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

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The green peach aphid, *Myzus persicae*, is a major pest worldwide. An examination of the impact of temperature, relative humidity and developmental stages of *M. persicae* on the efficacy of the whitefly mycoinsecticide Mycotal®, based on *Lecanicillium muscarium* and the effects of infection on aphid fecundity was evaluated under controlled conditions. Although this fungus can be grown at a broad range of temperatures (15-30$^{o}$C), the optimum temperature for control of *M. persicae* ranged between 20 and 30$^{o}$C. *L. muscarium* had high efficacy as a microbial control against *M. persicae* between 55 and 90% relative humidity. Total mortality of aphids treated with different spore dosages of *L. muscarium* varied according to the developmental stage: adults, fourth and third instar nymphs of proved more susceptible than first instar nymphs. Although the fungus did not affect the rate of nymph production, the reproductive period of aphids significantly decreased with increasing the spore dosage. Thus, total fecundity of treated aphids was 22.6 ± 1.1 and 31.6 ± 2.4 offspring per adult at the medium (644 ± viable spore/mm$^2$) and low (330 ± 40 viable spore/mm$^2$) dosages, compared with 45.7 ± 4.3 offspring per untreated aphid. The results suggest that *L. muscarium* has the potential as a biological control agent of *M. persicae*. However, fungal infection appears to have no sub-lethal effects on the fitness of the host's progeny.

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

## 1. Introduction

The green peach aphid, *Myzus persicae* Sulzer (Hemiptera: Aphidiae), is one of the most important and common aphid species of greenhouse and field crops worldwide (Blackman & Eastop, 2007). It is highly polyphagous infesting crops in over 40 plant 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, & Rasoulian,
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 &

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

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

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

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

## 2. Materials and methods

### *2.1. Plant material*

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

### *2.2. Rearing M. persicae*

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

### *2.3. Fungus application*

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

For each treatment, 1 ml of spraying suspension was applied once on each leaf 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$^{o}$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$^{o}$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

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

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

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

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

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

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

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

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

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

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

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

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

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

### *2.10. Statistical analysis*

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

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

## 3. Results

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

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

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

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

The developmental stage of *M. persicae* had a significant effect on the aphid corrected mortality when aphids were treated with either high, medium or low dosages of *L. muscarium*, with the highest mortality to adults, 4$^{th}$ instars and 3$^{rd}$ instars, compared with 1$^{st}$ instars 10 days post-spraying ($F_{3, 572}$ = 348.27, $P \leq 0.001$) (Figure 5). Spore dosage had a significant effect on aphid mortality at each aphid developmental stage, with the highest mortality occurring at the high dosage (880 viable spore/mm$^2$) . Aphid mortality was much lower in control treatments and ranged from 3 to 7.2%.

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

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

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

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

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

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

## 4. Discussion

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

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

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

Constant temperature had a significant effect on the efficacy of the fungus *L. muscarium*, with, however, the highest efficacy and infection occurring in a broad temperature range (20-30$^{o}$C) (Figure 3). Similarly to our results, Vu, Hong, & Kim (2007) found that *V. lecanii* caused the highest mortality of *M. persicae* in the same, broad temperature range (20-30$^{o}$C). Sharififard, Mossadegh, & Vazirianzadeh (2012) found that the efficacy of *B. bassiana* and *M. anisopliae* were also higher in the same, broad temperature range (20-30$^{o}$C) and that the $LT_{50}$ values reflected this finding, decreasing when temperature varied between 20-30°C. High numbers of fungus-infected aphids and the ability of the fungus to kill aphids quickly in a broad temperature range (20-30$^{o}$C) may be related to the ability of *L. muscarium* to germinate and colonise aphids rapidly (Yeo et al., 2003).

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

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

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

Results of this study showed that first instar nymphs of *M. persicae* were 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/$mm^2$) (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

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

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

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

The aphids' population was significantly affected by *L. muscarium* infection. The results showed that the aphid population exposed to the medium spore dosage (644 viable spore/$mm^2$), resulted in a significantly much smaller population size than the unexposed population (Figure 7A). The significant reduction in the population size of treated aphids was most likely to be caused by the impact of fungus treatment on the total fecundity of individual aphids as a result of a higher mortality rate or that nymph may become infected, once in contact with fungus-sporulation aphids. However, spraying the fungus only once was not sufficient to achieve a good level of control as the economic threshold level of *M. persicae* on Chinese cabbage was calculated to be 20 aphids per plant (Jeon, Kang, Kim, Yang, & Kim, 2008). This is in line with data of Xu & Feng (2002) who report that the size of an aphid population contaminated with *P.*

*delphacis* at a concentration of 112 ± 22.8 conidia/mm$^2$ was significantly smaller than those of the controls.

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

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**Table 1** Repeated measures analysis of variance of the effect of *L. muscarium* application on the population growth of *M. persicae* on Brussels sprouts and shoot dry 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 |

729 ***$P \leq 0.001$; **$P \leq 0.05$.

730

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.

735

736

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/mm$^2$.
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/mm$^2$.

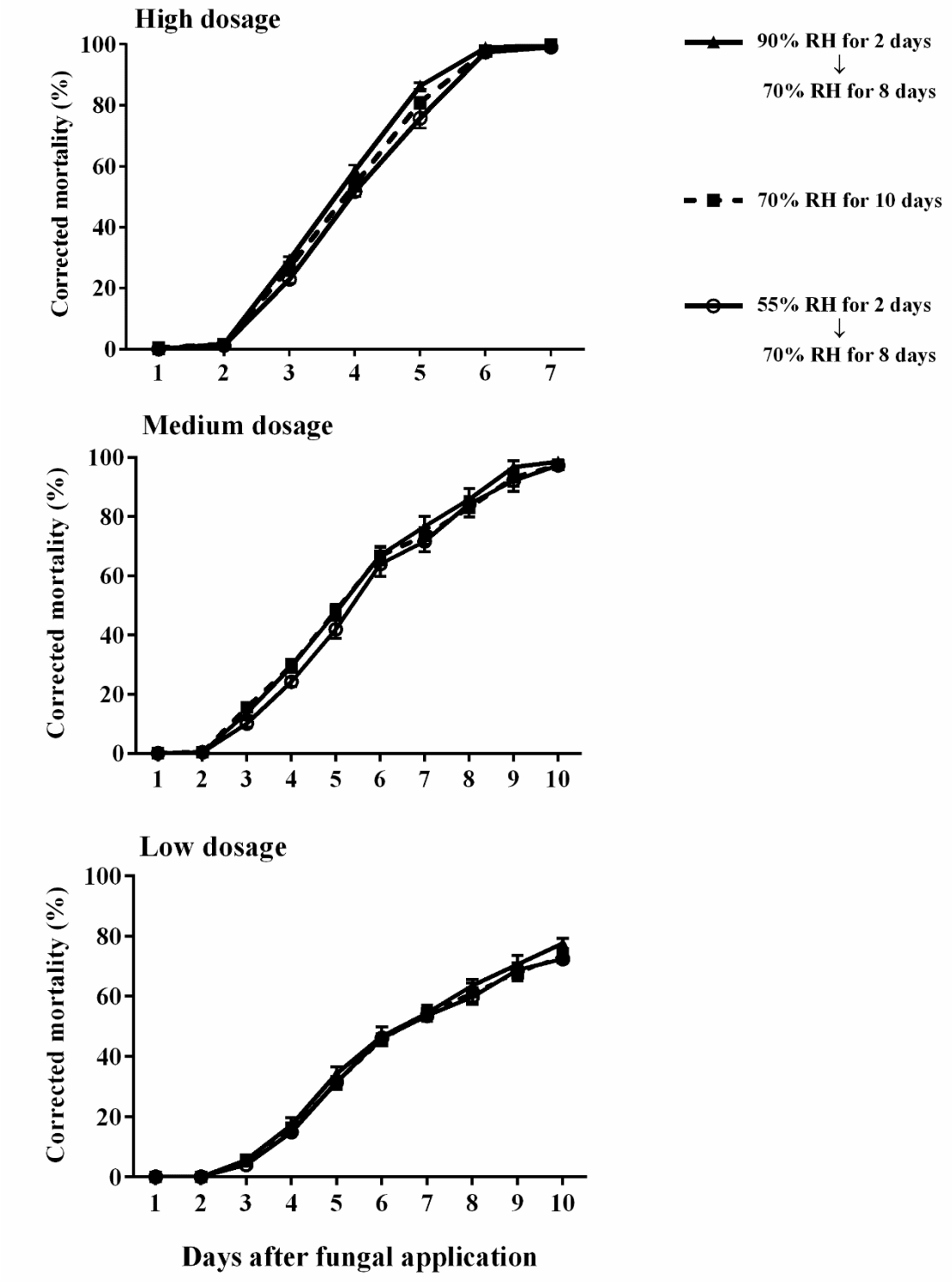
**Figure 3** The effect of temperature, spore dosage and period of time after application on the corrected mortalities (mean ± SE, n = 5) of *M. persicae* treated with *L. muscarium* after 10 days incubation at 15, 20, 25 or 30$^{o}$C. High, medium and low dosages were 880 ± 32, 664 ± 50 and 330 ± 40 viable spore/mm$^2$.

**Figure 4** The effect of temperature, spore dosage and period of time after application on the corrected mortalities (mean ± SE; n= 5) of *M. persicae* treated with *L. muscarium* after 3 days incubation at either 15, 20, 25 or 30$^{o}$C and then 7 days at 20$^{o}$C. High, medium and low dosages were 880 ± 32, 664 ± 50 and 330 ± 40 viable spore/mm$^2$.

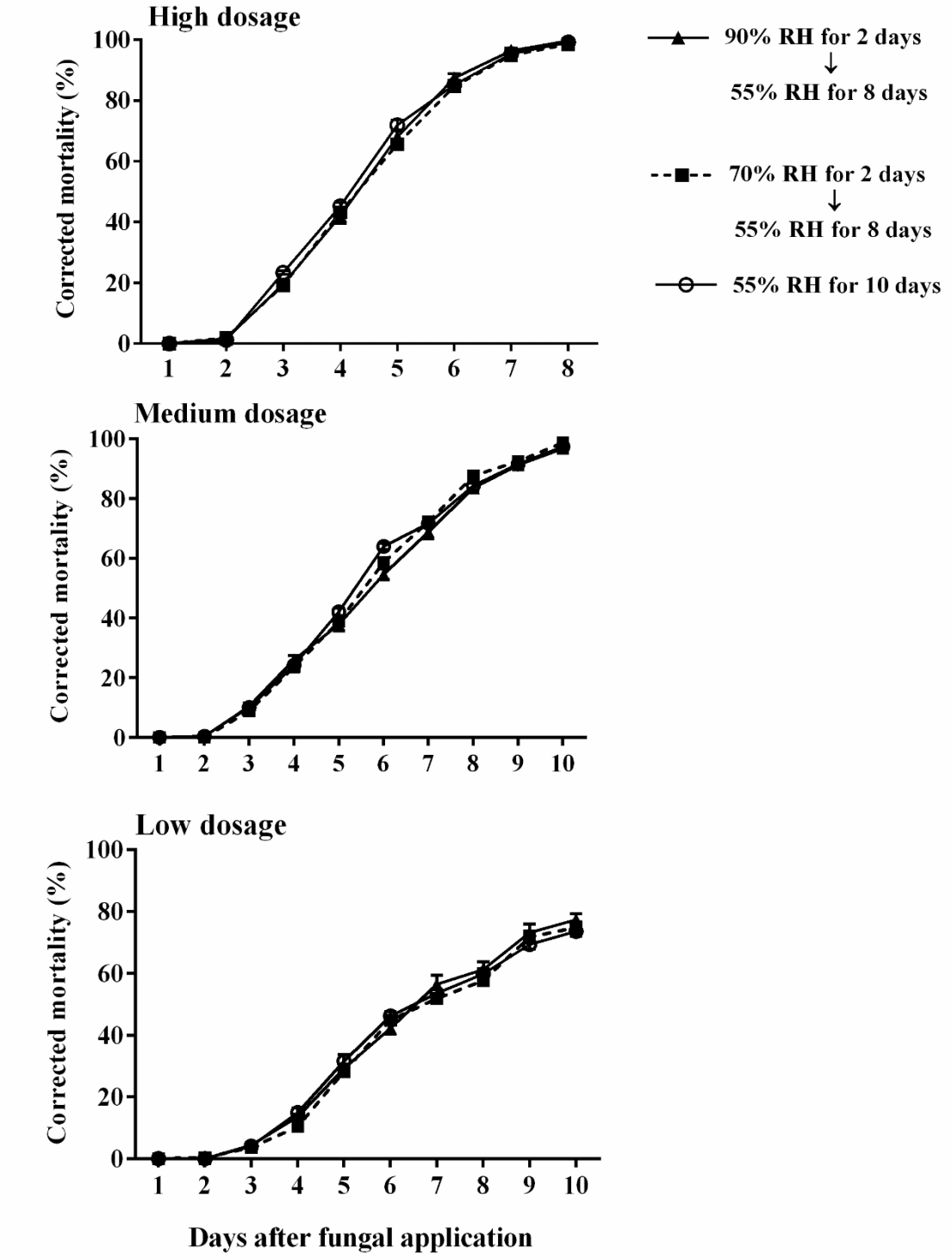
**Figure 5** Corrected mortality (±SE; n= 10) of first, third and fourth instar nymphs and adults of *M. persicae* sprayed with different spore dosages of *L. muscarium*. High, medium and low dosages were 880 ± 32, 664 ± 50 and 330 ± 40 viable spore/mm$^2$.

**Figure 6** Effect of fungal infection on the mean numbers of nymphs produced per aphid per day, mean length of reproductive period and mean fecundity of *M. persicae* after treatment with two spore dosages of *L. muscarium* at either adult, 3$^{rd}$ instar nymph or 1$^{st}$ instar nymphs, compared to the control. Different letters above columns indicate significant differences in the values at $P < 0.05$, using LSD test. Medium and low dosages were 664 ± 50 and 330 ± 40 viable spore/mm$^2$.

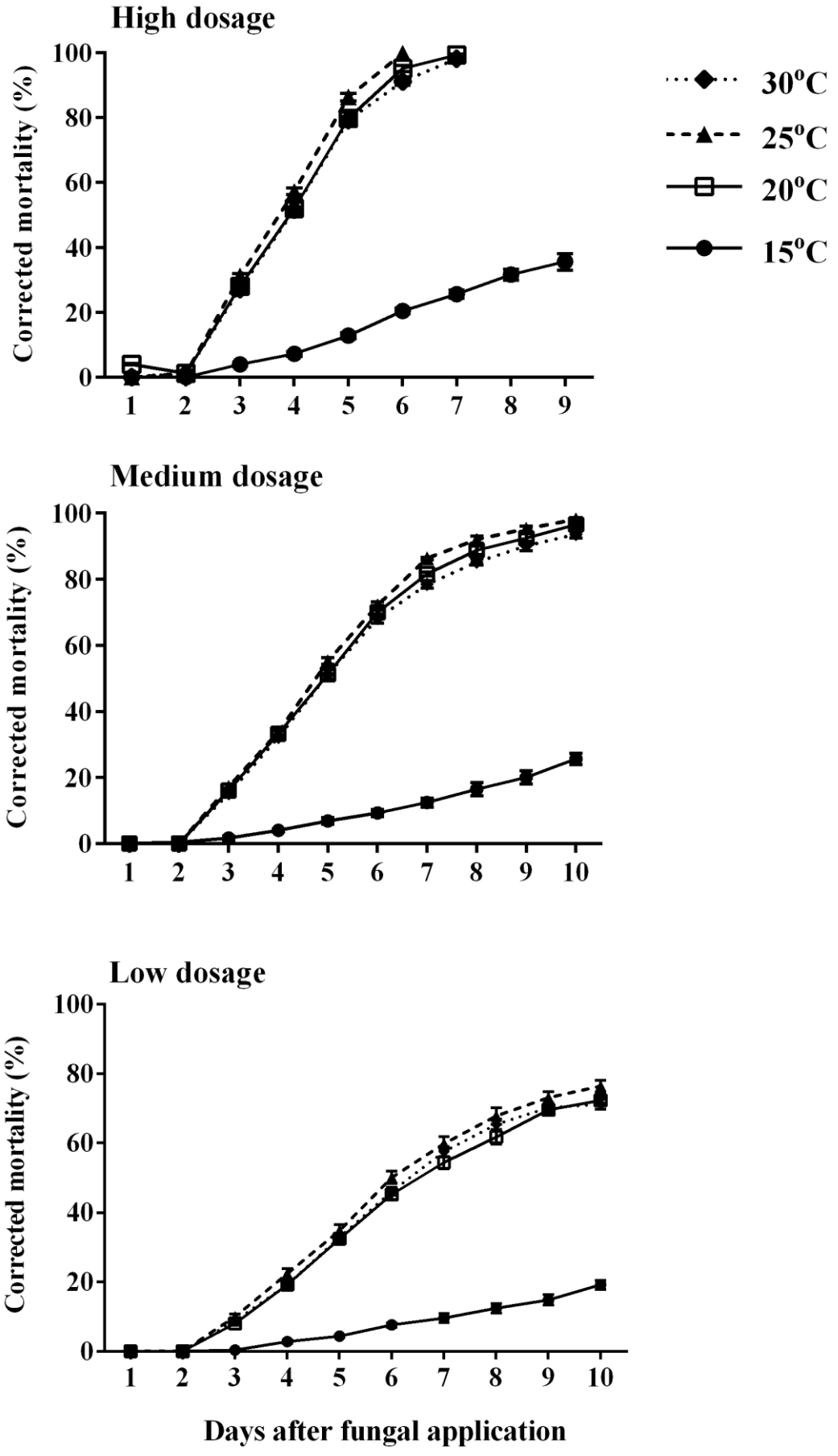
**Figure 7** The effect of *L. muscarium* application on (A) the population growth of *M. persicae* (mean ± SE; n = 8) and (B) shoot dry weight of aphid-infested plants (mean ± SE; n = 8) compared with the control treatment. Medium and low dosages were 664 ± 50 and 330 ± 40 viable spore/mm$^2$.


High dosage
Medium dosage
Low dosage
Corrected mortality (%)
Days after fungal application
90% RH for 2 days
↓
70% RH for 8 days
70% RH for 10 days
55% RH for 2 days
↓
70% RH for 8 days

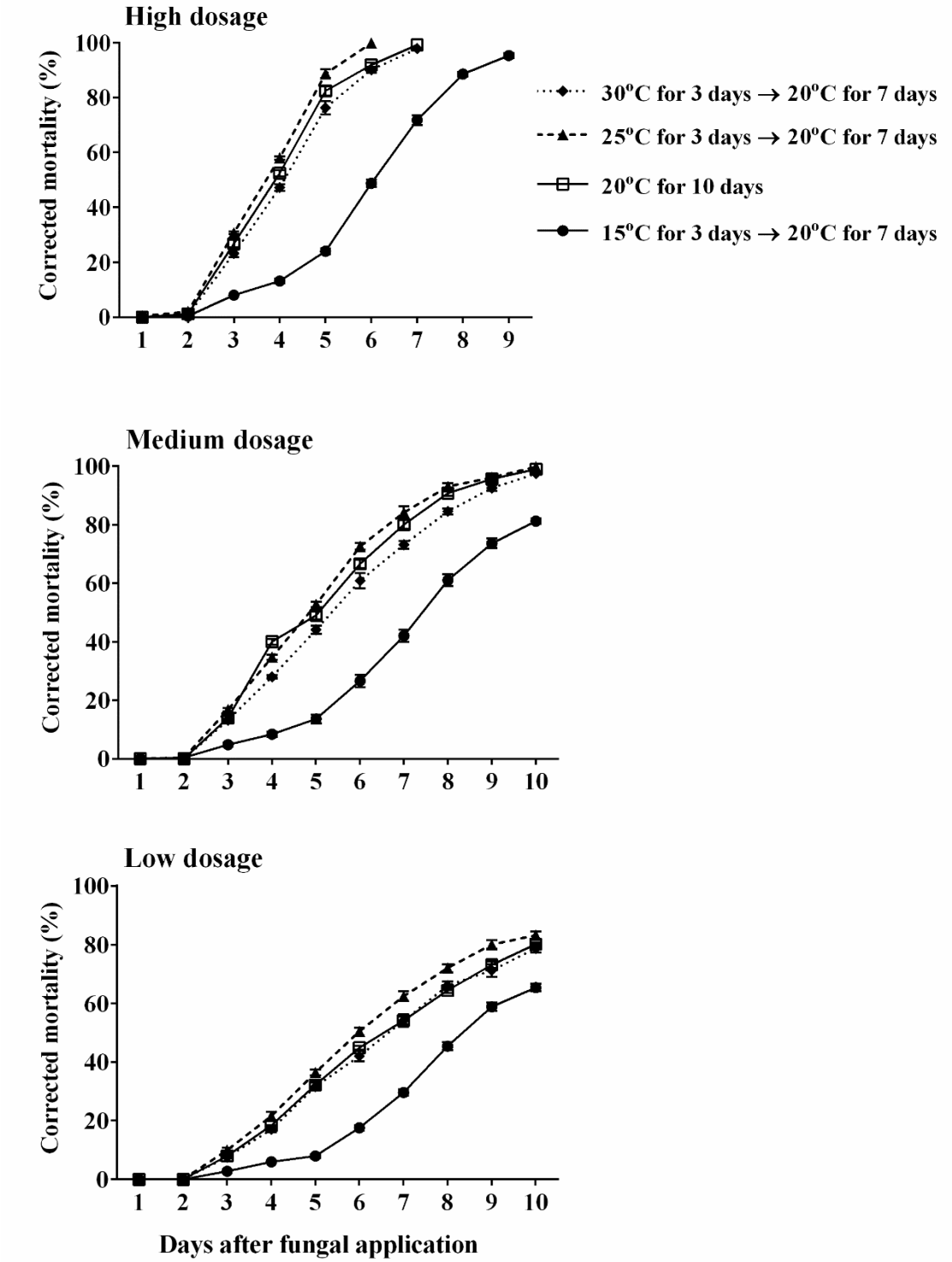
769


High dosage
Medium dosage
Low dosage
Corrected mortality (%)
Days after fungal application
90% RH for 2 days
↓
55% RH for 8 days
70% RH for 2 days
↓
55% RH for 8 days
55% RH for 10 days

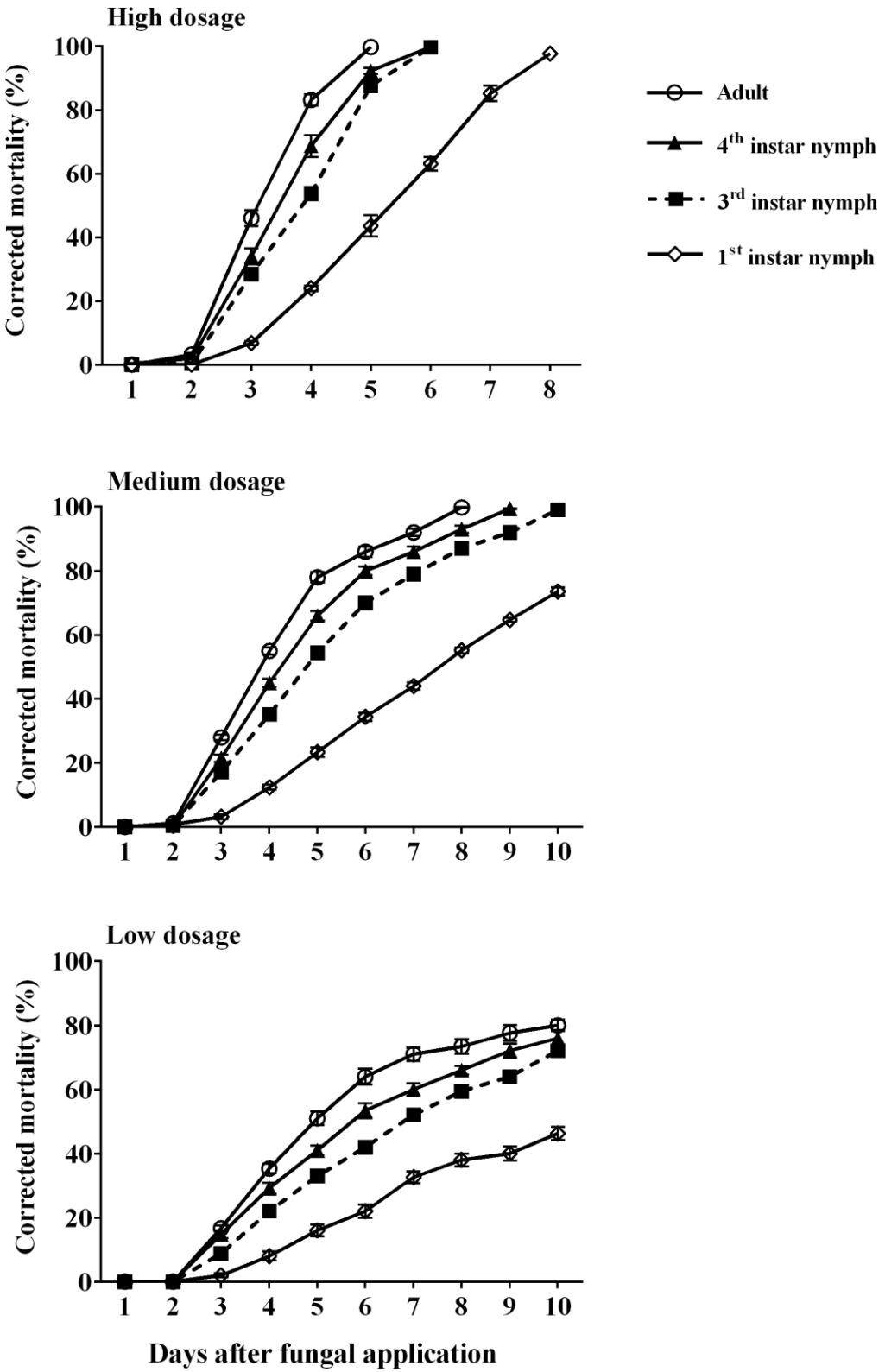
770


High dosage
Corrected mortality (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9
30°C
25°C
20°C
15°C
Medium dosage
Corrected mortality (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
Low dosage
Corrected mortality (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
Days after fungal application

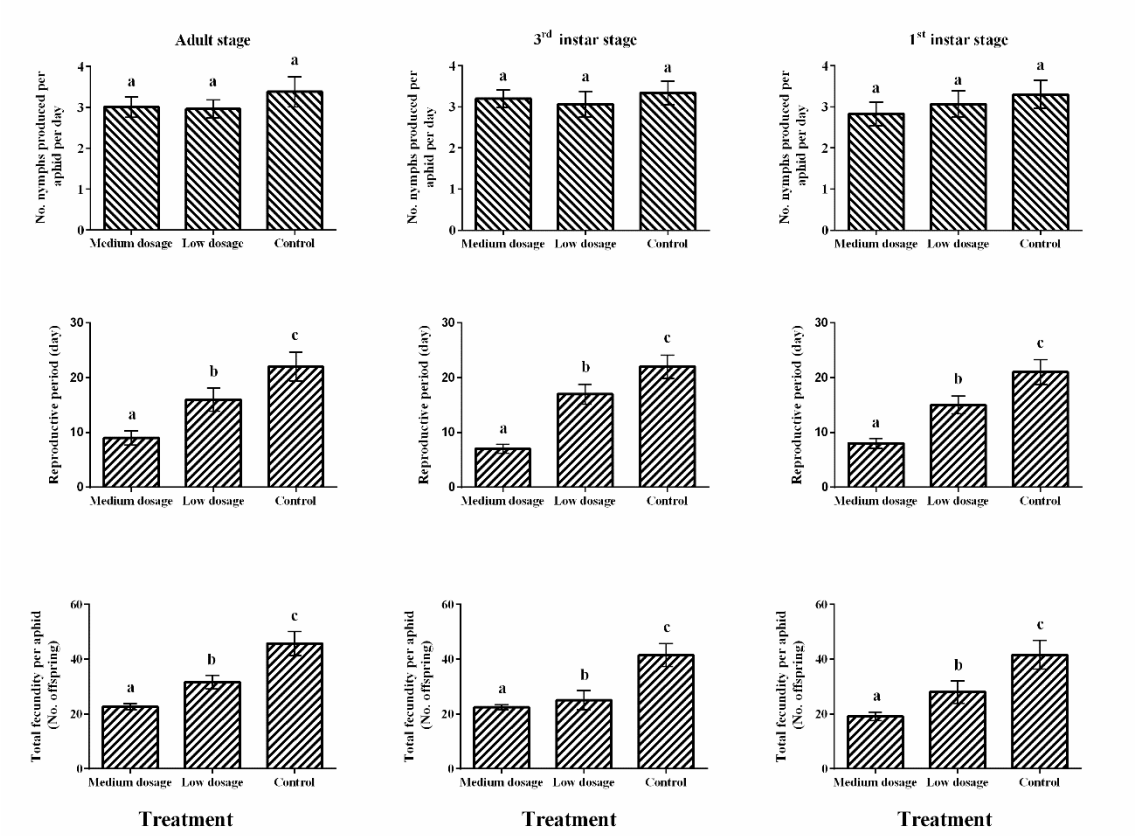
771


High dosage
Corrected mortality (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9
30°C for 3 days → 20°C for 7 days
25°C for 3 days → 20°C for 7 days
20°C for 10 days
15°C for 3 days → 20°C for 7 days
Medium dosage
Corrected mortality (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
Low dosage
Corrected mortality (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
Days after fungal application

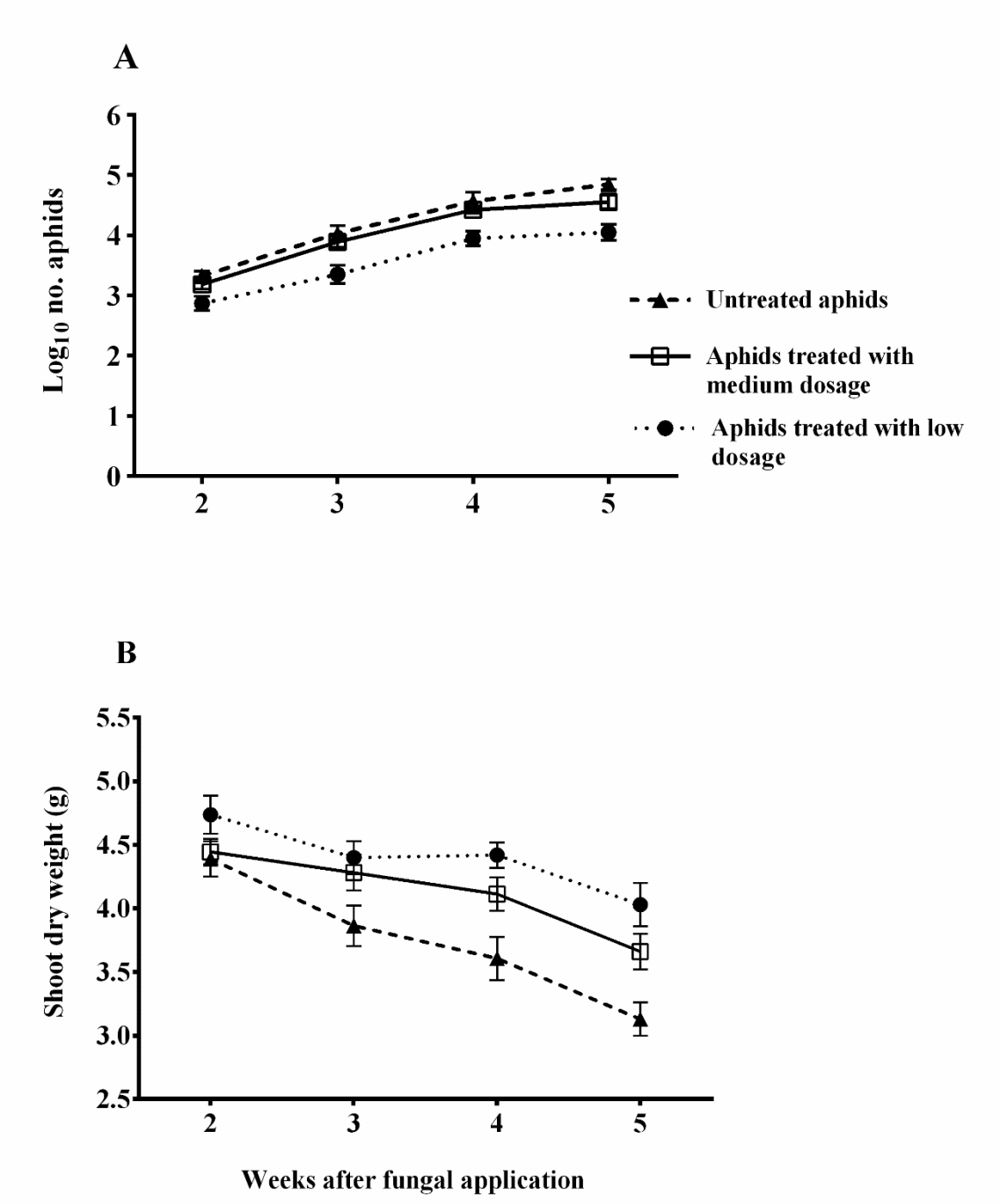
772


High dosage
Corrected mortality (%)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
Adult
4th instar nymph
3rd instar nymph
1st instar nymph
Medium dosage
Corrected mortality (%)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Low dosage
Corrected mortality (%)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Days after fungal application

773


Adult stage
3rd instar stage
1st instar stage
No. nymphs produced per aphid per day
Reproductive period (day)
Total fecundity per aphid (No. offspring)
Medium dosage
Low dosage
Control
Treatment

774


A
Log10 no. aphids
6
5
4
3
2
1
0
2
3
4
5
Untreated aphids
Aphids treated with medium dosage
Aphids treated with low dosage
B
Shoot dry weight (g)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2
3
4
5
Weeks after fungal application

775