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1 **Phoretic relationship between the myceliophagus mite *Microdispus lambi* (Acari:
2 *Microdispidae*) and mushroom flies in Spanish crops**

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12 Running title: Mushroom flies as vector of myceliophagus mites

13

14 **Abstract**

15 We studied the role played by the phorid *Megaselia halterata* (Wood) and the sciarid
16 *Lycoriella auripila* (Winnertz) in the phoretic dispersion of the myceliophagus mite
17 *Microdispus lambi* (Acari: Pygmephoridae). Twenty-four crops were monitorized
18 during 18 months in commercial mushroom farms in Castilla-La Mancha (Spain).
19 Adults of both species were collected weekly and the mites they carried were counted
20 and identified. Both phorids (19.6%) and sciarids (4.4%) carried the mite *M. lambi*. The
21 calculated load of each was 3.4 *M. lambi* mites per phorid and 1.9 per sciarid. The same
22 percentage of male and female phorid was used as vector, but the load was lightly
23 higher for females (1.86 mites per female compared with 1.48 mites per male).

24 A mean of 7.2% of the phorids examined in winter were vectors of *M. lambi*,
25 while in spring and autumn of the first year the average was higher than 22%. The mean
26 load did not vary significantly between seasons. Inside the mushroom farms, less than
27 10% of a small initial population of phorids carried mites (less than 2 mites per phorid).
28 As the cycle progressed, more than 35% of a larger population of emerging flies did so
29 (average 3.5 mites per phorid vector). At the end of the growth cycle, the flies may fly
30 off to colonize nearby farms, favouring the propagation of *M. lambi* from infected to
31 uninfected crops.

32 *Megaselia halterata* is the principal vector of *M. lambi* in the mushroom farms
33 of Castilla-La Mancha due to their high numbers, the high percentage carrying mites
34 and the number of *M. lambi* they carry.

35

36 **Key words:** *Megaselia halterata*, *Lycoriella auripila*, *Agaricus bisporus*, mushroom
37 mite, phoresis.

38 **Introduction**

39 The myceliophagus mite *Microdispus lambi* (Krczal) was detected for the first time in
40 Spain in the summer of 1996 (Ferragutet *et al.*, 1997). Since then this pest has become
41 widely dispersed among Spanish mushroom growing farms. Previously, the mite had
42 been described in New Zealand (Krczal, 1964), but had also been found in Australia and
43 China (Clift & Toffolon, 1981; Gao *et al.*, 1986). It can develop and reproduce only on
44 *Agaricus* species (Clift & Toffolon, 1981; Gao & Zou, 2001). Mite populations lead to
45 the slow disappearance of the mycelium and substantial yield losses, sometimes leaving

46 farmers with no mushrooms to harvest at all. In Shanghai, contaminated mushroom
47 spawn was a major source of mite infestation (Wu & Ma, 1988; Wu & Zhang, 1993),
48 while in Australia, *M. lambi* was found to be phoretic on sciarid and phorid flies (Clift
49 & Larsson, 1987).

50 A study of this pest in Spanish growing crops demonstrated that spawn, compost
51 and casing materials cannot be considered as sources of contamination by *M. lambi*
52 mite. Mite populations were detected on mushroom farms throughout the year, although
53 the incidence declined markedly during the winter. In the *Agaricus bisporus* growing
54 cycles, mites were first detected during the first flush but the initial infestation occurred
55 soon after the application of the casing layer. Mite infestations were initially detected at
56 the rear end of the room, near ventilation holes (Navarro *et al.*, 2004, 2010).

57 Dipteran species are some of the most serious arthropod pest problems affecting
58 the cultivation of *A. bisporus* throughout the world (Sandhu & Bhattal, 1987; Tibbles *et*
59 *al.*, 2005; Jess *et al.*, 2007; Erler *et al.*, 2009; Samshad, 2010). Mushroom yield losses
60 are either directly due to the larvae of mushroom flies feeding on mycelia or
61 mushrooms, or else due to other pests and diseases vectored by the flies (Erler & Polat,
62 2008). There is evidence of the transport of spores of different species of fungi by
63 phorid and sciarid flies (White, 1981; Geels *et al.*, 1988; Shamshad *et al.*, 2009;
64 Cloonan *et al.*, 2016). Similarly, both phorids as well as sciarids have been described as
65 vectors of mites (Clift & Toffolon, 1981; Clift & Larsson, 1987; Keumet *et al.*, 2015).
66 In Spain, the species of mushroom flies commonly found in mushroom farms have been
67 identified as *Megaselia halterata* (Wood) (Diptera: Phoridae) and *Lycoriella auripila*
68 (Winnertz) (Diptera: Sciaridae), with a phorid to sciarid ratio of 4:1 (Navarro *et al.*,
69 2002). The predominance of phorid flies over sciarids in mushroom growing farms has
70 been also described in Turkey (Erler & Polat, 2008) and in the Netherlands (Baars *et al.*,
71 2008). However, most authors that have studied mushroom flies describe sciarids as the
72 major mushroom arthropod pest (Jess *et al.*, 2007; Fletcher & Gaze, 2008; Shamshad,
73 2010; Andreadis *et al.*, 2016; Eui & Seo, 2016). In Spanish mushroom farms the highest
74 number of adult flies (phorids and sciarids) was collected in spring and autumn, while a sharp
75 decrease in numbers was observed in winter (Navarro *et al.*, 2002), a situation
76 also described in the literature by Jess *et al.* (2007) and Erler and Polat (2008).
77 However, contrarily to those described by Jess *et al.* (2007), sciarid flies were not
78 recorded throughout the year, but almost exclusively in spring. On the other hand, *M.
79 halterata* was continuously detected in Spanish mushroom farms during the two years

80 of this previous study (Navarro *et al.*, 2002). A search for immature stages of phorids
81 and sciarids in the substrates before filling of the farms and during the first few days of
82 the crop demonstrated that, contrary to that described in the literature (Jess *et al.*, 2007;
83 Fletcher & Gaze, 2008; Erler *et al.*, 2009), neither the compost and nor casing materials
84 can be considered as sources of contamination by phorids and sciarids in Spanish
85 mushroom farms (Navarro *et al.*, 2002, 2004).

86 Monitoring of the phorid and sciarid populations revealed that adult diptera
87 mainly fly into the mushroom farms during application of the casing layer, although
88 sometimes also during the incubation period. The usual route used by these species of
89 flies to the farm was through ventilation holes (Navarro *et al.*, 2002, 2004).

90 Phoresy is one of the ways that wingless arthropod can disperse by attaching
91 themselves to winged arthropods (Keum *et al.*, 2015). Thus, the dispersal of some
92 mushroom mites might possibly depend on insects, although phoretic host specificity
93 has not been clear in studies of most mushroom mites (Okabe, 2013). The aim of this
94 paper is to increase our knowledge of the role of sciarid and phorid flies as vectors of
95 the myceliophagus mite *Microdispus lambi*. It could also help to establish the way that
96 other mushroom pests infest growing farms. Accurate determination of the sources and
97 timing of infestations may provide an opportunity for an integrated pest management
98 control strategy within mushroom production facilities.

99

100 **Materials and methods**

101 The study was carried out over a period of 18 months at 24 growing farms of Castilla-
102 La Mancha (Spain) from March 1998 to August 1999, four crop cycles per season
103 (Spring1: C1-C4; Summer1: C5-C8; Autumn: C9-C12; Winter: C13-C16; Spring2:
104 C17-C20; Summer2: C21-C24). Each crop was located in a growing room (35x2.5x2
105 m) with a door for access at the front and a ventilation hole at the rear. Each crop was
106 entirely grown in a single room and completed within 70 days.

107 *Survey method*

108 For each farm, a black light lamp (60 cm, Philips TLD 18w/08, Holland), equipped with
109 a plastic sheet treated with a contact insecticide, was installed under the ventilation hole
110 in order to collect flies. Each farm was visited weekly. On each sampling day a
111 maximum of 48 flies was randomly collected in well-plates (IWAKI Glass, Japón) and

112 taken to the laboratory, where flies were identified (species and sex) by binocular
113 microscope and mites that were phoretic on them were also identified and counted. The
114 parameters defined for the study were the percentage of flies of each species carrying
115 *M. lambi* mites, and the average load, defined as the number of *M. lambi* mites
116 transported by each carrier fly (phorid or sciarid fly).

117 The factors studied were: species of fly (sciarid and phorid), sex (male and
118 female), seasonal period in which the crop was grown (spring 98, summer 98, autumn
119 98, winter 99, spring 99, and summer 99), and the stage of the mushroom growing
120 cycle. For this last category, the following growing stages were defined: before sowing
121 (filling: day 0), after incubation (day 20 approx.), after the primordia had formed in the
122 upper surface of the growing unit (induction: day 30 approx.), and after harvesting the
123 first flush (F1: day 41 approx.), second flush (F2: day 48 approx.), third flush (F3: day
124 56 approx.), fourth flush (F4: day 63 approx.) and fifth flush (F5: day 70 approx.).
125

126 *Data Analysis*

127 An analysis of variance (ANOVA) was applied to study the effect of sex in the role of
128 phorids as vectors of mites. Levene's test was used to check the homogeneity of
129 variance, and a natural AsinR transformation was used to account for the heterogeneity
130 of variance observed in the raw data related to the percentage of flies carrying mites.

131 Generalized linear models (GLM) were used to evaluate the effects of season
132 and stage of growing crop factors, and of the season * stage interaction on the
133 percentage of phorid flies carrying mites and on load variables (Gbur *et al.*, 2012). A
134 total of 216 observations were evaluated for each variable - a factorial treatment
135 consisting of 6 seasonal periods and 9 growth stages, with 4 replicates. To test whether
136 continuous variables fitted a normal distribution, data was examined using normal
137 probability plot, standardized skewness and kurtosis, and the Kolmogorov-Smirnov
138 test. A natural AsinR transformation was used to account some observed heterogeneity
139 of variance in the raw data of percentage of flies carrying mites. An SQRT
140 transformation was used to account some observed heterogeneity of variance in the raw
141 data of load. The effect of each particular season and growing stage on variables such as
142 percentage of phorids as vectors or load was tested using indicator variables (or dummy
143 variables) in multiple regression analysis (González-Ochoa *et al.*, 2004). These
144 indicator variables (predictor variables) were the different seasons (k-1 indicator or
145 dummy variables, k =6 levels of seasons), and the growing stages (k-1 indicator or

146 dummy variables, k =9 levels of growing stages), and the interaction of both. The
147 general linear statistic test (F-test, Neter *et al.*, 1996) was used to test some hypotheses
148 about regression coefficients. All the statistical analyses were performed using the
149 Statgraphics Centurion XV program (Statistical Graphics Corp., Princeton, NJ)

150

151 **Results and discussion**

152 *Phoretic role of sciarid and phorid flies*

153 8,927 flies were recovered from twenty-four farms with black light lamps (60 cm,
154 Philips TLD 18w/08, Holland): 7,196 phorids and 1,731 sciarids. In half of the farms
155 (C5, C9, C13, C14, C16, C17, C18, C20, C21, C22, C23 and C24) the presence of *M.*
156 *lambi* carrier sciarids was not detected (Figure 1). In the remaining farms (12 growing
157 cycles), the average percentage of phorids transporting *M. lambi* mites was always
158 higher than the percentage of sciarid carriers. This occurred even in the crop C4, in
159 which the number of examined sciarids was higher than that of phorids (566 sciarids
160 and 152 phorids, data not shown).

161 [Figure 1]

162 With regard to the average load of *M. lambi*, the number of *M. lambi* mites per
163 vector fly was higher for phorids than for sciarids in all of the twelve mushroom crops
164 where vector sciarid flies were detected (Figure 2). On one phorid vector, 41 *M. lambi*
165 mites were detected, whereas, in the case sciarid vectors, the maximum load detected
166 was 9 *M. lambi* mites.

167 [Figure 2]

168 In general terms, 19.6% of the phorids and 4.4% of the sciarid captured carried
169 *M. lambi* mites. The average load calculated was 3.4 *M. lambi* mites on each phorid and
170 1.9 mites on each sciarid vector.

171 The phoretic dispersion of mites on flies has been widely documented (Witch &
172 Snetsinger, 1971; Binns, 1972, 1973; Clift & Toffolon, 1981; Binns, 1982; Keum *et al.*,
173 2015). In the case of this myceliophage mite, Clift and Larsson (1987) demonstrated
174 that the phorid *M. halterata* was clearly a preferred host of *M. lambi* in Australian
175 mushroom crops, although they also found that *L. mali* (Fitch) could act as vectors of
176 this mite. In this current study the low percentage of *L. auripila* acting as vectors

suggests that there may be a lower level of importance of this species of sciarid fly in the phoretic dispersion of *M. lambi*. The lower number of total sciarid detected on farms could explain this fact, since the distribution of phoretic mites could be influenced by the availability of carriers (Glida *et al.*, 2003). However, the adaptive significance of the phoretic association between *M. lambi* and *M. halterata* could be, rather, that mushroom mycelium is the only source of food for both mushroom pests (Clift & Toffolon, 1981). Sciarids have less stringent nutritional requirement, consequently the mite-sciarid relationship may be weaker. However, the difference in the average load carried by phorids and sciarids also coincides with that described by Clift & Larsson (1987), although in their work, the values differ much more (9.1 and 2.9 mites per phorid and sciarid vector, respectively).

The greater presence in mushroom farms of *M. halterata* flies rather than *L. auripila* (ratio 4:1, Navarro *et al.*, 2002), together with a greater percentage of phorids carrying *M. lambi* and with a higher carried average load, lend weight to the importance of studying phorids as vectors in the phoretic dispersion of *M. lambi* in Spanish mushroom farms.

193 *Influence of sex in the role of phorid as vector*

194 Approximately one-third of the examined 7,196 phorids were males. The statistical
195 analysis of the data showed that there was no significant difference between the sexes in
196 the percentage of vector flies (19.4% for males and 19.7% for females; $F_{1,426} = 0.37$; $p =$
197 0.5437; LSD = 3.64; SED: 1.31), meanwhile the average size of the carried load (1.48
198 and 1.86 mites per males and females, respectively) was statistically higher for females
199 ($F_{1,426} = 4.34$; $p = 0.0378$; LSD = 0.36; SED: 0.13).

200 The number of *M. lambi* attached to *M. halterata* males and females (6.4 and
201 8.0, respectively) led Clift and Larsson (1987) to distinguish between non-dispersing
202 males and dispersing females for this phorid species. However, our work, with a much
203 higher number of examined fly, establishes much lower differences between sexes. It could
204 be due to the smaller size of the males rather than active discrimination.

205 *Percentage of phorid flies carrying mites*

206 The GLM developed to assess the effect of seasonal period interacting with that of stage
207 of the growing cycle on the percentage of flies carrying mites establishes that there was a
208 higher signification ($p < 0.0001$, F-test) in the influence of both factors, meanwhile

209 there was no signification for the interaction between the terms of “season” and “stage”
210 ($p > 0.05$, F-test) (Table 1).

211 [Table 1]

212 Multiple regression analysis (Table 2) showed that “winter99” and “spring99”
213 seasons were significant factors for “phorids as vector” variable, decreasing the
214 percentage mainly due to the decreasing populations of phorids and mites inside the
215 farms for winter, that which also influenced the levels of infestations in the next spring.
216 “Induction” stage also reduced the value due to the beginning of the emergence of the
217 first generation of phorids without a hard infestation of mites into the growing
218 substrates. Meanwhile “F3” and “F4” stages was also significant factors, but increasing
219 the value because, at this periods of time, mite population reached a very high level.

220 [Table 2]

221 *Load (number of M. lambi mites transported by each carrier phorid)*
222 Regarding to the load of mite on each vector phorid, the GLM developed to assees the
223 effect of seasonal period interacting with that of stage of the growing cycle on this
224 parameter demonstratedthat there was a higher signification ($p < 0.0001$, F-test) in the
225 influence of only “stage” factor. The p-values for “season” and for the interaction of
226 both factors were not significant ($p > 0.05$, F-test) (Table 1).

227 Multiple regression analysis (Table 2) showed that “filling” stage produces a
228 significant drop in the number of mites carried by each phorid vector, showing a very
229 low level of infestation at the beginning of the cycle. Meanwhile “casing”, “F4” and
230 “F5” stages produced a significant increment, associated clearly to the increasing level
231 of the mite infestation inside the growing substrates.

232

233 *Phoretic relationship between phorid fly and myceliophagus mite*
234 A study of mushroom pests in Spanish mushroom farmshas pointed to a direct
235 relationship between the myceliophagus mite *M. lambi* and the phorid fly *M. halterata*
236 (Navarro *et al.*, 2002, 2004, 2010). The progression of both pests in the growing crop is
237 represented in Figure 3a, while the progression of phoretic parameters during the
238 growth cycle studied in this paper is reflected in Figure 3b. Both figures show the
239 average values obtained for 24 crop cycles that were studied.

240 [Figure 3]

241 During the initial stages of spawn running, mites were not detected inside the
242 growing substrates, and a low number of phorid adults were observed in the farms (Fig
243 5a), since the compost shows low concentrations of mycelium and is not attractive to
244 oviposition (Smith *et al.*, 2006). During this time, less than 10% of phorid flies carried
245 *M. lambi* mites, and with a small number of mites per phorid (Fig 5b). During the casing
246 period a greater number of phorids entered the room due to the high concentration of
247 volatile substances, which would act as attractant (Grove & Blight, 1983; Pfeil &
248 Mumma, 1993; Tibbles *et al.*, 2005) (Fig 5a), and 20% of them carried mites, with an
249 average load of 2 mites per phorid (Fig 5b). Concurrently, oviposition by *M. halterata*
250 occurs, being stimulated by mycelium development (Jess *et al.*, 2017). Mites take
251 advantage of this to leave the vector and migrate to the compost, a substrate rich in food
252 sources.

253 The emergence of the first generation of flies developed inside of the growing
254 medium and those coming from eggs laid during the days of casing (Lewandoski *et al.*,
255 2012), starts with the first flushes (F1-F2) (Fig 5a). At that time, the population of mites
256 in the casing layer, a substrate from which most flies emerge (O'Connor & Keil, 2005),
257 is still very low (Navarro *et al.*, 2010). Therefore, only a small percentage (<10%) of the
258 high number of emergent flies (200-500 adults captured per plate and day) carries mites
259 and the average load is small (approximately 1.5 mites per phorid) (Fig 5b).

260 The third flush coincided with a peak in the population of phorids (almost 800
261 adults captured per plate per day). The incidence of *M. lambi* in the substrates is also
262 clearly greater (150-200 mites/sample, approx.), so a greater percentage of emerging
263 flies transporting mites was detected (15-20%), and with a high average load size (2-3
264 mites per phorid). Finally, in the final stages of the cycle (F4-F5), the presence of mites
265 in the cultivation substrates increases considerably (300-600 mites/sample) (Figure 5a),
266 and they can be observed in large numbers on the casing layer. Thus, not only the flies
267 that come from the casing but also those which continue to enter the farms are more
268 likely to carry mites. For this reason, the percentage of phorid vectors increases
269 considerably (up to 40% approx.), at the same time that an increase in the value of the
270 average load transported is detected, with almost 4 mites on each carrier phorid. After
271 the cycle, these flies, attracted by the volatiles from the growing mycelium of new

272 productive cycles, may colonise nearby crops, favouring the spread of *M. lambi* from
273 infected crops to uninfected farms.

274 Other studies describe this same behaviour in other phoretic species on diptera,
275 in which mites present in a substrate adhere to the diptero-vector at the moment in
276 which the adult emerges from the pupa (Binns, 1973). Bortolon *et al.* (2016) considered
277 that mites apparently attach themselves preferentially to females because after becoming
278 adults, the flies return to the substrate to lay their eggs. This differential phoretic role of
279 the sexes was not evident in this case, since the recorded differences could be due to the
280 smaller size of the male rather than active discrimination.

281 The monitoring of pest populations and the determination of potential infestation
282 sources are important prerequisites for establishing viable control strategies for crop
283 pests (Jess *et al.*, 2017). Sanitation and exclusion practices are vital to integrated pest
284 and disease management (Martín *et al.*, 2016), because such control will not only
285 minimises the risk of introductions from outside the farm but also reduces the chances
286 of pest spreading on the farm from affected to uninfected crops (Shamshad, 2010).
287 Insect pest control must be applied during the early stages of mushroom production to
288 avoid significant damage and consequent yield losses (Jess & Bingham, 2004).
289 Preventing flies from accessing the farm to lay their eggs, at least at the time of fruiting
290 induction, would delay the onset of the first generation of diptera from the growing
291 medium, while *M. lambi* infestation could be delayed and prevented from reaching high
292 levels. Likewise, interruption of the crop after harvesting the third flush would prevent
293 the spread of both pests.

294

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- 425

426 **Table 1.** R^2 , residual deviance, degrees of freedom and p-value for each variable in the
427 GLM. GLM was used for each parameter.

Parameter	R^2	Variable	Res. Des.	d.f.	p-value
Phorids as vector	56.92	Model	33471.6	53	0.0000
		Season	4727.0	5	0.0001
		Stage	19254.0	8	0.0000
		Season*Stage	9490.6	40	0.0504
Load	42.66	Model	29.39	53	0.0001
		Season	2.58	5	0.0712
		Stage	14.49	8	0.0000
		Season*Stage	12.33	40	0.1771

428

429

430 **Table 2.** Regression coefficients for predictor variables of the percentage of phorids as
431 vectors and load

	Phorids as vectors (%)	Load
Constant	18.7	1.8
Winter99	-13.6	
Spring99	-10.4	
Filling		-1.1
Casing		0.7
Induction	-11.1	
F3	18.1	
F4	22.9	0.8
F5	-11.2	1.7
(Autumn98)*(F3)		2.3
(Winter99)*(Casing)		-2.5
(Spring99)*(Induction)	17.1	

432

433

434 Figure legends

435

436 Figure 1. Percentage of flies of both species as vector of *M. lambi*, for each of the 24
437 growing crops.

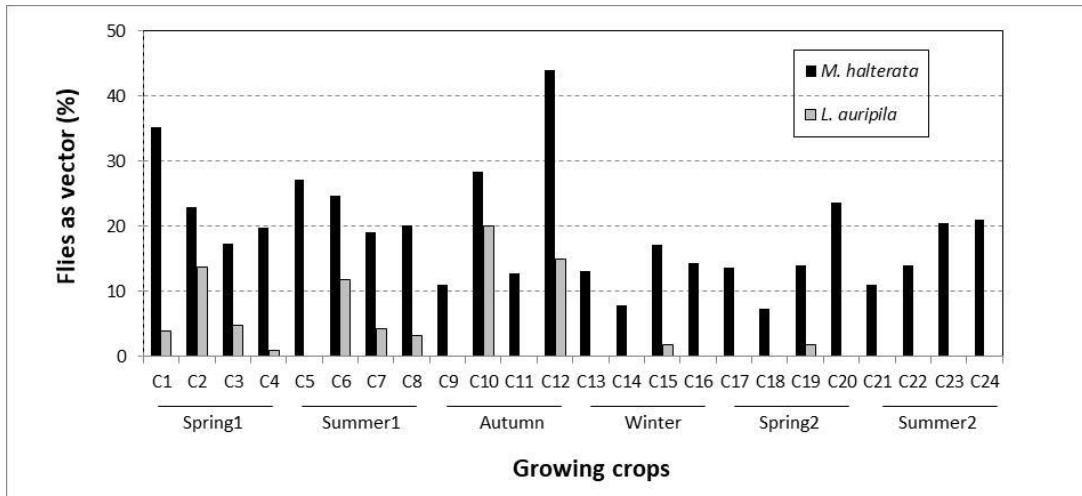
438

439 Figure 2. Load (number of *M. lambi* carried per phorid and sciarid) for each of the 24
440 growing crops.

441

442 Figure 3. a) Progression of the incidence of *M. lambi* (mites/20 g of substrate sample)
443 and *M. halterata* (adults captured per trap and per day) in the different periods of the
444 growth cycle. (b) Progression of the phorid as vector of *M. lambi* mites(%) and the load
445 (mite per vector phorid) in the different periods of the growth cycle.

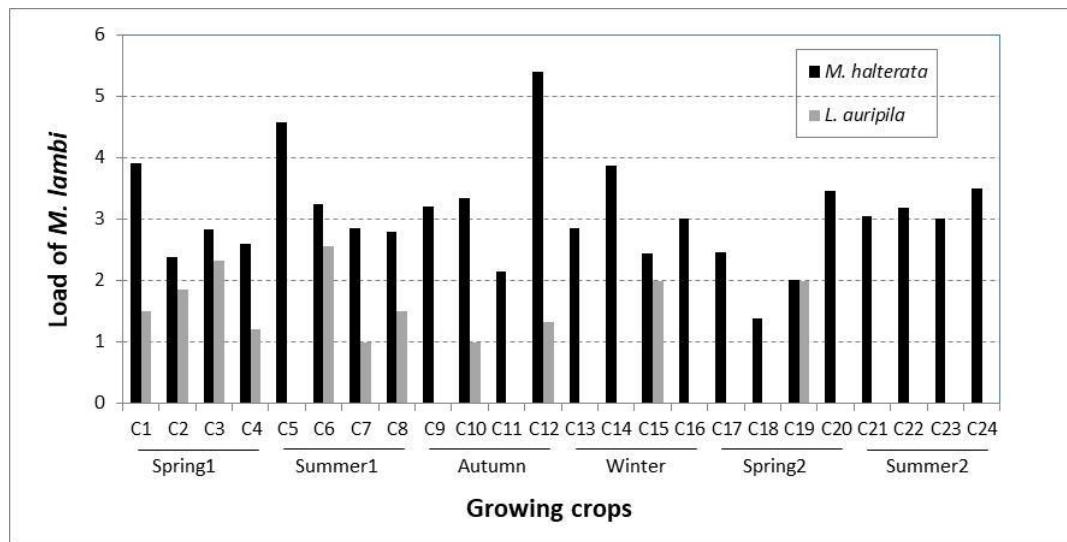
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448 Figure 1

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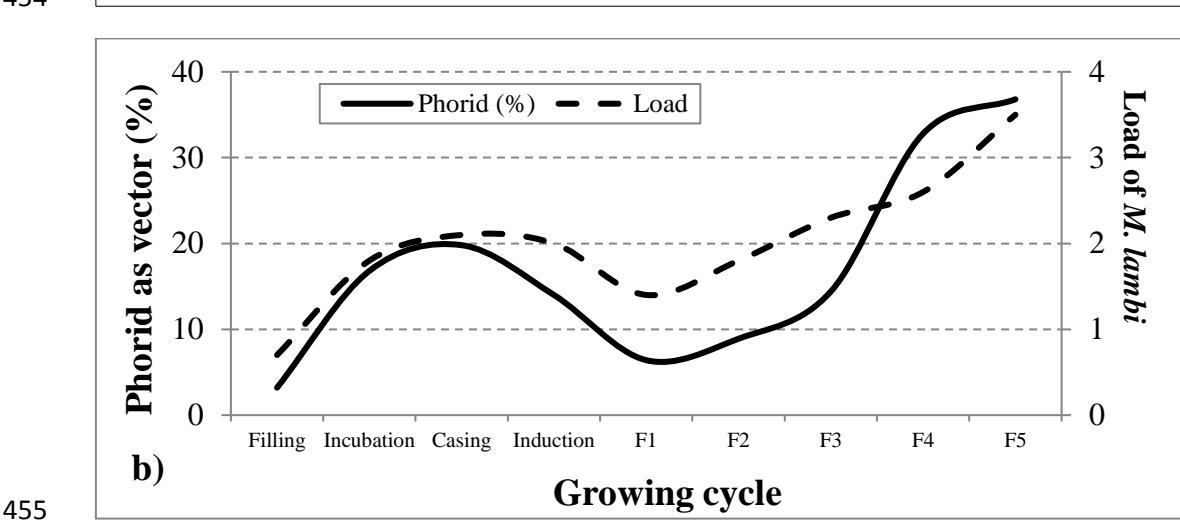
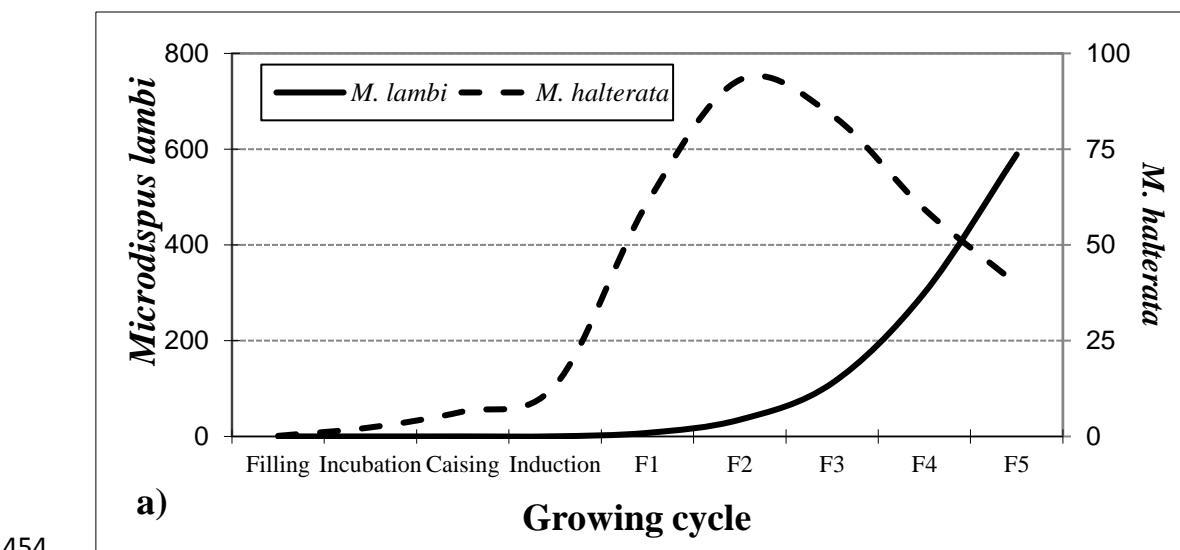


450

451 Figure 2

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453



456 Figure 3