Indigenous strains of *Beauveria* and *Metharizium* as potential biological control agents against the invasive hornet *Vespa velutina*

J. Poidatz, R. López Plantey, D. Thiéry

PII: S0022-2011(17)30258-6
DOI: https://doi.org/10.1016/j.jip.2018.02.021
Reference: YJIPA 7065

To appear in: *Journal of Invertebrate Pathology*

Received Date: 30 May 2017
Revised Date: 13 February 2018
Accepted Date: 28 February 2018

Please cite this article as: Poidatz, J., López Plantey, R., Thiéry, D., Indigenous strains of *Beauveria* and *Metharizium* as potential biological control agents against the invasive hornet *Vespa velutina*, *Journal of Invertebrate Pathology* (2018), doi: https://doi.org/10.1016/j.jip.2018.02.021

Indigenous strains of *Beauveria* and *Metharizium* as potential biological control agents against the invasive hornet *Vespa velutina*

Poidatz, J.[1]; López Plantey, R.[2] and Thiéry, D. [1]

1. UMR 1065 Santé et Agroécologie du Vignoble, INRA, Villenave d'Ornon, France.
2. Laboratory of Phytopathology, Institute of Agricultural Biology of Mendoza (IBAM), Mendoza, Argentina.

Corresponding author: Denis Thiery, UMR 1065 SAVE, INRA de Bordeaux, 71 rue Edouard Bourlaux, 33 882 Villenave d'Ornon Cedex, France, mail: denis.thiery@inra.fr.

Introduction

During the invasion process, alien species often miss parasites in invaded areas, because of reduced probability of their transport with the host and the unsuitability (climatic or biologic) of the invaded area for those parasites (Torchin *et al*., 2003). This reduced parasitism allows them, the alien species, to expand quickly and reach high population levels, which often results in damages (Torchin *et al*., 2002). Social insects are particularly good invaders, thanks to the adaptability provided by their life in society (Moller, 1996). Social insects are characterized by their group integration, the division of labor and the generation overlap; these characteristics are in favor of multiplied interactions, in particular in their nests. Indeed, members of the colony have to supply the nest with food, water and construction material foraged in the outside (Spradbery, 1973; Richter, 2000), and this enhances contamination transmission risks. Some ants developed prophylactic strategies to limit this risk (Cremer *et al*., 2007), like an ultra-specialization of the tasks to limit the interactions between extra and intra colony individuals, and limit direct queen and court interactions with the outside (Ugelvig and Cremer, 2007). For some bees, the use of chemicals to disinfect the colony structure from mandibular glands has been demonstrated (Cane *et al*., 1983), while for ants the use of formic acid is more favored (Stow and Beattie, 2008). However, the sociality level of Vespids is lower than for these bees or ants, the colonies being much smaller and less complex (Jeanson *et al*. 2007), and they might thus be more susceptible to infections.

Vespa velutina var. nigrithorax (Lepelletier, 1835) (Hymenopteran: Vespidae), native from East Asia, is an invasive predator of arthropods that was accidentally introduced in France around 2004. Since then, the “yellow legged hornet” spread in Europe: Spain, Portugal, Italy, Germany, Belgium, recently in England and in Scottland (Monceau and Thiéry, 2017). This species is a very efficient predator of pollinators, especially of honeybees, thus impacting both the apiculture and the global biodiversity directly and indirectly (Matsuura, 1988; Monceau *et*

al., 2014). *V. velutina* has an annual development cycle: a foundress initiates the nest in spring, the colony grows until the end of autumn when the new sexed (males and gynes) are produced. The colonies reach easily 4 000 individuals at this stage, and an estimation of the global population produced annually by a nest can reach 15 000 individuals (Rome *et al*., 2015). The nest is made by mixing plant fibres with water and saliva (Spradbery 1973) and closed with one entrance hole in its side. The nests are located mostly in open spaces (trees, brushes, under frames), and more rarely in closed places like roofs, holes *etc* (Monceau *et al*., 2014). The density of nests in invaded area can be impressive given the observed area (Monceau and Thiéry, 2016), and their destruction implies both material and qualified people. The methods that are currently used to limit the impact of *V. velutina* are a) trapping (for now nutrition-traps), in spring for foundresses, and in summer-autumn for apiaries protection by capturing hunters, b) physically protecting the apiaries by using nests, grills, and c) nest destruction, using chemical insecticides (powders of liquids) or Sulphur dioxide (gas). A significant impact of traps on non-target insects was already reported as well as their inefficiency (Beggs *et al*., 2011; Monceau *et al*., 2012; Monceau *et al*., 2013). The direct nest destruction methods by insecticide or gaseous Sulphur injection in the nest are efficient, but can have side effects on the environment if the nests are left in place after chemical treatment through food chain, and also for the applicator, with irritations and respiratory problems (H. Guisnel, Association Anti Frelon Asiatique, personal communication). Nevertheless, whatever the technique of nest control, locating the nests early in season, i.e. before predation on hives, remains the major unsolved limit, the colonies being discrete, numerous, often not accessible and well-hidden mostly in the trees foliage (Monceau *et al*., 2014).

Biological control is a long know methods in which microbial organisms control pest population by predation or parasitism in many countries (Lacey *et al*., 2015). Biological control, however, has not yet be examined with *V. velutina* about its risk on non-target

species, dispersion capacities nor non-adapted development cycle (Beggs *et al*., 2011; Monceau *et al*, 2014; Villemant *et al*., 2015). The possibility to use entomopathogenic fungi as an alternative method to the control with synthetic products has taken on some importance, emphasizing that practically all orders of the Insecta class are susceptible to be affected by entomopathogenic fungi (Alean, 2003; Rehner, 2005). On the other hand, a few studies on hymenopterans biocontrol by fungus exist: on micro-hymenopterans (Lord, 2001 (Bethylidae), Potrich *et al*., 2009 (Trichogrammatidae), Rossoni *et al*., 2014 (Braconidae), Agüero and Neves, 2014 (Scelionidae), Kpindou *et al*., 2007 (Encyrtidea), ants (Jaccoud *et al*., 1999; Tragust, 2013; Loreto and Hughes, 2016), and bees (most of the time for varroa treatment (Kanga *et al*., 2003; García-Fernández *et al*., 2008), or susceptibility (Conceição *et al*., 2014)); also Rose *et al*., 1999 and Harris *et al*., (2000) explored the potential control of such generalist entomopathogens on an invasive Vespidae, *Vespula vulgaris* (Vespidae), in New-Zealand.

Thirteen years after the introduction of *V. velutina* in France, the potential entomopathogenic fauna for this invasive hornet has not yet been studied, while we urgently need development of different biological control methods. This study aims to provide knowledge that could contribute to enlarge the panel of tools that can be used to control directly or indirectly the Asian hornet and limit its impact on bees, and also to reduce the risks on applicators.

The risks of contamination by infectious agents are significant in social insects and thus in hornets: they can be in contact with fungus in different ways, which inspired the modalities of inoculations and transfers of spores for us to complete this study: by direct contact with spores (rain, water), by walking on contaminated surfaces (ground, trees, preys, etc.), by eating contaminated food, and by trophallaxis or grooming with a contaminated individual from its colony. In this study we assessed the potential control efficiency of different indigenous

French isolates of entomopathogenic fungi that we inoculated to *V. velutina* by these different ways.

1. Material and methods

1.1 Insects collection

We collected individuals of *V. velutina* workers hunting in front of hives and in untreated nests. Before the experiment, the insects were maintained in groups in meshed boxes (10 x 20 x 10 cm) inside a climatic chamber at 23°C±1°C, 12h/12h light. They had *ad libidum* access to water and honey like in previous studies (Poidatz *et al*., 2017).

1.2 Fungus collection and multiplication

In the spring of 2015 a composite sampling of the first layer of the soil (20 cm) was made in the interrows of our experimental INRA vineyard (Villenave-d'Ornon, South West of France, 44°11847'30.4"N 0°34'36.9"W). This sampling consisted in the collection of 4 sub-samples per hectare, which were sieved up to 45 mesh and preserved at 4° C until use (Quesada-Moraga *et al*., 2007; Marques, 2012). A total of 20 sub-samples were collected for a surface of 5 hectares.

Afterwards, the bait insect technique (Asensio *et al*., 2003; Meyling, 2007; Tuininga *et al*., 2009) was carried out using L4 and L5 larvae instar of *Lobesia botrana* (Denis & Shiffermüller) (Lepidoptera: Tortricidae) which were placed in groups of 5 in Petri dishes with soil samples (3 replicates per one soil sample). The larvae were from the INRA laboratory colony isolate reared on artificial medium as described in Thiéry and Moreau (2005) (22°C, 60%HR, 16:8 lum.). The Petri dishes were placed at controlled temperature, humidity and photoperiod (24ºC, > 60% RH and 16:8). The dishes were observed daily and the individuals who manifested symptoms were transferred to a humid chamber in order to

favor the development and possible fructification of the entomopathogenic fungi. After isolating the fungi on this first bait, we screened them to see possible action on a hymenopteran, by inoculating them on adults of *Apis mellifera* (Hymenoptera: Apidae). By this technique were obtained the isolates of *Metarhizium robertsii* EF2.5 (2), EF3.5 (1), EF3.5 (2) and EF3.5 (4), that all had pathogenic action on hymenopterans. The growing and multiplication of all fungi took place in Petri dishes with OAC media (Oat 40g, Agar (PDA, BK095HA, Biokar) 20g, Chloramphenicol (SIGMA Aldrich, Germany) 50mg, QSP 1L) (Cañedo and Ames, 2004; Moino *et al.*, 2011; Marques, 2012) (darkness, room temperature).

The isolate of *Beauveria bassiana* BB came from Bretagne (North West of France). It was found directly in a foundress of *V. velutina* in spring 2016 (Poidatz *et al.*, in prep). After a rapid cleaning of the extern cuticle of the infected individual using a hypochlorite bath of 10 seconds, we cut the hornet in 3 parts that we placed in different Petri dishes on growing media OAC as described before. All isolates were purified by multi-passaging, i.e. multiple subculture of the fungi in Petri dishes for minimum 5 generations.

1.3 Inoculation methods

For assessing the potential control efficiency of the different isolates, we did different inoculation methods: direct contact: Direct, by contact with an inoculated surface: Contact, in the food: Food or inter-individual transfer: Transfer (Fig. 1).

All the petri dishes roofs were pierced with a thin needle for aeration (15 holes) a day before the experiment. The day of the experiment, maximum two hours before application, the spore suspensions were prepared under sterile conditions, and fixed at a concentration around 10^7 spores/ml of pure sterile water. As we treated very quickly the hornets after making the spore suspension, we didn't add any solvent in the suspension. For the control hornets in each modality, we used distilled water instead of spore suspension.

The hornets were cooled 20 min in falcon tubes that were put in ice, so they can be manageable during the fungus inoculation. The hornet workers don't survive very long when they were isolated (personal observation), probably because of social grooming lacks. We thus decided to leave them in groups after inoculation. For the three first treatment methods, the hornets were put in groups of five in each Petri dishes of 10cm diameter, that contained a thick filter paper on the ground, a cup with water in cotton, and a cup with food (candi sugar (glucose, fructose and saccharose) purchased from ®NutriBee propolis (Vétopharma)). For the fourth treatment method, c.a. contamination by transfer, we chose bigger pots (plastic honey pots, 9cm diam x 10 cm diam x 12 cm high) with a strip of embossed paper allowing the hornets to climb on it thus to avoid forced contacts. After placing the hornets in the different arenas, we waited 5-10 minutes for hornets wake up.

After inoculation, the boxes containing the hornets contaminated by the different modalities were all placed in a climatic chamber at 23°C±1°C, photoperiod of 12h.

Four repetitions of the bioassay were made: in October 2015 (10 individuals / modalities ((*Metarhizium r.* 4 isolates + control) x 4 inoculation methods); Nb individuals=200, N=2), in August 2016 (10 individuals / modalities ((all 5 isolates + control) x 4 inoculation methods); Nb individuals=240; N=2), the same in September 2016 (Nb individuals=240, N=2) and in October 2016 (Nb individuals=240, N=2).

- **Direct inoculation**

The hornets were contaminated by immersion (<1sec) in a spore suspension. The forceps used to manipulate the hornets for this method were first disinfected with ethanol (90%) then washed with water before switching from one isolate to another.

- **Inoculation by contact**

In this modality, 3ml of spore suspension was poured uniformly on the filter paper in the Petri dish using a pipette. The paper dried five minutes before the candy, the water and the hornets were put inside the box.

- **Inoculated food**

In this modality, 1ml of spore suspension was poured in 10mg of cooked tuna (from the market). The fish was left in the boxes only 24h to avoid hornet intoxication by potential bacterial development.

- **Inter hornets contamination, transfer**

Four hornets were placed in a pot as described above in 1.3. One extra individual was directly inoculated as described in "direct inoculation" paragraph, then placed on the opposite side of the box from the other hornets before reanimation.

1.4 Measured parameters

- *Mortality Index (MI)*

Each day after the inoculation, we removed the dead hornets from the different boxes and placed them individually in labeled hemolysis tubes closed by a cotton copper. We maintained the humidity of the tubes by adding distilled water in the copper using a pipette.

The isolated dead individuals were then observed each day for the fungus to emerge from the cuticle's intersections (Fig. 2.A, 2.B). The death of the hornets could be due to multiple factors (Fig. 2.C, 2.D, or bacterial infection, stress, etc.). Each death caused by entomo-pathogen infection was then counted, to correct the number of dead by treatment and obtain the number of dead by entomo-pathogenic infection per treatment.

$$\text{MI} = \frac{Control\ alive\ insect - Treatment\ alive\ insect}{Control\ alive\ insect}$$

- *Lethal time 50 (LT50)*

The LT50 is the moment after inoculation when 30% of the hornets died by infection.

1.5 Statistical analysis

All results have been analyzed in ANOVA with a test LSD Fisher (alpha= 0,05) using the software Infostat update 2016.

2. Results

No death of hornets due to entomopathogenic fungi was observed in the control.

2.1 Comparisons of the inoculation methods

For all the isolates, the most efficient modality concerning mortality was the direct inoculation, statistically more efficient than all inoculation modalities. The contact method was not different from the transfer method, and the transfer method was not different from the food method. This last treatment (food) was less efficient than the contact modality (LSD Fisher test) (Table 1).

Table 1: Average mortality and compared mortality in function of inoculation methods. Values with the same letter are not significantly different (p > 0,05) after parametric LSD Fisher test (alpha= 0,05; DMS= 0,12264; Error: 0,0746; gl: 151).

TREATMENT	***AVERAGE***	***N***	***SD***	
Food	*0.14*	*38*	*0.04*	*A*
Transfer	*0.26*	*38*	*0.04*	*A B*
Contact	*0.35*	*38*	*0.04*	*B*
Direct	*0.60*	*41*	*0.04*	*C*

2.2 Comparison on methods and isolates

For all strains the direct application method was the most efficient, and except in EF3.5(2), the contact method appeared to have mild effect. In EF2.5(2) and EF3.5(1) the transfer between individuals was also quite efficient, when in EF3.5(2) no differences between inoculation method could be assessed (Fig. 3).

No significant difference was observed between the different application methods in function of isolates and fungi in their LT50 (ANOVA).

- *Mortality.*

Considering all the inoculation methods, no difference could be found amongst the isolates virulence (ANOVA, $p = 0.31$). No difference could be found amongst the isolates for the direct inoculation method (ANOVA, $p = 0.14$), neither for the contact (ANOVA, $p = 0.24$) nor the transfer (ANOVA, $p = 0.47$) inoculation method, however, for the food inoculation method there was a difference (ANOVA, $p = 0.009$): the isolate EF3.5(2) was significantly more efficient (LSD Fisher test).

- *Lethal Time 50 (LT50).*

Table 2: Lethal time 50 of entomopathogenic isolates, i.e. time to kill 50% of the hornets, in function of the inoculation methods.

ISOLATE	**LT50 (DAYS, AVERAGE±SD)**
EF2.5(2)	5.68 ± 1.08
EF3.5(1)	5.86 ± 1.17
EF3.5(2)	5.49 ± 1.38
EF3.5(3)	5.41 ± 1.18

B	6.25 ± 0.67

No significant difference was observed between the different isolates and fungi in their LT50 (Table 2).

2.3 Comparisons of each method for each isolate

3. Discussion

This study offers two subjects, the first in the primary knowledge about the mode of infection of entomopathogenic fungi over *Vespa* species, and the second is the prospective of future control of *V. velutina*. We demonstrated that French indigenous entomopathogenic fungi coulb be developed for *Vespa* control by assessing their efficiency with different inoculation approach.

Microbial agents must be rigorously evaluated for reducing the potential environmental impact by its prescribed use. The main properties attributed to the use of entomopathogenic fungi are: strong specificity between pathogen-host, almost no presence of toxic residues, persistence in time after application, a lower cost than synthesis products, respect for biodiversity, but also a high potential as a source of metabolites for the creation of alternative phytosanitary products, etc… (Franco *et al.*, 2012).

Among the diversity of entomopathogenic fungi, the literature cites two gender as the most described and used in biological control: *Metarhizium* spp. and *Beauveria* spp. (both Ascomycota: Hypocreales) (Bidochka *et al.*, 1998; Bidochka and Small, 2005; Rehner, 2005). Both genders can have host specificity given the isolates and climatic conditions (Ignoffo,

1992; Rangel *et al*. 2015). The infection mechanism is quite similar in these two fungi, being first a phase of recognition and fixation of the spore to the insect host, its penetration in the insect tegument, then the evasion of the host immune defenses, the proliferation in the host body (provoking the host death), and finally the reemergence from the host and sporulation (Boucias and Pendland, 1991; Bidochka and Small, 2005; Ortiz-Urquiza and Keyhani, 2013). Isolates of *Metarhizium* are already on the market for biological control of pests, mostly lepidopteran and dipteran control (Appendix 1). *Beauveria bassiana* (Bals.) Vuill. has yet no host specificity connected to genetic described in the European clade (Rehner, 2005).

The reduction of adult hornet longevity by the application of the different isolates observed in the study is coherent with the study of Harris *et al*., 2000 on the same type of pathogens (2.1 to 5.6 days). The quite high variability in the mortality intra/inter sessions could be explained here by the fact that the hornets used in this study to be contaminated were savage individuals collected in the field, with unknown variation in their age, past, and therefore in their immune system and sensitivity to infection (Franceschi *et al*., 2000; Moret and Schmid-Hempel, 2000; Rolff, 2001; Doums *et al*., 2002). In the direct contamination treatment we observed the cumulated effect of both direct contamination and transfer between the adults.

The applied possibilities of these biocontrol methods of inoculation are numerous, but given our results, the one that seems best suited for *V. velutina* control is direct application of spore suspensions on and in the hornet colony. A direct treatment of nests could cumulate the effects of the “direct”, the “contact” and the “transfer” application modalities. Tests on nests have to be made to assess the isolates efficiency and to monitor the inoculum quantity needed given the nest size, as done in Harris *et al*. 2000 (effect on emergence rate and adult survival).

The “contaminated food” modality was not very efficient on the adults for all the tested isolates, but we have to note that the workers may be interested in not eating but collecting it for further use, i.e. mostly nutrition of the larvae as the prepared food was protein-made.

Adult hornets don't need proteins for their survival, they depend only on carbohydrates consummation (Spradberry, 1973; Richter, 2000). Monceau *et al*. 2014 showed that the roles concerning nest defense of *V. velutina* seem to develop with their age, and we can thus hypothesize that the attraction to protein food could also depend on age. To assess the control potential of this modality at the nest scale, further studies on the impact of contaminated food on hornet's larvae should be investigated, using different kinds of food.

We observed different responses of entomopathogenic fungal isolates according to the application methods to examine Trojan horse strategy. Trojan horse strategy is the method to use workers to bring a disturbing bait into their colonies, in our case using entomopathogens. It is the strategy after having fungal spray on nest. It could be envisaged as two different ways. First, by actively trapping and directly contaminating *V. velutina* workers with a spore suspension before releasing them so that they can return to their colony. Second, by using food bait contaminated with the fungi, that will be brought back to the larvae: but to do this, a selective "trap" must be fabricated, that will capture and let go hornet workers with the contaminated food, but not other species.

For potential biocontrol solution, a combination of several fungi isolates could thus be investigated. Moreover, the climatic requirements and adaption of isolates (García-Fernandez *et al*., 2008) were not evaluated in our study for the experimental purpose: a combination of several isolates adapted to different climatic conditions could thus overcome such eventual limits and enhance the biocontrol efficiency (Inglis *et al.*, 1995). Two risks could however be considered in the case of isolate combinations: the possibility of competition between fungal and possible decrease of efficiency, and the panel enlargement of non-target insects that could be contaminated.

The risk of transmission of such biological agents to other insects has not been measured yet but should be considered low, however the existence of the risk to infect non-targeted sources

(e.g. foraging sites of the hornets) must be kept in mind. The UV deactivation of spores potentially transported by the hornets may be very efficient (Ignoffo, 1992; Inglis *et al.*, 1995; Fernandes *et al*., 2015), and added with dehydration it could impact significantly the spore survival on non-targeted sources. Another point to check is the mobility and behavior of infected hornets which will determinate any possible dispersion of the contamination.

From an applied point of view, the efficiency of these isolates could be enhanced by adapted formulations, which could improve their infection efficiency, pathogenicity duration, climatic resistance, most of the time using carrier, natural or synthetic oils (Inglis *et al.*, 1995; Thompson *et al*., 2006; Fernandes *et al*., 2015; Hicks, 2016). All these factors make the formulation play an important role at the persistency of entomopathogenic fungi in the environment (Burges, 1998; Parker *et al.* 2015), where a good composition of additives could give a better way to hold the fungus species during time, even months after treatment.

To conclude, this is the first study exploring the potential efficiency of indigenous entomopathogens to biologically control the invasive Asian hornet *Vespa velutina*. We tested five isolates with different inoculation methods on workers, and found that they are efficient when applied directly. Some future work should be done on larvae and on whole nests, in different climatic conditions, to conclude about the potential treatment efficiency.

Author Contributions statement

JP, RLP and DT conceived the ideas and designed methodology; JP and RLP collected the data; JP and RLP analyzed the data; JP, RLP and DT wrote the manuscript.

Acknowledgements

This work was done during the PhD thesis of the first and the second author. We thank Olivier Bonnard and Salomé Gotreau for helping us capture the hornets used in the experiment. We thank Bayer Crop Science, represented by Dr. Bénédicte Laborie, for the first author financial support. We thank Mrs Avril Welwood for English revising.

Conflicting interest.

The authors declare no conflicting interest in this research

Appendix

Appendix 1: Examples of biological control agent formulations in the market, for *Metarhizium sp*. and *Beauveria bassiana*.

Fungi	***Metarhizium sp.***	***Beauveria bassiana***
commercial appellation	AGO BIOCONTROL METARHIZIUM 50®, BioGreen®, GREEN GARD®, BIO 1020®, Green Muscles® (Bidochka and Small, 2005)	BioPower®, Naturalis®, Biosoft®, Ostrinil®

References

Agüero, F.M.A., Neves, O.J., 2014. Selectivity of *Metarhizium anisopliae* to *Telenomus podisi* (Hymenoptera: Scelionidae). Investigación Agraria 16, 21–28.

Alean, I.R.I.N.A., 2003. Evaluación de la patogenicidad de diferentes hongos entomopatógenos para el control de la mosca blanca de la yucca *Aleurotrachelus socialis* Bondar (Homoptera: Aleyrodidae) bajo condiciones de invernadero. Grade work presented as final project for the Agricultural Microbiology and Veterinaty Grade, Pontifícia Universidad Javeriana. Facultad de Ciencias Básicas Microbiología Agrícola y Veterinaría, Colombia.

Arca, M., Mougel, F., Guillemaud, T., Dupas, S., Rome, Q., Perrard, A., Muller, F., Fossoud, A., Capdevielle-Dulac, C., Torres-Leguizamon, M., Chen, X.X., Tan, J.L., Jung, C., Villemant, C., Arnold, G., Silvain, J.-F., 2015. Reconstructing the invasion and the demographic history of the yellow-legged hornet, *Vespa velutina*, in Europe. Biological Invasions 17, 2357–2371. doi:10.1007/s10530-015-0880-9

Asensio, L., Carbonell, T., López-Jimenez, J.A. and López-Llorca, L.V., 2003. Entompathogenic fungi in soils from Alicante province. *Spanish Journal of Agricultural Research* 1, 37-45.

Beggs, J.R., Brockerhoff, E.G., Corley, J.C., Kenis, M., Masciocchi, M., Muller, F., Rome, Q., Villemant, C., 2011. Ecological effects and management of invasive alien Vespidae. BioControl 56, 505–526. doi:10.1007/s10526-011-9389-z

Bessa, A.S., Carvalho, J., Gomes, A., Santarém, F., 2016. Climate and land-use drivers of invasion: predicting the expansion of *Vespa velutina nigrithorax* into the Iberian Peninsula. Insect Conservation and Diversity 9, 27–37. doi:10.1111/icad.12140

Bidochka, M.J., Kasperski, J.E., Wild, G.A., 1998. Occurrence of the entomopathogenic fungi Metarhizium anisopliae and *Beauveria bassiana* in soils from temperate and near-northern habitats. Canadian Journal of Botany 76, 1198–1204.

Bidochka, M.J., Small, C., 2005. Phylogeography of Metarhizium, an insect pathogenic fungus. In Insect-Fungal associations: Ecology and evolution. Ed Fernando E. Vega,Meredith Blackwell, Oxford University press, London.

Boucias, D.G., Pendland, J.C., 1991. Chapter 5 : Attachment of Mycopathogens to cuticle. In "The fungal spore and disease initiation in plants and animals". G.T. Cole *et al*. (eds.). Springer Science + Business Media New York. Pp101-127.

Buczkowski, G., 2016. The Trojan horse approach for managing invasive ants: a study with Asian needle ants, *Pachycondyla chinensis*. Biological Invasions 18, 507–515. doi:10.1007/s10530-015-1023-z

Burges, H.D., 1998. Formulation of myoinsecticides. In H. S. Burges (Ed.), Formulation of microbial biopesticides: Benefical microorganisms, nematodes and seed treatments. Dordrecht: Kluwer Academic, 131-185.

Cane, J.H., Gerdin, S., Wife, G., 1983. Mandibular gland secretions of solitary bees (Hymenoptera: Apoidea): potential for nest cell disinfection. Journal of the Kansas Entomological Society, 199–204.

Cañedo, V. and Ames, T., 2004. Manual de laboratorio para el manejo de hongos entomopatógenos .V. Cañedo and T. Ames, Eds. *International Center of Potato*, Lima, Peru: CIP. http://doi.org/cip@cgiar.org, www.cipotato.org

Conceição, P. de J., Neves, C.M. de L., Sodré, G. da S., Carvalho, C.A.L. de, Souza, A.V., Ribeiro, G.S., Pereira, R. de C., 2014. Susceptibility of *Melipona scutellaris* Latreille, 1811 (Hymenoptera: Apidae) worker bees to *Beauveria bassiana* (Bals.) Vuill. Sociobiology 61, 184–188.

Cremer, S., Armitage, S.A.O., Schmid-Hempel, P., 2007. Social Immunity. Current Biology 17, R693–R702. doi:10.1016/j.cub.2007.06.008

Darrouzet, E., Gévar, J., Dupont, S., 2015. A scientific note about a parasitoid that can parasitize the yellow-legged hornet, *Vespa velutina nigrithorax*, in Europe. Apidologie 46, 130–132. doi:10.1007/s13592-014-0297-y

Doums, C., Moret, Y., Benelli, E., Schmid-Hempel, P., 2002. Senescence of immune defence in *Bombus* workers. Ecological Entomology 27, 138–144.

Fernandes, E.K.K.; Rangel, D.E.N., Braga. G.U.L.; Roberts, D.W., 2015. Tolerance of entomopathogenic fungi to ultraviolet radiation: a review on screening of strains and their formulation. Current Genetics, 19 May 2015. DOI: 10.1007/s00294-015-0492-z

Franceschi, C., Bonafè, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., De Benedictis, G., 2000. Inflamm-aging: an evolutionary perspective on immunosenescence. Annals of the New York Academy of Sciences 908, 244–254.

Franco, K., Rodríguez, S., Cervantes, J., Barranco, J., 2012. Enzimas y toxinas de hongos entomopatógenos, su aplicación potencial. Sociedades Rurales, Producción y Medio Ambiente, Mexico 12, 143-160.

García-Fernández, P., Santiago-Álvarez, C., Quesada-Moraga, E., 2008. Pathogenicity and thermal biology of mitosporic fungi as potential microbial control agents of *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of honey bee, *Apis mellifera* (Hymenoptera: Apidae). Apidologie 39, 662–673. doi:10.1051/apido:2008049

Grosso-Silva, J.M., Maia, M., 2012. *Vespa velutina* Lepeletier, 1836 (Hymenoptera, Vespidae), new species for Portugal. Arquivos entomolóxicos, 53–54.

Harris, R.; Harcourt, S.J.; Glare, T.R.; Rose, E.A.F. and Nelson, T.J., 2000. Susceptibility of *Vespula vulgaris* (Hymenoptera: Vespidae) to Generalist Entomopathogenic Fungi and their potential for wasp control. Journal of Invertebrate Pathologi 75, 251-258. DOI: 10.1006/jipa.2000.4928

Hicks, B.J., 2016. Optimization of *Beauveria bassiana* in a spray formulation against *Choristoneura fumiferana*. Canadian Journal of Forest Research 46, 543–547. doi:10.1139/cjfr-2015-0435

Ignoffo, C.M., 1992. Environmental Factors Affecting Persistence of Entomopathogens. The Florida Entomologist, 75(4):516-525.

Inglis, G.D.; Goettel, M.S.; Johnson, D.L., 1995. Influence of ultraviolet light protectans on persistence of the entomopathogenic fungus, *Beauveria bassiana*. Biological Control, 5(4):581-590.

Jaccoud, D. b., Hughes, W. o. h., Jackson, C. w., 1999. The epizootiology of a *Metarhizium* infection in mini-nests of the leaf-cutting ant *Atta sexdens rubropilosa*. Entomologia Experimentalis et Applicata 93, 51–61. doi:10.1046/j.1570-7458.1999.00561.x

Jeanson, R., Fewell, J. H., Gorelick, R., 2007. Emergence of increased division of labor as a function of group size. *Behav Ecol Sociobiol* 62, 289-298.

Kanga, L.H.B., Jones, W.A., James, R.R., 2003. Field trials using the fungal pathogen, *Metarhizium anisopliae* (Deuteromycetes: Hyphomycetes) to control the ectoparasitic mite, *Varroa destructor* (Acari: Varroidae) in honey bee, *Apis mellifera* (Hymenoptera: Apidae) colonies. Journal of economic entomology 96, 1091–1099.

Kpindou, O.K.D., Sinsin, B., Kooyman, C., 2007. Impact de *metarhizium anisopliae* var acridum, sur les hyménoptères : cas de *Anagyrus lopezi* (De Santis) et de *Cotesia Plutellae* (Kurdjumov). Annales des Sciences Agronomiques 9. doi:10.4314/asab.v9i1.43221

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: Back to the future. Journal of Invertebrate Pathology 132, 1-41.

López, S., González, M., Goldarazena, A., 2011. *Vespa velutina* lepeletier, 1836 (Hymenoptera: Vespidae): first records in Iberian Peninsula. EPPO Bulletin 41, 439–441.

Lord, J.C., 2001. Response of the Wasp *Cephalonomia tarsalis* (Hymenoptera: Bethylidae) to *Beauveria bassiana* (Hyphomycetes: Moniliales) as Free Conidia or Infection in Its Host, the Sawtoothed Grain Beetle, *Oryzaephilus surinamensis* (Coleoptera: Silvanidae). Biological Control 21, 300–304. doi:10.1006/bcon.2001.0942

Loreto, R.G., Hughes, D.P., 2016. Disease Dynamics in Ants, in: Advances in Genetics. Elsevier, pp. 287–306.

Makino, S. 'ichi, Kawashima, M., Kosaka, H., 2011. First record of occurrence of *Xenos moutoni* (Strepsiptera; Stylopidae), an important parasite of hornets (Hymenoptera: Vespidae: Vespa), in Korea. Journal of Asia-Pacific Entomology 14, 137–139. doi:10.1016/j.aspen.2010.09.001

Manley, R.; Boots, M. And Wilfert, L., 2015. Emerging viral disease risk to pollinating insects: ecological, evolutionary and anthropogenic factors. Journal of Applied Ecology, 52:331-340. DOI: 10.1111/1365-2664.12385

Marques, G., 2012. Biodiversidade functional do solo: qual o papel dos fungos entomopatogénicos na protecção da vinha? CITAB Presentation, University if Tràs-os-Montes e Alto Douro, Portugal 28th October.

Matsuura, M., 1988. Ecological study on vespine wasps (Hymenoptera : Vespidae) attaking honeybee colonies. 1. Seasonal changes in the frequency of visits to apiaries by vespine wasps and damage inflicted, especially in the absence of artificial protection. Appl. Ent. Zool. 23, 428-440.

Meyling, N.V., 2007. Methods for isolation of entomopathogenic fungi from soil environment. Laboratory manual, Department of Ecology, Faculty of Life Sciences, University of Copenhagen.

Moino, A., Sousa Cavalanti, R., 2011. Hongos y netmatodos entomopatógenos. Manual de biología de suelos tropicales. Ed. del Instituto Nacional de Ecología y Cambio Climático, Mexico. pp. 287-295.

Moller, H., 1996. Lessons for invasion theory from social insects. Biological Conservation 78, 125-142.

Monceau, K., Bonnard, O., Thiéry, D., 2012. Chasing the queens of the alien predator of honeybees: A water drop in the invasiveness ocean. Open Journal of Ecology 02, 183–191. doi:10.4236/oje.2012.24022

Monceau, K., Arca, M., Leprêtre, L., Mougel, F., Bonnard, O., Silvain, J.-F., Maher, N., Arnold, G., Thiéry, D., 2013. Native Prey and Invasive Predator Patterns of Foraging Activity: The Case of the Yellow-Legged Hornet Predation at European Honeybee Hives. PLoS ONE 8, e66492. doi:10.1371/journal.pone.0066492

Monceau, K., Bonnard, O., Thiéry, D., 2014. *Vespa velutina*: a new invasive predator of honeybees in Europe. Journal of Pest Science 87, 1–16. doi:10.1007/s10340-013-0537-3

Monceau, K., Thiéry, D., 2016. *Vespa velutina* nest distribution at a local scale: An eight-year survey of the invasive honeybee predator. Insect Science. doi:10.1111/1744-7917.12331

Monceau, K., Thiéry, D., 2017. *Vespa velutina* : current situation and perspectives. Atti Accademia Nazionale Italiana di Entomologia Anno LXIV, 137-142. (In press)

Moret, Y., Schmid-Hempel, P., 2000. Survival for immunity: the price of immune system activation for bumblebee workers. Science 290, 1166–1168.

Ortiz-Urquiza, A., Keyhani, N.O., 2013. Action on the Surface: Entomopathogenic fungi versus Insect Cuticle. Insects, 4:357-374. DOI: 10.3390/insects4030357

Parker, B.L., Skinner, M., Gouli, S.Y., Gouli, V.V., Tobi, D., Kim, J.S., 2015. Persistence of *Beauveria bassiana sensu lato* and *Metarhizium anisopliae sensu lato* in Vermont (USA) forest soil. Biocontrol Science and Technology 25, 768-788. doi: 10.1080/09583157.2015.1016895

Poidatz, J., Bressac, C., Bonnard, O., Thiéry, D. 2017. Delayed sexual maturation in males of *Vespa velutina*. Insect Science, DOI 10.1111/1744-7917.12452

Porporato, M., Manino, A., Laurino, D., Demichelis, S., 2014. *Vespa velutina* Lepeletier (Hymenoptera Vespidae): a first assessment two years after its arrival in Italy. Redia 97, 189–194.

Potrich, M., Alves, L.F.A., Haas, J., Silva, E.R.L. da, Daros, A., Pietrowski, V., Neves, P.M.O.J., 2009. Selectivity of *Beauveria bassiana* and *Metarhizium anisopliae* to *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae). Neotropical Entomology 38, 822–826. doi:10.1590/S1519-566X2009000600016

Quesada-Moraga, E., Navas-Cortés, J.A., Maranhao, E.A., Ortiz-Urquiza, A., Santiago-Álvarez, C., 2007. Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soil. Mycological research 111, 947-966.

Rangel, D.E.N., Braga, G.U.L., Fernandes, E.K.K., Keyser, C.A., Hallsworth, J.E., Roberts, D.W., 2015. Stress tolerance and virulence of insect-pathogenic fungi are determined by environmental conditions during colonial formation. Current Genetics. DOI: 10.1007/s00294-015-0477-y

Rehner, S.A., 2005. Phylogenetics of the genus *Beauveria*. In "Insect-Fungal associations: Ecology and Evolution". Ed Fernando E. Vega & Meredith Blackwell, Oxford University Press, pp 3-27.

Richter, M.R., 2000. Social wasp (Hymenoptera: Vespidae) foraging behavior. Annual review of entomology 45, 121–150.

Rolff, J., 2001. Effects of age and gender on immune function of dragonflies (Odonata, Lestidae) from a wild population. Canadian Journal of Zoology 79, 2176–2180. doi:10.1139/cjz-79-12-2176

Rome, Q., Dambrine, L., Onate, C., Muller, F., Villemant, C., García Pérez, A.L., Maia, M., Carvalho Esteves, P., Bruneau, E., 2013. Spread of the invasive hornet *Vespa velutina* Lepeletier, 1836, in Europe in 2012 (Hym., Vespidae). Bull Soc Entomol Fr 118, 21–22.

Rome, Q., Muller, F.J., Touret-Alby, A., Darrouzet, E., Perrard, A., Villemant, C., 2015. Caste differentiation and seasonal changes in *Vespa velutina* (Hym.: Vespidae) colonies in its introduced range. Journal of Applied Entomology 139, 771–782. doi:10.1111/jen.12210

Rose, E.A.F., Harris, R.J., Glare, T.R., 1999. Possible pathogens of social wasps (Hymenoptera: Vespidae) and their potential as biological control agents. NZ J Zool 26,179–190.

Rossoni, C., Kassab, S.O., Loureiro, E.D.S., Pereira, F.F., Costa, D.P., Barbosa, R.H., Zanuncio, J.C., 2014. *Metarhizium anisopliae* and *Beauveria bassiana* (Hypocreales: Clavicipitaceae) are Compatible with *Cotesia flavipes* (Hymenoptera: Braconidae). Florida Entomologist 97, 1794–1804. doi:10.1653/024.097.0455

Spradbery, J.P., 1973. Chap 6: Feeding and Foraging. In "Wasps. An account of the biology and natural history of solitary and social wasps". Ed Sidgwick & Jackson, London. Pp128-157

Thiéry, D., Moreau, J., 2005. Relative performance of European grapevine moth (*Lobesia botrana*) on grapes and other hosts. Oecologia 143, 548-557.

Thompson, S.R.; Brandenburg, R.L.; Arends, J.J., 2006. Impact of moisture and UV degradation on *Beauveria bassiana* (Balsamo) Vuillemin conidial viability in turfgrass. Biological Control, 39, 401-407.

Torchin, M.E., Lafferty, K.D., Kuris, A.M., 2002. Parasites and marine invasions. Parasitology 124. doi:10.1017/S0031182002001506

Torchin, M.E., Lafferty, K.D., Dobson, A.P., McKenzie, V.J., Kuris, A.M., 2003. Introduced species and their missing parasites. Nature 421, 628–630.

Tragust, S., Ugelvig, L.V., Chapuisat, M., Heinze, J., Cremer, S., 2013. Pupal cocoons affect sanitary brood care and limit fungal infections in ant colonies. BMC evolutionary biology 13, 1.

Tuininga, A.; Miller, J.; Morath, S.; Daniels, T.; Falco, R.; Machese, M.; Sahabi, S.; Rosa, D., Stafford, K., 2009. Isolation of Entomopathogenic Fungi from Soils and *Ixodes scapularis* (Acari: Ixodidae) Ticks: Prevalence and Methods. *J. Med. Entomol.* 46, 557-565.

Ugelvig, L.V., Cremer, S., 2007. Social Prophylaxis: Group Interaction Promotes Collective Immunity in Ant Colonies. Current Biology 17, 1967–1971. doi:10.1016/j.cub.2007.10.029

Villemant, C., Zuccon, D., Rome, Q., Muller, F., Poinar Jr, G. O., Justine, J. L., 2015. Can parasites halt the invader? Mermithid nemathodes parasitizing the yellow-legged Asian hornet in France. PeerJ 3:e947, DOI 10.7717/peerj.947

Stow, A., Beattie, A., 2008. Chemical and genetic defenses against disease in insect societies. Brain, Behavior, and Immunity 22, 1009–1013. doi:10.1016/j.bbi.2008.03.008

Figure Captions

Figure 1: The different inoculation methods used in the experimentation. Direct : direct inoculation of the hornets (orange), Contact : inoculated filter paper, Food : inoculated food (IF, orange), and Transfer : one inoculated individual (orange) with four uncontaminated individuals. In each box there are food (F) and water (W).

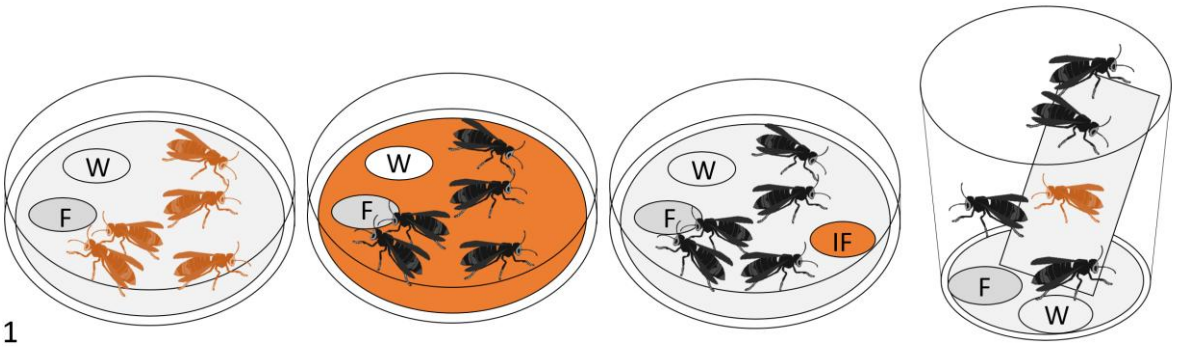
Figure 2: *V. velutina* workers infected by entomopathogens. A. An entomopathogenic fungus is making its way between the cuticle's segments of the abdomen of a hornet. B. Two contaminated hornets by *Metarhizium robertsii* (white mycelium, olive green spores)(top) and *Beauveria bassiana* sp. (white mycelium, cream spores)(bottom). A dead hornet with opportunistic fungus that is growing on its surface (*Penicilium sp.* C, *Aspergilus sp.* D).

Figure 3: Lethality of entomopathogens isolates in function of the inoculation methods, i.e. percentage of dead hornets by entomopathogen infection. (ANOVA tests).The vertical bar over the column represent standard deviation (SD).

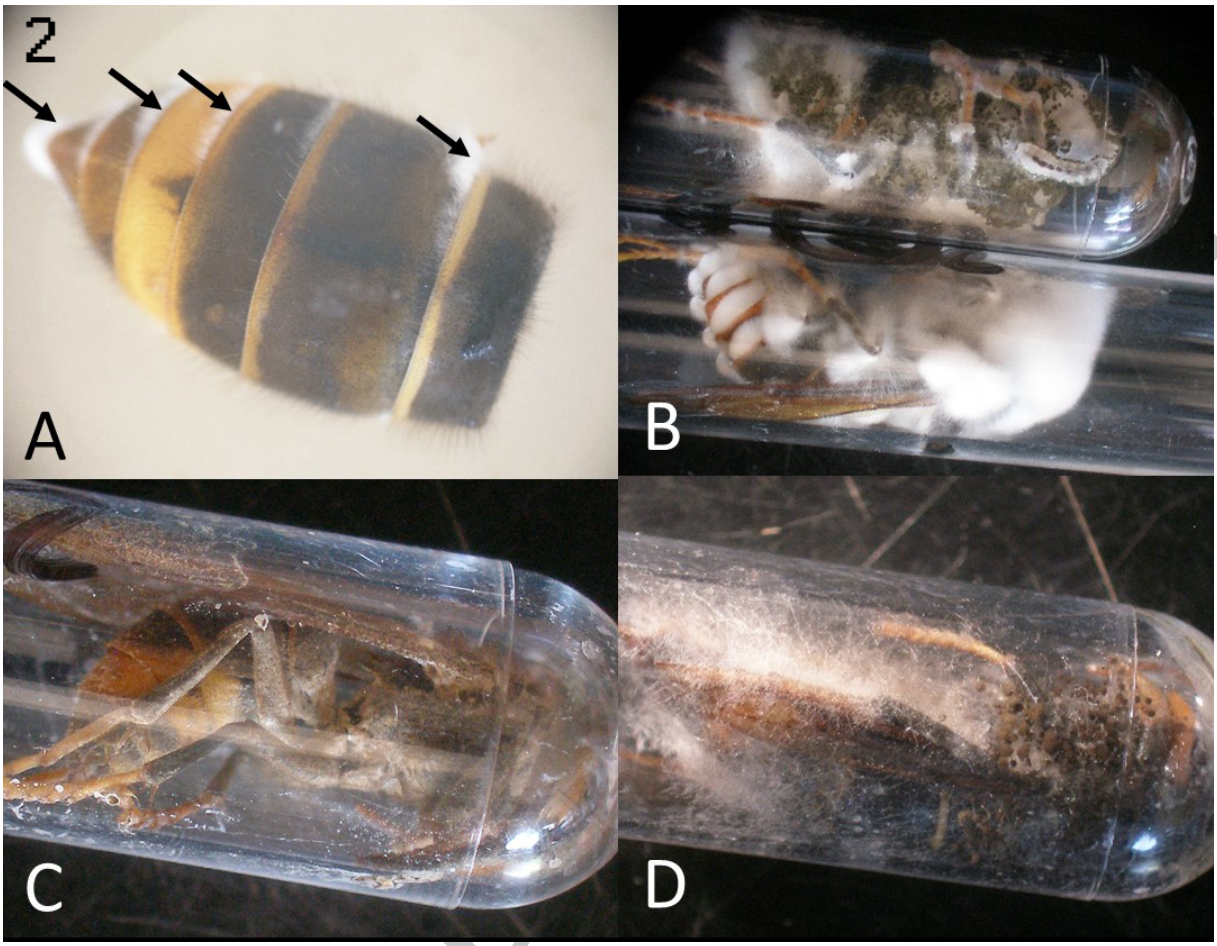
Table captions

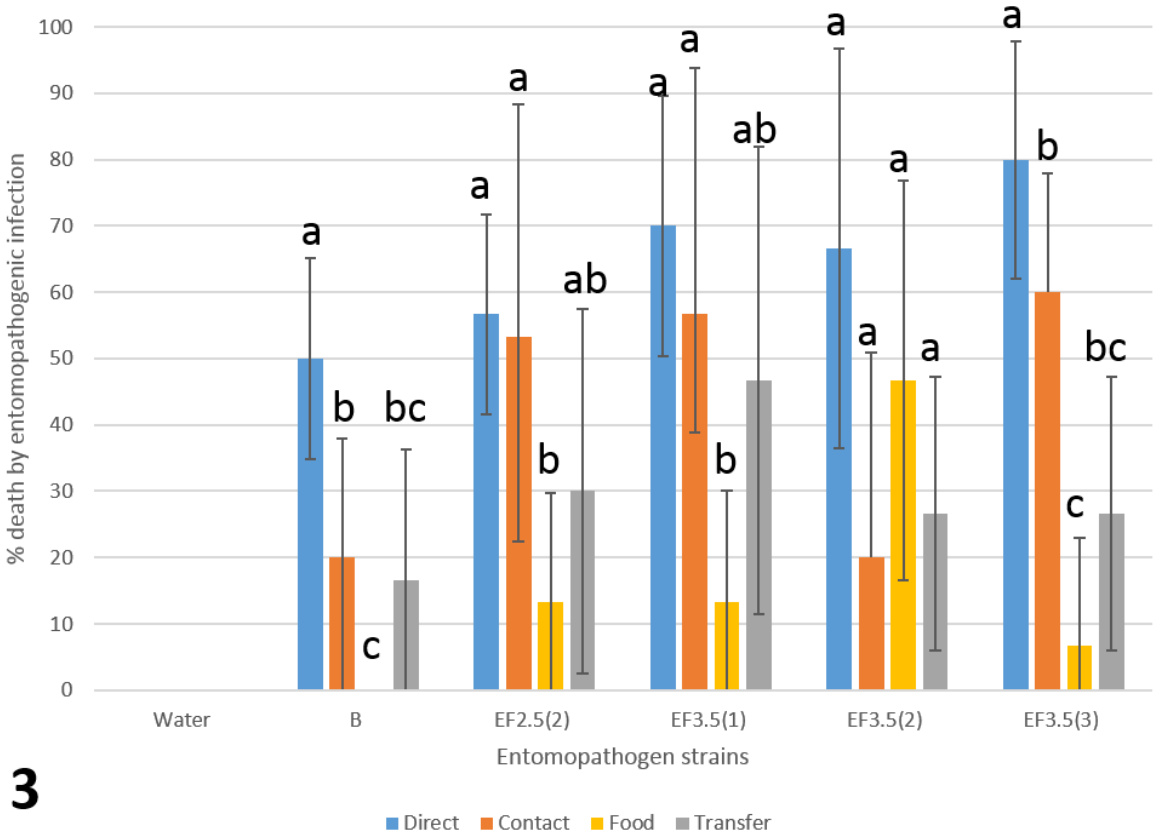
Table 1: Average mortality and compared mortality in function of inoculation methods. Values with the same letter are not significantly different ($p > 0,05$) after parametric LSD Fisher test (alpha= 0,05; DMS= 0,12264; Error: 0,0746; gl: 151).

Table 2: LT50 of entomopathogens isolates, i.e. time to kill 50% of the hornets, in function of the inoculation methods.



1





3

Highlights :

- Adults of the invasive *Vespa velutina* can be contaminated by indigenous isolates of *Beauveria bassiana* and *Metarhizium robertsii*.
- The inoculation by direct immersion of *V. velutina* in spore solution is more efficient for lethality than by contact on contaminated surface, consumption of contaminated food or inter-individuals transfer.
- There is no drastic difference among isolates and inoculation methods concerning fungal virulence.
- High fungus susceptibility of *V. velutina* in this work showed the high potential of biological control as alternative to traditional nest traetment.