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

J. Poidatz[a], R. López Plantey[b], D. Thiéry[a,*]

[a] *UMR 1065 Santé et Agroécologie du Vignoble, INRA, ISVV, Villenave d'Ornon, France*
[b] *Laboratory of Phytopathology, Institute of Agricultural Biology of Mendoza (IBAM), Mendoza, Argentina*

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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. Indeed to destroy nests, applicators currently use large spectrum insecticides, which is too costly or dangerous to applicators and also to the environment, affecting non-targeted arthropods (one period). Studying the potential interest of biological control methods may help to propose alternatives in *V. velutina* control. We present here the bioassays in which we assessed the potential control efficiency of different indigenous French isolates of entomopathogenic fungi. We inoculated adults *V. velutina* by different ways: being directly, by walking on a contaminated surface, in the food, or by inter-individual transfers. We tested differences between the isolates and the application methods using two parameters mortality and LT50. The direct inoculation method was the most efficient modality, then the contact, transfer and food. Considering all contamination methods, there was no difference on susceptibility or mortality among different isolates. Still the LT50 was quite short in all isolates (average 5.8 ± 0.44d), and their virulence was quite high: we conclude that there is high potential in using such entomopathogens as a biological control agent against *V. velutina*.

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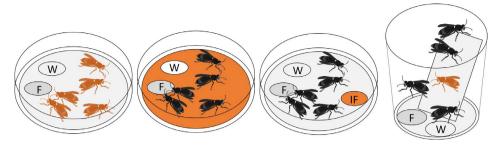
Fig. 1. The different inoculation methods used in the experimentation. Direct: direct inoculation of the hornets (orange), Contact: inoculated filter paper, Food: inoculated food (IF, orange), and Transfer: one inoculated individual (orange) with four uncontaminated individuals. In each box there are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

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 et al., 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.

2. Material and methods

2.1. 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 ($10 \times 20 \times 10$ cm) inside a climatic chamber at $23\,°C \pm 1\,°C$, $12\,h/12\,h$ light. They had *ad libitum* access to water and honey like in previous studies (Poidatz et al., 2017).

2.2. 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 ha.

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 (24 °C, >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 40 g, Agar (PDA, BK095HA, Biokar) 20 g, Chloramphenicol (SIGMA Aldrich, Germany) 50 mg, 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 s, 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.

2.3. 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 10 cm 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, 9 cm diam × 10 cm diam × 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 min 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 12 h.

Four repetitions of the bioassay were made: in October 2015 (10 individuals/modalities ((*Metarhizium r.* 4 isolates + control) × 4 inoculation methods); Nb individuals = 200, N = 2), in August 2016 (10 individuals/modalities ((all 5 isolates + control) × 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 (<1 s) 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, 3 ml 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, 1 ml of spore suspension was poured in 10 mg of cooked tuna (from the market). The fish was left in the boxes only 24 h 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.

2.4. 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
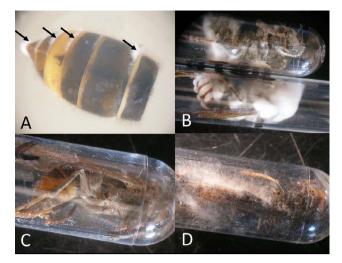

Fig. 2. *V. velutina* workers infected by entomopathogens. A. An entomopathogenic fungus is making its way between the cuticle's segments of the abdomen of a hornet. B. Two contaminated hornets by *Metarhizium robertsii* (white mycelium, olive green spores)(top) and *Beauveria bassiana* sp. (white mycelium,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

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. 2A and B). The death of the hornets could be due to multiple factors (Fig. 2C and 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.

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

- Lethal time 50 (LT50)

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

- Statistical analysis

All results have been analyzed in ANOVA with a test LSD Fisher (alpha = 0.05) using the software Infostat update 2016.

3. Results

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

3.1. 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

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: 00746; gl: 151).

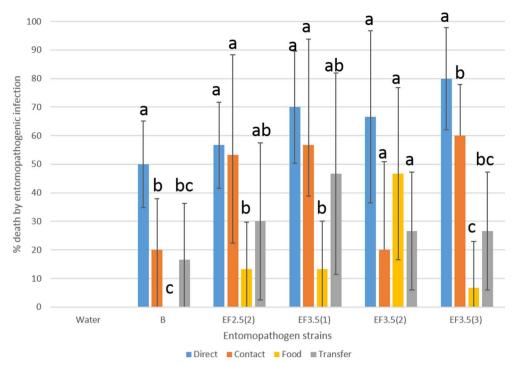| Treatment	Average	N	SD	
Food	0.14	38	0.04	A
Transfer	0.26	38	0.04	A B
Contact	0.35	38	0.04	B
Direct	0.60	41	0.04	C

Fig. 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).

food method. This last treatment (food) was less efficient than the contact modality (LSD Fisher test) (Table 1).

3.2. 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)

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

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

3.3. Comparisons of each method for each isolate

4. 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 A). *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 (2.1–5.6 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 (Spradbery, 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-Fernández 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.

5. 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.

Conflict of interest

The authors declare no conflicting interest in this research.

Appendix A. 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®

Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2018.02.021.

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