AARHUS
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DEPARTMENT OF AGROECOLOGY

Effect of temperature on the growth rate of different isolates of *Verticillium dahliae* from different *Spinacia oleracea* L. hosts

45 ECTS Master Thesis

Agro-Environmental Management

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May, 2017

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Preface

This 45 ECTS thesis completes my MSc in Agro-Environmental Management, conducted from 2016 to 2017 at the Research Centre Flakkebjerg Aarhus University. The experiment and their corresponding analysis were planned in collaboration with my supervisors, Lise Deleuram and Mogens Nicolaisen.

This thesis is divided in six sections: Introduction and Background: general introduction about the reasons of the project with a review literature about similar studies which included topics that were not evaluated in this project to give a better understanding of the importance of *Verticillium dahliae* in crop production as well possible ways to control/prevent this soilborne pathogen. Also, a small introduction about the use of different methods of measuring was made. Materials and Methods: all methodology and instruments used as well statistical analysis conducted to evaluate significance of the data. Results: presentation of results from the experiments with associated statistical analysis. Discussion: overall discussion of the results assessing possible errors of experiment as well as possible expectations for the results. Conclusions: overall conclusions of the study with an attempt of answer to aim and hypotheses. Future perspectives: proposed topics and studies for the future which are essential for this research area and possible others.

In this study, it was used several techniques connecting the interaction between pathogens and environmental factors as well as techniques of measurement. Since, my previous academic formation has always been centred in biology, more specifically evolutionary and developmental biology, this thesis gave me an opportunity to extend my knowledge to new research areas. The possibility of the connection between these two research areas will give me knowledge to develop several approaches in the future culminating in different research projects which will be a great advantage for my professional life.

Acknowledgements

Firstly, I would like to thank my supervisors Senior Adviser Lise Deleuran and Section Manager Mogens Nicolaisen for all the support, guidance and advice which were the pillars on the development of this thesis.

Also, to Postdoc Rumakanta Sapkota, I give my deepest thankfulness for helping me conducting the experiment and guiding me with techniques and processes and for teaching me some valuable knowledge.

To Senior Scientist, Birte Boelt, I would like to thank for guiding me in my most desperate times which was essential for keeping me on track.

To Professor Lindsey du Toit, I give my sincere gratitude for sending the isolates of *V. dahliae* which were crucial for the develop of this study.

To everyone in Flakkebjerg who welcomed me with open arms, helped me and was somehow involved in this thesis, I would like to express my deepest gratefulness.

A special acknowledgement go to my partner, Víctor García Palacios, whom was a constant and irreplaceable support.

Also, a special acknowledgement go to my friend Carmina Cabral for showing me Denmark in the first place, for helping in so many levels and for being a constant reminder of reality.

Finally, I could not fail to express my gratitude to my parents, Vicência and Albino, which were a constant help by listening to all problems and achievements even though they are not related to this research area.

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Abstract

Assessing the effects of temperature on the growth rate of different isolates of *Verticillium dahliae* could be a way of investigate the influence of this soilborne pathogen. In this study, measurements of colony radial area at different temperatures (12º, 17º, 24º and 30º) were made during three weeks which were used to calculate fungal growth rate. Posterior, it was assessed the validation of the method used comparing it with a different method. Results showed that there is a possible effect of temperature on the growth rate of *V. dahliae*. Differences were higher at 24º and 17º. Less differences were observed at 12º and 30º showed lower levels of growth rate. In general, there was fungal growth rate at all temperatures. On the other hand, differences between methods were observed suggesting that one method could, possibly, be better for conducting measurements. Temperature could have an effect on the development and growth on this soilborne pathogen which suggests that changes in climate could lead to a more problematic occurrence of this pathogen. Nevertheless, more research needs to be conducted to assess the interaction of climatic factors and pathogen activity, especially in the case of *V. dahliae*. Only then, successful management strategies can be developed to control and prevent this pathogen.

Summary

This study was conducted using knowledge from two research areas: environmental management and plant pathology. By combining the efforts of these two areas, it makes possible to assess the influence of environmental changes, more specifically, climate changes on pathogen activity in agricultural systems.

Verticillium dahliae is a soilborne pathogen that can have a negative impact on crop production. The fact that this pathogen has a wide range of hosts combined with a strong survival on the soil (resting structures called microsclerotia can survive in the soil for up to 10 years) only hinders its possible management. In addition, Verticillium wilt (disease caused by this pathogen) can be symptomless or have symptoms similar to other soilborne pathogens which makes it difficult to identify. Denmark is the one of the major exporters of spinach seeds in the world. Since 2005, spinach seed lots have been reported with *V. dahliae* which makes it a great concern for Danish seed companies. Moreover, crop production is necessary to meet food demands in the world indicating that problems caused by this pathogen can have tragic consequences. Several studies assessed how climatic effects could influence *V. dahliae,* suggesting that research of about these interactions are essential to develop mitigation strategies against this soilborne pathogen.

The aim of this study was to assess the effect of temperature on the growth rate of different isolates of *V. dahliae*. An experiment was conducted in vitro using different isolates from different countries and from different spinach hosts. The following measurements were made: colony growth rate for each isolate in four different temperatures (12º, 17º, 24º and 30º) during three weeks. Growth rate was calculated using colony radial measurements. In addition, validation of the used method of measurement was made by comparing with other method of measurement.

The main results were: temperature was a possible effect on the growth rate of the different isolates. It was possible to see growth rate at all temperature. Higher growth rates were observed at 24º, followed by 17º, then 12º and finally 30º showed lower growth rate. Also, differences were possible to see between isolates even in the same temperature as well differences between isolates of the same and different countries.

Based on the results, it was possible to conclude that: temperature has a possible effect on growth rate of *V. dahliae* and that effect is different in the different isolates. This suggests that with climate change could influence the incidence of *V. dahliae* as well as

variability of its isolates. These changes will only make the develop of mitigation strategies more challenging.

The insights of this study can contribute to future researches. Since it seems to be an effect of temperature on the develop and growth rate, more studies could be conducted to assess how these interactions will be happen. However, to do that, it is essential to conduct more studies about the biology aspects of *V. dahliae*. Efforts should be made in using in vitro experiments that could be used to develop possible management of *V. dahliae* in the field. Development of mitigation strategies without a proper knowledge of the pathogen could lead to unsuccessful practices.

Taking in consideration the insight of this study, new perspectives of temperature effect on growth rate of pathogens could influence the management in agricultural systems. Therefore, studies could be conducted at a rhizosphere level, to comprehend more the interactions that could happen in this region. Also, the study of climatic factors and their interaction with life cycle of some microorganisms is essential to predict change that could possible happen. Also, studies about effect of temperature on aggressiveness and infection rate should be conducted. Assessing the effect of possible combination effects of climatic factors could also be intriguing. Moreover, studies about microsclerotia (considered that most problematic stage of *V. dahliae*) is essential to have a better understanding of this pathogen. Therefore, research should prioritize studies about the biology of this pathogen. Only with a better knowledge of it, develop of successful, sustainable and long-lasting management practices can be possible.

Introduction and Background

In a world where the growth of population is increasing at a fast rate, leading to an increasing food demand, agricultural development and management is of the utmost importance (Tilman et al., 2011). Problems in agriculture such as water stress, desertification reducing the amount of arable land, pests and pathogens negative effect on crops, to name a few, have a direct effect in the population worldwide (Green et al., 2005). Therefore, a good knowledge of agro-systems is extremely important (Mäder et al., 2002). Although, intensive agriculture can be a good solution for the increasing food demand by promoting the increase of crop yields, it also has negative effects on the environment, while a sustainable agriculture is considered a better solution because it can have a positive effect on crop yields with a less impact on the environment (Mäder et al., 2002). In agriculture, a fertile soil can promote several factors such as availability of sufficient nutrients for crop growth, support a large biotic community, provide a good soil structure and permit organic matter decomposition processes without any disturbances (Mäder et al., 2002).

Soil Microbial Ecology

The soil holds one of the most important pools of biological activity of the entire planet, where many interactions and processes occur, e.g. decomposition, which are important for the sustainability of food networks and ecosystem services (Maron et al., 2011). Although some studies (Bender et al., 2016; Griffiths et al., 2016) have been developed to assess and quantify the soil biodiversity, the knowledge about the role of it is still very imperfect. The range of soil biodiversity is vast, it goes from small and microscopic bacteria and fungi to bigger organisms such as ants, earthworms and moles (Bardgett and van der Putten, 2014). One ecosystem can support millions of species with new species being found daily (Bardgett and van der Putten, 2014) which makes it difficult to assess the function of all species in the soil. Nevertheless, studies about soil biodiversity have increased in the last decade (Bardgett and van der Putten, 2014). Some studies try to comprehend the distribution in space and time of soil microbial communities (Fierer and Jackson, 2006; Paul, 2014; Strecker et al., 2016), others try to assess how responses of soil communities to global change can have an effect on plant communities, food network interactions and biogeochemical cycles (Delgado-Baquerizo et al., 2014; He et al., 2016). Moreover, the increase in of soil biodiversity research is correlated with the increase agricultural systems research due to the consciousness of the important interaction

between soil biodiversity, ecosystem services and goods to human society (Bardgett and van der Putten, 2014).

Fungi

Amongst soil biodiversity, fungi have a great important in ecosystem functioning dynamics e.g. having influence in nutrient dynamics (Vogt et al., 1986), having a role in soil aggregation (Rashid et al., 2016); creating a symbiotic association with plants called mycorrhiza (Legay et al., 2016) or having an important role in decomposition processes (Banerjee et al., 2016). Nevertheless, some species of fungi in the soil are pathogenic and are involved in the spread of diseases both above and below ground (Chapelle et al., 2016). The total number of fungi in the world is still controversial (Hawksworth, 2001) with a current estimated number of 1,5 million species with only 70000 described (Hawksworth, 2001). There is still uncertainties between the interaction of fungi, soil and plants, with some studies (Barea et al., 2002; Pii et al., 2015; Rillig et al., 2015) being developed about these interactions.

Fungi, Soil, Plants and Microbiome Interaction

Interactions between species within the same ecosystem is complex and challenging to study due to the vast number of associations between them. For that reason, it is normal to reduce these interactions into pairwise interaction between species, e.g. mutualism between plants and mycorrhizal fungi (de León et al., 2016), interactions between plants and nematodes (Manosalva et al., 2015) or even interactions between plants and herbivores (Borer et al., 2014) to name a few. Although pairwise interactions seem to solve the complexity of studying interaction between species, it can also be challenging if, for example, a third species appears compromising the initial study. Therefore, when conducting a pairwise interaction study between species, it is essential to know as much as possible about those species and their interaction with the habitat.

Plants have interactions with an incredible large number of organisms, from viruses to arthropods or even other plants. Some of these interactions can be beneficial while others can be extremely harmful.

The rhizosphere is considered the zone that is influenced by plants roots where there exist an astonishing number of organisms and for that reason is considered one of the most complex ecosystem on the planet (Jones and Hinsinger, 2008). A vast range of species belonging to different groups can be found in the rhizosphere included archaea, fungi and

oomycetes, protozoa, viruses, nematodes, arthropods, bacteria and algae (Grayston et al., 1998; Hinsinger et al., 2009; Mendes et al., 2014; Philippot et al., 2013). The majority of these organisms takes part in a complex food network that uses nutrients released by the plant called rhizodeposits or root exudates (Lloyd et al., 2016). It is these root exudates that are the responsible for regulating the diversity of microorganisms and their activity in the rhizosphere. Many studies (D'ALESSANDRO et al., 2014; Egamberdieva and Lugtenberg, 2014; Glick, 2014; Panke-Buisse et al., 2015) suggest that microorganisms can influence plants by enhancing seed germination and vigour, growth and development of the plant, nutrition, prevent diseases and improve productivity. Although many studies are being conducted to improve knowledge of the plant microbiome, it is still a long process to decipher all the possible interactions.

It is possible to categorise the organisms in the rhizosphere as the ones that have beneficial effects on plants (Pii et al., 2015) and the ones that have negative impact on plants (Adam et al., 2016). The beneficial organisms include nitrogen-fixing bacteria, mycorrhizal fungi, mycoparasitic fungi, protozoa and other, biocontrol microorganisms (Pii et al., 2015). The organisms with negative impact on plants include pathogenic fungi, oomycetes, nematodes, bacteria and the ones called human pathogens which are related to the propagation of opportunistic human pathogenic microorganisms on plants (Berg et al., 2013).

In relation to microorganisms present in the rhizosphere that have negative impact on plants, these are considered soilborne plant pathogens which are known for causing great yield loss in crops. It is possible to separate two groups of soilborne pathogen, the nematodes and the fungi including true fungi and oomycetes. Studies about soilborne pathogens are still very scarce due to the necessity of understanding their interaction with plants, e.g. how do they infect the plant (Azcón-Aguilar, 1997; Putten et al., 2013). Nevertheless, some examples of these interactions were recently reported. Fungi and oomycete pathogens need several triggers from host plat to establish in the rhizosphere e.g. root exudates can affect the dormancy of fungal spore as well as abiotic factor such as pH (Van Long et al., 2017; Wang et al., 2015). Various compounds present in root exudates such as phenolic compounds can affect germination of pathogenic fungi at low concentrations (Dey and Kuhad, 2014; Yang et al., 2015), yet when these concentrations increase the effect is the opposite (inhibitory effect) (Pizzolitto et al., 2015; Schmidt et al., 2014). For example, phenolic acids present in root exudates inhibit spores germination

of *Verticillium dahliae* and trigger plant defence responses (Cheng et al., 2017). Also, alkaloids seem to have a role by inhibiting mycelium growth of several soilborne plant pathogens (Zheng et al., 2017) such as some species of *Fusarium* and *Trichoderma sp.*. However the manipulation of the alkaloid composition of plants by microorganisms in the soil can have an ecological negative impact due to, possibly, attracting specialist herbivores aboveground and discouraging generalist herbivores (Kant et al., 2015; Zhou et al., 2016). Saponins can also have an effect on soilborne plant pathogens by causing formation of pores and promoting the loss of membrane stability in the fungi pathogens (de Sain and Rep, 2015).

Thus, more studies are necessary to comprehend the interactions that occur in the rhizosphere between soilborne pathogens and plants and between different soilborne pathogens. The knowledge about these interaction is extremely important for developing control and/or preventive measures against theses soilborne pathogens.

Verticillium

Verticillium is a genus that belongs to the division of Ascomycota and includes several plant pathogen species responsible for causing vascular wilts (Pegg and Brady, 2002) in both temperate and subtropical climates (Fradin and Thomma, 2006; López-Escudero and Mercado-Blanco, 2011). *Verticillium* spp can have a mycelium hyaline both simple or branched, septate and multinucleate, having ovoid to elongated conidia produced in long phialides with a spiral shape around the conidiophores called “verticillate” which is the responsible for the name of the genus.

Two species are considered the most notorious, *V. dahliae* and *V. albo-atrum*, causing major annual crop losses around the world (Ashworth Jr et al., 1972; Paplomatas et al., 1992). Yield losses caused by these species can be different e.g. potato crops can have losses from 10-50% (Johnson et al., 1987) and lettuce can reach up to 100% of losses (Vallad et al., 2006). These *Verticillium* species pose serious problems in crop production because of their soil habitat, their ability to survive in the soil for years and the wide range of host that can carry on the infection from year to year. As a consequence, farmers can experience enormous economic losses in their crop production. In the genus *Verticillium*, other plant pathogenic species can be found including *V. tricorpus*, *V. nigrescens*, *V. nubilum* and *V. theobromae*.

Nevertheless, *V. dahliae* and *V. albo-atrum* are the most problematic plant pathogenic species of this genus with both species showing speciality in individual isolates. *V. dahliae* is considered the type species of the genus *Verticillium* (Inderbitzin et al., 2011) and the most abundant worldwide.

V.dahliae can be divided into six groups based on vegetative compatibility (Puhalla and Hummel, 1983). Vegetative compatibility consists in the ability of the hyphae to fuse by the creation of a combined heterokaryotic mycelium. If there is a formation of this viable heterokaryotic mycelium between colonies it is possible to say they belong to the same Vegetative Compatibility Group (VCG). Vegetative compatibility is regulated by one or more nuclear genes that can control the conclusion of hyphal anastomosis (fusion between branches of the same or different hyphae) (Moore et al., 2011). Members of the same VCG have the same vegetative compatibly alleles (Moore et al., 2011). First, there is a pre-contact in which, if there is cytoplasmic compatibility, there will be cytoplasmic exchange beyond the first hyphal compartments. However, vegetative compatibility tests of self/non-self recognition only occur after hyphal anastomosis. If the colonies do not belong to the same VCG, all the cells that were involved in anastomosis are killed. This process prevents the transfer of nuclei and other genetic material between strains of different VCG. However, if the process is slow the hyphal compartments where anastomosis is happening can be attacked by a virus or cytoplasmic plasmid and the process is interrupted. Figure 1 shows a diagram of the process mentioned above.

This process of anastomosis is used by *Verticillium* to ensure the increase of genetic diversity within the species, since there is not an exchange of genetic information through sexual reproduction. Despite the economic impact of this pathogen in crop production, there is still scarce knowledge about the molecular mechanism to cause disease of *Verticillium* spp., which makes it difficult to develop preventive or controlling measures towards these species.

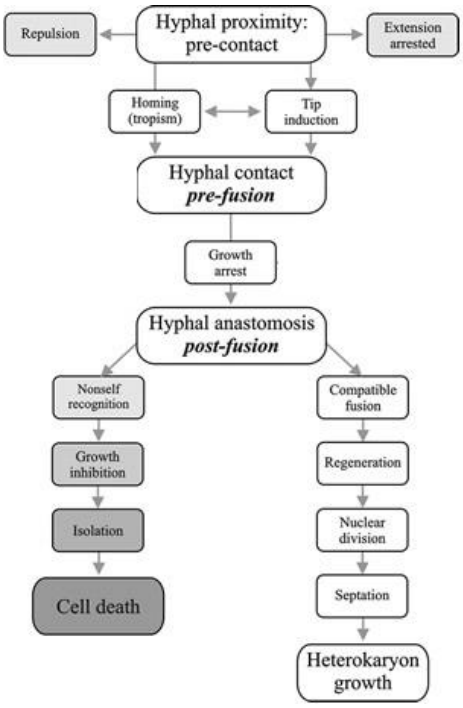

Figure 1: Diagram representing the development of hyphal interaction when controlled by the vegetative compatibility system. It is possible to point out three steps: hyphal proximity: pre-contact, hyphal contact: pre-fusion, hyphal anastomosis: post-fusion Adapted from (Moore et al., 2011)

Host Range and Symptoms

Verticillium spp. can infect more than 200 plant species from annual and perennial crops to trees and shrubs *V. dahliae* is responsible for causing wilt in vegetables such as artichoke, eggplant, pepper, potato and tomato, fruits such grapevine, olive and strawberry, flowers, sunflower, fibre crops such as cotton and flax and woody perennials (Bhat and Subbarao, 1999) while *V. albo-atrum* can cause wilt mainly in alfalfa, hop, soybean, tomato and potato (Inderbitzin et al., 2011). Also, many weeds with showing symptoms or not, can be *Verticillium* hosts (Doğan et al., 2014). It seems that, so far, monocotyledonous plants are non-hosts of *Verticillium* spp. In Denmark, *V. dahliae* is a main concern in spinach seed production (Sapkota et al., 2016) while in Portugal and Spain *V. dahliae* has a negative impact on the production of olive trees (López-Escudero and Mercado-Blanco, 2011).

It is possible to described several symptoms caused by *Verticillium* isolates, some showing more host speciality e.g. the case of *V. dahliae* isolates from mint and cocoa and *V. albo-atrum* isolates from alfalfa and hops (Correll et al., 1988; Iglesias-Garcia et al., 2013; Radišek et al., 2006; Resende et al., 1994). Since, symptoms can be different for each host, there is no unique symptoms to describe Verticillium wilt. Also, despite the name, wilt is not always associated with *Verticillium* infection. Although infection can occur in the beginning of the growing season, wilt symptoms can appear in the end of the

growing season or even not appear at all (Johnson and Dung, 2010). Initial symptoms include infected leaves, mainly oldest shoots due to acropetal invasion (from base to apex), in tomato some of lower leaves can present a yellowish colour as tips and edges start to die, causing a lesion in shape of a V (Figure 2D) (Isaac and Harrison, 1968). As well, leaves can develop yellow spots at first that will turn necrotic and brown showing veins with a brown or purple colour (Figure 2A,B,C) (Buhtz et al., 2016).

Normally, this wilting process can occur at warmer periods of the day with recovery at night (lower temperatures) (Buhtz et al., 2016). Also, drought or other stress conditions can increase the development of the wilt symptoms (Sun et al., 2016). Annual plants can, often, survive the season, however can also suffer chlorosis, stunt and early senesce and thus, loss of yield. In plant stems, it is possible to describe a brown discoloration of vascular tissues (Figure 2E,F). Another reason for the challenging of scoring disease symptoms of Verticillium wilt is the fact that *Fusarium* species (another plant pathogenic fungus) can cause similar symptoms. Finally, nematodes infection can enhance this disease by creating wounds in the roots which will not only increase the fungal penetration but also release root exudates into the surrounding areas, increasing the germination of this fungus and therefore increasing Verticillium wilt development.

Figure 2: Some examples of symptoms caused by Verticillium wilt. (A) Leaf necrosis and wilt in potato; (B) Wilt in young peppermint leaves; (C) Necrosis (left) and chlorosis (right) in peppermint leaves; (D) V-shaped lesions in leaves of tomatoes; (E) Healthy vascular tissues (left) and vascular discoloration (right) in maple stems; (F) Longitudinal section of stem with vascular discoloration (top) and healthy stem (bottom) of potato Adapted from (Allen, 2006; Berlanger and Powelson, 2000)

Disease cycle

Both *V. dahliae* and *V. albo-atrum* attack plants through roots. *V. dahliae* has only one cycle and production of inoculum during the growing season (monocyclic disease) (Zhou et al., 2006). Life cycles of both species are similar and can be divided into three phases: dormant, parasitic and saprophytic phase.

The dormant phase is defined by the germination of resting structures in the soil (microsclerotia), in response to root exudates (Tsror, 2011), with each microsclerotium being able to germinate multiple times and therefore increasing the chances of infection. Hyphae growing from the microsclerotia colonizes the root surface and cortex (Schnathorst, 1981). This process of infecting the root means that the life cycle is starting its parasitic phase. For being able to infect the vascular tissues (xylem), the fungus need to cross the endodermis (physical barrier) (Klosterman et al., 2009). Hence, the fungus penetrates the root tip where the endodermis is not yet formed or in places where there are wounds (caused by other organisms) (Back et al., 2002; Bowers et al., 1996), avoiding this physical barrier . Then, the fungus penetrates in the vascular tissues where it produces abundant conidia, a process called budding (Jiménez-Díaz et al., 2012), which will be translocated into the vascular system of the host. After this, sporulation happens which will permit the start of another infection cycle (Jiménez-Díaz et al., 2012). This parasitic phase can happen between 2 to 5 days, from the entering of the fungus in the xylem until the sporulation (Mace, 2012).

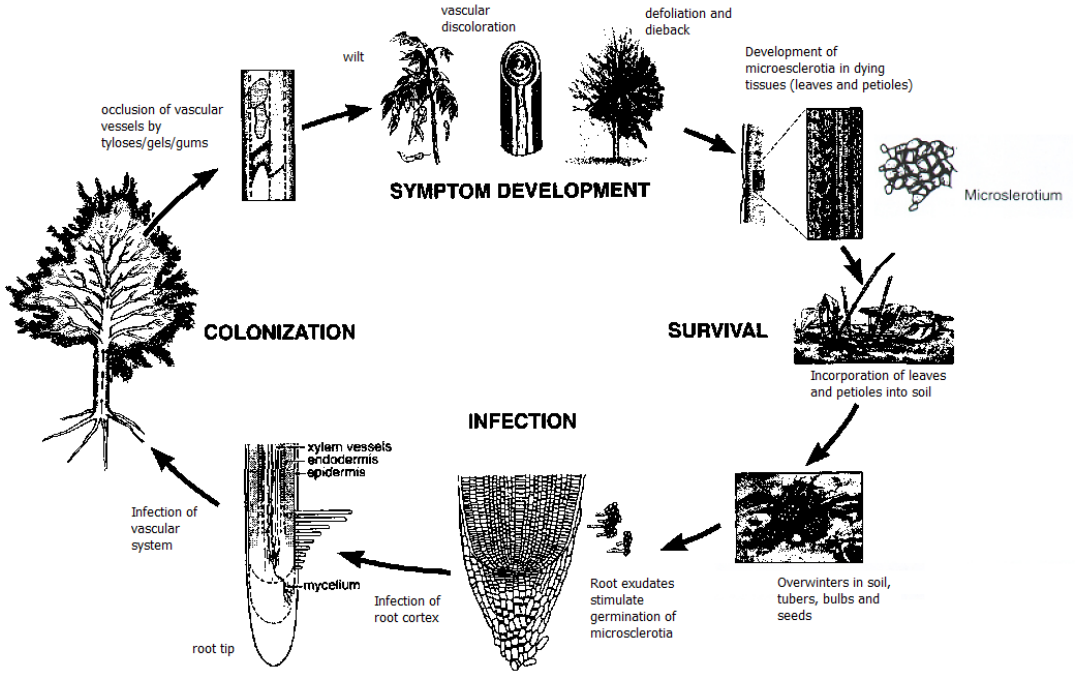

Figure 3: Illustration of the *Verticillium dahliae* disease cycle Adapted from (Hiemstra, 1998)

The saprophytic phase occurs during tissue necrosis or plant senescence (Perry and Evert, 1983). In this phase, not only the vascular tissues are infected but also shoots and roots of the plant (Perry and Evert, 1983). It is in this phase that differences *V. dahliae* produces microsclerotia that will be release in the soil after the decomposition of the plant where they can survive for up to 10 years (Fradin and Thomma, 2006; Perry and Evert, 1983). Both species can overwinter not only in perennial hosts but also in tubers, bulbs or seeds (Sapkota et al., 2016; Wu and Subbarao, 2014). Figure 3 shows an illustration of the disease cycle explained above.

The special case of spinach in Denmark

Denmark is the world's producer of hybrid spinach (*Spinacia oleracea* L.) seed. Since 2005, *V.dahliae* has been reported in commercial seed lots which made this soilborne pathogen a concern for seed production (du Toit et al., 2005). Seed production companies in Denmark are extremely interested in studies about *V. dahliae* due to its enormous negative impact on spinach seed production and subsequently on the quality of the produced seeds. As mentioned previously, *V. dahliae* can be transmitted through seeds or through the soil infecting new hosts. Once in the soil, microsclerotia of *V. dahliae* can survive for many years which compromises crop production of spinach and other crops. Since spinach are harvest before bolting and Verticillium wilt symptoms mainly appear after bolting, infection is difficult to identify in fresh-market spinach (du Toit et al., 2005). Therefore, more studies about *V. dahliae* are essential to improve its management and counter-attack its negative impacts in crop production.

Difficulties in the management of *Verticillium*

The intensity of the disease is a function between the interaction of the host, the pathogen and the environment over time (Scholthof, 2007). The disease intensity in both *V. dahliae* and *V. albo-atrum* is controlled by the amount and efficacy of initial inoculum and the span of time in which the host and the pathogen interact (Bejarano-Alcázar et al., 1995). Therefore, management of *Verticillium* wilt should have a focus on reducing this initial inoculum which will influence the development of the disease.

Some practices that can possibly reduce initial inoculum include soil fumigation, soil solarisation, crop rotation, the use catch crops, green manures and organic soil amendments and irrigation (Davis et al., 1990; Jiménez-Díaz et al., 2012; Klosterman et al., 2009; Larkin et al., 2011; Njoroge et al., 2009). In addition, the use of resistant cultivar can have an effect by reducing the colonization of the plant and therefore the spread of

the pathogen (López-Escudero et al., 2004; Vallad and Subbarao, 2008). However, more studies to develop resilient management tactics are required.

Regarding the use of resistant cultivars, this tactic can be effective and efficient in reducing the effects of Verticillium wilt. Some studies presented the positive effects of this resistant cultivars in potatoes (Davis et al., 1983; Secor and Gudmestad, 1999), tomatoes (Fradin et al., 2009; Paternotte and Van Kesteren, 1993) and olive trees (López-Escudero et al., 2004; Martos-Moreno et al., 2006). Nevertheless, the use of this resistant cultivars is not common due to its economic value compared with other varieties (Pegg, 1984; Smith et al., 1995). Because most fungicides seem to not have an impact on Verticillium wilt, resistance could be a way of controlling this disease. Still, economic interests are also involved which makes the use of these resistant cultivars debateable. Moreover, even if these resistant cultivars are effective for Verticillium wilt, they could be susceptible to other diseases.

Soil fumigation, may reduce the inoculum of *Verticillium* spp. however it is extremely expensive and restricted by governmental laws (Easton et al., 1975; Powelson and Carter, 1973). The fumigants are placed in a specific depth in the soil to eliminate microsclerotia (Easton et al., 1975; Powelson and Carter, 1973). Although the fumigants can have a positive impact in the present growing season, they fail to eliminate all the inoculum in the soil which will promote the infection of the posterior crops by microsclerotia. In addition, there are still scarce information of this soil fumigation in the beneficial soil organisms in the soil which can also create some debate (Easton et al., 1975; Xiao et al., 1998).

Soil solarisation can also be an effective method. The soil surface is covered with a transparent plastic film which will reduce the spreading of *Verticillium* in fields (López-Escudero and Blanco-López, 2001; Pullman et al., 1981). There is still limited information about the mode of action but it is presumed that the effect is caused, in part, by the increase of temperature and stimulation of antagonistic organisms (López-Escudero and Blanco-López, 2001; Pullman et al., 1981). Moreover, it is a method that can be used only in hot and dry climates and can be influenced by factors such as soil texture, soil temperature, soil moisture, rainfall, the amount of clouds (López-Escudero and Blanco-López, 2001; Pullman et al., 1981). Nevertheless, the need of covering large areas of the field with plastic can entail some negative environmental impacts and it can be extremely difficult to monitor its consequences.

Crop rotation can be effective in reducing the soil population of *V. albo-atrum*, assuming the use of non-susceptible crops for three to four years, yet the same pattern in observed in *V. dahliae* (Njoroge et al., 2009; Xiao et al., 1998). The reason for this is the presence of microsclerotia of *V. dahliae* in the soil. Also, microsclerotia can germinate in the roots of weeds or non-susceptible crops and then infect susceptible hosts (Njoroge et al., 2009; Xiao et al., 1998). Because of the wide range of host, some cross-pathogenicity of *V. dahliae* populations of different hosts can happen (Njoroge et al., 2009; Xiao et al., 1998). By contrast, crop rotation can increase crop yield by reducing the incident of Verticillium wilt (Njoroge et al., 2009; Xiao et al., 1998). Choosing the right crop rotation as well the management of weeds is essential for assuring the efficient of this tactic (Njoroge et al., 2009; Xiao et al., 1998).

The use of catch crops which include the use of green manures and organic soil amendments in the field before planting can also be an effective means to reduce Verticillium wilt (Davis et al., 1994; Pinkerton et al., 2000). Some examples of catch crops that can supress Verticillium wilt include barley, broccoli, maize, winter pea (Davis et al., 1994; Pinkerton et al., 2000). Nevertheless, information about the effectiveness of catch crops is still scarce and more studies need to be conducted. In addition, using green manures and organic soil amendments may be economical disadvantageous if there is a need of a full season for the green manure crops before planting the desirable crop (Davis et al., 1994; Pinkerton et al., 2000).

Irrigation studies (López-Escudero and Blanco-López, 2005; Wheeler et al., 2012; Xiao and Subbarao, 2000; Xiao et al., 1998) presented that preserving soil moisture up to 70-75% during vegetative growth of the crop, increasing to 80% during the tuber initiation could reduce the root infection by *V. dahliae* in irrigated fields. However, reducing the available of oxygen in saturated soils can lead to a decrease in the natural defences of the host (López-Escudero and Blanco-López, 2005; Xiao et al., 1998). In addition, different types of irrigation can affect the intensity of Verticillium wilt (López-Escudero and Blanco-López, 2005; Xiao et al., 1998). Surface drip irrigation can decrease the incidence of Verticillium wilt (Land et al., 2017) while irrigation using sprinkler systems can increase the incidence of this disease (Jefferson, 2002).

In conclusion, more studies are essential to assess the management of *Verticillium*, having a better understanding of tactics for preventing and controlling it as well as the impacts of those tactics.

Climate change effect on the host, the pathogens and their interactions

Climate change can have an impact in plant disease management (Chakraborty and Newton, 2011). Studies related to climate change can be limited due to short-range, analysis of the effect of only one or two climate factors and the use of conditions different from field. However, it is possible to infer that climate change has an effect in the host, the pathogen and the host-pathogen interactions (Altizer et al., 2013; Anderson et al., 2004). Also, this effect will be different on different ecosystems worldwide which can become a challenge in assessing its main impacts (Leemans and Eickhout, 2004).

Plant responses to climate change will occur whether the pathogen is present or not. If there is the presence of the pathogen, changes in the plant will affect their interaction e.g. increasing of leaf area can lead to an increase of foliar pathogens (Fuhrer, 2003; Mcelrone et al., 2005) as well changes in root size could increase the presence of soilborne pathogens (Pritchard, 2011; Walther et al., 2002). Also, abiotic stress such as temperature and drought could have an impact on plant susceptibility to pathogen (Gilman et al., 2010) or promote the increase of plant defences leading to an increase of resistance (Ashraf and Foolad, 2007).

Studies (Ahuja et al., 2010; Engelbrecht and Kursar, 2003; Grime et al., 2000) showed that drought could lead to both increased and decreased plant resistance. Also, an increase of CO_2 levels would lead to an increase of foliar density which would promote more infection rates by foliar pathogens (Mitchell et al., 2003). The increase of temperature could have both positive and negative impacts, decreasing plant stress during the cold seasons and increasing plant stress during warm seasons (Ahuja et al., 2010; Walther et al., 2002). In addition, increase of temperature could also promote symptoms such as wilting, leaf folding and changes in synthesis of proteins, enzymes and other compounds which could increase susceptibility to pathogens (Foden et al., 2009; Stillman, 2003). Finally, the increase of ozone concentrations could affect the plant by altering the chemical composition of leaf surface which would increase the infection by foliar pathogens (Ainsworth et al., 2012; Fuhrer, 2003). Also, the increased ozone concentration could increase the attack of fungi in the soil (Caldwell et al., 2007; Castro et al., 2010).

About the effect of climate change on the pathogen, the consensus is that temperature is the factor that will have more effect on the pathogen, e.g overwintering with higher temperatures in autumn/winter would increase the survival of the pathogen (Ayres and

Lombardero, 2000; Bergot et al., 2004). Also, the increase of temperature will lead to a geographical expansion of the pathogen leading to the infection of more hosts (Parmesan, 2006; Semenza and Menne, 2009) and more opportunities for the hybridization of the pathogen (Anderson et al., 2004; Parmesan, 2006). Moreover, temperature governs the rate of reproduction which indicates that higher temperatures will probably lead to a faster reproduction of pathogens (Chakraborty et al., 2000; Gregory et al., 2009). Also, longer seasons will also provide more time for the evolution of the pathogen (Parmesan, 2006; Rosenzweig et al., 2001). Climate change could also influence the type of reproduction e.g. change to sexual reproduction rather than asexual reproduction which would increase the evolution of the pathogen (Roos et al., 2011). Nevertheless, more studies need to be conducted to assess the possible effects of climate change on pathogens development.

In conclusion, there is still a long path to go to assess the effect of climate change in the host, the pathogen and their interactions. Despite being challenging that does not make it impossible and more studies need to be conduct to investigate these topics

The importance of choosing the right method of measurement

Measurement of the growth rate of fungi can provide important information that can be used for several aspects of mycology e.g. measuring colony growth area (Matcham et al., 1984). In this study, it was used a method of measurement that was thought to be the ideal one. Nevertheless, while conducting the experiment, an alternative method was present. This lead to enquiry if the right method was being used and lead to research to verify the validation of the first method.

The design of an experiment can be challenging due to several choices that need to be made. One of them is the use of the right instrument of measurement. Not always the right instrument is used which can lead to errors and other complications. Therefore, testing the measures made by two different instruments can increase the performance of an experiment.

Videometer (Carstensen and Folm-Hansen, 2006) and Tagarno (Tagarno, Horsens, Denmark) are two different instruments that can have several uses for different studies (Bodevin et al., 2009; Dissing et al., 2009; Röper et al., 2015) e.g. assessing the area of a specific object. VideometerLab4 is camera system specialises in spectral imaging that is used for determination of colour, texture and chemical composition on surface of 90 x 90 mm per image. It uses a combination of illumination, camera and computer software with

digital image analysis and statistic. VideometerLab4 is normally used for measurements of samples e.g. counting of microbial colonies, counting of seeds, combining up to 20 different wavelengths in a high-resolution spectral image. Tagarno is a digital microscope with a digital microscopy camera that permits the visualisation of small objects in high definition without distortion, delay or interference. It can be a powerful instrument for measurements of areas and for high quality observations of samples. However, it needs a good calibration before starting the measurements/observation. It also permits the manual selection of the desirable area or point to measure. Since, both instruments can be used for measuring samples it is essential to verify if one will have a better performance than the other to select the method that will produce less errors and complications.

Aim and hypothesis

Soil microbial communities are likely to shift with climate change e.g. temperature and CO_2 levels are important factors in driving microbial communities. However, studies about the effect of climatic factors are difficult to conduct, especially in the field. Therefore, assessing the effects of climatic factors can be challenging. *Verticillium dahliae* is a soilborne disease and a potential threat to crop production. In Denmark, this fungal pathogen has a major impact in spinach seed production. Thus, how climatic factors affect the development of *Verticillium* spp. in both soil and plant is of great interest for the Danish spinach seed crop companies. Nevertheless, few studies have been conducted to assess the effect of temperature on this pathogen. The **aim** of this project was to study the effect of climate (temperature) on the development of *Verticillium dahliae* (growth rate) at an individual level, using different isolates from different countries. While conducting this experiment, a small experiment was initiated to compare two methods of measurements of colonisation of selected *Verticillium dahliae* isolates to explore if there were signifncat difference between them. **The main hypothesis** was that "there is a possible effect of temperature on the growth rate of the different isolates of *V. dahliae*" (experiment 1) and "there is a significant difference between measurements of two different methods (experiment 2).

Materials and Methods

Experimental setup. In Experiment 1**,** to assess the effect of temperature, an experimental setup was made which predicted the use of 20 isolates of *V. dahliae* in four different temperatures (12º, 17º, 24º and 30º) with 4 replicates for each isolate. Therefore, the final experimental setup used was a total of 320 isolates (20 isolates x 4 temperatures x 4 replicates).

Isolates of Verticillium spp. In Experiment 1, a collection of 20 isolates of *V. dahliae* from different spinach hosts were used. The isolates were pre-selected from a larger collection. The selection was performed to ensure that the isolates had different VCG and different countries of origin (Table 1), showing diversity. The collection included isolates recovered from spinach seed lots produced in Denmark, the United States, The Netherlands, Chile, Italy and France and were provided by Professor Lindsey du Toit from the Washington State University (du Toit et al., 2005). After arrival of isolates, each was placed in a petri dish and incubated for four days at 24º to verify the status of isolates and the possible existence of contaminations.

Table 1: Origin of the isolates of *Verticillium dahliae* from spinach host used in Experiment 1 (n.d. = not determined)

Species	Isolate code	Host of Origin	Country of Origin	VCG	Ref. Paper	Notes
Verticillium dahliae	24	Spinach	United States	n.d		Heavy mycelium
Verticillium dahliae	88	Spinach	Denmark	2B		Microesclerotia very heavy
Verticillium dahliae	92	Spinach	Denmark	4B		Microesclerotia light
Verticillium dahliae	119	Spinach	Denmark	2B		Microesclerotia very heavy and dense
Verticillium dahliae	433	Spinach	The Netherlands	4B		Microesclerotia medium
Verticillium dahliae	623	Spinach	Italy	n.d		Microesclerotia heavy
Verticillium dahliae	630	Spinach	Denmark	n.d		Microesclerotia medium
Verticillium dahliae	662	Spinach	Chile	n.d		Microesclerotia very light
Verticillium dahliae	666	Spinach	France	n.d		Microesclerotia heavy
Verticillium dahliae	582a	Spinach	Chile	n.d		Microesclerotia heavy
Verticillium dahliae	584a	Spinach	Chile	n.d		No Microesclerotia on original plate
Verticillium dahliae	588a	Spinach	Chile	n.d		Microesclerotia light
Verticillium dahliae	WSU 107	Spinach	Denmark	2B	Iglesias-Garcia et al., 2013	
Verticillium dahliae	WSU 139	Spinach	United States	4B	Iglesias-Garcia et al., 2013	
Verticillium dahliae	WSU 210	Spinach	Denmark	2B	Iglesias-Garcia et al., 2013	
Verticillium dahliae	WSU 311	Spinach	Denmark	4B	Iglesias-Garcia et al., 2013	
Verticillium dahliae	WSU 359	Spinach	Denmark	4B	Iglesias-Garcia et al., 2013	
Verticillium dahliae	WSU 509	Spinach	The Netherlands	4B	Iglesias-Garcia et al., 2013	
Verticillium dahliae	WSU 523	Spinach	The Netherlands	2B	Iglesias-Garcia et al., 2013	
Verticillium dahliae	WSU 541	Spinach	The Netherlands	2B	Iglesias-Garcia et al., 2013	

Isolates inoculation. In Experiment 1, each isolate of *V. dahliae* was placed on one petri dish with PDA (4g of potato extract equivalent to 200 g of infusion from potatoes, 20 g dextrose and 15 g Agar, final pH: 5.6 ± 0.2 at 25°C) + Chloramphenicol. Chloramphenicol was used to prevent the contamination of the plate by bacteria. PDA plates were prepared by dissolving 39 g of PDA in 1L of distilled water with the addition, using size filters, of 0.05 g of Chloramphenicol for 1L of prepared media. Then, the preparation was autoclaved, poured and inoculated into plates. A total of 320 petri dishes were prepared. Fungal discs of around 1 cm were cut by using a sterile cork-borer and placed on the surface of the culture media, previously prepared, with five replicates per each isolate. An alcohol burner was also for flame sterilisation of the cork-borer to prevent contamination of the petri dishes. The inoculated plates were incubated at four different temperatures in four different incubators: 80 petri dishes (4 rep x 20 isolates) at 12ºC, 80 petri dishes at 17ºC, 80 petri dishes at 24ºC and 80 petri dishes at 30ºC. The incubation time was three weeks. Laboratory incubators (Mochizuki et al., 2015) can provide a controlled, contaminant-free environment where it is possible to regulate conditions such as temperature, humidity, and CO_2, light/dark conditions. For this experiment, temperature was regulated in each incubator at a 24-hour constant dark light regime, other factors such as relative humidity or CO_2 were not studied.

Fungal growth analysis. In Experiment 1, during incubation time, the plates with isolates of *V. dahliae* were observed for assessing the degrees of growth, in terms of the radial area (mm2), that each fungal mycelium had grown. Measurements were made by using VideometerLab4 instrument. The measurements were made by the software of this instrument by applying a contrast of colours between the growth area and the PDA medium, creating a "blob" (desirable area with fungal growth) with calculated area was exported to Microsoft Excel (2016) (Fig. 4).

Figure 4: Illustration showing an example of the method for assessing colony radial area of *Verticillium dahliae* isolates using Videometer. The petri dish was placed on the Videometer. Software distinguished desirable area (colony radial area) from undesirable area (medium). Desirable area was selected ("blob") and the software calculated the required parameters, in this case, colony radial area. Result was then exported to Microsoft Excel with respective units, in this case, mm^2.

Measurements were recorded each week during three weeks to observe the growth rate. For radial measurements, a linear regression of a plot was made using colony radial area (mm^2) against time (days). Since radial measurements fit a linear model, the growth rates were used as the main parameter to assess the effect of temperature on fungal growth. It was determined in which period the maximum growth rate occured (from the three radial measurements) for each isolate and subsequently the absolute growth rate was calculated. Absolute growth rate was calculated following Eq. 1 (Pietikåinen et al., 2005):

$$K_r = (a1 - a0)/(t1 - t0) \quad [1]$$

where a_1 and a_0 are final and initial colony radial area at initial (t_0) and elapsed time (t_1). Absolute growth rate was expressed as mm^2 per day.

Subsequent, a small experiment (Experiment 2) was conducted by randomly selecting 50 petri dishes of inoculated isolates in the last week of incubation. These 50 inoculated petri dishes were measured using VideometerLab4 and Tagarno instruments to do a comparison between methods. Measurements in Tagarno were made by calibrating the instrument for the desirable size (colony radial area). After that, a picture was taken by the software followed by a manual selection of the desirable colony area of the isolate by drawing lines in the picture around the desirable area (Fig. 5).

Figure 5: Illustration showing an example of the method for assessing colony radial area of *Verticillium dahliae* isolates using Tagarno. The petri dish was placed on the Tagarno. The digital microscope acquired a picture which was transfer to the software of the instrument on the computer. Measurement was made by manually rounded, with lines, the desirable area (colony radial area). After rounded the desirable area, result was presented in the middle in respective units, in this case mm^2.

Statistical analysis. All statistical analyses were operated by using R software (version 3.4.0) (R Core Team Development 2016).

In Experiment 1, analyses of variance were made on absolute growth rate. Analyses included: the effect of temperature on absolute growth rate of each *V. dahliae* isolate, the effect of temperature and isolate on absolute growth rate as well as their interaction and the effect of isolate on absolute growth rate at each temperature. Also, it was analysed if there was an effect of country on the absolute growth rate as well the effect of the interaction between country*temperature. Analyses of variance were conducted by using One-way and Two-way ANOVA and following GLM procedure. After analysis of variance, it was made a post-hoc analysis for mean comparison at a 95% confidence level using Fisher's Least Significant Difference (LSD) test. Means were not considered significantly different from each if $P > 0.05$.

In Experiment 2, a Bland-Altman plot (Dewitte et al., 2002) was created for estimating the agreement between the two methods of measurement. This plot displays the difference (A-B) or the ratio (A/B) between the measures against their average (A+B)/2)) called a difference plot, which is a scatter plot rotated 45 degrees clockwise. It is considered more informative than a scatter plot because it assesses the difference and the magnitude of the measurements. Instead of a statistical test, this plot intends to demonstrate both typical differences between measures and any patterns such differences may show. Although, a simple linear correlation shows strength of the linear association, it fails to but do establish how closely the two measures agree. Therefore, a high correlation does not necessarily imply that there is a good agreement between the two methods which makes Bland-Altman plot more ideal to assess the agreement. A horizontal line representing the bias is drawn at $\bar{d}$. Also, additional horizontal lines, known as limits of agreement, are added to the plot at $\bar{d} - 1.96 * S_d$ and $\bar{d} + 1.96 * S_d$, where S_d is the standard deviation. The d is given by differences of measurements formed as d = (A – B), as metioned above. Naturally, 1.96 represents the z-value that is used to form 95% limits of agreement.

Results

Validation of the method

In Experiment 2 (small experiment), it was compared two methods of measurement using the same isolate samples. It was plot a scatter to see the correlation between the two methods (Fig. 6).

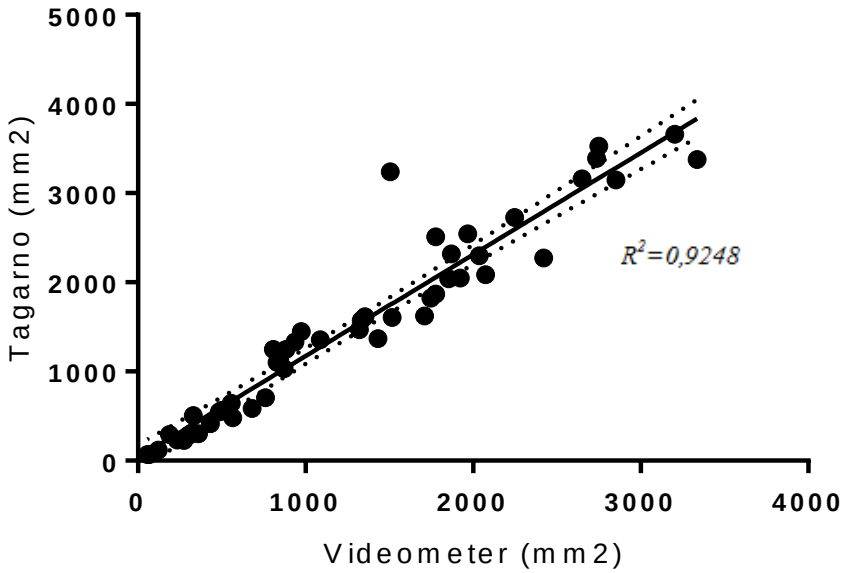

Figure 6: Scatter plot showing correlation between Videometer and Tagarno, N=50, R^2=0,92 ((P<0.0001)

Although there is a strong correlation between methods it does not imply that there is an agreement between them, as mentioned above. It is most improbable that the different methods will agree exactly, giving the exact result for all individuals. Therefore, the purpose it is to assess how much does the new method (Tagarno) differ from the old (Videometer) and see if this implys that substituting the old one for the new one would cause misinterpretation of the data.

Figure 7 shows a Bland-Altman plot with differences of measurements against their average. However, it is possible to see that the plot suggests a relationship between the differences and the average (magnitude), it starts narrow and then wides as magnitude increases. This suggest that variability of the differences increases with magnitude of measurements (non-constant variability). This is reflected in the wide Limits of Agreements presented on Fig. 7. In order to be able to make a Bland-Altman plot, it is assumed that the differences do not vary in any systematic way over the range of measurement which means that differences should not depend on the mean. Therefore, a

transformation of the measurements was made to remove the relationship and a Bland-Altman plot with ratio of measurements against average was made (Figure 7).

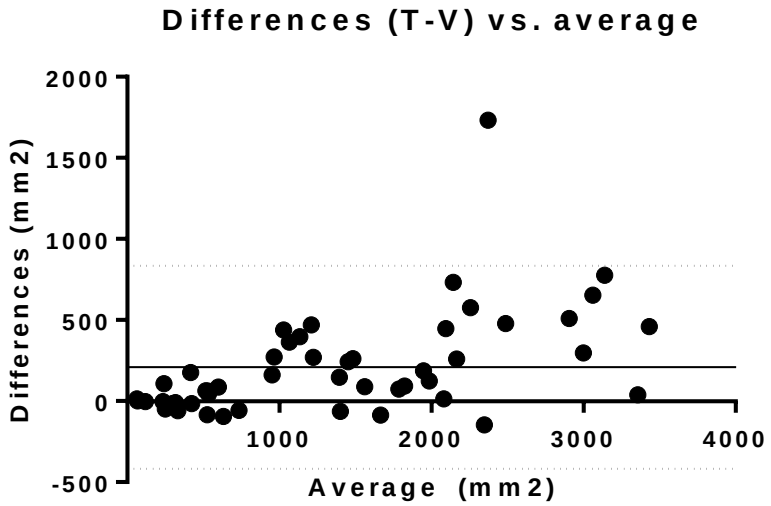

Figure 7: Bland and Altman plot of the data obtained from 50 paired samples analysed with Videometer and Tagarno (Bias = -208,4; Lower 95% Limite of Agreement = -418; Upper 95% Limit of Agreement= 834,7).

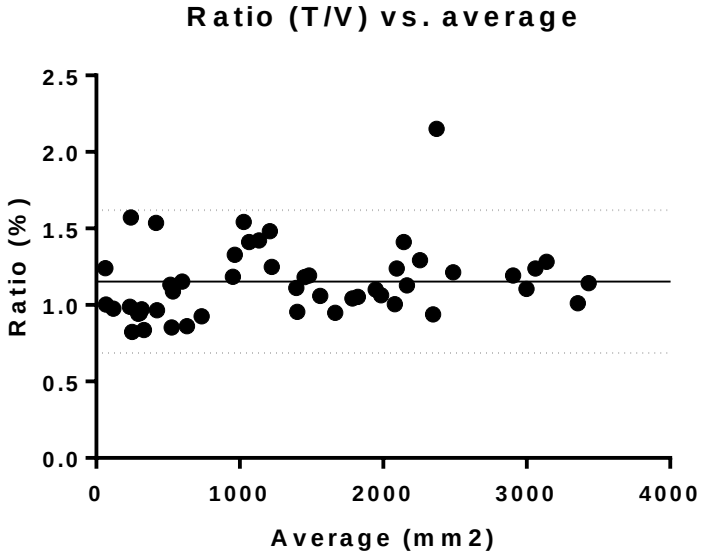

Figure 8: Bland and Altman Plot of the data obtained from 50 paired samples analysed with the Videometer and the Tagarno (Bias = 1,15; Lower 95% Limite of Agreement = 0,69; Upper 95% Limit of Agreement= 1,61).

Table 2: Fit differences of Bland-Altman plot with ratio against magnitude

Parameter	Estimate	95% CI	SE
Mean ratio	1,1526	1,08489 to 1,22034	0,03370
95% Lower LoA	0,6856	0,56905 to 0,80208	0,05798
95% Upper LoA	1,6197	1,50315 to 1,73618	0,05798
SD	0,2383		

Table 2 shows the fit differences of the Bland-Altman plot with ratio against magnitude of measurements. In this analysis report, it is possible to see that Tagarno measured 15% higher than the Videometer, the limits of agreement suggest that Tagarno method may measure from 7% to 61% above the Videometer method for 95% of the measurements. A hypothesis test with Pearson's r was made to see if the mean ratio was different from 0 (P<0,0001). Since it was significantly different from 0, adjusting 15% of the measurements below in the Tagarno method could make them more close to the measurements of the Videometer method.

Effect of temperature on growth rate of *V. dahliae* isolates

Table 3: Effect of temperature on radial growth rate of *Verticillium dahliae* isolates

Isolates	Country	Radial growth rate (mm2 per day)*			
		12°C	17°C	24°C	30°C
119	Denmark	61.7 (±4.7)^{b}	98.3 (±9.9)^{c}	152.8 (±21.6)^{a}	26.2 (±8.1)^{d}
24	United States	111.6 (±6.1)^{c}	193.5 (±8.5)^{b}	198.1 (±39.6)^{a}	12.6 (±2.6)^{d}
433	The Netherlands	54.8 (±7.1)^{c}	183.7 (±19.3)^{b}	270.3 (±44.2)^{a}	24.4 (±8.3)^{d}
582a	Chile	54.9 (±3.9)^{c}	100.1 (±10.9)^{b}	153.0 (±18.3)^{a}	33.6 (±14.8)^{d}
584a	Chile	75.1 (±7.0)^{c}	283.7 (±31.5)^{a}	263.0 (±14.8)^{b}	28.3 (±4.3)^{d}
588a	Chile	89.2 (±11.3)^{c}	231.4 (±43.1)^{a}	211.0 (±31.8)^{b}	29.4 (±5.0)^{d}
623	Italy	27.1 (±1.8)^{c}	79.6 (±9.2)^{b}	123.0 (±21.5)^{a}	8.1 (±4.2)^{d}
630	Denmark	71.5 (±5.6)^{c}	94.9 (±5.6)^{b}	233.9 (±40.4)^{a}	15.7 (±2.3)^{d}
662	Chile	70.0 (±7.4)^{c}	111.2 (±14.5)^{b}	151.3 (±14.8)^{a}	20.7 (±3.1)^{d}
666	France	48.1 (±1.3)^{c}	126.6 (±10.2)^{b}	133.0 (±9.1)^{a}	30.0 (±10.6)^{d}
88	Denmark	64.1 (±6.0)^{c}	166.7 (±15.2)^{a}	136.3 (±8.4)^{b}	30.5 (±12.3)^{d}
92	Denmark	39.8 (±6.3)^{c}	137.6 (±25.1)^{b}	204.0 (±6.6)^{a}	34.6 (±8.1)^{d}
WSU107	Denmark	58.7 (±7.2)^{b}	122.8 (±15.8)^{a}	121.5 (±13.0)^{a}	22.1 (±6.9)^{c}
WSU139	United States	56.0 (±11.2)^{c}	137.3 (±34,1)^{a}	110.3 (±19.0)^{b}	22.7 (±7.0)^{d}
WSU210	Denmark	74.6 (±3.4)^{c}	137.0 (±8.2)^{b}	382.5 (±25.1)^{a}	6.8 (±0.9)^{d}
WSU311	Denmark	74.6 (±5.4)^{c}	131.6 (±12.5)^{b}	169.7 (±27.6)^{a}	41.5 (±6.9)^{d}
WSU359	Denmark	53.0 (±4.1)^{c}	122.4 (±18.1)^{b}	225.8 (±14.2)^{a}	45.3 (±4.6)^{d}
WSU509	The Netherlands	65.0 (±9.6)^{c}	155.2 (±5.8)^{b}	160.4 (±11.0)^{a}	51.4 (±3.9)^{d}
WSU523	The Netherlands	74.4 (±8.1)^{c}	170.6 (±7.0)^{b}	195.5 (±5.0)^{a}	8.3 (±3.4)^{d}
WSU541	The Netherlands	59.1 (±3.7)^{c}	120.2 (±17.0)^{b}	133.6 (±16.5)^{a}	15.0 (±6.8)^{d}

* Linear growth rates. Mean of 4 replicates with S.E.
The growth rates in the same row followed by the same letter are not significantly different (P < 0.05) according to Fisher's Least Significant Difference (LSD)

Table 3 shows that the absolute radial growth rate fluctuated from 6.8±0.9 mm^2 per day (WSU210 at 30°C) to 382.5±25.1 mm^2 per day (WSU210 at 24°C). It was observer that all isolates grew between 12°C to 30°C. The "optimal temperature" was in majority 24°C, followed by 17°C then 12°C and 30°C showing the less growth rate. Nevertheless, isolates at 30º showed less differences in the growth rate compared with the other temperatures. In addition, 4 isolates showed maximum growth rate at 17°C and 15 isolates showed maximum growth rate at 24°C. One isolate (WSU107) did not show significant differences of maximum growth rate between 17°C and 24°C, therefore it was not possible to assess where the maximum growth rate occur between these two temperatures. Table 3 also shows that the effect of temperature was different in the isolates from different countries e.g. isolate 623 from Italy showed a growth rate of 27.1±1.8 mm^2/day at 12º compared with isolate 119 from Denmark which showed a growth rate of 61.7±4.7 mm^2/day at 12º. At the same time, it was possible to establish differences between isolates from the same country e.g. isolate WSU311 from Denmark showed a growth rate of 41.5±6.9 mm^2/day at 30º while isolate WSU210 also from Denmark showed a growth rate of 6.8±0.9 mm^2/day at 30º. Moreover, isolate WSU509 showed the greater growth rate at 30º with 51,4±3,9 mm2/day compared to the other isolates while isolate 24 showed the greater growth rate at 12º with 111,6±6,1 mm2/day. Isolates 584a, 588a, 88 and WSU139 showed a maximum growth rate at 17º (Fig. 11) while the rest of isolates (except isolate WSU107) showed a maximum growth rate at 24º (Table 3).

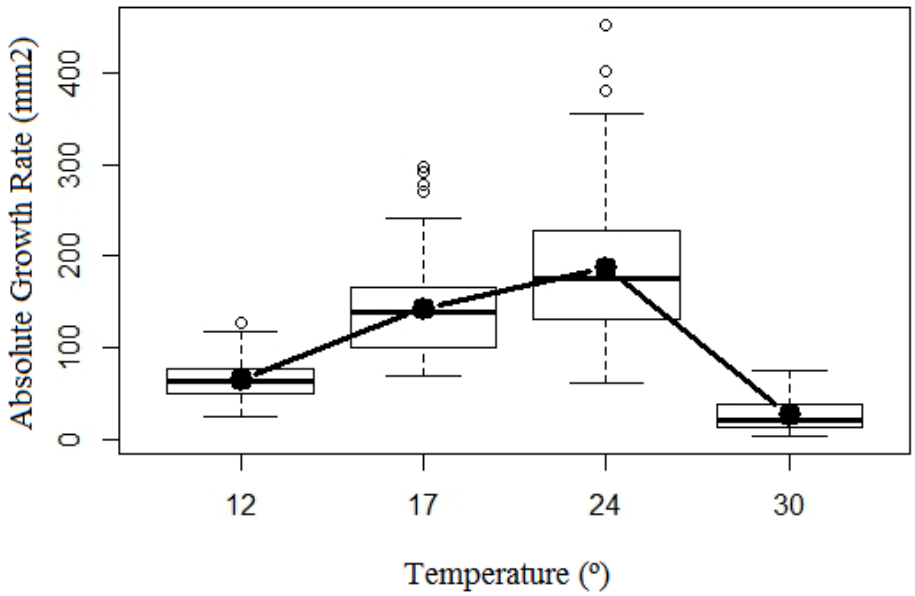

Figure 9: Boxplot representing the effect of temperature on absolute growth rate of *Verticillium dahliae* isolates. Black dots represent the means of absolute growth rate of all isolates in each temperature (64.49 at 12ºC, 143.23 at 17ºC, 186.44 at 24ºC and 25.42 at 30ºC) Means were significantly different from each other according to Fisher's Least Significant Differences (P <0.05)

Temperature (F = 1231.18; df 4, 238; P < 0,0001) and isolate (F = 11.51; df 19, 238; P < 0.0001) effects were significant with a strong interaction between temperature*isolate (F = 5.94; df 57, 238; P <0,0001) for *V. dahliae* isolates (Fig.9). In addition, it was possible to see a country effect (F=4.61; df 5, 294; P < 0.0001). However, the interaction between temperature*country was less significant (F=1.60; df 15, 294; P < 0.1) which indicates that the effect of the interaction of temperature and country is not possible to establish in this study.

Comparisons of the absolute growth rate at each temperature suggest that even a possible significant difference between isolates was found even at temperatures considered ideal for growth rate (e.g. isolate effect at 17°C F=59.11, df 20, 59; P < 0,0001 and at 24ºC F=73.28; df 20, 59 P < 0,0001). Nevertheless, it was possible to see that differences between isolates were greater at 12ºC (F=107,56, df 20, 60; P<0,0001) and lower at 30º (F=11,81, df 20, 59; P < 0,0001) (Table 3, Fig. 10).

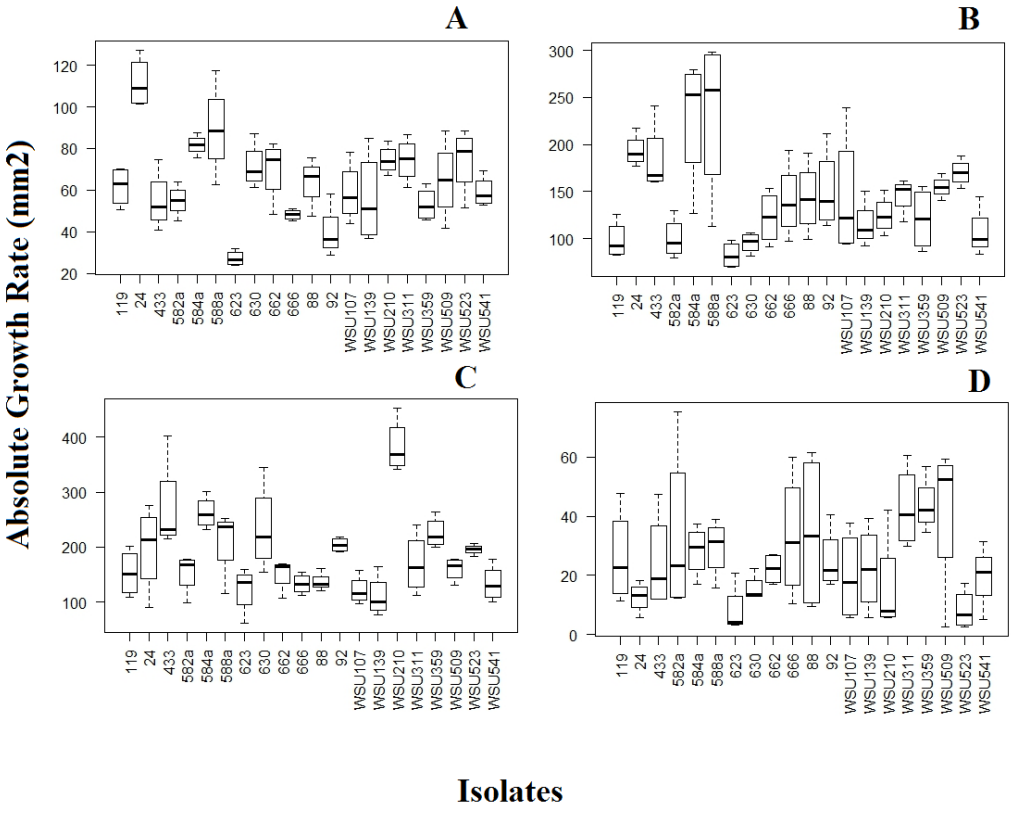

Figure 10: Boxplots representing differences in the absolute growth rate of the different isolates of *Verticillium dahliae*. A) at 12Cº; B) at 17ºC; C) at 24ºC and D) at 30ºC. Table 1 gives a detail information of each isolate.

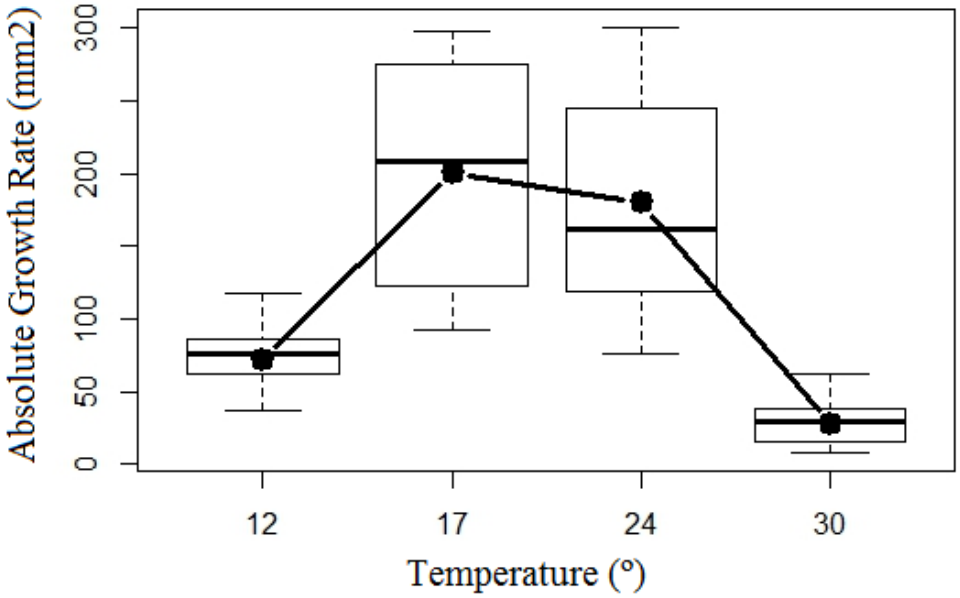

Figure 11: Boxplot representing the effect of temperature on absolute growth rate of *Verticillium dahliae* isolates that had higher growth rates at 17ºC (584a, 588a, 88, WSU139). Black dots represent the means of absolute growth rate of all isolates in each temperature (72.70 at 12ºC, 200.47 at 17ºC, 180.11 at 24ºC and 27.71 at 30ºC) Means were significantly different from each other according to Fisher's Least Significant Differences (P <0.05)

Despite having a higher absolute growth rate at 17º, isolates 584a, 588, 88, WSU139 also show a great variability at 24º. Temperatures 12º and 30º continue to show lower absolute growth rates with the latter showing smaller values and less variability between isolates.

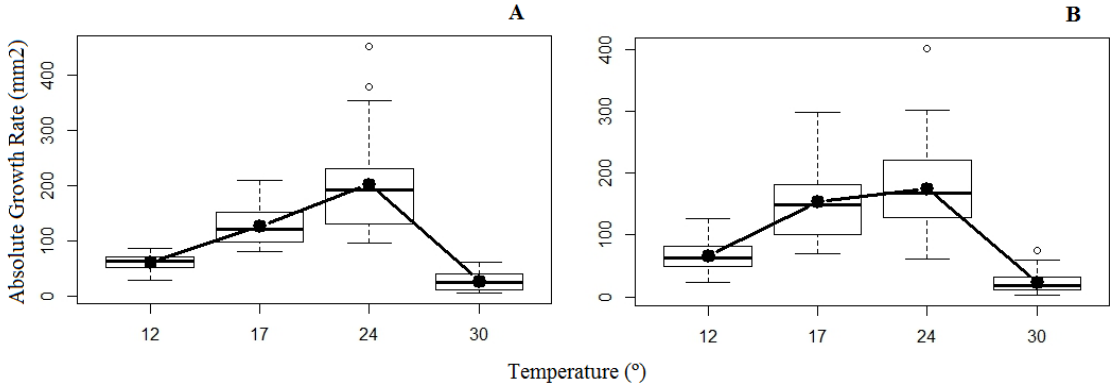

Figure 12: Boxplot representing the effect of temperature on absolute growth rate of *Verticillium dahliae* isolates **A)** from Denmark (88, 92, 119, 630, WSU107, WSU210, WSU311, WSU359) and **B)** from other countries. Table 1 shows a information about isolates. Black dots represent the means of absolute growth rate of all isolates in each temperature **A)** Means were 62.23 at 12ºC, 126.84 at 17ºC, 203.30 at 24ºC and 27.84 at 30ºC **B)** Means were 65.98 at 12ºC, 153.32 at 17ºC, 175.20 at 24ºC and 23.78 at 30ºC. Means were considered significantly different from each other according to Fisher's Least Significant Differences (P <0.05).

It was possible to establish significant differences between isolates of *V. dahliae* from Denmark compared with isolates from other countries. The higher growth rate for both was observed at 24º, followed by 17º then 12º and finally 30º. Nevertheless, isolates from Denmark showed less variability at 12ºC, 17ºC and 30ºC compared with the rest of the isolates. By contrast, isolates from Denmark showed higher variability at 24º compared with isolates from other countries. As well the mean of isolates from Denmark (203.30 mm2) was higher compared with isolates from other countries (175.20 mm^2). Differences between means of absolute growth rate at 17ºC and 24ºC were greater in isolates from Denmark (126.84 mm^2 and 203.30 mm^2, respectively) compared with isolates from other countries (153.32 mm^2 and 175.20 mm^2, respectively).

Discussion

Validation of the method.

When conducting a study, the question about what method of measurement should be used is always common between researchers. This matter is important because the answer to this question will decide the course of the study and can be a thinner line between success and failure (Holman and Gajda, 1994). The two methods studied were significantly different from each other with Tagarno showing measurements 15% higher than Videometer. A study conducted by Myers (1978) showed that there were variations in the mearument using Tagarno. Therefore, since measurements with Tagarno were made manually (area was selected by drawing lines around the growth area), this can suggest that Tagarno is less precise than Videometer. A study conducted by Lay-Ekuakille *et al.* (2014) showed that measurements using Videometer could improve the precision of measurement. In addition, Tagarno method could also be considered more subjective than Videometer e.g. the area that is selected can be different between researchers which would increase the error of measurement. Also, the visual perception of two researchers could influence the selection of the desirable area (Bland and Altman, 2007).

Although an error of 15% higher measurements could be considered not significant for some researchers, this error could make the decision between measurements with or without a strong statistical significance. Other researchers would infer that Tagarno could be use by adjusting its measurements 15% lower to be closer to measurements of Videometer. This would be ideal if the Videometer method was a time consuming instrument which was not the case.

Furthermore, despite being a good method to assess agreement between two methods, Bland-Altman plot system does not say if the agreement is sufficient or suitable to use a method or the other indifferently (Bland and Altman, 1986; Dewitte et al., 2002). It only quantifies the bias and the limits of agreement in which 95% of the differences between one measurement and other are included. The bias between these two methods is significant (line of equality is within the confidence interval of the mean difference) (Fig. 8). However, biologically it is not possible to define if the agreement interval is to wide or sufficiently narrow for the purpose in question. One way to contour this is to define a priori which are the limits of agreement expect based on biologically criteria and then see

if the limits are exceeded or not. Also, since Videometer is not considered a "reference" method, it is not possible to assess if one method is better than the other (Tagarno) because there is not a possibility to specify which one provides the right value for measurement (Bland and Altman, 1986).

Improvements could be made in this study to promote significant and more powerful results. The number of measurements used N=50 was probably too small for the purpose of this study. Therefore, an increase of measurements e.g. N=300 could improve the study and give a better agreement between the two methods. The CI and LoA indicate the uncertainty in the estimates. The wide intervals are related to a small sample size and large variance of differences. In this way, even with an optimistic interpretation, the agreement can be considered unacceptable for the reasons above. The use of repeatability or variation of repeated measurements on the same object under identical conditions over a short period is important because it has a direct effect on the agreement of the methods tested. In order to assess repeatability, two or more measurements should be done on each method on each object. If one method has poor repeatability, the agreement of both methods will also be poor. If both methods have poor repeatability, the agreement will even be worse. When comparing two methods, if the first one has a poor repeatability, even a perfect second method would not agree with it. When using repeated measurements, it is essential to consider the reduction in standard deviation due to averaging of the measurements. Standard deviation must be considered and adjusted if necessary. Repeatability of the measurements between methods was not assembled in this study which could have influence the results. Therefore, repeated measurements for both Videometer and Tagarno methods should be made to assess the agreement between them.

Effect of temperature on growth rate of *V. dahliae* isolates

To have a better understanding between climatic factors and survival of a pathogen is essential to establish the tenacity of the infection (Lukas et al., 2014). The effect of temperature on radial growth of *V. dahliae* was studied. Most of the isolates of *V. dahliae* studied had an optimal temperature at 24ºC (Table 3; Figure 9). Pegg and Brady (2008) demonstrated that optimal growth of *V. dahliae* isolates in vitro were between 22º and 25º. As well both studies conducted by Fayazalla *et al.* (2008) and Subbarao *et al.* (1995) placed optimal temperature around 20º-25º. This study comes to an agreement with these studies, yet demonstrating that the range of optimal growth rate could be extended to 17º-25º. On the other hand, some studies (Soumia et al., 2014; Wheeler, 2015) established

that *V. dahliae* grows in both temperate and sub-tropical environments, yet there is still no agreement in classifying the optimal temperature conditions for growth of this soilborne pathogen in the field.

Moreover, growth rate responses to temperature varied between isolates, some showed wide ranges between temperatures while others showed more restrictedness (Table 3; Figure 9). In this study, isolates from Denmark showed less variability at 12ºC, 17ºC and 30ºC compared with isolates from other countries (Fig. 12). By contrast, these isolates more variability at 24ºC compared with isolates from other countries (Fig. 12). Studies (ElSharawy et al., 2015b; Wheeler and Johnson, 2016) have demonstrated that *V. dahliae* isolates can have different behaviour in the field e.g. showing different disease symptoms amongst hosts. A study conducted by Tyvaert *et al.* (2014) showed that an isolate of *V. dahliae* could even protect against Verticillium wilt caused by *V. longisporum* in cauliflower. Another study conducted by Gharbi (2015) showed that there was an association between the genetic diversity of the isolates and their geographic origin in olive orchards, however an association between genetic diversity and virulence patterns was not possible to establish. Therefore, temperature effect could even influence more this variability of behaviour between isolates. Whether these changes would have positive or negative impacts it is not known. In addition, studies (Brown et al., 2014; Heitman, 2015) revealed that effect of temperature could even go further and affect evolutionary and developmental characteristics. Nevertheless, evolution aspects were not analysed in this study.

A study conducted by Deacon (2006) showed that fungi can tolerate temperatures below optimal conditions if other growth requirements were met e.g. nutrients. This effect was not shown in *V. dahliae*; however, it was possible to see that in temperatures below and above optimal condition there was a significant growth rate (Table 3, Figure 9) which could indicate that even under optimal conditions, this soilborne pathogen will still grow. Also, at 12ºC there was higher differences between isolates which could suggest a possible survival of the pathogen at temperatures below optimal conditions. Nevertheless, in this study it was not assess what are the minimum and maximum temperatures in which is possible to see a growth rate. A study conducted by Devaux and Sackston (1966) showed that growth of *V. dahliae* occurred at 30º C, however the production of microsclerotia was inhibited. In this study, it was not assessed the effect of temperature on the formation of microsclerotia. However, a study conducted by Willetts (1971)

reported that sclerotia appear to have a survival under cold conditions rather than under hot conditions. This effect was not yet described in *V. dahliae*.

Furthermore, in this study it was not demonstrated how temperature could affect the infection rate or if it would be possible to establish an effect at all. This is important to comprehend because even if temperature influences the growth rate of the pathogen, it could have a little or even no influence on the infection rate. Some studies about fungal pathogens such as *Pseudogymnoascus destructans*, *Fusarium* spp. and *Bipolaris sorokiniana* (Granett et al., 2015; Langwig et al., 2015; Lendenmann et al., 2016; Manici et al., 2014) demonstrated that an increase of temperature can influence the growth rate and consequent the infection rate. Yet, this effect was not demonstrated in *V. dahliae*. Therefore, there are no evidences suggesting that a higher growth rate caused by the increased temperature would initiate higher infection rates in this soilborne pathogen.

Assessing effects of climatic factors is always complex because of several reasons explained before. Given that this study was made in vitro, results of the same experiment made on the field can turn out differently. Even if temperature affects the growth rate in vitro, in the field the combination of different climatic factors e.g. CO_2, UV radiation, precipitation could display a different effect. Some studies (A'Bear et al., 2014; Ghini et al., 2015) demonstrated that climatic factors can increase or decrease the effect of the other or even neutralise each other e.g. levels of CO^2 or ozone can counteract the effect of temperature.

Predicting the fungal pathogen ability to sustaining its population in the field conditions is difficult. There are interactions such as competition with other organisms and responses of the host plant to infection that can affect the survival of the fungus. Some studies (Depotter et al., 2016; Frederick et al., 2017) demonstrated that temperature effect could influence the interactions between pathogen and host and also with other microorganisms. A study by Calderón (2014) showed that soil temperature can determine the reaction of olive cultivars to *V. dahliae* pathotypes. In addition, a study conducted by Tjamos (1987) showed that soil solarisation could possibly influence *V. dahliae* by enhancing the survival of antagonists of this pathogen. The activity of these antagonists could inhibit the germination of microsclerotia or cause the death of *V. dahliae*. However, it is not known to what extend these effects of interactions between pathogen, host and other organisms may happen.

A study conducted by Xu *et al.* (2012) showed that temperature could possibly influence on the aggressiveness of the pathogen. Also, a study made by Reusche (2014) showed that temperature could also have a possible effect on the aggressiveness of the disease symptoms. Moreover, aggressiveness in vivo and in vitro tests of some fungi revealed that the highest growth rate was correlated with the highest disease index (ElSharawy et al., 2015a). As demonstrated by this study, if temperature influences the growth rate, it could also indicate that it would influence the disease index. However, this effects were not reported in *V. dahliae.*

Knowing that the temperature was an effect on the development of the pathogen can lead to more studies to assess possible changes in the life cycle caused by temperature. For example, in some fungi of *Ascomycota* phylum temperature can have an effect on the reproduction by changing from asexual to sexual reproduction (Garrett et al., 2014; Newsham et al., 2016). This effect has not yet been demonstrated in *V. dahliae*.

Additionally, knowing that there is an effect of temperature on *V. dahliae* could improve the develop of management strategies to control and prevent Verticillium wilt. Studies made by Calderón et al. (2014) and Pullman et al. (1981) showed that changing the temperature on the soil could influence the incidence of the disease by reducing the number of inoculum density in the soil. A study conducted by Tjamos (1987) reported that soil solarisation had a long-term effect in controlling *V. dahliae* for a period of 2 or 3 years. However, knowledge about the applicability of soil solarisation and its effects on crops is still very scarce. Moreover, a study conducted by Morello (2015) showed that the use of moist hot air treatments in olive trees at 42-44°C for 6–12 h eradicated the pathogen, without compromising the viability of the plants.

Improvements on this study could be made to increase the significance of the results. The selection of 20 isolates for a study like this might be considered a small sample size. One way to improve would be increase the sample size of *V. dahliae* isolates e.g. to 50 isolates of *V. dahliae*. In this way, differences in growth rate could be more significantly pronounced. Also, the use of 4 replicates could be reflected on showed standard error (SE) in the different means. This could be counteracted by increasing the number of replicates. In addition, increasing the number of treatments (temperature) could increase the significance of the effect of temperature. e.g. the use of 8 temperatures in a range of 10º to 40º with 5º of difference, which was not possible in this study due to the lack of temperature chambers. Moreover, more measurements (daily or hourly) could improve

the significance of the observed growth rate showing less standard error. Nevertheless, these improvements would not be possible to do within the time span of this study. Yet, they could be used for improving studies in the future.

Conclusion

In conclusion, the present study showed that, by placing different isolates of *V. dahliae* in four different temperatures, there was a possible effect of the temperature on the growth and development of each isolate. This was evidenced by different growth rates in each temperature, suggesting that an effect was present. Too, it was possible to see a difference of growth rate even in the same temperature, suggesting the variability of isolates of *V. dahliae.* Likewise, growth also happened in temperatures which are considered extreme to *V. dahliae* (12ºC and 30ºC), suggesting that *V. dahliae* could have tolerance toward temperatures below optimal conditions. Thus, this study presented results that support the idea that variability in the climate (temperature) could possibly have an effect on the growth of the pathogen. This effect comes in evidence with other studies suggesting the same results.

Since Denmark is the major supplier of spinach seeds and has a temperate environment, this study could suggest that the increase of temperature in the next years could possibly increase the growth rate of the pathogen in the field. If temperature in Denmark in the spinach growing season will rise, this will provide a wide range of temperatures that will be favourable to the pathogen development. Whether this could affect the aggressiveness and the infection rate of this pathogen was not yet assessed. Studies about the effect of temperature in microsclerotia fn spinach seeds were not assessed in this study.

Subsequent, this study could establish the effects of climate change on pathogen activity and directly contributing to development of mitigation strategies that could prevent and control this soilborne pathogen.

Furthermore, this study permit the advantage of interdisciplinary research approach, combining environmental management with microbiological study of the *V. dahliae* and plant pathology.

Additionally, the comparison of two methods of measurement demonstrated that significant differences can happen, implying that altering methods without testing their agreement could result in erroneous results. Therefore, this study suggests that assessing the agreement between two methods of measurements is important in the decision of selecting the right method for an experiment.

Future perspectives

The insights provided by this study can promote a range of further research regarding the interaction between pathogens and environmental components. New research should focus on the effect caused by temperature on the pathogens and their growth and development.

Moreover, it is essential to carry on researches about the role of *V. dahliae* on agro-systems and how it affects crop production. To conduct favourable management practices based on decreasing negative impacts on the rhizosphere by soilborne pathogens in this research area, the focus should be located, firstly, on laboratory experiments using natural microbiota. Although, field experiments are difficult and challenging, they are necessary to understand the interaction between hosts and pathogens and further adapt to management practices against these soilborne pathogens.

The results of this study were obtained by using 20 isolates of *V. dahliae* from different countries, the use of more isolates of *V. dahliae* could potentially produce different results. Therefore, further studies with focus on using a higher number of isolates to assess the effect of temperature could provide more insight. Although the isolates used in this study were selected based on different countries and VCG to show a more diversity of isolates, this study did not establish an effect on this factors. Therefore, further research should also focus on using isolates based on VCG and see if there is a temperature effect on isolates from different VCG. In addition, research to assess if temperature could influence the anastomosis of isolates from the same VCG could improve the knowledge about this pathogen. Moreover, this study was based on isolates from spinach due to the major interested from Danish seed companies to understand the interaction between spinach and *Verticillium* spp. Hence, further studies could focus on establishing an effect of temperate on isolates of different species that are pathogenic e.g. the effect of temperature on the growth of isolates of both *V. dahliae* and *V. albo-atrum*. Since both species have a wide range of hosts, it could be also pertinent to see the effect of temperature on different isolates from different hosts and from different countries.

Based on the observed growth rates at low and high temperatures (12ºC and 30ºC, respectively), research could focus on the adaptation and evolution processes of this pathogen. Since some studies suggested that climatic factors can alter the behaviour and life cycle of some microorganisms, it could be interesting to assess if there would be

changes in *V. dahliae*. In addition, adaptation studies of *V. dahliae* could promote a better understanding of development and activity of this soilborne pathogen. Also, research about tolerance, if present, of *V. dahliae* to low or high temperatures could be conducted in order to assess its behaviour at temperatures below optimal conditions.

Many studies showed that climatic factors can influence aggressiveness and infection rate. Hence, research should also focus on the possible effect of temperature on aggressiveness and infection rate caused of *V. dahliae* isolates. Furthermore, many studies established the effect of isolates on decreasing the symptoms of other isolates from the same or different species. Hence, since this study also showed that isolates had different responses to a determined effect, research could focus on assessing if isolates could counteract the effect of others, endorsing the use of these isolates as biological control.

Although it is certain that the incidence of climate events is increasing, there are still little knowledge about the possible effects of this increase. Nevertheless, many studies are being conducted to predict these possible effects. Predicting the impacts of climate change is challenging, especially because laboratory experiments can only focus on one or two factors at a time. This comes as a constraint because in the field these climatic factors do not occur separately but together. Therefore, research should focus on trying to assess the combination of more than two factor. Only then a more realistic approach could be made about the effects of climate change.

So far, studies have shown that *Verticillium* wilt can endorse yield loss in crop production. However, assessing the effect of this disease is challenging because sometimes it can be symptomless or show symptoms similar to other pathogens. In addition, in some crop harvesting can occur before the early sign of symptoms which can difficult the diagnosis of this disease. Therefore, research could focus on trying to assess what triggers the starting of symptoms and see if factors such as temperature could have an effect on that. The results of these studies could then be adapted to management practices that could reduce the incidence of this disease.

Meanwhile, microsclerotia is the most problematic stage of *V. dahliae* life cycle, since it can survive in the soil for up to 10 years. Therefore, research regarding the effect of temperature on microsclerotia of different isolates of *V. dahliae* could be conducted. Also, studies could try to assess the possible combination of effects such as temperature and

moisture on microsclerotia. In this way, possible management strategies could be adapted that would promote the decrease of microsclerotia in the soil.

Lastly, there is still scarce knowledge about the biology of *V. dahliae*. For example, there is still no agreement about the optimal conditions for growing of this pathogen in the field e.g. the optimal conditions for the germination of microsclerotia. As well, there is little knowledge about the reproduction of *V. dahliae*. Hence, research should, primarily, focus on the biology of this pathogen and assess its main physiology and molecular aspects before trying to develop mitigation strategies to prevent and control it. Only then, the develop of more successful, sustainable and enduring management practices of this pathogen will be possible.

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Appendix

While conducting the experiment of the effect of temperature on growth rate of *V. dahliae* isolates another experiment was started. This experiment had the aim to see the effect of temperature on the infection of *Arabidopsis thaliana* using different isolates of *V. dahliae*. Nevertheless, time constrains led to the impossibility obtain results and finish this experiment on time which consequently led to a halt of it. Some of its procedure will be presented in here.

The aim of this experiment was to assess the effect of temperature on the infection of isolates of *V. dahliae* in *Arabidopsis thaliana*. The main hypothesis was that "there is an effect of temperature on the infection of *V. dahliae* in *Arabidopsis thaliana*"

Experimental setup. To assess the effect of temperature, an experimental setup was made which predicted the use of two wild types of *Arabidopsis thaliana* (Col-0 and Ler-0) and one double mutant B2B3 (susceptible), 2 isolates of *V. dahliae (*789 and 812*)* + control (distilled water) in three different temperatures (16ºC, 23ºC, 28º) with 5 replicates for each isolate + control. Therefore, the final experimental setup used was a total of 135 plants (3 genotypes x 3 isolates (2 isolates + control) x 5 temperatures x 3 replicates).

Isolates and formation of spores. Isolates of V. dahliae used were 789 and 812 provided by Flakkebjerg Research Centre. Table 1 shows information about these isolates. Fungal discs of 1 cm of each *V. dahliae* isolate were placed on different glass bottles (around five glass bottles per isolate) with Potato Dextrose Broth (4 g of potato infusion from 200 g potatoes, 20 g of dextrose, final pH: 5.1 ± 0.2 at 25°C). PDB glass bottles were prepared by dissolving 24g of medium in 1L of distilled water, mixed and then autoclaved. After adding fungal discs, each glass bottle was placed on a laboratory shaker in a dark room with a constant temperature of 17ºC for stimulate the production of spores.

Table 1*:* Origin of the isolates of *Verticillium dahliae* used in this experiment

Species	Isolate code	Host of Origin	Country of Origin	VC G	Ref. Paper
Verticillium dahliae	789	Olive	Spain	4B	Collado-Romero et al., 2006
Verticillium dahliae	812	Olive	Cyprus	2A	Collado-Romero et al., 2007

Cultivation of plants. It was used two wildtypes of *Arabidopsis thaliana*, Col-0 (Colombia), the most used wilt type of *A. thaliana* and Ler-0 (Landsberg erecta), the second most used wilt type, from the seed lot N22625 and N76164, respectively provided by Flakkebjerg Research Centre. It was also used a double mutant B2B3, a mutant with reduced auxin levels, of *Arabidopsis thaliana*. *Arabidopsis thaliana* was grown in 12-cm-diameter plastic pots filled with soil. After watering the soil, five distributed seeds were sown on the surface of each pot, to increase the probabilities of germination without compromising the area for germination. Approximately 450 (90 x 5 seeds) plants were sowed for genotypes Col-0 and Ler-0 according to the experiment setup (2 genotypes x 3 isolates (2 isolates + control) x 5 replicates x 3 temperatures). Additionally, 15 plants of double mutant B2B2 of *Arabidopsis thaliana* were sown, however due to lack of seeds, it was only used as a positive control. Pots were then covered with plastic and kept up to three to four days in a hibernation room at 4ºC before placing in a greenhouse during two to three weeks for growing.

Inoculation of plants. Plants were inoculated two to three weeks after sowing, after showing four to five leaves. For inoculum, it was used glass bottles, previously mentioned, which were filtrated, centrifugated and quantified for the concentration of spore. Isolate of *V. dahliae* with code 789 had a concentration of $3,7 \times 10^6$ and isolate of *V. dahliae* with code 812 had a concentration of $1,9 \times 10^6$. Then, it was left one germinated plant per pot and extract the others, with 90 pots each having 90 plants. It was made two small holes right next to the plant, without damage it and then with the help of a micropipete of 1000 μm it was inoculated 1 mL (0,5 mL on each hole) of inoculum per plant. 30 plants were inoculated with distilled water (control), 30 plants were inoculated with inoculum of *V. dahliae* isolate code 789 and 30 plants were inoculated with inoculum of *V. dahliae* isolate code 812. After inoculation, plants were placed in three growth chambers each with different temperatures, 16ºC, 23ºC and 28ºC during three weeks.

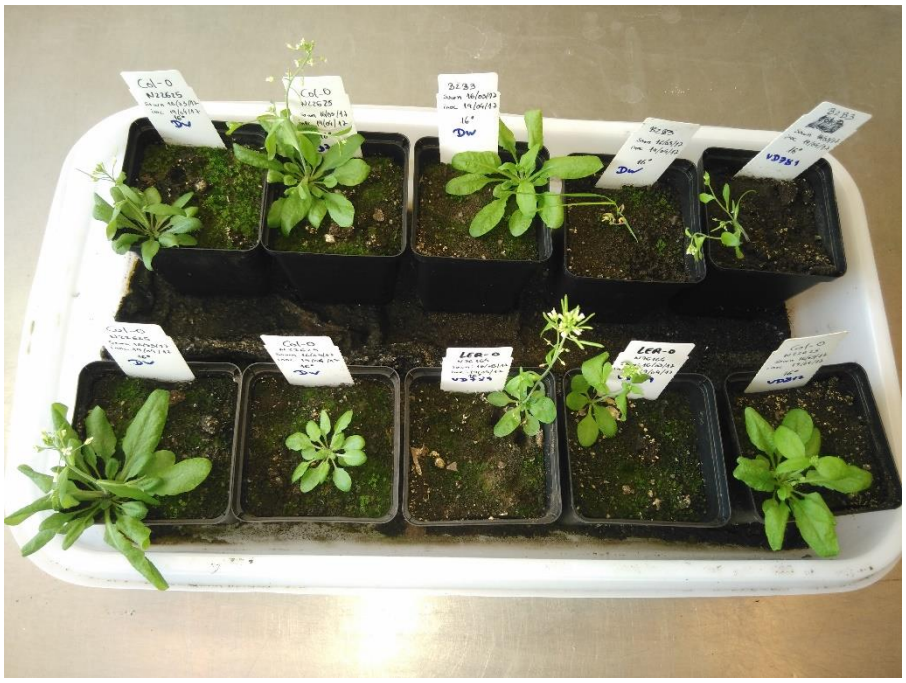

Figure 1: Example of *Arabidopsis thaliana* plants inoculated with isolates of V. dahliae (789 and 812) and distilled water and placed in growth chamber at 16º after three weeks

Figure 2: Example of *Arabidopsis thaliana* plants inoculated with isolates of *V. dahliae* (789 and 812) and distilled water and placed in growth chamber at 28º after three weeks

Sampling and DNA extraction. After the period on the growth chambers, separation of root material from stem material was made, using the latter for DNA extraction. Before DNA extraction, stem material from each plant was place for 1h in -80ºC, then placed for 3 days in a freeze dryer for water removal.

After sampling and placement in freeze dryer due to time constrains it was necessary to end the experiment, as mentioned above.

The next step would be to place the samples in the Geno/Grinder for preparation for DNA extraction. After that, a quantitative polymerase chain reaction (qPCR) was going to be made on each sample of stem material to quantify the presence of infection by *V. dahliae* isolates. Since the symptoms caused by *V. dahliae* are similar to symptoms of other pathogens, using a qPCR would testify the presence of *V. dahliae* on the samples.