

Effect of temperature, relative humidity and aphid developmental stage on the efficacy of the mycoinsecticide Mycotal® against Myzus persicae

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1 **Effect of temperature, relative humidity and aphid developmental**
2 **stage on the efficacy of the mycoinsecticide Mycotal® against *Myzus***
3 ***persicae***

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18 The green peach aphid, *Myzus persicae*, is a major pest worldwide. An examination of
19 the impact of temperature, relative humidity and developmental stages of *M. persicae*
20 on the efficacy of the whitefly mycoinsecticide Mycotal®, based on *Lecanicillium*
21 *muscarium* and the effects of infection on aphid fecundity was evaluated under
22 controlled conditions. Although this fungus can be grown at a broad range of
23 temperatures (15-30°C), the optimum temperature for control of *M. persicae* ranged
24 between 20 and 30°C. *L. muscarium* had high efficacy as a microbial control against *M.*
25 *persicae* between 55 and 90% relative humidity. Total mortality of aphids treated with
26 different spore dosages of *L. muscarium* varied according to the developmental stage:
27 adults, fourth and third instar nymphs of proved more susceptible than first instar
28 nymphs. Although the fungus did not affect the rate of nymph production, the
29 reproductive period of aphids significantly decreased with increasing the spore dosage.
30 Thus, total fecundity of treated aphids was 22.6 ± 1.1 and 31.6 ± 2.4 offspring per adult
31 at the medium ($644 \pm$ viable spore/mm²) and low (330 ± 40 viable spore/mm²) dosages,
32 compared with 45.7 ± 4.3 offspring per untreated aphid. The results suggest that *L.*
33 *muscarium* has the potential as a biological control agent of *M. persicae*. However,
34 fungal infection appears to have no sub-lethal effects on the fitness of the host's
35 progeny.

36 Keywords: temperature, relative humidity, Mycotal®, developmental stage, total fecundity,
37 reproduction rate

38 **1. Introduction**

39 The green peach aphid, *Myzus persicae* Sulzer (Hemiptera: Aphidiae), is one of the
40 most important and common aphid species of greenhouse and field crops worldwide
41 (Blackman & Eastop, 2007). It is highly polyphagous infesting crops in over 40 plant
42 families (Blackman & Eastop, 2008) causing damage by direct feeding (Saljoqi, 2009),

43 the development of sooty mould on honeydew (Miller, Gubler, Laemmlen, Geng, &
44 Rizzo, 2004) and the transmission of over 100 plant virus diseases (Dedryver, Le Ralec,
45 & Fabre, 2010).

46 Potential biological control methods for *M. persicae* include the use of predators
47 such as ladybird beetles (Dixon, 2000) and parasitoids (Perdikis, Lykouressis,
48 Garantonakis, & Iatrou, 2004). A further potential method for *M. persicae* biocontrol
49 agent is the use of entomopathogenic fungi (Ashouri, Arzanian, Askary, & Rasoulia,
50 2003; Xu & Feng, 2002; Yeo, Pell, Alderson, Clark, & Pye, 2003). Several
51 entomopathogenic fungi, including *Lecanicillium* spp. (Hypocreales: Ascomycete), play
52 an important role in biological control of aphids (Kim, Goettel, & Gillespie, 2007).
53 *Lecanicillium* spp. also have activity against many phytopathogenic fungi, including
54 powdery mildews (Miller et al., 2004). In laboratory conditions, *Lecanicillium* spp. have
55 been reported to be more virulent against aphid species when compared to *Beauveria*
56 *bassiana*, *Isaria fumosorosea* and *Metarhizium anisopliae* (Åsman, 2007; Loureiro &
57 Moino Jr, 2006). Worldwide, at least two products based on *Lecanicillium longisporum*
58 have been developed commercially to control aphids; Vertalec® and Vertirril WP
59 1300®. However, in the UK, the strain of *L. longisporum* marketed as a Vertalec® is no
60 longer commercially available (Faria & Wraight, 2007). Little is known about the
61 efficacy of Mycotal® (*L. muscarium*) which is on the UK market to control whitefly
62 species, against *M. persicae* (Hall, 1982). To evaluate the potential of Mycotal® against
63 *M. persicae* studies on aspects influencing the ability of this commercial formulation to
64 control aphids are required.

65 The most important aspects on the ability of entomopathogenic fungi to infect
66 and colonise host insects are the effect of temperature (Yeo et al., 2003), relative
67 humidity (Milner & Lutton, 1986) and developmental stage of aphid host (Kim &

68 Roberts, 2012) . Temperature and relative humidity can have a significant impact on the
69 rate of infection and time to death of insects treated by affecting conidial germination,
70 and germ tube development and penetration through the host cuticle (Shi, Feng, & Liu,
71 2008). Furthermore, entomopathogenic fungi infect their host via direct penetration of
72 the cuticle and since juvenile aphids shed their cuticle in the moulting process at
73 relatively short intervals (about 2 days) (Diaz & Fereres, 2005) juveniles may thereby
74 reduce their susceptibility to fungal infection compared to adults. The differences may
75 also be related to cuticle biochemical composition, physiological characteristics and/or
76 method of fungal application, as shown for other arthropods (Kirkland, Cho, &
77 Keyhani, 2004; Tang & Hou, 1998).

78 Another important aspect is the effect of the pathogen infection on the rate of
79 nymph production and the length of reproductive time of aphids. Studies by Xu & Feng
80 (2002), Baverstock, Roy, Clark, Alderson, & Pell, (2006), Roditakis, Couzin, Franks,
81 & Charnley, (2008), Gurulingappa, McGee, & Sword, (2011) and Shrestha, Enkegaard,
82 & Steenberg, (2015) have reported significant reductions in reproduction rate and the
83 reproductive period after treatment with entomopathogenic fungi. Reduction in the rate
84 of nymph production by a fungus-infected host could be attributed to histological and
85 cytological injuries to the ovaries, thus affecting follicle development or causing their
86 degeneration (Sikura, Sikura, & Trebesava, 1972). Infection by entomopathogenic fungi
87 can also result in reduced food intake in aphids, which could be a contributory factor in
88 the drop in reproductive rate (Roditakis et al., 2008). Competition with the fungus for
89 host resources and a negative impact of the fungus on host physiology such as through
90 tissue invasion or production of toxic metabolites, may also important in reducing the
91 reproductive output (Furlong, Pell, & Reddy, 1997).

92 None of the above mentioned aspects have been investigated for Mycotal® in

93 relation to the green peach aphid. Such information may help us predict the ability of
94 (Mycotal[®]) to reduce the aphid population level in the field and glasshouse under
95 realistic conditions. This study therefore aimed to investigate (1) the efficacy of the
96 commercial mycoinsecticide (Mycotal[®]) against *M. persicae* at different regimes of
97 temperatures and humidity, (2) the susceptibility of different instar nymphs and adults
98 of *M. persicae* to this product, (3) the effects of fungal infection on the fecundity of
99 individual aphids and aphid population growth and (4) the effects of fungal infection on
100 the maturation time and the rate of nymph production of the host aphid's progeny.

101 **2. Materials and methods**

102 ***2.1. Plant material***

103 Brussel sprouts, *Brassica oleracea* var. *gemmifera* L. cultivar Bedford-Winter Harvest
104 (Suttons Seeds, Paignton, UK) were established for the *M. persicae* cultures and for the
105 experimental work. This cultivar has been identified as *M. persicae* susceptible
106 (Qayyum, 1999), and ensured a regular supply of large numbers of aphids throughout
107 the year. Loam-based potting compost (John Innes No. 2: Roffey Brothers,
108 Bournemouth, UK) was used.

109 ***2.2. Rearing M. persicae***

110 The green peach aphid, *M. persicae*, was collected initially from cabbage plants in the
111 experimental garden at the University of Reading, in 2013. The aphids were identified
112 under a light compound microscope, using both cabbage aphid and polyphagous aphid
113 keys (Blackman & Eastop, 2000). *Myzus persicae* was reared on Brussels sprouts plants
114 5-6-weeks old in 45 x 45 x 45 cm cages at 21± 2°C and with 16:8 h daily photoperiod
115 for several generations. Plants were replaced every 2 weeks with healthy 5-6 week-old
116 plants.

117 ***2.3. Fungus application***

118 A formulation of *Lecanicillium muscarium* (Mycotal® by Koppert Biological Systems,
119 Berkel en Rodenrijs, the Netherlands) (Aqueel & Leather, 2013) was used. The product
120 is based on conidia of the KV01 strain isolated from a dead *T. vaporariorum* adult from
121 a glasshouse in the UK (Fargues et al., 2003). This mycoinsecticide which contains 10^{10}
122 spore g^{-1} was kept at 4°C until use. A stock suspension was prepared by mixing 100 g of
123 wettable Mycotal® powder in 1000 ml of sterile water and 0.02% Tween 80 (BDH
124 Chemicals Ltd, Poole, UK) in 2000 ml beakers then agitating for 30 minutes on a
125 magnetic stirrer. The resulting dosage (high dosage) was either used as prepared or
126 diluted 100-fold and 10000-fold to produce the medium and low dosage, respectively.
127 The viability and density of spores for each dosage was determined using the procedure
128 described by Poprawski & Jones (2000). 1ml of either the high, medium or low dosages
129 was sprayed on plates with 1.5% Sabouraud dextrose agar (SDA). The plates were
130 sealed with parafilm and incubated at 20°C, $90 \pm 2\%$ RH and a photoperiod of 16:8 h
131 (L:D). After 24h, four $0.05mm^2$ microscope fields ($400\times$ magnification) observed on
132 each plate, the number of germinated and non-germinated spores was counted and
133 counts were averaged per $1mm^2$. The plates treatments were replicated 5 times for each
134 dosage. Germination was considered positive when the length of germ tube was at least
135 half of the spore length. The dosage of spores that the aphids received in each treatment
136 was evaluated by counting the number of viable spores per mm^2 . The mean number \pm
137 standard error of high, medium and low dosages for *L. muscarium* were 880 ± 32 , $644 \pm$
138 50 and 330 ± 40 viable spore/ mm^2 , respectively. The viability of the *L. muscarium*
139 spores used in the bioassays exceeded 89%.

140 For each treatment, 1 ml of spraying suspension was applied once on each leaf
141 containing aphids, using a 750 ml trigger water sprayer (Ampulla, Hyde, UK). The

142 application was made with a distance of 15 cm between the sprayer nozzle and plant
143 leaf, to ensure that the fungal application covered the whole leaf and all aphids.

144 **2.4. Production of known-age third instar nymphs of *M. persicae***

145 In order to obtain a uniform age of third instar nymphs of *M. persicae* for use in all
146 experiments, adults were transferred from stock culture onto 6-week-old Brussels
147 sprouts plants (20 adults per plant) using two 3-cm-diameter clip cages and allowed to
148 produce nymphs for 1 day in a growth chamber at 20°C, 75 ± 2% RH and a photoperiod
149 of 16:8 h (L:D) . The adults were then removed and the offspring counted (50 nymphs
150 per plant) and allowed to develop on the plants for 4 additional days before each
151 experiment. If the number of nymphs was less than 50 per plant, the adults were kept on
152 the leaves for another day until they produced 50 nymphs.

153 **2.5. Effect of relative humidity (RH) on *L. muscarium* efficacy**

154 Two laboratory experiments were carried out on different dates to determine the effect
155 of relative humidity on the efficacy of *L. muscarium* against third instar nymphs . In
156 both experiments a uniform age of aphid nymphs (4-5 days old) was obtained as
157 described above. In addition, the high, medium and low dosages of Mycotal[®] were
158 sprayed onto Brussel sprouts plants each harbouring 50 third instar nymphs. Control
159 plants were sprayed with 0.02% sterile aqueous Tween 80.

160 In the first experiment, the treated plants were arranged in three groups (five
161 plants per spore dosage), and then each transferred to one of three different plant growth
162 chambers (Fitotron-Weiss Technik Ltd, Leicestershire, UK) at 20°C, 16:8 h (L:D) at
163 one of the following relative humidity (55, 70 and 90%) for 2 days. Subsequently, the
164 treated plants in the growth chambers at 55 and 90% relative humidity were transferred
165 to the growth chamber at 70% RH for 8 days. This relative humidity level was based on
166 the average daily high, moderate and low relative humidities in the field in the UK

167 (Climatological station, 2013). Relative humidity within the experimental growth
168 chambers remained relatively stable and never varied more than 4% from the target
169 humidity while chambers were sealed. To prevent aphids moving between treated
170 plants, they were kept in the 3-cm-diameter clip-cages post spraying. To minimize the
171 impact of horizontal transmission caused by spores discharged from cadavers killed
172 with *L. muscarium* on estimation of the efficacy of *L. muscarium*, the dead aphids were
173 removed daily for 10 days. Dead aphids were surface sterilised by rinsing twice with
174 70% ethanol for 30 seconds and then with sterilised distilled water and thereafter placed
175 on water agar (3g of agar L⁻¹ of water) in Petri dishes for 5 days, to confirm infection by
176 *L. muscarium* (Aiuchi et al., 2012). A cadaver was regarded as having died from
177 infection by this fungus if conidia of *L. muscarium* were recovered from it.

178 The second experiment repeated the procedure of the first experiment, except
179 that after spending 2 days at 55, 70 or 90% RH the treated plants were transferred to a
180 growth chamber at 55% RH for 8 days. This relative humidity (55%) was used because
181 it has been recorded as a lower average RH in the field in the UK (Climatological
182 station, 2013). The experiments were repeated once on a different date.

183 **2.6. Effect of temperatures on *L. muscarium* efficacy**

184 Two laboratory experiments were carried out on different dates to determine the effect
185 of temperature on the efficacy of *L. muscarium* against third instar nymphs. In both
186 experiments, a uniform age of aphid nymphs (4-5 days old) was obtained as described
187 above. The high, medium and low spore dosages of Mycotal[®] were each sprayed onto
188 Brussel sprouts plants each harbouring 50 third instar nymphs. The control group was
189 sprayed with 0.02% sterile aqueous Tween 80 only. After treatment, the plants were
190 kept at room temperature for 1 h to dry.

191 In the first experiment, the treated plants were arranged in four groups (five
192 plants per spore dosage and control), and then each transferred to one of four different
193 plant growth chambers (Fitotron-Weiss Technik Ltd, Leicestershire, UK) at $75 \pm 2\%$
194 RH, a photoperiod of 16:8 h (L:D) and one of the following temperatures: 15, 20, 25, or
195 30°C for 10 days. These temperatures were chosen to represent an average daily low
196 and high temperature experienced under summer field conditions in the UK. To prevent
197 aphids moving between treated plants, the aphids were kept in the 3-cm-diameter clip-
198 cages post-spraying. Dead aphids were counted and removed daily for a period of 10
199 days at each temperature. The method described in section 2.5 was used to confirm
200 infection by *L. muscarium*.

201 The second experiment followed the method described in the first experiment
202 above, except that treated plants were placed in four growth chambers at $75 \pm 2\%$ RH, a
203 photoperiod of 16:8 h (L:D) and one of the following temperatures: 15, 20, 25, or 30°C
204 for 3 days. Subsequently, all plants were transferred to a plant growth chamber at 20°C
205 for 7 days. The experiments were repeated once on a different date.

206 **2.7. Effect of aphid developmental stages on the efficacy of *L. muscarium***

207 This experiment was carried out under controlled conditions in a growth chamber
208 (Sanyo, Gallenkamp, UK) at 20°C , $75 \pm 2\%$ RH and a photoperiod of 16:8 h (L:D). In
209 order to obtain a uniform age of different developmental stages of *M. persicae* (adults,
210 4th instars, 3rd instars and 1st instars), adults were transferred from a stock culture of *M.*
211 *persicae* onto 6-week-old Brussel sprouts plants (20 adults per plant) using two 3-cm-
212 diameter clip-cages and allowed to produce nymphs for 10, 7, 5 or 1 days before
213 bioassay to allow all four developmental stages to be available for experimental use on
214 the same treatment date. The adults were then removed and the offspring counted (50
215 nymphs per plant) and allowed to develop on the plants for 9, 6, 4 or 0 additional days

216 before beginning the experiment in the growth chamber at 20°C, 75 ± 2% RH and a
217 photoperiod of 16:8 (L:D) h.

218 The high, medium and low spore dosages of Mycotal® were each sprayed onto
219 Brussels sprouts plants which had either 50 first instar nymphs, third instar nymphs,
220 fourth instar nymphs or adults. The control group was sprayed with 0.02% sterile
221 aqueous Tween 80 only. After treatment, the plants were kept at room temperature for 1
222 h to dry and then returned to the same growth chamber with conditions as described
223 above. Dead aphids were counted and removed daily for a period of 10 days post
224 spraying. The method described in section 2.5 was used to confirm infection by *L.*
225 *muscarium*. For each aphid developmental stage, five replicates were carried out at each
226 spore dosage and for the control. This experiment was repeated in the same growth
227 chamber on a different date.

228 ***2.8. Effect of L. muscarium infection on individual aphids and their progenies***

229 This experiment was carried out to determine the effect of fungal infection on the rate of
230 nymph production and the length of reproductive time of aphids. The method described
231 in section 2.7 was used to obtain a uniform age of adult, 3rd and 1st instar nymphs. The
232 medium and low spore dosages of Mycotal® were each sprayed once onto infested
233 leaves of Brussel sprouts plants which had either 50 first instar nymphs, third instar
234 nymphs or adults. Control treatments were sprayed with 0.02% sterile aqueous Tween
235 80 only. After fungal treatment, the plants were kept at room temperature for 1 h to dry
236 and then transferred to the same growth chamber under the same conditions described
237 above for 1 day. After this period a single exposed aphid which had been inoculated
238 with either of the treatments or control was selected randomly and transferred (20
239 individuals of each aphid stage per treatment) with a fine camel hair brush onto healthy
240 Brussel sprouts plants using 2-cm-diameter clip-cages. Plants were then transferred to

241 the same growth chamber in the same conditions described above. Each leaf clip cage
242 was carefully opened and inspected daily until death and mortality and nymph
243 production by each individual were recorded. And all offspring and dead aphids were
244 removed. The method described in the relative humidity assays was used to confirm
245 infection by *L. muscarium*. Offspring of fungus-treated third and first instar nymphs
246 were also counted when they reached adult stage and started reproducing.

247 To determine the effect of fungal infection on the number of nymphs produced
248 from the progeny of infected aphids, offspring of fungus-treated and untreated adults
249 (20 first instar nymphs per treatment which were obtained from above experiment) were
250 selected randomly and transferred individually with a fine camel hair brush onto healthy
251 Brussel sprouts plants and contained in a 2-cm-diameter clip-cage in the plant growth
252 chamber at 20°C, 75 ± 2% RH and a photoperiod of 16:8 h (L:D) . They were allowed
253 to develop on the plants until they became adults and started reproducing. The number
254 of nymphs produced by these first generation adults was recorded daily for the number
255 of days equal to the length of the aphid's development period (birth until first
256 reproduction). The same procedure was repeated with offspring produced from fungus-
257 treated and untreated third instars and first instars when the latter reached adult stage
258 and started reproducing. The generation time (T) was calculated as the time in days
259 between the birth of an aphid and the birth of its first offspring (Al-Naemi & Hatcher,
260 2013). The experiment was repeated once on a different date.

261 ***2.9. Effect of *L. muscarium* infection on aphid populations***

262 This experiment was carried out to assess the effect of *L. muscarium* infection on aphid
263 population development. The method described in section 2.7 was used to obtain a
264 uniform age of adult aphids. Ninety-six Brussels sprouts plants (6-7 weeks old) were
265 infested with 1 or 2-day-old adult aphids (25 adults per plant) using 3-cm-diameter clip-

266 cages for 1 day. After this period, the clip-cages were removed and three treatments
267 each with 32 plants were used, one treatment received 1 ml of the medium dosage (644
268 ± 50 viable spore/mm²) per plant, one received 1 ml of the low dosage (330 \pm 40 viable
269 spore/mm²) per plant and one was a control and only sprayed with 0.02% sterile
270 aqueous Tween 80. After treatment, the plants were kept at 21 \pm 2°C for 1 hour to dry.
271 To prevent aphids from escaping, the plants were covered individually with perforated
272 plastic bags (Al-Naemi & Hatcher, 2013). They were then transferred to the growth
273 chamber at 20°C, 75 \pm 2 % RH, a photoperiod of 16:8 h (L:D) and at 2, 3, 4 and 5
274 weeks after fungal treatment a subset of 8 plants of each treatment were randomly
275 selected and destructively harvested, removing all aphids. All plants (only the shoots)
276 were dried at 80°C for 2 days to constant weight and weighed. Population growth rate
277 was calculated using the formula population growth rate = $(N_t - N_0) / (t - t_0)$, where N_t is
278 the number at time t , N_0 is the initial number, and t_0 is the initial time (Pianka, 2011).
279 The experiment was repeated once on a different date.

280 **2.10. Statistical analysis**

281 Statistical analyses were conducted using GenStat (version 16; VSN International,
282 Hemel Hempstead, UK). Normality of data distribution was estimated using a Shapiro-
283 Wilk test (W-test). The data were transformed using arcsine square-root when it was
284 necessary to meet the assumption of normality. The effect of temperature, relative
285 humidity and developmental stages were assessed by repeated measures analyses of
286 variance. Two-way ANOVA was used to compare between two laboratory experiments
287 to determine the effect of relative humidity. Aphid mortality was corrected for natural
288 death in the control using Abbott's formula (1925), which calculates the proportion of
289 aphids killed by the fungus alone. The total fecundity data for individual aphids treated
290 with two different dosages of *L. muscarium* and their progenies were analysed

291 separately by two-way ANOVA. Unbalanced repeated measurements ANOVA was
292 used to determine the effect of fungal application on the rate of nymph production per
293 day because the high spore dosage (880 ± 32 viable spore/mm²) caused 100% mortality
294 after 10 days of treatment compared to low conidial concentration and control. Repeat-
295 measurements ANOVA was used to determine the effect of fungal application on the
296 population size and dry weight of plants 2, 3, 4, and 5 weeks after application,
297 compared to untreated plants. Mean comparisons were performed using LSD test at 5%
298 level of significance ($P < 0.05$).

299 **3. Results**

300 **3.1. Effect of relative humidity (RH) on *L. muscarium* efficacy**

301 In the first experiment, aphid mortalities caused by the same conidial concentration
302 when placed at 55, 70 or 90% RH for 2 days and then 8 days at 70% RH were not
303 significantly different ($F_{2, 436} = 1.33$, $P = 0.265$), with the highest corrected mortality
304 (100%) caused by the high dosage (880 viable spore/mm²) of *L. muscarium* after 6 days
305 in all treatments. The medium (644 viable spore/mm²) and low (330 viable spore/mm²)
306 dosages were also effective but required longer to achieve a high level of aphid
307 mortality in comparison (Figure 1). The interaction between relative humidity, spore
308 dosage and time after application was also not significant ($P > 0.05$). Aphid mortality in
309 control treatments ranged between 5.6 and 8%.

310 In the second experiment, aphid mortalities when subjected to 55, 70 or 90% RH
311 for 2 days and then 8 days at 55% RH, were not significantly different ($F_{2, 452} = 2.17$, P
312 $= 0.115$) at any of the tested spore dosages, with the highest level of corrected mortality
313 (100%) caused by a high dosage of *L. muscarium* after 8 days in all RH treatments. The
314 medium and low spore dosages were also effective, but required a longer period of time
315 to achieve a high level of aphid mortality (Figure 2). The interaction between relative

316 humidity, spore dosage and time after fungal application was also not significant ($P >$
317 0.05). Aphid mortality in control treatments ranged between 4 and 7.6%.

318 The level of aphid mortality was not significantly different between the two
319 experiments ($F_{1, 1108} = 0.06, P = 0.814$), and the period of time required to achieve a
320 high level of aphid death was also not significantly different between the two
321 experiments for each spore dosage ($F_{1, 1108} = 0.24, P = 0.626$).

322 **3.3. Effect of temperatures on *L. muscarium* efficacy**

323 In the first experiment, results indicated that temperature had a significant effect on the
324 aphid corrected mortality caused by *L. muscarium* 10 days post-spraying ($F_{3, 600} =$
325 1633.03, $P \leq 0.001$), with the lowest mortality found in aphids incubated at 15°C
326 (Figure 3). The effect of spore dosage of *L. muscarium* and time after application on the
327 percentage of aphid corrected mortality was significant ($P \leq 0.001$), with 100%
328 mortality recorded for aphids treated with the high dosage (880 viable spore/mm²) and
329 incubated at 20, 25 and 30°C 6 days after treatment. The interactions between
330 temperature, fungus concentration and time after application were significantly different
331 ($P \leq 0.001$). Aphid mortality in control treatments ranged between 3 and 6%.

332 Results of the second experiment showed that incubation of fungus-treated
333 aphids at different temperatures for 3 days and then 7 days at 20°C had a significant
334 effect on the period of time required to obtain a high level of aphid mortality ($F_{3, 596} =$
335 311.97, $P \leq 0.001$). For instance, 100% aphid mortality occurred 6 days after treatment
336 with the high dosage (880 viable spore/mm²) when aphids were incubated at 25 or 30°C
337 for 3 days and then at 20°C for 10 days., This was 3 days earlier compared with aphids
338 incubated for 3 days at 15°C and at 20°C afterward (Figure 4). The interactions between
339 temperature, spore dosage and time after application were significantly different ($P \leq$
340 0.001). Aphid mortality in control treatments ranged between 2 and 7%.

341 **3.4. Effect of aphid developmental stages on the efficacy of *L. muscarium***

342 The developmental stage of *M. persicae* had a significant effect on the aphid corrected
343 mortality when aphids were treated with either high, medium or low dosages of *L.*
344 *muscarium*, with the highest mortality to adults, 4th instars and 3rd instars, compared
345 with 1st instars 10 days post-spraying ($F_{3, 572} = 348.27, P \leq 0.001$) (Figure 5). Spore
346 dosage had a significant effect on aphid mortality at each aphid developmental stage,
347 with the highest mortality occurring at the high dosage (880 viable spore/mm²). Aphid
348 mortality was much lower in control treatments and ranged from 3 to 7.2%.

349 **3.5. Effect of *L. muscarium* infection on fecundity of individual aphids and their**
350 ***progenies***

351 There was no significant effect of treatment on the rate of nymph production when *L.*
352 *muscarium* was sprayed on 1st instar nymphs, 3rd instar nymphs and adults, compared to
353 the control (fungal concentration: $F_{2, 134} = 2.07, P = 0.211$; exposed stage: $F_{2, 134} = 1.22$
354 $P = 0.299$). However, the reproductive period of aphids significantly decreased with
355 increasing the number of viable spores per mm² of *L. muscarium* ($F_{2, 134} = 133.17, P \leq$
356 0.001). The total fecundity of aphids differed significantly among spore dosages of *L.*
357 *muscarium* ($F_{2, 179} = 41.89; P \leq 0.001$), with the lowest fecundity found in those aphids
358 treated with the medium dosage (644 viable spore/mm²) (Figure 6). Aphid
359 developmental stage at the time of exposure showed a significant effect on the total
360 fecundity of aphids ($F_{2, 179} = 4.63; P \leq 0.001$) and the interaction between aphid
361 developmental stage and spore dosage of *L. muscarium* as factors affecting the total
362 fecundity of infected aphids was not significantly different ($P \leq 0.001$).

363 Maturation time of progenies produced from fungus-treated aphids at different
364 stages was 9.1 ± 0.4 days, but not significantly different compared to progenies
365 produced from untreated aphids ($F_{2, 175} = 0.59, P = 0.557$), The rate of nymph

366 production of the progenies of fungus-treated and untreated aphids was 3.4 ± 0.3
367 nymphs, but not significantly different compared to progenies produced from untreated
368 aphids ($F_{2, 89} = 0.18$, $P = 0.832$). The mean fecundity did not differ between progeny of
369 fungus-treated aphids and untreated aphids ($F_{2, 179} = 0.30$, $P = 0.738$).

370 **3.6. Effect of *L. muscarium* on aphid populations**

371 Aphid colonies treated with *L. muscarium* were significantly smaller than those of the
372 control, with untreated plants having 5 fold more aphids than the aphid colonies treated
373 with the medium dosage (644 viable spore/mm²) (Table 1 and Figure 7A). In all
374 treatments, the number of aphids varied significantly during the time post-treatment,
375 with the highest number of aphids recorded 5 weeks post treatment and the interaction
376 between treatments and the time after treatments were also significantly different ($P \leq$
377 0.001). The rate for the increase of the aphid population depended on both spore dosage
378 and the time after fungal treatment (Table 2). The results indicated that shoot dry weight
379 of untreated plants was reduced by about 30% compared with plants treated with the
380 fungus at the medium dosage during the time after application (Table 1 and Figure 7B).

381 **4. Discussion**

382 **4.1. Effect of temperature, relative humidity and aphid developmental stage on the** 383 **efficacy of *L. muscarium***

384 Most of the published efficacy data for this commercial product relate to control of
385 whiteflies. Cuthbertson & Walters (2005) reported that the application of Mycotal[®]
386 against sweet-potato whitefly *Bemisia tabaci* under laboratory conditions resulted in
387 90% mortality after 7 days. In addition, Fatiha, Ali, Ren, & Afzal (2007) reported that
388 the mortality caused by different isolates of *L. muscarium* against third instar of *B.*
389 *tabaci* varied between 87 and 56% after 8 days. The present study has shown that the
390 application of Mycotal[®], against *M. persicae* within the laboratory resulted in a high

391 level of mortality and it shows potential as an important biological control agent of *M.*
392 *persicae*.

393 Constant temperature had a significant effect on the efficacy of the fungus *L.*
394 *muscarium*, with, however, the highest efficacy and infection occurring in a broad
395 temperature range (20-30°C) (Figure 3). Similarly to our results, Vu, Hong, & Kim
396 (2007) found that *V. lecanii* caused the highest mortality of *M. persicae* in the same,
397 broad temperature range (20-30°C). Sharififard, Mossadegh, & Vazirianzadeh (2012)
398 found that the efficacy of *B. bassiana* and *M. anisopliae* were also higher in the same,
399 broad temperature range (20-30°C) and that the LT₅₀ values reflected this finding,
400 decreasing when temperature varied between 20-30°C. High numbers of fungus-
401 infected aphids and the ability of the fungus to kill aphids quickly in a broad
402 temperature range (20-30°C) may be related to the ability of *L. muscarium* to germinate
403 and colonise aphids rapidly (Yeo et al., 2003).

404 Laboratory bioassays under alternating temperature showed that although
405 incubation at a lower temperature (15°C) for 3 days caused a significant reduction in the
406 efficacy of *L. muscarium* against aphids, the aphid mortality occurred as quickly when
407 the treated plants were transferred at 20°C (Figure 4). This may be explained by lower
408 conidia germination and/or increasing the death rate of fungal conidia at 15°C. There
409 are few studies that have investigated the effect of fluctuating temperatures on the
410 efficacy of entomopathogenic fungi on aphids. Feng, Poprawski, Nowierski, & Zeng
411 (1999) found that the efficacy of *Pandora neoaphidis* against the pea aphid
412 *Acyrtosiphon pisum* was greater at a constant temperature (20°C) than at alternating
413 temperatures (5.5-18.9°C). Although we did not investigate the effect of daily
414 fluctuating temperature, trials of the effect of fluctuating temperatures on the efficacy of
415 entomopathogenic fungi could be more useful and accurate than constant temperatures

416 in predicting the efficacy and speed of kill of *L. muscarium* against *M. persicae* under
417 glasshouse or field conditions, as they may be more representative of the conditions that
418 *L. muscarium* will be exposed to in actual crop conditions.

419 Varying relative humidity between 55-90% had no significant effect on the
420 corrected mortality of aphids caused by *L. muscarium* or the speed of death (Figure 1
421 and Figure 2). This may be due to the fact that relative humidity may not be the same as
422 the humidity in the microenvironment immediately next to the insect or leaf surface
423 (Willmer, 1986). Wraight et al. (2000) reported that the insect host and leaf substrate
424 together contribute to create a zone of high humidity that supports germination,
425 colonisation and sporulation of entomopathogenic fungi. These results confirmed the
426 findings of other researchers (Doberski, 1981; Ferron, 1977) who demonstrated that
427 infection of some pest species by entomopathogenic fungi may occur at low as well as
428 high relative humidities (30 to 75%). Contrary to the findings of these authors, and
429 those of this study, Hsiao, Bidochka, & Khachatourians (1992) reported that when
430 relative humidity was reduced for a period of time, some isolates of *Lecanicillium* spp.
431 had lower levels of efficacy. Milner & Lutton (1986) also reported that conidia of *L.*
432 *lecanii* required 100% relative humidity for at least 3 days before removal to 70%
433 humidity to obtain about 90% mortality of *M. persicae*. Sukhova (1987), tested different
434 isolates of *V. lecanii* and *B. bassiana* against whitefly populations and also reported
435 high relative humidity (80-90%) was required to achieve 100% mortality of whitefly.
436 The differences between the findings of these previous studies and the experiments
437 detailed here may be due to differences in the bioassay procedures, target insect pests
438 and/ or whether conidia or blastospores were used as the inoculum.

439 Results of this study showed that first instar nymphs of *M. persicae* were
440 generally less susceptible to infection with *L. muscarium* than all other stages tested

441 (adults, fourth instar nymphs and third instar nymphs). Moreover, the corrected
442 mortality in all aphid developmental stages was dependent on fungal dose, with the
443 highest mortality occurring at the high dosage (880 viable spore/mm²) (Figure 5). These
444 results are in agreement with previous studies including lower mortality caused by
445 different conidial concentrations of *L. attenuatum* in first instar nymphs of *A. gossypii*
446 as compared with other developmental stages reported by Kim & Roberts (2012). The
447 lower susceptibility of young instars of insects to infection by entomopathogenic fungi
448 has been reported to be related to fast and frequent moulting (Kim & Roberts, 2012;
449 Wekesa, Knapp, Maniania, & Boga, 2006), which may be explained by changes in
450 cuticle biochemical composition during development such as the presence of toxic
451 compounds which may inhibit spore germination (Kirkland et al., 2004), or low
452 numbers of spores attaching to the cuticle of early instar nymphs as a result of their
453 small body size (Tang & Hou, 1998). We suggest that the lower fungus-mortality of
454 first instar nymphs of *M. persicae* may be due to multiple moulting (within about 2 days
455 after fungal application) causing fewer spores to adhere to the new cuticle, perhaps also
456 in association with a low germination percentage on the cuticles of first instar nymphs.
457 Decreased conidial adhesion was observed by Kim & Roberts (2012), who reported that
458 the number of fungal conidia adhering to cuticles of young instar nymphs of *A. gossypii*
459 declined with increasing time after treatment due to moulting. In addition, the smaller
460 body size of first instar nymphs may have played a role in the lower infection with *L.*
461 *muscarium* by proportionate decline of the conidial load.

462 ***4.2. Effect of fungal infection on individual life history and on population*** 463 ***development of M. persicae***

464 The main objective of these studies was to gain information on the role played by *L.*
465 *muscarium* on the daily fecundity, total fecundity, development time and r_m value of

466 fungus-treated aphids and their progenies. These results led to the rejection of the
467 hypotheses that individuals of *M. persicae* treated with *L. muscarium* produce nymphs
468 at a different rate compared to untreated aphids and that offspring produced by fungus-
469 treated aphids have a high chance of acquiring the fungus before birth. Although the
470 pathogen had a significant effect on overall aphid fecundity, this effect was caused by
471 increasing aphid mortality which resulted in reducing the length of reproductive time of
472 fungus-treated aphids (Figure 6). The fact that fungal infection has no negative effect on
473 the rate of nymph production per day may be a result of the host diverting resources to
474 defence and then reproduction as a strategy to increase fitness. Furthermore, the
475 selection pressure of generalist hypocrealean fungi like *L. muscarium* may be too weak
476 to modify the behaviour of the aphids, compared to host-specific entomopathogenic
477 fungi like *Pandora neoaphidis* (Roy, Baverstock, Chamberlain, & Pell, 2005). The
478 results obtained in the present study are consistent with Wang & Knudsen (1993) and
479 Kim (2007) who reported that daily fecundity of individual aphids did not decrease due
480 to fungal treatment but total fecundity significantly decreased due to increasing level of
481 aphid mortality rather than by differential reproduction rates of individuals. However,
482 indirect effects of entomopathogenic fungi on aphid reproduction seem to depend on
483 aphid species and/or the fungal species or isolate. Thus, Askary, Carriere, Belanger, &
484 Brodeur (1998) found a negative impact on reproduction of potato aphids *Macrosiphum*
485 *euphorbiae* treated with Vertalec[®] and Shrestha et al. (2015) found a negative effect on
486 the daily rate of nymph production of *N. ribisnigri* treated with either high, medium or
487 low conidial concentrations of *B. bassiana*.

488 In spring and summer, parthenogenetic females of *M. persicae* do not lay eggs
489 but give birth to larvae. In this case, the development of an aphid begins when its
490 mother is still an embryo. Consequently, the embryos inside an adult parthenogenetic

491 aphid carry embryos themselves. This so-called telescoping of generations which is a
492 major reason for the high intrinsic rate of increase in aphid populations and the
493 agricultural harm that they can cause (Kindlmann & Dixon, 1989) as this reproductive
494 strategy gives aphids approximately a threefold reproductive advantage (Dixon, 1990).
495 Thus, it might be expected that fungal infection would have an impact on fecundity
496 beyond the first generation of offspring. However, our results showed that fungal
497 infection of aphids has no negative effects on their progenies' mean development time,
498 total fecundity and r_m -value. Fungal infection, therefore, appears to affect the host
499 aphid's reproduction numerically but does not have a physiological effect on the
500 developing progeny. These results were in agreement with Baverstock et al. (2006) who
501 reported that the intrinsic rate of increase did not differ between progeny of fungus-
502 infected aphids and uninfected aphids when they were infected by either *P. neoaphidis*
503 or *B. bassiana*.

504 The aphids' population was significantly affected by *L. muscarium* infection. The
505 results showed that the aphid population exposed to the medium spore dosage (644
506 viable spore/mm²), resulted in a significantly much smaller population size than the
507 unexposed population (Figure 7A). The significant reduction in the population size of
508 treated aphids was most likely to be caused by the impact of fungus treatment on the
509 total fecundity of individual aphids as a result of a higher mortality rate or that nymph
510 may become infected, once in contact with fungus-sporulation aphids. However,
511 spraying the fungus only once was not sufficient to achieve a good level of control as
512 the economic threshold level of *M. persicae* on Chinese cabbage was calculated to be 20
513 aphids per plant (Jeon, Kang, Kim, Yang, & Kim, 2008). This is in line with data of Xu
514 & Feng (2002) who report that the size of an aphid population contaminated with *P.*

515 *delphacis* at a concentration of 112 ± 22.8 conidia/mm² was significantly smaller than
516 those of the controls.

517 It can be concluded that the spores of *L. muscarium* can survive at low
518 temperatures (15°C) and have the ability to start germination and infection after a few
519 days when they are placed at optimum temperatures between 20°C and 30°C. Also, *M.*
520 *persicae* can be infected with *L. muscarium* by incubation at range of relative humidity
521 (55- 90%). Fungal infection does not have a physiological effect on the developing
522 progeny. However, the determination that *L. muscarium* had no significant impacts on
523 the daily reproduction of individual aphids. Based on these results, it is expected that
524 the commercially available mycoinsecticide Mycotal[®], could be an appropriate
525 biological control agent and can be included in integrated pest management
526 programmes designed for controlling the populations of green peach aphids under the
527 field conditions during the summer season in the UK. However, frequent spraying may
528 be required to keep the aphid number lower than economic threshold level. Further
529 investigations on the impact of daily fluctuation in temperature through the day and
530 night hours and the potential impact of this entomopathogenic fungus on other
531 biocontrol agents, such as parasitoids and predators, will be required.

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726 **Table 1** Repeated measures analysis of variance of the effect of *L. muscarium*
727 application on the population growth of *M. persicae* on Brussels sprouts and shoot dry
728 weight of aphid-infested plants.

Source	DF	SS	
		Aphid population	Dry weight of plant
Spore dosage	2	6.902***	6.794**
Time after treatment	3	27.051***	10.376**
Spore dosage*Time	6	0.282***	0.994
Residual	63	0.437	39.040

Total	95	34.799	67.787
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729 *** $P \leq 0.001$; ** $P \leq 0.05$.

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731 **Table 2** Population growth rate of exposed aphids during the time after application

732 compared to unexposed aphids.

Treatment (viable spore/mm ²)	Population growth rate (%) during the weeks after application		
	Week 2-3	Week 3-4	Week 4-5
Dosage (644 ± 50)	121.7 ± 26.7b	309 ± 18.6c	83.7 ± 13.9a
Dosage (330 ± 40)	447.2 ± 23.9a	668 ± 23c	620.3 ± 41.6b
Control	618 ± 37.7a	925.2 ± 42.5b	949.6 ± 42.2c

733 Means followed by different letter(s) in each column are significantly different at $P <$

734 0.05, using LSD test.

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737

738 **Figure legends**

739 **Figure 1** The effect of relative humidity, spore dosage and days after fungal application
 740 on the corrected mortalities (mean ±SE, n = 5) of *M. persicae* treated with *L. muscarium*
 741 after 2 days incubation at either 55, 70 or 90% RH and then 8 days at 70% RH. High,
 742 medium and low dosages were 880 ± 32, 664 ± 50 and 330 ± 40 viable spore/mm².

743 **Figure 2** The effect of relative humidity, spore dosage and days after fungal application
 744 on the cumulative mortalities (±SE) of *M. persicae* treated with *L. muscarium* after 2
 745 days incubation at either 55, 70 or 90% RH and then 8 days at 55% RH. High, medium
 746 and low dosages were 880 ± 32, 664 ± 50 and 330 ± 40 viable spore/mm².

747 **Figure 3** The effect of temperature, spore dosage and period of time after application on
748 the corrected mortalities (mean \pm SE, n = 5) of *M. persicae* treated with *L. muscarium*
749 after 10 days incubation at 15, 20, 25 or 30°C. High, medium and low dosages were 880
750 \pm 32, 664 \pm 50 and 330 \pm 40 viable spore/mm².

751 **Figure 4** The effect of temperature, spore dosage and period of time after application on
752 the corrected mortalities (mean \pm SE; n= 5) of *M. persicae* treated with *L. muscarium*
753 after 3 days incubation at either 15, 20, 25 or 30°C and then 7 days at 20°C. High,
754 medium and low dosages were 880 \pm 32, 664 \pm 50 and 330 \pm 40 viable spore/mm².

755 **Figure 5** Corrected mortality (\pm SE; n= 10) of first, third and fourth instar nymphs and
756 adults of *M. persicae* sprayed with different spore dosages of *L. muscarium*. High,
757 medium and low dosages were 880 \pm 32, 664 \pm 50 and 330 \pm 40 viable spore/mm².

758 **Figure 6** Effect of fungal infection on the mean numbers of nymphs produced per aphid
759 per day, mean length of reproductive period and mean fecundity of *M. persicae* after
760 treatment with two spore dosages of *L. muscarium* at either adult, 3rd instar nymph or 1st
761 instar nymphs, compared to the control. Different letters above columns indicate
762 significant differences in the values at $P < 0.05$, using LSD test. Medium and low
763 dosages were 664 \pm 50 and 330 \pm 40 viable spore/mm².

764 **Figure 7** The effect of *L. muscarium* application on (A) the population growth of *M.*
765 *persicae* (mean \pm SE; n = 8) and (B) shoot dry weight of aphid-infested plants (mean \pm
766 SE; n = 8) compared with the control treatment. Medium and low dosages were 664 \pm
767 50 and 330 \pm 40 viable spore/mm².

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