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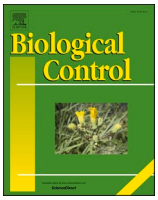


Can the African weaver ant be used as a vector of entomopathogenic fungi to bolster the biological control of tephritid fruit fly pests?

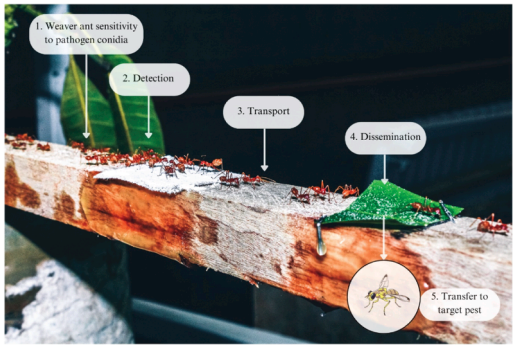
Thibault Nève de Mévergnies [a,b,*], Samba Diop [a,c], Massamba Diakhaté [a], Claire Detrain [d], Frédéric Bouvery [e], Thierry Brévault [f,g], Anaïs Chailleux [a,b]

[a] CIRAD, UPR HortSys, Biopass, Centre de recherche ISRA-IRD, Dakar, Senegal
[b] HortSys, Univ Montpellier, CIRAD, Montpellier, France
[c] ED-SEV, UCAD, Dakar, Senegal
[d] Université Libre de Bruxelles, Unit of Social Ecology, Belgium
[e] INRAE, PSH, Avignon, France
[f] CIRAD, UPR AIDA, Biopass, Centre de recherche ISRA-IRD, Dakar, Senegal
[g] AIDA, Univ Montpellier, CIRAD, Montpellier, France

HIGHLIGHTS

- Weaver ants were evaluated as vectors of entomopathogenic fungi to control fruit flies.
- Ants successfully loaded, transported, and disseminated pathogenic conidia.
- Significant fruit fly mortality was achieved in controlled conditions.
- Entomovectoring is, however, limited in time and space.
- Integration with other control methods is promising, but challenging.

GRAPHICAL ABSTRACT



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ABSTRACT

Entomovectoring is an environmentally friendly pest control strategy where insects act as precision vectors of a biocide to target pest populations through phoretic dispersal. While bumblebees are the only insects used commercially for this purpose, other insect species, including ants, offer untapped potential. The arboreal weaver ant, *Oecophylla longinoda*, known for its beneficial predatory role in production crops, could be used as a vector of the entomopathogenic fungus, *Metarhizium anisopliae* to bolster control of the invasive oriental fruit fly *Bactrocera dorsalis*. In this study, we set up a series of experiments under laboratory and mesocosm conditions to investigate the feasibility of using this ant as an entomovector. Results showed that while *M. anisopliae* was intrinsically pathogenic to the weaver ants, they were able to detect its presence and adjust their behavior according to its concentration. Despite exposure, the ants effectively protected themselves through social immunity behaviors. Furthermore, weaver ants auto-inoculated themselves with conidia by walking over contaminated areas and subsequently dispersed conidia along their trails. Although the density of dispersed conidia declined over time

* Corresponding author at: CIRAD, UPR HortSys, Biopass, Centre de recherche ISRA-IRD, Dakar, Senegal.
E-mail address: tneve@hotmail.fr (T. Nève de Mévergnies).

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and distance from the inoculation zone, up to 36% of fruit flies were killed when left to roam on mango trees where conidia had been disseminated by the weaver ants. While the integration of weaver ants as pathogen entomovectors could enhance pest fruit fly control when combined with other strategies, several challenges are yet to overcome before field applications.

1. Introduction

Entomovectoring is a control strategy relying on the interspecific horizontal transfer of microbial control pathogens from insect vectors to target pests through direct or indirect contact. It has been developed for biological control of invertebrate or microbial pests and proven successful for crop protection in both field and greenhouse situations (Mommaerts and Smagghe 2011; Mommaerts et al. 2011; Hokkanen et al. 2015). Compared to large-scale spraying of biocides, entomovectoring theoretically boasts the advantages of lower costs and labor as well as reduced non-target side effects due to its more precise pathogen dissemination (Smagghe et al. 2020). The objective is to generate an epizootic disease in a target pest population through phoresis of a pathogen by a vector organism. The success of this method therefore lies in the interactions between the vector, the pathogenic agent and the target pest, as well as the non-target risk to the environment and human health (Mommaerts and Smagghe 2011). Critical components of this strategy include selecting a vector resilient to the pathogen, optimizing methods to load the vector with the pathogen, and establishing the carrying and dispersal capacities of the pathogen by the vector to know the crop surface that can be protected per vector and per time unit. Further, evaluating the encounter rates between the pathogen and the target pest, but also non-target organisms, as well as the persistence of the pathogen in the environment, is crucial before large-scale field application

Among entomovectors, hymenopterans from the Apidae family are the most studied due to their appropriate morphological (e.g., hairy body) and behavioral (e.g., foraging traits) characteristics, but also because they are already broadly commercialized as pollinators (Peng et al. 1992; Kovach et al. 2000; Mommaerts et al. 2008, 2011; Smagghe et al. 2013). However, Apidae vectors are limited to protecting flowers, leaving many crop pests that attack other plant organs unaddressed. As a result, there has been growing interest in exploring other insect species as potential vectors. For instance, the use of a sciarid fly species as a vector of a microbial control agent against plant fungal pathogens appears promising (Kapongo et al. 2020). Tephritid fruit fly sterile males have also been investigated as intraspecific vectors of entomopathogenic conidia to boost SIT programs (Chailleux et al. 2023; Diop et al. 2024). Similarly, horizontal transmission of the fungus *Metarhizium anisopliae* (Metschnikoff 1879) (Ascomycota: Hypocreales) in mosquito populations has proven efficient to generate significant mortality in opposite-sex individuals following copulation (Scholte et al. 2004; Garza-Hernández et al. 2015). Aphid predators, such as coccinellids, lacewings, and the predatory bug *Orius laevigatus* (Fieber 1860) can also vector conidia, bolstering mortality in their prey aphid populations (Roy et al. 2001; Pell and Vandenberg 2002; Ekesi et al. 2005; Down et al. 2009; Zhu and Kim 2012). In many production systems, ants (Hymenoptera: Formicidae) are present and play key ecological roles due to their abundance, multitrophic interactions and ecosystem engineering capacities (Hölldobler and Wilson 1990). In addition, they often have extended foraging areas as well as efficient immune systems, promising traits for entomovectoring. *Formica fusca* L. 1758 scavenger ants have been successfully used as vectors to disseminate a specific entomopathogen to control a pest grasshopper (Kistner et al. 2015). Also, the mutualistic relation between *Lasius niger* (L. 1758) and phytophagous aphids was exploited to vector fungal conidia directly from the ants to aphid colonies, resulting in a 68, 30, and 3.7 % aphid mortality under laboratory, semi-field, and field conditions respectively (Bird et al. 2004). Despite these promising findings, further research is required to assess the broader potential of ants as entomovectors in pest management strategies.

Enhancing the pest control efficiency of already established ant biocontrol agents through additional vectoring of an entomopathogenic fungus could be further attempted. In this respect, the arboreal African weaver ant, *Oecophylla longinoda* Latreille, 1802, is a dominant generalist predator long established as an efficient biocontrol agent with little non-target adverse effects (Van Mele 2008; Thurman et al. 2019; Nève de Mévergnies et al. 2021). Weaver ants have received increasing attention in recent decades as they mitigate damage from the highly invasive oriental fruit fly, *Bactrocera dorsalis* (Hendel, 1912), which generates heavy losses in mango orchards (Van Mele et al. 2007; Diamé et al. 2015; Vayssières et al. 2016; Mekonnen et al. 2021). Although a detrimental effect of the presence of the weaver ant is its capacity to raise pest mutualistic hemipterans, supplementing the ants with a sucrose solution has proven efficient to change its behavior and limit this drawback (Chailleux et al. 2019; Correa et al. 2023). In parallel, spraying of the entomopathogenic fungus, *Metarhizium anisopliae*, in orchards or in laboratory conditions also induced higher *B. dorsalis* fruit fly mortality at all life stages (Ekesi et al. 2011; Ali 2014). This fungal pathogen is often used as a biocontrol agent due to its ubiquitous natural occurrence, its broad range of insect targets, its simplicity to store and produce as well as its safety to vertebrates (Zimmermann 1993, 2007; Roy and Pell 2000; Shah and Pell 2003; Vega et al. 2012). Therefore, we hypothesized that a combination of the weaver ant and the entomopathogenic fungus *M. anisopliae* would increase fruit fly *B. dorsalis* mortality in production orchards. Because the weaver ants forage on all the mango tree organs such as the fruits, where female fruit flies deposit their eggs, entomovectoring of the fungus by the ants could be an optimal delivery strategy to maximize encounter of this pathogen with the flies. However, no literature exists concerning the potential of this ant to detect, carry and disperse microbial control agents such as *M. anisopliae* fungal conidia.

Therefore, we designed a series of semi-controlled experiments in microcosm and mesocosm conditions to evaluate the potential of this method for future pest management strategies. First, we evaluated the pathogenicity of *M. anisopliae* to the weaver ants and their capacity to detect and avoid these pathogenic conidia, this to assess the possibility for an auto-inoculation system. Then, we investigated the carrying and dissemination capacities of the conidia by the ant through time and space. Finally, we simulated plant-mediated (from ant to plant to fly) pathogen transmission and measured the mortality in the target fruit fly pest. These results provide new insights into the feasibility of integrating ants into entomovectoring strategies as part of sustainable pest management.

2. Materials and methods

2.1. Biological system

Leaf nests of *O. longinoda* weaver ant colonies were collected in mango orchards in the Niayes area in Senegal during the 2018 and 2019 rainy seasons. For each replicate of the following experiments, a different weaver ant colony was used. For each treatment in each replicate, different nests of the same colony were used. We made sure nests between replicates belonged to different colonies by displacing at least three individuals from one nest to the other and observing if an aggressive behavior towards alien conspecifics was shown. All collected nest were similar in size (volume) and ant activity, by visually

evaluating major and minor individual abundances on their surface. After the experiments carried in 2019 which concerned the auto-inoculation and conidia dissemination of the pathogen by the ants, all ant individuals were counted in 36 different nests. They hosted an average of 376 ± 22 (mean ± SE) major and 611 ± 49 (mean ± SE) minor individuals. Collected nests were placed individually in large plastic boxes (25 cm x 15 cm x 15 cm) and brought back to the experimental site the same day. Boxes were then individually placed on the soil of young potted mango trees (around 1 m high, known as the "Nunkourouni mangot" variety) under net houses, protected from rainfall by a transparent plastic roof. Potted trees were unique to their ant nests. Boxes were then opened, allowing ants to build new nests in the potted mango trees. They were given water, a 1 M sucrose solution, and canned tuna for fat and proteins. The potted trees were then placed in larger bowls that contained water and liquid soap, forming a moat preventing ants from escaping their new habitat (Fig. 1). The potted trees were isolated from the moat containing the soap and water by placing a plastic bowl of a height of approximately 10 cm underneath the pot. For at least 72 h, ants were allowed to settle and build new nests in the young trees. They were left on their host mango tree surrounded by the moat for the following experiments.

The entomopathogenic fungus used was a strain of *M. anisopliae* commercialized by Real IPM in Kenya, known as Met69 OD, and known to be highly pathogenic to *B. dorsalis* (Chailleux et al. 2023). Pure conidia were provided as dry powder. We evaluated the conidia's germination rate from five replicates, which was over 80 % after 24 h at 25 °C in Petri dishes.

Fruit fly *B. dorsalis* larvae were collected from mangoes in Sangalkam (Niayes, Senegal) orchards during July 2018. Infested mangoes were placed in cups inside 5 L recipients containing sterilized sand and enclosed by a fine mesh net. After ten days in a greenhouse, the sand was sieved to collect the fly pupae, which were placed in a 50 cm x 50 cm x 50 cm fine mesh cage along with a sucrose solution, yeast extract, and water. Ten days after their emergence, the flies were sexually mature and were allowed to oviposit on bananas for two days. We used bananas to rear the flies due to their year-round availability (in contrast to mango) and suitability as *B. dorsalis* hosts (Rwomushana et al. 2008). Bananas were then placed in a cup on sterile sand until pupation, and the above procedure was repeated to obtain subsequent generations.

2.2. Pathogenicity of m. Anisopliae conidia to ants

Weaver ant foragers were randomly sampled on the potted mangoes and placed for 30 min in Petri dishes (9 cm diameter) containing 0.01 g of *M. anisopliae* conidia (0.13 mg/cm^2). We evaluated their capacity to survive infection by this entomopathogen depending on their isolated or group status as well as the composition of the latter. To do so, a first experiment consisted of isolating inoculated individuals from the major caste. These are the most obvious targets as entomovectors because, in contrast to minor workers, they do most of the foraging thus most suited for vectoring of a pathogen. Fifteen major ants (five individuals from three different colonies) were isolated after their inoculation in the Petri dish as described above. They were individually placed in an arena with a sapodilla (*Manilkara zapota*) branch, water, and agave syrup *ad libitum*.

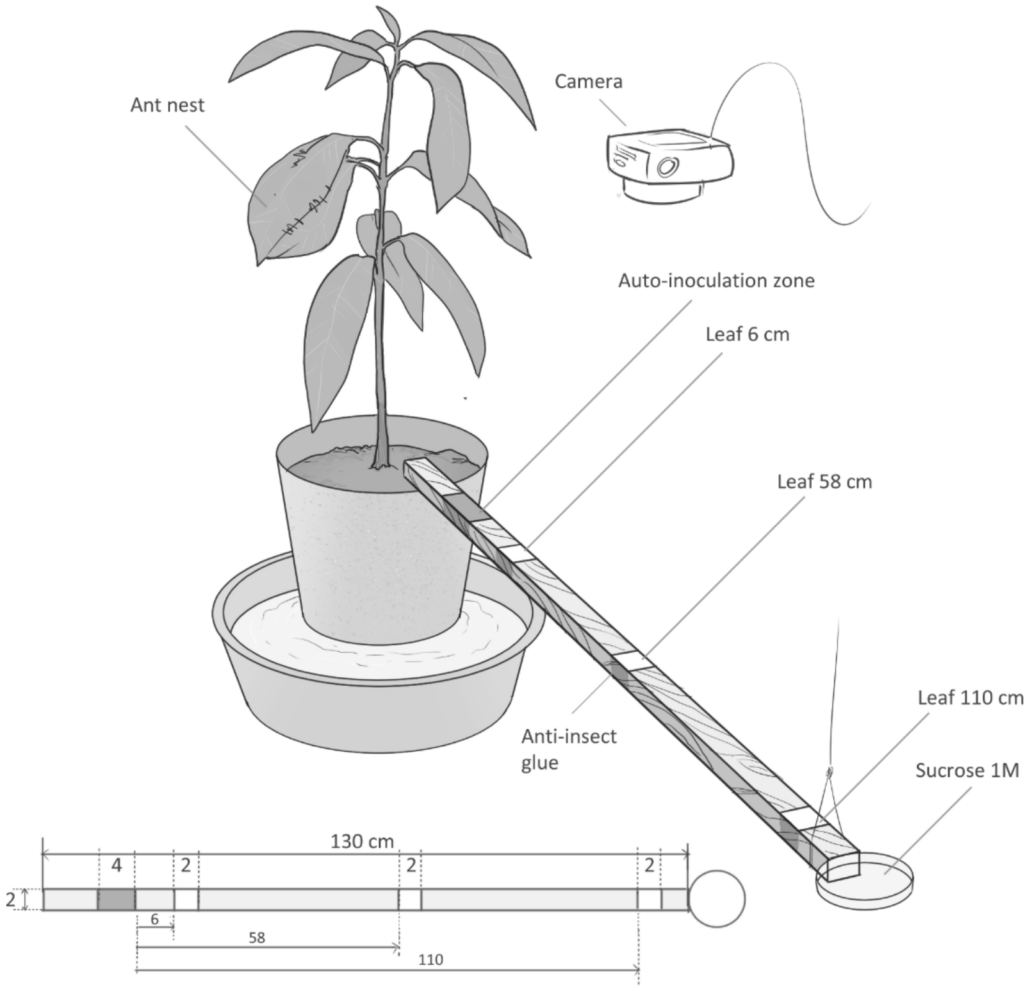

Fig. 1. Experimental design to assess the ability of the weaver ants to detect, avoid, carry, and disperse *Metarhizium anisopliae* conidia through time and space.

The arena design was adapted from Biondi et al. (2012) and consisted of two overlapping plastic cups (600 ml each) generating two separated areas (Fig. S1). The upper cup contained the sapodilla leaves, with a central hole at the bottom to allow the sapodilla branch stems to reach the water in the cup placed underneath. This allowed to keep the sapodilla watered whilst preventing the ants from falling and drowning in the water (Fig. S1). The ants were left in these arenas at 25 °C for 30 days, and their mortality was assessed daily. A control treatment was done with 15 isolated non-inoculated ants (five individuals from each of the same three colonies as the inoculated ones). In a second experiment, their survivability in group was tested. The same inoculation method in a Petri dish as described above was used, but the treatment groups consisted of (i) five major individuals kept together and (ii) five major and five minor individuals (total of 10 individuals) kept together. In this latter situation, the mortality of major and minor ants was recorded separately. This experiment was replicated three times using ants from three different colonies, and a treatment with non-inoculated ants was again used as a control. We did not evaluate a group treatment of five minor individuals together as these seldom leave the nests to forage and are thus not the target entomovectors.

2.3. Auto-inoculation and conidia dissemination by ants

2.3.1. Experimental design

For the evaluation of the potential of the entomovectoring of *M. anisopliae* conidia by the weaver ant, we created a closed arena system where weaver ants were forced to come into contact with this fungus pathogen whilst foraging towards a 1 M sucrose solution (Fig. 1). A wooden bridge (130 cm × 2.5 cm × 2 cm) was connected from the potted mango tree trunk base hosting the ant nest to the sucrose solution. Each unique mango tree and ant nest combinations were used only once then discarded. At 10 cm from the base of the mango tree trunk, a linen tissue (4 × 2 cm) was placed on the bridge, on which was distributed *M. anisopliae* conidia −hereafter called the 'auto-inoculation zone' (Fig. 1). On this auto-inoculation zone, *M. anisopliae* conidia were distributed alone or in formulation with corn starch powder (Maizena^{TM}) as an adjuvant, following six different treatments: control, corn starch (25 mg/cm^2 of corn starch only), low inoculum (1.25 mg/cm^2 of conidia), low inoculum + corn starch (1.25 mg/cm^2 of conidia and 1.25 mg/cm^2 of corn starch together), high inoculum (12.5 mg/cm^2 of conidia), high inoculum + corn starch (12.5 mg/cm^2 of conidia and 12.5 mg/cm^2 of corn starch). We used the corn starch formulation because of its known aggregating effect on conidia, thus expected to increase their numbers transported by vectors (Al-Mazra'Awi et al., 2007; Mommaerts and Smagghe 2011; Smagghe and Diaz 2012). Formulations were prepared in 9 cm Petri dishes, in which linen tissue were placed and then firmly shaken by hand to homogenize dispersal of the conidia. In the case of high inoculum concentrations, conidia and corn starch remaining after shaking the petri dish were deposited directly on the tissue once placed on the bridge. On the bridge, three mango leaf pieces (2 cm x 2 cm, cleaned with distilled water) were placed at 6, 58, and 110 cm from the auto-inoculation zone (Fig. 1) to assess conidia dispersal. Before any experiment, ants were deprived of food for 24 h by removing access to the sucrose solution and any remaining tuna pieces, this to stimulate their foraging behavior. Once the experiment started by connecting the bridge, observations and measures (see below) were done at defined times over two days. All bridge experiments were replicated nine time, each replicate using a different colony. Per replicate, six nests of the same colony were thus used for the six different treatments, each nest implemented on a unique potted mango tree.

2.3.2. Detection and avoidance of conidia

We placed Logitech C920 HD webcams above the auto-inoculation zone on the bridge for three treatments: control, corn starch, and high pathogen inoculum + corn starch. This subsample was done due to logistic constraints (camera availability and time). We selected treatments we considered most representative to analyze the behavior of the ants when facing pathogen conidia. We replicated each of the three treatments five times. To assess the detection of the fungal conidia and its potential repellant effect on the ants, we first measured the total duration of antennations (antennation time) made by ten randomly selected individuals in each treatment (in total 150 individuals) to the auto-inoculation zone. Second, we recorded the time spent by these ants in a 4 cm x 2 cm bridge area just before the auto-inoculation zone (indecision time). Their chosen exit path was then noted, whether by stepping on the inoculated tissue or doing a U-turn back towards the potted mango tree. All these observations were made using the BORIS open-source software (Friard and Gamba 2016). Next, we quantified the foraging dynamics of the weaver ant in all treatments by counting the number of ants crossing a virtual line (both ways) at the end of the bridge, two centimeters away from the sucrose solution dispenser. This ant traffic was counted for 3 min at defined times (10 min, 20 min, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h) after the beginning of the experiment. On the following day (exactly 24 h after the start of the experiment), these counts were done again at the same time intervals. The average temperature during this experiment was 32.8 (± 2.17) °C, and the mean luminosity was 79.4 (± 50.9) lux.

2.3.3. Load and transport of conidia

To evaluate the capacity of the weaver ants to load themselves with conidia, we delicately captured two ants with tweezers at four defined times after the start of the experiment (40 ± 20 min; 5 h ± 20 min; 24 h 40 ± 20 min; and 29 h ± 20 min). The time variability was due to waiting for an ant to cross the tissue. Ants were captured on the foraging bridge directly after crossing the auto-inoculation zone and individually placed in 1.5 ml Eppendorf tubes containing 1 ml distilled water. One drop of polysorbate surfactant Tween 80 was added to allow solubilization of the conidia in the water. Each tube containing an individual ant was then vortexed for at least 2 min to detach the conidia from the ants' cuticle. Then, the number of conidia per ant was counted under a microscope using a Malassez cell, which is a hemocytometer with a first irregular grid of 200 μm x 250 μm rectangles, further subdivided in 40 x 50 μm rectangles with a depth of 100 μm.

2.3.4. Dissemination of conidia

After leaving their nests and crossing the auto-inoculation zone, all ant foragers were forced, using anti-insect glue to prevent their dispersion, to walk on the leaves placed at the three different positions on the bridge to reach the food source. These leaves were collected after six hours of foraging by the weaver ants on the first day, then replaced on the next day at 10:00 am (24 h after the start of the experiment), and left again for six hours of foraging time by the weaver ants. Collected leaves from both days were placed individually in 50 ml Falcon tubes containing 10 ml of distilled water and two drops of Tween 80. They were vortexed for at least 2 min to detach the conidia, which were then counted using a Malassez cell under a microscope. We transformed these numbers into conidia density per area unit (with leaf surface of 4 cm^2).

2.4. Plant-mediated vectoring to fruit flies

The two following experiments had the objective to evaluate the horizontal interspecific transfer from the ant vector to the target fruit fly pest, using mango organs (leaf or fruit) or whole tree as intermediate pathogen hosts.

2.4.1. Microcosm experiment

Groups of five and groups of ten major ant individuals were placed separately in a Petri dish containing pure *M. anisopliae* conidia (0.13 mg/cm^2) that were evenly spread by hand shaking the Petri dish before inserting the ants. A control (no conidia) was done for every treatment. After 30 min in the Petri dish, ants were transferred and allowed to roam for two hours in a plastic box (15 cm x 10 cm x 10 cm) containing either

half a mango fruit (cut side sealed against the plastic box) or two mango leaves. The mango fruit and leaves were then transferred without the ants to a new clean box in which six (three males and three females) *B. dorsalis* fruit flies were placed for 24 h with a source of water. Afterward, the flies were transferred to another sterile 20 cm x 20 cm x 10 cm box with water, yeast, and sucrose solutions. Their mortality was subsequently assessed every day for two weeks. Five replicates were done per treatment (group size x plant organ), with ants from five different colonies.

2.4.2. Mesocosm experiment

A greenhouse experiment was carried out to evaluate the potential to inoculate weaver ants with fungal conidia using sugar feeder devices under more realistic conditions. The sugar feeder device consisted of a 60 ml polypropylene jar filled with a 1 M sucrose solution. Its lid was perforated in the center to allow a hydrophilic cotton wick to be inserted, absorbing the sucrose solution. A Petri dish was then glued to the jar lid, perforated in the center to let the cotton wick pass, giving access to the ants to the sucrose solution. Next, a round piece of linen tissue of a diameter of 9 cm (identical to the Petri dish surface) was positioned on the Petri dish. This tissue was used again as an auto-inoculation zone on which the ants were forced to walk to reach the sucrose solution absorbed by the cotton wick in the center of the Petri dish. On this tissue, *M. anisopliae* conidia were evenly dispersed following different treatments. Two different pathogen formulations were tested, both with 12.5 mg/cm^2 of conidia on the auto-inoculation tissue but formulated with or without corn starch (also 12.5 mg/cm^2). A control treatment (tissue only) was also done. This feeder and auto-inoculation device was placed on a new young potted mango tree free from weaver ants, with some leaves trimmed to discourage the ants from building a new nest upon colonization. A tree containing a weaver ant nest was then connected by direct foliage contact to the tree with the feeder device. Ants then started exploring this new foraging ground. To reach the cotton wick soaked by the sucrose solution, they had no choice but to walk on the auto-inoculation linen tissue zone. The three treatments (conidia only, conidia with corn starch, and blank control) were replicated 13 times, each one with distinct ant colonies. Once the experiment was started by connecting the trees together, the weaver ant traffic on the tree trunk bearing the feeder was measured by counting ants crossing a virtual line (both ways) after 1 h, 4 h, 24 h, and 48 h. After 48 h of allowing the ants to roam freely and disseminate the fungal conidia on the tree organ after contamination on the feeder and auto-inoculation device, the two trees were disconnected. All ants were removed from the supposedly contaminated tree that hosted the device. Then, we carefully placed an insect-proof net over this tree. Next, we introduced three adult female flies aged between eight and twelve days old under the net enclosing this tree. A humidified cotton was placed as a source of water for the flies. The three flies were left for 24 h under the nets on the contaminated trees, then collected and individually placed in insect boxes with water, yeast extract powder (Alfa Aesar, Kandel, Germany), and sugar. Their mortality was then observed daily for 14 days. Dead flies were then dipped in 70 % alcohol and distilled water for external sterilization, making sure that conidia that develop post mortem were internal to the fly body. Fly cadavers were kept for a week on humid sponges in Petri dishes to diagnose whether their death was due to *M. anisopliae* infection through the observation of conidiogenesis.

2.5. Statistical analyses

2.5.1. Pathogenicity of conidia to ants

Longevity analyses were carried out using Cox proportional hazard models, with the colony of origin of the weaver ants accounted for the 'cluster' parameter of the survival package (Therneau et al. 2021). A first model was fitted to evaluate major individual longevity using their group composition (isolated (hereafter called solo), in a group with majors only, or in a group with minors and majors) and the type of treatment (control or contaminated with conidia) as factors. A second model was then fitted to evaluate the survival of both major and minor individuals in groups depending on their caste (minor or major) and their contamination status.

2.5.2. Detection and avoidance of conidia by ants

To compare behavioral (indecision time and antennation time) parameters allowing the weaver ants to detect and avoid the pathogen across the different auto-inoculation treatments (control, conidia and corn starch 12.5 mg/cm2 each, or corn starch only 25 mg/cm2), we used generalized linear models (GLM) fitted with a gamma distribution and a log link. When necessary, a post hoc Tukey test was done for pairwise comparisons between treatments. Next, we fitted a GLM model with a binomial distribution, followed by post hoc Tukey pairwise comparisons to evaluate the probability of an individual weaver ant doing a U-turn when facing the auto-inoculation zone. Repeated measures of ants' traffic (number of ants crossing a virtual line) were analyzed using generalized estimating equations (GEE, package *geepack* (Halekoh et al. 2006)) fitted with a Poisson distribution. This allowed the random effects generated by the repeated measures on the same weaver ant populations to be accounted for by defining the replicate as "id" in the model. The correlation structure of the GEE model was set to exchangeable, as we estimated that the correlation coefficients between each individual in their respective colony were identical. The implemented explanatory variables were the conidia concentration (0, 1.25 or 12.5 mg/cm^2), the corn starch amount (0, 1.25, 12.5 or 25 mg/cm^2), and the time since the start of the experiment.

2.5.3. Load and transport of conidia by ants

The capacity of the weaver ants to load themselves with conidia after walking over the auto-inoculation zone was assessed using GEE with a Poisson distribution and a correlation structure of the observations set to be exchangeable. Explanatory variables were the conidia concentration (1.25 or 12.5 mg/cm^2), the corn starch amount (0, 1.25 or 12.5 mg/cm^2), and the time (20 min, 5 h, 24 h 20 min, and 29 h) since the start of the experiment. Control treatments without conidia were used to verify that there was no undesired contamination of the ants.

2.5.4. Dispersal of conidia by ants

A GEE model evaluated the transfer of the conidia from ants to leaves. The predicted variable was the conidial density found on the leaves. The initial conidia concentration (1.25 or 12.5 mg/cm^2) placed on the auto-inoculation zone, the time since the start of the experiment (6 or 30 h), and the distance from the auto-inoculation zone were fitted as explanatory variables.

2.5.5. Plant-mediated vectoring to the fruit flies

The survival of fruit flies in the controlled microcosm experiment was estimated using a Cox proportional hazard model. In this first experiment, the inoculation status (control or contaminated) of the weaver ants that had roamed on the support, their group size (five or ten individuals), and the nature of the support itself (mango fruit or mango leaf) were considered explanatory variables. Then, in the semi-controlled mesocosm experiment, we used a Cox model to evaluate the survival of the fruit flies following transmission of the pathogen *M. anisopliae* conidia deposited on the potted mango trees by the weaver ant. Models were fitted with the treatment (control, pathogen + corn starch at 12.5 mg/cm^2 each or pathogen only at 12.5 mg/cm^2) as the explanatory variable. In all models (GLM, GEE, or Cox), we did an analysis of the variance (ANOVA, type II or III accordingly, package "car") to determine the significance of the variables. When differences were significant, Tukey post-hoc multiple comparisons tests (package *multcomp* (Hothorn et al. 2008)) were done to compare within-group means. All tests were considered significant at $P < 0.05$, and all analyses were carried out in R using the Rstudio (R Core Team 2022) interface.

3. Results

3.1. Pathogenicity of conidia to ants

Overall, the survival of the major weaver ant individuals was significantly impacted by the interaction between their group composition and their contamination status with *M. anisopliae* conidia (Fig. 2; Cox, $\chi2 = 29.6$, df = 2, $P < 0.001$). In solitary, all major ants died before the 30-day observation period, whether contaminated or not. In groups of only major individuals, their mortality was significantly higher when they were contaminated compared to individuals in the control group (Cox, $\chi2 = 4.21$, df = 1, $P = 0.04$). However, when in a more realistic group including both minor and major ants, all major individuals survived the 30-day observation period, whether or not they were contaminated. In this case, however, there was a significant effect on the minor ant's mortality in the presence of the pathogen, detected by the interaction between the ant caste and their contamination status (Cox, $\chi2 = 17.3$, df = 2, $P < 0.001$).

3.2. Auto-inoculation and conidia dissemination by ants

3.2.1. Detection and avoidance of conidia

When leaving their host mango tree towards the sucrose solution, ant workers spent a similar amount of antennation time (antennae in contact) with the linen tissue representing the auto-inoculation zone, regardless of the treatment (control, pathogen + corn starch or corn starch only) (Fig. 3. A; GLM, $\chi2 = 1.64$, Df = 2, $P = 0.440$). Ants spent significantly more time (17.08 ± 13.15 s) being indecisive before crossing the auto-inoculation zone when inoculated with 12.5 mg/cm^2 of pathogenic conidia and corn starch compared to the control (12.13 ± 7.32 s) and to the corn starch only (25 mg/cm^2) (10.22 ± 7.34 s) (Fig. 3. B; GLM, $\chi2 = 14.2$, Df = 2, $P < 0.001$ and post hoc Tukey test). Compared to the control, the probability of an ant doing a U-turn in front of the auto-inoculation zone was not affected by the presence of corn starch only but significantly increased in the presence of pathogenic conidia (12.5 mg/cm^2) (GLM, $\chi2 = 51.85$, df = 1, $P < 0.001$). An individual ant facing pathogenic conidia was 25 % more likely to make a U-turn than when facing a pathogen-free tissue covered or not with corn starch.

The ant foraging dynamics on the bridge was significantly affected by the interaction between the pathogen concentration on the auto-inoculation zone as well as the time since the start of the experiment (Fig. 4. GEE, $\chi2 = 8.2$, df = 2, $P = 0.016$). In the first hour, ants were the most active regardless of the pathogen concentration compared to the rest of the observation period. The lower the pathogen concentration, the more active the ants were during that initial period. The addition of corn starch did not affect the number of ants foraging on the bridge.

3.2.2. Load and transport of conidia

The maximum average number of conidia found on ants was 449.3 (± 78.5) x 10^3 for the high pathogen concentration (12.5 mg/cm^2) in the first 20 min since the start of the experiment. The pathogen load (number of conidia) on the ants was significantly affected by the pathogen concentration ($\chi2 = 40.2$, df = 1, $P < 0.001$) placed on the auto-inoculation zone and the time since the beginning of the experiment ($\chi2 = 50.6$, df = 3, $P < 0.001$). Whatever the day and the time of sampling, the weaver ant load was always significantly higher for the high (12.5 mg/cm^2) conidia concentration on the auto-inoculation zone (Fig. 5). Adding corn starch to the pathogen did not significantly increase the load on the ants.

3.2.3. Dissemination of conidia

The triple interaction between the pathogen concentration placed on the auto-inoculation zone, the time since the start of the experiment, and the distance of the leaf on the foraging bridge from the auto-inoculation zone had a significant effect on the number of conidia dispersed by the weaver ants on the mango leaves (Fig. 6. GEE, $\chi2 = 13.4$, df = 2, $P = 0.001$). The maximum average density of conidia found on leaves after

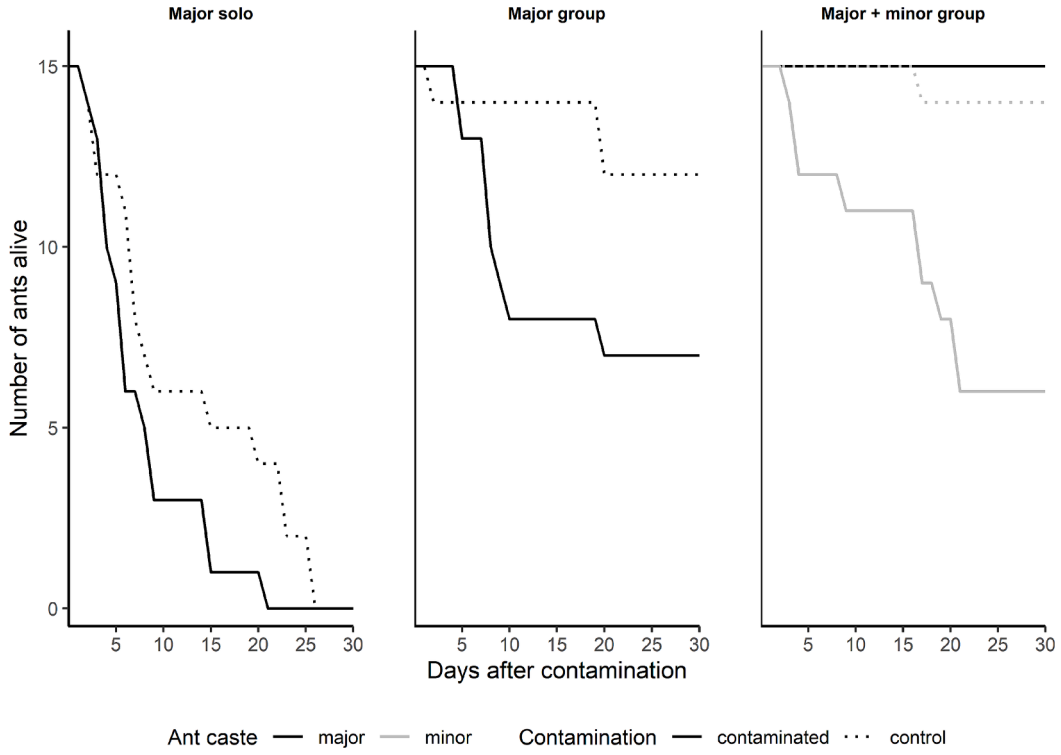

Fig. 2. Number of weaver ant workers alive over time depending on their isolated or group status and the group composition. In the major + minor group, survival was recorded independently for each caste (major ants are represented by the black lines, minor ants by the grey lines). Ants contaminated in Petri dish with *M. anisopliae* conidia are represented by the continuous line, and non-contaminated (control) individuals by the dashed lines.

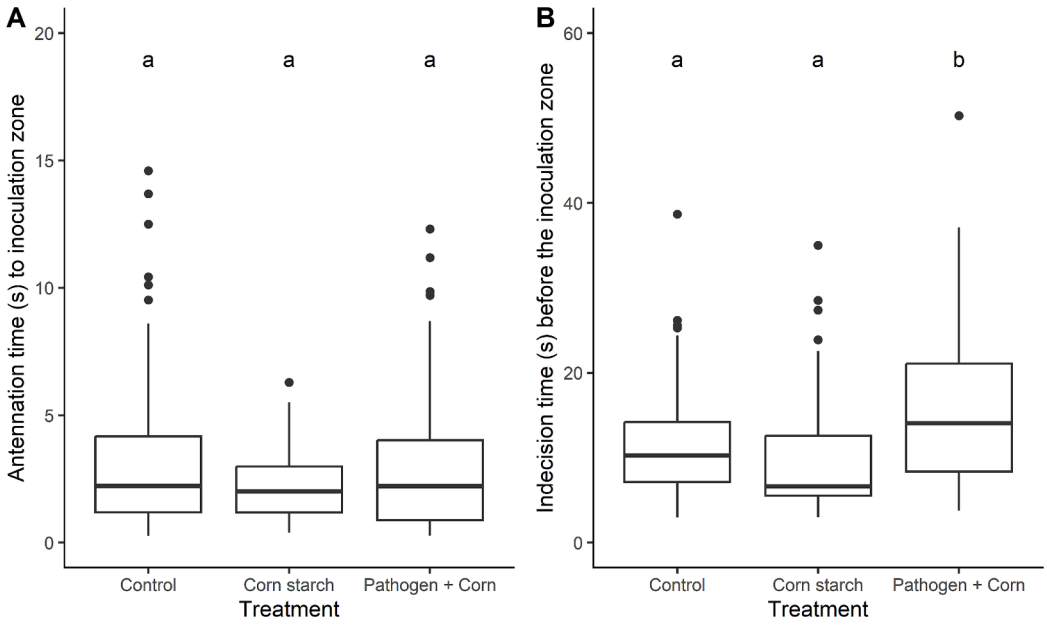

Fig. 3. A. Ant antennation time to auto-inoculation zone depending on the presence of corn starch, pathogen + corn starch or a blank control. B. Indecision time spent by the ants in the upstream zone in front of the auto-inoculation zone with the same treatments. Treatments bearing different letters are significantly different at $P < 0.05$ (post hoc Tukey pairwise comparisons) inside each figure. $N = 50$ per group.

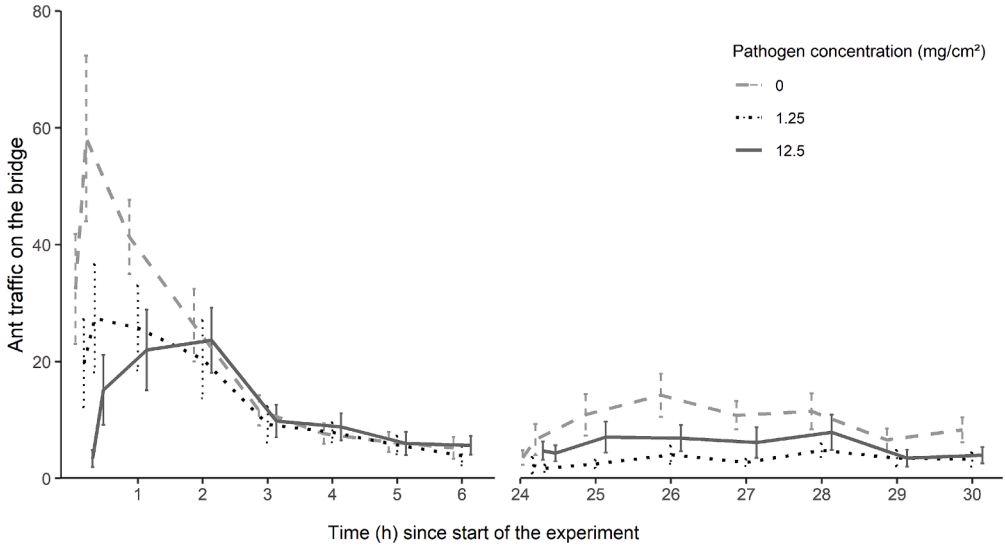

Fig. 4. Ant traffic (mean ± SE) over 3 min on the bridge over time (h) depending on the pathogen conidia concentration placed on the auto-inoculation zone (grey dashed line: 0 mg/cm^2 , black dotted lined: 1.25 mg/cm^2 , grey full line: 12.5 mg/cm^2). Data are pooled for the treatments with and without adding corn starch, as its addition did not affect traffic.

dispersal by the foraging weaver ants was 891.3 (±102.3) x 10^3 for the high pathogen concentration (12.5 mg/cm^2) on the first time period (6 h after the start of the experiment) at the first leaf position (6 cm) from the auto-inoculation zone (Fig. 6). With the same pathogen concentration and time period, there were, on average, over eight times fewer conidia on the leaf at the second position (58 cm from the auto-inoculation zone). At the same position, for the same pathogen concentration, but at the end of the experiment (30 h since the start), there were over three times fewer conidia on the leaf. Whatever the position and the time since the beginning of the experiment, there was always less conidia found on the leaves with the low pathogen concentration (1.25 mg/cm^2).

3.3. Plant-mediated vectoring to fruit flies

3.3.1. Microcosm experiment

The survival of the fruit flies was significantly impacted by the presence of conidia dispersed by the weaver ants (Fig. 7; Cox, $\chi 2 = 33.90$, $df = 1$, $P < 0.001$) left to roam on the supports before introducing the flies. Also, the sex of the flies had a significant impact on their survival ($\chi 2 = 4.40$, $df = 1$, $P = 0.035$). When the ants had dispersed pathogen conidia, 24.2 % of fruit fly individuals died compared to the control showing less than 1 % mortality. In presence of the pathogen, mortality of the female flies reached 35 % mortality, significantly higher than that of the males which had 13.3 % mortality. The type of vegetal organ (leaf or mango) used as support for the transfer of the pathogen conidia and the group composition of the weaver ant group (five or ten

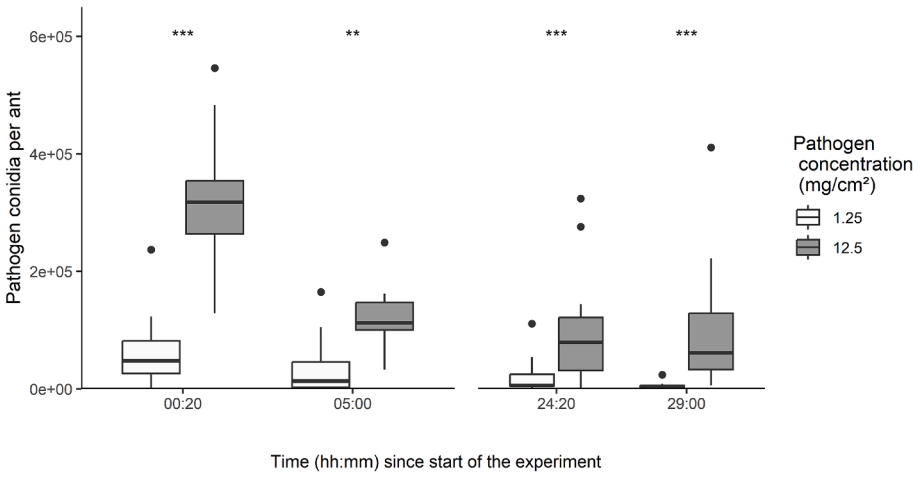

Fig. 5. Number of pathogen conidia recorded on ants depending on the time since the start of the experiment and on the pathogen concentration (white is 1.25 mg/cm^2 , grey 12.5 mg/cm^2) placed on the auto-inoculation zone. Data was pooled per pathogen concentration, regardless of whether or not corn starch was added, as it had no impact on the ant conidial load. Asterisks represent significant differences in conidia loads on the ants depending on the two pathogen concentrations for each time stamp since the start of the experiment (Tukey post hoc; *** $P < 0.001$; ** $P < 0.01$). N = 16 per group.

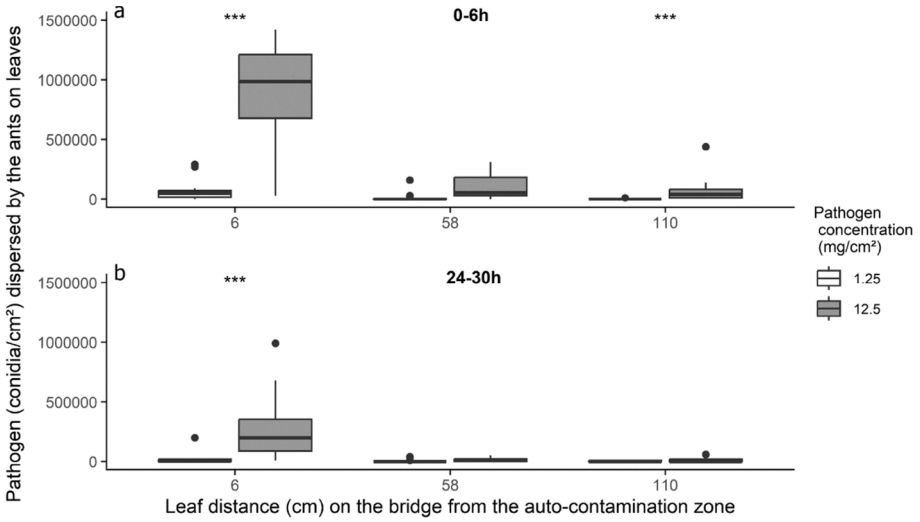

Fig. 6. Density of *M. anisopliae* conidia (conidia/cm^2) dispersed by the ants during six hours on the leaves at increasing distance from the auto-inoculation zone (6, 58, and 110 cm) depending on the concentration (low 1.25 mg/cm^2 in white and high 12.5 mg/cm^2 in grey) of conidia placed on the auto-inoculation zone and on the day with (a) first day and (b) second day. Asterisks represent significant differences between dispersed conidia densities depending on the pathogen concentration (1.25 or 12.5 mg/cm^2 ; Tukey post hoc; *** $P < 0.001$). N = 16 per group.

individuals) evaluated had no impact on the survival of the flies.

3.3.2. Mesocosm experiment

The ant foraging dynamics (number of ants counted during three minutes) on the tree trunk were the sugar and pathogen dispenser device was placed were significantly impacted by treatment used on this device (GEE, $\chi2 = 14.29$, df = 2, $P < 0.001$), but not by the time since the beginning of the experiment. There was no significant difference between the control and the pathogen + corn starch treatments (12.5 mg/cm^2 each) on the ant foraging dynamics. However, the pathogen-only (12.5 mg/cm^2) treatment resulted in a significantly higher ant activity than the other treatments throughout the observation period (Tukey post hoc test, z-value = 3.38, P = 0.002). The ant activity on the trunk was highest one hour after the start of the experiment in the pathogen-only treatment, with, on average, 25 (±7) ants observed crossing a virtual line in three minutes. Next, the number of ants recruited to the feeder was significantly impacted by the interaction between the time since the start of the experiment and the treatment used on the auto-inoculation zone of the dispenser device ($\chi2 = 74.4$, df = 6, $P < 0.001$). The ant recruitment to the feeder was highest in the control treatment four hours after the start of the experiment, with an average of 7 (±3) ants observed on the feeder. After an initial delay during the first two observation periods (one and four hours), the average number of ants recruited to the feeder was higher in the pathogen-only treatment than the control and pathogen + corn starch treatments.

After removing the ants from the mango trees and leaving female fruit flies for 24 h under a net on those trees, three out of 39 (7.69 %) of the flies from the control treatment died without developing mycelium during the 14 days following inoculation (Fig. 8). The treatment significantly impacted female fruit flies' survival (Fig. 8; Cox, $\chi2 = 9.51$, df = 2, P = 0.009). Indeed, ten (25.6 %) flies, and fourteen (35.9 %) flies died during the observation period following contact with mango trees inoculated by foraging ants previously exposed respectively to pathogen conidia alone (12.5 mg/cm2) or formulated with corn starch (12.5 mg/cm2 each). The pathogen + corn starch formulation proved the only treatment to significantly impact fruit flies' survival compared to the

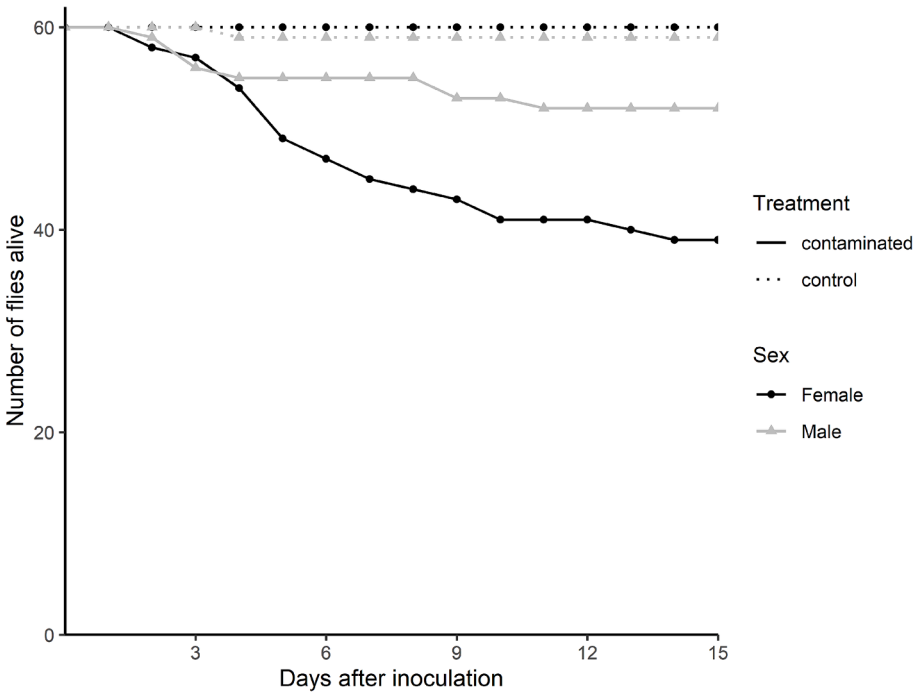

Fig. 7. *Bactrocera dorsalis* fruit fly survival depending on their sex (females illustrated by black lines with round shapes; males by grey lines with triangle shapes) and the treatment used on the weaver ants (full line contaminated; dotted line control) that were left to roam on a transfer support before flies were introduced. Data is shown pooled for both supports (mango and leaf) and for the group composition (groups of 5 or 10 individuals) of the weaver ants that were left to roam, as none of these variables had any impact on the fly survival (see text).

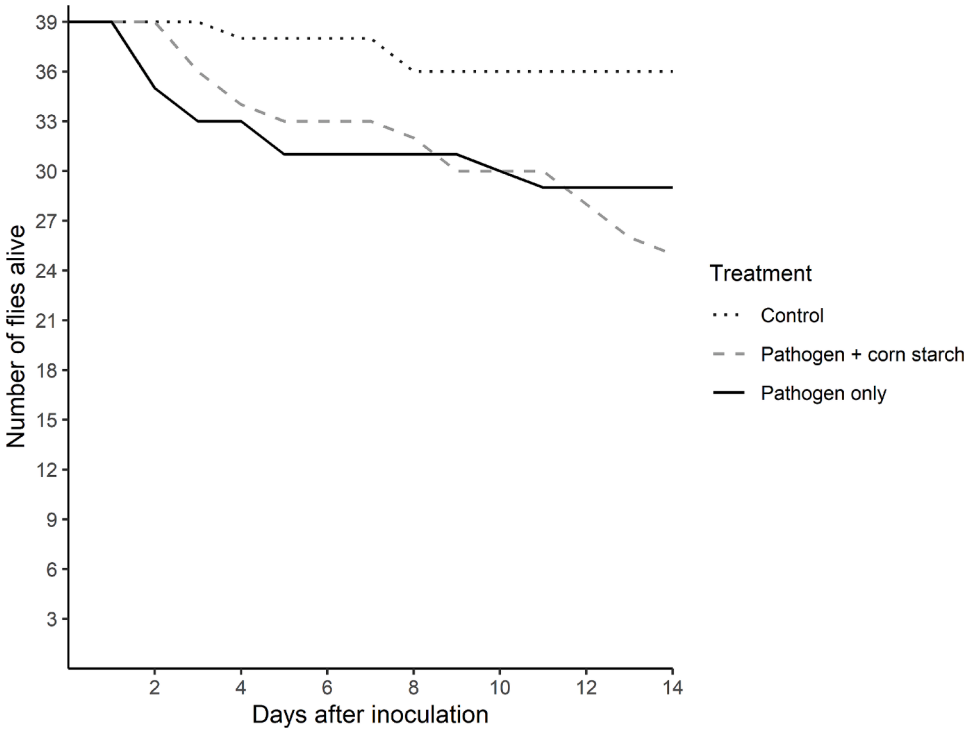

Fig. 8. Female *Bactrocera dorsalis* survival depending on the treatment (control dotted black line, pathogen + corn starch (12.5 mg/cm^2 each) dashed grey line, pathogen only black full line) and the days after inoculation.

control (Tukey post hoc, z-value = 3.08, P = 0.006). Of the 24 flies that died following treatments with pathogen, we observed conidiogenesis on seven (29.2 %) cadavers after one week of observation.

4. Discussion

Under controlled conditions, our study demonstrated that the weaver ant Oecophylla longinoda can effectively entomovector pathogen Metarhizium anisopliae conidia to the oriental fruit fly Bactrocera

dorsalis. Although ants were able to detect the presence of the pathogen, they physically contacted and loaded themselves with the conidia, and subsequently survived inoculation when in the presence of other colony members. Then, we observed increased mortality in B. dorsalis following plant-mediated transfer of the pathogen in our microcosm experiments. However, with the pathogen formulations and the inoculation methods that we evaluated, entomovectoring was limited to unrealistic small spatial and temporal scales. Indeed, (i) after 6 h of foraging by the ants we found a significant reduction of the number of conidia on the leaf only 52 cm away from the auto-inoculation zone compared to the one at 6 cm; and 24 h later, the number of conidia found on this leaf was three times lower. Then, (ii) the mortality rate of the flies in the more realistic mesocosm experiment where whole young potted mango trees were used to assess interspecific conidia transmission was low.

Regarding pathogenicity to the vector, we noticed that the survival of the weaver ant major individual workers was mainly driven by their isolated or group status rather than by the contamination with the *M. anisopliae* pathogen conidia. Whether contaminated or not, major ants were indeed very sensitive to isolation stress and rapidly died when maintained individually, as observed in other ant species (Boulay et al. 1999; Wang et al. 2016). In a group composed of major ants only, there was a significant mortality observed when individuals were contaminated by pathogen conidia. However, when in a larger and more realistic group with both minor and major individuals, the presence of the pathogen conidia had no impact on the survival of the major individuals. These results highlight that (i) individual and limited social immunity are not enough to prevent the disease's onset, and that (ii) caste specialization probably explains the social immunity mechanisms leading to higher major worker survival (Tranter and Hughes 2015). Social immunity integrates behavioral traits used to limit the spread of diseases (Cremer et al. 2007; Cremer et al., 2018), such as grooming or chemical disinfection using acidic venom in *Oecophylla* ants (Tranter and Hughes 2015). Ants exhibit sophisticated social immune strategies illustrated by their capacity to learn and adjust their behavior according to the contamination risk (Cremer et al. 2007; Walker and Hughes 2009; Yanagawa et al. 2009; Stroeymeyt et al. 2014; Konrad et al. 2018). These integrate self- and allo-grooming behaviors that physically reduce body pathogen load, which were both observed by major and minor ant individuals in our experiments. Although allo-grooming from minor individuals towards the major workers is probably the major reason explaining survival of the latter, survival of the minor individuals themselves was significantly impacted by the presence of the pathogen conidia. Aside behavioral traits specific to each caste that suggesting this increased allo-grooming from minor towards major workers, caste-specific resistances to pathogens are also known (Poulsen et al. 2006; Koch et al. 2013; Quque et al. 2022). In weaver ants, major individuals are mainly responsible for foraging activities thus benefit from increased resistances to pathogens encountered in the environment. Nevertheless, cleaning from the minor ants upon returning to their colony might be vital to their survival when their pathogen load is high. In our experiments, minor individuals were themselves directly exposed to pathogens when forced to walk in an inoculated Petri dish, which would probably never occur in nature as they seldom leave their woven leaf nests (Way 1954). Overall, the resistance provided by the weaver ants' social immunity means that (i) inoculated major workers will survive contamination and not cause an epidemic in their colony, and that (ii) they could be used as entomovectors of this pathogen only if they are re-inoculated regularly, as each return to the colony results in cleaning by minor worker congeners.

Next, we observed that weaver ants were capable to auto-inoculate themselves with *M. anisopliae* conidia by walking in contaminated areas, even if they could detect its presence. From a behavioral perspective, both their indecision time and the number of U-turns when facing a zone inoculated with pathogen conidia were significantly increased compared to the control or corn starch alone treatments. A higher quantity of conidia further increased this reluctance, as observed by the initial latency in ant traffic on the bridge when confronted to the highest pathogen concentration. This was specifically linked to the pathogen presence and not the presence of any physical particles as corn starch, dispersed at the same concentration and with a similar texture (dry powder), did not generate any barrier effect. These results thus acknowledge the ability of the weaver ants to detect the pathogen conidia but also its concentration (Hölldobler and Wilson 1990; Yanagawa et al. 2009; Tranter et al. 2015). Further research is needed to investigate the detection mechanisms which appear to depend on the pathogen and the ant species (Tranter et al. 2015). For example, Pereira et al. (2020, 2021) showed that the ant *Myrmica rubra* L. 1758 did not detect and avoid areas contaminated with *Metarhizium brunneum* Petch, 1935. In our study, although the pathogen was clearly detected, the weaver ants did gradually cross the inoculated zone when motivated by a sucrose solution, and after around two hours its foraging activity reached the same level to that of the control. This gradual increase in activity in the presence of the pathogen could be explained because (i) a motivating chemical recruitment trail is deposited by certain pioneer foragers, (ii) a physical track with fewer pathogen conidia appeared on the auto-inoculation zone due to the repeated passage of weaver ants, and (iii) disinfection of the area possibly occurred through repeated acid excretion (Oi and Pereira 1993; Tragust et al. 2013; Tranter and Hughes 2015; Brütsch et al. 2017). We observed that even when a high quantity of pathogen conidia was placed on their route resulting in most members of the weaver ant colony being indecisive, certain individuals instantly crossed this inoculated zone. They subsequently found the sucrose solution and rapidly returned to the colony while depositing recruiting chemical trails, motivating other members to cross the conidia-inoculated tissue. This high-risk behavior of pioneer ants may counterbalance the indecisive behavior of others and could be explained by a differential sensitivity of ant individuals to detect the pathogen (Pereira and Detrain 2020).

Ants walking over the auto-inoculation zone with the highest concentration carried around 4.5×10^5 conidia on their bodies 20 min after initiating the experiment, down to around five times less after 24 h. In our situation, the addition of corn starch did not significantly increase this pathogen load, in contrast to previous findings (Mommaerts and Smagghe 2011; Mommaerts et al. 2011). In terms of load comparison, Smagghe et al. (2013) reported an average acquisition of 9.3×10^6 *M. anisopliae* conidia per bumblebee (*Bombus terrestris* L. 1758) after walking on a dispenser saturated with 10^7 conidia. This represents 20 times more conidia load per insect than in our study. In contrast to many Formicidae including the weaver ant which have rather slick bodies held high above the ground when walking, Apidae possess numerous setae and are more prone to touching surfaces with their bodies whilst on the ground, favoring retention of micro-particles (Michener et al. 1978; Smagghe et al. 2020; Temmermans and Smagghe 2022). However, Chailleux et al. (2023) showed that the LD50 (lethal dose that causes the death of 50 % of individuals in a group) at seven days was 1.69×10^3 conidia of *M. anisopliae* per *B. dorsalis* fly. The number of conidia dispersed by the weaver ants on the leaves in our experiment was between 6×10^4 conidia/cm^2 and 5.8×10^5 conidia/cm^2 depending on the distance from the inoculation zone, the time since inoculation and the dose of pathogen used. While still theoretically enough for lethal contamination of flies, (i) these still need to be transferred from the leaves to the flies, (ii) our experiment was done at a much smaller scale than that of a production mango tree which larger surface will decrease this density, and (iii) number of conidia found on leaves rapidly decreased over time and space.

In our experimental evaluation of the pathogen transfer and impact to *B. dorsalis*, we observed a significant increase in its mortality when in contact with mango tree organs after vectoring of *M. anisopliae* conidia over tree surfaces in both micro- and mesocosm conditions. After contamination, the lifespan of females was shorter than that of males in the microcosm experiment, which could be explained by the fact that females were seeking oviposition sites and thus spent more time on the

mango tree organs, increasing probability of contact with pathogen conidia. However, in the mesocosm experiment, only around 30 % of dead flies exposed to the pathogen showed conidiogenesis, suggesting that the experimental conditions themselves caused an increase in their mortality. Because these results were obtained under constrained experiments, where weaver ants, *M. anisopliae*, and target pest flies were forced to interact in limited spatial and temporal frames, we expected higher contamination and mortality rates of the target flies. From what we observed in the above results and our knowledge of the weaver ant's ecology, we predict much lower encounter rates between the three organisms in field conditions. For instance, the weaver ants use extensive territories on and out of their host tree (Hölldobler 1979; Hölldobler and Lumsden 1980), which, despite ensuring an extended surface dispersal of the conidia, would reduce the number of conidia dispersed per surface area.

In conclusion, although in our controlled conditions we observed that weaver ants were capable of physically contacting, carrying and dispersing quantities of *M. anisopliae* conidia sufficient to achieve significant fruit fly mortality at small scales, the use of this entomovectoring technique for field pest management strategy appears challenging. By itself, this strategy would not be sufficient to ensure B. dorsalis pest control in production orchards. The weaver ants' poorly adapted physical characteristics, their social immunity behaviors that include pathogen avoidance, grooming and chemical disinfection will altogether hamper pathogen vectoring in real conditions. Precision entomovectoring could nevertheless be viable as a curative treatment at critical crop phenological stages. For example, the encounter rate of conidia transferred from the vector to the target pest is expected to significantly increase during the mango fruiting period, when ants patrol on fruits and can directly encounter and predate female fruit flies seeking to oviposit (Vayssières et al. 2015). Increasing conidial load on the vector ant to bolster interspecific horizontal transfer to the target pest in the field requires to design an efficient dispensing device (see Maccagnani et al., 2020) and to optimize pathogen formulation (Barton et al. 2006). Combining sugar delivery with a pathogen dispenser device could be optimal, as in addition to fruit flies, scale insects will probably also be impacted by the pathogen. Concerning the pathogen formulation, techniques such as coating have proven successful to increase their appetence or reduce any repellent effect (Wang and Powell 2004; Gracia-Garza et al. 2007; Jackson et al. 2010), though this might prevent the ant vector from its detection with possible consequences on their fitness. Also, although the ants themselves negate the impact of the pathogen on their survival, this is probably not the case for other beneficial or neutral organisms that occur in the mango orchards. A non-target risk assessment linked to an entomovectoring strategy of *Metarhizium aniospliae* using weaver ants should therefore be conducted before any transfer to field applications.

Ethic statement

The authors declare no conflict of interest.

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CRediT authorship contribution statement

Thibault Nève de Mévergnies: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Samba Diop:** Methodology, Investigation. **Massamba Diakhaté:** Investigation. **Claire Detrain:** Writing – review & editing, Validation, Supervision, Resources, Methodology. **Frédéric Bouvery:** Methodology, Investigation, Conceptualization. **Thierry Brévault:** Writing – review & editing, Validation, Methodology, Funding acquisition, Conceptualization. **Anaïs Chailleux:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2025.105722.

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