# Factors involved in the early events of spore germination by *Botrytis cinerea.*

Salem Nassr[1] and Radwan Barakat[2 *]

[1,2] Department of Plant Production and Protection, Faculty of Agriculture Hebron University, P.O.Box 40, Hebron, Palestine

**ABSTRACT**

*Botrytis cinerea* is a necrotrophic fungal plant pathogen distributed worldwide. The early stages of epidemiology namely spore germination is a topic of great interest among researchers. In the current study, the effect of various physical, chemical and nutritional factors on germination of *Botrytis cinerea* conidia were studied *in vitro*. Results showed that there was no particular influence of spore age (5-14 days) on germination in 10 mM fructose. In addition, germination-self-inhibition was found to be associated with increased spore concentrations (above $4.5\times10^5$ conidia/ml) without significant differences between fungal isolates. When setting different pH values in the medium, conidial germination of *Botrytis cinerea* was impaired by pH values below 6 and above 8. However, germination of *Botrytis cinerea* was strongly enhanced (>90% after 24 hours) in the presence of sugars (i.e. fructose, sucrose and glucose) at concentrations above 100 mM, whilst the cations ($Ca^{2+}$, $Mg^{2+}$, $K^{+}$, and $Fe^{2+}$ ) had no visible influence on conidial germination at a wide range of concentrations (0.001-1mM). With other additives and in the presence of inorganic nitrogen forms (i.e. NH4 and NO3), conidial germination responded similarly with no particular influence on germination, whilst germ tube growth and elongation increased progressively with increasing concentrations of both N-forms.

*Key Words: Botrytis cinerea, conidial germination, early event, germ tube*

## 1. INTRODUCTION

*Botrytis cinerea* Pers. ex. Fr. is the causal agent of gray mold. The name of the sexual stage or teleomorph is *Botryotinia fuckeliana* (de Bary) Whetzel, but the ascocarps are rarely observed under field conditions (Polach and Abawi 1975 ). *Botrytis cinerea* is a filamentous, heterothallic Ascomycete exhibiting great variability in mycelial growth rate, conidial germination, pathogenicity, incidence of sporulation structures, production of sclerotia, and resistance to anti-*Botrytis* chemicals (Grindle 1979; Lorbeer 1980; Di Lenna et al. 1981; Kerssies et al. 1997). The early events of plant's infection by plant pathogenic fungi are essential for disease initiation and progress. Such early events (adhesion, conidial germination, and formation of external infection structures) were intensively studied lately on *Botrytis cinerea* throughout several studies (Doehlemann et al. 2006; Klimple et al. 2002; Schumacher et al. 2008).

Conidial germination of *Botrytis cinerea* is induced by different physical and chemical signals, including the presence and quality of nutrients in particular sugars such as fructose (Kosuge and Hewitt 1964; Blakeman 1975). Conidial germination in most filamentous fungi requires the presence of low-molecular-mass nutrients such as sugars, amino acids and inorganic salts (Carlile and Watkinson 1994). Along with germination and after conidial adhesion, different mucilages are secreted and assist in anchoring of the germ tube and appressorium to the host surface. Several groups of proteins have been suggested to assist in germ tube and appressorium attachment and to mediate the exchange of early signalling between the fungus and the plant (Prins et al. 2000).

Conidia of *Botrytis cinerea* are typically nutrient-dependent; they do not readily germinate in sterile water, and they usually require an exogenous input of nutrients for germination. In addition, it has been proposed that nutrient-dependent conidia of phytopathogenic fungi may use germination-stimulating compounds from a host plant as an alternative chemical cue when nutrient concentrations are too low for conidial germination (Filonow, 2002). In addition, diverse carbon sources (mono- and disaccharides, acetate) are effective at low concentrations (10 mM) to induce

* Tel: 02 2220995 ext. 153 Fax: 02 2229303 Email: radwanb@hebron.edu

54 germination in *Botrytis cinerea*. Rich media such as malt extract induced rapid germination and
55 early germ tube branching. Induction of conidial germination by nutrients, in particular sugars, is
56 well known in saprotrophic fungi (Osherov and May, 2000). The mechanism of nutrient sensing by
57 *Botrytis cinerea* conidia is unknown. As diverse sugars and acetate induce germination with similar
58 efficiency, it appears unlikely that nutrient sensing occurs by plasma membrane proteins (Forsberg
59 and Ljungdahl, 2001). Conidia are also able to germinate on inert artificial surfaces; various amino
60 acids plus sugars efficiently induced germination of conidia, while mineral salts such as ammonium
61 and phosphate were effective only in the presence of low concentrations of sugars (Blakeman,
62 1975). On cuticular surfaces, however, dry-inoculated conidia can germinate at high humidity in the
63 absence of liquid water (Prins *et al.,* 2000). Surface hydrophobicity, together with surface hardness,
64 is well known to induce germination of *Botrytis cinerea* conidia in the absence of nutrients
65 (Osherove and May, 2000). The current study has illustrated the effect of such several physical and
66 chemical factors on germination of *Botrytis cinerea* conidia.
67
68 **2. METHODS**
69
70 **2.1 FUNGAL ISOLATES AND COMMERCIAL CULTURE MEDIUM**
71 *Botrytis cinerea* wild type isolates used throughout this study were provided by the Plant Protection
72 Research Center (PPRC) fungal collection at Hebron University. The first isolate, (PBC1) was
73 isolated from infected beans (*Phaseulous vulgaris* L.) growing under greenhouse in Hebron. The
74 second isolate, (PBC3) was isolated from infected grape berries (*Vitis vinefera L.*) growing in an
75 open field in Hebron. Following isolation, the two isolates were grown on PDA medium and kept at
76 20±1 ºC under continuous light.
77 After 12 days, and when cultures sporulated, 5mm mycelium plug from each isolate culture was
78 taken and placed in a fresh PDA culture plate; 24 hours later, one freely emerging conidium was
79 transferred into another plate to get monosporic cultures. The monosporic cultures were grown on
80 PDA medium amended with 10% homogenized bean leaves. Plates were then kept under
81 continuous light in an incubator at 20±1 $^{\circ}$C for the coming experiments.
82 The third isolate used was B05.10 which is a universal known strain. It was derived from the wild-
83 type isolate SAS56 by treatment with benomyl for haploidization (Quidde *et al.,* 1999). This putative
84 haploid wild type isolate B05.10 was provided by the lab. of Prof. P. Tudzynski (University of
85 Munster, Germany).
86
87 **2.2 CONIDIAL CONCENTRATION**
88 The influence of conidial concentration on germination assays of *Botrytis cinerea* isolates was
89 assessed in a 24 well Sarstedt microtitre plate (Sarstedt, Newton. USA), according to (Doehlemann,
90 2006). Two plates of PDA medium amended with 10% w/v homogenized bean leaves were
91 inoculated with 100 µl of conidial suspension ($1 \times 10^6$ conidia/ml) from PBC3, PBC1 and B05.10
92 isolates. The inoculum was spread over the surface of the medium with the aid of a glass rod.
93 After 11 days, conidia were harvested from each plate by 10 ml of sterile distilled water. Conidia
94 were then filtered through a Nytex membrane to remove traces of mycelia and placed in a sterile
95 plastic vial for each isolate.
96 Spore suspension was then washed three times with 10 ml of SDW and centrifuged (IEC Centra-
97 CLD) for 3 minutes at 3000 rpm. The concentration of the conidial suspension was determined by a
98 haemocytometer and diluted to the final concentrations of $4 \times 10^5$, $2.5 \times 10^4$, $5 \times 10^3$ and $2.5 \times 10^3$
99 conidia/ml. Spherical glass coverslips - 15mm (Roth, Karlsruhe, Germany) were placed in the
100 bottom of each well of the 24-welled microtitre plate. A 25 µl of each concentration were placed in
101 the bottom of the well to which 475 µl of 10mM D-Fructose solution were added to reach a final
102 volume of 500 µl and according to (Doehlemann , 2006). Plates were then incubated in the dark at
103 20$^{o}$C±1 and conidial germination counted after 5 hours of incubation. Each treatment consisted of 4
104 replicates (wells) and 100 randomly selected conidia were counted in each of the 4 wells under an
105 inverted microscope. A conidium was considered as germinated when the germ tube was visible.
106 **2.3 AGE OF CONIDIA**
107 The influence of conidial age on germination of *Botrytis cinerea*-isolate B05.10 conidia was
108 assessed. The isolate B05.10 was grown on plates containing potato dextrose agar (PDA)
109 amended with 10% homogenized bean leaves. Four plates of PDA medium were inoculated with 5
110 mm mycelium plug from a newly growing mycelium (two days old), and incubated at 21 $^{\circ}$C and

111 continuous light. Conidia were then harvested after 7, 9, 10, 12, and 14 days with 10 ml of SDW,
112 and filtered through a Nytex membrane to remove traces of mycelia.
113 Spore suspensions were then washed three times with 10 ml of SDW and centrifuged (IEC Centra-
114 CLD) for 3 minutes at 3000 rpm; supernatant was discarded each time. Conidial concentrations
115 were then determined with the aid of a haemocytometer [Tiefe Depth Protondeur 0.200 mm] and
116 fixed at $2.5\times10^4$ Conidia/ml. Spherical glass coverslips (15mm, Roth, Karlsruhe. Germany) were
117 placed on each well of the 24-welled microtitre plate. Conidia (25 µl of each age ) were placed in
118 the bottom of the well. Fructose was prepared and suspended in liquid Gamborg B5 basal salt
119 mixture (GB5) (Duchefa Biochem. BV, Haarlem, The Netherlands; Art: G0209.0050) to reach a final
120 concentration of 10 mM. After that, 475 µl of the 10mM fructose+GB5 solution were added to reach
121 a final volume of 500 µl. Sarstedt plates were then incubated in the dark at 20±1 ºC.
122 Using the same selected conidial ages, germination was monitored on a hydrophobic surface;
123 polypropylene film was placed at the surface of a glass slide. Slides were then placed on a moist
124 filter paper inside closed sterile petri dishes. Conidial suspension was prepared from the isolate
125 B05.10 and fixed at a concentration of $1\times10^5$ Conidia/ml. The surfaces were then inoculated with 4
126 separate droplets of conidial suspension 25 µl each and then placed in an incubator. A completely
127 randomized design was used, each treatment consisted of 4 replicates (wells); germinated spores
128 were counted out of 100 randomly selected spores under an inverted microscope.
129

130 **2.4 MICROCLIMATE PH**

131

132 The influence of microclimate pH on germination of *Botrytis cinerea*, isolate B05.10 was determined
133 in 1mM fructose solution. Fructose solutions were prepared and adjusted to pH ranges starting from
134 3, 4, 5, 6, 7, 8, 9 and up to 10 using 1M NaOH and 1M HCl. Conidia of *Botrytis cinerea* (B05.10)
135 were harvested from 10 days old sporulating cultures grown previously on (PDA+Beans) medium
136 with SDW and conidial concentration was fixed at $2.5\times10^4$ conidia/ml. Spherical glass coverslips
137 were placed in the bottom of each of the 24 wells of the Sarstedt microtitre plate. After that, 25 µl
138 spore suspension was placed in the middle of each well and 475 µl of Fructose solution were added
139 to reach a final volume of 0.5 ml. A completely randomized design was used with 3 replicates for
140 each treatment. Numbers of germinated conidia were recorded after 5 hours.
141
142

143 **2.5 SUGARS**

144

145 The role of carbon sources in conidial germination of *Botrytis cinerea* was investigated using three
146 sugars: Fructose, Glucose and Sucrose in 5 molar concentrations 1µM, 10 µM, 100 µM, 1mM and
147 10mM. Sugar solutions were prepared in DW and sterilized in the autoclave for 30 minutes at
148 127°C. *Botrytis cinerea* was grown on (PDA+10% beans) and incubated at 21°C and continuous
149 light for ten days. Spore suspensions from the isolates B05.10, PBC3 and PBC1 were prepared
150 using SDW and adjusted to a final concentration of $2.5\times10^4$ conidia/ml. Spherical glass coverslips
151 were placed in the bottom of each of the 24 wells of the Sarstedt microtitre plate. Spore suspension
152 (25µl) was placed in the middle of each well and 475 µl of each sugar treatment were added to
153 reach a final volume of 0.5 ml. A completely randomized design was used with 4 replicates for each
154 treatment. Numbers of germinated conidia were recorded after 5 and 25 hours.
155

156 **2.6 SALT CATIONS**

157

158 The role of the cations, $Ca^{2+}$, $Mg^{2+}$, and $Fe^{2+}$ in conidial germination of *Botrytis cinerea* was
159 investigated. Ca ($CaCl_2$), Mg ($MgCl_2$), and Fe (FeSO4.7H2O) were prepared into 6 concentrations
160 (0.001M 0.01M 0.1M 1mM 10mM and 1M). Solutions were prepared in distilled water and sterilized
161 in the autoclave for 30 minutes at 127°C. *Botrytis cinerea* was grown on (PDA+10% beans) and
162 incubated at 21°C and continuous light for ten days. Conidial suspensions from the isolates B05.10
163 and PBC3 were harvested by SDW. Conidia were then filtered through Nytex membrane and
164 washed three times to remove traces of mycelium. The concentration was adjusted to a final
165 concentration of $1\times10^3$ conidia/ml. Spherical glass cover slips were placed in the bottom of each of
166 the 24 wells of the Sarstedt microtitre plate. Spore suspension (25 µl) was placed in the middle of
167 each well and 475 µl of each treatment were added to reach a final volume of 0.5 ml. A completely
168 randomized design was used with 4 replicates for each treatment. Numbers of germinated conidia

169 were recorded after 40 hours of incubation at 21°C. At the same time, the average germ tube length
170 of 10 random germinated conidia (replicates) was recorded.
171

172 **2.7 INORGANIC NITROGEN FORMS (NH4 AND NO3)**

173 The effect of the nitrogen forms, $NH4^{+}$ and $NO3^{-}$ on conidial germination of *Botrytis cinerea* was
174 studied. The procedure is the same as that of the previous section. NH4 (NH4Cl), NO3 (NaNO3)
175 was used as source of the cations. The spore concentration was set to $1\times10^{3}$ conidia/ml. A
176 completely randomized design was used with 4 replicates for each treatment. Numbers of
177 germinated conidia were recorded after 25 hours of incubation at 21°C. At the same time, the
178 average germ tube length of 10 random germinated conidia (replicates) was recorded.
179

180 **2.8 STATISTICAL ANALYSIS**

181 The data of all experiments were analyzed statistically using analysis of variance (one way ANOVA)
182 and Fisher least significant difference (LSD) test with the aid of (Sigma stat 2.0 for Windows®
183 statistical package program, SPSS Inc., Chicago, IL, USA).
184

185 **3. RESULTS**

186 **3.1 THE EFFECT OF CONCENTRATION OF CONIDIA ON GERMINATION OF**
187 ***BOTRYTIS CINEREA* CONIDIA**

188 The influence of spore concentration of *Botrytis cinerea*-isolates B05.10, PBC1 and PBC3 on
189 conidial germination was determined in 10mm fructose solution (Fig. 1). Results showed that
190 conidial germination rates decreased with increasing spore concentrations. The highest germination
191 rate was recorded at the spore concentration ($2.5\times10^{3}$ conidia/ml) for all isolates. Generally, there
192 was no significant difference in germination rates between the three *Botrytis cinerea* isolates. it was
193 evident that the three isolates responded similarly in which germination rates decreased with
194 increasing spore concentrations (Figure 1 and 2: c, d, and e).
195

196 **3.2 THE EFFECT OF AGE OF CONIDIA ON GERMINATION OF *BOTRYTIS CINEREA***
197 **CONIDIA**

198 Spore age could be another factor involved in early conidial germination in fungi. The influence of
199 conidial age of *Botrytis cinerea* (B05.10) on germination percentage was investigated. No
200 significant difference in germination percentages was found between different conidial ages in sugar
201 amended with Gamborg' B5- salt mixture (GB5). Conidial germination percentages, however, were
202 significantly reduced in older conidia (67% after 14 days) compared to younger conidia (91% after 5
203 days) when germination was tested on a hydrophobic surface (polypropylene) (see table 1 and 2).
204

205 **3.3 THE EFFECT OF MICROCLIMATE PH ON GERMINATION OF *BOTRYTIS CINEREA***
206 **CONIDIA**

207 The influence of microclimate pH on germination of *Botrytis cinerea* conidia was assessed on
208 Sarstedt plates. *Botrytis cinerea* conidia were able to germinate well at ph values ranging from 6-8;
209 the highest germination rate was obtained at pH 7. However, B05.10 conidia germinated poorly at
210 pH= 3 and 10. The experiment was repeated twice. Data on the average germination rates in
211 different microclimate pH are presented in Figure 3 and 4.
212

213 **3.4 THE EFFECT OF SUGARS ON GERMINATION OF *BOTRYTIS CINEREA* CONIDIA**

214 The influence of the sugars (Fructose, Sucrose and Glucose) on conidial germination of *Botrytis*
215 *cinerea* was tested in various concentrations (Fig.5 and 6). Results showed that germination of
216 conidia was stimulated in sugars in various proportions according to various concentrations
217 compared to SDW. Sucrose was the best in inducing conidial germination even after 5 hpi only
218 recording 87% compared to glucose 18% and fructose 59%. Two sugars (sucrose and glucose)
219 have induced high germination rates (>90%) after 24 hours of incubation at the highest
220 concentration used (10mM) . The concentration (100 µM) was the breaking point for most sugars
221 to induce significant increases in conidial germination.
222
223

224 **3.5 THE EFFECT OF CATIONS ON GERMINATION OF *BOTRYTIS CINEREA* CONIDIA**

225 The cations $Ca^{2+}$, $Mg^{2+}$, $K^{+}$, and $Fe^{2+}$ had no influence on conidial germination of *Botrytis cinerea*
226 isolates (B05.10 and PBC1) at the relatively low concentrations used (0.001-1mM). At 10mM
227 concentration, however, Fe reduced germination dramatically. At higher concentrations (>10mM),
228 all cations showed toxicity and totally inhibited conidial germination. Concerning germ tube
229 elongation, only Fe was able to enhance germination at low concentrations, but as concentration
230 increased germ tube elongation decreased until totally inhibited at high concentrations (>10mM). All
231 the other cations ($Ca^{2+}$, $Mg^{2+}$ and $K^{+}$ ), however, showed no influence on germ tube elongation at all
232 concentrations tested (Fig. 7)
233

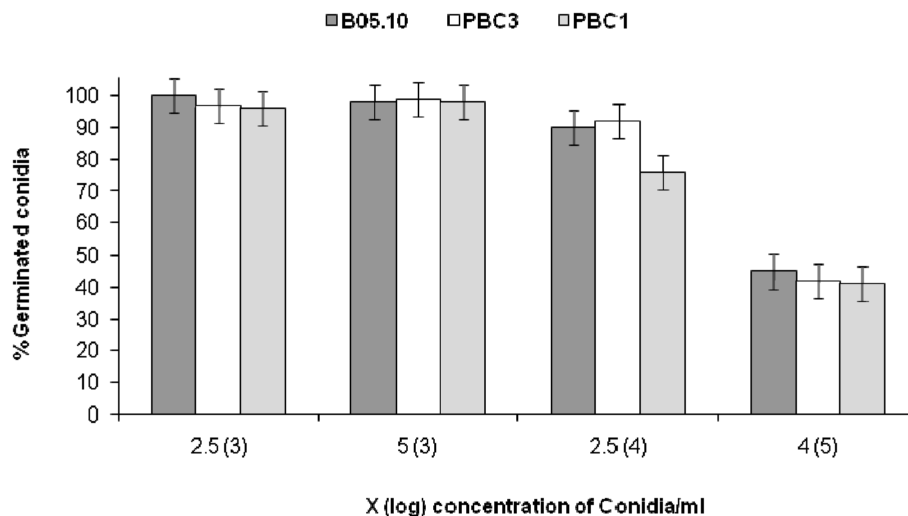
234 **3.6 THE EFFECT OF INORGANIC NITROGEN FORMS ON GERMINATION OF**
235 ***BOTRYTIS CINEREA* CONIDIA**

236 The effect of NH4 and NO3 on germination of *Botrytis cinerea* (B05.10 and PBC1) conidia and germ
237 tube lengths was investigated (Fig. 8). Inorganic nitrogen forms had no influence on germination
238 percentages of *Botrytis cinerea* isolates at all concentrations tested. However, germ tube length
239 growth was dramatically influenced by both nitrogen forms positively; germ tube length increased by
240 almost 99% at the highest concentration of NH4 (1M) compared to the control (SDW). NH4 form of
241 nitrogen enhanced germ tube growth to a larger extent than NO3 form of N for both *Botrytis cinerea*
242 isolates. Both *Botrytis cinerea* isolates responded almost similarly in respect to percentage
243 germination and germ tube growth.
244

245 **4. DISCUSSION**

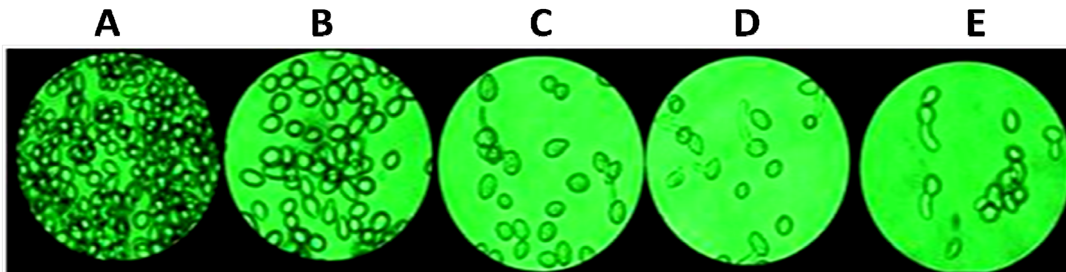
246 The ability of fungi to adhere to and germinate on leaves and other substrata is well documented
247 and is thought to represent an important early event in plant-microbe interactions (Braun and
248 Howard 1994; Jones 1994). Spore germination in *Botrytis cinerea* follows a developmental
249 sequence of spore swelling, localized outgrowth of the germ tube and subsequent polarized growth
250 of the new hyphae. It was noted that, conidial germination rates of *Botrytis cinerea*-isolates
251 decreased with increasing spore concentrations without significant differences between isolates. At
252 concentrations above $4\times10^{5}$ conidia/ml, conidia were unable to germinate and appeared in clots.
253 Sharrock, et al. 2001 found that conidia of *Botrytis cinerea* exhibit a self inhibition strategy during
254 germination at high concentrations ($1\times10^{6}$ conidia/ml) or more. It is assumed that at high
255 concentrations, conidia tend to produce specific germination and/or growth inhibitors regardless of
256 the richness of the substrate. Furthermore, several germination-self-inhibitors in other fungal
257 species such as *Puccinia, Uromyces, Colletrotrichum, Dictyostelium, Fusarium* and *Aspergillus* were
258 investigated and reports showed that these inhibitors can be volatile or non-volatile (Allen 1955;
259 Bacon et al. 1973; and Barrios-Gonzales et al. 1989). It was also concluded that self-inhibitors can
260 affect other fungal processes, such as prevention of appressorium induction which make conidial
261 germination unlikely to occur.
262 Spore age could be another factor involved in early conidial germination in fungi. It was found that
263 conidial germination was significantly reduced in older conidia (67% after 14 days) compared to
264 younger conidia (91% after 5 days) when germination was tested on a hydrophobic surface
265 (Polypropylene). However, no difference was noticed when spores germinated in Fructose and GB5.
266 This suggests that nutritional factors may mask the effect of age and older conidia can germinate as
267 well as younger conidia if the growth substrate was supplied with appropriate nutritional source.
268 Using different germination conditions, Shirashi et al., 1988 found that young *Botrytis* conidia, in
269 general, germinated well at 20℃ compared to old conidia.
270 As for pH, conidial germination was significantly impaired at high and/or low values (below 6 and
271 above 8). Conidia germinated well at pH ranging from 6-8 with the highest germination rate at
272 pH=7. In this direction, fungi very often can dynamically alter the local pH to fit its enzymatic
273 arsenal, with the level of pathogenicity being related to the efficiency of the pH change. (Prusky et
274 al. 2001). Generally, *Botrytis cinerea* is classified among acidic fungi (Prusky and Yakoby, 2003)
275 and similar to other pathogenic fungi, such as *Penicillium expansum*, *P. digitatum, P. italicum*, and
276 *Sclerotinia sclerotiorum* that use tissue acidification in their attack (Vautard and Fevre, 2003). This
277 investigation, however, was restricted to the conidial germination in vitro. The ability of *Botrytis*
278 *cinerea* to germinate at various pH values emphasizes the previous findings stating that *Botrytis*
279 spp. change the medium or site pH to facilitate the enzymatic activities.
280 Nutritional supplements, namely sugars are considered rich nutrients; germination of *Botrytis*
281 *cinerea* conidia was stimulated in the three different sugars (fructose, sucrose and glucose) at
282 various concentrations compared to the control (SDW). Almost all sugars have induced full

283 germination (100%) after 24 hours of incubation at the highest concentration used (10mM) knowing
284 that the concentration (100 µM) was the (breaking point) for all sugars to induce significant increase
285 in conidial germination. Sugars at relatively low concentrations (i.e 10mM) induced early swelling of
286 conidia and enhanced early germ tube branching. In this direction, it has been shown that Fructose
287 induced germination of *Botrytis cinerea* conidia more efficiently than any other monosaccharide
288 (Blakeman, 1975). Germination induction by sugars was concentration dependent, and fructose
289 was more effective than glucose. Similarly and among sugars, fructose has been pointed out as the
290 best inducer of germination in *Botrytis cinerea*, being more effective than glucose and other hexoses
291 or disaccharides (Blakeman, 1975). One explanation for the particular important activity of fructose
292 in conidial germination could be that this sugar is preferentially taken up by a fructose-specific
293 transport system. This is surprising since glucose is usually the most efficient hexose not only as a
294 nutrient, but also as a signalling compound (Doehlemann et al. 2005). Using almost the same
295 protocol for germination, Doehlemann, et al. 2006 found similar results after incubation for 24
296 hours. Induction of conidial germination by nutrients, in particular sugars, is well known in
297 saprotrophic fungi (Osherov and May 2000). In rich media, most fungi germinate quickly, including
298 phytopathogens such as *Fusarium solani, Colletotrichum graminicola* and *Colletotrichum*
299 *gloeosporioides* (Ruan et al. 1995; Chaky et al. 2001; Barhoom and Sharon, 2004).
300 The mechanism of sugar sensing by *Botrytis cinerea* conidia is unknown. As diverse sugars and
301 acetate induce germination with similar efficiency, it appears unlikely that nutrient sensing occurs by
302 plasma membrane proteins (Forsberg and Ljungdahl, 2001).
303 Regarding the addition of salt cations and from looking at the results, it was obvious that the tested
304 cations ($Ca^{2+}$, $Mg^{2+}$, $K^{+}$, and $Fe^{2+}$ ) had no influence on conidial germination at a wide range of
305 concentrations (0.001-1mM). However, at high concentrations (>10mM), germination declined
306 sharply, especially with $Fe^{2+}$ which suggests a level of toxicity induced at high concentrations. It is
307 very likely that conidia before germination are not affected at low concentrations of cation availability
308 in the growth substrate. However, after germination, germ tube growth becomes more sensitive to
309 a wide range of cation concentrations in the growth media. $Fe^{2+}$ seems to provide an important
310 nutritional source for germ tube growth at low concentrations (0.001 M). Barakat and Almasri, 2009
311 (unpublished data) found that at high concentrations (i.e. 1M) all these cations inhibited germination
312 of *Botrytis* conidia and the level of toxicity varied between isolates. Shirani and Hatta (1987), found
313 that at the concentration ($5\times10^{4}$ conidia/ml) conidial germination of *Botrytis cinerea* was optimum
314 (100%) in the presence of $Ca^{2+}$ ($CaCl_2$) and was relatively high (66%) in $Mg^{2+}$ ($MgSO_4$) at the
315 concentrations (0.1-0.7 g/liter). Conidial germination responded almost similarly to nitrogen forms.
316 While N-forms had no influence on germination, germ tube growth and elongation responded
317 positively with increasing concentrations of both forms. This suggests that conidia may depend
318 more on available energy inside the spore to germinate but after germination, germ tube growth
319 greatly depends on nutritional elements available in the growth substrate.
320
321–338




339 **Figure 1.** Effect of spore concentration on conidial germination rates of *Botrytis cinerea* isolates
340 grown on (PDA+10% bean leaves) medium and incubated in 10mM Fructose at 20±1 ºC

341 under continuous light after 20 hours of incubation. (vertical bars represent LSD= 5.49,
342 n=3).
343
344
345
346




347
348
349
350 **Figure2.** *Botrytis cinerea* (B05.10) conidial germination at different concentrations of conidia at
351 200X. Conidial concentrations: (A), $5\times10^6$ conidia/ml; (B), $1\times10^6$ conidia/ml; (C), $4\times10^5$
352 conidia/ml; (D), $2.5\times10^4$ conidia/ml and (E), $5\times10^3$ conidia/ml.
353
354

355 **Table 1.** Influence of conidial age on germination of *Botrytis cinerea*-isolate B05.10 after 20 hours
356 of incubation in 10 mM fructose solution+GB5
357

| Age of B05.10 culture (days) | % Germination |
|---|---|
| **5** | 97a |
| **7** | 95a |
| **10** | 96a |
| **12** | 95a |
| **14** | 93a |

358
359 Means followed by the same letter in the same column are not significantly different (*P*= 0.064).
360 GB5: Gamborgs B5-basic salt mixture.
361
362

363 **Table 2.** Influence of conidial age on germination of *Botrytis cinerea* conidia isolate B05.10 after 20
364 hours of incubation on polypropylene surface.
365

| Age of B05.10 culture (days) | % Germination |
|---|---|
| **5** | 91 a |
| **7** | 84 ab |
| **10** | 92 a |
| **12** | 78 bc |
| **14** | 67 c |

366 -Means followed by the same letter in the same column are not significantly different
367 (LSD=11.309, n=4). GB5: Gamborgs B5-basic salt mixture.
368
369

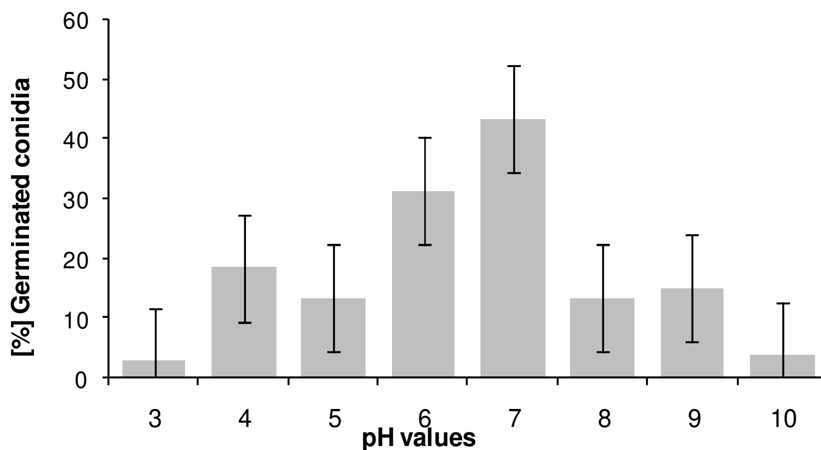



**Figure 3.** Influence of microclimate pH on conidial germination of *Botrytis cinerea*-isolate B05.10 in 1mM fructose solution after 5 hours of incubation. (LSD = 9.020, n=3).

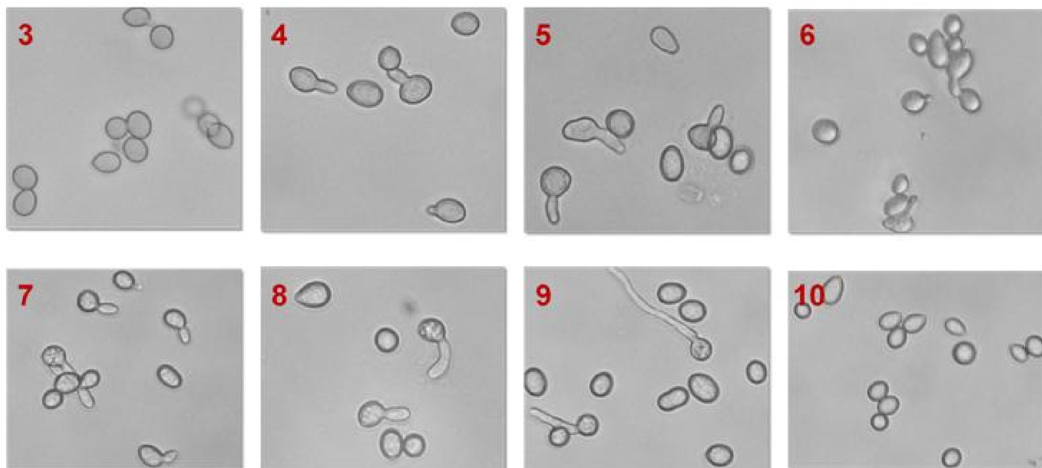
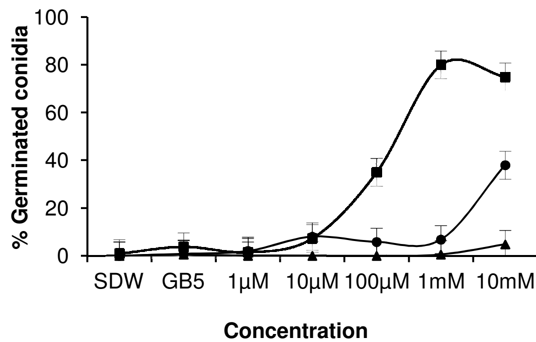


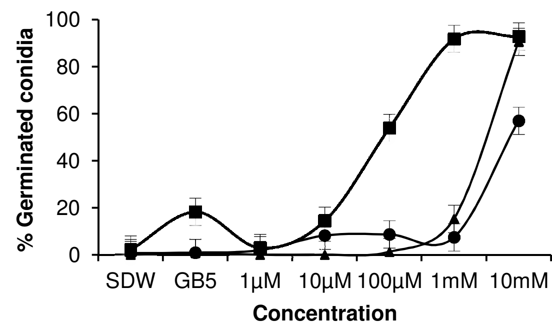

**Figure 4.** Conidial germination of *Botrytis cinerea* –isolate B05.10 under different pH values in 1mM fructose solution at 200 X .

| 5 hpi | 24 hpi |
| --- | --- |

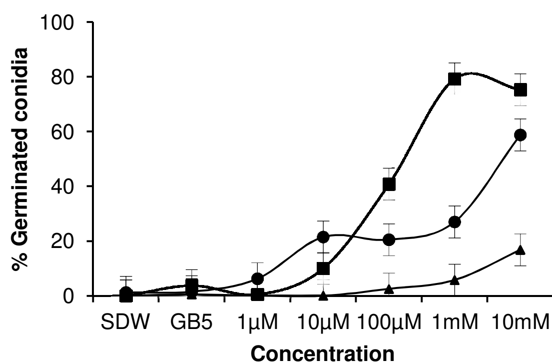
Fructose  Sucrose  Glucose

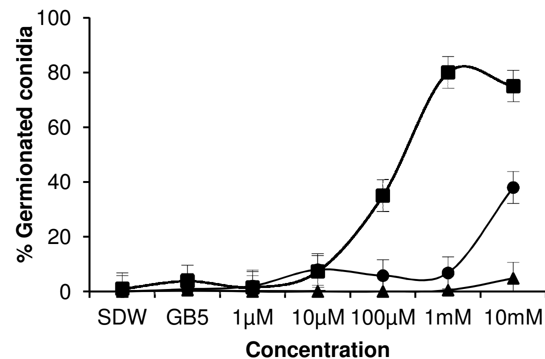
***Botrytis cinerea* (B05.10)**



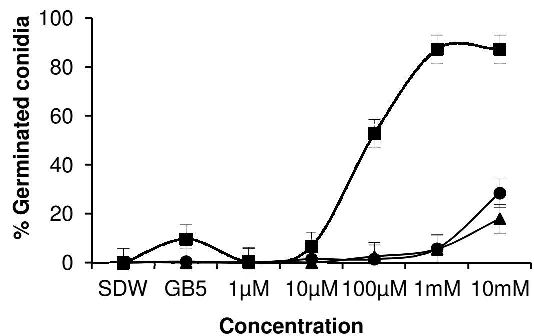




***Botrytis cinerea* (PBC3)**




***Botrytis cinerea* (PBC1)**



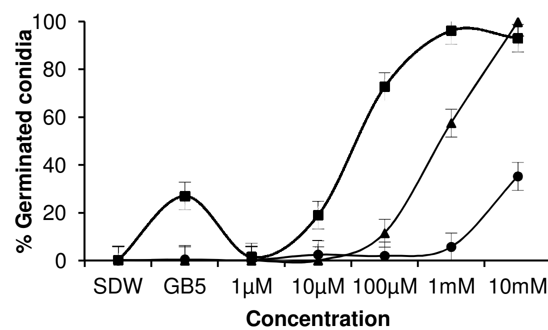
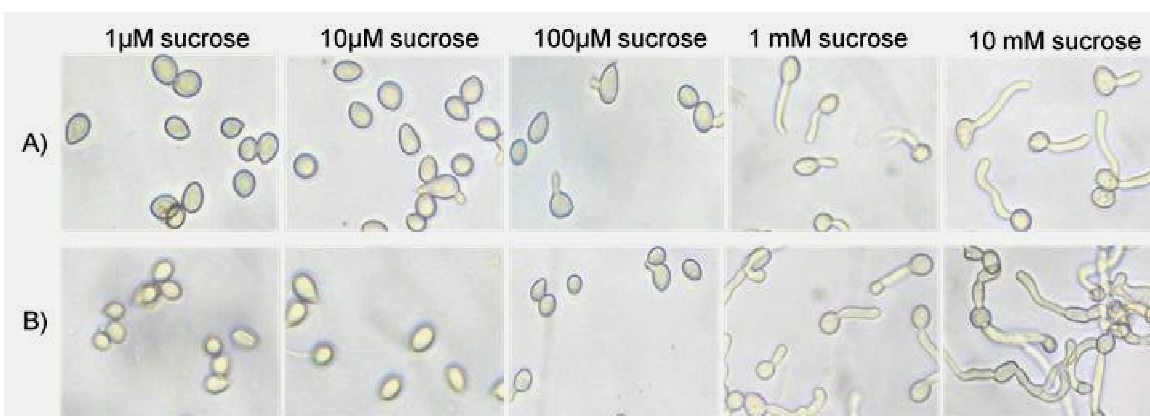
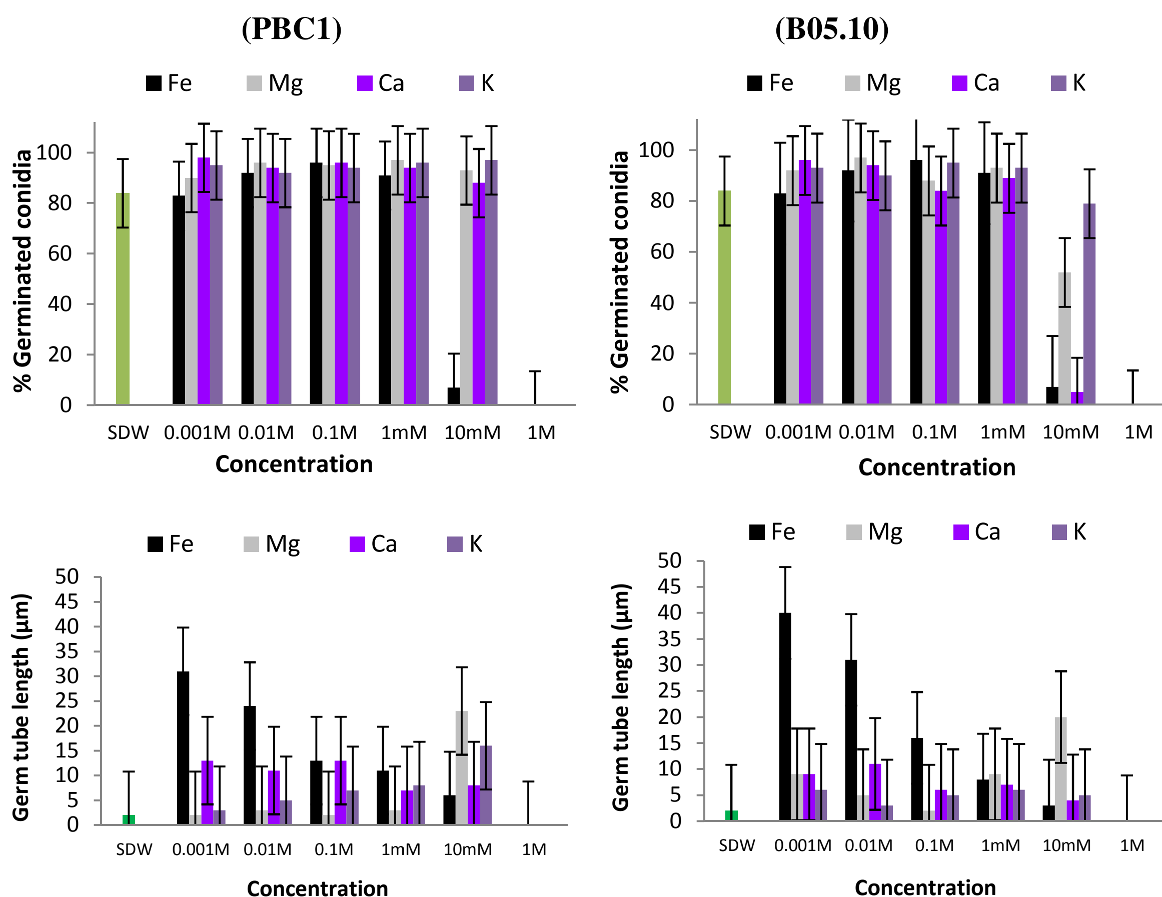




**Figure 5.** Influence of Fructose, Sucrose and Glucose solutions on germination of *Botrytis cinerea* conidia. (LSD=10.168, n=4, p<0.001). Experiment was done after 5 and 24 hours of incubation in various concentrations at 20±1 ºC. SDW: sterile distilled water; GB5: Gamborg's B5 basic salt mixture; hpi: hours post inoculation.

417

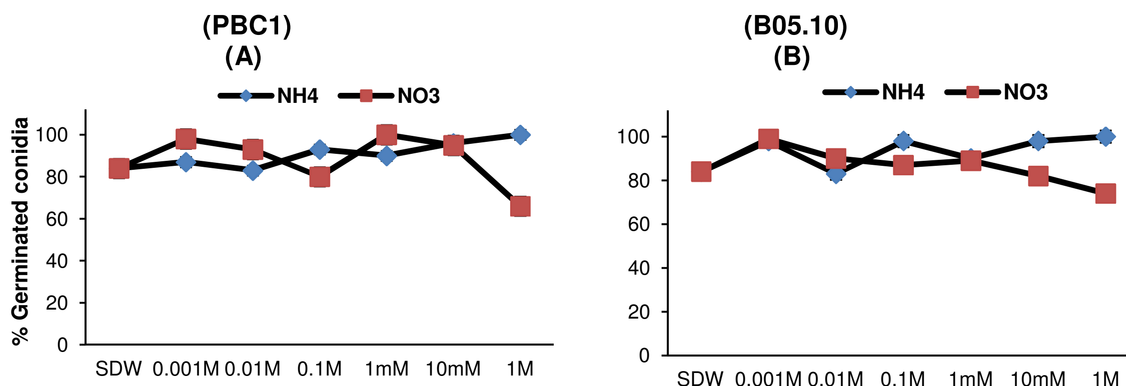



418 **Figure 6.** Conidial germination of *Botrytis cinerea* (B05.10) in different concentrations of sucrose.
419 A): after 5 and B): after 24 hours at 200X.
420
421
422




423
424

425 **Figure 7.** Influence of $Ca^{2+}$, $Mg^{2+}$, $K^{+}$, $Fe^{2+}$ in various concentrations on conidial germination and
426 germ tube elongation of *Botrytis cinerea* after 40 hours of incubation. Conidial
427 germination (LSD=13.527, n=4) ; Germ tube elongation (LSD=8.815, n=10).
428
429
430
431
432
433
434



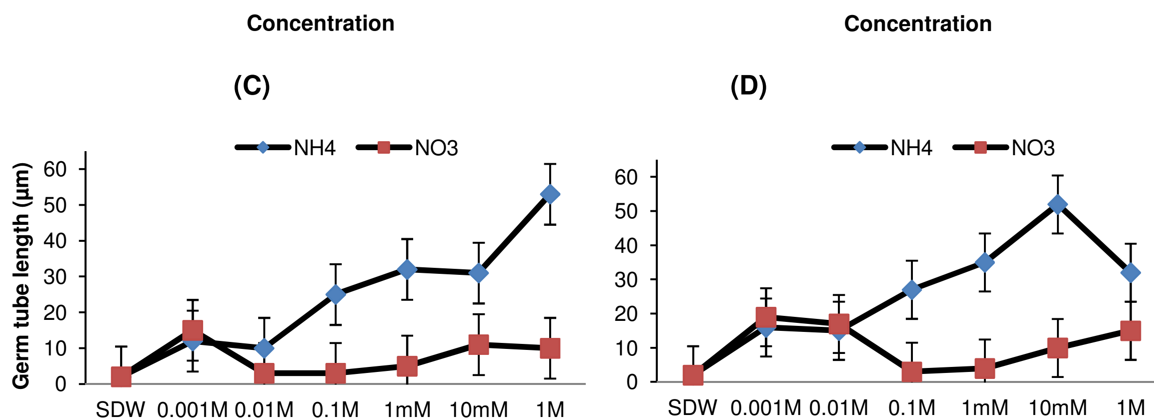




**Figure 8.** Influence of NH4 and NO3 in various concentrations on conidial germination and germ tube elongation of *Botrytis cinerea* PBC1 (A, C), and B05.10 (B, D). Differences between means of germination percentages were not significant; bars in (B, D) represent the standard error of the mean with LSD=8.489.

**Acknowledgment**

The authors acknowledge the financial support provided by the Deutsche Forschungsgemeinschaft (DFG) - grant number (Tu50/15).

470
471 **References**
472


473 1. Allen PJ. The role of a self-inhibitor in the germination of rust uredospores. Phytopathology.
474 1955; 215: 259-266.
475
476 2. Bacon CW, Sussman AS, Paul AG. Identification of a self-inhibitor from spores of
477 *Dictyostelium discoideum*. Journal of Bacteriology. 1973;113: 1060-1063.
478
479 3. Barhoom S, Sharon A. cAMP regulation of 'pathogenic' and 'saprophytic' fungal conidia
480 germination. Fungal Genetics and Biology. 2004; 41:317-326.
481
482 4. Barrios-Gonzales J, Martinez C, Aguilera A, Raimbault M. Germination of concentrated
483 suspensions of spores from *Aspergillus niger*. Biotechnology. 1989;11: 551-554.
484
485 5. Blakeman JP. Germination of *Botrytis cinerea* conidia in vitro in relation to nutrient
486 conditions on leaf surfaces. Transaction British Mycological Society. 1975; 65:239-247.
487
488 6. Braun EJ, Howard RJ. Adhesion of fungal spores and germlings to host plant surfaces.
489 Protoplasma. 1994;181: 202-212.
490
491 7. Carlile MJ, Watkinson SC. The Fungi. Academic Press. London.UK. 1994
492
493 8. Chaky J, Anderson K, Moss M, Vaillancourt L. Surface hydrophobicity and surface rigidity
494 induce conidia germination in *Colletotrichum graminicola*. Phytopathology. 2001; 91: 558-
495 564.
496
497 9. Di Lenna P, Marciano P, Magro P. Comparative investigation on morphological and
498 physiological features of three isolates of *Botrytis cinerea*. Phytopathologische Zeitschrift
499 1981; 100: 203-211.
500
501 10. Doehlemann G, Berndt P, Hahn M. Different signaling pathways involving a Gα-protein,
502 cAMP and a MAP kinase control germination of *Botrytis cinerea* conidia. Molecular
503 Microbiology 2006;59: 821-835.
504
505 11. Doehlemann G, Molitor F, Hahn M. Molecular and functional characterization of a fructose
506 specific transporter from the gray mold fungus *Botrytis cinerea*. Fungal Genetics and
507 Biology. 2005;42: 601-610.
508
509 12. Filonow AB. Mycoactive acetate esters from apple fruit stimulate adhesion and germination
510 of conidia of the gray mold fungus. Journal of Agricultural Food Chemistry 2002; 50: 3137-
511 3142.
512
513 13. Forsberg H, Ljungdahl PO. Sensors of extracellular nutrients in *Saccharomyces cerevisiae*.
514 Current Genetics. 2001; 40: 91-109.
515
516 14. Grindle M. Phenotypic differences between natural and induced variants of *Botrytis cinerea*.
517 Journal of General Microbiology. 1979; 111: 109-120.
518
519 15. Jones EB. Fungal adhesion. Mycological Research. 1994; 98: 961-981.
520
521 16. Kerssies A, Zessen AI, Wagemakers CM, Van Kan J. Variation in Pathogenicity and DNA
522 polymorphism among *Botrytis cinerea* isolates sampled inside and outside a glasshouse.
523 Plant Disease.1997; 81: 781-786.
524
525 17. Klimple A, Gronover C, Stewart J, Tudzynski B. The adenylate cyclase (BAC) in *Botrytis*
526 *cinerea* is required for full Pathogenicity, Molecular Plant Pathology. 2002;6:439-450.
527

528 18. KosugeT, Hewitt WB. Exudates of grape berries and their effect on germination of conidia
529 of *Botrytis cinerea*. Phytopathology. 1994; 54:167-172.
530
531 19. Lorbeer JW. Variation in *Botrytis* and *Botryotinia*. In: Coley-Smith JR, Verhoeff K, Jarvis
532 WR. (eds.). The biology of *Botrytis*. Academic Press Inc. (London) LTD. (pp 19-40). 1980.
533
534 20. Osherov N, May G. Conidial germination in *Aspergillus nidulans* requires RAS signaling and
535 protein synthesis. Genetics. 2000; 155: 647-656.
536
537 21. Polach FJ, Abawi GC. Occurrence and biology of *Botryotinia fuckeliana* on beans in New
538 York. Phytopathology. 1975; 65: 657-660.
539
540 22. Prins TW, Tudzynski P, von Tiedemann A, Tudzynski B, ten Have A, Hansen ME, et al.
541 Infection strategies of *Botrytis cinerea* and related necrotrophic pathogens. In Fungal
542 Pathology (Kronstaad JW, ed). Dordrecht: Kluwer Academic Publishers 2000; (pp. 33-64).
543
544 23. Prusky D, McEvoy JL, Leverentz B, Conway WS. Local modulation of host pH by
545 *Colletotrichum* species as a mechanism to increase virulence. Molecular Plant-Microbe
546 Interactions. 2001;14:1105-1113.
547
548 24. Pruskyi, D., and N. Yakoby, 2003. Pathogenic fungi: leading or led by ambient pH?
549 Molecular Plant Pathology 4, 509-516.
550
551 25. RuanY, Kotraiah V, Straney DC. Flavonoids stimulate spore germination in *Fusarium solani*
552 pathogenic on legumes in a manner sensitive to inhibitors of cAMP-dependent kinase.
553 Molecular Plant-Microbe Interactions. 1995; 8: 929-938.
554
555 26. Scharrock K, Henzell R, Parry F. Self-inhibition of germination of *Botrytis cinerea* conidia.
556 The Horticulture and Food Research Institute of New Zealand. Hamilton, New Zealand.
557 2001; (pp25-26).
558
559 27. Schumacher J, Kokkelink L, Huesmann C, Jimenez-Teja D, Collado I, Barakat R, et al. The
560 cAMP-dependent signaling pathway and its role in conidial germination, growth and
561 virulence of the grey mould *Botrytis cinerea* . Molecular Plant-Microbe Interactions.
562 2008;21: 1443-1459.
563
564 28. Shirani N, Hatta T. Mineral salt medium for the growth of *Botrytis cinerea* in vitro. Annals of
565 Phytopathological Society of Japan. 1987; 53: 191-197.
566
567 29. Shirashi, M, Fucotomi M, Akai S. Influence of temperature and conidium age on mycelium
568 growth rates of *Botrytis cinerea* fr. Pers. Annals of the Phytopathological Society of
569 Japan.1988; 40: 230-235.
570
571 30. Vautard-Mey G, Fevre M. Carbon and pH modulate the expression of the fungal glucose
572 repressor encoding genes. Current Microbiology. 2003; 46: 146-150.
573
574
575
576
577
578
579
580
581
582

583 **COMPETING INTERESTS**
584
585 "Authors declares that no competing interests exist.".
586
587 **AUTHORS' CONTRIBUTIONS**
588
589 Authors may use the following wordings for this section: " 'Author 1' designed the study, performed
590 the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. 'Author 2'
591 managed the analyses of the study, read and approved the final manuscript."
592
593 **CONSENT (WHERE EVER APPLICABLE)**
594 **NOT APPLICABLE**
595
596 **ETHICAL APPROVAL (WHERE EVER APPLICABLE)**
597
598 **NOT APPLICABLE**
599