AXE 3 : VERS DES OPTIONS DE BIOCONTRÔLE DE *VESPA VELUTINA* ?

Dans l'**Axe 1**, nous avons évoqué le fait que le contrôle des reproducteurs chez *V. velutina* serait difficile à mettre en place, sachant (1) que les mâles et les gynes sont produits en grande quantité dans chaque colonie, (2) que les mâles ont des réserves de spermatozoïdes largement excédentaires, chacun d'eux suffit largement en théorie pour féconder une voir plusieurs femelles (Article 1), (3) que les fondatrices ont un développement très précoce au printemps et sont très fertiles (Manuscrit 2) et que leur capture n'impacte que de façon très marginale le niveau de population global. Une autre stratégie, plus axée sur le contrôle des colonies doit donc, selon nous, être envisagée. Je me suis donc intéressée dans cet **Axe 3** à des options de biocontrôle *via* en particulier l'usage de champignons entomopathogènes. Les résultats présentés dans l'**Axe 2** renforcent cette approche, en particulier sur le rayon d'action moyen et maximum des fourrageuses et leur rythme quotidien d'activité (Manuscrit 3), et sur la quantification des échanges par trophallaxie entre les différents membres de la colonie au cours de son développement (Manuscrit 4), dépendants de sa structure et du type de nourriture distribué.

Nous allons maintenant dans ce dernier axe **évaluer certaines méthodes de contrôle biologique applicables à *V. velutina*.** Comme développé en introduction, la destruction des nids se fait actuellement majoritairement par intervention humaine, mais sachant que la détection et l'accès aux nids est souvent difficile, nous envisageons ici de travailler sur une méthode permettant d'introduire naturellement des pathogènes dans les colonies.

V. velutina infecté par *B. bassiana* Photo de R. Lopez Plantey.

C.1 La lutte biologique

La lutte biologique est définie, entre autres, par l'OILB (Organisation Internationale de Lutte Biologique) http://www.iobc-global.org/ comme étant l'« Utilisation par l'Homme d'ennemis naturels tels que des prédateurs, des parasitoïdes ou des agents pathogènes pour contrôler des populations d'espèces nuisibles en dessous d'un seuil de nuisibilité ». Mais une définition beaucoup plus large a été adoptée par l'EPA (Agence de Protection Environnementale des Etats Unis) pour décrire les biopesticides. Ces derniers peuvent être dérivés de matériaux biologiques naturels classés en trois catégories : les biopesticides microbiens (bactéries, champignons, virus, algues *etc*.), les biopesticides d'origine végétale et les biopesticides biochimiques (substances naturelles sans effet toxique direct, mais un effet sur la croissance ou la reproduction) (Suty 2010).

Plusieurs stratégies de lutte biologique peuvent ainsi être mises en place et sont listées ci-après (liste inspirée de Suty 2010).

- **La lutte biologique par acclimatation (classique):** consiste à introduire de manière **inoculative mais non renouvelée** puis de **favoriser l'installation à long terme** d'un agent de biocontrôle dans un nouvel environnement, pour lutter contre une espèce cible exotique invasive. Par exemple, suite à la pullulation de jacinthes d'eau invasives *Eichhornia crassipes* sur des grands lacs africains, un charançon *Neochetina spp*. issu de l'aire native de la jacinthe a été introduit, ce qui a permis aux lacs de retrouver leur équilibre naturel (Wilson *et al.* 2007).
- **La lutte biologique inoculative** : le but de cette stratégie est de maintenir un niveau de population d'un ravageur sous le seuil de nuisibilité pendant la durée d'une culture, en effectuant des **petits lâchers réguliers de l'agent de biocontrôle en prévention et tout le long de la saison de production**. Un *baculovirus* (OpMNPV) est par exemple utilisé de cette manière pour lutter contre les populations de chenille à houppes du sapin, *Pseudotsuga menziesii* (Mirbel) (Hunter-Fujita *et al.* 1998). Ces baculovirus ne sont létaux que pour les stades larvaires, mais les adultes peuvent servir de vecteurs.

- **La lutte biologique inondative** : cela consiste en des lâchers de **grandes quantités d'agents de biocontrôle de manière plus curative**. On applique ici par exemple l'agent à une population de ravageurs problématique. Cette méthode est le plus souvent mise en place en agriculture, comme par exemple avec *Bacillus thuringiensis* pour lutter contre les processionnaires du pin, les noctuelles défoliatrices en culture maraichères, et les tordeuses de la vigne (ex Sanahuja *et al.* 2011).
- **La lutte biologique de conservation** : elle consiste à **favoriser les populations locales naturelles d'agents de biocontrôle** afin d'en augmenter l'efficacité dans la lutte contre les ravageurs. Les agents de biocontrôle sont déjà présents sur site, et l' objectif consiste donc à les attirer dans les parcelles à protéger, mais aussi à diminuer leur mortalité, favoriser leur reproduction et leur efficacité biologique. Cela passe généralement par l'aménagement d'habitats semi-naturels comme sources de refuges ou de nourriture, l'adaptation des pratiques culturales (diminution de pesticides ou de pratiques destructrices comme les labours ou le désherbage chimique pouvant perturber les agents) et le contrôle des prédateurs des agents (DeBach & Rosen 1991).

De manière générale, l'objectif des méthodes de lutte biologique n'est pas l'éradication d'espèces nuisibles, concept rarement envisageable en agriculture, mais bien la diminution de leurs populations en dessous de leur **seuil de nuisibilité**[1] (Figure 25).

[1] Seuil de nuisibilité : le niveau des populations d'organismes nuisibles qu'un agriculteur peut admettre sans grand risque pour sa récolte, ou sans que la dépense en pesticides ne dépasse le gain de récolte.

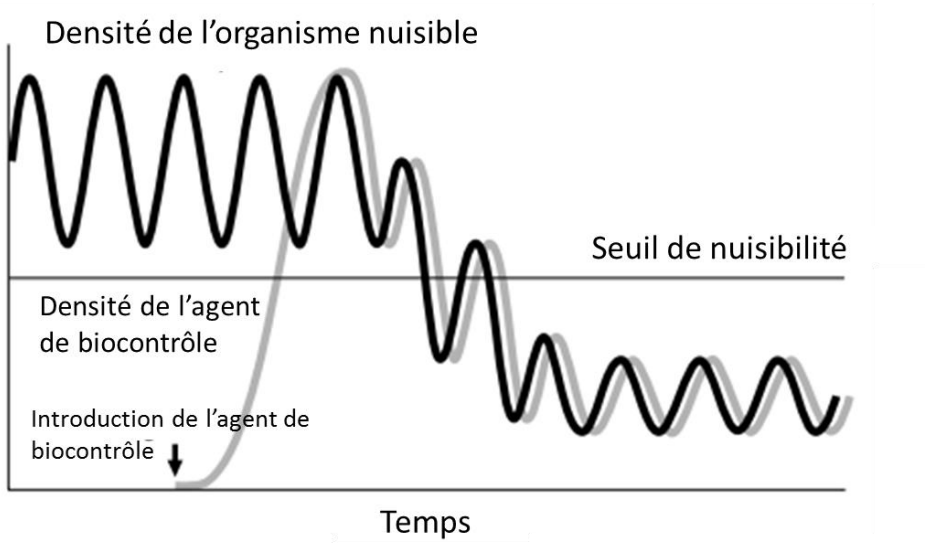

Figure 25 : Représentation schématique du fonctionnement recherché d'un programme de biocontrôle, où la population de l'organisme à contrôler diminue de manière à rester sous le seuil de nuisibilité suite à l'introduction de (ou de l'ensemble) d'agent(s) de biocontrôle. (Adapté de Briese 2000).

C.1.1 La lutte microbiologique entomopathogène

Les agents microbiens (bactéries, virus ou champignons) sont bien connus depuis les travaux de Louis Pasteur sur la flatide du ver à soie en fin de XIX ème siècle. Ces agents ont alors été très tôt utilisés en lutte biologique contre les insectes ravageurs de cultures (Le Moult 1890) et couramment invasifs. Depuis le début des années 2000, à l'aire de la prise de conscience de l'écologie et de l'importance de la durabilité dans le contrôle de nuisibles, l'usage de pathogènes d'insectes en lutte biologique est un champ de recherche en constante expansion (Lacey *et al.* 2015).

Suivant les espèces, les agents microbiens de biocontrôle sont présents dans différents compartiments environnementaux (sol, air, eau) et peuvent infecter leur hôte soit par ingestion, soit par contact avec la cuticule (St Leger et al. 1988a, b) ou en entrant par d'autres orifices (Figure 26) (St Leger 1993, Hajek & St Leger 1994). Le pathogène se multiplie alors dans l'hôte en lui causant des dommages par destruction des tissus, par septicémie ou toxémie entraînant sa mort plus ou moins immédiate. Tous ces micro-organismes possèdent des formes de résistance leur permettant de persister dans

l'environnement et de perpétuer leur cycle de vie (Fargues & Robert 1985, Bidochka *et al.* 1998, Meyling & Eilenberg 2007).

Parmi les micro-organismes utilisés en lutte biologique, appartiennent à plus de 700 espèces de microchampignons entomopathogènes (Starnes *et al.* 1993) qui jouent un rôle important dans la régulation naturelle des populations d'insectes (Ferron 1978, Wraight & Roberts 1987, Butt *et al.* 2001). La plupart des champignons entomopathogènes appartiennent au sous-taxon des *Mastigiomycotina, Zygomycotina, Ascomycotina* et *Deuteuromycotina*. Le plus grand nombre d'entre eux se trouve dans la classe des Zygomycètes, mais les plus utilisées en lutte biologique proviennent des Deuteromycètes (*Fungi imperfecti*). Les espèces des genres *Beauveria, Metharizium, Verticillium, Erynia, Hirsutella, Entomophtora* et *Entomophaga* sont les plus utilisées en lutte biologique (Wraight & Roberts 1987, Goettel & Inglis 1997). Le principal avantage des micro-champignons est leur aptitude à infecter l'hôte **par ingestion ou par simple contact à tous les stades, œuf, larve et adulte** (Hajek & St. Leger 1994). Ils peuvent être produits en masse à moindre coût et peuvent être appliqués avec les méthodes conventionnelles. Les principaux facteurs limitant l'utilisation en champ des microchampignons sont abiotiques : en effet, la plupart des spores des souches classiquement utilisées sont sensibles aux UV (la formulation peut améliorer cet aspect Inglis *et al.* 1995), ont besoin d'une certaine humidité pour se développer (Inglis *et al.* 2000), et réagissent souvent mal aux fortes oscillations de température (Inglis *et al.* 1999). Cela renforce l'importance de tenir compte de l'**écologie des champignons** en eux-mêmes et de favoriser autant que possible des souches d'entomopathogènes locales dans le développement de stratégies de lutte, car elles seront adaptées naturellement aux contraintes climatiques de leur environnement d'utilisation (Vega *et al.* 2009).

C.1.2 Les mécanismes infectieux des entomopathogènes

Le mode d'infection des champignons entomopathogènes se divise en quatre étapes distinctes qui sont **l'adhésion, la germination, la différentiation, et la pénétration**. L'**adhésion** est caractérisée par un mécanisme de reconnaissance et de compatibilité des conidies avec les cellules

tégumentaires de l'insecte (Vey *et al.* 1982, Boucias & Pendland 1991, Bidochka & Small 2005, Ortiz-Urquiza & Keyhani 2013). Cette phase se scinde en deux étapes distinctes : la première, passive, où l'attachement à la cuticule est réalisée grâce à des forces hydrophobiques et électrostatiques (Butt 1990, Boucias & Pendland 1991) et la seconde, active, caractérisée par la production d'un mucilage qui va engendrer une modification épicuticulaire (Wraight *et al.* 1990) aboutissant à la germination (Figure 26).

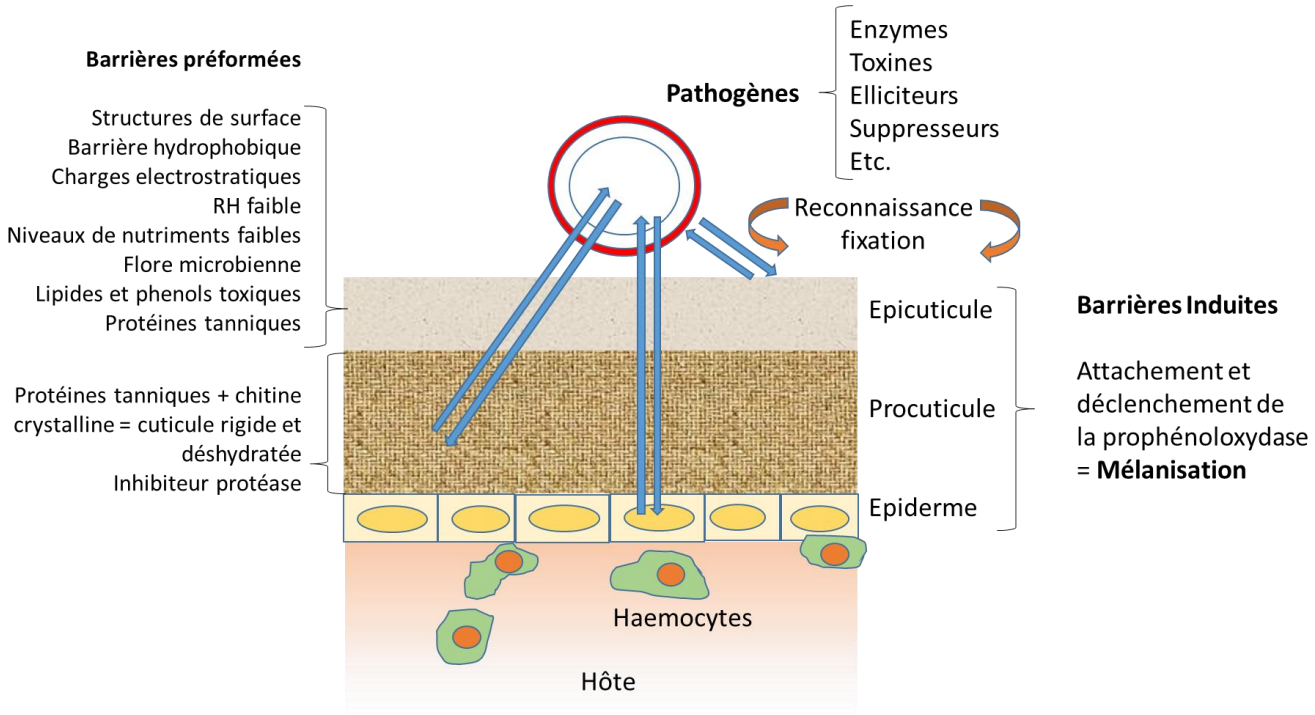

Figure 26 : Schéma du mécanisme infectieux illustrant les composantes majeures des interactions entre les insectes et les pathogènes durant la pénétration dans la cuticule. (Inspiré de St Leger 1993).

Après la phase d'adhésion, la **germination** est dépendante des conditions environnantes (Vega *et al.* 2009) et aussi de la physiologie de l'hôte, telle que la composition biochimique de la cuticule de l'hôte, qui peut favoriser ou inhiber la germination (Smith & Grula 1982, St Leger *et al.* 1989, Butt 1990, Butt & Becket 1994, Butt *et al.* 1995). L'avant dernière phase est la **différenciation** caractérisée par la production d'**appressorium**, structures terminales servant de point d'encrage, de ramollissement de la cuticule et à favoriser la **pénétration**.

C.2 La technique du Cheval de Troie

Là où le piégeage massif d'ouvrières ou de reproducteurs, principalement des reines, s'est révélé relativement inefficace sur le long terme chez les vespidés invasifs, l'usage d'appâts empoisonnés, *i.e.* la technique du « Cheval de Troie », a quant à elle fait ses preuves (ex avec *V. vulgaris* et *V. germanica* en Nouvelle Zélande, plus de 99% d'efficacité Beggs *et al.* 2011).

La technique du Cheval de Troie est une méthode qui consiste à faire rentrer un perturbateur ou un parasite dans la colonie, *via* des ressources rapportées par les ouvrières à la colonie. Cette technique est classiquement utilisée pour lutter contre différents insectes sociaux (blattes, fourmis), en fournissant des appâts traités avec différentes molécules insecticides afin qu'ils soient ramenés puis distribués au groupe ou accumulés dans la colonie. L'usage de cette méthode de lutte a par exemple été conseillé pour la gestion de fourmis invasives *Pachycondyla chinensis* dans Buczkowski 2016. Les doses utilisées se doivent d'être non létales pour les ouvrières vectrices, afin qu'elles puissent avoir le temps de rapporter l'appât empoisonné jusqu'à leur colonie : on recherchera alors un effet d'accumulation dans le couvain. Harris *et al.* 2011 ont envisagé l'utilisation de pathogènes d'insectes dans ce genre d'appâts contre des guêpes, afin de limiter l'impact environnemental de cette technique. En effet, dans le cas d'usage d'insecticides dans ce genre d'appâts, on risque d'assister à une accumulation de molécules toxiques en grande quantité dans un nid dont la localisation resterait inconnue. Le nid restant alors accessible à de nombreux autres organismes (oiseaux, mammifères, autres insectes) qui s'intoxiqueraient en mangeant ou en dégradant les individus traités, et des résidus se retrouvant alors dans l'environnement (eau, sol).

C'est pourquoi dans le but de développer une méthode de cheval de Troie à la fois efficace et écoresponsable, nous avons choisi de travailler sur différents micro agents de lutte biologique. L'impact potentiel des agents microbien de biocontrôle doit toutefois être évalué

rigoureusement, pour limiter les risques sur les organismes non-cibles, et optimiser leur mode d'application.

C.3 Recherche d'agents de biocontrôle pour agir contre *V. velutina.*

C.3.1 Présentation générale des agents de lutte étudiés

La **pathogénicité de l'inoculum** sporal, la **spécificité de l'hôte** et **l'origine** (natif, alien) sont des paramètres important dans le choix d'un l'isolat fongique pouvant être utilisé en lutte biologique. Dans le cadre de la recherche d'agents de lutte biologique contre *V. velutina*, nous avons utilisé différents isolats natifs d'entomopathogènes appartenant à deux espèces très fréquemment utilisées en lutte biologique : *Beauveria bassiana* et *Metarhizium robertsii*. Nous avons travaillé avec des champignons présents dans des échantillons de sol, isolés sur des larves de l'eudémis de la vigne (*Lobesia botrana)* à l'automne 2015 dans des parcelles de vigne INRA (*Metarhizium*), et sur une souche isolée directement sur une reine de *V. velutina* en sortie d'hibernation au printemps 2016 en Bretagne (*Beauveria*). (Pour plus de détails, voir les Manuscrits 5 et 6)

C.3.2 Méthodes d'identification de souches d'entomopathogènes

C.3.2.1 Observations morphologiques

Dans un premier temps, une observation de l'insecte infecté doit être effectuée (Goettel & Inglis 1997): on observe la texture du mycélium, sa forme, sa couleur, puis la coloration des spores. Ensuite un montage de fragments fongiques entre lame et lamelle dans une goutte de bleu de méthylène permet d'observer les structures de sporulation de plus près (taille, forme des spores, forme des

hyphes). Grace à ces premières observations, on peut généralement obtenir l'identification de la famille du champignon (Wraight & Roberts 1987).

Les espèces de *B. bassiana* produisent des colonies avec un mycélium blanc cotonneux (Figure 27.A) très dense, et des spores transparentes. Les conidies ou spores (Figure 27.B) sont soutenues par de long filament en zigzag qui sont des hyphes transparents et septaux avec un diamètre de 2.5 à 25 µm. Les *M. robertsii* produisent des colonies avec un mycélium blanc poudreux et des spores vert olive plus ou moins sombres (Figures 27 C, D).

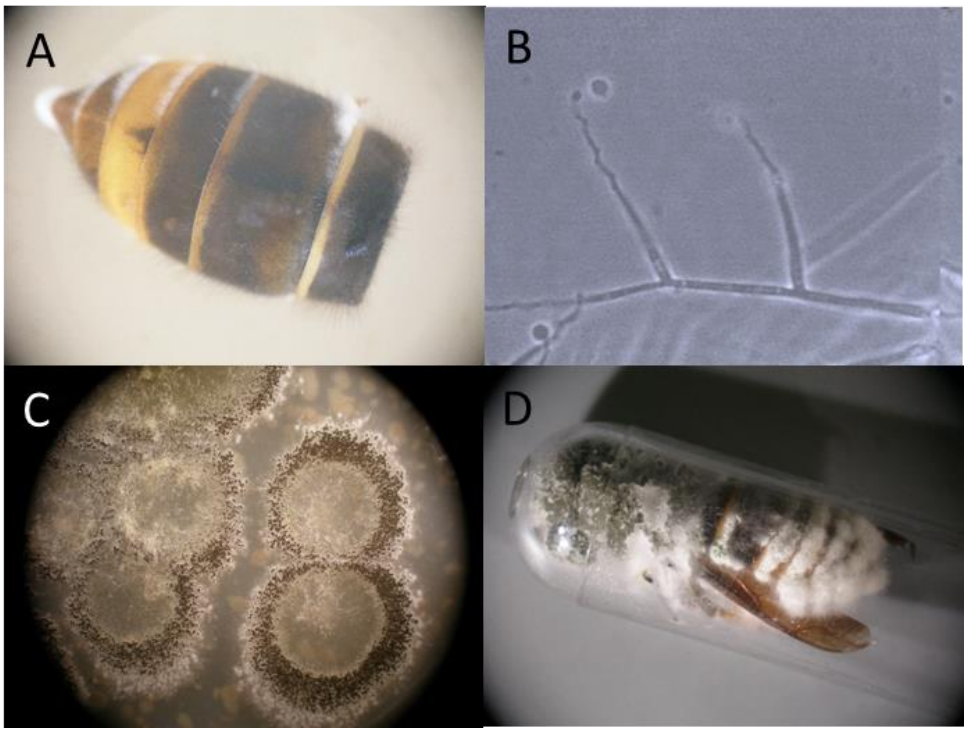

Figure 27 : A. Mycélium de *Beauveria bassiana* sortant d'entre les tergites de l'abdomen d'un *V. velutina* (loupe binoculaire X45). B. Hyphes de *B. bassiana* (Microscope) (photo de Kouassi 2001), C. Culture de *Metarhizium robertsii* sur gélose, les spores marron-vertes sont bien visibles. D. Frelon attaqué par des colonies de *M. robertsii*, le mycélium est blanc poudreux et les spores vertes olive. (Photos J. Poidatz).

C.3.2.2 Analyses génétiques

Comme indiqué précédemment, nous avons identifié un isolat de *B. bassiana* provenant d'une fondatrice de *V. velutina* capturée au printemps 2016 en Bretagne. Afin de valider l'identité du champignon proposée après sa description morphologique, nous avons réalisé une analyse génétique

(St Leger & Joshi 1997), par PCR classique sur fragments TEF de son ADN. Ces parties d'ADN sont les plus intéressantes à observer pour une identification précise des souches isolées, et sont bien plus robustes que les ADN mitochondriaux pour identifier ces organismes (Rehner *et al.* 2011). Les analyses réalisées sont détaillées dans le manuscrit en cours de préparation Manuscrit 5.

Manuscrit 5 (Shortnote): Description of a strain of Beauveria bassiana naturally parasitizing the bee predator Vespa velutina in France

Juliette Poidatz, Rodrigo Javier Lopez Plantey, Leslie Daraigne & Denis Thiéry

***Manuscrit en cours de préparation**, pour soumission dans Journal of Invertebrate Pathology*

Introduction

Insects can be parasitized by different fungus, belonging mainly to two genders: *Metarhizium* and *Beauveria*. 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, mycelium development, and after the host death, sporulation outside the host's body (Meyling & Eilenberg 2007). The pathogenicity of a fungus strain depends on different parameters (1) *host specificity*, some strains being more or less specified to some insect orders, (2) *growth speed*, (3) *lethality*, e.g. the probability to kill the host, and (4) *ultimate climatic conditions* (e.g. temperature, humidity). All these parameters are key to describe pathogenic fungi.

The yellow legged hornet *Vespa velutina* is an invasive predator of bees accidentally introduced in France from East China in 2004 (see Monceau *et al.* 2014a for a review). Since then, its expansion in Europe is quite consequent, this alien species being present in Portugal, Spain, Italy, and recently in 2016, England and Germany. This hornet is characterized by its strong predation on pollinators, especially honeybee, which it hunts in large amount (Monceau *et al.* 2013b, c). *V. velutina* enhances the stress level of the honeybee colony (Tan *et al.* 2007) and can sometimes directly cause its death, by predating honeybees, or indirectly, by decreasing the colony defences and resources for hibernation (Matsuura 1988, Monceau *et al.* 2014a). *V. velutina* lives in huge nests containing several thousands of individuals at the end of fall, and these colonies are often hard to find, because often hidden in tree canopies, bushes or buildings (Monceau *et al.* 2014a). Mass trapping, direct hives protection with nests, entrance grids etc, foundresses trapping and nest destruction are the current control methods used to limit *V. velutina* impact on beehives… but these are costly, not efficient enough (Monceau *et al.* 2012, D. Decante ITSAP pers. com. 2015), and moreover, the products classically used for nest

destruction (insecticides powders, sulfur dioxide) are dangerous for both the environment and the applicator. More ecologic and safe control methods are needed in this situation. Biological control of invasive species with native organisms, which are well therefore adapted to their application environment (Vega *et al.* 2009), could be a good solution here. This method was for example efficient in New Zealand, where native strains of *Serratia* were used to control the grass grub *Costelytra zealandica* (Jackson *et al.* 1993).

A few natural occurring organisms that attack *V. velutina* have been described in France, being other insects (*Conops vesicularis* (Conopidae) (Darrouzet *et al.* 2015), and the nematode *Pheromermis vesparum* (Villemant *et al.* 2015) or viruses ((IAPV) Manley *et al.* (2015) and (DWV) (A. Dalmon *et al.* in prep)).

We describe here a native strain of *Beauveria bassiana* isolated from a queen of *V. velutina* caught in Bretagne and naturally parasitized.

Material and methods

- **Fungus isolation**

A foundress of *V. velutina* naturally parasitized by a fungus was captured in an alimentary trap at the end of May 2016 in Brest (Britain, North-West France). The insect was externally disinfected by using paper towel imbibed with Calcium hypochlorite. The insect was then cut in pieces, and the different parts were distributed amongst 3 Petri dishes on OAC medium, so the fungus could grow. White mycelium began to emerge from the hornet cuticle's intersections (Figure 1 A, B).

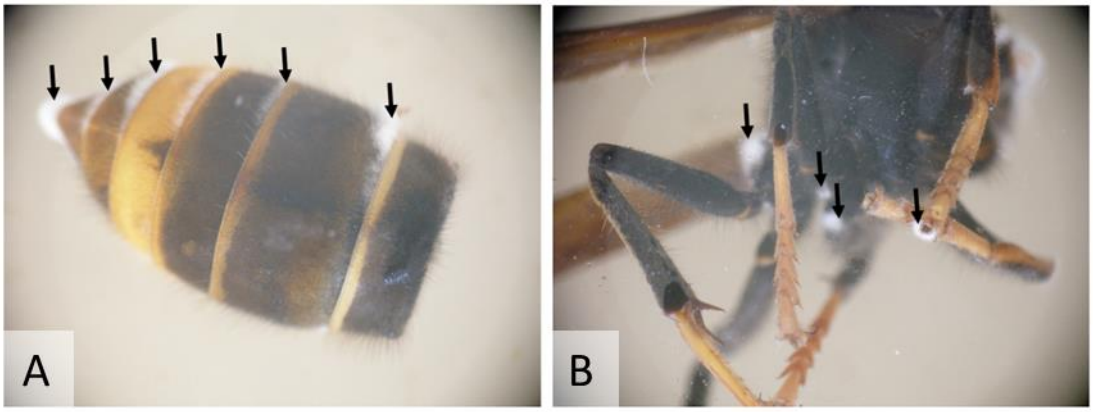

Figure 1. White mycelium of *Beauveria b.* began to emerge from the hornet cuticle's intersections in the abdomen (A) and the head and thorax (B) of the *V. velutina* queen (black arrows).

The fungus was then 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.

- **Macroscopic description** (Figure 2 A).

- Colony Shape : regular round
- Colony aspect : white cottony compact
- Colony edges : well-defined
- Conidia color : colorless

- **Microscopic observation**

A fungus preparation colored by methylene blue was made on a microscopic lamella, then we observed the fungus structure (x40) to make another diagnostic of the species (Figure 2 B). Under microscope we found conidiophores as sympodulospores (conidia positioned in zig-zag).

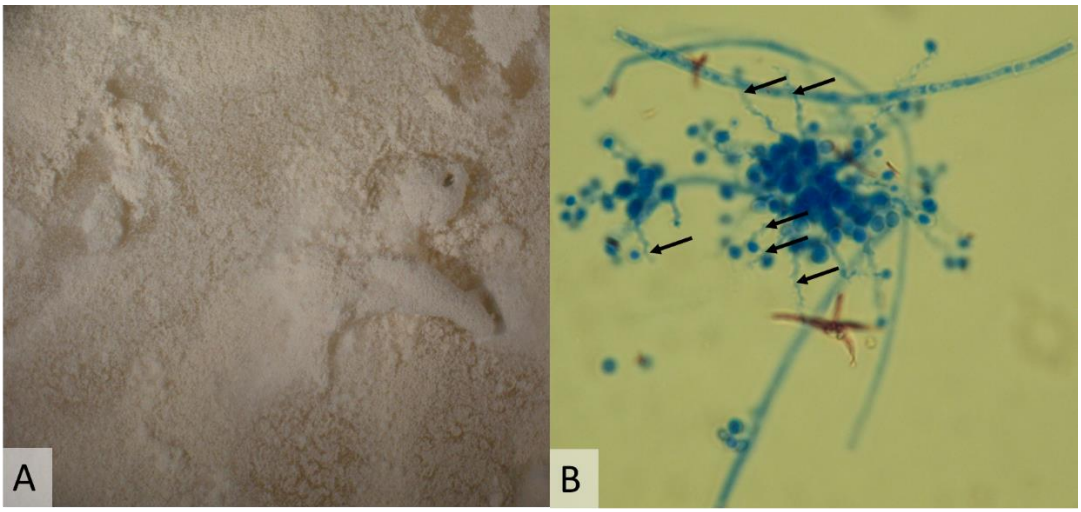

Figure 2. A. *Beauveria bassiana* mycelium growing on agar (x1.5). (Picture J. Poidatz). B. Microscopic observation of the *B. bassiana* structures colored with Methylene blue. The black arrows indicate the position of the conidiophores as sympodulospores (x1500) (Picture R. Lopez Plantey).

- **Genetic analysis**

- **DNA extraction**: after the lyophilisation of the mycelium samples, their DNA was extracted using the technique described by Zolan & Pukkila (1986) without Proteinase K.
- **PCR:** We concentrated the DNA at two different concentrations for the PCR: 20 and 50µL/ml. Thanks to macro and microscopic observations, we had a strong presumption on the fungi to be *Beauveria sp*. Therefore we chose the primers TEF-exon 983F for the fungi DNA amplification (Rehner *et al.* 2011).

983F: GCYCCYGGHCAYCGTGAYTTYAT

2218R: ATGACACCRACRGCRACRGTYTG

We used 0.2µL of primer 938F and 0.2µL of 2218R, with 0.2µL of dNTPs, 1.5µL of PCR Buffer 10x, 0.45µL of MgCl2, 11.31µL milli q water, 0.04µL of Taq and 1.1µL of DNA. A PCR Touchdown was made (Table 1), *i.e.* with a diminution of 1°C in each 9 first cycles. The PCR products were validated by an electrophoresis migration (Agarose 2%).

Table 1: description step by step of the PCR process (Rehner *et al.* 2011)

Step	***Temperature***	***Time***	***Cycles***
Denaturation	94ºC	2 minutes	
Denaturation	94ºC	30 seconds	
Appariement	66ºC (-1ºC/cycle)	30 seconds	9
Elongation	72ºC	1 minute	
Denaturation	94ºC	30 seconds	
Appariement	56ºC	30 seconds	36
Elongation	72ºC	1 minute	
Elongation	72ºC	10 minutes	
Conservation	14ºC	∞	

Forward and reverse DNA sequencing were made by the company Genewiz (Takeley, Essex CM22 6TA United Kingdom).The sequences analysis were made by using the Software Mega7® with the option alignment muscles. We then made a Maximum Likelyhood Tree with Mega7®.

- **Koch Postulate validation**

After isolation of the fungi from the different parts of the infected queen we inoculated the fungus to 15 workers of *V. velutina* in summer 2016, by immersion of <1 second in a 10^7spores/ml solution. The survival over time of the infected hornets was assessed daily during one week. As hornet are social insect and cannot survive alone very long, the inoculated individuals were maintained in group of five in 10cm diameter Petri dishes in a climatic chamber at 23°C±1°C, 12h/12 Lum. To confirm the cause of death, once an individual was found dead, it was put in an hemolysis tube closed with a humid cotton copper: we check for fungus growth on these individuals every day after.

Results

The white mycelium indicated us that this fungus was a *Beauveria sp.* Under microscope, we observed the conidiophore with sympodulospores (conidia positioned in zig-zag) and small round spores (Fig 2b), so we supposed this fungus was included in the species *B. bassiana.*

The DNA migration on agarose of the PCR products revealed strong signals for the 50ng/µL DNA concentrated products, but at 20 ng/µL the signals were very light, therefore we choose to genotype only the first. After sequencing and sequence cleaning, the results of the muscle alignments are illustrated in the tree Figure 3. The fungi matches at 100% with other *B. bassiana* strains, confirming previous observations (Table 2).

Table 2: Genetic proximity of the TEF 1α fragments analyzed with other *B. bassiana* strains.

GENBANK	SPECIES	AUTHORS	JOURNAL / INSTITUTION
HQ881006	*B. amorpha*	Rehner, S.A. *et al.*	Mycologia 103 (5), 1055-1073 (2011)
HQ880994	*B. australis*	Rehner, S.A. *et al.*	Mycologia 103 (5), 1055-1073 (2011)
JQ867149	*B. bassiana*	Cai, Y. & Huang, B.	Anhui Provincial Key Laboratory, Anhui Agr. Univer., China (2012)
HQ880974	*B. bassiana*	Rehner, S.A. *et al.*	Mycologia 103 (5), 1055-1073 (2011)
HQ880976	*B. brongniartii*	Rehner, S.A. *et al.*	Mycologia 103 (5), 1055-1073 (2011)
JN689371	*B. lii*	Zhang, S.L. *et al.*	Mycotaxon 121, 199-206 (2012)
HQ881002	*B. varroae*	Rehner, S.A. *et al.*	Mycologia 103 (5), 1055-1073 (2011)
HQ881020	*Cordyceps militaris*	Rehner, S.A. *et al.*	Mycologia 103 (5), 1055-1073 (2011)

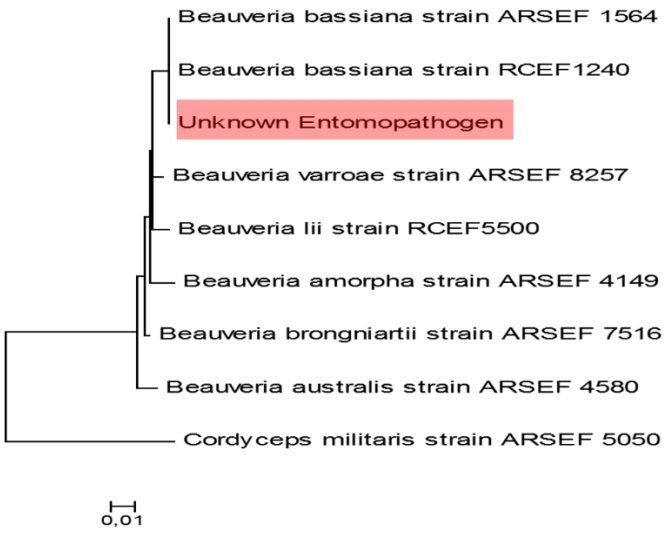

Figure 3: Genetic tree of the analyzed 1-α fragments and their genetic proximity with other fungi (MEGA 4, Genebank). The unknown fungus is on the same bar than the other B. bassiana, what means that they are 100% identical.

The Koch postulate was here successfully validated by succeed in reproducing the symptoms of the initial infected foundress of *V. velutina* on 10 of the 15 infected *V. velutina* workers.

Discussion

This is the first time 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).

There are several ways an entomopathogenic fungus could be used to control *V. velutina*, and several tests should be run to assess which one will be the more suitable for this strain. First, we observed a good capacity of the spores of this fungus to parasitize adults of *V. velutina*, so we already know that this strain could be used as a potential direct control product that could be applied in the nest the same way other chemical product currently are. Second, we should evaluate the potential of this strain as a

"Trojan horse" control agent: meaning the parasite is hidden in an attractive and selective bait that will be brought back to the colony by workers, distributed amongst colony members and by accumulation inside the colony will lead to its collapse. To do so, we need to evaluate the potential impact of this strain on hornet larvae and their development (Rose *et al.* 1999), but we also have to be sure that its odours are non-repulsive for workers (Mburu *et al.* 2013), or that the odour could be hidden thanks to the formulation and the bait, so the workers will bring it back to their nest and distribute it.

It could also be interesting to look at the impact of the infection on the hornet behaviours, as they may reduce their foraging activity or social behaviour before being incapacitated by the fungus growth (Baverstock *et al.* 2010). Analysis of activity rhythm and foraging tracks durations will be analysed in further studies using the RFID technique in order to evaluate to each extend early infection of workers would affect their foraging behaviour.

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).

Acknowledgements

We thank Jessica Wallance (UMR 1065 SAVE, BSA) for her advices on PCR analysis.

C.4 Evaluation de l'efficacité de certains isolats d'entomopathogènes généralistes sur *V. velutina*

C.4.1 Biocontrôle d'adultes de *V. velutina*

Afin d'évaluer l'efficacité potentielle des souches d'entomopathogènes isolées comme agents de biocontrôle de *V. velutina*, des bio-essais ont été mis en place dans un premier temps sur des ouvrières, entre 2015 et 2016. Généralement, les articles publiés présentent leurs résultats fondés sur une technique d'inoculation : immersion dans une solution de spores, ou pulvérisation aérosol de la solution de spores (Goettel & Inglis 1997). Mais nous avons choisi dans notre étude de tester plusieurs méthodes d'application mimant les différents mécanismes de contamination des frelons asiatiques suite à un traitement. Cette approche avait déjà été celle de Harris *et al.* 2000 sur *V. vulgaris*. En plus du traitement direct par immersion où les adultes sont trempés quelques secondes dans une solution de spores, nous avons testé un traitement de contact (*i.e.* par contact entre l'adulte et une surface contaminée sèche), dans de la nourriture (dans notre expérience nous avons mis du poisson), et par transfert entre individus (un individu contaminé par groupe de cinq). Nous avons utilisé deux critères classiques d'évaluation des souches d'entomopathogènes: **la LT50** *i.e.* le temps mesuré *a posteriori* nécessaire pour tuer 50% des individus exposés, et la **létalité,** correspondant au pourcentage de morts par infection par le champignon après une semaine. Pour valider le fait que les morts étaient bien dues à une attaque de l'entomopathogène, les individus morts ont été isolés dans des tubes à hémolyse tous les jours, et leur état était surveillé: l'apparition du mycélium de l'entomopathogène entre les plaques chitineuses de l'insecte permettait de confirmer l'infection. Pour rappel, un entomopathogène n'est pas saprophyte, *i.e.* il n'infecte donc pas un hôte déjà mort.

Manuscrit 6: Indigenous strains of Beauveria and Metharizium as potential biological control agents against the invasive hornet Vespa velutina

Juliette Poidatz, Rodrigo López Plantey, Denis Thiéry

*Soumis à **Journal of Invertebrate Pathology** (juin 2017)*

Abstract

Alien species often miss parasites in their invaded area, and this is the case in *Vespa velutina*. This invasive hornet predator of bees was accidentally introduced in Europe from East China in 2004. The control of this species is still problematic, because it's too costly or dangerous for the applicator. Studying the potential interest of biological control methods may help to propose alternatives in *V. velutina* control. We present here bioassays in which we assessed the potential control efficiency of different French isolates of entomopathogenic fungi that we inoculated to adults of *V. velutina* by different ways, being direct inoculation, contact on a contaminated surface, contaminated food, or by inter-individual transfers. We tested differences between the isolates and the application methods using two parameters being lethality and aggressiveness. The direct inoculation method was the most efficient modality, then the contact, transfer and food. Considering all contamination methods, there was no differences in the LT50 or lethality between the different isolates. Still the aggressiveness was quite short in all isolates (average 5.8 ±0.44days), and their efficiency is quite high: we conclude that there is high potential in using such entomopathogens as a biological control agent against *V. velutina*, first at least to replace currently occurring direct chemical treatments, and then potentially to be integrated in future Trojan horse strategies .

Keywords: Asian hornet, *Beauveria bassiana, Metarhizium robertsii*, biological control.

Introduction

During the invasion process, alien species often miss parasites in their invaded area, 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 allow 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, Raveret Richter 2000), and this enhances contamination transmissions risks. Some ants developed prophylactic strategies to limit this risk (reviewed in 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 & 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 & 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 (Monceau *et al.* 2013b, 2014a, Arca 2015). Since then, the "yellow legged hornet" spread in Europe: Spain (López *et al.* 2011), Portugal (Grosso-Silva & Maia 2012, Bessa *et al.* 2016), Italy (Porporato *et al.* 2014), Germany (Rome *et al.* 2015), Belgium (Rome *et al.* 2013), recently in England (2016) and in Scottland (March 2017) (reviewed in Monceau & 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, Tan *et al.* 2007, Monceau *et al.* 2014a). *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 papier-mâché made, by mixing plant fibres with water and saliva (Spradbery 1973), closed, with one unique enter 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* (reviewed in Monceau *et al.* 2014a). The density of nests in invaded area can be impressive given the observed area (Monceau & Thiéry 2017), and their destruction implies specific equipment 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 or liquids) or Sulphur dioxide (gas). A significant impact of traps on non-target insects was already demonstrated as well as their inefficiency (Beggs *et al.* 2011, Monceau *et al.* 2012, Monceau *et al.* 2013c). 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 (food chain), and also for the applicator, with irritations and respiratory problems (H. Guisnel, personal comunication). 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.* 2014a).

Biological control is a long known method where organisms control pests by predation or parasitism (Lacey *et al.* 2015). *V. velutina* is parasitized by the tachinid fly *Xenos Moutoni* (Dipteran) in Korea (Makino *et al.* 2011); in France, the endoparasitoïd *Conops vesicularis* (Conopidae) (Darrouzet *et al.* 2015), and the nematode *Pheromermis vesparum* (Villemant *et al.* 2015) were found parasitizing *V. velutina*. According to Manley *et al.* (2015) the Israeli acute paralysis virus (IAPV) can affect *V. velutina*, but also the Deformed Wings Virus (DWV) (A. Dalmon *et al.* in prep). No application in biological control could however be yet envisaged with these species on *V. velutina*, because of low

efficiency, not yet evaluated risks on non-target species and dispersion capacities or non-adapted development cycle (Beggs *et al.* 2011, Monceau *et al.* 2014a, 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). 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, but also a high potential as a source of metabolites for the creation of alternative phytosanitary products (toxins for example), etc… (Franco *et al.* 2012).

Among the diversity of entomopathogenic fungi, the literature cites two genders as the most described and used in biological control: *Metarhizium* spp. and *Beauveria* spp. (both Ascomycota: Hypocreales) (Bidochka *et al.* 1998, Bidochka & 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 & Pendland 1991, Bidochka & Small 2005, Ortiz-Urquiza & Keyhani 2013).

Isolates of *Metarhizium* are already on the market for biological control of pests, mostly lepidopteran and dipteran control (Annexe 1). *Beauveria bassiana* (Bals.) Vuill. has yet no host specificity connected to genetic described in the European clade (Rehner 2005).

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 & Neves 2014 (Scelionidae), Kpindou *et al.* 2007 (Encyrtidea)), ants (Jaccoud *et al.* 1999, Tragust 2013, Loreto & 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, *V. 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 French isolates of entomopathogenic fungi that we inoculated to *V. velutina* by these different ways.

Material and methods

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).

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 and Shiffenmüller) (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 & 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. By this technique were obtained the isolates of *Metarhizium robertsii* EF2.5 (2), EF3.5 (1), EF3.5 (2) and EF3.5 (4). The growing and multiplication of all the fungi took place in Petri dishes with OAC media (Oat 40g, Agar (PDA, BK095HA, Biokar) 20g, Chloramphenicol (SIGMA Aldrich, Germany) 50mg, QSP 1L) (Cañedo & 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.

Inoculation methods

For assessing the potential control efficiency of the different isolates, we did different inoculation methods: **direct inoculation**, by contact with a **contaminated surface**, in the **food**, or **inter-individual transfer** (Fig 1).

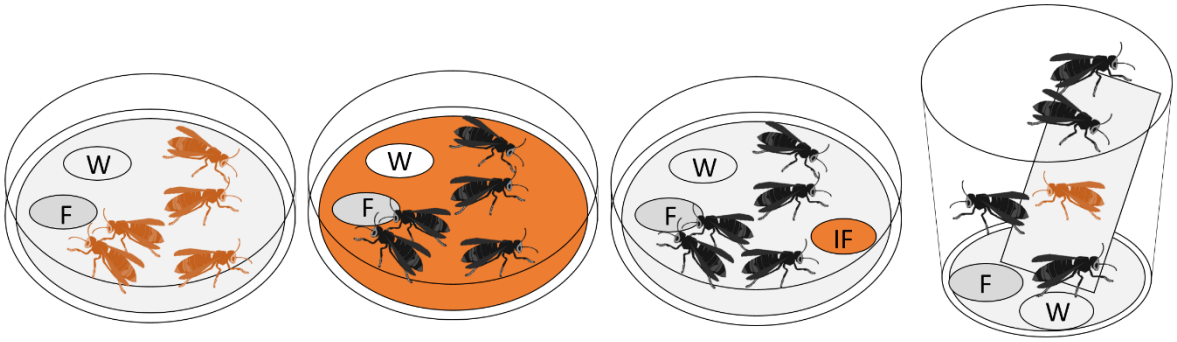

Figure 1: The different treatments used in the experimentation. From left to right: direct contamination of the hornets (orange), contaminated filter paper, contaminated food (IF, orange), and one contaminated individual (orange) with four uncontaminated individuals. In each box there are food (F) and water (W).

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. The control was distilled 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 (candy sugar (glucose, fructose and saccharose) purchased by ®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 all the hornets to wake up, and we removed and replaced dead individuals due to temperature shock or to drowning.

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); N=2, Nb individuals=200), in August 2016 (10 individuals / modalities (all 5 isolates + control x 4 inoculation methods); N=2, Nb individuals=240), the same in September 2016 (N=2, Nb individuals=240) and in October 2016 (N=2, Nb individuals=240).

- **Direct contamination**

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.

- **Contamination 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.

- **Contaminated food**

In this modality, 1ml of spore's solution 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 contaminated as described in "direct contamination" paragraph, then placed on the opposite side of the box from the other hornets before reanimation.

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 (Figures 2.A, 2.B). The death of the hornets could be due to multiple factors (Figures 2.C, 2.D, or bacterial infection, stress, etc.).

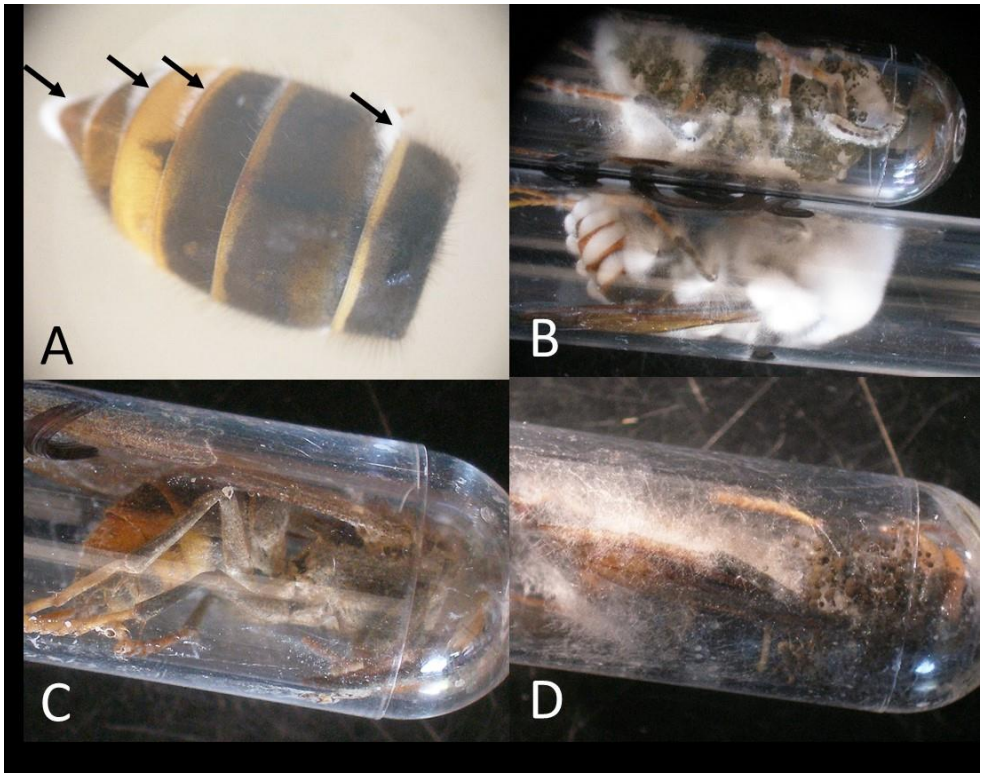

Figure 2: *V. velutina* workers infected by entomopathogens. A. An entomopathogenic fungus is making its way between the cuticle's segments of the abdomen of a hornet. B. Two contaminated hornets by *Metarhizium robertsii* (white mycelium, olive green spores)(top) and *Beauveria bassiana* sp. (white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

Each death caused by entomo-pathogen infection was then reported, to correct the number of dead by treatment and obtain the number of dead by entomo-pathogenic infection per treatment.

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

- *LT50 measurement*

The LT50 is the moment after inoculation when 50% of the hornets died by infection.

Statistical analysis

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

Results

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

Comparisons of the inoculation methods

For all the isolates, the most efficient modality concerning lethality 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) is different from the contact modality (LSD Fisher test) (Table 1).

Table 1: Average lethality and compared lethality 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***	***E.E***			
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*

No significant difference was observed between the different isolates and fungi in their aggressiveness (ANOVA).

Comparison of the isolates

- *Lethality.*

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 LT50.*

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

ISOLATE	M±SD (DAYS)	LT50 (DAYS)
EF2.5(2)	5,68 ± 1,08	6
EF3.5(1)	5,86 ± 1,17	6
EF3.5(2)	5,49 ± 1,38	5
EF3.5(3)	5,41 ± 1,18	6
B	6,25 ± 0,67	6

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

Comparisons of each method for each isolate

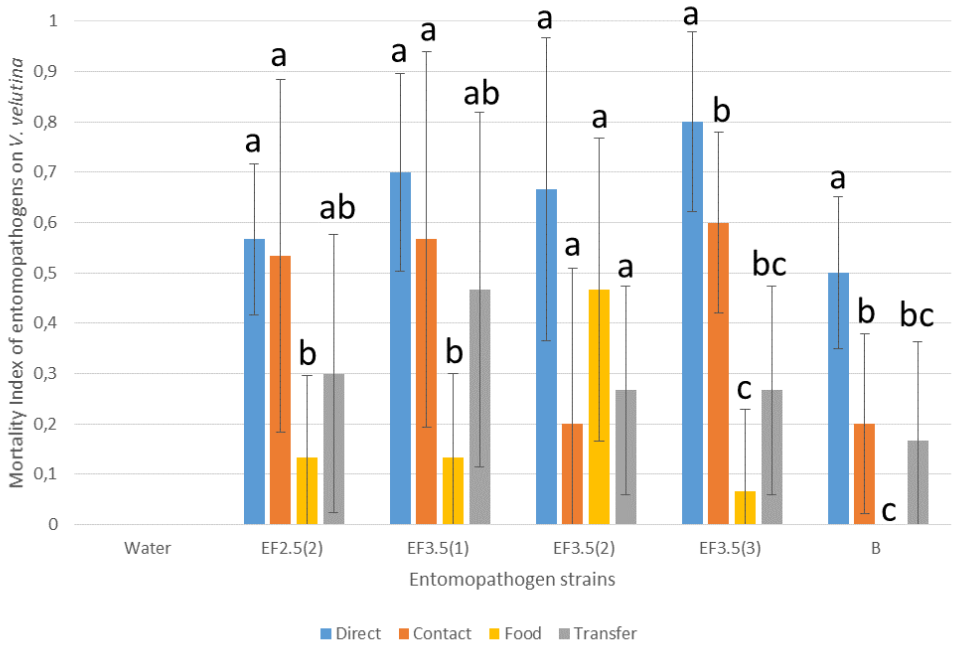

Figure 3: Lethality of entomopathogens isolates in function of the inoculation methods, *i.e.* percentage of dead hornets by entomopathogen infection (ANOVA tests).

For all strains the direct application method was the most efficient, and except in EF3.5(2), the contact method appeared to be also interesting. 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.

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

Discussion

This study may offer at least two issues, first in the theoretical knowledge on the entomopathogenic mode of action of fungi on Vespa species, and second on the perspective of future application to control *V. velutin*a populations. We here highlighted different entomopathogenic fungi that could

potentially be developed for *Vespa* control, and we assessed their efficiency with different inoculation modalities.

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 wild, with unknown variation in their age, past, and therefore in their immune system and sensitivity to infection (Franceschi *et al.* 2000, Moret & 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 solutions on and in the hornet colony. Indeed, 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 keep in mind that we chose protein food as a vector here, and therefore just a proportion of the workers may be interested in eating or collecting it for further use, *i.e.* mostly nutrition of the larvae. Adult hornets don't need proteins for their survival, they depend only on carbohydrates consummation (Spradbery 1973, Raveret Richter 2000). Monceau *et al.* 2013a showed that the roles concerning nest defense of *V. velutina* individuals seem to evolve with their age, and we can thus hypothesize that the attraction for 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.

After having tested the fungi sprayed on nests, a potential **Trojan horse strategy** using entomopathogens could be envisaged in two different ways. First, by actively trapping and directly contaminating *V. velutina* workers with a spore solution before releasing them so they 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 built that will capture and let go hornet workers with the contaminated food, but not other species.

We observed different responses of the entomopathogenic isolates according to the application methods. For a better efficiency of a potential biocontrol solution, a combination of several fungi isolates could thus be envisaged. Moreover, we used here homogeneous climatic conditions for the experimentation purpose, thus we did not consider the climatic requirements and adaptations of the isolates we tested (García-Fernandez *et al.* 2008): 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 assessed in the case of isolate combinations: the possibility of **competition** between isolates and possible decrease of efficiency, and the **panel enlargement of non-target insects** that could be contaminated.

While classical neurotoxic insecticides are, most of the time, quite instantaneously lethal, the infected hornets died from 5±0.44 days after infection, enhancing this way theoretically the potential risk of transmission of the fungi to other insects. This risk, not measured yet, may however appear to be very low in our case because: (1) once contaminated, hornets self-clean themselves and one-another by grooming, decreasing this way the potential transported inoculum (and increasing the intra-colonial transfer), (2) 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 spores survival on exposed surfaces visited by *V. velutina* workers: flowers (collection of nectar), wood (collection of wood pulp for nest construction), apiaries walls or flight board (for honeybee hunt), (5) fungi development may impact the mobility of infected hornets, decreasing this way their dispersion and potential contamination capacity (6) the fungi will not produce spores in high quantity before its total colonization of the host body (e.g. its host death).

While most of chemical insecticide used to treat hornets would accumulate in soil, water and other organisms by trophic network, infected hornets 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.

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 plays an important role for the persistency of entomopathogenic fungi in the environment (Burges 1998, Parker *et al.* 2015), when 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 indigeneous entomopathogens to biologically control the invasive Asian hornet *Vespa velutina*. We tested five different isolates with different inoculation methods on workers, and found very efficient isolates especially when applied directly. Some further work will now 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, DT and RLP 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 Bénédicte Laborie, for the first author financial support. The authors declare no conflict of interest. The first author's funding sponsor had no role in any steps of the study (design, data collection, analyses, writing).

Conflicting interest.

The authors declare no conflicting interest in this research

Appendice

Table 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®

C.4.2 Evaluation de l'efficacité de molécules insecticides et de champignons entomopathogènes sur les larves de *V. velutina*

Cette expérimentation préliminaire est détaillée en **Annexe 3.1**

Résumé

Une stratégie de type Cheval de Troie chimique ou biologique pourrait être mis en place dans le but d'un contrôle efficace des colonies de *V. velutina*. Dans cette expérimentation, nous avons voulu tester l'impact de l'ingestion de **trois insecticides** (D, F et S)[2] et de **deux isolats d'entomopathogènes** (*B. b*assiana et *M. robertsii*) (EP) (EP BB et EP 3.5(1)) sur la survie de larves de *V. velutina*. Le choix des insecticides testés a été fait suivant leur mode d'action, direct ou perturbateur de mue, et les entomopathogènes choisis présentaient des résultats intéressants sur frelons adultes dans nos précédents travaux. Deux concentrations ont été testées pour chaque produit insecticide, à la dose homologuée officiellement pour un usage agricole et à une dose dix fois plus élevée. Pour chaque modalité, une trentaine de larves de *V. velutina* de stades 4-5 provenant d'une même colonie ont été testées. Chacune des 300 larves était nourrie 3 fois par jour et était maintenue dans sa galette à 23°C durant la durée de l'expérience. Chaque larve a reçu à la becquée 5µL de solution de produit ou de spores, ou seulement de l'eau pour les témoins. Puis nous avons suivi l'évolution de l'activité des larves à 24h pour celles testées par des produits, ainsi qu'à 72h pour les EP, dont le développement demande d'avantage de temps, suite à une stimulation mécanique. Les témoins ont très bien survécu à l'essai, le produit S a eu une létalité totale à 24h à la dose x10 mais aucun effet à la dose homologuée. A l'inverse, le produit F a été létal pour plus de la moitié des larves appliqué à sa dose homologuée, mais n'a eu aucun effet à dose x10. Pour finir, le produit D n'a eu aucun effet quelle que soit sa dose d'application. Concernant les entomopathogènes, des émergences d'adultes inopinées dans les galettes testées durant l'expérience ont provoqué la mort d'une grande partie des larves, dont les exsudats étaient leur seule source de nourriture sucrées. Les EP étant incapables de se développer dans des insectes morts dans les premiers stades infectieux, nous ne pouvons rien conclure de ces modalités. Cet aspect pourra facilement être amélioré en tuant les nymphes avant de démarrer l'expérience. Cette expérimentation a permis de faire un premier screening de produits, mais surtout de développer une méthode intéressante pour de futurs essais sur couvains de *V. velutina*. (Pour plus de détails, voir la Discussion de la thèse.)

[2] L'identité de ces trois insecticides est confidentielle, ils sont donc mentionnés sous les noms de code D, F et S.