Formulated *Ampelomyces quisqualis* CPA-9 applied on zucchini leaves: influence of abiotic factors and powdery mildew mycoparasitization

Anna Carbó, Neus Teixidó, Josep Usall, Cristina Solsona, Rosario Torres*

IRTA, Postharvest Programme, Edifici Fruitcentre, Parc Científic i Tecnològic Agroalimentari de Lleida, Parc de Gardeny, 25003 Lleida, Catalonia, Spain.

*Corresponding author:

Address: Postharvest Program, Institute for Food and Agricultural Research and Technology (IRTA), Edifici Fruitcentre, Parc Científic i Tecnològic Agroalimentari de Lleida, 25003 Lleida, Catalonia, Spain

Phone number: 973003430

e-mail: rosario.torres @irta .cat

Anna Carbó, ORCID: 0000-0002-3363-8479
Neus Teixidó, ORCID: 0000-0002-1676-3593
Josep Usall, ORCID: 0000-0002-0856-3508
Cristina Solsona, ORCID: 0000-0002-0595-372X
Rosario Torres, ORCID: 0000-0002-1806-9626

26   **Abstract**

27   Even though the potential of the fungus *Ampelomyces quisqualis* against powdery mildew has been largely

28   demonstrated, the efficacy of the commercialised product AQ10 Biofungicide is not consistent. Recently,

29   a solid formulation of *A. quisqualis* strain CPA-9 that included biodegradable coatings on its composition

30   was developed to overcome the major shortcomings of the biocontrol agents applied under practical

31   conditions. The aims of the present study were to show the compatibility of CPA-9 with different

32   phytosanitary products, and to confirm the potential of this novel formulation in different approaches: (i)

33   under different conditions of temperature (20 and 30 ºC) and relative humidity (40, 60 and 85 %) on

34   different surfaces (glass and zucchini leaves), (ii) after different rainfall episodes, and (iii) verifying

35   *Podosphaera xanthii* parasitization by dried conidia of CPA-9. It was demonstrated that CPA-9 was

36   compatible with several phytosanitary products, so it might be included in integrated management

37   programmes. Moreover, the solid formulation showed better resilience than non-formulated conidia, both

38   applied on a glass surface and on zucchini leaves. Adherence of both treatments on zucchini leaves did not

39   show significant differences after simulated rainfall and all tested concentrations of dried conidia were able

40   to produce pycnidia in *P. xanthii* hyphae. Therefore, the developed fluidised-bed spray-dried formulation

41   of *A. quisqualis* CPA-9 together with coatings compounds has all the makings of becoming a biocontrol

42   product, although their efficacy under practical conditions should be assessed.

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45   *Keywords***:** biocontrol; dehydration; *Podosphaera xanthii*; temperature; relative humidity.

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Introduction

Fungi of genus *Ampelomyces* are considered the major antagonist of *Erysiphales* (powdery mildew) infections (Sucharzewska et al. 2012). The biocontrol potential of *Ampelomyces quisqualis* against powdery mildew has been largely demonstrated (Angeli 2013; Gautam and Avasthi 2016; Gilardi et al. 2017, 2008; Legler et al. 2016), and the isolate M-10 of the fungus *A. quisqualis* was commercialised under the name AQ10 Biofungicide® as water dispersible granules (Ecogen, Inc, Langhorne, PA, USA) (Sztejnberg 1993, 1991). However, the commercialised product showed contradictory results in different trials (Kiss 2003; Shishkoff and McGrath 2002) and it is still necessary to find consistent and effective strains to apply under practical conditions (Angeli et al. 2012).

In fact, despite of biocontrol agents (BCAs) are used either at pre- and postharvest, applications at postharvest used to be more effective (Sharma et al. 2009), and even some BCAs only could be used at postharvest (Ippolito and Nigro 2000). At field conditions BCAs are exposed to uncontrollable abiotic stress, whereas postharvest conditions are clearly defined and usually constant. The use of edible coatings during the formulation process or the enhancement of stress adaptation are possible approaches to overcome the weakness of biocontrol products during preharvest applications (Usall et al. 2016). Integrated management programmes (IMP) were also suggested to improve the inconsistent performance of BCAs at different crop conditions (Romero et al. 2007), although in this case, BCA compatibility with commonly used phytosanitary products is fundamental.

Abiotic factors, such as temperature and relative humidity fluctuations, rain wash-off, or solar radiation, influenced the resilience and subsequent efficacy of the BCAs. High relative humidity is required to maintain the efficacy of *Ampelomyces* mycoparasites, and different additives as paraffin oil or Tween 20 were added to *Ampelomyces* conidial suspensions to overcome this limitation (Kiss 2003). AQ10 Biofungicide® also requires high relative humidity to be effective (Romero et al. 2007) and the biocontrol efficacy was enhanced with the addition of the wetting agent AddQ (Ecogen, Inc, Langhorne, PA, USA) (Elad et al. 1998). However, obtained results with AddQ were also contradictory because the additive only improved the BCA efficacy in some conditions (Brand et al. 2009), and it could reduce powdery mildew when it was used without the BCA (Shishkoff and McGrath 2002).

The benefits of applying bacterial and yeast BCAs with films or coatings to improve their performance under practical conditions were verified in several studies (Aloui et al. 2015; Calvo-Garrido et al. 2013; Cañamás et al. 2011; Marín et al. 2016; Parafati et al. 2016). AQ10 Biofungicide® required repeated

applications to be effective against powdery mildew (Gilardi et al. 2012; Sztejnberg 1993) and the manufacturer recommended its application every 6-8 day time intervals. The improvement of the dispersion and the adherence of the BCAs implied a better colonisation and survival on plant material (Droby et al. 2009) and it could allow for the same efficacy with less applications. Coating forming compounds also could increase spore adhesion to plant surface and prevent wash-off by rain (Greaves et al. 1998; Slininger et al. 2003). Promising results were obtained with two solid formulations of the BCA *Candida sake* CPA-1 developed by the addition of biodegradable coatings using a fluidised-bed spray-drying system. Both solid formulations improved the survival of the BCA under stress conditions (Carbó et al. 2017) and their efficacy was demonstrated at field conditions in organic wine grapes (Carbó et al. 2019).

The efficacy of the strain CPA-9 of *A. quisqualis* was recently demonstrated against powdery mildew on zucchini plants (Carbó et al. 2020). Additionally, the conidial production of CPA-9 was optimised in liquid media (Carbó et al. 2020) and one formulation based on biodegradable coatings was developed for CPA-9 using a fluidised-bed spray-drying system (Carbó et al. *non published data*). Despite of CPA-9 biocontrol product showed optimistic results, is still necessary to check the viability under different abiotic conditions, and then to test the efficacy of the formulation under practical conditions.

The aim of the present study was to verify the potential of the biodegradable coatings-based solid formulation of the biocontrol agent CPA-9 for its use under practical conditions against powdery mildew on cucurbits. The specific objectives were: (i) to check in vitro compatibility of CPA-9 with phytosanitary products commonly used against powdery mildew to value possible IMPs; (ii) to evaluate the resilience of CPA-9 formulation at different temperature and relative humidity conditions, both on an inhert surface and on zucchini leaves; (iii) to determine the effect of simulated rainfall at 100 mm h^{-1} and different rain intensities after the application of CPA-9 treatments on zucchini leaves; and (iv) to verify the *P. xanthii* parasitization by CPA-9 formulation product applied at different doses on zucchini leaves.

Materials and methods

Microbial antagonist

The strain CPA-9 of *A. quisqualis* was isolated from pumpkin leaves at IRTA (Lleida, Catalunya, Spain), deposited at the Colección Española de Cultivos Tipo (CECT-20749) at the University of Valencia (Burjassot, Spain) and it was patented by LAINCO, S.A. (Rubí, Spain) (Garriga et al. 2014). CPA-9 was

maintained in Glycerol 20% and in Criobilles tubes (Criobilles AEB 400100, AES Laboratory, Comburg, France) at -80 ºC for long term storage. The fungus was transferred to malt extract agar medium (MEA: malt extract, 30 g/l; peptone, 5 g/l; and agar 15 g/l) plates under a daily 12-h photoperiod (black light UV/dark at 25/18 ºC), and it was sub-cultured approximately every 10-15 days.

Conidial production was carried out in 500-mL Erlenmeyer flaks with 75 mL of modified potato dextrose broth (PDB: potato peptone, 4 g/l; glucose, 20 g/l; and glycerol, 25 g/l) and incubated in the dark without agitation, at 25 ºC for 11 days. The initial concentration of Erlenmeyer flasks was adjusted at 10^5 conidia/ml (Carbó et al. 2020). Then, a suspension of $2\text{-}3 \times 10^8$ conidia/ml was prepared and it was dehydrated with the fluidised-bed spray-drying system (Hüttlin GmbH, Bosch Packaging Technology, Stuttgart, Germany) to obtain the fluidized-bed spray-dried formulation (150 g of conidia suspension; native potato starch, 208 g; pregelatinised potato starch: 42 g as carrier and 3.5 g as binder; skimmed milk, 15 g; and sucrose, 15 g) used in the present study.

In vitro compatibility of CPA-9 with pesticides commonly used to control pests and diseases on cucurbits

Compatibility of the biocontrol agent CPA-9 was tested with several preharvest phytosanitary products (fungicides and insecticides) commonly used against pests and diseases on cucurbits (Table 1). A suspension of CPA-9 was prepared at 10^7 conidia/ml, then 0.5 ml were transferred to a glass tube containing 4.5 ml of water amended with Tween 80 (one drop per litre), and other 0.5 ml of the initial suspension were transferred to glass tubes that contained 4.5 ml of each chemical product at field application dose, as recommended by the manufacturer (Table 1). Vial tubes were maintained at 25 ºC for 30 min to allow contact between the fungus and the products. Samples from vials were taken just after the addition of CPA-9 and after the time of contact to obtain the reduction of conidia. Viability of CPA-9 was obtained by serial dilutions of samples plated on MEA. Colonies were counted after 6 days of incubation at 25 ºC. The test was repeated twice.

Table 1 Active matters, target disease and doses of the phytosanitary products tested for their compatibility in *vitro* with *A. quisqualis* CPA-9

Active matter	Target pest/disease	Recommended dose (w/v or v/v)	Reduction of viability (%)
Kresoxim-methyl 50% w/w [WP]	Powdery mildew	0.03%	95*
Myclobutanil 12.5% w/v [EC]	Powdery mildew	0.08%	100*

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Polioxyne (B) 2% w/v [SL]	Powdery mildew	0.25%	4
Sulphur 72% w/v [SC]	Powdery mildew	0.6%	0
Trifloxystrobin 50% w/w [WP]	Powdery mildew	0.015%	91*
Cymoxanil 3% w/w with Copper sulphate (Bordeaux mixture) 22.5% w/w [WP]	Downy mildew	0.4%	100*
Folpet 50% w/v [SC]	Downy mildew	0.4%	100*
Copper hydroxide 50% w/w [WP]	Downy mildew	0.35%	0
Copper oxychloride 50% w/w [WP]	Downy mildew	0.4%	0
Copper calcium sulphate 20% w/w [WP]	Downy mildew	1%	100*
Abamectin 1.8% w/v [EC]	Insecticide	0.1%	0
Azadirachtin 3.2% w/v [EC]	Insecticide	0.1%	0
Bacillus thuringiensis var. kurstaki 64% w/w [WP]	Insecticide	0.5%	0
Chlorpyrifos 48% w/v [EC]	Insecticide	0.2%	100*
Flufenoxuron 10% w/v [DC]	Insecticide	0.1%	88*
Imidacloprid 20% w/v [SL]	Insecticide	0.1%	0
Lambda cyhalothrine 20% w/v [CS]	Insecticide	0.08%	0
Piridaben 20% w/v [CE]	Insecticide	0.1%	0
Spinosad 48% w/v [CS]	Insecticide	0.025%	0

141   * Active matters considered incompatibles with CPA-9.

142   Resilience of CPA-9 on an inert surface at different temperature and relative humidity conditions

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144   Glass Petri plates with a diameter of 100 mm were used as inert surface to evaluate the resilience of non-

145   formulated and formulated CPA-9 on an inert surface without nutrients. Resilience of the fluidised-bed

146   spray-dried formulation was evaluated after 18 h and 24 h of incubation at 20 ℃ and 30 ℃, and at 40, 60

147   and 85% of relative humidity (RH). A suspension of each treatment of CPA-9 was prepared approximately

148   at 10^7 conidia/ml. The exact amount of conidia was counted in a Thoma-Zeiss counting chamber and

149   viability was determined with conidia germination as described Carbó et al. (2020). Three ml of the

150   suspensions were sprayed on glass Petri plates using a Potter spray tower. Three replicates were sprayed

151   for each treatment and condition of temperature and RH. Then, plates were left to dry at room temperature

152   and placed in plastic boxes, which were sealed with plastic bags to maintain the required RH. Relative

153   humidity conditions were achieved introducing one flask with 50 g of specific inorganic salts and another

154   flask with water inside the plastic boxes: (i) sodium bromide and 20 mL of water to achieve 40% RH; (ii)

155   sodium chloride and 20 ml of water to achieve 60% RH; and (iii) potassium chloride and 50 ml of water,

156   for 85% RH. External data loggers (Testo 175H1, Testo Inc., Sparta Township, NJ, USA) were introduced

157   to each box to monitor temperature and RH during incubation. Three plates for each formulation were also

158   sprayed to know the viable conidia of CPA-9 without incubation.

159   After incubation, 1.5 ml of water amended with Tween 80 were added to each Petri plate to recover the

160   conidia. Then, conidia were counted in a Thoma-Zeiss counting chamber and viability was determined with

161   the germination of conidia as described Carbó et al. (2020) although in this case plates were incubated for

162   40 h.

163   The rate of conidial viability was calculated as Log (N/N_0), where N_0 represents the true concentration of

164   the prepared treatments, and N the concentration of viable conidia after the sprayed (0 h) or after the

165   incubation period at different conditions (18 and 24 h). At 0 h, results were not influenced by temperature

166   or RH, although it was interesting to evaluate the survival of CPA-9 after application. When no viable

167   conidia were recovered, 0.01% of germination was considered to calculate N.

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169   Resilience of CPA-9 on zucchini plants at different temperature and relative humidity conditions

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171   Non-formulated CPA-9 and the fluidised-bed spray-dried formulation of the fungus were applied on

172   zucchini plants (variety "Black Beauty") grown up in plastic pots to evaluate conidial viability after

173   incubation at 20 ºC and 30 ºC, and 40, 60 and 85% RH. Three plants for each condition were treated at $2 \times$

174   10^6 conidia/ml with pressurised spray bottles and once plants were dried, they were incubated in climatic

175   chambers programmed at the corresponding temperature. Different values of RH were maintained by

176   placing the plants inside a sealed plastic chamber with a dehumidifier (FDC32S, FRAL, Carmignano di

177   BR., PD, Italy) and external data loggers (Testo 175H1, Testo Inc., Sparta Township, NJ, USA) were used

178   to monitor the RH and the temperature throughout the assay. One leaf of each plant was detached to evaluate

179   conidial viability, which was assessed after 2, 5 and 7 days at 20 ºC, and after 2 and 3 days at 30 ºC. An

180   equilateral triangle with each side being 5.5 cm (15.125 cm^2) was removed from the leaf and placed into a

181   sterile plastic filter bag containing 3 ml of water amended with Tween 80. Then, the bags were homogenised

182   in a Stomacher blender (Masticator Basic 400 mL, IUL SA, Barcelona, Catalonia, Spain) for 4 min, they

183   were left to settle for 2 min, and finally they were homogenised for 2 min more. Viable conidia were counted

184   by serial dilutions plated on MEA and incubated at 25 ºC for 6 days. The rate of conidial viability was

185   calculated as Log (N/N_0), where N_0 represents the viable conidia recovered from leaves just after the

186   applications, and N the viable conidia recovered from leaves after incubation periods at different conditions.

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188   Rainfastness of CPA-9 formulations on zucchini leaves

190 The wash-off of CPA-9 caused by simulated rainfall was evaluated with non-formulated CPA-9 and with

191 the dried formulation. The treatments were applied with pressurised spray bottles at 2×10^6 conidia/mL on

192 zucchini plants (variety "Black Beauty"). A rain intensity of 100 mm/h was simulated as described Calvo-

193 Garrido et al. (2014) and three rain volumes were evaluated: 20, 60 and 120 mm. Four plants were treated

194 with each treatment, although one of them was used as control to express results of rainfastness in relation

195 to a treated plant but non-exposed to simulated rainfall.

196 Sampling was carried out as described previously to evaluate the resilience of CPA-9 on zucchini plants

197 (Section 2.4). Briefly, one leaf of each plant was taken, an equilateral triangle with each side being 5.5 cm

198 was removed of the leaf, and it was homogenised with water amended with Tween 80. Quantification of

199 conidia was carried out with serial dilutions plated on MEA and incubated at 25 ºC for 6 days. The rate of

200 wash-off was calculated as Log (N/N_0), where N_0 represents the viable conidia recovered from control leaves

201 (treated but non-exposed to rainfall), and N the viable conidia recovered from leaves exposed to rainfall

202 events.

204 Mycoparasitization of CPA-9 formulation applied at different doses on zucchini leaves against powdery

205 mildew

207 *Podosphaera xanthii* 04/05 was used in the present study to check the mycoparasitization of formulated

208 CPA-9 applied at different doses. *P. xanthii* 04/05 was isolated in IRTA (Lleida, Catalonia, Spain) from

209 cucurbit leaves and, as a biotroph parasite, it was maintained by growing on zucchini cotyledons (variety

210 "Black Beauty") as described Carbó et al. (2020).

211 Zucchini plants (variety "Black Beauty") were grown from seeds in a growth chamber under a photoperiod

212 adjusted to be as similar as possible to real conditions. Third and fourth leaves of zucchini plants were

213 detached and placed in double Petri dishes with their petioles immersed in 50% Hoagland's solution. The

214 nervation of leaves were infected with *P. xanthii* using an eyelash, then plates were placed in a plastic box

215 and incubated in the growth chamber for 5 days prior the application of CPA-9 treatments.

216 Mycoparasitization of the fluidised-bed spray-dried formulation of CPA-9 was assessed at different doses:

217 (i) 5×10^5 conidia/ml; (ii) 10^6 conidia/ml; and (iii) 5×10^6 conidia/ml. CPA-9 treatment was compared with

218 a control (water), and mycoparasitism was observed at $10 \times$ and $40 \times$ magnification with a stereoscopic

219   microscope after 7 days. Incidence of powdery mildew was also evaluated as the incidence disease index

220   (IDI), calculated as: $[[(a \times 0) + (b \times 1) + (c \times 2) + (d \times 3)] / 3n] \times 100$, where numbers designate the relative

221   surface of the leaf covered with powdery mildew (0: no powdery mildew; 1: only few spots; 2: a half of

222   leaf or more; 3: all surface practically covered); letters indicated the amount of leaves assigned at each

223   group; and n was the total amount of infected leaves. Each replicate consisted of five leaves.

224

225   Statistical analysis

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227   Data were analysed by one-way ANOVA and differences at $P <0.05$ were considered significant. LSD

228   Student's t test was used when only two means had to be compared, and the Tukey's test was used to

229   compare more than two means. CPA-9 populations expressed as conidia/mL or conidia/g were transformed

230   to logarithmic values prior to ANOVA. All analysis were performed with JMP13 software (SAS Institute

231   Inc., Cary, NC).

232

233   **Results**

234   *In vitro* compatibility of CPA-9 with pesticides commonly used to control pests and diseases on cucurbits

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236   Results of the reduction of viability showed that CPA-9 was compatible with the most of tested

237   phytosanitary products (Table 1). It was also observed that reduction of viability values were placed in the

238   extremes, being non-existent or negligible reduction for compatible active matters, whereas viability were

239   totally or practically totally lost for incompatible products. Intermediate values were not observed for any

240   tested active matter.

241   Eight active matters were incompatible with CPA-9. Specifically, six fungicides: kresoxim-methyl 50%

242   w/w [WP], myclobutanil 12.5% w/v [EC], trifloxystrobin 50% w/w [WP], cymoxanil 3% w/w with copper

243   sulphate (Bordeaux mixture) 22.5% w/w [WP], folpet 50% w/v [SC], and copper calcium sulphate 20%

244   w/w [WP]; and two insecticides: chlorpyrifos 48% w/v [EC], and flufenoxuron 10% w/v [DC] were

245   incompatible.

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247   Resilience of CPA-9 under different conditions of temperature and relative humidity

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249   1. Fluidised-bed spray-dried formulation of CPA-9 applied on an inert surface

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251   The combined effect of different temperatures and relative humidity on *A. quisqualis* CPA-9 incubated on

252   an inert surface is represented in Figure 1. After 18 h and 24 h of incubation, significantly better survival

253   of the fungus was always obtained with the solid formulation than with non-formulated cells.
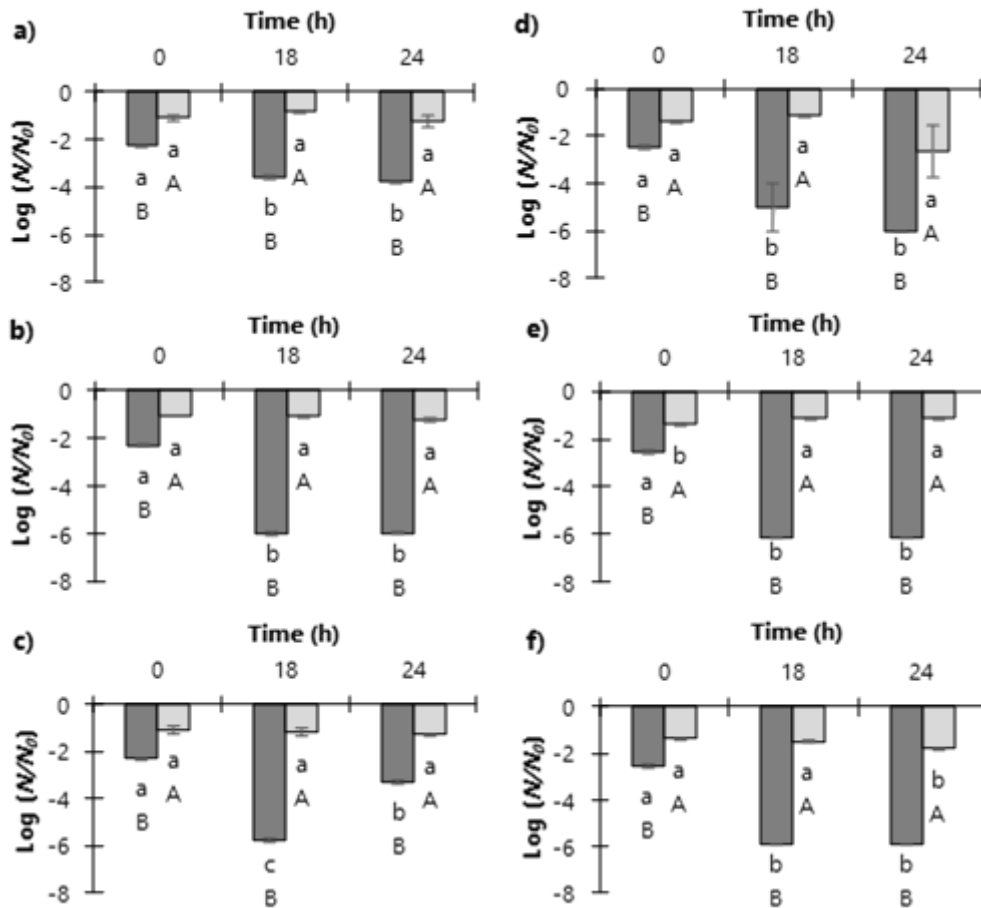
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256   **Figure 1.** Resilience of different *A. quisqualis* CPA-9 treatments on an inert surface (glass) incubated under
257   controlled conditions of temperature and RH during 24 h: (a) 20 °C and 40% RH; (b) 20 °C and 60% RH;
258   (c) 20 °C and 85% RH; (d) 30 °C and 40% RH; (e) 30 °C and 60% RH; and (f) 30 °C and 85% RH.
259   Resilience of non-formulated *A. quisqualis* (■); and fluidized-bed spray-dried formulation (□) were
260   tested. Values are the mean of three replicates and vertical bars indicate standard error of the means.
261   Uppercase letters indicate significant differences among CPA-9 formulations within time; different
262   lowercase letters indicate significant differences among time within CPA-9 formulations. Means
263   separations were considered significant at $P < 0.05$.
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265
266   Specifically, the reduction obtained with the formulated CPA-9 was always <2.61 Log, whereas the rate of

267   survival of non-formulated CPA-9 decreased until 6.12 Log. Even when CPA-9 was not influenced by

268   incubation temperature or RH (0 h), the rate of survival of fluidised-bed spray-dried formulation with

269   biodegradable coatings was significantly higher than for non-formulated CPA-9.

Regarding the survival of CPA-9 over time, non-formulated CPA-9 decreased greatly over time of incubation, mainly during the first 18 h. In contrast, the decrease of the solid formulation over time was less pronounced, and fluidized-bed spray-dried formulation only showed significant differences over time after 24 h of incubation at 30 ºC and 85% RH (F=8362,086; df=3; P=0,001) (Fig. 1f). Surprisingly, the survival of non-formulated CPA-9 significantly improved after 18 h and 24 h of incubation at 30 ºC and 60% RH (18h: F=27404,28, df=4, P<0,001; 24h: F=450000, df=4, P<0,0001) (Fig. 1e), despite of differences were around 0.2 Log.

Survival of formulated CPA-9 showed the same trend regardless the relative humidity during the incubation time. In contrast, it was no possible to observe a trend for non-formulated CPA-9, mainly due to the high reductions of viable conidia obtained, although the lowest reduction was obtained after 24 h at 20 ºC and 85% RH (-3.28 Log).

2. Fluidised-bed spray-dried formulation of CPA-9 applied on zucchini plants

The results of the survival rate of CPA-9 applied on zucchini plants and incubated at different conditions of temperature and RH are shown in Figure 2. Solid formulation of CPA-9 allowed for significantly higher viability of the fungus after 7 days of incubation at 20 ºC at both 40% (F=51,3375; df=4; P=0,0056) and 60% RH (F=20,4625; df=5; P=0,0106) (Fig. 2a and 2b) and after 3 days at 30 ºC and 40% RH (F=8,3282; df=5; P=0,0447) (Fig. 2c). In contrast, no differences were observed among treatments after incubation at 30 ºC and 60% RH (2 days: F=0,3407; df=5; P=0,5908; 3 days: F=9,4143, df=4, P=0,0546) (Fig. 2d).

The reduction of viability was 0.31 Log (N/N_0) for fluidized-bed spray-dried formulation incubated 7 days at 20 ºC and 60% RH. The highest reductions on CPA-9 viability were obtained at 20 ºC and 40% RH with non-formulated cells, when rate of survival decreased rapidly to 1.2 Log (N/N_0) after 5 days and decreased to 1.3 Log (N/N_0) after 7 days. In the same conditions, the solid formulation decreased 0.56 Log (N/N_0) after 5 days, and 0.34 Log (N/N_0) after 7 days. Comparing both RH, at 20 ºC survival od CPA-9 was better when it was incubated at 60% RH, whereas at 30 ºC it was not possible to observe a trend, probably due to the short duration of experiment.

No results of CPA-9 survival on zucchini plants incubated at 85% RH were obtained because the plant withered before two days of assay. The same occurred when plants were incubated at 30 ºC for more than three days.

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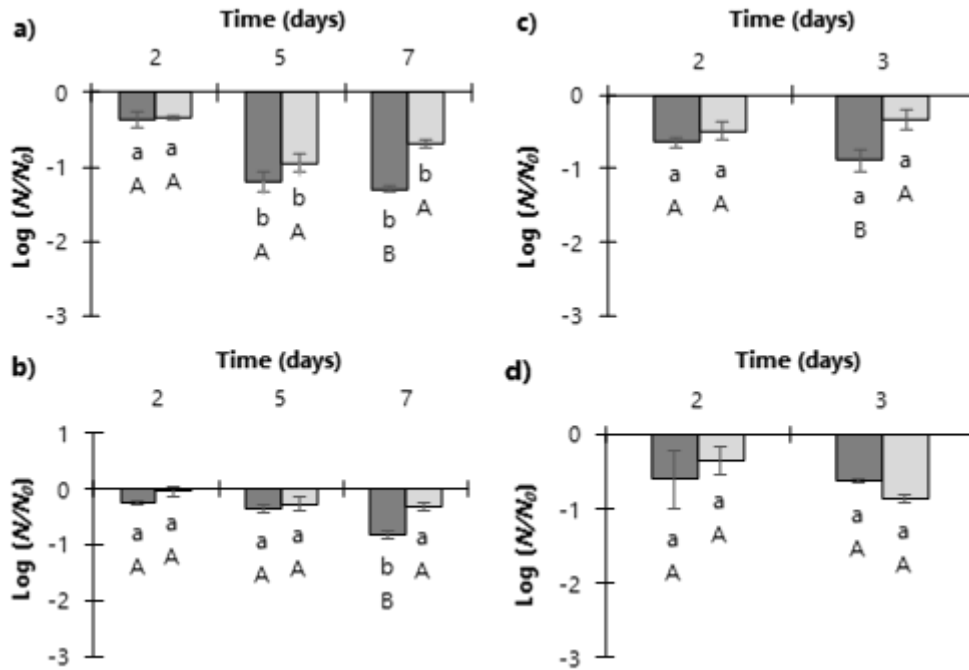
Fig. 2 Resilience of different *A. quisqualis* CPA-9 treatments on zucchini plants incubated during 7 days under controlled conditions of temperature and RH: (a) 20 ºC and 40% RH; (b) 20 ºC and 60% RH; (c) 30 ºC and 40% RH; and (d) 30 ºC and 60% RH. Resilience of non-formulated *A. quisqualis* (■); and fluidized-bed spray-dried formulation (□) were tested. Values are the mean of three replicates and vertical bars indicate standard error of the means. Uppercase letters indicate significant differences among CPA-9 formulations within time; different lowercase letters indicate significant differences among time within CPA-9 formulations. Means separations were considered significant at $P < 0.05$.

Adherence of CPA-9 treatments on zucchini leaves after simulated rainfall

Rainfastness of the different treatments of CPA-9 applied on zucchini plants is shown in Figure 3. No significant differences were observed among treatments (20 mm: F=2,6798, df=8, P=0,1474; 60 mm: F=4,0967, df=6, P=0,1076; 120 mm: F=2,0064, df=6, P=0,2492) neither rain volumes (non-formulated: F=0,1298, df=5, P=0,7369; fluidized-bed spray-dried formulation: F=0,0042, df=6, P=0,9510), although non-formulated CPA-9 appeared to be more resistant to rain wash-off than the solid formulation.

In general, results showed a trend between rain volume and rain wash-off, so rainfastness of the treatments decreased at higher rain volume. Nevertheless, despite of no significant differences were observed, it could be perceived some tendencies depending on the treatment. In this sense, the wash-off of non-formulated cells did not differ after 20 mm or 60 mm of rain (-0.4 Log (N/N_0)) and only increased after 120 mm of rain; whereas in the case of fluidized-bed spray-dried formulation, CPA-9 cells decreased gradually from -0.5 Log (N/N_0) after 20 mm of rain to -1.1 Log (N/N_0) after 120 mm.

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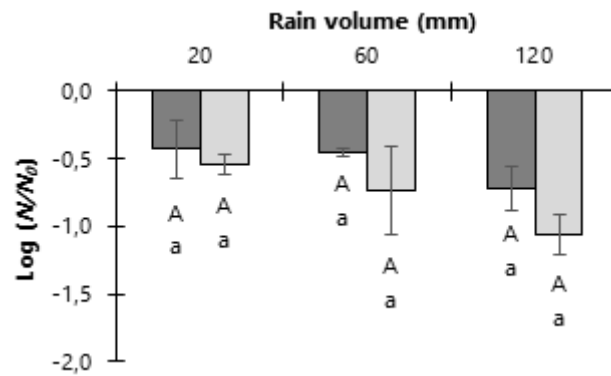
Fig. 3 Adherence of *A. quisqualis* CPA-9 fluidised-bed spray-dried formulations on zucchini leaves after simulated rainfall at 100 mm h^{-1} and different rain volumes (mm). Three rain volumes were tested for each CPA-9 application: non-formulated *A. quisqualis* (■); and fluidized-bed spray-dried formulation (☐). Values are the mean of three replicates and vertical bars indicate standard error of the means. Uppercase letters indicate significant differences among CPA-9 formulations within rain volume; different lowercase letters indicate significant differences among rain volumes within CPA-9 application. Means separations were considered significant at *P* <0.05.
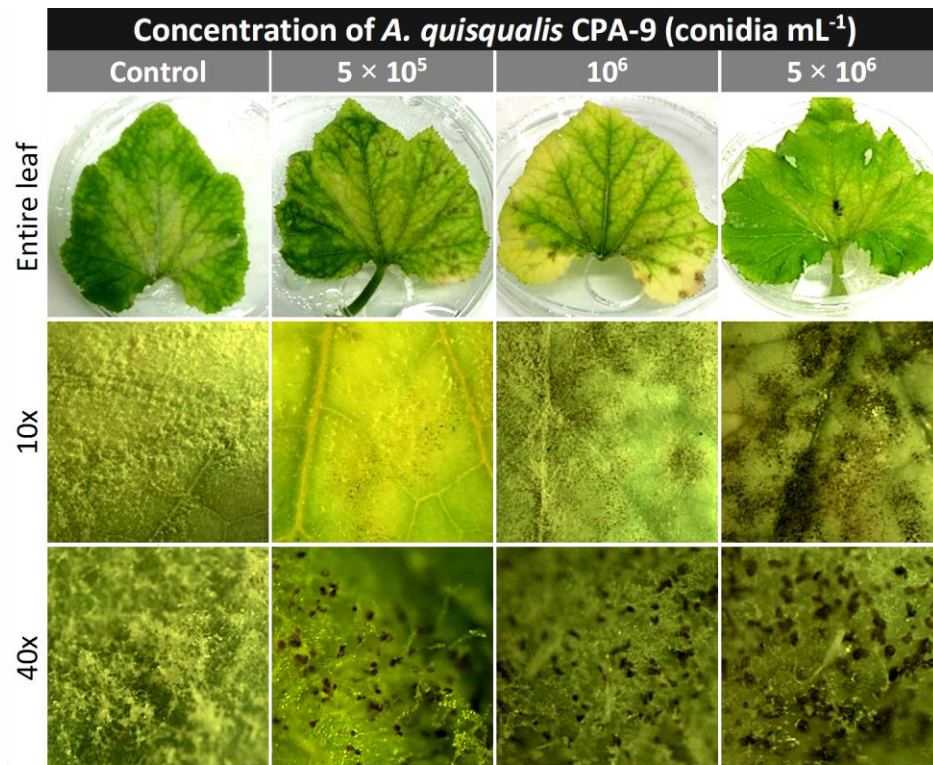
CPA-9 mycoparasitization on zucchini leaves artificially infected with powdery mildew

P. xanthii parasitization by different doses of fluidised-bed spray-dried CPA-9 was observed at multiple magnification levels (Fig. 4). The solid formulation showed a high parasitization level, which could be observed directly by naked eye by means of brownish spots on the leaves. At 10 and 40 × magnification, brownish spots parasitization appeared as numerous tiny dark points that generally formed aggregates. Control leaves were covered with a whitish powdery mildew, and as expected, parasitization was not found at any magnification level.

Parasitization was observed at all tested concentrations; the presence of the pycnidia is influenced by the concentration of the suspension used in the mycoparasitic tests. In fact, at 10 × magnification it was clearly observed a larger amount of pycnidia when 5×10^6 conidia/ml were applied on zucchini leaves.

Fig. 4 Representative images of the fluidised-bed spray-dried formulation of *A. quisqualis* CPA-9 mycoparasitization of *P. xanthii* on zucchini leaves (variety "Black Beauty") after 7 days of the treatment and applied at different concentrations. Control was treated with water. Zucchini leaves were artificially infected with *P. xanthii* 5 days before the application of the treatments.

Discussion

The present study represents another step towards the confirmation of the potential of the fluidised-bed spray-dried formulation of CPA-9. In most studied situations, the performance of the solid formulation under stress conditions resulted better than for non-dehydrated CPA-9, and no significant differences were observed among treatments with respect to rainfastness despite of biodegradable coatings were water soluble. Moreover, CPA-9 compatibility with the most commonly used pesticides was satisfactorily tested, and *P. xanthii* parasitization by dehydrated CPA-9 was confirmed applying different concentrations of CPA-9 conidia on zucchini leaves.

The substitution of synthetic chemical pesticides with biocontrol agents is a key aspect for integrated management programmes (IMP) (Pertot et al. 2017), and BCA compatibility with other phytosanitary products is indispensable. A disease control strategy, based on reduction in the number of overwintering chasmothecia and targeted application of fungicides in spring against ascosporic infections of grapevine

362 powdery mildew has been also proposed (Caffi et al. 2013). CPA-9 was compatible with the most of tested

363 fungicides and insecticides, therefore to include CPA-9 in an IMP, it might be necessary to consider that

364 the BCA is incompatible with kresoxim-methyl, myclobutanil, trifloxystrobin, cymoxanil with copper

365 sulphate, folpet, copper calcium sulphate, chlorpyrifos, and flufenoxuron. Germination of AQ10

366 Biofungicide® also was inhibited with kresoxim-methyl, trifloxystrobin, or folpet, when the fungicides were

367 applied at field doses recommended by the producer (Schweigkofler 2006). Interestingly, CPA-9 was

368 compatible with sulphur, whereas AQ10 was incompatible with it (Schweigkofler 2006). Other ABCs also

369 showed incompatibility with cymoxanil with copper sulphate (Calvo-Garrido et al. 2017).

370 The addition of low doses of fungicides together with the biocontrol product could be another approach to

371 be considered in relation to the compatibility of BCAs (Sharma et al. 2009), and in this way the tested doses

372 of applied fungicides could be reduced to gain compatibility with CPA-9. Additionally, the direct contact

373 (*in vitro*) of the BCA with the phytosanitary products could be more harmful than the contact on the plant

374 surface (*in vivo*), as occurred for *C. sake* CPA-1 (Calvo-Garrido et al. 2017). Shishkoff and McGrath (2002)

375 did not observe significant differences in powdery mildew reduction when applied AQ10 alone or AQ10

376 with myclobutanil at 10 μg/mL. The combination of AQ10 with 250 μg/ml of myclobutanil also provided

377 interesting results (Gilardi et al. 2012), whereas in the present study, a dose of 100 μg/ml resulted fully

378 incompatible. However, previous cited studies did not confirm the germination of CPA-9 conidia combined

379 with the fungicide, and from our point of view the reduction of powdery mildew infection could be

380 produced by the fungicide, even though the BCA germination was inhibited.

381 Regardless of the tested conditions, solid formulation of CPA-9 generally showed better resilience than

382 non-formulated fungus, both in an inert surface and applied on zucchini plants. As might be expected,

383 survival of CPA-9 was better on zucchini leaves surface due to the nutrient's contribution from the plant,

384 although the highest differences between formulated and non-formulated CPA-9 were observed when

385 applied on the inert surface. In fact, biodegradable coatings were included in the solid formulations to

386 improve the resilience and efficacy of the BCA under unfavourable conditions (Carbó et al. 2017).

387 Accordingly, variation on CPA-9 resilience tended to be lower at high relative humidity (60-85%) and

388 warm temperature (30 ºC), which conditions were described as favourable for *A. quisqualis* performance

389 (Angeli et al. 2017; Kiss 2003; Romero et al. 2007).

390 Biodegradable coatings had a positive effect on the resilience of the BCA, especially under stress

391 conditions. In contrast, results obtained after simulated rainfall were better for non-formulated CPA-9. This

might be explained because biodegradable coatings that constitute the solid formulation are soluble compounds that are easily washed-off by rainfall. Furthermore, CPA-9 conidia were concentrated in the production medium which contains potato dextrose broth and glycerol. Glycerol is considered a plasticizer (Ribeiro et al. 2007; Rodríguez et al. 2006) and, together with glutinous matrix or enzymes of *A. quisqualis*, it could be the responsible of the conidia adhesion to leaves skin. This effect loss importance in formulated conidia because biodegradable coating components solubility. Despite rainfastness of CPA-9 solid formulations were similar of obtained for film-forming formulations of the BCA *C. sake* CPA-1 (Carbó et al. 2017), adhesion of CPA-9 conidia after simulated rainfall might be enhanced with an establishment period before rain (Calvo-Garrido et al. 2014; Gotor-Vila et al. 2017). In addition, both CPA-9 formulations are focused to control powdery mildew on cucurbits, which are mostly cultivated on greenhouses where rain wash-off does not happen.

Angeli et al. (2012) suggested that the level of *A. quisqualis* parasitization depends on the strain; notwithstanding, parasitization by dried CPA-9 was verified in the present study because some of the weaknesses of solid formulations are the cell damage (Fu and Chen 2011) and the high cell mortality (Dukare et al. 2018) produced during dehydration and rehydration process. Fortunately, all tested concentrations of fluidised-bed spray-dried CPA-9 conidia parasitized *P. xanthii* structures, although more CPA-9 pycnidia were observed at higher concentrations. Fluidised-bed spray-drying process is characterised to allow dehydration of cells without high-temperature heat damage (Carbó et al. 2017).

To sum up, developed formulation of CPA-9 is suggested as promising biocontrol product against *P. xanthii* on zucchini plants. However, bringing a new product to the market is the most difficult stage in the development of a biocontrol product (Spadaro and Droby 2016), and several objectives still need to be faced. For example, storage and packaging conditions might be optimised to improve the formulations shelf life, which was evaluated only until five months (Carbó et al. unpublished data); moreover, it would be necessary to test the biocontrol efficacy of the products under field conditions, both greenhouse and open field crops.

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