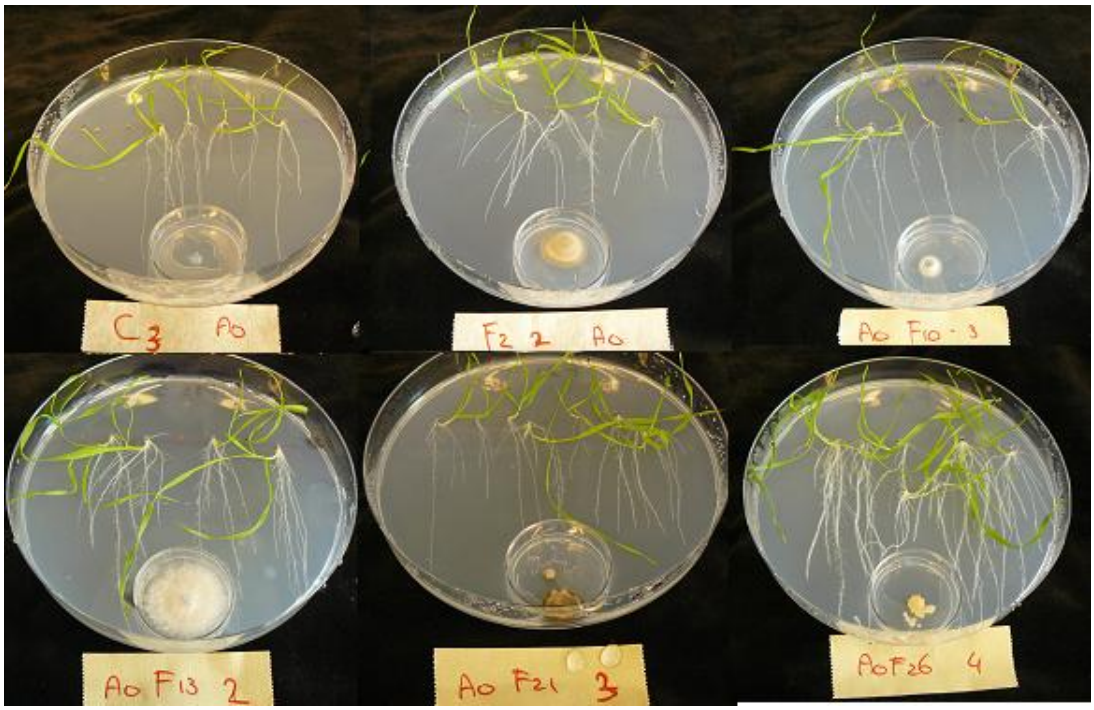

Figure 17: Pictures taken from the separation experiment of the A. odoratum plants separated from the different fungi (From left to right: Control, F2, F10, F13, F21, F26). Codes are fungal treatment (Table 1)

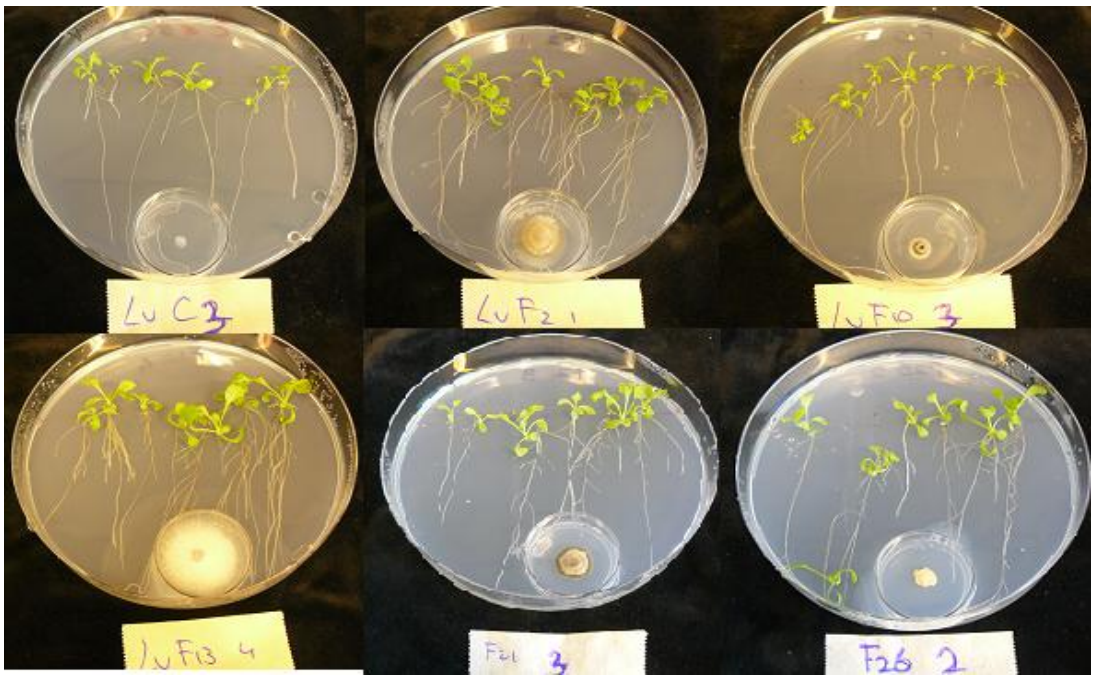

Figure 18: Pictures taken from the separation experiment of the L. vulgare plants separated from the different fungi (From left to right: Control, F2, F10, F13, F21, F26). Codes are fungal treatment (Table 1)

"Big" plate assay

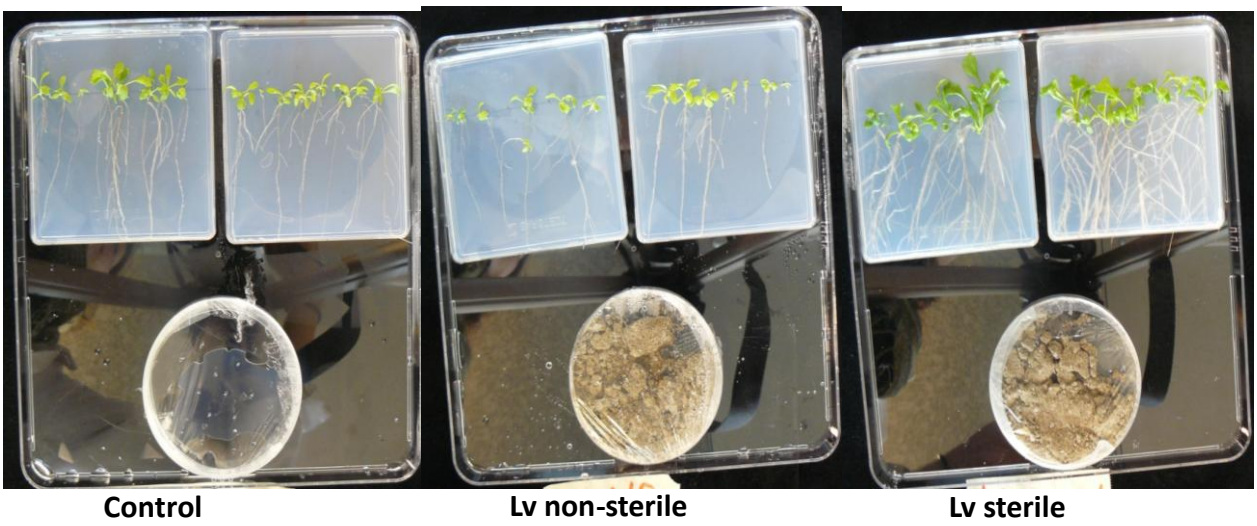

Figure 19: Pictures taken of the "big" plate assay of Leucanthemum vulgare.

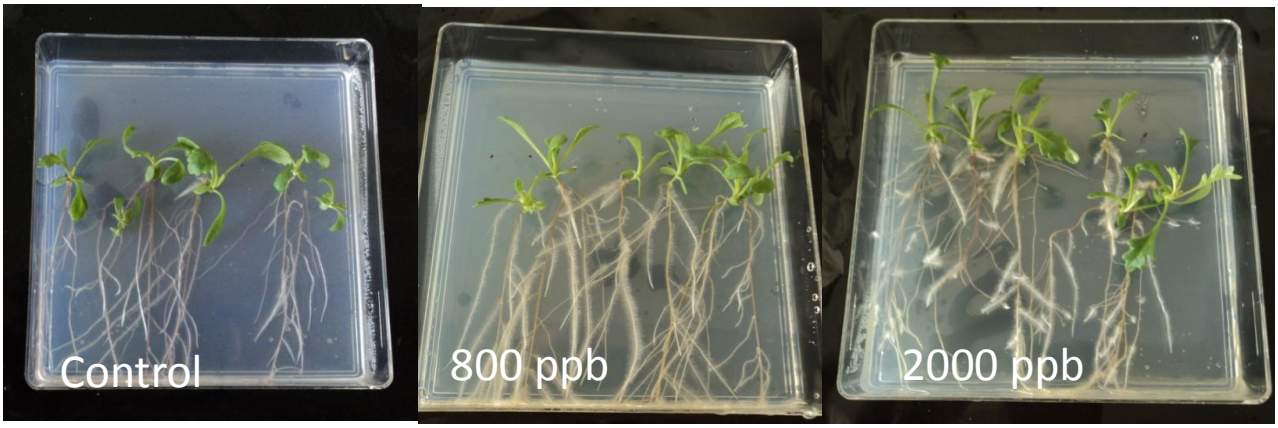

Figure 20: pictures taken from the L. vulgare plants grown under different concentration of ethylene.

Appendix 2.

Separation assay trial

The separation experiment was first performed with only five of the seven fungi which were later on chosen to be investigated in this project (Fig 20 & 21). The results from the 'trial' experiment are shown below. Due to infection (the seeds of *A. odoratum* were not sufficiently sterilized) some plates had to be thrown away, other contained small opportunistic fungi on the plates and these might have been able to influence the plant growth as well. Still some results of this trial correlate to the results found on the final separation experiment (Fig. 5 &6). Most fungi promote the growth of both plant species and *P. chrysanthemicola* is causing the highest promotion for *L. vulgare*.

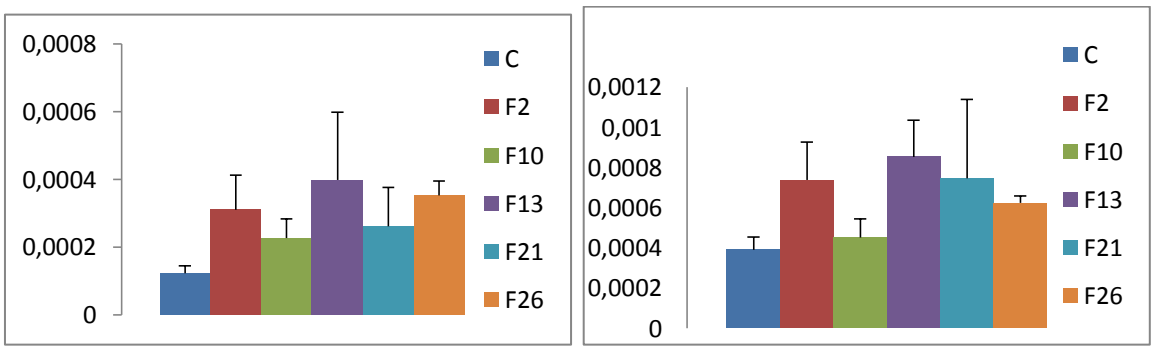

Figure 21: Mean biomass of the roots (left) and shoot (right) of A. odoratum plants grown on 0.5 MS agar with the fungi growing separately on 0.5 MS + sucrose. Data are means + S.E., N=3, except for C & F26: N=4. Codes are fungal treatment (Table 1).

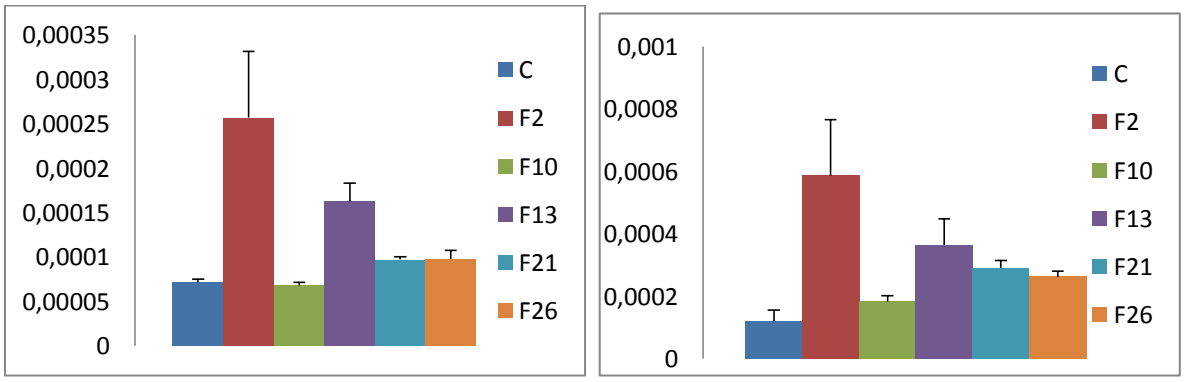

Figure 22 : Mean biomass of the roots (left) and shoot (right) of L. vulgare plants grown on 0.5 MS agar with the fungi growing separately on 0.5 MS + sucrose. Data are means + S.E., N=4. Codes are fungal treatment (Table 1).

'Double' separation assay

Since *Fusarium oxysporum* (F13) grows very fast and in some cases the hyphae grew out of the small petridish and infected the agar underneath on which the plants were growing, we decided to physically separate the fungi and plants more. So in this experiment the plants were grown on 0.5MS without sugar on a smaller petridish (92 x 16 mm) which was glued with agar in the bigger round petridish (145 x 20 mm) together with the small petridish (35 x 10mm) containing 0.5 MS+ sucrose and a fungal plug. However the growth of especially *A. odoratum* was inhibited by the lack of space in the smaller petridish. Furthermore kanamycin (antibiotic) and delvocid (fungicide) were added to the medium for *A. odoratum* and it seemed the plants suffered from these compounds since the blades were white; nearly all chlorophyll had disappeared from the leaves (Fig 24). Therefore it was decided to leave these results out of the report.

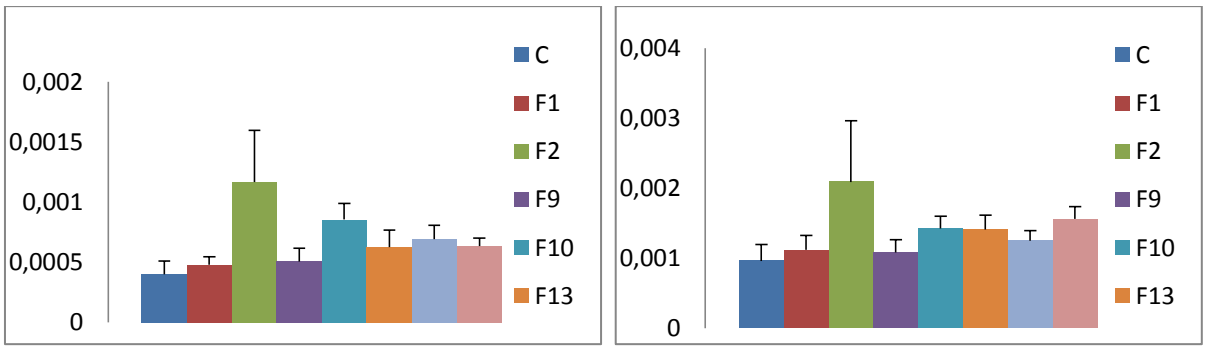

Figure 23: Mean biomass of the roots (left) and shoot (right) of A. odoratum plants grown on 0.5 MS agar (size petridish: 92 x 16mm) with the fungi growing separately on 0.5 MS + sucrose. Data are means + S.E., N=6, except for F1 & F10; N=5. Codes are fungal treatment (Table 1).

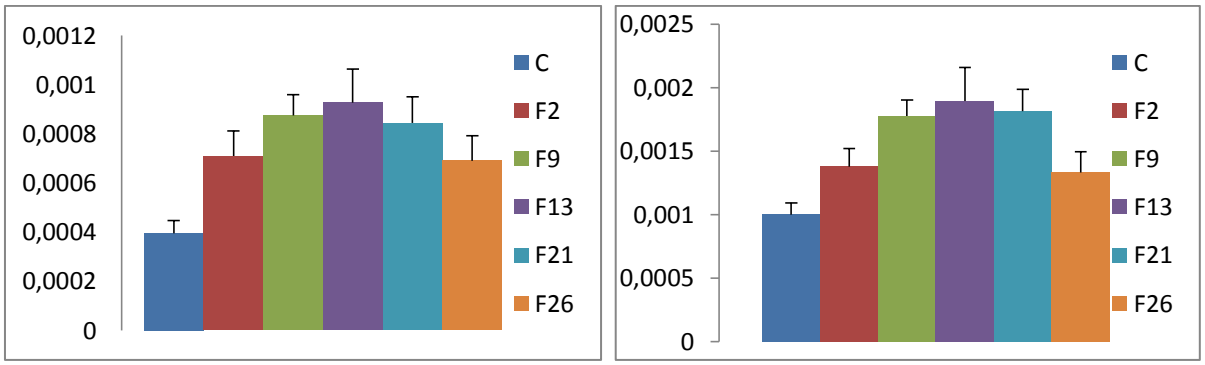

Figure 24: Mean biomass of the roots (left) and shoot (right) of L. vulgare plants grown on 0.5 MS agar (size petridish: 92 x 16mm) with the fungi growing separately on 0.5 MS + sucrose. Data are means + S.E., N=4, except for F13; N=3. Codes are fungal treatment (Table 1).

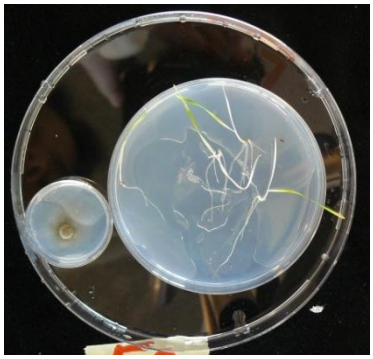

Figure 25: picture taken from one of the plates of the 'double' separation experiment. The blades are coloured white due to the lack of chlorophyll possibly caused by delvocid.

Fungi assay

The conditioned soil of the monocultures of both *A. odoratum* and *L. vulgare* contained a diverse fungal community and its influence on plant growth would not only be directly to the plant, but some effects could have been caused by the interaction between the fungi (Whipps, 1987). To investigate the interactions between our fungal species on 1/5 PDA agar, we grew them together in different combinations. Since *F. oxysporum* (F13) is a very fast grower, we decided to test the effects of that fungus on the other fungi (Fig. 25). *P. Chrysanthemicola* (F2) and *P. inflorescentiae* (F21) are slow growers, so we decided to test the effects of these two fungi on the other fungi (Fig. 26) and as a last test *C. udugawae* (F1), *G. incrustans* (F9), *G. cylindrosporus* (F10) and *P. cucumerina* (F26) were grown together with the target fungus in the middle (Fig. 27). Some of the interactions that stand out is the effect of *G. incrustans* (F9) on the different fungi (Fig. 26 and Fig. 27) show a dark line where F9 comes in contact with the other fungi and as can be seen from Fig. 27, the fungus will grow over *C. udugawae* (F1) and *P. cucumerina* (F26). Another striking feature is the fact that both *P. chrysanthemicola* (F2) and *P. inflorescentiae* (F21) will not touch eachother when grown together (Fig. 26).

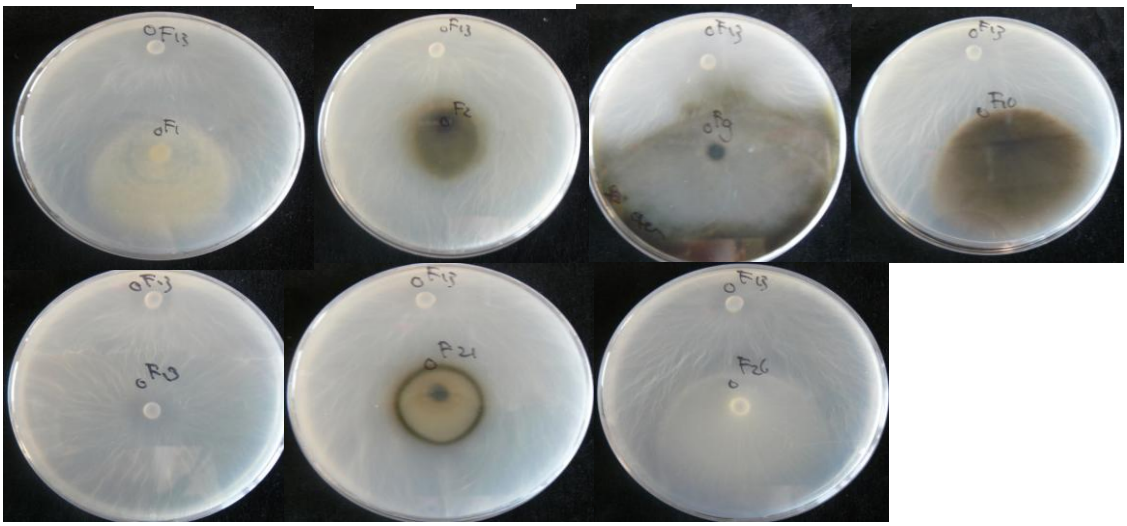

Figure 26: The interactions between F. oxysporum (F13) on the different fungi (from left to right: F1, F2, F9, F10, F13, F21, F26 codes are fungi (Table 1))

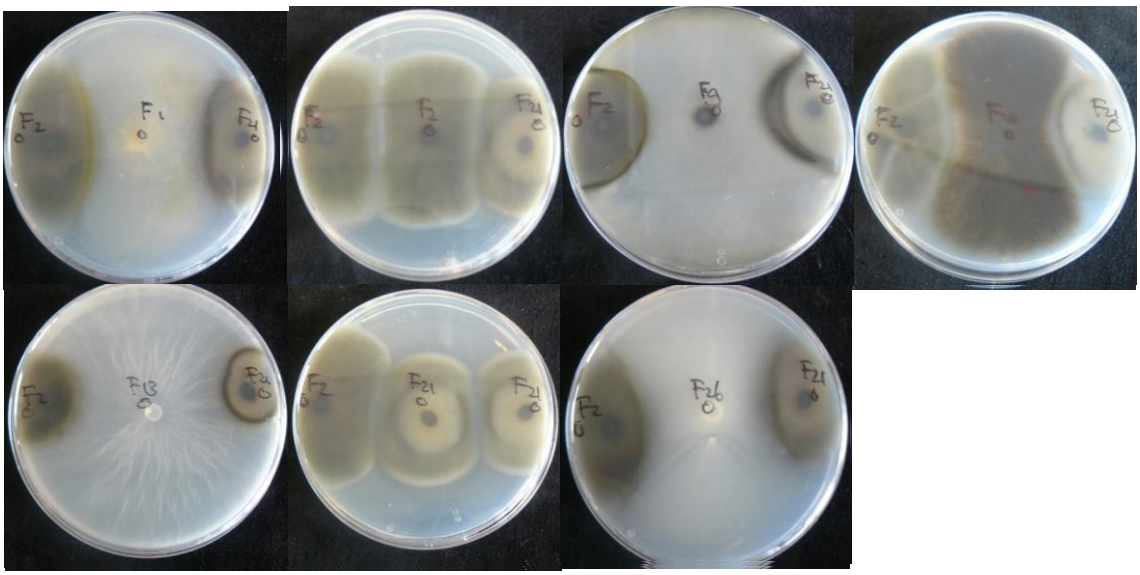

Figure 27: The interactions between P. chrysanthemicola (F2)and P. inflorescentiae (F21) on the different fungi (from left to right: F1, F2, F9, F10, F13, F21, F26 codes are fungi (Table 1))

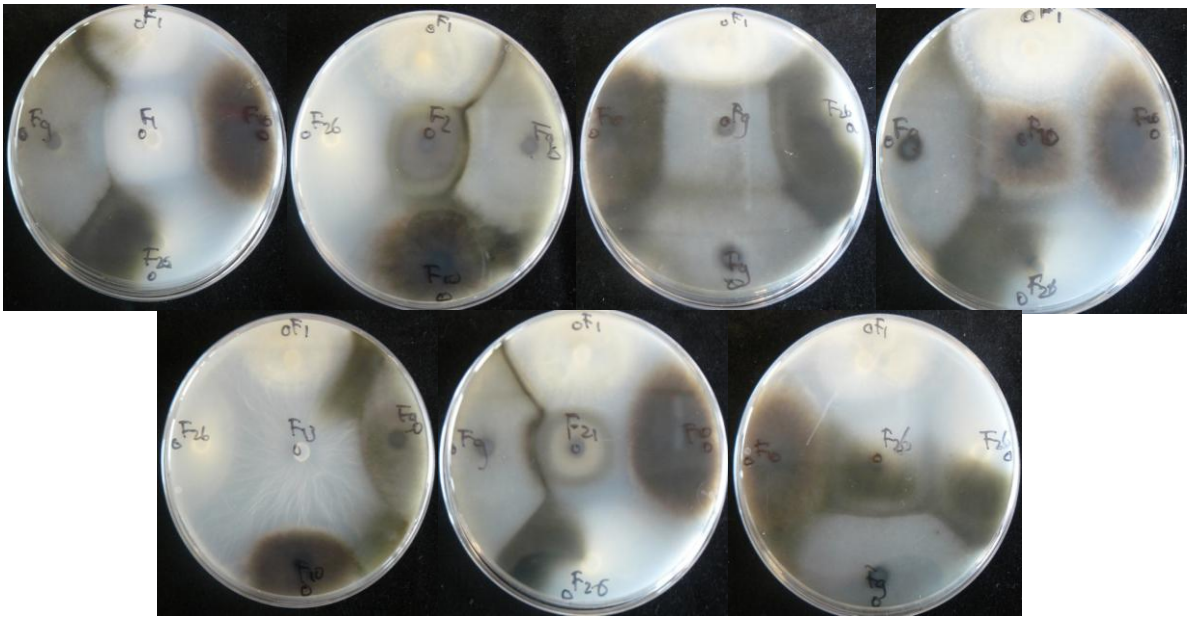

Figure 28: The interactions between C. udugawae (F1), G. incrustans (F9), G. cylindrosporus (F10) and P. cucumerina (F26) on the different fungi (from left to right: F1, F2, F9, F10, F13, F21, F26 codes are fungi (Table 1))