

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

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13 **ABSTRACT**

14 **The *Cucurbitaceae* is a genetically diverse group of plants containing several important**
15 **commodity crops in many parts of the world such as cucumber, pumpkin and melon. In**
16 **the last decades, fruit rot caused by *Stagonosporopsis spp.* became a major disease in both**
17 **field grown and greenhouse grown cucurbits. Yield losses due to *Stagonosporopsis* can**
18 **show seasonal peaks up to 30%. Despite its economic importance, only limited**
19 **information is available about growth characteristics of *Stagonosporopsis***
20 ***cucurbitacearum*. Our *in vitro* studies with different media indicated an optimal growth**
21 **rate of the fungus within the range of pH 5 to pH 6. Independent of the carbon source**
22 **(sucrose, glucose, dextrose, fructose) alkalization of 1-3,5 pH units was noticed under both**
23 **carbon deprivation and excess. The observed pH modulation could not always be related**
24 **with a more favourable growth environment. The key factor influencing both pH**
25 **modulating capacity and growth showed to be the nitrogen source. Supplying nitrate,**
26 **ammonium or a combination of both, the environmental pH respectively increased,**
27 **decreased or remained stable. In addition to a pH elevating effect nitrate supply did also**
28 **stimulate growth whilst growth on ammonium containing media was seriously affected.**
29 **This research highlights the importance of the nitrogen source in the growth and**
30 **regulation of environmental pH by fungi and adds in our understanding of *S.***
31 ***cucurbitacearum* pathogenicity.**
32

33 **Key words:** *Didymella bryoniae*, *Stagonosporopsis cucurbitacearum*, internal fruit rot,
34 *Cucurbitaceae*, pH modulation
35

36 **INTRODUCTION**

37 The Cucurbitaceae is one of the most genetically diverse group of plants containing several
38 important commodity crops in many parts of the world, such as cucumber (*Cucumis sativus* L.),
39 pumpkin (*Cucurbita* spp.), melon (*Cucumis melo* L.), and watermelon (*Citrullus lanatus*)
40 (Robinson & Decker-Walters, 1997). Production of cucurbits is under jeopardy by a fungal
41 disease caused by *Stagonosporopsis cucurbitacearum* which can infect at least 12 genera and
42 23 species of the Cucurbitaceae, and occurs in temperate regions including North America,
43 Europe, Asia and New Zealand. First reports of this disease in cucurbits in Europe date back to
44 1823. The pathogen may originally have been introduced to Europe on papayas brought from
45 the Americas (Keinath, 2011). It is still an important field disease in subtropical and tropical
46 growing areas, and a serious concern for glasshouse cucumber growers in temperate regions
47 since production intensification in the late 1960's (Corlett, 1981, Robinson & Decker-Walters,
48 1997). Nearly all growers in temperate regions are confronted with this problem and average
49 yield losses are estimated at an average of 5% with seasonal peaks in heavy rainfall periods up
50 to 30% (Keinath, 2000).

51 Over the years, the nomenclature and classification of the pathogen has been subject to
52 change. Initially, in 1881, Heinrich Rehm classified the teleomorphic phase of the fungus to the
53 genus *Didymella*. In 1949; Chiu and Walker re-identified it as *Mycosphaerella melonis*.
54 Following DNA sequencing by Aveskamp, Gruyter and Verkley in 2010, the anamorphic phase
55 of the fungus appeared to belong to the genus *Stagonosporopsis* instead of *Phoma* (Stewart et
56 al., 2015). In an infection site, the anamorph *Stagonosporopsis cucurbitacearum* (Fr.)
57 Aveskamp, and teleomorph *Didymella bryoniae* (Auersw.) Rehm, can occur at the same time.
58 *Stagonosporopsis* is a typical member of the ascomycetes and can produce both ascospores and
59 conidiospores which can both cause infections. The initial infections can occur on all plant
60 parts, except the roots, and are typically caused by airborne ascospores. The pathogen causes a
61 variety of symptoms, including leaf spots, stem cankers, vine wilt and black fruit rot (Van
62 Steekelenburg, 1982). Spores are usually produced within four to eight days after initial
63 infection, causing a new cycle of secondary infections shortly after (Miller et al., 2010). Within
64 24 h after germination, infection hyphae and appressoria are produced. The hyphae produce
65 various enzymes such as amylase, lipase, protease, urease and polygalacturonase, causing plant
66 cells to degrade and thereby creating moist lesions (Tsay et al., 1990). *Stagonosporopsis* can
67 infect the fruit in several ways. The first type of infection is characterized as external fruit rot,
68 and results from infections on mechanical injuries. The second type of externally invisible
69 infection is known as internal fruit rot, which occurs when airborne spores attach on the stigma
70 of the pistil. *In vitro* germination studies have indicated that 50 % of the spores can germinate
71 within a time frame of just 9 h, whilst four hours later germination was nearly 100 % (Van
72 Laethem et al., 2019). Germination *in vivo* is followed by penetration of the pathogen through
73 the style into the ovary tissues which occurs two days after inoculation (DAI) (De Neergaard,
74 1989, Van Steekelenburg, 1986). When infections remain latently in the fruit the fungus is
75 quiescent for a certain period and can eventually become active after a period of time
76 (McPherson, 2014).

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

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

101 glutamine. In the absence of these compounds, less easily assimilated nitrogen sources such as
102 nitrate, urea, uric acid, amines, amides, purines, and pyrimidines may be used (Marzluf,
103 1997; Wong et al., 2008). Assimilation of these nitrogen sources results in formation of
104 ammonium which will be converted first to glutamate via glutamate dehydrogenase, and then
105 to glutamine via glutamine synthetase (Tudzynski, 2014). Efficient regulation mechanisms are
106 needed to coordinate activation and repression of genes that are involved in the metabolism,
107 perception and transportation of nitrogen-containing substances. Furthermore, nitrogen
108 availability has a critical role in the pathogenicity of fungi to plants (Wiemann & Tudzynski,
109 2013). Recent studies by Prusky et al. (2016) and Bi et al. (2016) stated that the balance between
110 gluconic acid and ammonia accumulation in the host tissue is relying on carbon availability.
111 *Colletotrichum gloeosporioides*, *Penicillium expansum*, *Fusarium oxysporum* and *Aspergillus*
112 *nidulans* have the ability to secrete small pH-modulating molecules as a key factor in the
113 acidification or alkalization of their environment. Acidification by the secretion of organic acids
114 occurred under carbon excess (60 g/L sucrose), while alkalization by active secretion of
115 ammonia was induced under limited carbon (< 5 g/L sucrose) (Bi et al., 2016). Because of the
116 similar type of response in several fungi this carbon response seemed sugar-concentration
117 dependent rather than pathogen dependent. The pH-sensor system is driven by a zinc
118 transcription factor, PACC, which promotes the transcription of genes expressed in alkaline
119 environments and represses others at acidic pH. Enzymes, antioxidants and transporters
120 belonging to the same gene family are part of the acid-expressed, or the alkaline-expressed
121 groups (Alkan et al., 2013; Ment et al., 2015). Bi et al. (2016) suggested that the balance of
122 secreted ammonia and gluconic acid regulate the expression of genes induced by PACC. It is
123 clear that PACC controls genes under dynamic pH conditions, and allows fungi to adapt to
124 these changing environments. This adaptation might be crucial to ensure the expression of the
125 necessary genes contributing to pathogenicity, and that the products -cell wall degrading
126 enzymes, transporters, antioxidants- are present at a pH that is optimal for their activity (Alkan
127 et al., 2013; Prusky & Yakoby, 2003; Fernandes et al., 2017).

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

138

139 **MATERIALS AND METHODS**

140 **Fungal isolate and growing media**

141 *Stagonosporopsis cucurbitacearum* isolate 04.14/006 was isolated from infected cucumbers on
142 a Belgian cucumber plantation by the Sint-Katelijne-Waver Research Station for Vegetable
143 Production, Belgium (PSKW) and identified by Scientia Terrae, Sint-Katelijne-Waver,
144 Belgium. To obtain a stock, the isolate was stored at -80 °C in the fungal collection of the
145 Laboratory of Sustainable Plant Production and Protection, KU Leuven, Geel. In preparation to
146 perform experiments, the isolate was maintained and propagated on Potato Dextrose Agar
147 (PDA, Biokar Diagnostics, Beauvais Cedex, France) at 23 °C in a 12 h light (PPFD: 10 μmol
148 $\text{m}^{-2} \text{s}^{-1}$) / 12 h dark regime for 14 days.

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

151 also selected to conduct the experiments. To this minimal medium (MM), based on the Czapek-
152 Dox recipe by Leslie and Summerel (2006), 20 g of sucrose and 2 g NaNO₃ was added per litre
153 medium. For the experiments conducted on solid media, 15 g agar (Bacteriological Agar Type
154 E, Biokar Diagnostics, Beauvais Cedex, France) per litre was added to the different minimal
155 media. PDA was used as the reference medium.

156

157 **Effect of pH**

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

170

171 **Effect of carbon source**

172 To investigate the effects of carbohydrates on the ability of *Stagonosporopsis* to alter the pH
173 and pathogenicity, different amounts and types of sugars were used in the MM. The
174 determination of the growth responses under limited and excess sugar conditions was realised
175 by adding 0, 5, 20 and 60 g of sucrose to the MM. Different types of sugars were also tested in
176 this experiment by using either 20 g of sucrose, glucose, dextrose or fructose. PDA was always
177 used as a reference. The original pH values of the media were measured after autoclaving at 55
178 °C. Mycelial disks of the fungus were transferred on the plates, and after 5, 7 and 14 days of
179 incubation at 23 °C in the dark, the media of three different petri dishes for every condition
180 were melted to measure pH again at 55°C. Plates without mycelial disks were used as a control
181 to account for possible time effects. To obtain the growth rate (mm.day⁻¹), the slopes of the
182 colony size in ten different petri dishes for each treatment, measured daily in the 14 days of
183 incubation, during the linear growth phase against time were calculated by simple linear
184 regression.

185

186 **Effect of nitrogen source**

187 To consider whether the nitrogen source had an effect on the ability of the fungus to adjust the
188 pH and pathogenicity, the MM was adjusted with three types of nitrogen in the same molar
189 concentration equivalent to 2 g NaNO₃ per liter. This resulted in media supplemented with
190 either 1.56 g (NH₄)₂SO₄ or 1.88 g NH₄NO₃. Mycelial disks of the fungus were transferred to
191 the middle of the plates, and after 14 days of incubation at 23 °C in the dark, the media of three
192 different petri dishes for every condition were melted to measure the final pH at 55°C. Empty
193 plates were used again as a control. To obtain the growth rate (mm.day⁻¹), the slopes of the
194 colony size in ten different petri dishes for each treatment, measured daily in the 14 days of
195 incubation, during the linear growth phase against time were calculated by simple linear
196 regression.

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198 **Statistical analyses**

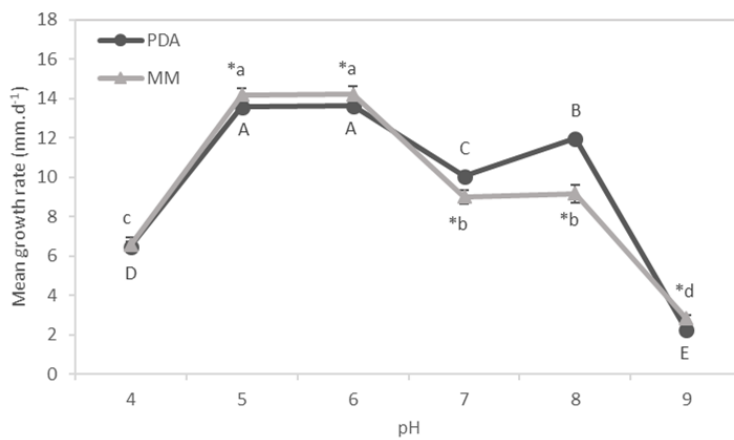
199 To obtain the growth rate (mm.day⁻¹) of each condition, the slopes of the colony size during the
200 linear growth phase against time were calculated by simple linear regression. Analysis of

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

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 207 **RESULTS**

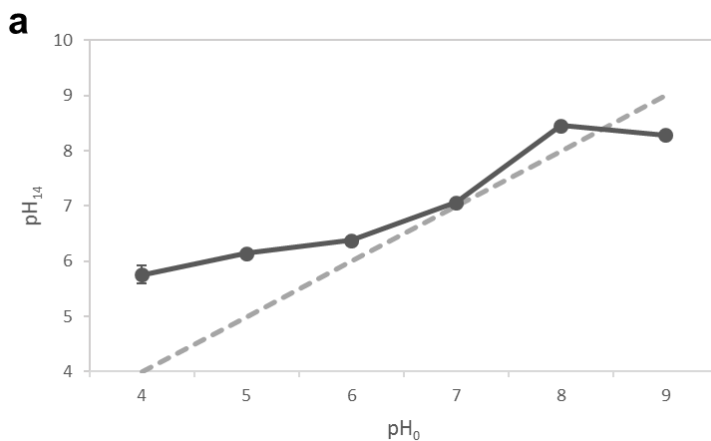
208 **pH**

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

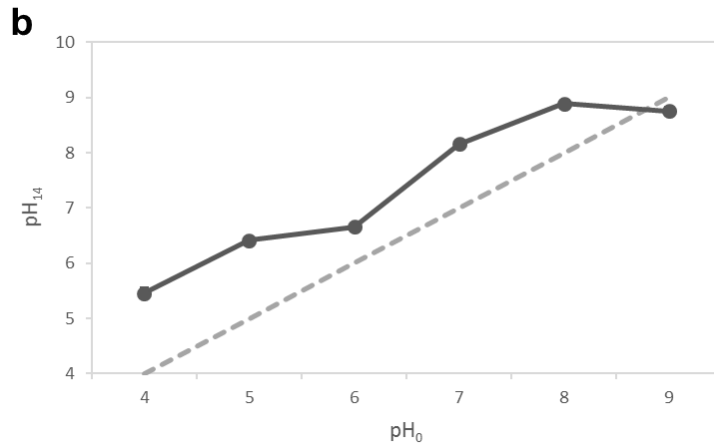


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 214 Figure 1: Plots of colony growth rate (mm.day⁻¹) versus pH for *S. cucurbitacearum* on PDA (●) and MM (▲)
 215 (n=10). Different capital and small letters are significantly different at P=0.05 according to Tukey's post hoc test
 216 for different conditions on PDA and MM respectively. According to the student T-test (CI=99%), points of the
 217 growth on MM marked with * are significantly different from growth on PDA.
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219 In the presence of the different pH buffers *S. cucurbitacearum* was still able to alter the pH of
 220 the different media. In general, after 14 days of incubation, an alkalinisation of the media
 221 occurred with increases between 0.06 ± 0.02 and 1.76 ± 0.16 units, and 0.66 ± 0.01 and $1.46 \pm$
 222 0.10 units on PDA and MM respectively. Only at the most alkaline condition i.e. pH 9 an
 223 acidification of both PDA and MM occurred (0.71 ± 0.02 and 0.24 ± 0.08 units respectively).



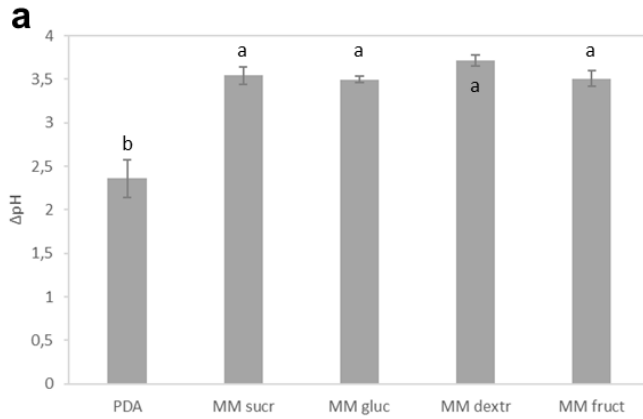
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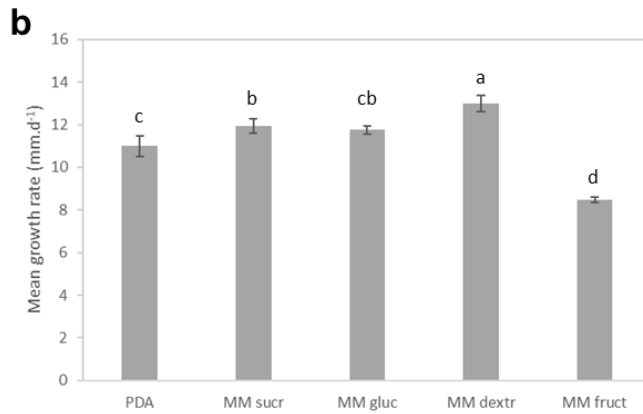
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 226 Figure 2: pH modulation of a) PDA and b) Minimal Medium caused by *S. cucurbitacearum*. The different initial
 227 pH values were created with the use of buffers. The pH was measured again after 14 days of inoculation (n=3).
 228 Values represent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

229 **Carbon source**

230 Without a pH buffer present in the growing medium the fungus was able to raise the pH from
 231 5.34 to 7.70 ± 0.22 after 14 days of incubation on PDA. On MM the initial pH increased with
 232 approximately 3.5 units irrespective of the type of carbohydrate ($P > 0.01$) (Figure 3a). The mean
 233 growth rate was situated around $12 \text{ mm} \cdot \text{d}^{-1}$ when sucrose and glucose were used as carbon
 234 sources ($P > 0.01$). In the presence of dextrose, the growth rate was $\sim 10\%$ faster whilst fructose
 235 caused a reduction in growth rate of $\sim 30\%$ compared to sucrose or glucose ($P < 0.01$) (Figure
 236 3b). Sucrose was further selected as carbon source for more detailed experiments investigating
 237 the influences of sucrose concentration (Figure 4a). Without addition of sucrose only a limited
 238 increase of < 1 pH unit manifested. Carbon addition caused an increase of > 2 pH units, which
 239 seemed generally independent of the amount of added sugar. Alkalinization mainly occurred
 240 within the first three days. After 14 days, the pH values of the media had increased significantly
 241 from an original pH around 5 to $\text{pH } 5.93 \pm 0.01$ without sugar addition and $\text{pH } 7.87 \pm 0.03$, pH
 242 8.14 ± 0.04 , $\text{pH } 8.07 \pm 0.09$ in the presence of 5, 20 and 60 g sucrose per liter respectively. The
 243 plates without fungal mycelium did not show any significant alkalinisation as the pH was
 244 approximately 0.04- 0.24 units lower at the end. Mean growth rate linearly increased with the
 245 sugar concentration except for a similar growth rate of $10.17 \pm 0.27 \text{ mm} \cdot \text{day}^{-1}$ ($P > 0.01$) for 0
 246 and 5 g sucrose per liter medium (Figure 4b). In the presence of 20 g and 60 g sucrose per liter,
 247 the growth rate was respectively $\sim 20\%$ and $\sim 55\%$ higher compared to growth on sucrose-free
 248 medium.



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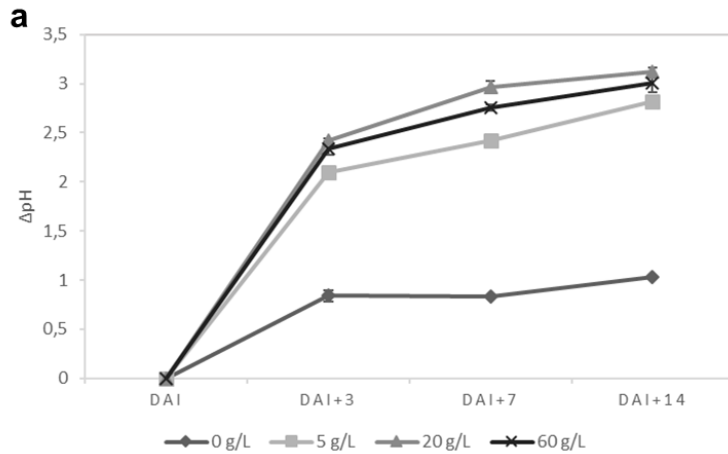
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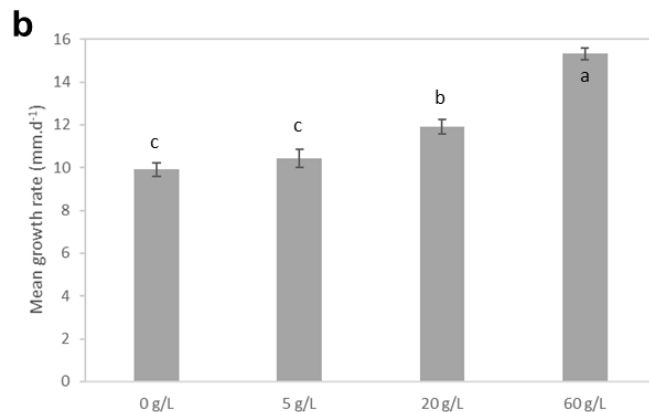
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Figure 3: Effects of different carbon sources (20 g/L) in Minimal Medium (MM) a) on the induction of medium alkalisation by *S. cucurbitacearum*, b) on the mean growth rate (mm.day⁻¹) of the mycelium, measured daily for 7 days (n=10). Potato Dextrose 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.



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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 \pm 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.

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Nitrogen source

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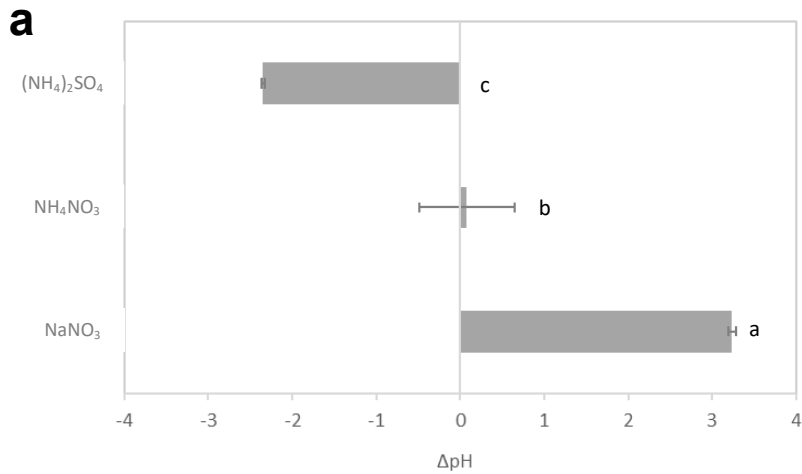
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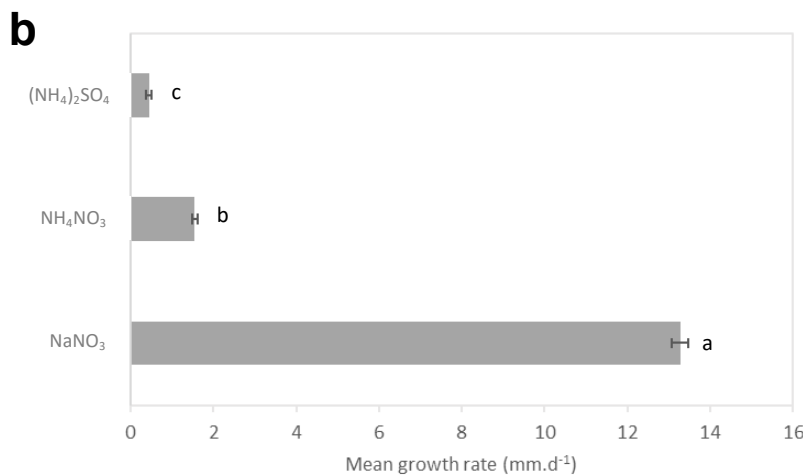
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Under a fixed sucrose concentration of 20 g/L, the source of mineral nitrogen in MM also seemed to play an important role in pH modulation (Figure 5a). A combination of both nitrogen sources supplied as ammonium nitrate did not cause a significant change (0.08 ± 0.56 units) of the original pH of the medium, i.e. \sim 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 alkalisation 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).



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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.

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DISCUSSION

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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.

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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

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

318 Bi et al. (2016) reported that the amount of tryptone, as a nitrogen source in the growth medium,
319 was not related with the environmental pH either, but numerous reports have indicated the
320 importance of the nitrogen status in fungal infection (Jurick et al., 2012; Tavernier et al., 2007;
321 Snoeijers et al., 2000). When considering different mineral nitrogen sources, instead of the
322 amount of nitrogen, it was very obvious that *S. cucurbitacearum* could either acidify or alkalis
323 its environment dependant on the nitrogen source provided. Although the three media offered
324 the same molar amount of nitrogen, the effect on both growth and pH was clearly different. The
325 mycelial growth rate of *S. cucurbitacearum* was fastest on the nitrate medium at 13.28 ± 0.20
326 mm per day with a concomitant alkalisation of 3.24 ± 0.05 units. On the ammonium nitrate
327 medium, growth rate was reduced to 1.55 ± 0.07 mm per day without any pH modulation.
328 Mycelium production was found to be lowest on the ammonium medium, i.e. just 0.44 ± 0.06
329 mm per day with a final pH 2.35 ± 0.02 units lower due to acidification. In *Coprinopsis*
330 *phlyctidospora* He and Suzuki (2003) also reported an increase in the final pH of media supplied
331 with nitrate, asparagine and urea, and a decrease when ammonium was used. Since ammonium
332 is taken up by the fungus as ammonia, a hydrogen ion is left behind, causing an acidification of
333 the medium (Griffin, 1981). Some fungi fail to continue using ammonium due to an alteration
334 of the pH in solution or toxicity of ammonia in the alkaline state (Morton & MacMillan, 1954)
335 but the use of ammonium salts of weak acids can provide enough buffering capacity (Griffin,
336 1981). However, utilization of the ammonium salts such as NH_4Cl , NH_4NO_3 and $(\text{NH}_4)_2\text{SO}_4$
337 have already been reported to cause a rapid drop in the pH and concomitant decrease in mycelial
338 growth of *Scopulariopsis brevicaulis*, saprotrophic and ectomycorrhizal fungi (Morton &
339 MacMillan, 1954; Jongbloed et al., 1990; Yamanaka, 1999; Cooke & Whipps, 1993). Chloric,
340 nitric and sulfuric acid are all strong acids with a $\text{pK}_a < 1$, and cannot provide a sufficient
341 buffering. In general fungi prefer to use energetically favored nitrogen sources such as
342 ammonium before assimilating nitrate (Moore-Landecker, 1996; Wong et al., 2008).
343 Assimilation of nitrate results in formation of ammonium which will be converted afterwards
344 to glutamine (Tudzynski, 2014). However, our studies indicate that *S. cucurbitacearum* clearly
345 prefers nitrate above ammonium and as such belongs to the nitrate fungi, like *Aspergillus*
346 *nidulans* and several *Fusarium* species (Keller, 1996; Pfanmüller et al., 2017). The majority of
347 fungi are able to assimilate nitrate because it is the most abundant nitrogen ion present in plants
348 (Siverio, 2002; Song et al., 2007; Gorfer et al., 2011; Schinko et al., 2013). This assimilation is
349 an energy-consuming process and therefore, nitrate is considered as unfavorable nitrogen
350 source that is only used when the preferred sources are not available, which explains why the
351 majority of fungi are characterized by high growth on ammonium and reduced growth on nitrate
352 (Pfanmüller et al., 2017). In cucumber fruit nitrate levels are rather low, because nitrate is
353 assimilated into amino acids and as such transported via the phloem to the developing fruit
354 (Marschner et al., 1996).

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

375

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379

380 **Compliance with ethical standards**

381 The authors declare that they have no conflict of interest. They ensure that this article does not
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383

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