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ORIGINAL PAPER

# Potential shortfalls of using entomopathogenic fungi for boosting the sterile insect technique to control the oriental fruit fly, *Bactrocera dorsalis*

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## Abstract

Entomovectoring relies on the dissemination of biocides by insects to control plant pests and diseases. Current research aims at coupling entomovectoring with the Sterile Insect Technique (SIT). Such boosted-SIT is a promising technique to control the Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera, Tephritidae), an invasive pest that dooms African and Asian fruit-producers and is invading Europe. Here, we investigated empirically the potential of boosting the SIT using spores of the entomopathogenic fungus, *Metarhizium anisopliae*. Laboratory bioassay confirmed the transmission potential of the fungus from inoculated males to males and females, with subsequent reductions in survival and fecundity. Inoculation, like sterility, nonetheless reduced male mating success. Semi-field tests (i.e., large outdoor cages) revealed greater costs of fungal inoculation on male competitivity than observed in the laboratory. Combined with effects of inoculation on male survival, these costs led to a lower reduction in female reproduction in the presence of inoculated sterile males compared to plain sterile males. As tested here, boosting the SIT with *M. anisoplae* spores to control *B. dorsalis* could reduce its efficacy. The encouraging transmission patterns, however, suggest that technical improvements may render the boosted-SIT effective in some, if not all, ecological contexts.

**Keywords** Entomovectoring · SIT · Attract and kill · Mating success · Horizontal transmission · *Metarhizium anisopliae*

## Introduction

Advances toward environmentally sound crop protection require the development of science-based innovative biocontrol technologies. To this end, research efforts can focus on the selection of new biocides but also more effective application methods. The entomovector technology relies on the use of insects to transport and disseminate biocides for the control of crop pests and plant diseases (Mommaerts and Smagghe 2011). Compared to spray applications, a small quantity of biocide is needed to reduce damages caused by pests or diseases (Hokkanen et al. 2015), thereby decreasing production costs and non-target effects. Initially, this technology has been designed using pollinators as vectors in the context of biological control of plant diseases (e.g., Peng et al. 1992; Butt et al. 1998). An innovative version of this technique relies on the use of insect pests to disseminate biocides within a target pest population of the same species (Flores et al. 2013; Bouyer and Lefrançois 2014; Bouyer et al. 2016). In this line, wild males or mass-reared sterile

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males used for SIT could be effective vectors (Scholte et al. 2004; Toledo et al. 2007, 2017), a strategy referred to "the attract and kill technic" for the first, and as boosted-SIT for the last. Entomovectoring of fungal entomopathonenic spores by sterile males has shown some potential in coffee-growing areas in Guatemala where sterile males of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera, Tephritidae), inoculated with *Beauveria bassiana,* transmitted spores to 44% of the captured wild flies (Flores et al. 2013).

The Oriental fruit fly, *Bactrocera dorsalis* (Diptera, Tephritidae), is the most economically important invasive fruit fly species in the tropics (Vayssières et al. 2014). It has caused severe fruit production losses in mango and citrus orchards since its introduction into the African continent in 2003 (Ekesi et al. 2006). This quarantine insect can also lead to critical impacts to export markets (Vayssières et al. 2014). The entomovector technology could be effective to control fruit flies as far as the lethal effect of the biocide allows vectors to disseminate it into the target pest population before dying (Soper 1978, Skadeland 1981). Entomopathogenic fungi are relevant candidates as biocide due to their mode of transmission and virulence (Hywel-Jones & Gillespie 1990; Dimbi et al 2013). They hold greater potential as biocontrol agents for adult fruit flies than bacteria or viruses which must be ingested to be effective (Lacey et al. 1994). Unlike, fungus spores adhere to the insect cuticle, germinate and drill the cuticle for mycelium invasion the insect body (Shah and Pell 2003). In addition, they are transmitted during social interaction (Hansen and Henrik 2019). *Metarhizium anisopliae* (Metschnikoff.) is one of the most widely used entomopathogenic fungus in pest control, particularly fruit control (Zimmermann 2007; Dimbi et al. 2013). Ekesi et al. (2002, 2011) showed the efficacy of the entomopathogenic fungus, *M. anisopliae* in controlling *B. dorsalis* fruit flies. In addition, significant horizontal transmission of fungal spores between flies was observed in *C. capitata* (Quesada-Moraga et al. 2008; Dimbi et al. 2013). Wild and sterile mass-reared males are vector candidates since (i) autodissemination techniques ("attract and kill") can be designed using male attractants, herein methyl-eugenol (precursor of the male sexual pheromone), and (ii) SIT has already been implemented for *B. dorsalis* (Orankanok et al. 2007; Shelly et al. 2010; Chailleux et al. 2021). Chailleux et al. (2023) provide the first steps by testing the fungus on adult flies, both on the fertile Senegalese population and sterile flies from the IAEA. They found high mortality levels with variable delay to death depending on the temperature, which is promising for entomovectoring uses in this biological model.

The use of wild or sterile males coated with fungal spores is expected to trigger an epizootic in the target population by contamination of conspecifics (males and females), particularly through interactions during lek and mating behaviors (Gaugler et al. 2012). Emlen and Oring (1977) defined lek as a "communal display area where males congregate for the sole purpose of attracting and courting females and to which females come for mating", which is a common behavior in Tephritidae. Such an approach has been investigated (Faye et al. 2021; Maniania and Ekesi 2013; Toledo et al. 2017) but knowledge of the importance and conditions of spore transfer is still lacking to improve the technology. Horizontal transmission of *M. anisopliae* among *B. dorsalis* fruit flies has not been studied to date, as opposed to *Ceratitis* spp. (Dimbi et al. 2013; Quesada-Moraga et al. 2008), but the results are hardly transposable to other fruit fly species which differ in their morphology and mating behavior. Moreover, the cumulative effect of irradiation and spore inoculation on the sexual competitiveness of sterile males compared to wild males (Rull et al. 2012b; Schutze et al. 2015) has not been documented. Similarly, vertical transmission, i.e., transmission from parents to offspring (Benoit and Yoder 2004), is not known. Previous studies have only investigated the effect of fungal inoculation on female fertility (Dimbi et al. 2013). In the present study, the objectives were (i) to check the occurrence of vertical transmission of fungal spores of *M. anisopliae*, and (ii) to evaluate the importance of spore horizontal transmission from males (fertile or sterile ones) to females and competitors and the subsequent effects on survival and reproduction, under laboratory and semi-field cage conditions.

## Materials and methods

### Insects

A wild strain of the oriental fruit fly, *B. dorsalis,* was obtained by collecting and incubating infested mangoes from the Niayes area in Senegal in 2019. Fruit flies were reared in the laboratory in 45 × 45 × 45 cm insect cages (BugDorm, Taiwan) at 27 °C and 70% RH. Field-collected flies (~ 100 individuals/year) were annually added to the lab-reared population. Water, sugar, and yeast extract powder (Alfa Aesar, Kandel, Germany) were provided ad libitum to adult fruit flies, whereas fresh mature bananas were provided as oviposition substrate. After 48 h, bananas were incubated in plastic bins containing sterilized sand. Pupae were then placed in a rearing cage until adult emergence.

Sterile flies were provided by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (Seibersdorf, Austria). The sterilization (80 Gy) and shipping procedure have been described by Chailleux et al. (2021). After pupae reception (batches of approximately 2000 pupae), they were immediately placed in a rearing cage. Emergence rates were about 95 ± 2%. To ensure the use of unmated flies in the experiments, individuals were sorted by sex within three days after emergence. Male and female flies were then

placed in different rearing cages until they were sexually mature (10–14 days).

## Fungal spores

The entomopathogenic fungal strain of *M. anisopliae,* MET69OD, was provided as pure free spore powder by Real IPM (Kenya). Spore viability was checked in the laboratory before each experiment by assessing the germination rate. Spores were suspended in distilled water with one drop of Tween 80 and diluted to 1 $10^{-5}$ g/ml. The solution was applied on SDA medium (Sabouraud Dextrose Agar) at 27 °C for 24 h. Then, the number of germinated spores per 100 was counted under a binocular microscope and considered conformed if above 70%.

## Vertical transmission

### Inoculation of mated females

Virgin fertile females from the laboratory strain (10–14 day-old) were used in this experiment. To ensure the use of mated females, 10 females were mixed with 10 males in 30 × 30 × 30 cm cages, and mating was assessed by visual observation at dusk. Pairs were moved in a new cage during 12 h. Mated females were then inoculated with fungal spores using an inoculation device with cylindrical shape (10 × Ø 6 cm), the inside of which was covered in velvet, containing 0.32 g of spores (spore powder contained 1,888 $10^{10}$ spores. $g^{-1}$ [Chailleux et al. 2023], total number of spores/velvet surface = [0.32 × 1,888 $10^{10}$]/[6 × 3.14 × 10] = 3.21 $10^{7}$ spores per $cm^2$) during 3 min (Dimbi et al. 2013). Females were then placed in transition cages for 3 h to avoid the contamination of test cages due to intense auto-grooming.

### Internal transmission

Five replicates of clean 30 × 30 × 30 cm cages with 10 females each were set up as control (non-inoculated females) and treatment (inoculated females). An artificial oviposition device consisting in a perforated bottle lined with paper soaked in mango juice (26 × 17 cm) was offered to females. Eggs were collected daily for 4 days as most flies died after this time. They were recovered by rinsing the paper with water and filtering with a black cloth. The number of eggs was counted using a magnifying glass (×4). Every day, 30 eggs were randomly sampled from the oviposition device.

Eggs were surface sterilized with a bleach solution at 9° and rinsed with distilled water (Yoder et al. 2008). They were deposited in a banana peel with a brush to monitor hatching at 27 °C for 48 h. Non-hatched eggs were incubated during 7 days in Petri dishes containing a moistened sponge to observe mycelium development. Larvae were placed in small boxes (3 × 3 × 4 cm) containing a 1 $cm^2$ piece of banana. Dead larvae were counted daily. Once dead, they were incubated under the same conditions as eggs to observe mycelium development on their bodies.

Hereafter, fecundity is defined as the number of eggs, while fertility is defined as egg hatching percentage.

### External transmission

The same experimental design was led but recording only the first day of oviposition and with one additional treatment. In addition to the control (non-inoculated females), two treatments were carried out: inoculated females whose eggs were sterilized, and the new treatment, inoculated females whose eggs were not sterilized. In the new treatment, the eggs collected were nor rinsed neither surface sterilized to maintain spores potentially transmitted to eggs through external contacts with the female. Four replicates were led per treatment, and thirty eggs oviposited the first day after contamination were incubated per replicate as described above.

## Horizontal transmission

### Laboratory experiments

- Effect of male inoculation and sterility on mating success

  Males (either sterile of fertile) were inoculated in the morning with spores by spending 3 min in a cylindrical inoculation device (7.5 × Ø 4.5 cm) containing 0.1 g of spores (1.78 $10^{7}$ spores per $cm^2$). They were then placed in 30 × 30 × 30 cm transition until the start of the experiment. Four treatments were set up: (i) uninoculated fertile males (fertile control), (ii) uninoculated sterile males (sterile control), (iii) inoculated fertile males, and (iv) inoculated sterile males. At 4 PM, 10 males of each treatment were transferred to different cages (30 × 30 × 30 cm) containing 10 females. On the same day, the number of matings was recorded at dusk using a red-light lamp. Pairs of mating individuals were transferred to Petri dishes to record the duration of mating. Seven to 11 replicates of each of the four treatments were performed.
- Spore transmission from males to females

  Mated and unmated females from the previous step were individually monitored, in entomological boxes with water, sugar, and yeast extract powder, for 15 days at 27 °C. Mortality of fertile and sterile males was also recorded. Dead flies were incubated in Petri dishes containing moistened sponges at 27 °C for 7 days to determine whether mortality was due to fungal infection. Besides, 10 mated females from cages containing inoculated fertile or sterile males were sampled to evaluate their spore load. The number of spores was assessed

using a Malassez cell with an optical microscope after vortexing the solution of an Eppendorf tube containing the fly immersed into 1 ml of water and a Tween 80 droplet.

- Effect of male inoculation and sterility on mating success in a competitive situation

  Uninoculated fertile males (hereafter called "competitors") and females were grouped with either (i) inoculated fertile males, (ii) inoculated sterile males, or (iii) uninoculated sterile males (hereafter called "test males"). Ratios 10:10:10 (females: competitors: test males) were used and compared to a control with only uninoculated fertile males (20) and females (10). In the early morning, before inoculation, males were marked on the thorax with acrylic paint (Posca markers) with a different color according to their status. They were grouped with females at 4 PM, and matings were recorded at dusk. Ten to 14 replicates were performed.

#### Field cage experiments

Experiments were carried out at the end of the rainy season between September and October and during the dry season between December and March (2019 and 2020). Three competitive situations were designed according to the test male type: (i) inoculated fertile males, (ii) inoculated sterile males, and (iii) uninoculated sterile males. Ratios 20:20:20 (females: competitors: test males) were used. Two control treatments with females with only sterile males, or only fertile males (females: males, 20:40) were also set up. Each treatment was replicated 5 times. Males were inoculated with an inoculation device (10 × Ø 6 cm) containing 0.32 g of spores (3.21 $10^7$ spores per cm2) for 3 min. As previously mentioned for laboratory experiments, they were allowed to groom in transition cages. Test males were then placed in large field cages (200 × 200 × 200 cm) according to the treatment. Cages were made of a metallic structure covered with a net, and a roof made of transparent sheets was protecting the experiment from rain. Four small mango trees (one meter tall) were placed in each cage to provide flies with mating sites (Toledo et al. 2007). Water, sugar, and yeast extract were provided as a food source.

- Effect of male inoculation and sterility on mating success

  As in the laboratory experiment, the males were color-marked according to their status prior to the experiment. The flies were left in the field cages, and mating observations were made at dusk during three successive days.
- Survival of females and competitors

  The flies were left in the field cages after mating observations until the experiment lasted 10 days in total. Every day, dead male and female flies were collected. For treatments with inoculated test males, dead flies were cleaned with alcohol 70% and rinsed with distilled water, and then incubated at 27 °C in Petri dishes containing moistened sponge during 7 days, to determine whether mortality was due to fungal infection.
- Effect of inoculation and sterility on female fecundity and fertility

  Four artificial oviposition sites, as described previously, were introduced in each cage to collect eggs. Eggs were daily recovered. After counting, eggs were incubated in Petri dishes at 27 °C for 48 h to monitor hatching.

### Statistical analyses

All statistical analyses were performed using R software (R Core Team, 2020) version 4.0.5. For internal vertical transmission, a Generalized Estimated Equation (GEE) adapted to repeated data over time (geepack package [Halekoh et al. 2006]) fitted with a Poisson distribution was used to analyze the effect of inoculation and days on female oviposition. A GEE fitted with a binomial distribution was used to analyze the effect of female inoculation and days on egg hatching and on the percentage of dead larvae. For external vertical transmission, Generalized Linear Models (GLMs) with binomial distribution were used to analyze egg hatching and larval mortality, with the treatment as a factor.

For horizontal transmission in the laboratory, GLMs with Poisson distribution were used to analyze the number of spores on inoculated males and receiver females depending on the male types (sterile or fertile). A Cox model (survival package [Terry et al. 2000, Therneau 2022]) plus pairwise comparisons using a Log-Rank tests (survminer package [Kassambara et al. 2021]) were used to analyze female survival depending on their three situations: control (with uninoculated fertile males), mated with inoculated males or unmated but in cages with contaminated males. A Cox model plus pairwise comparison using a Log-Rank tests was also used to analyze the survival of males depending on male type. GLMs with a binomial distribution were used to assess the effect of sterility and inoculation on mating success. GLMs with a gamma distribution were used to analyze the effect of sterility and inoculation on mating duration. Pairwise comparisons between treatments were done using a post hoc test (Multcomp package [Hothorn et al. 2008]) with Tukey tests.

Field cages results were analyzed using GLMMs (Generalized linear mixed model) (lme4 package [Bates et al. 2015]). Analyses were carried out separately to compare control cages hosting only one type of males on one side, and cages testing competitive situations hosting test males and competitors on the other side. Male type of the tested males and days were introduced as a fixed effect and replicate as a random effect. Models analyzing mating success,

mortality, and egg hatching were fitted with a binomial distribution, while the model analyzing the number of oviposited eggs was fitted with a Poisson distribution. Pairwise comparisons between treatments were done using a post hoc test (Multcomp package [Hothorn et al. 2008]) with Tukey tests.

A p value $< 0.05$ was considered statistically significant, and a value of $0.05 < P < 0.1$ was considered marginally significant.

## Results

### Vertical transmission

#### Internal transmission

Although female fecundity was significantly reduced over days (significant treatment*day interaction in the GEE, $\chi^2 = 29.4$, df = 1, $P < 0.001$) due to spore inoculation (GEE, $\chi^2 = 106.6$, df = 1, $P < 0.001$) (Fig. 1), no fungal mycelium was observed on incubated eggs and larvae obtained from inoculated females. In addition, neither egg hatching (GEE, $\chi^2 = 0.48$, df = 1, $P = 0.490$) nor larval survival (GEE, $\chi^2 = 0.16$, df = 1, $P = 0.685$) were affected by female inoculation. The overall percent of eggs that hatched was 42.2% ± 36.6 (mean ± SE), and the overall percent of larvae that died was 30.6% ± 3.8 (mean ± SE).

#### External transmission

No significant effect of the treatment was observed on egg hatching on the first day of oviposition (GLM, $\chi^2 = 5.39$, df = 2, $P = 0.067$) nor on the larvae from those eggs (GLM, $\chi^2 = 0.23$, df = 2, $P = 0.891$). The overall egg hatching percent was 73.3% ± 4.91 (mean ± SE) and the overall percent of larvae that died was 25% ± 3.6 (mean ± SE).

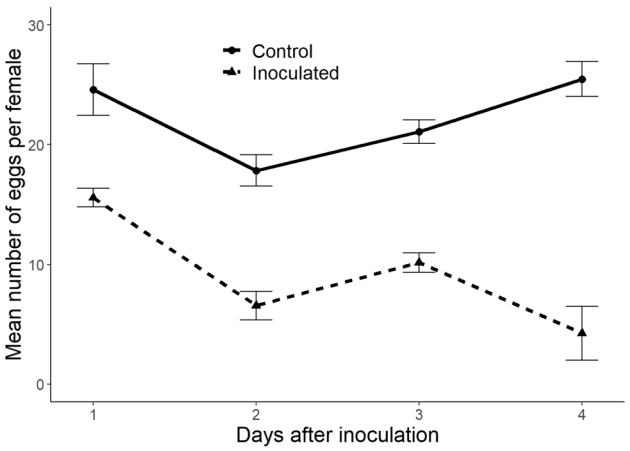


**Fig. 1** Mean number of eggs laid per female per day according to spore inoculation. Bars represent standard errors

### Horizontal transmission

#### Laboratory experiments

- Effect of male inoculation and sterility on mating success

  Sterility and inoculation did not affect the mating success of males on the day of inoculation (GLMs, $\chi^2 = 0.92$, df = 1, $P = 0.337$ and $\chi^2 = 0.19$, df = 1, $P = 0.664$, respectively) (Table 2). The overall mating success on the first day was 46.3% ± 2.6 (mean ± SE). Inoculation did not significantly affect mating duration (GLM, $F_{(1,126)} = 1.81$, $P = 1.181$), but sterility did (GLM, $F_{(1, 127)} = 11.8$, $P < 0.001$). The interaction between sterility and inoculation was marginally significant (GLM, $F_{(1, 125)} = 3.59$, $P = 0.060$). The mating duration of inoculated sterile males was the shortest (Fig. 2).
- Spore transmission from males to females

  Three hours after inoculation, the mean number of spores was significantly higher (mean ± SE: 697 200 ± 268 400), for fertile males than for sterile males (mean ± SE: 414 286 ± 158 663) (GLM, $\chi^2 = 590.76$, df = 1, $P < 0.001$). However, inoculated fertile males and sterile males transferred similar spore numbers to females during mating, 2818 ± 1119 and 3100 ± 737 spores, respectively (GLM, $\chi^2 = 0.14$, df = 1, $P = 0.707$). Inoculation significantly reduced male survival (Cox model, $\chi^2 = 78.7$, df = 1, $P < 0.001$), but survival of inoculated fertile males and inoculated sterile males were not significantly different (pairwise comparison, df = 1, $P = 0.080$)

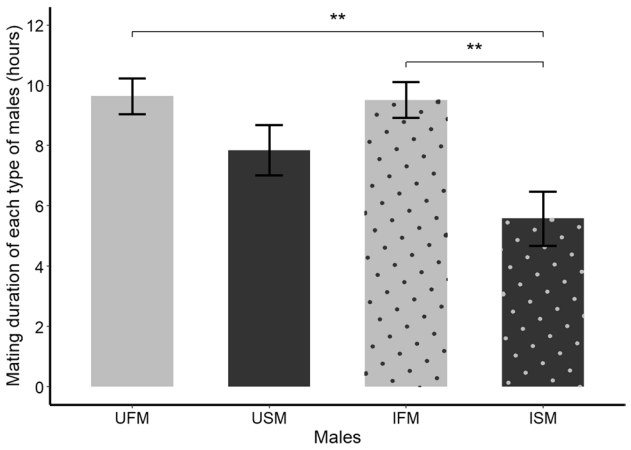


**Fig. 2** Mating duration according to male types. Light gray represents fertile males and dark gray sterile ones. Dotes represent inoculation. UFM: Uninoculated Fertile Males, USM: Uninoculated Sterile Males, IFM: Inoculated Fertile Males, ISM: Inoculated Sterile Males. Bars represent standard errors. "*" indicates statistically significant differences in pairwise comparisons (* < 0.05, ** < 0.01, *** < 0.001)

(Fig. 3A). None of the females in the control treatments died within 15 days. In contrast, when caged with inoculated males, 32.1% of females that mated with inoculated males and 17.4% of non-mated females died within the same period (Fig. 3B). This difference between mated and non-mated females was not statistically significant (pairwise comparison, $P = 0.105$), while difference with control females was (pairwise comparison, mated female-control: $P < 0.001$, none-mated female-control: $P = 0.002$). After incubation, 88.2% of the dead mated females and 100% of the dead non-mated females developed mycelium.

- Effect of male inoculation and sterility on mating success in a competitive situation

  When competing with uninoculated fertile males (i.e., "competitors"), inoculation and sterility significantly reduced male mating success (GLM, $\chi^2 = 18.35$, df = 1, $P < 0.001$, and $\chi^2 = 12.14$, df = 1, $P < 0.001$, respectively). There was no significant interaction between sterility and inoculation (GLM, $\chi^2 = 0.40$, df = 1, $P = 0.524$) (Fig. 4).

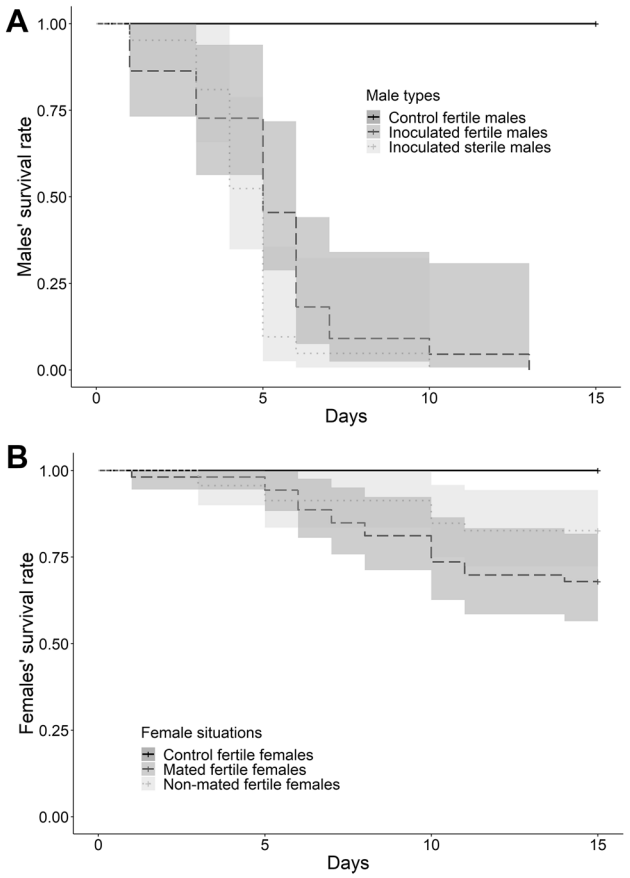


**Fig. 3** Survival of **A** males and **B** females with confidence interval

## Field cage experiments

- Effect of male inoculation and sterility on mating success

  In the control cages, where uninoculated males competed with the same type of males, fertile male mated significantly more than sterile ones (GLMM, $\chi^2 = 10.19$, df = 1, $P = 0.001$), independently of the day (GLMM, $\chi^2 = 2.59$, df = 1, $P = 0.108$). However, mating success tends to decrease across time for both, being the highest the second day for fertile males (mean ± SE, 8.0% ± 0.9) (Fig. 5.A). In a competitive situation, test males' situation significantly affected the mating success but in interaction with days (GLMM, $\chi^2 = 7.61$, df = 2, $P = 0.022$) (Fig. 5.B). The inoculated males showing the lowest mating success the first day (mean ± SE, IFM: 2.5% ± 2.5, ISM: 4.0% ± 1.87). The mating success of sterile males rapidly decreased, either inoculated or not (Fig. 5.B) while the one of the inoculated males slightly increased. The overall highest mating success per day was obtained third time (once on day one and twice on day two) by uninoculated fertile males replicates and was of 17.5%.
- Survival of females and male competitors

  After 10 days, the survival of fertile control males (79.5 ± 4.3 % alive males) was significantly higher than that of sterile control males (52.0 ± 10.3 % alive males) ($\chi^2 = 26.89$, df = 1, $P < 0.001$), but female survival was the same in both sterile and fertile male control ($\chi^2 = 0.110$, df = 1, $P = 0.740$) with 83.0% ± 3.5 alive females. On the contrary, test males significantly affected the survival of their competitors (uninoculated fertile males) (GLMM, $\chi^2 = 20.81$, df=2, $P < 0.001$) and females (GLMM, $\chi^2 = 8.52$, df=2, $P = 0.014$). Inoculation of test males significantly increased the mortality of competitors (pairwise comparison USM-IFM: $P = 0.089$ and USM–ISM: $P = 0.011$) and of females (pairwise comparison USM-IFM: $P = 0.007$ and USM–ISM: $P < 0.001$) (Figure 6). The lowest survival of females after 10 days was found with inoculated sterile males (mean ± SE, 68.0% ± 7.0 alive females). After incubation, almost half of dead females presented mycelium (mean ± SE, 48.7 ± 9.0 % and 49.5 ± 2.9 % with fertile and sterile inoculated test males, respectively). Similarly, the presence of mycelium was detected on almost half of the incubated dead competitor males (48.3 ± 3.9 % and 49.7 ± 3.2 % with fertile and sterile inoculated test males, respectively).
- Effect of inoculation and sterility on female fecundity and fertility

  In the control cages, the number of eggs oviposited was significantly lower (GLMM, $\chi^2 = 666.59$, df = 1, $P < 0.001$) when males were sterile than fertile (mean of the total ± SE, 398.0 ± 74.8 and 807.0 ± 54.3, respectively) (Fig. 7.A). The test males in competitive situa-

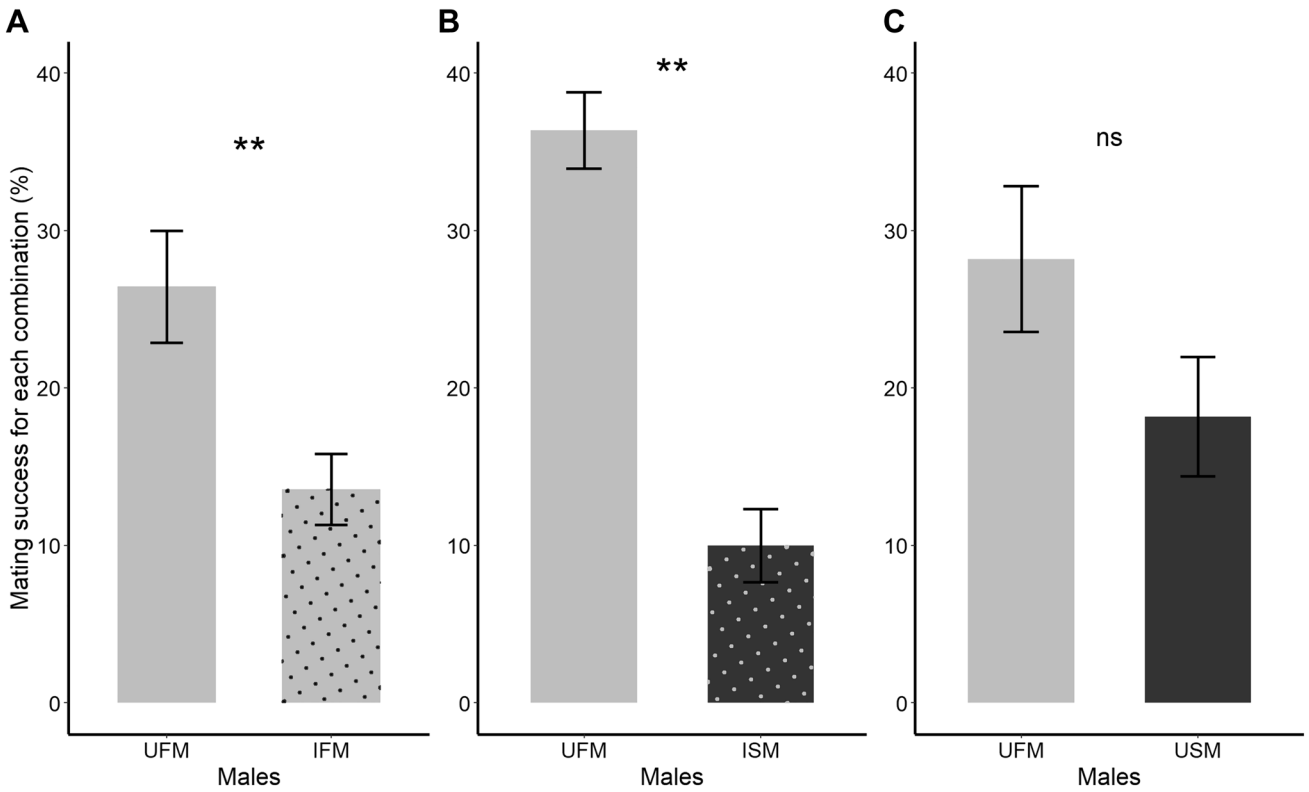


**Fig. 4** Mating success of males (percentage of males that mated) in each male combination. Light gray represents fertile males and dark gray sterile ones. Dotes represent inoculation. **A** Competitors (UFM) + Inoculated Fertile Males (IFM), **B** competitors (UFM) + Inoculated Sterile Males (IFM), and **C** competitors (UFM) + Uninoculated Sterile Males (USM). Bars represent standard errors. "*" indicates statistically significant differences with binomial GLMs (* < 0.05, ** < 0.01, *** < 0.001, ns: not significant)

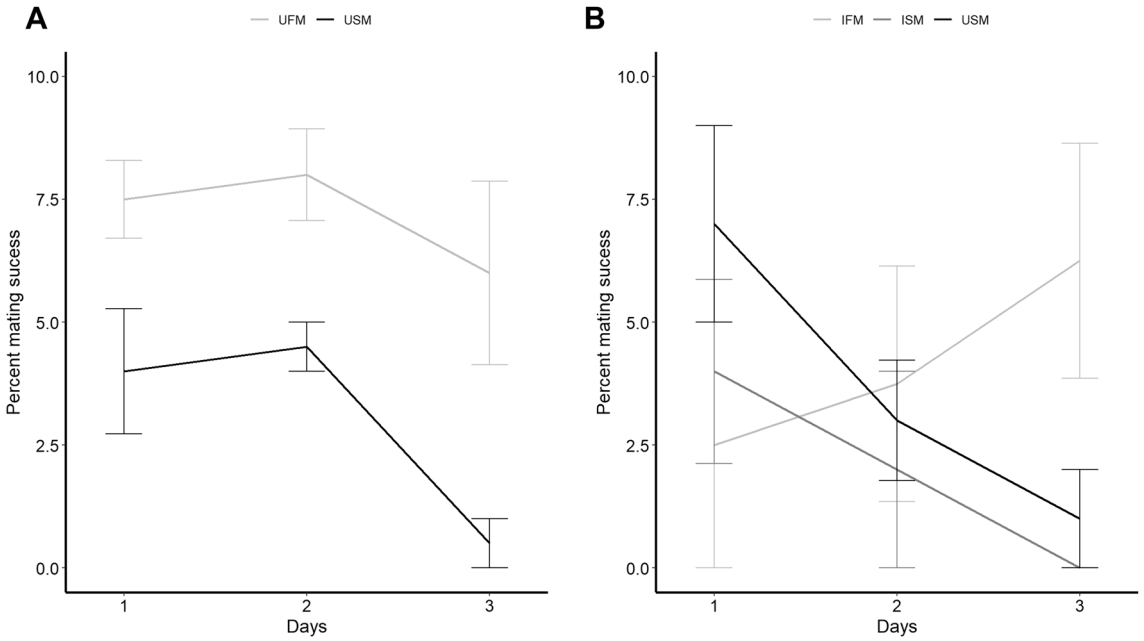


**Fig. 5** Mating success of males (percentage of males that mated) under field cage conditions in **A** control cages, and **B** when competitors (Uninoculated Fertile Males UFM) were placed with test males: IFM (Inoculated Fertile Males), ISM (Inoculated Sterile Males), and USM, (Uninoculated Sterile Males). Bars represent standard errors

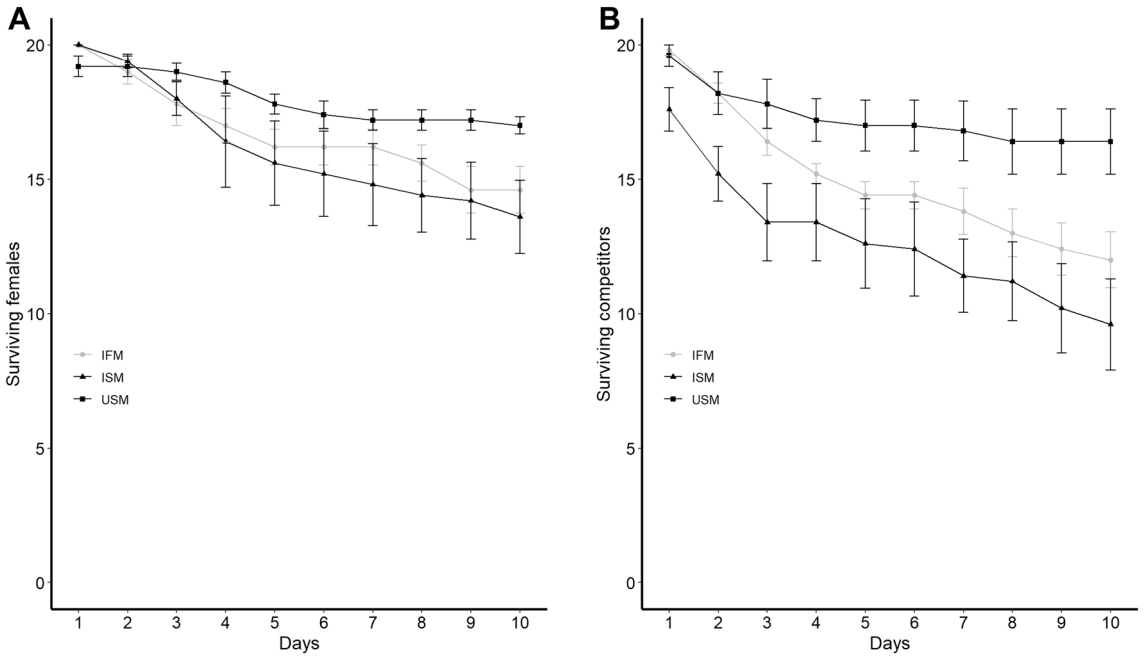


**Fig. 6** Survival of **A** females and **B** competitors according to the status of test males, IFM (inoculated fertile males), ISM (inoculated sterile males), and USM, (uninoculated sterile males)

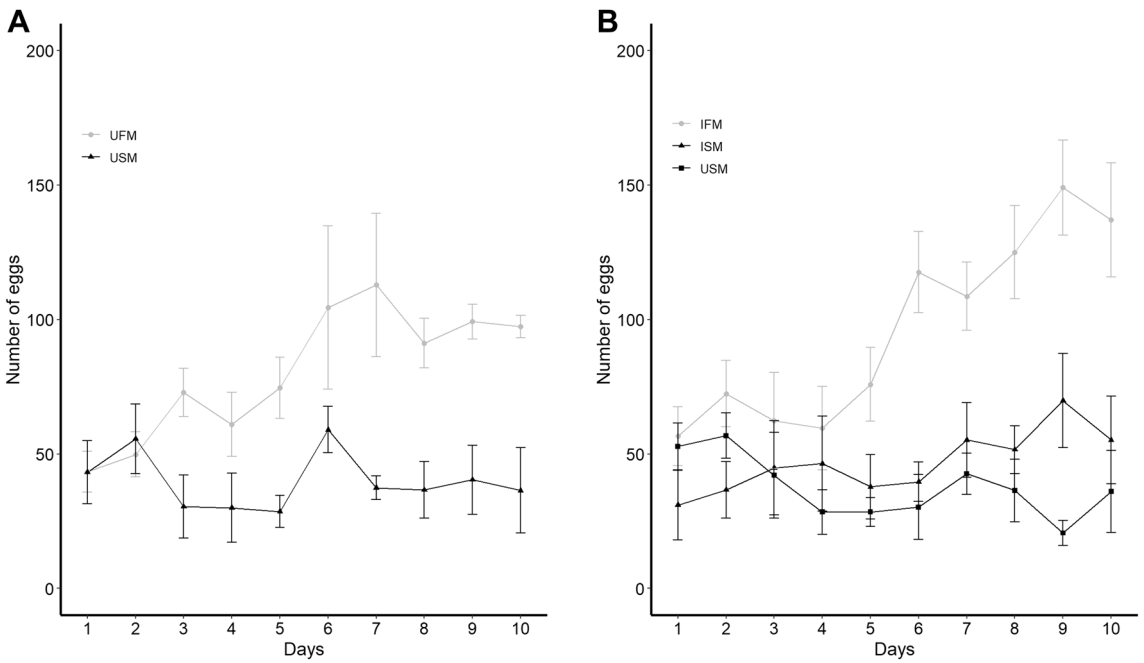


**Fig. 7** Mean (±SE) number of eggs per cages per day in **A** control cages (UFM: Uninoculated Fertile Males and USM: Uninoculated Sterile Males), and **B** competitive situation, according to test males, IFM (Inoculated Fertile Males), ISM (Inoculated Sterile Males), and USM (Uninoculated Sterile Males). Bars represent standard errors

tion significantly affected the number of eggs (GLMM, $\chi^2 = 1571.02$, df = 2, $P < 0.001$) (Fig. 7.B). The lowest number of eggs was observed for uninoculated sterile males in competitive situation, while the highest was observed for inoculated fertile males in competitive situation (mean of the total ± SE, 375.0 ± 17.8 and 964.0 ± 98.2, respectively).

In the control cages, none of the eggs hatched when males were sterile, while for fertile males hatching percent increased across time ending at 62.6% ± 6.46 (mean ± SE) (Fig. 8.A). In competitive situation, test males significantly affected hatching percent (GLMM, $\chi^2 = 453.3$, df = 2, $P < 0.001$) (Fig. 8.B). It decreased across time for uninoculated sterile males competing with competitors until 4.05% ± 1.87 (mean ± SE) after 10 days, while it increased across time for inoculated fertile and sterile males competing with competitors (mean ± SE, IFM: 52.6% ± 7.64 and ISM: 71.3% ± 8.83 after 10 days).

## Discussion

Our experiments shed light on the potential of *M. anisopliae* fungal spores and entomovectoring by wild males through autodissemination or released mass-reared sterile males in SIT programs (i.e., boosted-SIT) to control the Oriental fruit fly. In both techniques, the direct effect of fungal spores transmitted by inoculated males to wild individuals, and the epidemics that may follow inoculation, should result in effective control. However, these two processes necessitate high mating rates between inoculated males and wild females, as well as the frequent transmission of spores to conspecifics. In return, this secondary inoculation should induce death or sub-lethal effects sufficient to impair population growth. Fungal epidemics may further proceed if inoculated females transmit the fungus to their offspring. Each of these steps that we investigated is discussed below.

We did not observe vertical transmission of *M. anisopliae* from females to their offspring nor internal neither external. Indeed, inoculation of gravid females with fungal spores did not affect egg hatching nor larvae survival. It is also possible that a low quantity of fungus transmitted might be undetectable with our bioassay. More advanced tools such as molecular biology may allow fungus detection in eggs and larvae or even on adults of the first generation. Yoder et al. (2019) studied the vertical transmission of the entomopathogenic soil fungus *Scopulariopsis brevicaulis* using a molecular method to detect fungus on eggs of the winter tick, *Dermacentor albipictus*. They found that about 40% of eggs and larvae were inoculated with the parasite. However, our bioassay proved that the quantity transmitted, if any, was not enough to be pathogenic for the offspring. Inoculation of females decreased oviposition, probably starting when females triggered the disease and become ill (Zimmermann 2007). Sookar et al. (2014) showed similar results with females of *B. cucurbitae* and Dimbi et al. (2013) with *Ceratitis* spp. when inoculated with *M. anisopliae*.

The first laboratory experiments in small cages showed, under a non-competitive situation, mating success of sterile males from the IAEA strain (inoculated or not) with females of the Senegalese strain in a similar proportion with that of males from the Senegalese strain (inoculated or not) on the day of inoculation. The absence of inoculation effect on the

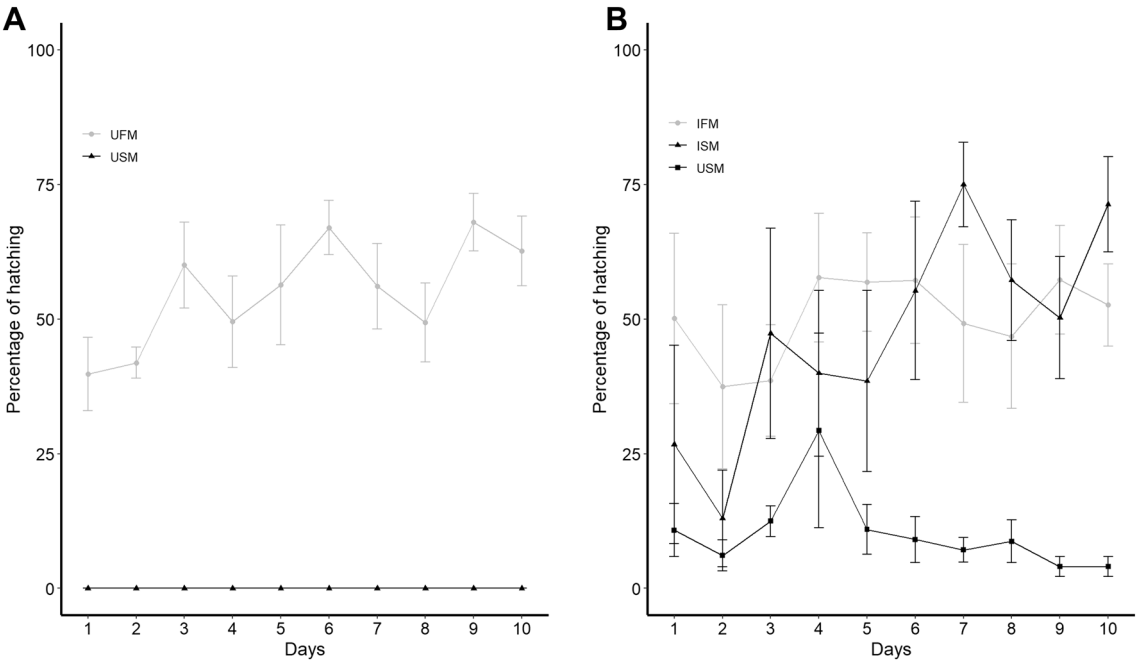


**Fig. 8** Mean percent of egg hatching in **A** control cages (UFM: Uninoculated Fertile Males and USM: Uninoculated Sterile Males), and **B** competitive situation according to test males, IFM (Inoculated Fertile Males), ISM (Inoculated Sterile Males), and USM (Uninoculated Sterile Males). Bars represent standard errors

mating success on the day of inoculation suggests the presence of spores on males did not affect their behavior nor that of females, probably because males were not ill yet, as spores need at least 5 h to start germination (Hywel-Jones & Gillespie 1990; Rangel et al. 2004). One experimental bias that may hide some difference in the mating success across male types is that small cages strongly increase the encounter rate compared to natural conditions (Cruickshank et al. 2001). However, the inoculated sterile males had a shorter mating duration compared to that of the other types of males, indicating some trouble in their behavior. In a competitive situation, both sterility and spore inoculation decreased the mating success of males. Irradiation, mass rearing, and shipping conditions may affect sterilized flies' performance along time (Rull et al. 2012a). In *Anastrepha obliqua* (Diptera: Tephritidae), laboratory flies mated less than wild flies in field cages, irrespectively of the irradiation level they received, highlighting that a decrease in mating success may be attributed to rearing conditions rather than to irradiation (Rull et al. 2012b). In addition, behavioral changes owing to rearing conditions can somehow result in mating incompatibility with wild populations (Rull et al. 2012b). Inoculated males probably started being sick during the three days of observation (Zimmermann 2007), then becoming less and less competitive until death (Armsworth et al. 2006; Thaochan and Ngampongsai. 2018; Ramírez y Ramírez et al. 2022). As a result, the males that were both sterile and inoculated had the lowest mating success. Laboratory experiments showed that inoculated flies allocated much time to auto-grooming, which left less time to courtship and mating (S.D. personal observation). Lastly, as fruit fly females generally select one male from several suitors (Hendrichs et al. 2002), they might be able to detect sick males (Beltran-Bech and Richard 2014) or fungus volatiles (Zang et al. 2024). Yet, other studies on tephritid flies showed that inoculation does not affect mating success in small cages until the fifth day after inoculation (Dimbi et al. 2009; Thaochan and Ngampongsai 2018). The virulence of the *M. anisopliae* strain, the lower inoculation levels of flies, or cage size, could explain this difference with our results. During mating, fertile and sterile inoculated males transferred spores to females in similar amount (around 3000 spores). In these experiments, 34% of mated females and 17% of unmated females died, indicating that only few matings transferred enough spores to induce mortality. This could be due to a lack of spore transfer or to the inadequate placement of spores on females (Gillett-Kaufman and Kimbrough 2009). Indeed, Chailleux et al. (2023) showed that the LT 50 (lethal time 50, i.e., number of days to kill 50% of flies) kept small, 4.85 days, with 6100 spores per fly, but reach 22.9 days with 5600 spores per fly. Mortality of unmated females indicate that inoculation can occur directly through unsuccessful mating attempts or indirectly through contaminated surfaces. Toledo et al. (2006) showed similar results with 41.6% of mated *C. capitata* females and 14.2% of unmated females inoculated with *B. bassiana* in small cages.

In field cages, despite poor mating success, male inoculation reduced the survival of females and male competitors. The survival of competitors was slightly lower than that of females, suggesting that male-male interactions in leks were a significant way of transfer. Flores et al. (2013) made the same observation when releasing sterile males in coffee plantations to control *C. capitata* in Chimaltenango, Guatemala. In our study, there was no significant difference in horizontal transmission of fungal spores by fertile and sterile males to females and competitors. Male sterility reduced egg hatching as expected (Robinson 2005), but also female oviposition. By contrast, spore inoculation of males did not reduce female oviposition nor egg hatching. This result can be explained by the low mating success of inoculated fertile and sterile males (< 10%) leading to a small proportion of inoculated females and living a room to their competitors. In other words, boosting the sterile males with spore did not impact reproduction, but even reduced the effect of sterility as hatching rates were lower in the absence of spores.

To summarize, our experiments showed that inoculated sterile males transmitted spores to other males and females during courtship and mating, but induced low mortality among the target population. In addition, inoculated sterile males showed lower survival and mating success than non-inoculated sterile males. This low performance probably explains why plain sterile males had greater effect on female fertility than when they were inoculated. These results suggest fungal virulence may be counterproductive for spore dissemination by sterile males. The two different scales of our experimental designs highlighted the importance of conducting further research in realistic environmental conditions in terms of space and time. Using an agent-based simulation model, Diouf et al. (2022) showed that the boosted-SIT could be promising but that additional data on transmission parameters are needed. Our results partially fill this gap.

Several strategies may be levered in order to counterbalance or avoid the fitness decrease in inoculated male. This might be reached by (i) bettering male mating success by feeding them with methyl eugenol (a plant-borne compound whose metabolites are used in the synthesis of the male sex pheromone) (Shelly and Dewaire 1994), (ii) selecting pathogens or other *Metarhizium* strains with greater latency or sex-specific effects (Duneau et al. 2024), (iii) increasing male spore load with an adequate dispenser or adding an adjuvant to spores' powder to increase both male load and transfer, or (iv) releasing inoculated sterile males in specific demographic contexts where even moderate effects on females may have wide effects on reproduction, for example

during the off-season when populations are numerically low and concentrated in a few sparse reservoirs. Taken together, our results also suggest another approach: combining the autodissemination technique ('attract and kill') with the use of sterile males pre-fed with methyl eugenol. This may also be a good option to preserve the efficacy of sterile males and combine it with the pathogen transfer from fertile males to females (Barclay et al. 2014).

## Author contributions

S. Diop, A. Chailleux, T. Brévault conceived research. S. Diop and F. Dosso conducted experiments. S. Diop and A. Chailleux analyzed data. S. Diop, A. Chailleux, T. Brévault, S. Fellous, A. Sohel, E. Deletre wrote the manuscript. A. Chailleux and T. Brévault secured funding. All authors read and approved the manuscript.

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## Declarations

**Conflict of Interest** The authors have not disclosed any competing interests.

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