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Biocontrol potential of *Ampelomyces quisqualis* strain CPA-9 against powdery mildew: conidia production in liquid medium and efficacy on zucchini leaves

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Abstract

Ampelomyces quisqualis has been reported as a promising biocontrol agent (BCA) against powdery mildew and it was commercialised as AQ10 Biofungicide®. However, isolated strains showed inconsistent results when applied at practical conditions, and research to find a thoroughly effective strain is still in progress. Besides, asexual spores of the BCA are required to parasitize powdery mildew, although is complex to produce a high amount of conidia. The present study aimed to select of an effective strain of *A. quisqualis* against powdery mildew on zucchini leaves, and to test its conidiation in liquid media. Efficacy of several isolates from cucurbits were tested and the most effective was identified as *A. quisqualis* CPA-9, which reduced powdery mildew on zucchini leaves up to 61.5% when it was applied at 2.5×10^5 conidia/mL. Conidial production of CPA-9 in liquid media was optimised, testing several growing conditions such as medium, agitation, or incubation time. Asexual reproduction was induced by modifying the a_w of the media or reducing agitation. A concentration of 7.3×10^7 conidia/mL was achieved with 500-mL Erlenmeyer flasks containing 75 mL of potato dextrose broth modified with 2.5% (w/v) of

glycerol incubated at 25 ºC for 11 days at dark and without agitation. Produced conidia of CPA-9 maintained the biocontrol efficacy against *P. xanthii* on zucchini plants, reducing the disease incidence up to 83%.

Keywords: conidiation; *P. xanthii*; cucurbits; fungi; mycoparasitation; biological control

1. Introduction

Powdery mildew is probably the most common and widespread disease of cucurbits, being *Podosphaera fusca (synomin Podosphaera xanthii)* the main causal agent of this infection in many countries around the world (Bélanger et al., 2002; Křístková et al., 2009). *P. xanthii* is an obligate biotrophic parasite which develops as a whitish powdery on leaf surfaces, petioles and stems (Pérez-García et al., 2009).

The two main methods for powdery mildew control are the use of resistant cultivars and the application of fungicides (Kiss et al., 2004). Nevertheless, the use of resistant cultivars is limited (Kiss et al., 2001), and repeated fungicide treatments against *P. xanthii* often caused the development of resistance (Gilardi et al., 2012). For that reasons, together with environmental and health concerns of fungicides, biological control agents (BCAs) have been studied for a long time as a safety alternative to control powdery mildew, being fungi of the genus *Ampelomyces* the major antagonist of *Erysiphales* (powdery mildews) fungi (Sucharzewska et al., 2012).

Ampelomyces quisqualis is a specific mycoparasite of *Erysiphales* (Angeli et al., 2013) which is considered an endoparasitic fungus whose conidia (pycnidiospores or spores, as other authors called) penetrate into *P. xanthii* and forms pycnidia within powdery mildew structures (Romero et al., 2003; Sztejnberg and Galper, 1989). The mycoparasite restricts the number of *P. xanthii* haustoria, whereby the nutrient uptake of the pathogen is reduced. *A. quisqualis* also reduces the disease multiplication due to the limitation of the amount of powdery mildew conidiophores and conidia (Romero et al., 2003).

The growing of the fungus *A. quisqualis* is considered to be slow (Kiss et al., 2004), and despite of formation of pycnidia on culture media is possible, the conidiation is relatively poor (Angeli,

57 2013). Asexual reproduction is required to obtain *A. quisqualis* conidia, which are the
58 reproductive spores (Sztejnberg, 1993), and liquid fermentation is preferred for large scale
59 production of conidia due to it is an extensively studied methodology which permits an economic
60 and relatively short process (Zaki et al., 2018). However, vegetative growth prevails during
61 submerged growth in rich liquid medium for other studied ascomycetes (Adams et al., 1998).
62 Different media or supplementary substances has been recently studied to improve conidiation
63 and germination of *A. quisqualis* produced in liquid fermentation (Angeli, 2013; Angeli et al.,
64 2017; Saharan et al., 2013) but is still difficult to achieve a high concentration of conidia in the
65 formulated products.

66 Conidiation is determined by the metabolic state of the cell, which is influenced by its own
67 biological rhythms and also by the environment (Steyaert et al., 2010a). In this way, several
68 studies have been focused on the induction of asexual reproduction by exposing the fungi to
69 environmental cues (Adams et al., 1998; Boualem et al., 2014; Steyaert et al., 2010b). It has been
70 observed that the conidia of several fungus species germinate directly and generate new conidia
71 without forming mycelia when they were exposed to stress conditions; this process was defined
72 as microcycle conidiation (Jung et al., 2014).

73 One *A. quisqualis* strain isolated in Israel was formulated, registered and commercialized in
74 several countries as a biocontrol product under the name AQ10 Biofungicide® (Ecogen, Inc, USA)
75 (Sztejnberg, 1993, 1991) against powdery mildew. However, the efficacy results obtained with
76 AQ10 Biofungicide® use are contradictory. Some experiments resulted in unsatisfactory levels of
77 biocontrol (Dik and Verhaar, 1998; Kiss, 2003; Shishkoff and McGrath, 2002). Other trials
78 required high relative humidity to be effective (Romero et al., 2007), or repeated applications
79 were necessary to control the disease (Sztejnberg, 1993). Therefore, there is still noteworthy
80 interest in finding strains of *A. quisqualis* species more effective than the existing biofungicide
81 strain (Angeli et al., 2012).

82 The potential of *A. quisqualis* as a biocontrol agent against powdery mildew has been thoroughly
83 documented (Angeli et al., 2013; Gautam and Avasthi, 2016; Gilardi et al., 2017, 2012, 2008;
84 Zhao et al., 2012). However, mass production and formulation of the fungus to achieve a high

amount of conidia for biocontrol purposes is a crucial step before commercialization (Kiss et al., 2004). For this reason, production of conidia of the isolates should be at least preliminary verified to consider its suitability as a potential biocontrol agent.

This research has firstly focused on the search and selection of a new and effective isolate of *A. quisqualis* against powdery mildew on zucchini leaves. Then, the conidiation ability of this new strain was studied in liquid media to value its suitability for future optimisation of dried formulations of this BCA. The specific objectives of the present study were: (i) to select an effective isolate of *A. quisqualis* against powdery mildew; (ii) to determine the required dose of *A. quisqualis* conidia to control powdery mildew on zucchini leaves; (iii) to make a characterisation of growing media to achieve a high concentration of conidia; and finally, (iv) to test the efficacy of produced *A. quisqualis* conidia against *P. xanthii* on zucchini plants.

2. Materials and methods

2.1. The biocontrol agent

Different strains of *Ampelomyces* spp. were isolated from pumpkin, zucchini and cucumber leaves affected with the typical symptoms of powdery mildew infection. All the leaves were collected from different fields located in the North-East of Spain (Lleida, Catalonia) and examined with a stereoscopic microscope to select those leaves which presented brownish intracellular pycnidia of *Ampelomyces* spp. The pycnidia found were isolated and transferred every 10 days to malt extract agar medium (MEA: malt extract, 30 g/L; peptone, 5 g/L; and agar 15 g/L) plates incubated under a daily 12-h photoperiod of black light UV at 25 ºC and 12 h dark at 18 ºC. In total, eleven strains with a similar phenotypical morphology of *Ampelomyces* spp. were isolated.

Once the best strain was selected, it was kept in Glycerol 20% and in Criobilles tubes at -80 ºC for long term storage. When required, the strain was sub-cultured on MEA plates and incubated in the conditions described before during 10 to 15 days. Then, the fungus was periodically sub-cultured at most three times. The selected strain, renamed as CPA-9, was identified as *A. quisqualis* by the University of León (Spain) and it was deposited at the Colección Española de Cultivos Tipo (CECT-20749) at the University of Valencia (Burjassot, Spain). This strain has patented by LAINCO, S.A. (Rubí, Spain) (Garriga et al., 2014).

113 **2.2. The pathogen**

114 *P. xanthii* strain 04/05 isolated from cucurbit leaves was used in this study to test the efficacy of
115 the biocontrol agent. The biotroph parasite was maintained by growing on zucchini cotyledons
116 (variety "Black Beauty") as described Álvarez and Torés (1997) with some modifications.
117 Briefly, cotyledons were disinfected thrice with an $HgCl_2$ solution (0.1% wv) during two minutes
118 each time. Then, cotyledons were left to dry at room temperature and introduced in plates with a
119 specific medium to maintain them (CTM: sucrose, 40 g/L; benzimidazol, 40 mL/L; and agar 2.4
120 g/L). The petioles of the cotyledons were embedded in the medium and a pinch of powdery
121 mildew mycelium was spread on their surface with an eyelash to avoid damages in the leaves.
122 Plates were incubated under a daily photoperiod, which consisted of 12 h with white light at
123 21 ºC and 12 h in the dark at 18 ºC. The process was repeated approximately every 15 days to
124 maintain the strain.

125 **2.3. Efficacy of different *Ampelomyces* spp. isolates on zucchini plants**

126 Experiments to test the efficacy of different *Ampelomyces* spp. isolates on zucchini plants were
127 conducted in an experimental greenhouse property of IRTA, which was located in Lleida
128 (Catalonia, Spain). Zucchini plants (variety "Black Beauty") were raised in seedling trays in a
129 nursery-like greenhouse with a daily 16-h photoperiod of light 24 ºC and 8 h dark at 19 ºC. After
130 3 weeks, plants with the first leave completely developed were transplanted into bigger plastic
131 pots (16 L) and placed in the experimental greenhouse.

132 Conidia of *P. xanthii* 04/05 grown in cotyledons were blown on the surfaces of the second and
133 third leaves of the plant to simulate a natural infection. Biocontrol treatments were applied twice
134 at 10^7 conidia/mL: (i) when powdery mildew infection appeared on the surface of the leave, and
135 (ii) seven days after the first application. Biocontrol efficacy was estimated 7 days after the second
136 application with the incidence disease index (IDI), which was calculated as: $[[(a \times 0) + (b \times 1) +$
137 $(c \times 2) + (d \times 3)] / 3n] \times 100$, where numbers indicate the relative surface of the leaf covered with
138 powdery mildew (0: no powdery mildew on leaf; 1: only few spots of powdery mildew; 2: a half
139 of leaf or more; 3: all surface of leaf practically covered); letters are related to the amount of
140 leaves assigned at each category; and *n* was the total number of inoculated leaves.

141 ### 2.4. Effective dose of *A. quisqualis* strain CPA-9 against powdery mildew

142 The surface of detached leaves of zucchini plants (variety "Black Beauty") were infected with
143 *P. xanthii* grown in cotyledons. Second and third leaves of 3-week-old zucchini plants were
144 detached and placed in double Petri plates with their petioles immersed in Hoagland's solution
145 diluted to 50%. Then, the pathogen was blown on the leaves and plates were incubated in a plant
146 growth chamber during 7 days before the treatment application. Chlorotic leaves and those that
147 presented advanced or retarded infection were discarded to achieve a homogeneous batch. Control
148 leaves were treated with sterile water and *A. quisqualis* CPA-9 was applied at three
149 concentrations: (i) 2.5×10^5 conidia/mL; (ii) 1×10^6 conidia/mL; and (i) 1×10^7 conidia/mL.
150 Biocontrol efficacy was estimated 5 days after the treatments and calculated as the incidence
151 disease index (IDI) detailed before (Section 2.3). Each replicate consisted of seven leaves and the
152 trial was repeated twice.

153 ### 2.5. Production of *A. quisqualis* CPA-9 in liquid medium

154 #### 2.5.1. Inoculation and conidia counts

155 Inoculum was prepared with *A. quisqualis* CPA-9 grown in MEA plates during 10 to 15 days.
156 Colonies mixed with sterile water were grinded with a sterile porcelain mortar and pestle. Then,
157 the concentration of conidia was counted in a Thoma counting chamber and the required volume
158 was inoculated in 250-mL Erlenmeyer flasks containing 50 mL of media to achieve the initial
159 concentration of 10^5 conidia/mL. Obtained cultures after incubation were grinded with an
160 homogeniser-disperser Miccra D-9 (Miccra GmbH, Heitersheim, Germany) for 5 min at 21000
161 rpm to facilitate the rupture of the pycnidial wall and to release the conidia from the pycnidia,
162 then conidia were counted in a Thoma counting chamber.

163 #### 2.5.2. Characterisation of media to increase conidia production

164 Different liquid media were considered to achieve a high concentration of *A. quisqualis* CPA-9
165 conidia: (i) soluble potato starch (SPS: soluble potato starch, 30 g/L); (ii) potato extract glucose
166 broth (PEGB: potato extract, 100 mL/L; and glucose, 50 g/L); (iii) modified potato extract broth
167 adjusted at 0.98 a_w with glycerol (MPEB: potato extract, 100 mL/L; and glycerol, 92 g/L); (iv)
168 potato dextrose broth (Scharlau, Sharlab SL, Barcelona, Spain) (PDB: potato peptone, 4 g/L; and

169 glucose, 20 g/L); (v) modified potato dextrose broth adjusted at 0.98 a_w with glycerol (MPDB:
170 potato peptone, 4 g/L; glucose, 20 g/L; and glycerol, 20 g/L); (vi) concentrated potato dextrose
171 broth (CPDB: potato peptone, 10 g/L; and glucose, 50 g/L); and (vii) diluted potato dextrose broth
172 (DPDB: potato peptone, 3 g/L; and glucose, 15 g/L). Four flasks per medium were inoculated
173 with *A. quisqualis* CPA-9, and one flask was used to control external sources of contamination.
174 Inoculated flasks were incubated in the dark at 25 ºC and 150 rpm during 15 days. Conidia were
175 counted after 6, 10, 13, and 15 days of incubation, and one Erlenmeyer flask was used at each
176 time.

177 **2.5.3. Modified media with glycerol to simulate stress conditions**

178 The a_w of the best medium obtained in the first approach was modified to produce stress during
179 the growth of *A. quisqualis* and induce asexual reproduction. Media were modified with the
180 addition of different amounts of glycerol: 1%, 1.5%, 2%, 2.5%, 5%, 7%, and 9.2% of glycerol
181 (w/v). Non modified medium was used as negative control and 250-mL Erlenmeyer flasks were
182 incubated in the dark at 25 ºC and 150 rpm during 7 days. After incubation, conidia were released
183 and counted as described before (Section 2.5.1).

184 **2.5.4. Impact of agitation conditions during incubation**

185 The effect of agitation during the incubation period was also evaluated with the best media
186 obtained in the previous experiment. Three Erlenmeyer flasks were incubated for each agitation
187 condition: (a) 0 rpm; (ii) 75 rpm; and (iii) 150 rpm. One flask was used as contamination control.
188 The counts of conidia were done after 7, 10, and 15 days of incubation in the dark at 25 ºC as
189 described before (Section 2.5.1).

190 **2.6. Optimisation of flask and medium volumes to increase conidia production**

191 Different flasks and media volumes were tested after the selection of the media and the agitation
192 conditions: (i) 250-mL Erlenmeyer flask with 50 mL of medium (250/50); (ii) 500-mL
193 Erlenmeyer flask with 75 mL of medium (500/75); (iii) 500-mL Erlenmeyer flask with 100 mL
194 of medium (500/100); (iv) 1000-mL Erlenmeyer flask with 100 mL of medium (1000/100); (v)
195 1000-mL Erlenmeyer flask with 150 mL of medium (1000/150); (vi) 75-mL cell culture flask
196 with 50 mL of medium (75/50); (vii) 75-mL cell culture flask with 75 mL of medium (75/75);

197 and (viii) 300-mL cell culture flask with 200 mL of medium (300/200). Conidia counts were done
198 after 7 and 11 days of incubation at 25 ºC and the agitation speed selected previously. The
199 germination of conidia was measured to estimate the viability of *A. quisqualis* 22/2005 as
200 described Buron-Moles *et al.* (2012) for *Penicillium* spp. with some modifications. Three 10-µL
201 droplets of each growing condition adjusted to 5×10^6 conidia/mL were inoculated on MEA plates
202 with 50 ppm of gentamicin to inhibit bacterial growth. Plates were incubated at 25 ºC for 26 h,
203 then each drop was aseptically removed using a sterile pipette tip. The three agar disks (7 mm
204 diameter) corresponding to each drop were placed into a sterile Petri dish and conidia germination
205 was stopped by adding 3 mL of ammonia onto a filter paper placed on the cover of each plate.
206 Once conidia germination was stopped, agar disks were stored at 4 ºC until microscopic
207 examination. Each treatment consist on three disks and three replicates of fifty single conidia per
208 disk were examined (150 conidia per replicate and 450 conidia per treatment). Conidia were
209 considered germinated when the germ tube was equal or longer than the length of the
210 ungerminated conidium. The variable was expressed as the percentage of germination.

211 **2.7. Efficacy of produced *A. quisqualis* CPA-9**

212 Efficacy of produced *A. quisqualis* CPA-9 was confirmed against powdery mildew on zucchini
213 plants (variety "Black Beauty") and compared with infected leaves treated with water (control).
214 Four leaves of each plant, excluding the first leaf, were infected with powdery mildew. *P. xanthii*
215 was spread on the leaf nervation using an eyelash and infected plants were incubated in a plant
216 growth chamber for four days. Then, plants were sprayed with water (control) and *A. quisqualis*
217 CPA-9 produced with the optimised methodology at 10^7 conidia/mL. The incidence disease index
218 (IDI) was calculated six days after treatment as described before (Section 2.3).

219 **2.8. Statistical analysis**

220 Data were analysed by one-way ANOVA and differences at $P < 0.05$ were considered as
221 significant. In general, means separation were obtained by Tukey's test, although LSD Student's
222 *t* test was used when only two means had to be compared. The results of conidia per mL were
223 transformed to logarithmic values prior analyses. Data analysis was performed using JMP 13
224 software (SAS Institute Inc., Cary, NC).

225 **3. Results**

226 **3.1. Efficacy of different isolates of *A. quisqualis* against powdery mildew on zucchini**
227 **plants**

228 The biocontrol efficacy of *Ampelomyces* spp. isolates against powdery mildew on zucchini plants
229 is shown on Figure 1. All tested isolates reduced the incidence disease index, although only four
230 of them significantly reduced the powdery mildew incidence: 14/2005, 21/2005, 22/2005, and
231 25/2005. Despite no significant differences were observed among these isolates, incidence
232 reductions ranged from 60% to 74%, being the isolates 25/2005 (71%) and 22/2005 (74%) the
233 most effective against *P. xanthii.*
234 However, it was necessary to select one isolate to continue with the assays, and this experiment
235 was repeated with the isolates 22/2005 and 25/2005. Both isolates were compared again with a
236 control treated with water but no significant differences were observed between the isolates (F =
237 42.1512; df = 2; P = 0.0003) (data not shown). Notwithstanding, incidence reduction was also
238 higher with the isolate 22/2005 (66.7%) than with the 25/2005 (47.6%). Moreover, the obtained
239 results also showed that reduction values were more repetitive with the isolate 22/2005. Both
240 observations were considered to discern between both isolates and the isolate 22/2005 was
241 selected to continue with the assays. The selected isolate was identified as *A. quisqualis* and
242 renamed CPA-9 strain.

243 **3.2. Effective dose of *A. quisqualis* CPA-9 to control powdery mildew on zucchini leaves**

244 Three doses of *A. quisqualis* CPA-9 were tested against powdery mildew on zucchini leaves and
245 compared with a control treated with water (Figure 2). All tested concentrations significantly
246 reduced the disease incidence from 61.5% to 88.5% compared with the control. No significant
247 differences were observed among concentrations, although a trend of increasing incidence
248 reduction at higher doses of CPA-9 was observed. Results suggested that the efficacy of CPA-9
249 was dose-dependent. Apparently, a high concentration of conidia controlled easily the powdery
250 mildew infection.

251 **3.3. Conidia production of *A. quisqualis* CPA-9 in liquid medium**

252 **3.3.1. Characterisation of medium to increase conidia production**

253 Seven liquid media containing potato derivatives were tested after 6, 10, 13, and 15 days to
254 produce conidia of *A. quisqualis* CPA-9 (Table 1). The highest production of conidia were
255 achieved 15 days after the inoculation with modified potato dextrose broth adjusted at 0.98 a_w
256 with glycerol (1.4×10^7 conidia/mL), and with diluted potato dextrose broth (8.6×10^6
257 conidia/mL). Other media achieved at most 4.0×10^5 conidia/mL (CPDB after 10 days), whereas
258 the majority of tested media could not produce conidia (concentration $< 5.0 \times 10^3$ conidia/mL).
259 Therefore, production of conidia was higher when potato dextrose broth was modified to produce
260 a stress during the fungus growth, either modifying the a_w or diluting the media. If not, vegetative
261 growth of *A. quisqualis* prevailed and only mycelia was observed.
262 From this data, potato dextrose broth with different concentrations of glycerol were tested to
263 promote asexual reproduction of *A. quisqualis* (Figure 3a). The highest concentration of conidia
264 was achieved with a concentration of 2.5% (w/v) of glycerol in the medium (1.2×10^7 conidia/mL)
265 which corresponded to 0.976 a_w. Good results were also obtained with 2% (w/v) of glycerol (0.98
266 a_w), although the concentration of conidia sharply decreased with lower or higher concentrations
267 of glycerol. Water activity values higher than 0.98 only permitted mycelium growth, whereas
268 *A. quisqualis* could be too much stressed when $a_w < 0.97$.
269 Potato dextrose broth with 2.5% (w/v) of glycerol was used to continue the optimisation of
270 medium. Different conditions of agitation during incubation were also tested to evaluate the effect
271 on the asexual reproduction of the fungi (Figure 3b). The best results were obtained after 10 days
272 without agitation of Erlenmeyer flasks during the incubation, achieving a concentration of $6.9 \times$
273 10^7 conidia/mL. At 75 or 150 rpm, the highest concentrations were also achieved after 10 days,
274 although the values were lower, 3.9×10^7 and 4.8×10^7 conidia/mL, respectively. Visual
275 differences were also observed between static and dynamic growth. A brownish and thin layer of
276 *A. quisqualis* was formed over the medium without agitation, whereas CPA-9 growth as small
277 spheres inside the medium when flasks were agitated.

278 3.3.2. Optimisation of flask volume to medium volume ratio to increase conidia 279 production

280 Potato dextrose broth modified with 2.5% (w/v) of glycerol was selected for *A. quisqualis* CPA-
281 9 production at 25 ºC for 10 days at dark and without agitation. Different flasks and medium
282 volumes were tested to evaluate the effect of the air volume and the growing surface of
283 *A. quisqualis* when it was incubated without agitation (Figure 4). The significantly highest
284 concentration of conidia obtained with Erlenmeyer flasks 500/75 after 11 days of incubation
285 raised to 7.3×10^7 conidia/mL. At these conditions, conidia germination for *A. quisqualis* was of
286 84.8%, concentration of viable conidia was 6.2×10^7 conidia/mL, and the total amount of viable
287 conidia in the Erlenmeyer flask was approximately 4.5×10^9 conidia.
288 In general, germination of conidia was higher after 7 days of incubation, when ranged from 81.2%
289 to 98.7% (data not shown). After 11 days, germination values ranged from 64.0% to 88.7% (data
290 not shown) although concentration of conidia were usually higher, being concentration of viable
291 conidia also higher. The worst results were obtained with cell culture flasks 300/200, obtaining
292 2.3×10^6 conidia/mL after 7 days of incubation, and 2.1×10^6 conidia/mL after 11 days.

293 #### 3.3.3. Efficacy of *A. quisqualis* CPA-9 against powdery mildew

294 Efficacy of *A. quisqualis* CPA-9 produced at the optimised conditions (500-mL Erlenmeyer flasks
295 with 75 mL of potato dextrose broth modified with 2.5% (w/v) of glycerol incubated at 25 ºC for
296 11 days at dark and without agitation) were tested against powdery mildew on zucchini plants
297 (Figure 5). Conidia of CPA-9 significantly reduced the disease incidence of powdery mildew up
298 to 83% (Figure 5a). Treated leaves were quite healthy and only few spots of powdery mildew
299 were observed on some leaves (Figure 5c). In general, the surface of the treated leaves were
300 entirely green with the exception of the areas where *A. quisqualis* parasitised *P. xanthii,* which
301 were yellowish. In contrast, control leaves treated with water appeared highly damaged after 13
302 days of the infection and all the surface of the leaves was practically covered with powdery
303 mildew (Figure 5b).

304 ## 4. Discussion

305 Despite of *A. quisqualis* is reported as a promising BCA to control powdery mildew, few studies
306 have been published regarding its conidial production or formulation because the information are
307 usually kept secret by industry (Angeli et al., 2017). Moreover, when a new effective isolate is

308 selected, its conidia production needs to be confirmed to consider the isolate as a potential BCA
309 because generic diversity could determine the fungi development (Kiss et al., 2004).
310 The strain CPA-9 of *A. quisqualis* significantly reduced *P. xanthii* incidence on zucchini plants.
311 Specifically, incidence reduction achieved with CPA-9 was around 70% compared with the
312 control. Angeli et al. (2012) tested the ability of *A. quisqualis* to reduce conidiation of powdery
313 mildews in vivo, achieving reductions of *P. xanthii* on cucumber that ranged from 23 to 93%,
314 therefore, high reductions were also obtained with some of these isolates. Efficacy of other
315 biocontrol agents against powdery mildew has been also tested: different *Bacillus subtilis* strains
316 reduced powdery mildew incidence on melons from 44 to 58% (Romero et al., 2007); other
317 isolated *Bacillus* spp. reduced the incidence of downy mildew by 42% on cucumber (Sun et al.,
318 2013); the BCAs *Serratia marcescens*, *Clonostachys rosea*, and *Trichothecium roseum*, reduced
319 powdery mildew on zucchini by 53%, 61%, and 68%, respectively (Tesfagiorgis et al., 2014); and
320 a high reduction of *P. xanthii* was obtained with *Pseudozyma aphidis*, which reduced powdery
321 mildew leaf coverage by 83% on cucumber plants (Gafni et al., 2015).
322 A single dose of *A. quisqualis* CPA-9 applied at 2.5×10^5 conidia/mL controlled effectively
323 powdery mildew infection on zucchini leaves, and no significant differences were observed
324 among different applied doses. However, CPA-9 showed a trend between the dosage and the
325 efficacy, the higher dose, the better efficacy, suggesting that it could be interesting to apply a
326 higher dose of *A. quisqualis* to control highly developed infections of *P. xanthii*, whereas a lower
327 dose of the BCA will be enough to control an emerging infection. The commercialised AQ10
328 Biofungicide® is generally applied at 10^6 conidia/mL to control low powdery mildew infection
329 levels (Sztejnberg, 1991), and the application should be repeated at 6-8 days intervals. However,
330 Pertot et al. (2008) applied 0.08 g/L of AQ10 Biofungicide® (corresponding approximately to 4
331 $\times 10^8$ conidia/mL) every 10 days to partially supress powdery mildew on strawberries. Grapevine
332 powdery mildew was significantly reduced with pre- and post- harvest applications of AQ10
333 Biofungicide® at 5×10^9 conidia/mL (Caffi et al., 2013). In contrast, Romero et al. (2003) applied
334 AQ10 Biofungicide® at 5×10^5 conidia/mL to significantly reduce powdery mildew on detached
335 melon leaves under controlled conditions and in early stages of infection. Other studies also tried

336 to find new effective strains of *A. quisqualis* which were more aggressive and applied the BCA
337 at 10^6 conidia/mL with other substances to promote its germination (Angeli et al., 2013).
338 A high amount of conidia is required to develop a potential biocontrol product, for this reason it
339 was necessary to check the ability of the selected isolate to produce conidia in liquid media. The
340 highest production of *A. quisqualis* CPA-9 conidia was achieved with potato dextrose broth with
341 2.5% (w/v) of glycerol to modify the a_w of the medium. The incubation of 75 ml of medium in
342 500-mL Erlenmeyer flasks, without agitation, for 11 days at dark, resulted in the highest
343 production: 6.2×10^7 viable conidia/mL. Previous studies modified the a_w and the nutrient
344 concentration of growth media to improve the low a_w tolerance of *Candida sake* (Teixidó et al.,
345 1998a, 1998b). In the present work, stress conditions, such as the reduction of the a_w of the
346 medium or the static incubation, favoured the asexual reproduction of the fungi and consequently
347 the production of conidia increased. However, the growing conditions need to be adapted to each
348 isolate, and other *A. quisqualis* strains could require other conditions to produce ripe pycnidia and
349 release high concentrations of conidia. For example, Sztejnberg and Galper (1989) also described
350 PDB as the most productive liquid media, achieving approximately 10^6 viable conidia/mL after
351 incubating 100 mL of medium in 250-mL Erlenmeyers flasks for 9 days at 25 ºC and with shaking
352 at 122 rpm. Conidia production in a 2-L fermentor increased from 10^4 to 10^6-10^7 conidia/mL after
353 9 days of incubation at 25 ºC, 1 L/min of aeration and 200 rpm of agitation (Sztejnberg and Galper,
354 1989). Recent studies also produced *A. quisqualis* conidia in liquid sugar-based medium,
355 producing 3.8×10^7 conidia/mL in a 5-L fermenter after 15 days (Angeli et al., 2017).
356 Once agitation during incubation was avoided, the production became a liquid surface
357 fermentation (LSF), and conidial production was directly related to the surface area of the liquid
358 (Lopes et al., 2018). Therefore the ratio of flask/medium volume was optimised, being 75 ml of
359 medium in 500-mL Erlenmeyer flasks the best ratio. Despite of many fungi are grown in a solid
360 state (SSF) (Montesinos, 2003) to achieve better production, LSF could be advantageous in
361 several areas compared with SSF: a large amount of solid waste could be avoided in the industrial
362 production (Lopes et al., 2018); downstream processing of LSF became easier because a thin layer

363 of *A. quisqualis* conidia growth on the medium and it could be removed in one piece; and LSF
364 could be easily sterilised, reducing the risk of contamination during fungus growth.
365 Besides produced concentration, germination of *A. quisqualis* could also be considered as an
366 important factor because the conidial germination could determine the virulence of the BCA
367 against powdery mildew (Angeli et al., 2013). In the present study, the conidial germination of *A.*
368 *quisqualis* CPA-9 produced in the best conditions was near 85%. This value could be considered
369 very high taking into account that none stimulatory substances were added to increase the
370 germination. Angeli et al. (2017) achieved a conidial germination for *A. quisqualis* of 98% after
371 48 h of incubation with the addition of shrimp shell as a stimulatory substance.
372 Produced conidia of *A. quisqualis* CPA-9 significantly reduced *P. xanthii* on zucchini plants,
373 achieving a reduction around 80%. Despite of some studies suggested that produced conidia with
374 liquid state fermentation could differ in efficacy from those produced in a solid substrate (Zaki et
375 al., 2018), in the present study, CPA-9 maintained its efficacy after being produced in liquid
376 media. Treated zucchini leaves appeared healthy but with some yellow areas after *A. quisqualis*
377 CPA-9 controlled the infection. The yellowing is caused due to the reduction of photosynthesis
378 produced by powdery mildew infection (Pérez-García et al., 2009), and these symptoms were also
379 observed when cucumber leaves were treated with endophytic bacteria or even with chemical
380 antifungals (metalaxyl-mancozeb) (Sun et al., 2013).

381 ## 5. Conclusions

382 The present study introduces the strain CPA-9 of *A. quisqualis* as a potential BCA against
383 powdery mildew on zucchini plants. Conidiation of CPA-9 on liquid media was improved
384 reducing the water potential of the growing culture medium, and germination of produced conidia
385 was tested in different flasks with different media volumes. Moreover, the efficacy of produced
386 conidia was very satisfactory against *P. xanthii* on zucchini plants. The results obtained suggested
387 that the strain CPA-9 was a promising BCA to be applied against *P. xanthii,* on zucchini plants.
388 However, is still necessary to obtain a solid formulation which efficacy could be totally
389 comparable with other *A.* quisqualis-based products as AQ10, which is a water dispersible
390 granule. Future dried formulations of *A. quisqualis* CPA-9 could improve the handling and the

391 shelf-life of the fungus. Moreover, some problems of *A. quisqualis* consistency controlling
392 powdery mildew under field conditions, such as the requirement of warm temperatures (Angeli
393 et al., 2017) or relative humidity values above 80% (Kiss, 2003; Romero et al., 2007), could be
394 faced with the addition of biodegradable coatings in the dried formulation, as it has been done for
395 other BCAs (Carbó et al., 2017). Thus, future studies should be addressed to obtain a solid
396 formulation which improves *A. quisqualis* CPA-9 efficacy under practical conditions.

397

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559 **Table 1** Conidia formation of *A. quisqualis* CPA-9 in liquid medium after 6, 10, 13 and 15 days
560 of incubation at 25 ºC, 150 rpm and darkness conditions. Erlenmeyer flasks of 250 mL containing
561 50 mL of tested growing medium were used in this assay.

Medium	**Incubation days**			
	6	10	13	15
Soluble potato starch (SPS)	1.8×10^5	9.0×10^4	$< 5.0 \times 10^3$	$< 5.0 \times 10^3$
Potato extract glucose broth (PEGB)	$< 5.0 \times 10^3$	1.0×10^4	$< 5.0 \times 10^3$	$< 5.0 \times 10^3$
Modified potato extract glucose broth (MEGB)	5.0×10^4	$< 5.0 \times 10^3$	$< 5.0 \times 10^3$	$< 5.0 \times 10^3$
Potato dextrose broth (PDB)	5.0×10^3	1.0×10^5	$< 5.0 \times 10^3$	$< 5.0 \times 10^3$
Modified potato dextrose broth (MPDB)	1.2×10^5	3.8×10^5	5.0×10^6	1.4×10^7
Concentrated potato dextrose broth (CPDB)	2.0×10^4	4.0×10^5	7.0×10^4	$< 5.0 \times 10^3$
Diluted potato dextrose broth (DPDB)	1.0×10^4	$< 5.0 \times 10^3$	$< 5.0 \times 10^3$	8.6×10^6

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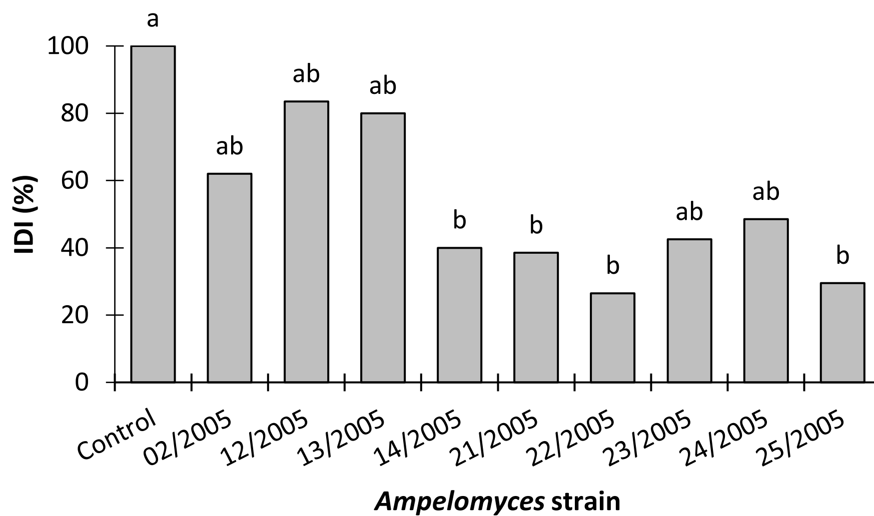
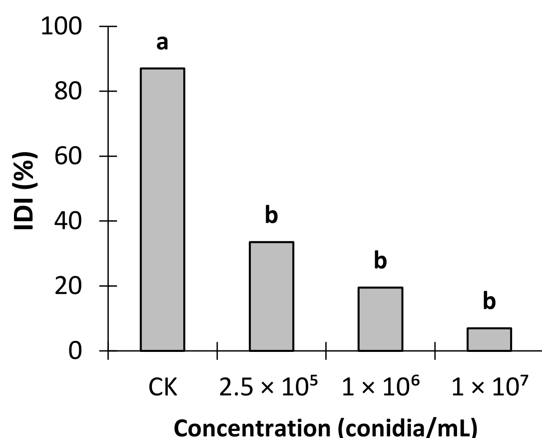


567

568 **Fig. 1** Efficacy of different *Ampelomyces* spp. isolates on zucchini plants (variety "Black beauty")
569 artificially infected with powdery mildew (*P. xanthii*). Biocontrol treatments were applied twice
570 at 10^7 conidia/mL: (i) after the apparition of powdery mildew infection, and (ii) seven days after
571 the first application. Control was treated with water and efficacy was estimated as the incidence
572 disease index (IDI) after seven days of the last treatment. Columns with different letters indicate
573 significant differences ($P < 0.05$) according to Tukey's test.
574



575

576 **Fig. 2** Efficacy of *A. quisqualis* strain CPA-9 on zucchini leaves (variety "Black beauty")
577 artificially infected with powdery mildew (*P. xanthii*). Biocontrol treatment was applied at
578 different concentrations seven days after the infection. Control was treated with water and
579 efficacy was estimated as the incidence disease index (IDI) after five days of the treatment.
580 Columns with different letters indicate significant differences ($P < 0.05$) according to Tukey's
581 test. Each replicate consisted of seven leaves and the trial was repeated twice.

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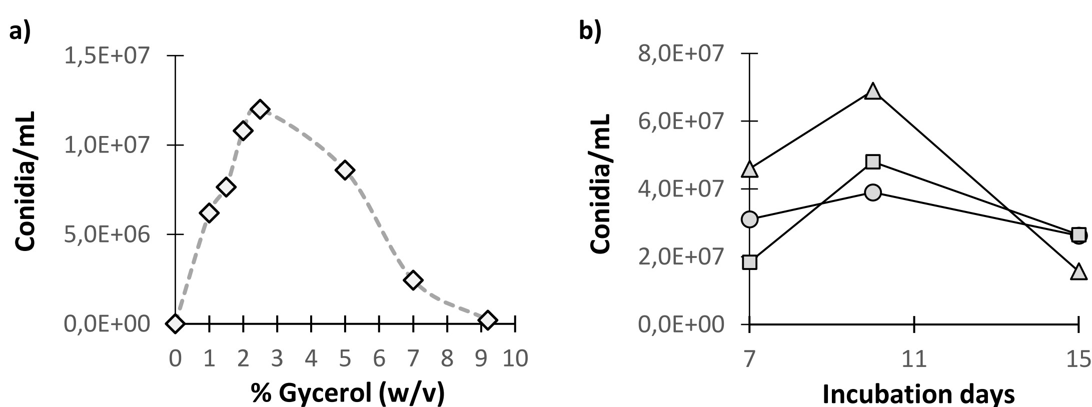

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591 **Fig. 3** Preliminary assays to consider conidia formation of *A. quisqualis* CPA-9: (a) in liquid
592 medium (PDB) with different concentrations of glycerol after 7 days of incubation in the dark at
593 25 ºC and 150 rpm; and (b) in liquid medium (PDB) with 2.5% of glycerol, with different agitation
594 conditions, specifically at 0 rpm (△), 75 rpm (○), and 150 rpm (□). In both cases, Erlenmeyer
595 flasks of 250 mL containing 50 mL of medium were inoculated at 10^5 conidia/mL.

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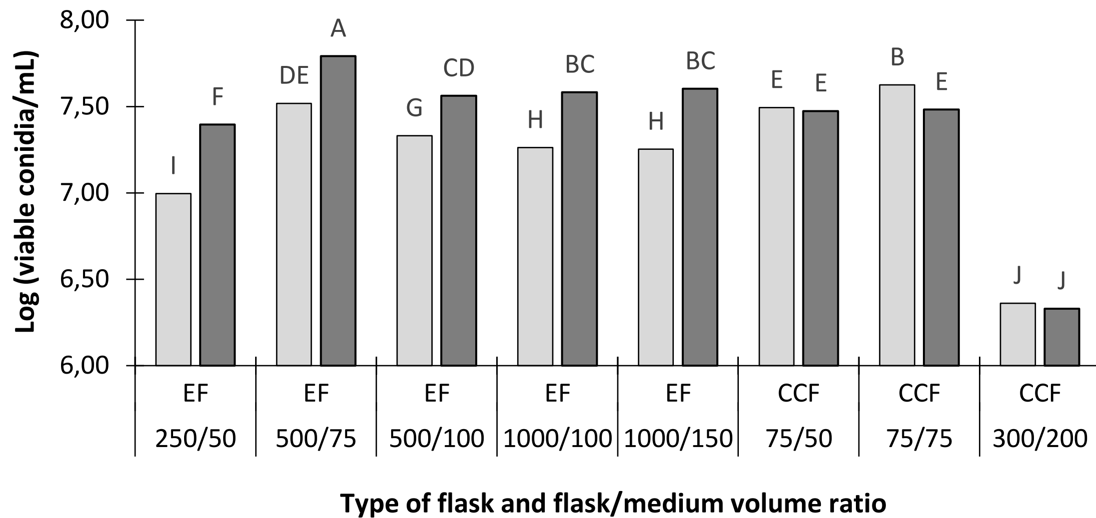

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598 **Fig. 4** Viable conidia production of *A. quisqualis* CPA-9 in liquid medium after 7 (□) and 11
599 days (■) of incubation at dark at 25 ºC without agitation. Erlenmeyer flasks (EF) and cell culture
600 flasks (CCF) of different volumes containing different volumes of PDB with 2.5 % of glycerol
601 were inoculated at 10^5 conidia/mL. Mean values of three replicates are represented and columns
602 with different letters indicate significant differences (P < 0.05) according to Tukey's test.
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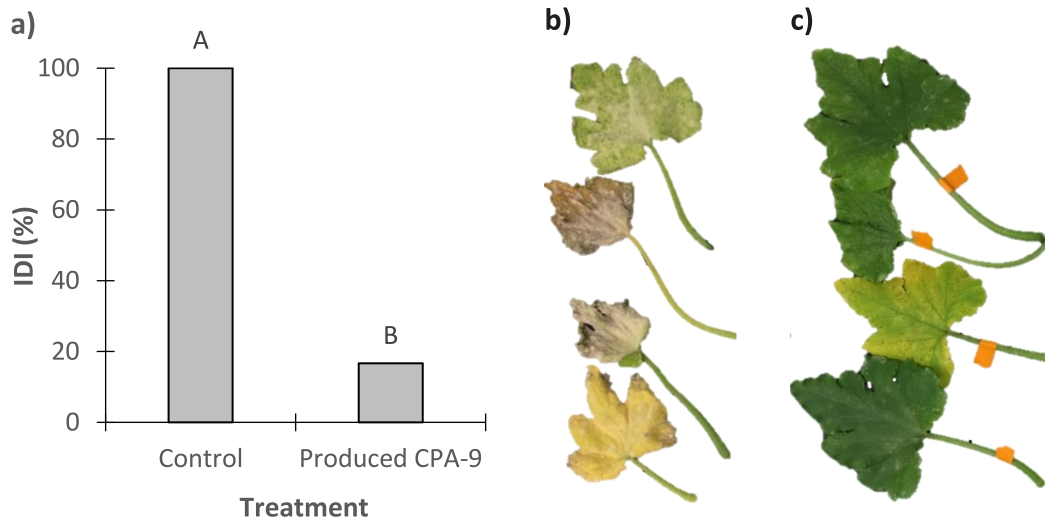

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609 **Fig. 5** Efficacy of produced *A. quisqualis* CPA-9 on zucchini plants (variety "Black beauty")
610 artificially infected with powdery mildew (*P. xanthii*): (a) Efficacy represented as IDI (incidence
611 disease index); (b) leaves treated with water (control) after six days of the treatment; and (c) leaves
612 treated with *A. quisqualis* CPA-9 at 10^7 conidia/mL after six days of the treatment. Both
613 treatments were applied seven days after the infection. Columns with different letters indicate
614 significant differences (*P* < 0.05) according to Student's *t* test.
615