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A *Beauveria bassiana* strain naturally parasitizing the bee predator *Vespa velutina* in France

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With 4 figures and 1 table

Abstract: *Vespa velutina* is a hornet predator of bees that was accidentally introduced in Europe from China in 2004. Since its arrival, it expanded through Europe, impacting both biodiversity and beekeeping. As there are currently no biological alternatives to chemical treatment of *V. velutina* nests, we need more studies on the development of potential biological control methods. We present here a further description of an indigenous strain of entomopathogenic fungi, *Beauveria bassiana* that was discovered naturally parasitizing a foundresses of *V. velutina* in France. The genus was identified by its macro and micro morphological characteristics and the specie by molecular techniques. It growing preferences were described according temperature conditions, and the potential in using such entomopathogens as a biological control agent against *V. velutina* was also discussed here, first at least to replace currently occurring direct chemical treatments, and then potentially to be integrated in future Trojan horse strategies.

Keywords: Asian hornet, Hymenoptera, *Beauveria bassiana*, biological control, entomopathology, fungus physiology

1 Introduction

Insects can be parasitized by many different fungi but most frequently by species classified in the hypocrealean genera *Beauveria* (Cordycipitaceae) and *Metarhizium* (Clavicipitaceae). These entomopathogenic fungi are characterized by a common infection mode, e.g. spore attachment to the insect cuticle (adherence and penetration (Shahid et al. 2012)), penetration of the mycelium in the insect, fungus development, and after the host death, sporulation outside the host's body (Meyling & Eilenberg 2007). The pathogenicity of a fungus strain depends on several different parameters, including (1) growth speed, (2) lethality, e.g. the probability to kill the host, and (3) ultimate climatic conditions (e.g. temperature, humidity); some strains being more or less specified to some insect orders. All these parameters are keys to describe pathogenic fungi.

Vespidae are generalist predators, which alimentary bolus can vary depending on their prey abundance, given season cycle (Spradbery 1973, Harris 1991). Therefore, they are capable to adapt to most environments (Richter 2000). Besides, vespids are social insects, they have an annual development cycle, and are very prolific: all this parameters make them very good candidates for biological invasions (Moller 1996, Beggs 2001). Moreover, when arriving in a new area, invasive species often miss their parasites, making their population development rate even more high (Torchin et al. 2003).

The yellow legged hornet *Vespa velutina* is an invasive predator of bees accidentally introduced to France from East China in 2004 (see Monceau et al. 2014 for a review). Since its introduction, *V. velutina*'s range has rapidly expanded to include Portugal, Spain, Italy, England and Germany (Keeling et al. 2017, Monceau & Thiéry 2017). This hornet is characterized by its strong predation on pollinators, especially honeybee, which it hunts in large amount (Monceau et al. 2013a, b). Although *V. velutina*'s predatory attacks may destroy honeybee colonies outright (Tan et al. 2007), its attacks more often stress honeybee colony by reducing honeybee colony defences and their ability to acquire resources sufficient for hibernation survival (Matsuura 1988, Monceau et al. 2014). *V. velutina* has large colonies that may grow to include thousands of workers before their collapse at the end of fall and the new queens entering hibernation, but their colonies are difficult to locate as they are often hidden in tree canopies, shrubs or buildings (Monceau et al. 2014). Mass trapping, hives protection (using nets, entrance grids etc.), foundresses trapping and nest destruction using chemical

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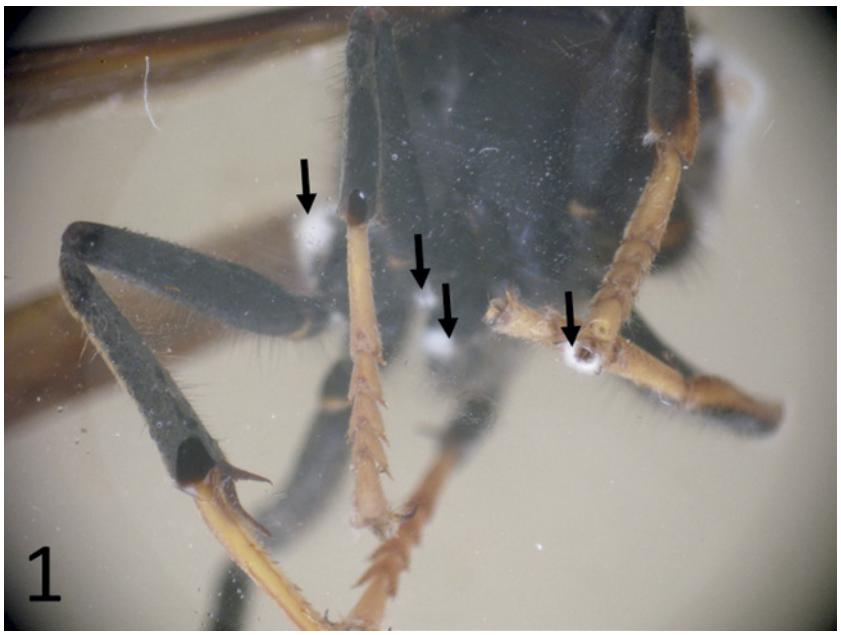

Fig. 1. White mycelium of Beauveria bassiana emerging from the hornet's cuticles intersections in the thorax of the Vespa velutina queen (black arrows) (Picture J. Poidatz).

pesticides are the current control methods used to limit *V. velutina* impact on beehives, but these are costly and insufficient for effective control (Monceau et al. 2012; D. Decante pers. com. 2015). Moreover, methods of chemical control classically used for nest destruction (insecticides powders, sulphur dioxide) are dangerous for both the environment and the applicator. More ecologic and safer control methods are needed. Biological control of invasive species with native organisms, which are well therefore adapted to the invaded area (Vega et al. 2009), could be a good solution here.

A few natural occurring organisms that attack *V. velutina* have already been described in France, including other insects such as the endoparasitic fly *Conops vesicularis* (Conopidae) (Darrouzet et al. 2015), the nematode *Pheromermis vesparum* (Villemant et al. 2015), or viruses such as the IAPV (Manley et al. 2015) and the DWV (A. Dalmon et al. unpublished). No application in biological control could however be yet envisaged with such species on *V. velutina*, because of low efficiency, and potential but not yet evaluated risks on non-target species and dispersion capacities or non-adapted development cycle (Beggs et al. 2011, Monceau et al 2014, Villemant et al. 2015). Entomopathogenic fungus could be very interesting to study and promising in this context (Poidatz et al. 2018a), being more adapted to biological control programs than macro-organisms (Butt et al. 2001).

A first study on the inoculation methods of entomopathogenic fungi to *V. velutina* was recently published, and one of the French native strains used in this study (Poidatz et al. 2018a), the *Beauveria bassiana* isolated from a queen of *V. velutina* caught in Bretagne that was found naturally parasitized, is described here. After a morphological and genetic description, its growth in function of temperature was also studied.

2 Materials and methods

2.1 Fungus isolation

A foundress of *V. velutina* naturally parasitized by a fungus was captured alive in a trap baited with sirop and beer at the end of May 2016 in Brest (Britain, North-West France), and send to the INRA laboratory (Bordeaux, France). After it death, the insect was externally disinfected by using paper towel imbibed with Calcium hypochlorite (50g/L). The insect was then cut in pieces, and the different parts were distributed amongst 3 Petri dishes on OAC medium ((Oat 40g, Agar (PDA, BK095HA, Biokar) 20g, Chloramphenicol (SIGMA Aldrich, Germany) 50mg, QSP 1L)), so the fungus could grow. White mycelium began to emerge from the hornet cuticle's intersections (Fig. 1).

The fungus was purified by multi-passaging on OAC media for two months. The fungus was then put in culture on top of cellophane paper on OAC medium Petri dishes, so the mycelium could be easily extracted by gently scraping the surface of the cellophane for DNA extraction.

2.2 Macroscopic and Microscopic characterization

Macroscopic observation was performed at naked eye on SDAY medium and discriminating the colony colour, shape

and typo of edge. Colonies growth for 7 days at 21 ºC and obscurity.

For microscopic characterization, a fungus preparation coloured by methylene blue was obtained on a microscopic lamella, then we observed the fungus structure (×40) to make a further diagnostic of the genus. Conidia´s size was assessed by measuring 100 conidia's diameter using a Nikon SMZ 1000 microscope for the observation and the software Image J v1.50i for the analysis (Fig. 2A).

2.3 Molecular identification

After the lyophilisation of the mycelium samples (12h), their DNA was extracted using the technique described by Zolan and Pukkila (1986) without Proteinase K. We used two different concentrations of DNA for the PCR: 20 and 50µL/µl. Concentration of DNA was measured by a DeNovix© DS-11 spectrophotometer®.

DNA amplification was performed by primers for a partial sequence of the nuclear protein-encoding gene, the translation elongation factor 1-alpha, named TEF-exon: 983F and 2218R (Rehner et al. 2011). Touchdown PCR conditions were fixed by the instructions of Rehner (2005); we used Taq DNA Polymerase (Silverstar®). The PCR products were validated by an electrophoresis migration (Agarose 2%).

Forward and reverse DNA sequencing were made by the company Genewiz (Takeley, Essex CM22 6TA United Kingdom). The phylogenetic analysis was carried by using the sequence from this study and additional sequences from NCBI GenBank® with a similarity degree over 99% and different species of the genus. We then built a phylogram by two methods, Maximum Likelihood (Tamura-Nei Model) and Maximum Parsimony (Subtree-Pruning-Regrafting Model) with Mega7®. All branch support was estimated by bootstrapping with 2000 replications and cut-off of at list 50% of probability.

2.4 Mycelium growth at different temperatures

We collected fungus plugs from similar age areas of the mycelium, and placed them in the centre of new Petri dishes on a MAC culture medium (Malt + Agar (PDA, BK095HA, Biokar) (2%) + Chloramphenicol (SIGMA Aldrich, Germany). Six growth temperatures were evaluated: 12 ºC, 16 ºC, 20 ºC, 22.6 ºC, 28 ºC in different climate chambers (LMS cooled incubators LMS 610XAP) and room temperature (21–25 °C). For sixteen days, all Petri dishes were placed at conditioned temperature and obscurity (dark boxes). For each temperature, ten replicates were done. The growth of the fungus was measured once each day by taking pictures of the Petri dishes. We thus assessed the mycelium area through image analysis using ImageJ v1.5 software. We then calculated the AUDPC (Area Under Disease Progress Curve) for each temperature and each replicate as described in Campbel & Madden (1990) using the R package "agricolae". After verifying for data normality by doing a Shapiro

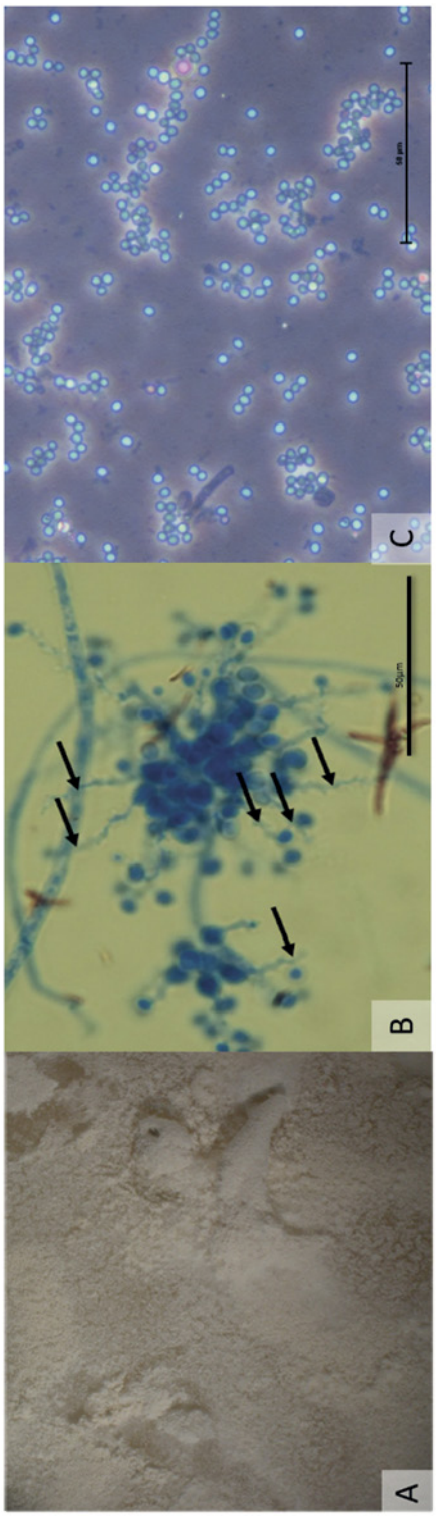

Fig. 2. A. *Beauveria bassiana* mycelium growing on agar (×1.5). (Picture J. Poidatz). B. Microscopic observation of the *B. bassiana* structures coloured with Methylene blue. The black arrows indicate the position of the conidiophores as sympodulospores (×1500) (Picture R. Lopez Plantey). C. Microscopic observation of our *B. bassiana* conidia coloured with Methylene blue, grown on PDA media, used for conidia size measurement (×1000).

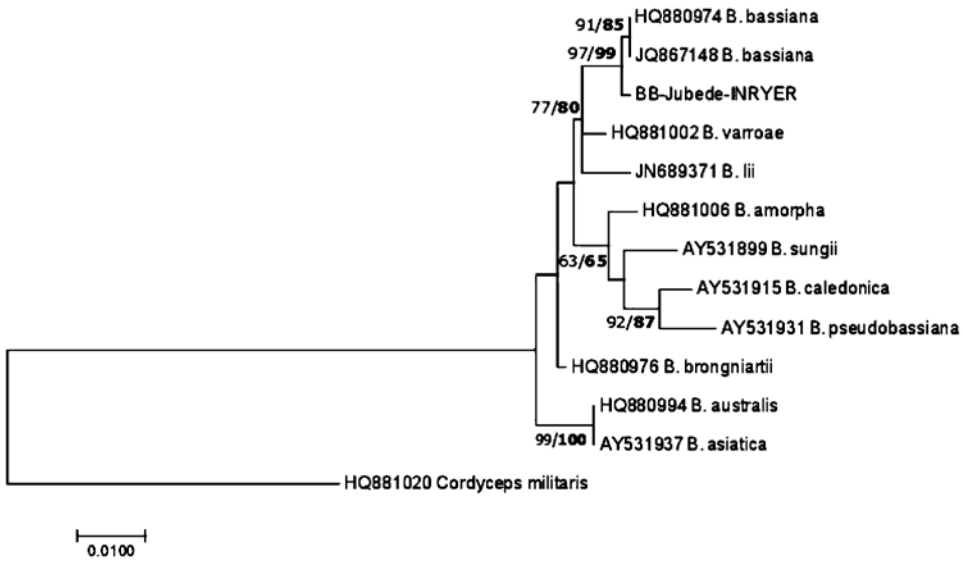

Fig. 3. Phylogeny of Beauveria spp. and isolated BB from joint Maximum Likelihood analysis of Translation Elongation Factor 1-alpha. Values above internal branches correspond to Maximum Likelihood and Maximum Parsimony (indicated in bold) (≥ 50).

test, we made an ANOVA and then a Tuckey HSD test for comparing each growing modality.

3 Results

3.1 Morphological description and genetic characterization

The colony shape is regular round, colony aspect is white cottony compact; colony edges are well-defined; and conidia are colourless. Macroscopic observations show round white colonies with defined edges and cottony aspect. Under microscope, these colonies presented uncoloured conidiophore and conidia disposed in zig-zag (sympodulospores) (Fig. 2b). Thanks to microscopic observations, conidia's diameter was assessed to be 2.91±0.29 micrometers.

A BLAST® analysis of the TEF-exon fragments shows a match with the specimen *B. bassiana* over a 99%, confirming previous observations. Phylogenetic analysis by Maximum Likelihood and Maximum Parsimony methods (Fig. 3) shows the strain BB-Jubede-INRYER close to the genotype of other evaluated strains of *B. bassiana* and on a different genotype that other *Beauveria* species listed below (Table 1).

3.2 Fungal growth in function of temperature

For the temperature 12 °C, one Petri dish was found contaminated by mites and was not kept in the analysis.

The *B. bassiana* strain growth was faster between 20 and 28 °C (Fig. 4), and significantly slower between 12 and 16 °C. The growing at 28 °C and at room temperature were very variable. The optimal growth temperatures seemed to be positioned between 20 °C and 22.6 °C.

4 Discussion

This is the first time that a native pathogen naturally parasitizing the invasive yellow legged hornet *V. velutina* is described. The infected foundress found in Britain probably contaminated itself during its hibernal diapause in ground or wood, or maybe during wood pulp collection for its nest construction, such pathogen's spores being present naturally in soil and wood for conservation (Meyling & Eilenberg 2007). The strain described here seems to be more adapted to intermediate temperature than fresh ones, what seems logical given that it was found in an oceanic temperate climate. The application of this strain for treatments using various methods (see Poidatz et al. 2018a), shall better take place when the temperatures are higher than 20 °C for a better fungal installation and dispersion.

Given this results, it could be interesting to associate several fungus strains with diverse optimal development temperature, in order to provide a more adapted biological control solutions for different ecological contexts.

Entomopathogenic fungus could be used to control *V. velutina* by several ways, once the suitable mode of application has been screened out by several assays. First, we have observed a good capacity of the spores of this fungus in liquid suspension to infect adults of *V. velutina*, such suspen-

Table 1. List of the fungal species used in this study for the genetic tree building.

GENBANK	SPECIES	ARSEF
HQ881006	*B. amorpha*	2641
AY531937	*B. asiatica*	4850
HQ880994 *HQ880995	*B. australis*	4598
HQ880974 * JQ867149	*B. bassiana*	1564
HQ880976 *HQ880991	*B. brongniartii*	617
AY531915	*B. caledonica*	2567
KC339709	*B. hoplocheli*	Bt 96
HQ881005	*B. kipukae*	7032
JN689371	*B. lii*	5500
HQ881015	*B. malawiensis*	4755
KU994833	*B. medogensis*	2898
AY531931	*B. pseudobassiana*	3405
AY531899	*B. sungii*	1685
HQ881002	*B. varroae*	8257
AY531920	*B. vermiconia*	2922
HQ881020	*Cordyceps militaris*	NC

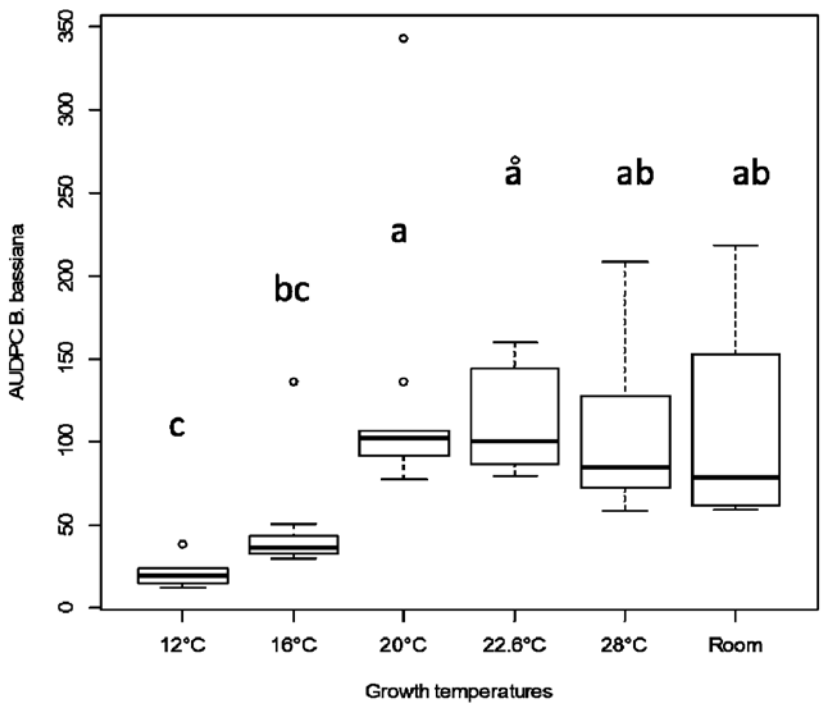

Fig. 4. Evolution of the AUDPC (Area Under Disease Progress Curve, Campbell & Madden (1990)) of the *B. bassiana* strain given the temperature.

sions could thus be sprayed on/inside the nests. As shown in Poidatz et al. (2018a) infection of workers through a short passage in an aqueous spore suspension could be an issue in using it as a "Trojan horse" method, meaning the parasite is hidden in an attractive and selective bait and bring back to the colony by the workers that have feed on the bait. This however must be assayed in field conditions to evaluate the suitable doses to be used. To do so, one needs to evaluate the potential impact of this strain on hornet larvae and their development (Rose et al. 1999).

In order to develop a control method based on such entomopathogenic fungal strains, the foraging behaviour of early infected workers should be carefully investigated to evaluate the potential dissemination of the spores to the preys (e.g. honeybees, bumble bees) and in the environment (Baverstock et al. 2010). Analysis of activity rhythm and foraging tracks durations by different tracking techniques or cartographic techniques as recently developed for *V. velutina* could be very instructive for this purpose (Kennedy et al. 2018, Poidatz et al. 2018b). An improvement of the potential of this strain as a control method for *V. velutina* could be done through its formulation, by making the spore more persistent, virulent or less sensitive to UV radiations (Burges 1998, Fernandes et al. 2015, Inglis et al. 1995).

Of course, several consistent tests for risks assessment must be done: indeed, given the chose inoculation method (spray, bait), the environmental risks for other insect species have to be evaluated. The choice of treatment localization, application method and application timing are clue. Unlike chemical insecticides that could accumulate in soil, water and other organisms by trophic network, infected insects could be eaten by mammals and birds without risks, this kind of pathogens being specific arthropod parasites, and are easily degraded by the local micro fauna.

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Conflicting interest

The authors declare no conflicting interest in this research

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