Significant ~~High~~ Mortality of eggs and young larvae of two Pine Processionary Moth species due to the entomopathogenic fungus *Metarhizium brunneum*

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Significant ~~High~~ Mortality of eggs and young larvae of two Pine Processionary Moth species due to the entomopathogenic fungus *Metarhizium brunneum*

Bioassays were conducted to determine the susceptibility of egg masses and young larvae of two pine processionary moth species, *Thaumetopoea pityocampa* and *Thaumetopoea wilkinsoni*, to two strains (ARSEF4556, V275) of the entomopathogenic fungus *Metarhizium brunneum*. Mortality of treated eggs by both strains ranged from 96 to 99% but not all of this was caused by *M. brunneum* since control groups also experienced ~~high~~ egg mortality due to saprophytic fungi. Still, larvae hatched in the laboratory from eggs treated with *M. brunneum* were all killed by this fungus, acquiring *M. brunneum* conidia, whereas larval mortality was 0% in the control groups. Young larvae of both pine processionary moth species were also highly susceptible to ARSEF4556 and V275 with larval mortality ranging between 94 and 100%, eight days post inoculation, with the vast majority of larvae being killed within the first 2-4 days. Larval mortality was dose-dependent. Results were consistent across the two pine processionary moth species, showing that the pathogenicity of *M. brunneum* to both eggs and young larvae might be promising for biological control of these insect pests. The study also showed that non-target parasitoids of pine processionary moth eggs were also susceptible to *M. brunneum*. Further work is required to understand and reduce the *M. brunneum* effect on non-target insects~~/organisms.~~

Keywords: Pine processionary moth, entomopathogenic fungi, *Metarhizium brunneum*, ovicidal activity, larval mortality, egg masses.

Introduction

Two species of pine processionary moth, the western *Thaumetopoea pityocampa* (Schiff.) and the eastern *Thaumetopoea wilkinsoni* Tams (Lep.: Thaumatopoeidae), are major pests of *Pinus* and *Cedrus* in Europe, North Africa and the Middle East (Battisti et al., 2015). Feeding damage by the larvae results in reduced and stunted tree growth and yield losses of pine nuts. In extreme situations, defoliation leads to host tree death (Jacquet et al., 2012). Peri-urban forests, urban trees and forest edges are especially at high risk of attack by pine processionary moth since they prefer border and isolated trees for oviposition and nest construction (Rossi et al., 2016).

Both *T. pityocampa* and *T. wilkinsoni* are univoltine and have similar life cycles. The short-lived adults emerge from pupae in the soil and mate in the summer. Each mated female moth lays between 70 and 300 eggs in highly conspicuous cylindrically shaped egg masses around pairs of needles at the tips of pine shoots. Each egg mass is 4-5 cm in length and is covered with the scales from the female anal tuft, which mimics the pine shoots. The delicate 1st instar larvae emerge 30-45 days after oviposition. In the spring, mature larvae descend the tree in processions in order to seek out suitable pupation sites in the soil.

The pine processionary moth is also a health risk since the urticating hairs of the larvae trigger severe allergic reactions in people and animals. The hairs contain a proteinaceous toxin, thaumetopoein, which elicits allergic reactions affecting the skin, respiratory system, mouth and eyes (Lamy et al., 1986; Vega et al., 2014). The urticating hairs are only produced by older (3rd-5th) instars, which live gregariously in silken nests (Battisti et al., 2015). The dart-like hairs are produced in large quantities in special abdominal pockets of the larvae and, when discharged, are transported considerable distances by the wind. The hairs may remain active in the environment for several months, when on the surface of tree trunks and soil. During the period when larvae are descending to find pupation sites that they are most dangerous as this is when the larvae are most likely to encounter humans with forest workers, eco-tourists, children and animals being at high risk.

The pine processionary moth control options are very limited. *Bacillus thuringiensis* (bacteria) and synthetic chemical diflubenzuron are the most widely used pesticides in forested areas, but these can give varied results depending upon the climate and larval instar (Battisti et al., 1998; Gatto et al., 2009). The potential exists to reduce oviposition through the use if sex pheromones in mating disruption and mass trapping programmes and through use of non-host volatiles (Halperin, 1985; Chenchouni *et al.*, 2010; Jactel *et al.*, 2011). In urban areas, ~~the same~~

insecticides may be used but these are not approved in many countries due to the risk they pose to human health and the environment. The Eco-trap, which is wrapped around tree trunks, captures larvae as they descend to seek out pupation sites in the ground (Martin, 2015). These traps are environmentally friendly but are costly to deploy on a large scale and do not preclude operator exposure to the urticating hairs. Targeting the eggs or early instar larvae would offer a safer and more efficient strategy particularly for the urban and semi-urban environment.

Entomopathogenic fungi have shown promise for the control of late instar *T. pityocampa* larvae (Er *et al*., 2007; Sevim *et al.*, 2010). As far as we are aware entomopathogenic fungi have not been tested against pine processionary moth egg masses and early larval stages. Pine processionary moth eggs are rarely infected by fungi, even though they are often exposed to a wide range of fungi inoculum. Deliberate exposure of mite (Shi and Feng, 2004), and insect eggs (Maniania, 1991; Samuels *et al.*, 2002; Ekesi *et al*., 2002; Lacey *et al*., 1999; Marannino *et al*., 2006) to entomopathogenic fungi inoculum such as *Metarhizium anisopliae* (Metschn.) Sorokin, *Beauveria bassiana* (Bals.-Criv.) Vuill. or *Isaria fumosorosea* Wize has been shown to significantly reduce egg viability. Targeting eggs of arthropod pest species with entomopathogenic fungi has several benefits. To suppress pest populations it is vital to control all pest developmental stages, being thus important to identify fungal strains that kill all stages including the egg stage of the pest. Where eggs are laid in clusters then destruction of the egg mass offers a cost effective method of pest control. Less inoculum and time is required to treat an egg mass than targeting the larvae once they have dispersed. For some pest species, such as *Haematobia irritans* (Linnaeus), entomopathogenic fungi fails to kill the eggs but are efficacious in killing the emergent larvae (Mochi *et al*., 2010).

The aim of the study was to determine the susceptibility of egg masses and young larvae of two different pine processionary moth species, *T. wilkinsoni* and *T. pityocampa*, to two strains of the entomopathogenic fungus *Metarhizium brunneum* Petch.

Materials and Methods

Fungal strains and preparation of inoculum

Trials were carried out in two countries with two different moth species, *T. pityocampa* in Portugal and *T. wilkinsoni* in Turkey to test the impact of species differences. Two strains of *Metarhizium brunneum* V275 (= Met52) and ARSEF4556, were used in this study. Details of their origin are summarised in Table 1. Air dried conidia of both strains were prepared using the methods outlined by Ansari and Butt (2011). Conidial concentration was determined using

a Thoma haemocytometer and the final concentration adjusted to $1x10^7$ conidia/ml in 100 ml 0.03% w/v Aqueous Tween® 80. Conidial viability was determined by inoculating $1x10^5$ conidia/ml spore suspension on Sabouraud dextrose agar (SDA) and evaluating the germination of 100 spores after 24 h of incubation at 25 ± 2ºC. Conidia viability always exceeded 90% for both strains of *M. brunneum*.

Table 1.

Collection of egg masses

Due to the difficulties in rearing and mating under laboratory conditions the pine processionary moth (Berardi et al., 2015; Branco et al., 2017), it was not possible to have egg masses produced in laboratory reason why egg masses were collected from the field. Egg masses of both the eastern (*T. wilkinsoni*) and western (*T. pityocampa*) pine processionary moth were collected between September and October 2015. Over 200 egg masses of the eastern pine processionary moth egg masses were collected from *Pinus brutia* Tenore and *Pinus nigra* J. F. Arnold in the Isparta and Antalya regions of Turkey. Egg masses and samples of *Pinus* were kept in ventilated plastic boxes (15 cm length X 20 cm width) lined with moist tissue to prevent the pine shoots from dehydrating. About 180 eggs masses of the western pine processionary moth were collected from *Pinus pinaster* Aiton and *Pinus pinea* L. trees in the Setubal Peninsula region, ca. 15-30 Km south of Lisbon. The egg masses were placed in separate glass test tubes, and kept at 25 ± 2 °C and 50–70% RH, until required. The egg masses were placed in separate glass test tubes, and kept at 25 ± 2 °C and 50–70% RH, until required. From the pine processionary moth egg masses collected from both countries two hymenopteran egg parasitoids species emerged *Baryscapus servadeii* Dom. and *Ooencyrtus pityocampae* Mercet. Egg masses parasitism rates ranged from 0 to 34% on *T. pityocampa* and from 0 to 6.2% on *T. wilkinsoni*.

Inoculation of egg masses with <u>Metarhizium brunneum</u>

<u>Thaumetopoea wilkinsoni</u> – The eastern pine processionary moth from Turkey

Egg masses consisted of two groups, relatively young eggs collected shortly after they were laid, and egg batches that were 15 days older. For both young and older eggs, two subgroups were generated, one in which eggs were denuded of the female tuft scales from the adult moth, to see if these interfered with the infection process, and another subgroup with intact scales. The scales were gently removed using sterile fine forceps under a dissecting microscope. The

egg masses with and without scales were treated with *M. brunneum* V275 and ARSEF4556. Individual egg masses were immersed for 5 s in 100 ml of conidial suspension of $1x10^7$ conidia/ml, then transferred to 9 cm diameter Petri dishes lined with moist sterile filter paper. There were two controls, the first consisted of 0.03% w/v Aqueous Tween® 80 only and the second consisted of no treatment. There were five replicates per treatment and the experiment was carried out twice for the young egg masses and once for the older egg mass. The Petri dishes were kept at 25 ± 2°C and 60 ± 5% RH with a 16:8 hours Light: Dark photoperiod. Egg masses were checked daily and the number of live larvae, dead fungal infected pine processionary moth larvae, and parasitoid infection recorded. Dead larvae were transferred to clean Petri dishes lined with moist filter paper to encourage external sporulation on mycosed larval cadavers.

Thaumetopoea pityocampa – The western pine processionary moth from Portugal

The egg masses were inoculated with *M. brunneum* V275 and ARSEF4556 as outlined above with slight modifications. The treatments included: (1) Egg masses dipped in liquid suspension of each strain of *M. brunneum* for 5 s using two doses: $1x10^6$ (n=5 replicates) and $1x10^7$ conidia/ml (n=25 replicates); (2) Egg masses "dusted" with dry conidia of each isolate (9 x 10^{10} conidia /g) (n=10, repeated twice); (3) Egg masses without any treatment (control group, n=10); (4) Egg masses dipped in 0.01% aqueous Tween® 80 (n=10). Treated egg masses were kept individually in aerated glass test tubes at 25 ± 0.5 °C and 60% RH. Egg hatching was monitored daily and the number of emergent live and dead larvae and presence of parasitoid infections recorded. The latter were incubated as described earlier to confirm infection by *M. brunneum*.

Additional studies were done to determine the susceptibility of the pine processionary moth egg parasitoids *B. servadeii* and *O. pityocampae* to *M. brunneum*. Briefly, 4-7 adult wasps were released in glass tubes in which egg masses inoculated with $1x10^7$ conidia/ml of V275 or ARSEF4556 had been placed. Parasitoids were provided 50% sucrose as a food source and monitored daily with dead insects being transferred to SDA+ 1% yeast extract to encourage fungal growth.

Susceptibility of early instars to <u>Metarhizium brunneum</u>

Thaumetopoea wilkinsoni

Pine needles were first surface-sterilized in 1% sodium hypochlorite for 2 min, 70% ethanol for 1 min and then washed three times in sterile distilled water. Sterilized pine needles were then dipped for 5 sec in 10 ml of a conidial suspension ($1x10^7$ conidia/ml) of V275 or ARSEF4556, or 0.03% Aqueous Tween only (control). Two treated pine needles were placed with ten 1st instar larvae in Petri dishes (9 cm) lined with moist sterile filter paper. There were five replicates per treatment, and the experiment was repeated on two different days. Larval mortality was checked daily for 10 days. Dead larvae were collected and placed into Petri dishes with moist filter paper to observe any fungal development.

Thaumetopoea pityocampa

Ten 2nd instar larvae of *T. pityocampa* were sprayed with a dose of either $1x10^5$ or $1x10^6$ conidia/ml of V275 or ARSEF4556. The control group was treated with 0.01% aqueous Tween 80 only. There were eleven replicates per treatment for all but larvae treated with ARSEF4556 at 10^6 conidia/ml where there were only six replicates. After treatment with conidia the larvae were gently transferred to transparent plastic cups (12 height x 10 diameter cm) containing freshly collected and sterilized pine needles inserted into moist floral foam to prevent dehydration. The cups were incubated at 23 ± 1°C, 12h light:dark photoperiod and monitored daily. Needles were replaced as needed. After 10 days, the dead and live larvae were counted. Dead larvae were incubated in Petri dishes, as described earlier, to encourage external sporulation of *M. brunneum*.

Statistical analyses

Thaumetopoea wilkinsoni

The proportion of dead eggs, in relation to the initial total number of eggs per egg mass, was compared among treatments using a Generalized Linear Model (GLM) with Binary response data, logit link function and robust model estimation. Results are presented as Wald Chi-Square (Wald Chi2) test and P values. Pairwise comparison among treatments were done with least significance deviance (α=0.05). Survival rate of 1st instar larvae till 8 days following treatments was estimated by a Kaplan-Meier procedure. The equality of survival distributions were tested among pairwise treatments using Log rank (Mantel-Cox) test.

Thaumetopoea pityocampa

Differences among the two treatments with the fungal strains and the control, on the proportion of hatched egg masses was analysed by Chi-square test or Fisher exact probability test. The

proportion of 2nd instar larvae infected by *M. brunneum* was tested by GLM with Binary response data and logit link function and pairwise comparison were done as described above. Mortality of the larvae (larval bioassay) was corrected by natural mortality observed for the control treatment according to Abbott's formula (Abbott, 1925).

All statistical analyses for both moth species were performed using IBM SPSS version 23.0 software (SPSS Inc., Chicago, IL).

Results

Thaumetopoea wilkinsoni

Metarhizium brunneum strains V275 and ARSEF4556 were highly pathogenic to both young and old eggs and emergent 1st instar larvae of *T. wilkinsoni* (Table 2). Larvae started to die within 2 days of inoculation, with the majority being killed 4-5 days post inoculation. Within 4-5 days of treatment, saprophytic fungi developed on some egg masses (with or without covering of adult moth scales) often leading to little or no eclosion.

Larvae emerged from young egg masses 15-20 days post treatment. The hatch rate in controls was 17-28% but zero if saprophytic fungi were present. The egg mortality for V275 and 4556 was 100% in all but six egg masses, whereas the average egg mortality ranged between 96-99% (Table 2). All larvae that hatched from eggs in the control groups survived, whereas there was 100% mortality in the *M. brunneum* treated group with all cadavers becoming mycosed (Table 2), however very few larvae emerged form treated eggs. The interaction between scale cover and treatment was not significant (Wald $Chi^2 = 0.897$, df =3, p=0.826) nor the presence or absence of scale cover (Wald $Chi^2 = 0.137$, df =1, p=0.712). There were significant differences in the proportion of egg mortalities among treatments (Wald $Chi^2 = 23.387$, df =3, $p<0.001$). Pairwise comparisons showed that all fungi treated groups had significantly higher mortality than control groups (Table 2). Of the entomopathogenic fungi treated groups, the strain V275 caused less mortality than the strain ARSEF4556, still in all cases mortality was above 96% and differences were not significant (Table 2).

Table 2.

Larvae emerged from older egg masses 4-5 days post-treatment, much earlier than from younger egg masses. Half of the older egg masses from control groups became contaminated

with saprophytic fungi with zero eclosion whereas the other half that escaped saprophytic fungi infection (with or without scales) hatched with all live larvae surviving during the observation 10 day period (Table 3). The hatch rate from egg masses (with and without scales) exposed to V275 and 4556 ranged from 0 to 96.2% with all emergent larvae being killed by *M. brunneum* and becoming mycosed (Table 3).

Table 3.

For the older egg masses, the scale cover was not significant (Wald Chi^2 = 0.172, df =1, p=0.678), nor the treatment (Wald Chi^2 = 3.458, df =3, p=0.326) or the interaction term (Wald Chi^2 = 1. 728, df =3, p=0.631). The average mortality varied across treatments, and ranged from 71% to 87% (Table 3).

Parasitoids emerging from egg masses were identified as *Ooencyrtus pityocampae*. Approximately 66 parasitoids emerged from the total of 80 egg masses in the control, and 26 parasitoids emerged from EPF treated young egg masses. All parasitoids that emerged from the *M. brunneum* treated egg masses died, and were susceptible to both *M. brunneum* strains. Only one egg mass from the older control group had parasitoids with only six adults emerging.

Thaumetopoea pityocampa

Immersion of egg masses resulted in hatch rates of 30% in aqueous Tween, 10% in spore suspension of *M. brunneum* V275, and 10% from immersion in ARSEF4556. The low hatch rate was attributed to saprophytic fungi and *M. brunneum* (Fig. 1). Differences among the three treatments were not significant (Chi^2= 3.060, df=2, p=0.216). Where hatchings were observed, eclosion was reduced to 1-5 larvae per egg mass (number of eggs per egg mass was about 70 to 130). From the entomopathogenic fungi treated groups, all larvae were infected with *M. brunneum*. Mycosed cadavers from the aqueous Tween control group were contaminated by *Aspergillus* sp., *Paecilomyces* sp., *Fusarium* sp. and *Beauveria bassiana*. Egg masses with no treatment (natural control) had 100% eclosion with 62-118 larvae per egg mass. In total, ten *Baryscapus servadeii* and four *O. pityocampae* parasitoids emerged from the control groups, whereas no parasitoids emerged from *M. brunneum* treated groups. For egg masses with parasitoids, mortality due to *M. brunneum* ARSEF4556 of *B. servadeii* and *O. pityocampae* was 55% and 72 %, respectively; whereas for V275 was 78% and 57%, respectively. *M. brunneum* emerged from all dead specimens.

Figure 1.

281 Eclosion from egg masses inoculated with dry conidia was 55% and 50% for ARSEF4556 and
282 V275, respectively. Hatching was 100% (10 out of 10) from untreated egg masses with the
283 proportion of hatched egg masses being significantly higher in the control compared with the
284 two entomopathogenic fungi treated modalities (Fisher Test; p =0.01). Of the newly emerged
285 larvae, the proportion which developed infection by *M. brunneum* during the two following
286 days was 13% ± 1.1 and 5% ± 0.7 for ARSEF4556 and V275 isolates, respectively. Differences
287 between the two isolates were significant (Wald Chi^2 = 42.722, df=1, P<0.001). No larvae from
288 the control group developed entomopathogenic fungal infection.

289 As regards the egg parasitoids, a total of 230 individuals of *B. servadii* and 46 *O. pityocampa*e
290 emerged from the egg masses treated with the dried conidia formulation. The proportion of
291 parasitoids that became infected by *M. brunneum* was 35% ± 5.9 and 66% ± 3.3 for 4556 and
292 V275 isolates, respectively. Differences between isolates in infection of parasitoids was
293 significant (Chi^2 = 20.957, P<0.001).

294 ***Susceptibility of early instar pine processionary moth to <u>M. brunneum</u>***

295 Both strains of *M. brunneum* caused 100% mortality of 1st instar larvae of the eastern pine
296 processionary moth 8 days post-inoculation whereas there was 0% mortality in the control
297 groups (untreated and aqueous Tween). Control groups differed significantly from both 4556
298 and V275 strain of *M. brunneum*, Chi^2= 225.048, df=1, p<0.001 and Chi^2= 230.054, df=1,
299 p<0.001, respectively. There were no statistical differences between the V275 and ARSEF4556
300 (Chi^2= 1.035, df=1, p=0.309), which had similar survival curves (Fig. 2). All dead larvae
301 showed successfully entomopathogenic fungus development and sporulation.

302 Total mortality of the 2nd instar larvae of the western pine processionary moth depended on the
303 dose and strain of *M. brunneum*, ranging from 84.5% ± 6.1, for *M. brunneum* strain V275 at 1
304 x10^5 conidia/ml, to 100% ± 0.0, for *M. brunneum* strain ARSEF4556 at 1 x 10^6 conidia/ml; the
305 corrected mortality was only slightly lower (Table 3). Between 89 and 96% of the dead larvae
306 became mycosed with *M. brunneum* (Table 3, Fig. 3). Control mortality was very low (Table
307 3).

308 Figure 2.

309 Table 4.

310 Figure 3.

Discussion

Targeting pine processionary moth egg masses before larval dispersal offers an effective way of controlling pine processionary moth. Once dispersed, far more inoculum needs to be applied which increases costs. This study shows that the eggs and newly emerged larvae of both species of pine processionary moth *T. pityocampa* and *T. wilkinsoni,* are highly susceptible to *M. brunneum* ARSEF4556 and V275, as it caused 100% mortality within a relatively short time of 7-10 days. Both ARSEF4556 and V275 have also been shown to be highly virulent strains for other pest species including thrips (Ansari et al., 2007), midges (Ansari et al., 2010) and mosquitoes (Greenfield et al., 2015). The pine processionary moth larvae appear to be naturally susceptible to entomopathogens (Vargas-Osuna et al. 1994; Er et al., 2007; Sevim et al., 2010; Draganova et al., 2013) with *B. bassiana* often reported as infecting larvae and pupae with variable results (Battisti et al., 2000; Sevim et al., 2010). This current study, is the first to test *M. brunneum* pathogenicity on pine processionary moth and to show that both pine processionary moth eggs and young larvae are susceptible to this entomopathogenic fungi.

The present study also demonstrated that both wet (aqueous Tween suspension) and dry conidia reduce egg hatch rates significantly enough to warrant investigation at the field level. What is perplexing is the high mortality of the egg masses of control groups treated with aqueous Tween 80 or kept in high humidity. Mortality was particularly high when the eggs masses were inoculated using the dipping method. One explanation is that hydration of saprophytic (*e.g. Aspergillus, Fusarium*) and entomopathogenic (*e.g. Beauveria*) fungi at the egg mass surface triggered spore germination and saprophytic fungi growth with egg death caused by the activity of hydrolytic enzymes, toxic metabolites and mechanical damage; as reported for other arthropods (Fernandes et al., 2003; Zhang et al., 2014; Santos et al., 2009). Rodrigues et al., (2015) reported that high humidity was essential for development of *M. anisopliae* on eggs of *Triatoma infestans* (Klug), and under drier conditions the eggs completely resisted infection. Similarly, Lord (2009) reported egg hatch was higher for stored product beetles exposed to *B. bassiana* at high humidity than low. In this present study, significant egg mortality (45-50%) was observed with dry conidia kept in aerated conditions with low humidity, presumably this is due to preformed pathogenicity determinants such as the protease Pr1 (Butt et al., 2016). Indeed, entomopathogenic fungi conidia have been shown to be active even before germination with most activity being attributed to the spore bound enzymes such lipases and proteases (Butt *et al*., 2013; Santi et al., 2010).

Mortality of the treated eggs depend on the fungal strain, dose, and formulation and environmental factors especially temperature and humidity (Anand and Tiwary, 2009; Fernandes et al., 2003; Maniania, 1991; Sabbour, 2015; Santos et al., 2009). In our study, pine processionary moth egg mortality was also affected by the age of the egg mass but not by the removal of the scales from the adult moth that usually cover the egg mass. In contrast, removal of scales from the egg masses of another lepidopteran *Spodoptera litura* Fab. resulted in 100% mortality when inoculated with either saprophytic (*e.g. Aspergillus, Fusarium*) or entomopathogenic (*e.g. M. anisopliae*) fungi but mortality was significantly lower if scales were left intact (Anand and Tiwari, 2009). Our study shows that young eggs are more susceptible to entomopathogenic fungi ovicidal activity than older egg masses. These observations are in accord with those reported for *Chilo partellus* Swinhoe, *T. infestans* and *Nilaparvata lugens* (Stål) where susceptibility of the eggs to entomopathogenic fungi decreased with egg age (Maniania, 1991; Rodrigues et al., 2015; Li et al., 2013). Well-developed embryos inside the eggs presumably do not provide the right cues to encourage fungal infection. It has also been shown that embryos have the ability to respond to microbes with immune responses (Gorman et al., 2004). Such responses may be partially responsible for the difficulty of infecting eggs, but the barrier presented by the egg chorion is the primary and probably most important barrier to infection (Campbell et al., 2016).

Entomopathogenic fungi application to egg masses appears to be a viable strategy to reduce the impact of pine processionary moth because the *M. brunneum* pathogen reduced egg viability and also infected any surviving, emergent neonate larvae. In the current study, we observed 100% mortality of emergent or young pine processionary moth larvae treated with *M. brunneum* independently of the age of the egg mass, whereas mortality in the control group was zero. Other studies (eg Mochi et al., 2010; Lord, 2009) have observed that independently of the susceptibility of the eggs to entomopathogenic fungi infection, emergent larvae are highly susceptible to infection. The larvae acquire conidia from the surface of eggs and immediate surroundings (Mochi et al., 2010; Lord, 2009). Since pine processionary moth live gregariously and larval survival depends on group activity, even if some larvae survived infection they could not survive alone. Larvae from different nests on the same tree tend to merge to produce larger colonies (Branco et al., 2008), which could facilitate horizontal transmission of inoculum between infected and healthy larvae from different egg masses. In practical terms, since egg laying is distributed over more than a one month period, and different stages are found at the same date on individual trees, in these insect species (Battisti et al,

2015), a treatment targeting only the larval stage would be less effective than one that would target both egg and larval stages at the same time.

The pine processionary moth egg parasitoids, *B. servadeii* and *O. pityocampae* also acquired conidia on emergence from the egg and became infected with *M. brunneum* V275 and ARSEF4556. Still, these results were obtained in laboratory conditions in which adult parasitoids were confined for several days with the treated egg masses, which is not the case in natural conditions. The susceptibility of parasitoids to entomopathogenic fungi appears to depend upon the fungal strain, parasitoid, and parasitoid host (Husberg and Hokkanen, 2001; Nielsen et al., 2005; Hansen and Steenberg, 2007). ~~Most often, entomopathogenic fungi work in concert with parasitoids to suppress pest populations (Hansen and Steenberg, 2007).~~ Some predators and parasitoids avoid hosts infected with entomopathogenic fungi (Butt et al., 2016). Rannback et al (2015) observed that *Trybliographa rapae* Westwood, a larval parasitoid of the cabbage root fly, *Delia radicum* (L.), laid more eggs in healthy than entomopathogenic fungi infected larvae. Parasitoids also vector entomopathogenic fungi carrying inoculum from infected to uninfected hosts (Oreste et al., 2016). Although entomopathogenic fungi like *M. brunneum* have been reported infecting non-target arthropods, most often the impact is either low or can be mitigated. For example, *M. brunneum* will kill predatory mites but the target pest species of spider mite (*Tetranychus urticae*) is even more susceptible allowing the two biological control agents to be used together with interactions being synergistic (Dogan et al., 2017).

In conclusion, this study demonstrates that *M. brunneum* strains have the potential to control early stages of pine processionary moth and thus stop them causing harm to trees and humans. Both wet and dry formulations of this fungus are effective ovicides and larvicides. The advantages of dry conidia formulations is that they are more amenable; they enable control of the pest in areas where it is difficult to access water to suspend the spores. A good microbial biological control agent must be able to reproduce on its host and it will be more effective if it allowed for horizontal transfer of inoculum among individuals to induce epizootics. The strains of *M. brunneum* tested here are clearly able to infect and sporulate on the sister species of pine processionary moth larvae from both Turkey and Portugal. Additional research is needed to determine the effectiveness of *M. brunneum* in the field. Further studies are recommended to carry out experiments with this fungus in nature. Application methods and long-term effects of the fungus in the forest ecosystem should also be investigated.

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Figure 1. Undetached egg mass of *T. wilkinsoni* (left) and detached egg mass of *T. pityocampa* colonised by saprophytic fungus (right).

Figure 2. Kaplan-Meier survival probability estimates (± SE) of 1st instar larvae of *T. wilkinsoni* at different time periods (up to 8 days) after application of *M. brunneum* strains V275 and 4556. Control groups had 100% survival.

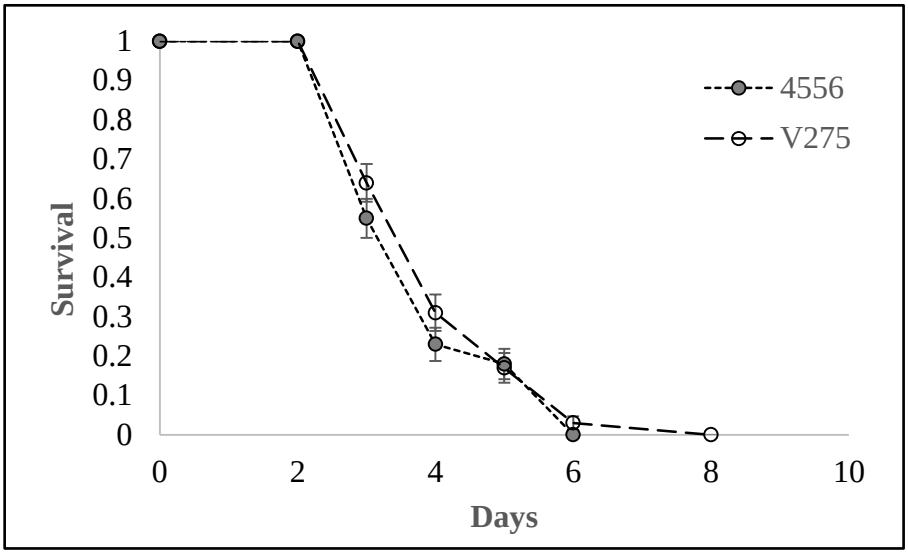

Figure 3. Larvae of healthy newly emerged *T. wilkinsoni* (left). Larvae of *T. pityocampa* infected with *M. brunneum*, most larvae are covered with white mycelium (Middle). Details of mycosed cadaver covered with green conidia of *M. brunneum* (Right).

Table 1. Origin of entomopathogenic fungi tested against the pine processionary moth larvae

Fungal isolate	**Original host**	**Geographic origin**
M. brunneum V275 (= Met52, BIPESCO 5)	*Cydia pomonella* (Lepidoptera: Tortricidae)	Austria
M. brunneum ARSEF[1] 4556	*Boophilus* spec. (Acari: Ixodidae)	USA

[1]The USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF)

Table 2. Mean percentage mortality of *T. wilkinsoni* young eggs and emergent 1st instar larvae following inoculation of egg masses with *M. brunneum* strains V275 and ARSEF4556 using a dose of 1 x 10^7 conidia/ml. Mortality was recorded over a 7 day period following larvae emergence. Letters indicate pairwise comparison among treatments, with least significance deviance (α=0.05), following GLM analysis with Binary response data

Treatment of pine processionary moth egg masses	**% Egg mortality** (±SE)	**% Mortality of emergent larvae** (±SE)
Eggs with scales *M. brunneum* (V275)	96.7±3.1 a	100±0
Eggs without scales *M. brunneum* (V275	96.1±3.5 a	100±0
Eggs with scales *M. brunneum* (4556)	97.8±2.2 a	100±0
Eggs without scales *M. brunneum* (4556)	99.1±0.9 a	100±0
Eggs with scales (Tween 80 control)	83.0±5.0 b	0±0
Eggs without scales (Tween 80 control)	79.5±6.2 b	0±0
Eggs with scales (untreated control)	81.6±6.1 b	0±0
Eggs without scales (untreated control)	72.0±10.3 b	0±0

Table 3. Mean percentage mortality of *T. wilkinsoni* older eggs and emergent 1st instar larvae following inoculation of egg masses with *M. brunneum* strains V275 and ARSEF4556 at 1 x 10^7 conidia/ml. Mortality was monitored over a 10 day period with the larvae being killed 4 (±2) days post inoculation. Letters indicate pairwise comparison among treatments, with least significance deviance (α=0.05), following GLM analysis with Binary response data

Treatment of old eggs	**% egg mortality** (±SE)	**% mortality of emergent larvae** (±SE)
M. brunneum (V275)	76.5 ± 12.2 a	100±0
M. brunneum (4556)	87.3 ± 6.0 a	100±0
Control (Tween 80)	70.6 ± 10.7 a	0±0
Control (Natural - untreated)	72.2 ±11.6 a	0±0

Table 4. Percentage mortality of 2nd instar larvae (% ± SE) of *T. pityocampa* 10 days after application of *M. brunneum* strains. Mortality data were corrected by using Abbott's formula. Dead larvae were incubated in Petri dishes for fungi sporulation and identification. Proportion of dead larvae with confirmed *M. brunneum* sporulation is provided in the last column. Different letters represent statistically significant differences amongst mortality (α=0.05).

Treatment	% Total Mortality	Corrected % Mortality	% of dead larvae with confirmed *M. brunneum* conidia
Untreated (Natural) control	2.7 ± 1.5 a		0 ± 0
Tween control	9.6 ± 2.8 b		0 ± 0
M. brunneum V275 10^5	84.5 ± 6.1 c	82.8 ± 6.7 a	89 ± 3.3 a
M. brunneum V275 10^6	98.3 ± 1.0 de	98.2 ± 1.2 b	96 ± 1.7 a
M. brunneum 4556 10^5	95.5 ± 3.1 d	94.5 ± 3.5 ab	91 ± 2.8 a
M. brunneum 4556 10^6	100 ± 0 e	99.3 ± 0 b	90 ± 4.0 a