Interactions between plant pathogens and their implications for crop protection.

Ayano Tanaka

Independent project in Biology (15 hp)
Swedish University of Agricultural Sciences, SLU
Department of Forest Mycology and Plant Pathology
Agronom-mark/växt
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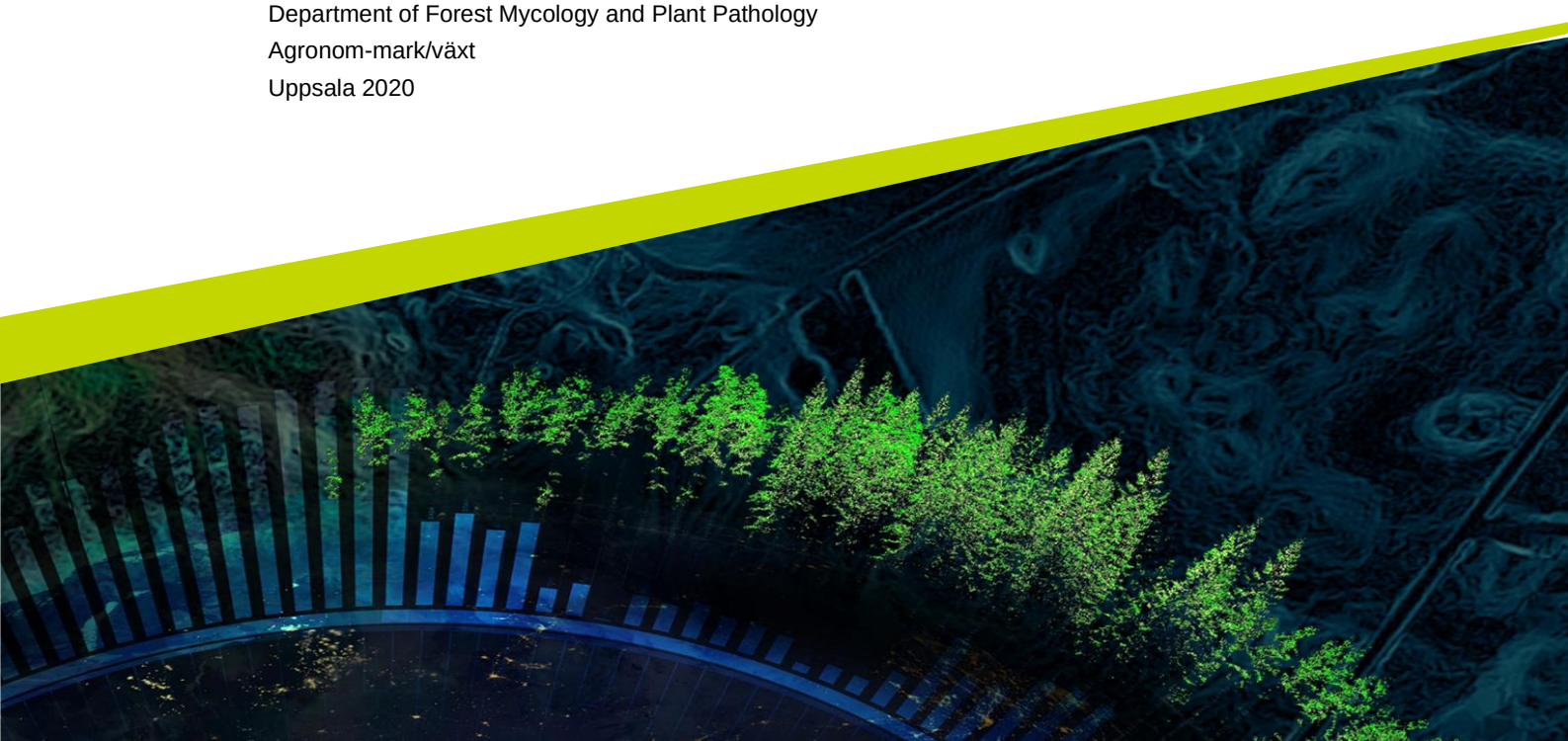

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Ayano Tanaka

Supervisor: **Salim Bourras, SLU, Department of Forest Mycology and Plant Pathology**
Assistant supervisor: Heriberto Vélëz, SLU, Department of Forest Mycology and Plant Pathology
Assistant supervisor: Mukesh Dubey, SLU, Department of Forest Mycology and Plant Pathology
Examiner: Hanna Friberg, SLU, Department of Forest Mycology and Plant Pathology

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Swedish University of Agricultural Sciences
Department of Forest Mycology and Plant Pathology

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Abstract

One plant may often be infected by more than one pathogen species. It is assumed that species which exploit the same resource have the potential to affect each other. Plant diseases where more than one pathogen is involved in the development of infection are usually termed as ¨disease complex¨. Different pathogen species causing similar symptoms on a single host plant species may complicate diagnosis and subsequent control, and besides, the complex interaction may alter the expression of host disease severity. However, the knowledge of multiple-pathogen interactions is still limited, and therefore recent epidemiologic studies have begun to focus on this subject. Pathogen-pathogen interaction varies due to different interaction mechanisms. For example, antagonism where one pathogen has a negative effect on the development of the other. Antagonistic interaction can often be divided into three different mechanisms. First, pathogens produce antimicrobial components, and therefore the development of the other pathogen is supressed. Second, pathogens induce systemic resistance by activating the defence mechanism in the plant-host. Finally, pathogens which out-compete the other pathogens because they are quicker in consuming nutrients and occupying the ecological niches. Another example of multiple-pathogen interactions is synergism, where one pathogen promotes the development of another, and thus it may result in more severe disease symptoms.

The objective of this study was to learn about pathogen-pathogen interactions in plant diseases via literature review and test a method for analysing the interactions *in vitro*, using plant pathogens from the *Fusarium* genus (*F. graminearum*, *F. culmorum* & *F. oxysporum*). The lab result showed that *F. oxysporum*, in the interaction setup, was quicker in consuming nutrients and occupied most of space on PDA at temperature 25°C, whereas *F. culmorum* seemed to be supressed. Hence, *F. oxysporum* seemed like a better competitor toward *F. culmorum* under the *in vitro* conditions tested. Compared to the traditional studies where focus is on only one pathogen, the study of multiple-pathogen interactions has just begun recently, and it is important to establish the experimental methods to understand disease complexes, the synergisms and antagonism in pathogen-pathogen interactions, leading to identification of pathogens in relation to the crop production system. This will require a holistic understanding of how a host responds to co-infection and how pathogens interact and coexist; therefore, this needs to involve the interdisciplinary research collaboration between bacteriologists, mycologists and virologists.

Keywords: Disease complex, Interaction, Antagonism, Synergism and *Fusarium spp*.

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1. Literature review

1.1. Introduction

One plant may often be infected by more than one pathogen species(Fitt *et al.*, 2006). The basic ecological assumption is that species exploiting the same resource have the potential to affect each other (Le May *et al.*, 2008). Plant diseases where more than one pathogen is involved in the development of infection are usually termed as ¨disease complex¨ (Bartoli *et al.*, 2015). disease complexes where different pathogen species cause similar symptoms on a single host plant species, may complicate diagnosis and subsequent control (Le May *et al.*, 2008; Bartoli *et al.*, 2015). However, studies of pathogenic microbes were mostly focused on single host-pathogen pairs until recently (Jesus Junior *et al.*, 2014; Bartoli *et al.*, 2015). The knowledge of pathogen-pathogen and host-multiple-pathogen interactions is scarce, and the combined effects of diseases on crop yield is still poorly understood (Jesus Junior *et al.*, 2014; Bartoli *et al.*, 2015). This complex interaction can alter the expression of plant disease severity. Thus, recent epidemiologic studies have begun to focus on this subject (Abdullah *et al.*, 2017). The structure of disease complexes is influenced by interactions between pathogenic species, host plants and abiotic and biotic environmental factors and therefore, the dynamics of diseases in the field, their severity and effect on yield are also influenced (Fitt *et al.*, 2006; Le May *et al.*, 2008). Therefore, understanding the interaction of multiple pathogen species/genotypes is crucial to develop effective disease control strategies (Bartoli *et al.*, 2015). The objective of this study is to learn about pathogen-pathogen interactions in plant diseases via literature review.

1.2. Material and methods

A literature survey was conducted by utilizing google scholar. The main search terms were 'disease complexes', and `interactions´. To understand the different interactions as `competition´, `coexistence´, 'synergism', and ‘antagonism’, I studied publications about well-known plant pathogens, for example, *Leptoshaeria maculans* and *L. biglobosa,* and *Fusarium spp.*

1.3. Limitation of Koch’s Postulates

During the late 19th century, a bacteriologist Robert Koch formulated a set of rules for confirming that an organism is the cause of a disease, and these rules became known as the ¨Kochs postulates¨ (Agrios, 2005).

1) The organism must be consistently associated with the lesions of the disease.
2) The organism must be isolated from the lesions and grown in pure culture.

3) The organism from pure culture must be re-inoculated into the healthy host and must cause the same disease as was originally observed.
4) The organism must be re-isolated into culture and shown to be identical to the organism originally isolated.

However, some of these postulates are not valid for multiple microbial infections (Bartoli *et al.*, 2015). For example, ¨re-inoculation of the pathogen¨ does not automatically causes disease if synergism is absent. Another postulate defined by Koch was that the pathogen should be isolated from diseased and not from healthy organisms. This is also challenged for plant diseases as for example, Pierce's disease of grapevine, where the bacterial pathogen *Xylella fastidiosa* can take over xylem vessels of plants for a long period of time without showing symptoms. Another problem related to his postulates are the nonculturable pathogens for which none of the Koch's criteria can be fulfilled (Bartoli *et al.*, 2015). The identification of disease associated with multiple pathogenic organisms and other issues suggests that Koch's postulates cannot be applied to all diseases.

1.4. Different types of interactions

1.4.1. Competiton

Pathogen-pathogen interaction varies due to different interaction mechanisms (Jesus Junior *et al.*, 2014; Bartoli *et al.*, 2015). Competition is generally divided into either interference or exploitative competition. These competition types are based on distinct behavioural traits. In an interference competition, one species interacts directly with another species via aggressive behaviour or other antagonistic means. In an exploitative competition, one species interacts indirectly with other species via competition for the the same resources. One species consumes resources more efficiently, thereby depleting the availability of the resource for the other species (Abdullah *et al.*, 2017).

For example, competition for space or nutrients, altered host susceptibility via induced resistance, or toxin production by one pathogen suppressing the development of the other (Le May *et al.*, 2008). Competition under such conditions may lead to selection for the more virulent species, or conversely (Abdullah *et al.*, 2017). Pathogens that can inhibit certain metabolic processes, such as antimicrobial toxin production, when the necessary nutrients are greatly reduced, may have a greater competitive advantage (Glenn *et al.*, 2008; Abdullah *et al.*, 2017). However, these compounds may also provide a competitive benefit to other opportunistic pathogens that do not have to carry the energetic costs for their production (Cornelis and Dingemans, 2013; Ghoul *et al.*, 2014).

A classic example of more aggressive forms of competition between pathogens that includes direct chemical exclusion, is tenuazonic acid secreted by the finger millet colonizing endophyte *Phoma sp.*, which supresses growth of several pathogens including the toxigenic fungus, *Fusarium graminearum* (Mousa *et al.*,

2015, 2016a). Competition can also occur indirectly, facilitated by the plant host through targeted defence mechanism against at least one pathogen (Kamilova *et al.*, 2008; Abdullah *et al.*, 2017).

1.4.2. Coexistence

In contrast to competition, cooperation, whereby pathogens positively interact, by providing mutualistic signals that are crucial for pathogenesis, or by functional complementation through the exchange of resources, which is obligatory for existence. An expected consequence of competition is a localized reduction in microbial diversity and simultaneous specialization of pathogenic microorganisms to various tissues or host species, which is called niche specialization, whereby pathogens can stably coexist through different times, place and resource use (Fitt *et al.*, 2006; Abdullah *et al.*, 2017). As an example, differences in disease onset resulted in temporal separation and stable coexistence between two related fungal pathogens of canola, *Leptoshaeria maculans* and *L. biglobosa* (Abdullah *et al.*, 2017). Niche specialization can reduce the severity of competition between pathogens and allowing coexistence (Fitt *et al.*, 2006), although pathogens separated by various niches within a plant may interact indirectly by stimulating a common host defence response. Nevertheless, on an evolutionary timescale, competition may result in exclusion, enabling species to coexist when arriving at various times (Fitt *et al.*, 2006). The section below shortly explains different types of interaction between pathogens.

1. Antagonistic interaction occurs when one pathogen inhibits or reduces the development of another.
2. Synergistic interaction occurs when there is some enhancement in the development of one or more interacting pathogens.
3. Additive interaction occurs when the development of one pathogen is not altered in the presence of another and vice versa.
4. Mutually exclusive interaction occurs when the development of all involved pathogens is reduced.

In this literature review, antagonistic and synergistic interaction are in focus.

1.5. Antagonistic interaction

Antagonism should make co-occurrence rarer than expected from individual species frequencies, where one pathogen has a negative effect on the development of the other (Le May *et al.*, 2008). Examples are (i) microorganisms which produce antimicrobial components, (ii) microbes which can induce systemic resistance by triggering the defence mechanism of the plant host, and (iii) microbes which can out-compete pathogens because they are quicker in consuming nutrients secreted by the plants and faster in occupying the ecological niches (Kamilova *et al.*, 2008)

Among the most studied endophytes are the antagonistic *F. oxysporum* strain such as *Fo*47 and CS-20, which are used as a biocontrol agent against several plant root pathogens including the pathogenic *F. oxysporum* (de Lamo and Takken, 2020). these antagonistic *F. oxysporum strains* produce antimicrobial compounds that enhance plant resistance to pathogenic *F. oxysporum* (Aimé *et al.*, 2013) However, a study of another antagonistic *F. oxysporum* strain MSA 35 is revealed that the presence of a consortium of ectosymbiotic bacteria belonging to *Serratia*, *Achromobacter*, *Bacillus* and *Stenotrophomonas* genera associated with the *F. oxysporum* strain MSA 35, and the association is crucial for the biocontrol properties of non-pathogenic *F. oxysporum* MSA 35 (Minerdi *et al.*, 2008). Furthermore, when the antagonistic fungus was cured of the associated bacteria, the biocontrol strain became pathogenic. This suggests that the antagonistic effect of *F. oxysporum* seems not a fungal trait, but it is due to the interaction with the ectosymbiotic bacteria (Minerdi *et al.*, 2008).

1.6. Synergistic interaction

Synergism among pathogens should lead to species co-occurrence (i.e., co-inoculation) more frequently than predicted, where one pathogen promotes the development of another (Le May *et al.*, 2008). Reports of the synergistic interaction are relatively rare compared to antagonistic interaction, but it is likely that synergism among different pathogens leads to more severe disease symptoms (Le May *et al.*, 2008). The synergistic interaction may also alter the occurrence and speed of epidemics (Jesus Junior *et al.*, 2014). Moreover, the synergistic interactions may increase crop damage, complicate the identification of the primary cause of diseases and its control. Therefore, understanding their interactions is important because the economic damage threshold for each pest can be significantly lowered by the presence of the interacting organism (Johnson 1990).

One example of synergistic diseases interaction is caused by a group of *Fusarium* species. More than 16 species of the *F. graminearum* species complex have been reported as the causal agent of Fusarium Head Blight (FHB) (Yli-Mattila *et al.*, 2009). Studies from Brazil show that the incidence of the species in FHB varies from one geographic region to another (Del Ponte *et al.*, 2015), yet the current knowledge is very limited regarding mechanisms that explain the geographic variation and occurrence of specific pathogens in plants affected by a particular disease complex. It is possible that such variations are associated to the

ecological preference of these pathogens. Besides, abiotic factors and cultural practice might also influence this variation in pathogen prevalence (Del Ponte *et al.*, 2015).

Another example of a complex disease caused by a group of *Fusarium species* is foot and crown rot on wheat. According to a study of Kuzdralinski *et al.* (2014), four important species of pathogens, *F. graminearum*, *F. culmorum*, *F. poae* and *F. sporotrichioides*, are associated with the disease, though their occurrence differs from one geographic region to another in Poland (Kuzdralinski *et al.*, 2014). It has also been reported that most fields in eastern Poland are exposed to the attack of at least one or two *Fusarium species*. The occurrence of *F. graminearum* was found to be correlated to the occurrence of *F. culmorum* and this result was also observable for *F. poae* and *F. sprotrichioides* (Kuzdralinski *et al.*, 2014).

1.7. New approaches for studies of disease complex

Metagenomic projects have in the last 10 years been combined with NGS (Next-Generation Sequencing) technologies and it has created innovative opportunities for studying the wide range of pathogens associated with a single host (Bartoli *et al.*, 2015; Abdullah *et al.*, 2017) Using these novel OMICs will help to better characterize complex diseases. Identification of the disease-causing agents are the primary step of management before performing suitable disease control strategies. Although the use of a chemical substance is still important, a more sustainable disease management can be achieved by the development of more long-term strategies (Bartoli *et al.*, 2015). Targeted disease control by using a chemical substance becomes limiting when more than one pathogenic organism is present and contribute to the disease severity, thus the application of the specific targeted substance may not necessarily result in effective disease control (Bartoli *et al.*, 2015). Therefore, it is important to study disease complexes, the synergisms and antagonism in pathogen-pathogen interactions. Disease complexes are also linked to abiotic factors, likely weather, climate, cultural practices and geography, thus it could be a difficult mission. For that reason, it is important to establish an experimental approach leading to identification of pathogens in relation to the crop production system.

Finally, a careful evaluation of the roles of all the microorganisms isolated from the infection sites needs to be estimated, as multi species interactions and consortia can be involved in formation and escalation of the disease. This will require a holistic understanding of how a host responds to co-infection and how pathogens interact and coexist (Abdullah *et al.*, 2017); therefore, this will need to involve the interdisciplinary research collaboration between bacteriologists, mycologists and virologists. Understanding the biology and molecular interaction of these inter-microbial processes may be important in defining new targets and strategies for more sustainable disease control (Bartoli *et al.*, 2015)

2. Interaction assay

2.1. Introduction

The genus *Fusarium* causes many plant diseases. Fusarium head blight (FHB), (also known as ear blight or scab), *Fusarium* foot rot, and seedling blight and wilt, are the most common fungal plant diseases caused by various *Fusarium* species (Brennan *et al.,* 2003). They do have preferences on host and host organs, however they are often not highly specialized, thus one species of *Fusaria* can infect several hosts, which results in crop yield losses and lower quality of products. Furthermore, they produce secondary metabolites that are toxic for humans and livestock, and contaminate cereal-grain products that become unsuitable for food, feed and malting (Corina *et al.,* 2013). Since many *Fusarium spp*. are involved in causing disease on a same host, it makes control more complicated (Bartoli *et al.,* 2015). It is not possible to confirm which specific fungal species has caused FHB from the visual symptoms alone. If there are more than one species, it is difficult to determine relationships between individual species in the FHB complex (Xu *et al.,* 2008). Environmental factors such as temperature and humidity have significant effect on occurrence of diseases associated by *Fusarium spp.* and the severity of the symptom (Brennan *et al.,* 2003; Xu *et al.,* 2008).

Fusarium graminearum is amongst the most frequent pathogenic species of *Fusaria* for cereals which has saprophytic and pathogenic phases, although other *Fusarium* species such as *F. poae*, *F. avanaceum* and *F. culmorum* can be found associated with FHB (Vaughan *et al.,* 2016). FHB caused by *F. graminearum* and among other *Fusaria* lineage is initiated by airborne inoculum of spores on flowering spikelets. *F. graminearum* is more predominant in warmer and humid countries (Brennan *et al.,* 2003). *F. graminearum* might require relatively higher temperature and longer periods of wet condition to infect than for subsequent colonization (Xu *et al.,* 2008). Studies *in vitro* showed that *F. graminearum* has optimal growth conditions in the temperature range around 25°C (Corina *et al.,* 2013; Brennan et al., 2003). Whereas *F. culmorum,* which is considered as secondary important species, is commonly found in cooler maritime regions of Europe, and the optimal growth temperature *in vitro* is between 20°C and 25°C (Brennan *et al.,* 2003; Vaughan *et al.,* 2016). Both *F. graminearum* and *F. culmorum* are reported at temperatures above 25°C and are favoured by humid periods longer than 24 hours (Brennan *et al.,* 2003).

Climate change is expected to affect agriculture differently in different places. Most European countries have experienced increases in surface air temperature today (Olesen *et al.,* 2002). An analysis of General Circulation model (GCM) simulations indicates that annual temperatures over Europe warm at a rate of between 0,1 and 0,4 per decade (Olesen *et al.,* 2002). Though warmer temperatures may contribute to higher crop production in some regions or allow the cultivation of new crop species in Europe would be advantageous, it may increase the need of

plant protection and the more frequent extreme weather events may cause lower harvest yield (Olesen *et al.,* 2002).

F. graminearum is most prevalent in Asia, America and Southern Europe, however, it has been recently found to spread toward Northern Europe, such as Sweden, Finland and Russia, and it has become a common species (Ferrigo *et al.*, 2016). Other species, such as *F. culmorum*, *F. avenaceum*, *F. langsethiae*, and *F. sporotrichioides*, are of secondary importance but, nevertheless, they may also take an important role in pathogenesis when the climatic condition is not optimal for the dominating species (Ferrigo *et al.,* 2016).

According to a field study by Xu *et al.* (2008), there was a positive correlation among six *Fusarium spp.* including *F. graminearum* and *F. culmorum,* which may result from indirect association facilitated by microclimatic conditions conducive to several species. A study by Kuzdralinski *et al.* (2014) showed that the presence of *F. graminearum* seemed to be related to the presence of *F. culmorum*. Interaction between *F. graminearum* and *F. culmorum* can be explained by their coexistence as a complex of main species on the same plant. However, most of interaction between species from the genus *Fusarium* had a character of growth inhibition (Kuzdralinski *et al.,* 2014).

F. oxysporum is one of the most represented fungal populations in the rhizosphere (Aimé *et al.,* 2013) and well-documented ability to persist without recourse to pathogenesis. However, some pathogenic *F. oxysporum* are very host specific attacking only one or a few species of plants, and in many cases, attacking only certain cultivars of that plant, which may be an exception as many other *Fusarium spp.* are not host specialized. *F. oxysporum* is a saprophytic fungus and able to grow and survive for long periods on organic matter in soil. It has a predominant asexual reproduction phase and invades and penetrates from roots (Gordon and Martyn, 1997; Fravel *et al.,* 2003; Michielse and REP, 2009).

It is not completely clear how climate differences affect the distribution of *Fusarium spp.* causing diseases, especially because of the close association with a preferred crop host, and how they currently differ in relation to infection efficiency and competitiveness mediated by the climate (Vaughan *et al.,* 2016). The objective of the experiment was to test a method for analysing the interactions *in vitro*, using three important plant pathogens from the *Fusarium spp.* lineage. I conducted growth competition assays on axenic agar media with three *Fusarium species* (*F. graminearum, F. culmorum, F. oxysporum*) to study their interactions. *Fusarium graminearum and Fusarium culmorum* are present ecologically in the same niche, whereas *Fusarium oxysporum* is from another niche. Each species was grown alone and in pair combinations at different temperatures (4°C, 25°C, and 37°C). Pictures were taken and their growth was measured using the image analysis software, ImageJ (Schneider, Rasband, and Eliceiri 2012).

2.2. Hypotheses

My hypotheses were that

1) all species on control setups should grow best at 25°C, but since *F. graminearum* is a widespread species and known to be the most abundant in several arable plants, it is expected to grow fastest and occupy the most area in the agar plates.
2) Even though *F. graminearum* is known to grow best at temperature 25°C, it may adapt better within a wide range of temperatures than other species.
3) Considering the predominance of *F. graminearum*, it may have antagonistic interactions toward other species.
4) While *F. culmorum* favours cooler climate, is considered of secondary importance and not the most dominant species, it is expected to grow slower than *F. graminearum* at 25°C.
5) *F. culmorum* is expected to grow less in higher temperatures such as 37°C, and should grow better at 4°C than other species.
6) *F. oxysporum*, has a different niche to *F. graminearum* and *F. culmorum*, (e.g., tomato, melon, bean, banana, cotton, and chickpea; Michielse and REP, 2009) and is known to be an aggressive pathogen species because of the predominant anamorphic phase. Since *F. oxysporum* occurs often in greenhouse cultured-crops and favours warmer climate, it should grow better at 37°C than another *Fusarium spp.*.

2.3. Materials and Methods

Strains of *Fusarium graminearum* (PH-1), *F. culmorum* (PV) *and F. oxysporum* (f.sp. radices-lycopersici (isolate ZUM 2407/IPO-DLO)) on agar plates were obtained from Mukesh Dubey at the Department of Forest Mycology and Plant Pathology at Swedish University of Agricultural Sciences (SLU). These fungal cultures were kept on potato dextrose agar (PDA; Full-strength, 39.0 g/L^{-1}; VWR International B.V., Amsterdam). To do this, I sub-cultured *F. graminearum, F. culmorum and F. oxysporum* by taking two pieces of agar with mycelium (approx. 2cm x 1cm) and placing them upside down on to the new plates of full-strength PDA. A total of nine petri plates (three plates for each *Fusarium spp.*) were inoculated. After the plates were sealed with parafilm, they were placed into plastic bags, which were then incubated at 25°C.

I made 2 L of half-strength PDA (19.5 g/L^{-1}) medium and prepared 54 cell culture plates (6-well, Sarstedt). Half-strength PDA and 6-well cell culture plates was chosen for the following reasons:

1) All the isolates can grow on half-strength PDA.
2) It is more transparent than full strength medium, so the growth should be easier to see on the scanned plates.
3) *F. graminearum* is a fast grower and makes fluffy mycelium, so hopefully the half-strength PDA would keep the "fluffiness" to a minimum to bring out the differences between the isolates better.
4) Using 6-well cell culture plates makes it easier to scan and saves time.

Two plugs (approx. ⌀ 0,2mm) of *Fusarium spp.* were placed in the same agar plate (approx. ⌀ 3,0cm) as shown in Fig. 1, and then the different sets of plates were incubated at 4, 25, and 37°C. Each setup had 18 replications at one temperature. The interactions were scanned daily for one to two weeks.

Table 1. Pairing scheme used in the experiment

	FG	**FC**	**FO**
FG	FG×FG(control)	FC×FG	FO×FG
FC	—	FC×FC(control)	FO×FC
FO	—	—	FO×FO(control)

FG = *F. graminearum*, FC = *F. culmorum*, FO = *F. oxysporum*.

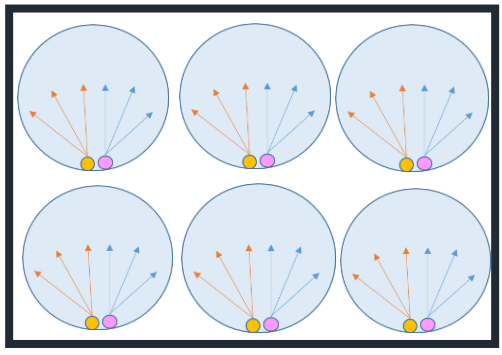

Figure 1. Set-up of interaction assay petri dishes. The dots represent the plugs of mycelium, with arrows indicating the expected direction of fungal growth.

To measure the area of both *F. oxysporum's* and *F. culmorum*'s mycelium, the RGB-colour feature in ImageJ was used. Most of pictures were measured by Grayscale channel 1 because it showed the edge of the mycelium most clearly. Grayscale channel 3 was used when the interaction zone between *F. oxysporum* and *F. culmorum* was unclear, because *F. culmorum* has darker yellow pigmentation. The darker yellow pigmentation was shown as black shadow on Grayscale channel 3, while *F. oxysporum* could be seen as light grey. The growth of area per day was compared within the same species from the control and interaction setups, and within two different species at the interaction setups.

2.3.1. Limitation of the experiment

This work has been done in the laboratory. The assay does not take into account the plant-host interaction or response toward the effects of the pathogen-pathogen interactions. The laboratory experiment may lead to different results than the field experiment, and the setups and microclimate conditions may also give different results. Furthermore, PDA has not the same composition as for example dead plant material.

2.4. Results and discussion

At 25 °C, both *F. culmorum* and *F. oxysporum* grew well on the agar plates (Fig 2). Experiments with *F. graminearum* at 25 °C did not show the results as expected. *Fusarium graminearum* was highly branched and tight, which made it difficult to determine the interaction with others. Therefore, the focus of the study is on *F. culmorum* and *F. oxysporum* at 25°C

Fusarium oxysporum produced pigmentation as pink (Fig 2a), *and F. culmorum* produced yellow pigmentation (Fig 2b). At the end of the experiment, *F. oxysporum* on the interaction plates had covered most of the area and seemed to out-compete *F. culmorum* (Fig 2d). Moreover, the pigmentation had become more intense. In some of the interaction plates, *F. oxysporum* showed an intense dark pink pigmentation near the interaction zone (Fig 2d). Also, in some of the interaction plates, *F. culmorum* showed a highly branched phenotype as visualized under the microscope (data not shown).

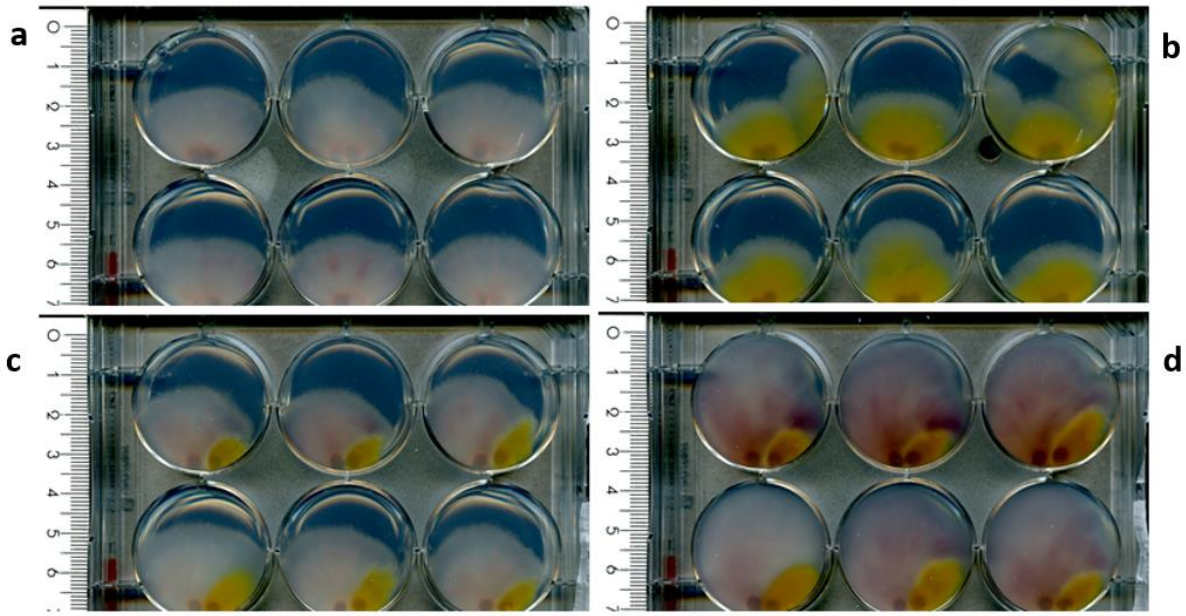

Figure 2. Scanned image plates for *F. oxysporum* and *F. culmorum at 25°C*. Top: Control plates for *F. oxysporum* (a) and *F. culmorum* (b) at day-three. Bottom: Interaction plates of *F. oxysporum* and *F. culmorum* at day-four (c). After the sixth day, *F. oxysporum* occupied most of the area in the plates and both species showed more intense pigmentation(d).

Grayscale channel 1 filter of the ImageJ which I used to measure the growth area per day (Fig 3). The Grayscale channel 1 showed the clearest edge of mycelium. Grayscale channel 3 was used when the interaction zone between *F. oxysporum* and *F. culmorum* was unclear, because *F. culmorum* has darker yellow pigmentation. The darker yellow pigmentation was shown as black shadow on Grayscale channel 3, while *F. oxysporum* could be seen as light grey.

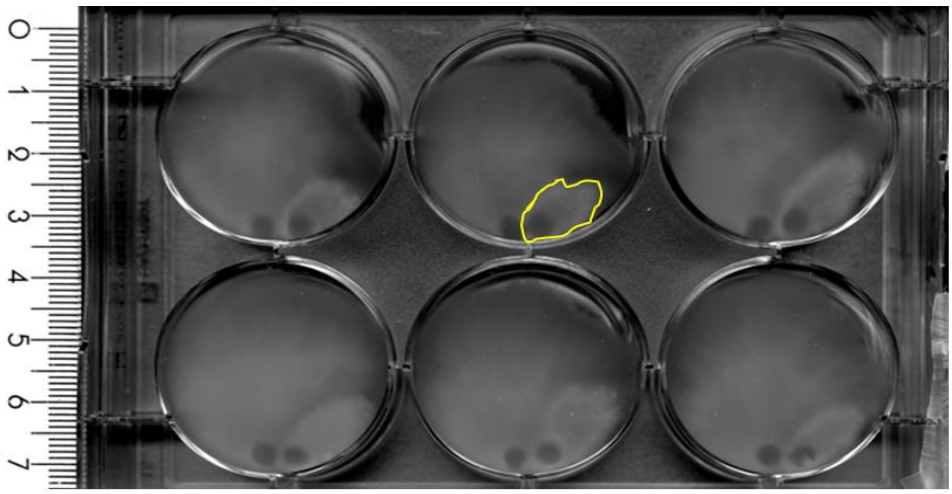

Figure 3. Interaction setup for *F. oxysporum* and *F. culmorum,* as shown by the Grayscale channel 1 filter of the ImageJ software. Because the Grayscale channel 1 filter showed the clearest edge of mycelium, it was used during the analysis.

Growth area per day were measured by using the ImageJ software, and converted to graphs (Fig 4). The growth of both fungi when they were on the interaction plates were scored by comparing them to the control plates. The graphs are showing the data collected starting from the third day, after the experiment was started. *Fusarium oxysporum* took over more than half area of medium in the interaction setups, by the end of the experiment. While *F. culmorum* grew much less than in the control setups (Fig 4). The rapid growth of *F oxysporum* occurred between the third and sixth day after experiment was set up (Fig 4). After the sixth day, *F. oxysporum* had covered most of the area on the plates (Fig 2d).

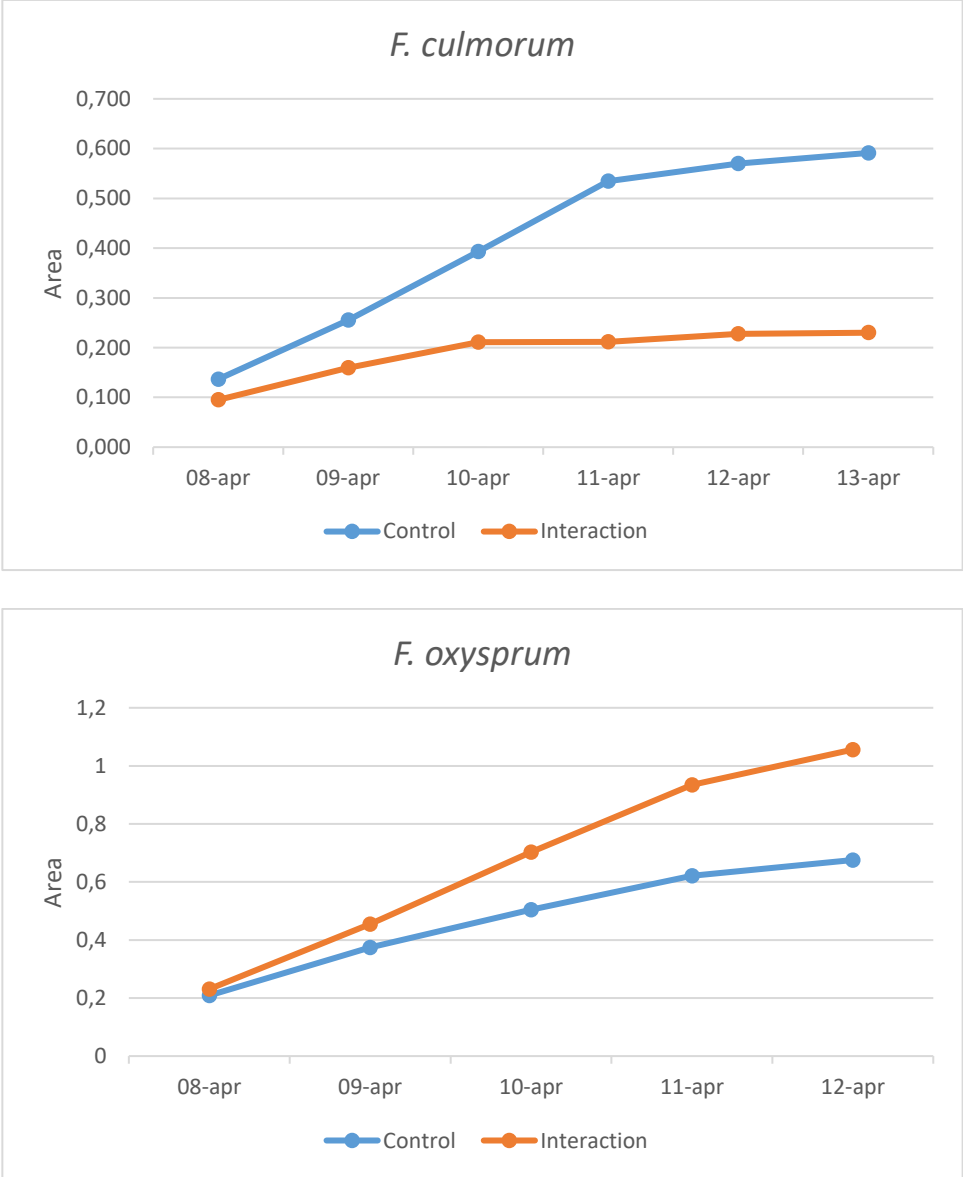

Figure 4. Top graph: Growth area per day of *F. culmorum* in control plates and during interaction setups. Bottom graph: Growth area per day of *F. oxysporum* in control plates and during interaction setups. The graphs are showing the data collected starting from the third day, after the experiment was started.

2.4.1. Interaction between *F. culmorum* and *F. oxysporum* at 25°C

Both *F. culmorum* and *F. oxysporum* on the control plates grew nicely. The growth rate of *F. culmorum* was slightly slower, but mostly the same as *F. oxysporum*. In addition, the control plates showed that *F. culmorum* and *F. oxysporum* are able to grow on the half-strength PDA on small plates. Both *F. culmorum* and *F. oxysporum* produced intensive pigmentation as yellow and pink respectively, thus it was easy to identify them morphologically. The growth of both fungi when they were on the interaction plates were scored by comparing them to the control plates. *Fusarium oxysporum* took over more than 3/4 area of medium in the interaction setups, by the end of the experiment, while *F. culmorum* grew much less than in the control setups (Fig 4). The rapid growth occurred between the third and sixth day after experiment was set up (Fig 4). After the sixth day, *F. oxysporum* had covered most of the area on the plates and seemed to out-compete *F. culmoru*m. Moreover, the pigmentation was more intense (Fig. 2d). Since two plugs were placed on every plate, the growth pattern for both *F. oxysporum* and *F. culmorum* during their interaction would be expected to look similar to the control plates, that is, each plug would occupy half the area. However, this was not the case, suggesting that *F. oxysporum* is able to inhibit *F. culmorum,* but more experiments are needed to validate these observations. According to a review of Xu *et al.* (2007), there seems to be no advantage for a competitive pathogen species, which are related to FHB, growing together with a weaker pathogenic species. That is, they do not colonise particularly well than when it grows alone. However, my interaction assay shows the opposite result. *Fusarium oxysporum* grew faster in the interaction assay than the control setups (Fig 4). Although, *F. oxysporum* is not related to FHB, hence that could be the reason to the difference in the results. In some of the interaction plates, *F. oxysporum* showed an intense dark pink pigmentation near the interaction zone (Figs 2d & 3). Also, in some of the interaction plates, *F. culmorum* showed a highly branched phenotype as visualized under the microscope (data not shown).

2.4.2. The growth of *F. graminearum* at 25°C

Fusarium graminearum did not grow as expected. With half-strength PDA in small petri plates, *F. graminearum* growth was highly branched and tight, which made it difficult to determine the interaction with others. It is difficult to grow different fungal species using the same medium because they can grow differently. Both, temperature and amount of nutrition in the medium can influence the morphology and the thickness of mycelium, and even the branching pattern. Nevertheless, it would be interesting to repeat the experiment and observe the interaction between *F. graminearum* and *F. culmorum since* they have the same niche, and since there are reports that their occurrence was correlated (Kuzdralinski *et al.,* 2014). In addition, a "tri-interaction" with *F. culmorum, F. graminearum* and *F. oxysporum,* would be interesting since these last two are known to be aggressive pathogens.

2.4.3. Growth and interactions at 4°C

All fungi placed at 4°C grew very slowly, and no interactions could be seen with the naked eye until the eleventh day. *Fusarium oxysporum* did not grow fast at 4°C, but upon microscopic examination, germinated conidiospores that had started to grow were visible at the third day. *Fusarium graminearum* and *F. culmorum* could grow even though it was very slow. Both fungi grew at the same speed and growth-area comparison between the interaction and the control plates looked similar, that is, each plug occupied half the area on the interaction and the control plates. Interestingly, *F. graminearum,* which is most dominant in Asia, America and Southern Europe, and requires relatively higher temperatures and humidity (Xu *et al.,* 2008), could even grow as good as *F. culmorum,* which is more adapted to cooler temperatures. Recently, *F. graminearum* has been reported to spread toward Northern Europe, such as Sweden, Finland and Russia, and it has become well common species (Ferrigo *et al.,* 2016). Therefore, it may be able to adapt quickly to different abiotic condition.

2.4.4. Growth and interactions at 37°C

Fusarium oxysporum could grow better at 37°C than at 4°C, even though it was slow, while the other species did not grow at all. As stated in the hypotheses (section 2.5), The pathogenic *F. oxysporum* is often found in greenhouse cultured-crops (e.g, tomato and melon) and crops grown in warmer countries (e.g., banana and cotton) (Michielse and REP, 2009). Therefore, *F. oxysporum* seems to be adapted to a warmer environment as shown by its ability to grow at 37°C. Unfortunately, the PDA medium dried out after two weeks and all fungi could not grow further. If the plates had been stored with a plastic bag so the humidity could be preserved longer, the experiment could have continued longer.

2.5. Remarks and technical limitation

The range of temperatures could be much narrower than 4°C, 25°C and 37°C, since at 4°C and at 37°C, the fungi grew very slowly or not at all. Thus, I could repeat the experiment at 8°C and 33°C, instead of 4°C and 37°C. Another drawback of using 37°C, was that the PDA medium dried out quickly. If the humidity could be preserved longer, the experiment can continue for a longer period. Nevertheless, I could still observe some growth for *F. graminearum* and *F. culmorum* at 4°C, and some for *F. oxysporum* at 37°C.

As already mentioned, *F. graminearum* did not grow as I expected at 25°C. With the half-strength PDA and in small petri plates, *F. graminearum* growth became highly branched and tight, which made it difficult to determine the interaction with the other species. *Fusarium graminearum* is known as a fast-growing species, but it may need enough space or enough nutrient. In a side experiment to test this, I could observe that *F. graminearum* grew as expected in a bigger petri plate using half-strength PDA or in smaller petri plates using full strength PDA. This would

suggest that *F. graminearum* does need either enough space or enough nutrient to grow properly. However, more testing is required to confirm this, since CO_2 concentration could also influence fungal growth.

Furthermore, the placement of plugs could give a different result, because *F. oxysporum* is usually a slow growing species and therefore, other species that grow faster could compete differently if plugs are placed opposite to each other instead of next to each other. Also, it would be interesting to observe the interaction zone when they are placed opposite to each other, instead of placing them next to each other and let them interact from the start. Using bigger petri plates could also give different results, but it would be easier to work during the placement of plugs. With bigger plates, the spreading of mycelium during the setup could be avoided, ensuring only one starting point. However, cell culture plates with six-wells were chosen because they made the scanning easier and the experiment more compact.

2.6. Conclusion

Regardless of the selection of methods, *F. culmorum* and *F. oxysporum* showed interaction at 25°C. *Fusarium oxysporum* grew fast and occupied more surface area on the plates than *F. culmorum*, which did not grow as in the control plates. Hence, *F. oxysporum* seems like a better competitor toward *F. culmorum* under the *in vitro* conditions tested. To state their interaction confidently and understand their interaction mechanism, more replicates and different experimental methods are required.

As mentioned above, the experiment at temperatures 4°C and 37°C did not perform as I expected, and the interactions between the species could not be observed. However, I could see their adaptability to different temperatures, that is, *F. oxysporum* could grow even at 37°C; whereas, *F. culmorum* and *F. graminearum* could grow at 4°C. It was surprising to see *F. graminearum*, which is most dominant in warmer countries, grow at cooler temperatures. However, *F. graminearum* has been recently reported to spread toward Northern Europe, suggesting that *F. graminearum* may adapt quickly to different abiotic condition. Because of climate change and temperatures increasing, *F graminearum* may be even more common in northern Europe in the near future.

One of my hypotheses could not be tested due to *F. graminearum* growth-problems at 25°C; although, it would be interesting to see the interaction between *F. culmorum* and *F. graminearum* since both occupy the same niche and they are often found as associated species causing FHB on cereal crops.

To confirm their interaction in terms of ¨antagonism¨ and ¨synergism¨, further experiments are needed. For example, analyses of enzymes, biomass and antimicrobial metabolites may give more detail information on how they interact with each other. Measuring growth rate and area does not tell the whole story, that is, even though it may grow faster, it may not mean is optimal growth.

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