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Research Article

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Attraction of the Invasive Hornet, *Vespa velutina nigrithorax*, by using *Bacillus* sp. BV-1 Cultures

Da Jung Lim[1†], Jeong Eun Lee[1†], Jin Sil Lee[2], Iksoo Kim[2] and In Seon Kim[1*]

[1]Department of Agricultural Chemistry,

[2]Institute of Environmentally Friendly Agriculture, Chonnam National University, Gwangju 61186, Korea

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ORCID
In Seon Kim
https://orcid.org/0000-0003-1061-6848

Abstract

BACKGROUND: The invasive hornet *Vespa velutina nigrithorax* has becomes a public concern in rural and urban South Korea. The technologies are necessary to develop a way to counter *V. velutina*. In an effort to develop a way to counter *V. velutina*, we found that a bacillus strain, named *Bacillus* sp. BV-1, produces volatile compounds that attract *V. velutina*.

METHODS AND RESULTS: Field trials of *V. velutina* attraction were performed using plates and traps containing BV-1 cultures grown on sugar medium. When the sugar medium and sugar-grown BV-1 cultures in the plates were placed close together, *V. velutina* visited preferably the plates with the BV-1 cultures. Significantly more *V. velutina* were caught in the traps containing BV-1 cultures than in those containing only sugar medium. Headspace solid-phase microextraction coupled with GC/MS analysis of BV-1 cultures detected 2-methyl-1-propanol, 3-methyl-1-butanol, 3-methylbutanoic acid, ethyl hexanoate, 2-pheylethanol, ethyl octanoate, and ethyl decanoate as the major volatiles.

CONCLUSION: BV-1 cultures were suggested as potential agents for managing *V. velutina* as they produce volatile compounds that attract the hornet.

*Corresponding author: In Seon Kim
Phone: +82-62-530-2131; Fax: +82-62-530-2139;
E-mail: mindzero@chonnam.ac.kr
†These two authors contributed equally to this work.

Key words: Hornet, Insect attraction, *Vespa velutina*, Volatile compounds, Wasp

Introduction

Vespa velutina is a honeybee predator that was accidently introduced into Busan, South Korea, in the early 2000s (Moon *et al.,* 2006). A positive correlation between the abundance of *V. velutina* and degree of urbanization indicates that this invasive hornet is adapted to urban environments (Lee *et al.,* 2012), contributing to its wide distribution throughout South Korea. The spread of *V. velutina* has becomes a social issue due to its impact on rural beekeepers and people in urban areas (Jung, 2012a; Park and Jung, 2016). According to a report by the National Fire Agency of South Korea, the number of emergency call-outs due to *V. velutina* has increased significantly since 2003, with 396,822 call-outs from 2015 to 2017. The National Fire Agency attributed four deaths to *V. velutina* stings in 2017, indicating that *V. velutina* is a threat to human health in urban areas. *V. velutina* is also a major threat to rural beekeeping businesses. Annually, *V. velutina* destroys more than 30% of the hives, which is equivalent to a honey production loss of 175 million dollars (Jung, 2012b). Consequently, research seeking ways to counter *V. velutina* has received much attention.

Techniques introduced to fight hornets include toxic bait (Harris and Etheridge, 2001), mites (Gerson *et al.,* 2003), entomopathogenic fungi (Poidatz *et al.,* 2018), parasitoids (Darrouzet *et al.,* 2015), and bait traps (Liang and Pietri, 2017). Of these, bait-trap methods are typically used because they are simple and inexpensive. Bait-trap methods use carbohydrate and protein foods containing volatile compounds to attract hornets. Demichelis *et al.* (2014) compared the use of different bait traps for studying social wasp populations using polyethylene bottles with different-colored caps containing sugar, syrup, vinegar, and essential oil, alone and in combination, in beer. They suggested that beer-bait traps filled with syrup in white- or yellow-capped bottles were effective for attracting *V. velutina*. A mixture of sweet and acidic baits in wine has been reported to be useful for capturing hornet queens in early March (Sim *et al.,* 2014). Honeybee comb extracts containing sugar syrup and beer were also used to trap *V. velutina* queens in southern South Korea from March to May (Choi *et al.,* 2015; Kang *et al.,* 2016).

Given the rapid spread of *V. velutina* in South Korea, it is important to develop various methods to control this hornet. In a search for *V. velutina* control methods, we screened microorganisms that produce volatile compounds related to the attraction of the hornet and found that bacillus strain *Bacillus* sp. BV-1 produced attractive metabolites. The metabolites were determined by headspace solid-phase microextraction coupled with GC-MS. Here, we report microbial metabolites as a bait that is attractive to *V. velutina*.

Materials and Methods

Microbial isolation

Microorganisms were isolated from a fermentation mixture containing sugar and oak tree that has been conventionally used in honeybee farms as a bait trap to counter *V. velutina*. According to honeybee keepers, acidic volatiles released from the mixture during storage process, suggesting microbial involvement in the process. To isolate microorganisms attributed to the volatiles, an aliquot (1 mL) of the mixture was suspended in 100 mL of mineral salt medium (MSM) containing 30% (w/v) and 50% (w/v) sugar as a carbon source and the following constituents (in grams per liter, pH 7.0): Na_2HPO_4, 2.0, KH_2PO_4, 1.0, $(NH_4)_2SO_4$ 0.4, $MgSO_4{\cdot}7H_2O$, 0.4. The suspension was incubated at 30℃ for 4 days on a shaking incubator at 150 rpm. Following microbial growth in the MSM, four different colonies by visual observation were obtained and purified by plating them repetitively on the MSM agar plates. Individual colonies were incubated in the MSM medium and subjected to further studies. Microbial identification was performed based on 16S rRNA sequence analyses, as described previously (Lim *et al.,* 2017).

Attraction bioassay

Field trials of for attraction of *V. velutina* were performed at a honeybee farm (Suncheon, Jeonnanm) in the early afternoon in August. Firstly, for attraction bioassays using plates, the plates (i.d. 9 cm) containing 20 mL of sugar medium and sugar-grown microbial cultures were placed on tables near honeybee hives. The plates were spaced apart (18 cm × 9.5 cm) from each other. The number of visits of *V. velutina* to the plates was counted by visual observation during the first 5 minutes of each attraction trial. Secondly, for attraction bioassay using traps, a model Version 3 Damok EcoTech (Suncheon, Jeonnam) hornet trap with holes allowing honeybees to escape was used. The traps containing 500 mL of sugar medium and sugar-grown microbial cultures were placed near honeybee hives and spaced 70 cm apart from each other. The bioassay was performed three times over a period of one hour from AM 13:00 to PM 16:00. The traps were transferred to a refrigerator at -20℃ to knockdown *V. velutina*, and the number of hornet workers caught in the traps were counted.

Identification of volatile compounds

Headspace solid-phase microextraction (SPME) coupled with gas chromatography mass spectrometer (GC/MS) was employed to identify volatile metabolites in microbial cultures (Fuchsmann *et al.,* 2015). For this, a Supelco SPME fiber assembly with a SPME PDMS/DVB fiber (65 ㎛, Supelco, Bellefontr, PA, USA) was conditioned and calibrated according to the manufacturer's introduction. The samples (10 mL) were placed in a 20 mL amber glass vial with a silicon/PTFE septum (Supelco) along with butyl isopropyl ketone as the internal standard (Sigma-Aldrich, St. Louis, MO, USA). The sample was equilibrated at room at 25℃ for 30 min, and the SPME fiber in the assembly was exposed to the samples for 10 min. The volatiles on the fiber was

desorbed onto the column (DB-5, 0.25 mm i.d. × 30 m in length, 1.0 μm film thickness) for 3 min through a splitless injector at 250℃. A Shimazu model QP2010 GC/MS system was employed in electron impact (EI) with 70 eV EI energy. The flow rate of carrier gas (He) was 1.0 ml/min, and the oven temperature was set for 2 min at 60℃, followed by ramping at a rate of 10℃/min to 250℃. GC/MS chemical library databases (Willey7, NIST27 and NIST147) were used to identify the volatile metabolites.

Results and Discussion

Among the isolates studied, the microorganism that showed the best attraction of *V. velutina* workers, designated *Bacillus* sp. BV-1 (BV-1), was examined for further study. The 16S rRNA sequence of BV-1 showed 98% similarity to that of *Bacillus jeotgali* YKJ-10 (Fig. 1). However, the strain YKJ-10 could not grow on the sugar medium, suggesting that BV-1 is an isolate different from YKJ-10. The growth of BV-1 was accompanied by the formation of bubbles after a 48-h incubation, demonstrating that BV-1 produced volatile metabolites during its growth.

Field trials of the attraction of *V. velutina* were performed using microbial cultures in plates and hornet traps. *V. velutina* workers were mostly attracted to the plates with microbial cultures grown on sugar medium (Fig. 2). During the first 5 minutes, *V. velutina* workers visited first the plates with microbial cultures grown on 50% sugar medium first. The *V. velutina* workers visited more frequently the plates with 50% sugar-grown cultures than the plates with 30% sugar-grown cultures, while the number of *V. velutina* workers that visited the 30% or 50% sugar solution without microbial cultures was not observed during the experiment. The field trials using hornet traps caught significantly more *V. velutina* workers in the traps with BV-1 cultures than in the traps with only sugar medium over the duration of the experiments (Table 1). These results suggest that BV-1 cultures contain volatile metabolites that attract the hornet.

To characterize the volatile metabolites produced by BV-1, GC/MS analysis coupled with headspace solid-phase microextraction was performed. The GC/MS analyses detected 2-methyl-1-propanol, 3-methyl-1-butanol, 3-methylbutanoic acid, ethyl hexanoate, 2-pheylethanol, ethyl octanoate and ethyl decanoate as the major volatile metabolites produced by the BV-1 cultures (Table 2), while no volatile metabolites were detected in the sugar medium without BV-1 cultures. Of the metabolites, 3-methyl-1-butanol was the most abundant based on the area and number of peaks. Other metabolites were detected depending on the sugar medium. These observations suggested that 3-methyl-1-butanol is likely the main metabolite attracting *V. velutina* workers.

V. velutina has becomes a public concern due to its impact on beekeepers in rural areas and people in urban areas. Therefore, much effort has been made to

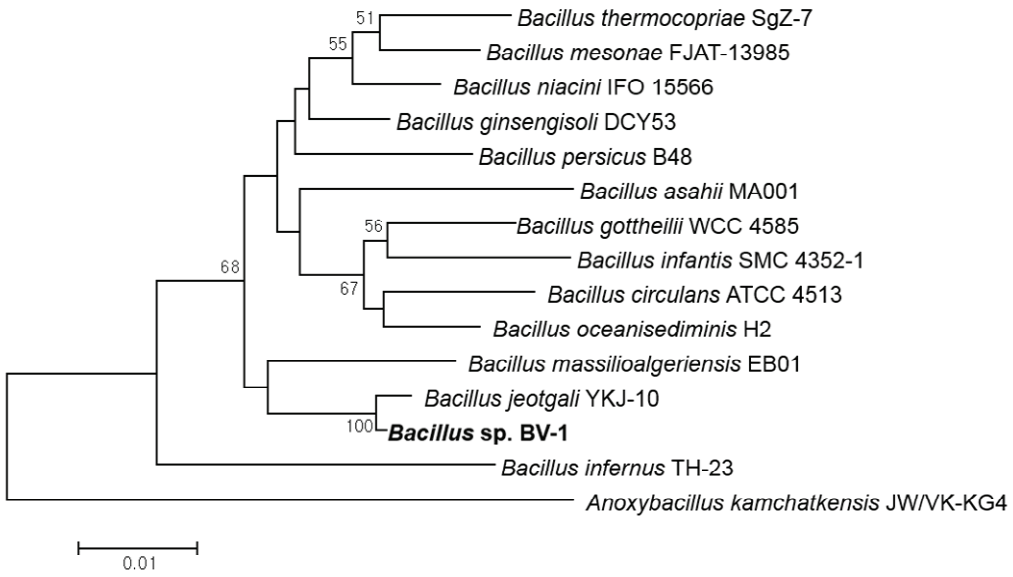

Fig. 1. Phylogenetic tree of *Bacillus* sp. BV-1 based on 16S rRNA sequence analysis.

Fig. 2. Field trials for the attraction of *Vespa velutina* workers using *Bacillus* sp. BV-1 cultures. 1. Blank, 2. 30% (v/v) sugar medium, 3. 50% (v/v) sugar medium, 4. Cultures grown on 30% (v/v) sugar medium, 5. Cultures grown on 50% (v/v) sugar medium.

Table 1. Number of *V. velutina* workers attracted and captured in traps containing sugar medium or sugar-grown *Bacillus* sp. BV-1 cultures

Attractant	Number of *V. velutina* workers		
	1st trial	2nd trial	3rd trial
SM 1[1)]	0	0	1
SM 2[2)]	0	0	1
BV-1 cultures grown on SM1	13	14	16
BV-1 cultures grown on SM2	18	20	25

[1)] 30% (v/v) sugar medium.
[2)] 50% (v/v) sugar medium.

Table 2. Chemical constitutes of volatile metabolites in sugar-grown Bacillus sp. BV-1 cultures

Chemical constitute	Abundance (peak area x 10^6)[1)]	
	Cultures grown on SM1[2)]	Cultures grown on SM2[3)]
2-methyl-1-propanol	0.94 ± 0.03	0.35 ± 0.01
3-methyl-1-butanol	13.2 ± 0.13	16.32 ± 0.08
3-methylbutanoic acid	-	0.16 ± 0.01
Ethyl hexanoate	3.04 ± 0.42	0.52 ± 0.05
2-phenylethanol	2.10 ± 0.09	4.76 ± 0.08
Ethyl octanoate	3.95 ± 0.38	1.30 ± 0.16
Ethyl decanoate	6.75 ± 2.29	0.95 ± 0.15

[1)] Means ± SD of triplicate.
[2)] 30% (v/v) sugar medium.
[3)] 50% (v/v) sugar medium.

develop effective strategies for managing this hornet. One commonly used method is bait traps that attract the hornet (Monceau *et al.,* 2013; Couto *et al.,* 2014; Wang *et al.,* 2014). Several methods using bait traps have been introduced for the effective management of *V. velutina* in Korea (Sim *et al.,* 2014; Choi *et al.,* 2015; Kang *et al.,* 2016), demonstrating that the main mechanism for attracting *V. velutina* is by a feeding response. However, these methods require quality control to reach consistent attraction efficiency. In Korea, beekeepers struggle to manage *V. velutina* each year to protect their hives. Consequently, the attraction agents should be prepared consistently in a commercial product for beekeepers.

In this study, we examined microbial cultures as a potential attractant of *V. velutina* workers. Field trials of BV-1 cultures combined with mechanical selection (*i.e.*, with holes allowing honeybees to escape) successfully attracted *V. velutina* workers. BV-1 produced 3-methyl-1-butanol as the major volatile metabolite attracting *V. velutina* workers. 3-Methyl-1-butanol is known to attract lepidopteran pests (Landolt & Alfaro, 2001; Landolt & Higbee, 2002; El-Sayed *et al.,* 2005). The combination of 3 -methyl-1-butanol and acidic chemicals also attracts pests (Day & Jeanne, 2001; Landolt *et al.,* 2007). 3-Methyl-1-butanol is an insect pheromone that acts synergistically with 2-pentanol to elicit a strong defensive reaction in the hornet (Ono *et al.,* 2003). These studies suggest that 3-methyl-1-butanol can play a role in pest management programs. Other minor volatiles detected in the BV-1 cultures, such as 2-methyl-1-propanol, 3-methylbutanoic acid, 2-phenyethnol, and fatty acid esters, are also insect chemical attractants and pheromones (Hossain *et al.,* 2008; Johnson *et al.,* 2009; Tóth *et al.,* 2002; Okumu *et al.,* 2010; Trhlin and Rajchard, 2011). These indicate that the volatile metabolites produced by BV-1 cultures relate to insect attraction. Our study does not rule out the possibility that BV-1 cultures producing 3-methyl-1-butanol would attract both hornets and honeybees, but the combination of cultures with mechanical selection (with holes allowing honeybees to escape) could successfully manage *V. velutina*, which would help the beekeeping business in Korea.

Note

The authors declare no conflict of interest.

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