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pH modulation of the environment by *Stagonosporopsis cucurbitacearum*, an important pathogen causing fruit rot in *Cucurbitaceae*.

S. Van Laethem[1] M. Frans[1] R. Aerts[1] J. Ceusters[1, 2]

[1] KU Leuven, Campus Geel, Faculty of Engineering Technology, Department of Biosystems, Research Group for Sustainable Plant Production and Protection, Kleinhoefstraat 4, 2440 Geel, Belgium (E-mail: sofie.vanlaethem@kuleuven.be, Phone: +3214 562310)

[2] UHasselt, Centre for Environmental Sciences, Environmental Biology, Campus Diepenbeek, Agoralaan Building D, 3590 Diepenbeek, Belgium

ABSTRACT

The *Cucurbitaceae* is a genetically diverse group of plants containing several important commodity crops in many parts of the world such as cucumber, pumpkin and melon. In the last decades, fruit rot caused by *Stagonosporopsis spp.* became a major disease in both field grown and greenhouse grown cucurbits. Yield losses due to *Stagonosporopsis* can show seasonal peaks up to 30%. Despite its economic importance, only limited information is available about growth characteristics of *Stagonosporopsis cucurbitacearum*. Our *in vitro* studies with different media indicated an optimal growth rate of the fungus within the range of pH 5 to pH 6. Independent of the carbon source (sucrose, glucose, dextrose, fructose) alkalization of 1-3,5 pH units was noticed under both carbon deprivation and excess. The observed pH modulation could not always be related with a more favourable growth environment. The key factor influencing both pH modulating capacity and growth showed to be the nitrogen source. Supplying nitrate, ammonium or a combination of both, the environmental pH respectively increased, decreased or remained stable. In addition to a pH elevating effect nitrate supply did also stimulate growth whilst growth on ammonium containing media was seriously affected. This research highlights the importance of the nitrogen source in the growth and regulation of environmental pH by fungi and adds in our understanding of *S. cucurbitacearum* pathogenicity.

Key words: *Didymella bryoniae*, *Stagonosporopsis cucurbitacearum*, internal fruit rot, C*ucurbitaceae*, pH modulation

INTRODUCTION

The Cucurbitaceae is one of the most genetically diverse group of plants containing several important commodity crops in many parts of the world, such as cucumber (*Cucumis sativus* L.), pumpkin (*Cucurbita* spp.), melon (*Cucumis melo* L.), and watermelon (*Citrullus lanatus*) (Robinson & Decker-Walters, 1997). Production of cucurbits is under jeopardy by a fungal disease caused by *Stagonosporopsis cucurbitacearum* which can infect at least 12 genera and 23 species of the Cucurbitaceae, and occurs in temperate regions including North America, Europe, Asia and New Zealand. First reports of this disease in cucurbits in Europe date back to 1823. The pathogen may originally have been introduced to Europe on papayas brought from the Americas (Keinath, 2011). It is still an important field disease in subtropical and tropical growing areas, and a serious concern for glasshouse cucumber growers in temperate regions since production intensification in the late 1960's (Corlett, 1981, Robinson & Decker-Walters, 1997). Nearly all growers in temperate regions are confronted with this problem and average yield losses are estimated at an average of 5% with seasonal peaks in heavy rainfall periods up to 30% (Keinath, 2000).

Over the years, the nomenclature and classification of the pathogen has been subject to change. Initially, in 1881, Heinrich Rehm classified the teleomorphic phase of the fungus to the genus *Didymella*. In 1949; Chiu and Walker re-identified it as *Mycosphaerella melonis*. Following DNA sequencing by Aveskamp, Gruyter and Verkley in 2010, the anamorphic phase of the fungus appeared to belong to the genus *Stagonosporopsis* instead of *Phoma* (Stewart et al., 2015). In an infection site, the anamorph *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, and teleomorph *Didymella bryoniae* (Auersw.) Rehm, can occur at the same time. *Stagonosporopsis* is a typical member of the ascomycetes and can produce both ascospores and conidiospores which can both cause infections. The initial infections can occur on all plant parts, except the roots, and are typically caused by airborne ascospores. The pathogen causes a variety of symptoms, including leaf spots, stem cancers, vine wilt and black fruit rot (Van Steekelenburg, 1982). Spores are usually produced within four to eight days after initial infection, causing a new cycle of secondary infections shortly after (Miller et al., 2010). Within 24 h after germination, infection hyphae and appressoria are produced. The hyphae produce various enzymes such as amylase, lipase, protease, urease and polygalacturonase, causing plant cells to degrade and thereby creating moist lesions (Tsay et al., 1990). *Stagonosporopsis* can infect the fruit in several ways. The first type of infection is characterized as external fruit rot, and results from infections on mechanical injuries. The second type of externally invisible infection is known as internal fruit rot, which occurs when airborne spores attach on the stigma of the pistil. *In vitro* germination studies have indicated that 50 % of the spores can germinate within a time frame of just 9 h, whilst four hours later germination was nearly 100 % (Van Laethem et al., 2019). Germination *in vivo* is followed by penetration of the pathogen through the style into the ovary tissues which occurs two days after inoculation (DAI) (De Neergaard, 1989, Van Steekelenburg, 1986). When infections remain latently in the fruit the fungus is quiescent for a certain period and can eventually become active after a period of time (McPherson, 2014).

Activation of quiescent biotrophic fungi can be impeded by different factors such as 1) an unsuitable environment for the activation of pathogenicity factors, 2) a lack of nutritional resources in the host, 3) the presence of inducible fungistatic or antifungal compounds in resistant unripe fruits (Prusky, 1996; Prusky & Lichter, 2008). The ability of pathogens to modulate the environmental pH seems to be an important early-acting factor in their activation. During fruit ripening and senescence, fruits undergo physiological changes, such as a decrease in hardness, cell wall remodelling, changes in sugar contents and changes in the ambient host pH, accompanied by a decrease in antifungal compounds. According to Prusky et al. (2013), these changes might represent a possible explanation for the transition from a quiescent state to a necrotrophic and/or pathogenic phase. In fungi, ambient pH is a regulator of growth and development and plays an important role in fungal pathogenicity (Fernandes et al., 2017). Many fungi can occur in a wide range of pH values because they have developed a complex regulatory system to sense and respond to changes in their environment (Bi et al., 2016). The fungal ability for local pH modulation has initially been described for the post-harvest pathogen *Colletotrichum gloeosporioides*, but also other pathogens, such as *Alternaria alternata*, *Botrytis cinerea*, *Monillia fructicola*, *Fusarium oxysporum and Penicillium spp*. have been reported to modulate their environmental pH (Prusky, 2001, 2004; Eshel et al., 2002; Manteau et al., 2003).

In 2002, Eshel et al. suggested that pH changes in a host are the result of a complex interaction of nitrogen and carbon availability, the initial pH of the fruit and tissue's buffer capacity. To produce compounds such as phospholipids, proteins, amino acids and chitin, fungi need organic or inorganic sources of nitrogen (Nicholas, 1965). Many fungi can use ammonium, urea, L-asparagine and nitrate as nitrogen sources (Morton & MacMillon, 1954; Pateman & Cove, 1967; Lewis & Fincham, 1970; Arima et al., 1972). Despite the ability to use many compounds, fungi prefer to use energetically favored nitrogen sources such as ammonium and

glutamine. In the absence of these compounds, less easily assimilated nitrogen sources such as nitrate, urea, uric acid, amines, amides, purines, and pyrimidines may be used (Marzluf, 1997; Wong et al., 2008). Assimilation of these nitrogen sources results in formation of ammonium which will be converted first to glutamate via glutamate dehydrogenase, and then to glutamine via glutamine synthetase (Tudzynski, 2014). Efficient regulation mechanisms are needed to coordinate activation and repression of genes that are involved in the metabolisation, perception and transportation of nitrogen-containing substances. Furthermore, nitrogen availability has a critical role in the pathogenicity of fungi to plants (Wiemann & Tudzynski, 2013). Recent studies by Prusky et al. (2016) and Bi et al. (2016) stated that the balance between gluconic acid and ammonia accumulation in the host tissue is relying on carbon availability. *Colletotrichum gloeosporioides*, *Penicillium expansum*, *Fusarium oxysporum* and *Aspergillus nidulans* have the ability to secrete small pH-modulating molecules as a key factor in the acidification or alkalization of their environment. Acidification by the secretion of organic acids occurred under carbon excess (60 g/L sucrose), while alkalization by active secretion of ammonia was induced under limited carbon (< 5 g/L sucrose) (Bi et al., 2016). Because of the similar type of response in several fungi this carbon response seemed sugar-concentration dependent rather than pathogen dependent. The pH-sensor system is driven by a zinc transcription factor, PACC, which promotes the transcription of genes expressed in alkaline environments and represses others at acidic pH. Enzymes, antioxidants and transporters belonging to the same gene family are part of the acid-expressed, or the alkaline-expressed groups (Alkan et al., 2013; Ment et al., 2015). Bi et al. (2016) suggested that the balance of secreted ammonia and gluconic acid regulate the expression of genes induced by PACC. It is clear that PACC controls genes under dynamic pH conditions, and allows fungi to adapt to these changing environments. This adaptation might be crucial to ensure the expression of the necessary genes contributing to pathogenicity, and that the products -cell wall degrading enzymes, transporters, antioxidants- are present at a pH that is optimal for their activity (Alkan et al., 2013; Prusky & Yakoby, 2003; Fernandes et al., 2017).

Although *Stagonosporopsis* has caused losses in cucurbits for more than 100 years, few studies have been performed on the physiological aspects of the pathogen. The current research is the first to suggest that *Stagonosporopsis cucurbitacearum* is able to modulate environmental pH. Based on the factors interacting in pH modulation (Eshel et al., 2002), this research describes how pH affects the fungal growth *in vitro* and in what amount *S. cucurbitacearum* is able to modulate it in a buffered environment. The effects of carbon and nitrogen availability on growth and pH modulation will be discussed. These results will lead to a deeper understanding of the pathogenicity of *S. cucurbitacearum* as the main pathogen of internal fruit rot in cucumber, which can contribute in the development of sustainable strategies to reduce/eliminate economic losses due to this disease.

MATERIALS AND METHODS

Fungal isolate and growing media

Stagonosporopsis cucurbitacearum isolate 04.14/006 was isolated from infected cucumbers on a Belgian cucumber plantation by the Sint-Katelijne-Waver Research Station for Vegetable Production, Belgium (PSKW) and identified by Scientia Terrae, Sint-Katelijne-Waver, Belgium. To obtain a stock, the isolate was stored at -80 °C in the fungal collection of the Laboratory of Sustainable Plant Production and Protection, KU Leuven, Geel. In preparation to perform experiments, the isolate was maintained and propagated on Potato Dextrose Agar (PDA, Biokar Diagnostics, Beauvais Cedex, France) at 23 °C in a 12 h light (PPFD: 10 µmol m^{-2} s^{-1}) / 12 h dark regime for 14 days.

PDA is the most widely used medium for growing fungi and bacteria. Due to its semi-synthetic composition, variations are possible in each batch. Therefore, a synthetic medium was

also selected to conduct the experiments. To this minimal medium (MM), based on the Czapek-Dox recipe by Leslie and Summerel (2006), 20 g of sucrose and 2 g $NaNO_3$ was added per litre medium. For the experiments conducted on solid media, 15 g agar (Bacteriological Agar Type E, Biokar Diagnostics, Beauvais Cedex, France) per litre was added to the different minimal media. PDA was used as the reference medium.

Effect of pH

To study the effects of the pH, both media were modified with a citric acid buffer to achieve pH 4.0. A phosphate buffer was used to reach pH values 5.0, 6.0 and 7.0, and a boric acid – borax buffer was added to both PDA and MM to obtain a pH of either 8.0 or 9.0 (Gomori 1955). The final acidity was measured with a Polyplast BNC electrode (Hamilton, Bonaduz, Switserland). Mycelial agar disks, with a diameter of 5 mm, from two weeks old *S. cucurbitacearum* colonies were aseptically transferred to the centre of a 9 cm petri dish containing 25 mL of medium. Daily growth, of the fungus in ten different petri dishes for each condition, was determined during the 14 days of incubation at 23 °C by measuring the colony size on two perpendicular axes with a digital calliper. To obtain the growth rate ($mm.day^{-1}$), the slopes of the colony size during the linear growth phase against time were calculated by simple linear regression. After 14 days of fungal growth, five petri dishes for both media and every pH-value were melted in a microwave and the pH was measured again at 55 °C.

Effect of carbon source

To investigate the effects of carbohydrates on the ability of *Stagonosporopsis* to alter the pH and pathogenicity, different amounts and types of sugars were used in the MM. The determination of the growth responses under limited and excess sugar conditions was realised by adding 0, 5, 20 and 60 g of sucrose to the MM. Different types of sugars were also tested in this experiment by using either 20 g of sucrose, glucose, dextrose or fructose. PDA was always used as a reference. The original pH values of the media were measured after autoclaving at 55 °C. Mycelial disks of the fungus were transferred on the plates, and after 5, 7 and 14 days of incubation at 23 °C in the dark, the media of three different petri dishes for every condition were melted to measure pH again at 55°C. Plates without mycelial disks were used as a control to account for possible time effects. To obtain the growth rate ($mm.day^{-1}$), the slopes of the colony size in ten different petri dishes for each treatment, measured daily in the 14 days of incubation, during the linear growth phase against time were calculated by simple linear regression.

Effect of nitrogen source

To consider whether the nitrogen source had an effect on the ability of the fungus to adjust the pH and pathogenicity, the MM was adjusted with three types of nitrogen in the same molar concentration equivalent to 2 g $NaNO_3$ per liter. This resulted in media supplemented with either 1.56 g $(NH_4)_2SO_4$ or 1.88 g NH_4NO_3. Mycelial disks of the fungus were transferred to the middle of the plates, and after 14 days of incubation at 23 °C in the dark, the media of three different petri dishes for every condition were melted to measure the final pH at 55°C. Empty plates were used again as a control. To obtain the growth rate ($mm.day^{-1}$), the slopes of the colony size in ten different petri dishes for each treatment, measured daily in the 14 days of incubation, during the linear growth phase against time were calculated by simple linear regression.

Statistical analyses

To obtain the growth rate ($mm.day^{-1}$) of each condition, the slopes of the colony size during the linear growth phase against time were calculated by simple linear regression. Analysis of

Variance (one-way ANOVA) followed by Tukey's post hoc test were performed on the data to investigate significances of differences ($\alpha = 0.01$) between the treatments. To determine differences between PDA and MM, 99% confidence intervals were calculated using Student's t-tests. SPSS (IBM© SPSS Statistics version 22.0, New York, USA) was used for all statistical analyses.

RESULTS

pH

Optimal growth of *S. cucurbitacearum* occurred between pH 5 and 6 with a reduced growth rate at pH 7 and 8 and negligible growth at pH 9. In the more acid region, at pH 4, growth was also drastically reduced, but was still approximately 3 times faster compared to growth at pH 9. There was no clear interaction between type of medium and pH (Figure 1).

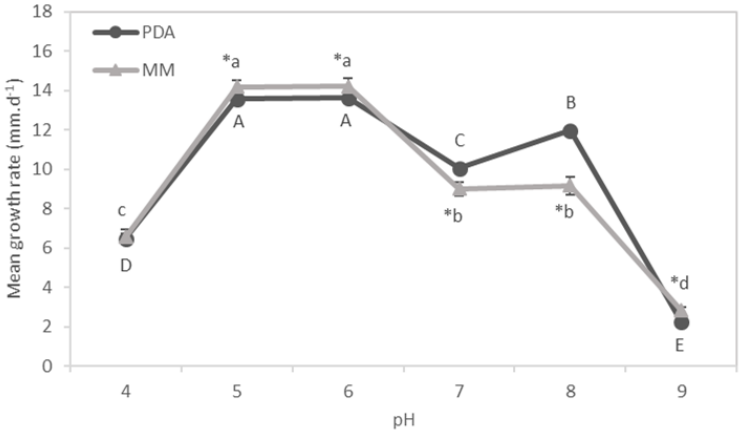
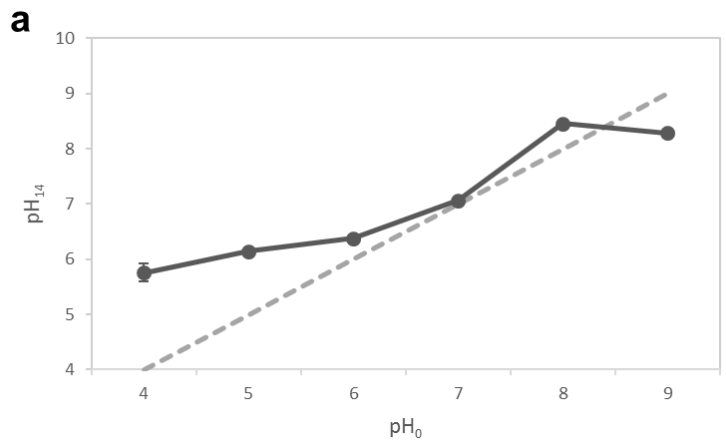


Figure 1: Plots of colony growth rate (mm.day^{-1}) versus pH for *S. cucurbitacearum* on PDA (•) and MM (▲) (n=10). Different capital and small letters are significantly different at P=0.05 according to Tukey's post hoc test for different conditions on PDA and MM respectively. According to the student T-test (CI=99%), points of the growth on MM marked with * are significantly different from growth on PDA.

In the presence of the different pH buffers *S. cucurbitacearum* was still able to alter the pH of the different media. In general, after 14 days of incubation, an alkalinisation of the media occurred with increases between 0.06 ± 0.02 and 1.76 ± 0.16 units, and 0.66 ± 0.01 and 1.46 ± 0.10 units on PDA and MM respectively. Only at the most alkaline condition i.e. pH 9 an acidification of both PDA an MM occurred (0.71 ± 0.02 and 0.24 ± 0.08 units respectively).



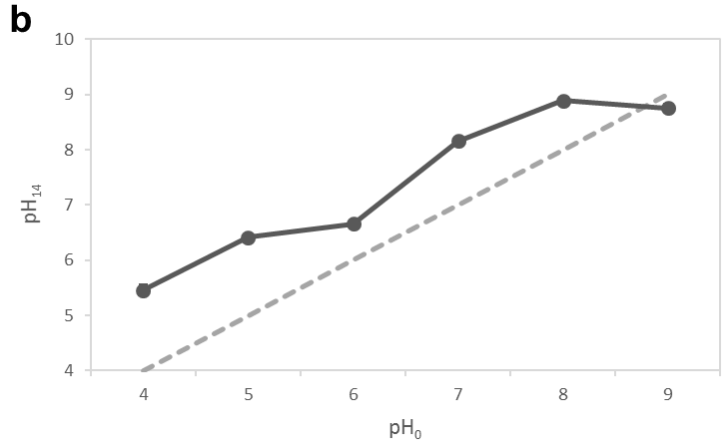


Figure 2: pH modulation of a) PDA and b) Minimal Medium caused by *S. cucurbitacearum*. The different initial pH values were created with the use of buffers. The pH was measured again after 14 days of inoculation (n=3). Values represent means ± SD. --- Indicates the pH without any modulation.

Carbon source

Without a pH buffer present in the growing medium the fungus was able to raise the pH from 5.34 to 7.70 ± 0.22 after 14 days of incubation on PDA. On MM the initial pH increased with approximately 3.5 units irrespective of the type of carbohydrate ($P>0.01$) (Figure 3a). The mean growth rate was situated around 12 $mm.d^{-1}$ when sucrose and glucose were used as carbon sources ($P>0.01$). In the presence of dextrose, the growth rate was ~10 % faster whilst fructose caused a reduction in growth rate of ~30 % compared to sucrose or glucose ($P<0.01$) (Figure 3b). Sucrose was further selected as carbon source for more detailed experiments investigating the influences of sucrose concentration (Figure 4a). Without addition of sucrose only a limited increase of <1 pH unit manifested. Carbon addition caused an increase of >2 pH units, which seemed generally independent of the amount of added sugar. Alkalization mainly occurred within the first three days. After 14 days, the pH values of the media had increased significantly from an original pH around 5 to pH 5.93 ± 0.01 without sugar addition and pH 7.87 ± 0.03, pH 8.14 ± 0.04, pH 8.07 ± 0.09 in the presence of 5, 20 and 60 g sucrose per liter respectively. The plates without fungal mycelium did not show any significant alkalinisation as the pH was approximately 0.04- 0.24 units lower at the end. Mean growth rate linearly increased with the sugar concentration except for a similar growth rate of 10.17 ± 0.27 $mm.day^{-1}$ ($P>0.01$) for 0 and 5 g sucrose per liter medium (Figure 4b).In the presence of 20 g and 60 g sucrose per liter, the growth rate was respectively ~20 % and ~55 % higher compared to growth on sucrose-free medium.

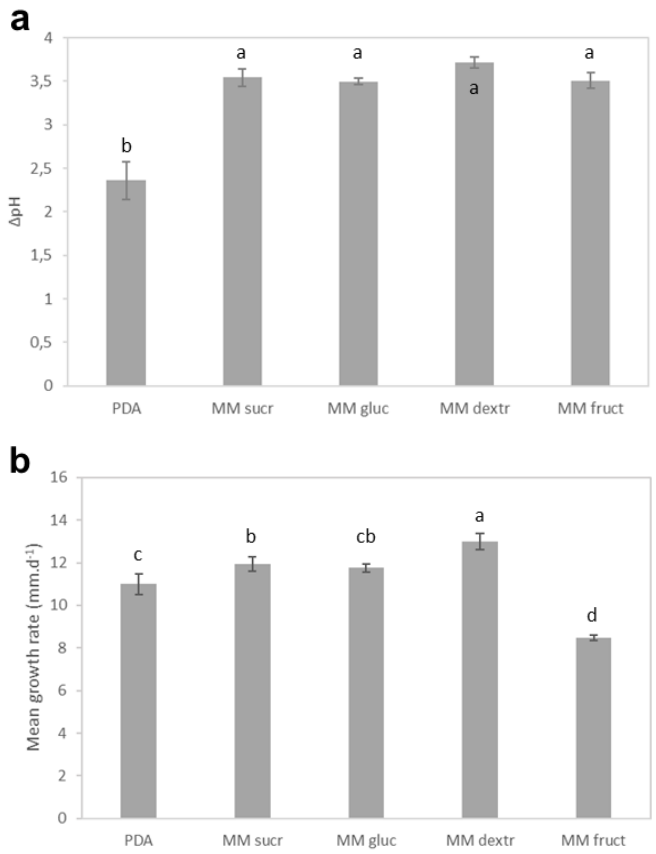
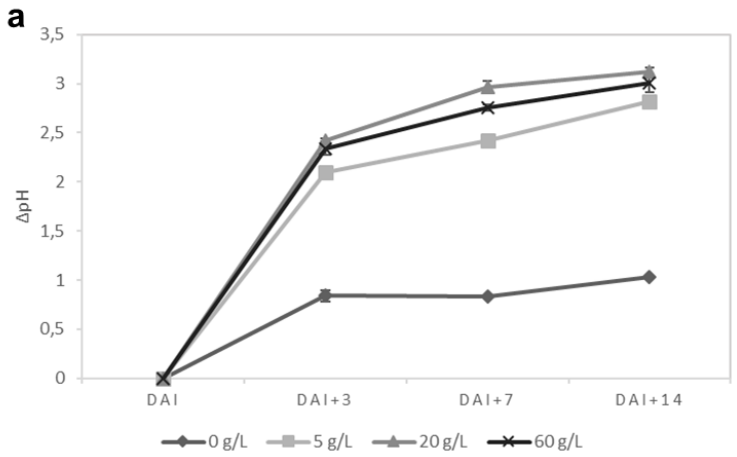


Figure 3: Effects of different carbon sources (20 g/L) in Minimal Medium (MM) a) on the induction of medium alkalinisation by *S. cucurbitacearum*, b) on the mean growth rate (mm.day^{-1}) of the mycelium, measured daily for 7 days (n=10). Potato Dextrose Agar (PDA) was used as reference. pH's were measured after autoclaving and again after 14 days of incubation at 55°C. Bars represent means ± SD of tree inoculations. Different letters are significantly different at P=0.01 according to Tukey's post hoc test for different conditions. Sucr = sucrose; gluc = glucose; dextr = dextrose; fruct = fructose.



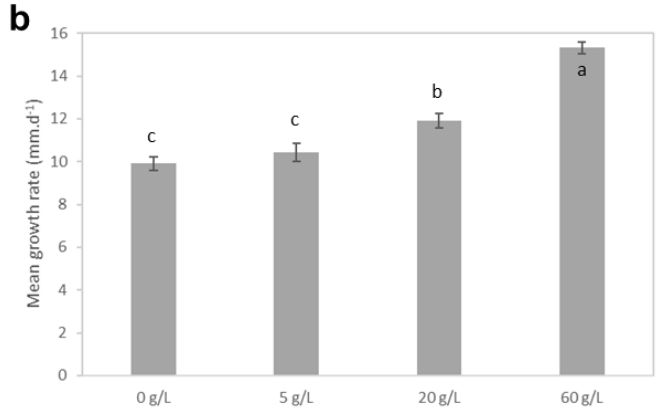


Figure 4: Effects of sucrose levels on a) the pH modulation by *S. cucurbitacearum*, and b) the mean growth rate (mm.day^{-1}) of the mycelium, measured daily for 7 days (n=10). The pH of Minimal Medium (n=3) containing 0, 5, 20 or 60 g/L sucrose was measured 3, 7 and 14 days after inoculation (DAI). Plates without the fungus were used as control. Values represent means ± SD of the inoculations. a) Statistical differences are not shown, but are discussed in the text. b) Different letters are significantly different at P=0.01 according to Tukey's post hoc test for different conditions.

Nitrogen source

Under a fixed sucrose concentration of 20 g/L, the source of mineral nitrogen in MM also seemed to play an important role in pH modulation (Figure 5a). A combination of both nitrogen sources supplied as ammonium nitrate did not cause a significant change (0.08 ± 0.56 units) of the original pH of the medium, i.e. ~pH 5. In the presence of ammonium sulphate, a significant acidification of 2.35 ± 0.02 units occurred, causing a strong decline of the pH to less than 3. In contrast, when sodium nitrate was provided as sole nitrogen source a significant alkalinisation of 3.24 ± 0.05 units occurred, which resulted in a pH>8.

Mean growth rates of *S. cucurbitacearum* were also clearly different in the presence of the different nitrogen sources (Figure 5b). The growth rate of 13.28 ± 0.20 mm.day^{-1} on the medium with sodium nitrate was clearly faster than growth rates registered on the other media (P<0.01). However, the fungus still showed a 3,5 times faster growth on the medium enriched with ammonium nitrate compared to ammonium sulphate (respectively 1.55 ± 0.07 and 0.44 ± 0.06 mm.day^{-1}).

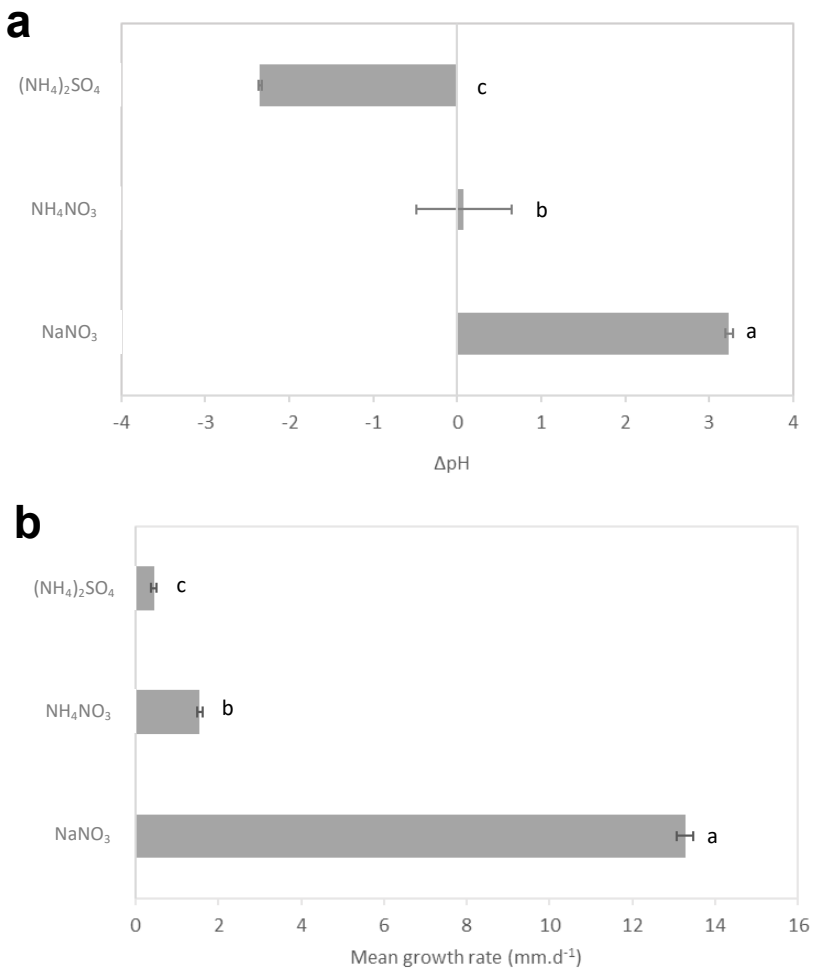


Figure 5: Characteristics of *S. cucurbitacearum* on Minimal Medium with different nitrogen sources. a) Modulation of the pH, measured after 14 days of inoculation (n=3). b) Mean growth rate (mm.day^{-1}) of the mycelium, measured daily for 7 days (n=10). Values represent means ± SD. Different letters are significantly different at P=0.01 according to Tukey's post hoc test for different conditions.

DISCUSSION

Changes in the surrounding microenvironment can have a significant impact on the development of fungi, including both spore germination and mycelial growth (Manteau et al., 2003; Frans et al., 2017). Our *in vitro* studies with both a synthetic custom-made medium (minimal medium) and general PDA medium indicated an optimal growth rate within the range of pH 5 to pH 6 for *Stagonosporopsis cucurbitacearum*, with a significantly reduced growth rate at pH 7 and 8 and negligible growth at pH 9. In the more acid region, at pH 4, growth was also drastically reduced, but was still approximately 3 times faster compared to growth at pH 9. Based on *in vivo* measurements on cucumber fruit showing a mean pH of 5.08 ± 0.08, it is indeed reasonable that this fungus favours acid conditions rather than basic ones. Even when grown in the presence of buffers the pathogen was still able to significantly alkalize its environment.

This alkalizing effect was found independent of the carbon source without any distinction between dextrose, sucrose, glucose and fructose, of which the latter two represent more than 75% of the total soluble solids in fruit (Hulme, 1971; Prusky, 1996). Whilst sucrose is transported from the leaves to the developing fruit, glucose and fructose are the main sugars in the meso- and endocarp tissues (6-12 mg.gFW^{-1}) with only a limited contribution of sucrose (0.3 mg.gFW^{-1}) (Handley et al., 1983; Schaffer et al., 1987). Our experiments showed that the presence of the afore mentioned carbon sources in the growth media brought about an increase

of the pH of the media with ~3.5 units. Acidification could not be initiated by changing the source of carbon. However, Bi et al. (2016) showed that in *Colletotrichum gloeosporioides*, *Penicillium expansum*, *Fusarium oxysporum* and *Aspergillus nidulans*, acidification by the secretion of gluconic acid was induced under carbon excess, i.e. 60 g/L, whilst alkalinisation with ammonia occurred under a deprivation of carbon, i.e. 5 g/L. In our experiments with *S. cucurbitacearum* significant alkalisation was observed under both carbon deprivation and excess. However, a sucrose concentration effect was present; without sucrose addition pH rose with ~1 unit after 14 days of incubation whilst a stronger alkalisation of ~3 pH units was observed when sucrose was supplied at either 5, 20 or 60 g/L. The difference of ~2 pH units between adding 0 and 5 g/L of sucrose to the medium did not affect growth rate as higher concentrations were needed to stimulate fungal growth. These results indicate that pH modulation might not always be related to carbon availability in order to create a more favourable growth environment.

Bi et al. (2016) reported that the amount of tryptone, as a nitrogen source in the growth medium, was not related with the environmental pH either, but numerous reports have indicated the importance of the nitrogen status in fungal infection (Jurick et al., 2012; Tavernier et al., 2007; Snoeijers et al., 2000). When considering different mineral nitrogen sources, instead of the amount of nitrogen, it was very obvious that *S. cucurbitacearum* could either acidify or alkalise its environment dependant on the nitrogen source provided. Although the three media offered the same molar amount of nitrogen, the effect on both growth and pH was clearly different. The mycelial growth rate of *S. cucurbitacearum* was fastest on the nitrate medium at 13.28 ± 0.20 mm per day with a concomitant alkalinisation of 3.24 ± 0.05 units. On the ammonium nitrate medium, growth rate was reduced to 1.55 ± 0.07 mm per day without any pH modulation. Mycelium production was found to be lowest on the ammonium medium, i.e. just 0.44 ± 0.06 mm per day with a final pH 2.35 ± 0.02 units lower due to acidification. In *Coprinopsis phlyctidospora* He and Suzuki (2003) also reported an increase in the final pH of media supplied with nitrate, asparagine and urea, and a decrease when ammonium was used. Since ammonium is taken up by the fungus as ammonia, a hydrogen ion is left behind, causing an acidification of the medium (Griffin, 1981). Some fungi fail to continue using ammonium due to an alteration of the pH in solution or toxicity of ammonia in the alkaline state (Morton & MacMillan, 1954) but the use of ammonium salts of weak acids can provide enough buffering capacity (Griffin, 1981). However, utilization of the ammonium salts such as NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$ have already been reported to cause a rapid drop in the pH and concomitant decrease in mycelial growth of *Scopulariopsis brevicaulis*, saprotrophic and ectomycorrhizal fungi (Morton & MacMillan, 1954; Jongbloed et al., 1990; Yamanaka, 1999; Cooke & Whipps, 1993). Chloric, nitric and sulfuric acid are all strong acids with a pKa < 1, and cannot provide a sufficient buffering. In general fungi prefer to use energetically favored nitrogen sources such as ammonium before assimilating nitrate (Moore-Landecker, 1996; Wong et al., 2008). Assimilation of nitrate results in formation of ammonium which will be converted afterwards to glutamine (Tudzynski, 2014). However, our studies indicate that *S. cucurbitacearum* clearly prefers nitrate above ammonium and as such belongs to the nitrate fungi, like *Aspergillus nidulans* and several *Fusarium* species (Keller, 1996; Pfanmüller et al., 2017). The majority of fungi are able to assimilate nitrate because it is the most abundant nitrogen ion present in plants (Siverio, 2002; Song et al., 2007; Gorfer et al., 2011; Schinko et al., 2013). This assimilation is an energy-consuming process and therefore, nitrate is considered as unfavorable nitrogen source that is only used when the preferred sources are not available, which explains why the majority of fungi are characterized by high growth on ammonium and reduced growth on nitrate (Pfanmüller et al., 2017). In cucumber fruit nitrate levels are rather low, because nitrate is assimilated into amino acids and as such transported via the phloem to the developing fruit (Marschner et al., 1996).

In conclusion, the current research has highlighted the importance of the nitrogen source in the growth and regulation of environmental pH by fungi such as *Stagonosporopsis cucurbitacearum*. In vitro, fungal growth is more influenced by the source of supplied nitrogen and the pH of the medium than the carbon source. Because of the importance of mechanisms of pH adaptation, more fundamental studies are needed to shed light on the entire network, from sensing the available nitrogen sources to the cascade of expression profiles in this metabolism. In vivo, cucumber fruit show a significant increase in soluble sugars from ~5 $mg.gFW^{-1}$ to ~20 $mg.gFW^{-1}$ upon ripening (Hu et al., 2009), potentially stimulating fungal growth but the availability of different nitrogen sources such as proteins, amino acids, nitrate and ammonium in the fruit (Ruiz and Romero, 1999) make the situation much more complex. Important key topics revealed by our *in vitro* work that need further detailed consideration *in vivo* in cucumber fruit encompass registration of the pH-evolution in pathogen-infected fruit and to determine whether the provision of different nitrogen sources can modulate pathogen development and growth. As such, increasing the knowledge of processes in fungi as a reaction to environmental stress and understanding the details of the mechanisms how fungi grow and change the specific extracellular pH, will add in our understanding and the management of decay of vegetables and fruit. Similar investigations with other isolates of *S. cucurbitacearum* are also encouraged to reveal possible isolate-host-environment interactions and to shed more light on similar diseases in other important commodity crops such as pumpkin (*Cucurbita* spp.), melon (*Cucumis melo* L.) and watermelon (*Citrullus lanatus*).

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Compliance with ethical standards

The authors declare that they have no conflict of interest. They ensure that this article does not contain any studies with human participants or animals. All authors consent to this submission.

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