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RESEARCH ARTICLE

# Volatiles from wasabi inhibit entomopathogenic fungi: implications for tritrophic interactions and biological control

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When wasabi plants are damaged by plant pathogens or herbivores they produce volatile allyl isothiocyanate (AITC) as a defense mechanism. In the current study, we conducted experiments to determine whether volatiles from damaged wasabi leaves or synthetic AITC also have negative effects on the beneficial entomopathogenic fungi, *Beauveria bassiana sensu lato* (Balsamo) Vuillemin and *Isaria fumosorosea* Wize, which contribute to regulation of a major pest of wasabi, the striated white butterfly, *Pieris melete* Ménétriès. Conidial germination of both fungi was inhibited when exposed to volatiles from macerated wasabi leaves; the concentration of AITC in the volatiles was also quantified. The inhibitory activity of macerated wasabi leaves was compared with that of synthetic AITC, demonstrating that the inhibitory effect of wasabi volatiles was due to the presence of AITC. These results indicate that AITC released from damaged wasabi leaves has the potential to inhibit fungal infection of insect pests in wasabi fields.

**Keywords:** wasabi; plant volatiles; allyl isothiocyanate; *Beauveria bassiana*; *Isaria fumosorosea*; *Pieris melete*

## 1. Introduction

Japanese horseradish, or wasabi (*Wasabia japonica* Matsumura), is a cruciferous plant widely used in Japanese cuisine, particularly as a pungent spice in sushi. Wasabi plants are grown for 2–3 years in rice paddy-like fields, filled throughout the year with shallow flowing water from mountain streams (Figure 1a). These conditions should be very suitable for biological control of herbivores using entomopathogenic fungi because high humidity is an absolute requirement for conidial germination and the establishment of infection (Hall 1981; Milner & Lutton 1986; Helyer et al. 1992; Luz & Fargues 1999). Furthermore, to avoid downstream water pollution, there are enforced restrictions on chemical applications in wasabi fields; this should also be beneficial for entomopathogenic fungi that can be susceptible to pesticides, particularly fungicides (Saito & Yabuta 1996; Shinohara et al. 2013).

The strong flavor of wasabi is due to the presence of allyl isothiocyanate (AITC), also called mustard oil, which is produced in plant tissues that have been damaged by biotic or abiotic stressors; the enzyme myrosinase hydrolyses allyl glucosinolate (sinigrin) producing the breakdown product, AITC (Rask et al. 2000; Wittstock & Halkier 2002; Wittstock et al. 2003). The compound is highly toxic to a wide range of organisms including various herbivores (Rask et al. 2000; Agrawal & Kurashige 2003; Wittstock et al. 2003; Eltayeb et al. 2010) and plant pathogens (Mayton et al. 1996; Oliver et al. 1999). As such, AITC contributes to the plant's defense strategy against herbivores and disease (Chew 1988; Louda & Mole 1991; Rask et al. 2000; Wittstock et al. 2003). Wasabi actually produces larger quantities of AITC in leaves, petioles, rhizomes, and roots, than other cruciferous plants (Kumagai et al. 1994; Sultana et al. 2003).

As AITC is toxic to plant pathogens (including fungi) it is possible that it could also have toxic effects on beneficial entomopathogenic fungi in the field; this may decrease their potential as biological control agents of herbivorous pests on wasabi. Therefore, to ensure successful biological control, fungal isolates with potential for augmentation in the field should be evaluated for their susceptibility to volatile AITC prior to use. To date, there have already been some studies describing interactions between other plant volatiles and entomopathogenic fungi (Brown et al. 1995; Baverstock et al. 2005; Hountondji et al. 2005); however, little is known about the effects of volatile AITC released from cruciferous plants on entomopathogenic fungi, with the exception of one study describing effects of synthetic isothiocyanates, including AITC, produced by cruciferous plants, on conidial germination and mycelial growth of *Metarhizium anisopliae* (Metschn.) Sorokin when incorporated into the culture medium (Inyang et al. 1999).

The purpose of our study was to examine the effects of volatiles released from wasabi leaves on conidial germination of entomopathogenic fungi that had been isolated from populations of the small white butterfly, *Pieris melete* Ménétriès, a serious pest of wasabi plants in Japan (Nakata 1963; Saito et al. 2010). Based on these results, we discuss the importance of AITC in terms of biological control and plant volatile-mediated tritrophic interactions between plants, herbivores, and their natural enemies.

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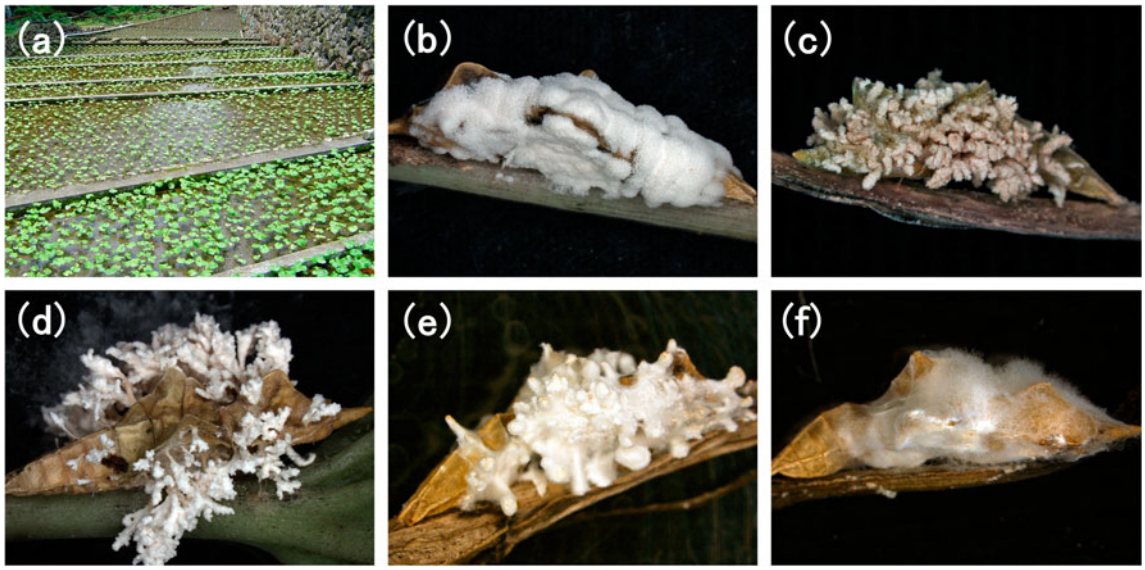


Figure 1. (a) A wasabi field. (b–f) Mycosed pupae of *P. melete* infected by *B. bassiana* s.l., *I. fumosorosea*, *I. tenuipes*, *I. cateniannulata*, and *Lecanicillium* sp., respectively.

## 2. Materials and methods

### 2.1. Collection of entomopathogenic fungi

In 2011 and 2012, *P. melete* larvae were collected from several wasabi fields in Shizuoka, Japan, and then reared in groups of 20–30 on leaves of kale, *Brassica oleracea* L. var. *acephala*, in containers (20 × 15 × 5 cm) at 25 ± 1°C and 16L:8D. Dead larvae and pupae were transferred to Petri dishes (9 cm diameter) with a wet filter paper and incubated for a week at 25 ± 1°C in darkness to induce fungal sporulation on cadavers. Entomopathogenic fungi were isolated from infected cadavers on to Sabouraud dextrose agar (SDA; Difco BD Bioscience, MD, USA) and all isolates were stored on slopes of culture medium in glass test tubes at −60°C prior to use in each experiment. Each isolate was identified according to morphological characteristics using the taxonomic keys for the genus *Beauveria* (Rehner et al. 2011), genus *Isaria* (Samson 1974; Shimazu 2001; Liang et al. 2005; Luangsa-ard et al. 2005), and genus *Lecanicillium* (Zare & Gams 2001, 2008).

### 2.2. Effect of volatiles from macerated wasabi leaves on conidial germination

For use in experiments, two isolates were selected from those collected in the field (see Section 3.1); these isolates were retrieved from storage, subcultured on SDA in Petri dishes (9 cm diameter), and incubated at 25 ± 1°C for 10 days in darkness. Conidial suspensions of each isolate were prepared by scraping the mycelium from plate cultures into sterile 0.1% Tween 80, agitating with a vortex mixer and then filtering through sterile muslin (0.2 mm mesh size). Conidial concentrations of the suspensions were determined using a Thoma haemocytometer and adjusted to $1 \times 10^7$ conidia $mL^{-1}$ by the addition of sterile 0.1% Tween 80. A 50-µL aliquots of suspension were placed, individually, on to 10-mL SDA in Petri dishes (9 cm diameter); the dishes, without their lids, were each suspended upside down beneath the lids of airtight glass chambers (12 cm diameter, 7 cm height, 0.79 L volume) using double-sided adhesive tape. The prepared chambers were used to expose conidia to volatiles from macerated wasabi leaves as described below.

Fresh wasabi leaves collected from the field were macerated for about 5 min in a food processor (SKH-A100, Tiger, Osaka, Japan) and then immediately 0, 1, 3, 5, or 10 g were placed in the base of each chamber; these quantities were calculated as 0, 1.3, 3.8, 6.3, and 13 g $L^{-1}$ chamber, respectively. The chambers were sealed using petrolatum and then incubated at 25 ± 1°C and 16L:8D. After 24 h, the conidia were fixed and stained with drops of lactophenol-cotton blue solution (Sigma-Aldrich, MO, USA); the germination rate of approximately 100 conidia in each replicate of each treatment was determined under a microscope, for both isolates. Conidia were considered to have germinated when the germ tube was at least half as long as the width of the conidium. Five replicate chambers were used for each treatment. The proportion of conidia that had germinated in each replicate was arcsine square-root transformed and submitted to factorial analysis for two factors, the quantity of wasabi and the fungal isolate, using a two-way analysis of variance. If there was an interaction between the two factors, multiple comparisons using a Bonferroni correction were made. These analyses were all done in the software package SPSS (2009).

### 2.3. Effect of synthetic AITC on conidial germination

The toxicity of synthetic AITC (99%, Wako Pure Chem., Osaka, Japan) was examined using the same method as described above in Section 2.2, except that 100 µL of acetone solution containing AITC was used instead of

macerated wasabi leaves. A series of different concentrations of AITC that had been selected from preliminary experiments (data not shown) were evaluated: 0, 6.3, 63, and 630 μg $L^{-1}$ chamber. Five replicate chambers were used for each treatment. Data were analyzed using the same methods as described above in Section 2.2.

### 2.4. Quantifying the concentration of AITC in volatiles from macerated wasabi leaves

AITC in volatiles from macerated wasabi leaves was quantified using a headspace sampling method in conjunction with solid phase microextraction (SPME). Three grams of macerated wasabi leaves (3.8 g $L^{-1}$ chamber) were placed in the bases of each of five of the glass chambers described in Section 2.2. An SPME fiber (DVB/CAR/PDMS, Sigma-Aldrich, Dorset, UK) was inserted through a 4-mm diameter hole (sealed with a rubber washer) into the sie of each chamber for 30 min at 25 ± 1°C. Each SPME fiber was then introduced into the injector of a gas chromatograph (3800GC, VARIAN, CA, USA) with a DB-WAX capillary column (60 m × 0.32 mm, 0.25 μm). Helium was used as the carrier gas (2 mL $min^{-1}$). Thermal conditions for the column were 35°C for 5 min, followed by a temperature increase from 35 to 120°C at a rate of 5°C $min^{-1}$ and then from 120 to 320°C at a rate of 15°C $min^{-1}$.

The concentration of AITC produced was quantified based on the area beneath the peak, which was estimated using a standard curve for AITC. Data for the standard curve were collected using 100-μL acetone solutions containing known concentrations of AITC (0, 6.3, 63, 630, and 6300 μg $L^{-1}$ chamber); there were three replicate chambers for each concentration and these data were subjected to linear regression to determine the relationships between peak areas and log doses using the software package SPSS (2009).

## 3. Results

### 3.1. Entomopathogenic fungi isolated from P. melete

A total of 1557 larvae of *P. melete* were collected from wasabi fields (Figure 1a) and five species of fungi were isolated from 67 cadavers (mainly pupae): *Beauveria bassiana sensu lato* (Balsamo) Vuillemin (six isolates; Figure 1b); *Isaria fumosorosea* Wize (three isolates; Figure 1c); *Isaria tenuipes* Peck (52 isolates; Figure 1d); *Isaria cateniannulata* (Z. Q. Liang) Samson & Hywel-Jones (eight isolates; Figure 1e); and *Lecanicillium* sp. (one isolate; Figure 1f). Three mycosed cadavers had mixed infections of two fungal species: these were *I. tenuipes* and *I. cateniannulata* (one cadaver) and *I. fumosorosea* and *I. cateniannulata* (two cadavers). Of the isolates collected, *B. bassiana* s.l. and *I. fumosorosea* had also been recorded in previous preliminary field surveys (Saito et al. 2010); for this reason one isolate of each of these species (BB006 and IF002, respectively), both with reliable spore production capacities, was selected for evaluation as it was representative of the most common species affecting *P. melete* in wasabi fields.

### 3.2. Effect of volatiles from macerated wasabi leaves on conidial germination

There was no significant difference in germination between the *B. bassiana* s.l. and the *I. fumosorosea* isolates in response to wasabi leaves ($F_{1,40}$ = 3.14, $P$ = 0.0839; Table 1). However, there was a significant negative effect of macerated wasabi leaves on germination of both isolates ($F_{4,40}$ = 3018.38, $P$ < 0.0001; Table 1). Both *B. bassiana* s.l. and *I. fumosorosea* conidia had a germination rate of 100% in the control, and *B. bassiana* s.l. conidia exposed to volatiles from 1.3 g $L^{-1}$, 3.8 g $L^{-1}$, and 6.3 g $L^{-1}$ of macerated wasabi leaves had germination rates of 100%, 2.7%, and 1.8%, respectively, while *I. fumosorosea* conidia exposed to the same levels of wasabi leaves had germination rates of 100%, 4.5%, and 0%, respectively. Neither isolates germinated when exposed to volatiles from 13 g $L^{-1}$ of macerated wasabi leaves. There was a significant interaction between the quantity of wasabi leaves and isolate ($F_{4,40}$ = 3.14, $P$ = 0.0245) but only in one treatment (6.3 g $L^{-1}$ of wasabi leaves) indicating a significant difference in germination rate between the two isolates at this quantity of wasabi leaves ($F_{1,40}$ = 15.71, $P$ = 0.0003).

### 3.3. Effect of synthetic AITC on conidial germination

There was no significant difference in germination of *B. bassiana* s.l. and *I. fumosorosea* isolates in response to synthetic AITC ($F_{1,32}$ = 1.82, $P$ = 0.1864; Table 2). However, there was a significant negative effect of synthetic AITC on germination of both isolates ($F_{3,32}$ = 1048.74, $P$ < 0.0001; Table 2). Both *B. bassiana* s.l. and *I. fumosorosea* conidia had a germination rate of 100% in the control. *Beauveria bassiana* s.l. conidia exposed to 6.3 μg $L^{-1}$ and 63 μg $L^{-1}$ of AITC had germination rates of 99.0% and 12.3%, respectively, and *I. fumosorosea* conidia exposed to the same levels of AITC had germination rates of 90.5% and 15.1%, respectively. Neither isolate germinated when exposed to volatiles from 630 μg $L^{-1}$ of AITC. There was a significant interaction between the concentration of AITC and the isolate ($F_{3,32}$ = 6.67, $P$ = 0.0013) but only in one treatment (6.3 μg $L^{-1}$ of AITC) indicating a significant difference in germination rate between the two isolates at this concentration of AITC ($F_{1,32}$ = 19.07, $P$ = 0.0001).

### 3.4. Concentration of AITC in volatiles from macerated wasabi leaves

The quantity of AITC contained in volatiles released from macerated wasabi leaves (3.8 g $L^{-1}$) was quantified by gas chromatography. The standard curve for AITC was determined as $Y = 86.5X - 104.3$ ($F_{1,10}$ = 36.73, $r^2$ = 0.786) for peak areas ($Y$) and log doses ($X$). Based on linear regression analysis, the concentration of AITC

Table 1. Percent germination of *B. bassiana* s.l. and *I. fumosorosea* conidia exposed to different quantities of macerated wasabi leaves for 24 h in a chamber.

| Quantity of macerated wasabi leaves (g $L^{-1}$ chamber) | Percent germination (mean ± SE) | |
|---|---|---|
| | *B. bassiana* s.l. | *I. fumosorosea* |
| 0 | 100 | 100 |
| 1.3 | 100 | 100 |
| 3.8 | 2.7 ± 1.2 | 4.5 ± 4.0 |
| 6.3 | 1.8 ± 0.5 | 0 |
| 13 | 0 | 0 |

released from the sample leaves was 45 ± 5 μg $L^{-1}$ (mean ± SE).

## 4. Discussion

There have been some studies describing interactions between plant volatiles and entomopathogenic fungi. For example, *Neozygites tanajoae* Delalibera Jr., Humber & Hajek produced more conidia when exposed to volatiles from cassava leaves damaged by mites (Hountondji et al. 2005). *Pandora neoaphidis* (Remaudière & Hennebert) Humber increased the number of conidia germinating on broad bean plants damaged by aphid feeding (Baverstock et al. 2005) and, in another study, demonstrated that conidial germination of *P. neoaphidis* was inhibited by the volatiles released from tobacco plants in response to either aphid attack or artificial damage (Brown et al. 1995). These reports indicate that plant volatiles influence interactions between herbivores and entomopathogenic fungi in different ways, and that understanding these interactions is essential for the development of successful biological control strategies using fungal agents.

In the present study, we have demonstrated that volatiles from artificially damaged wasabi leaves inhibited germination of *B. bassiana* s.l. and *I. fumosorosea* conidia (Table 1). This is likely to be as a result of the plant volatile AITC, because AITC levels in 3.8 g $L^{-1}$ of macerated leaves, which caused low germination rates (2.7% and 4.5% for *B. bassiana* s.l. and *I. fumosorosea*, respectively; Table 1), were estimated at 45 μg $L^{-1,}$ and a similar concentration (63 μg $L^{-1}$) of synthetic AITC also led to low germination rates (12.3% and 15.1% for *B. bassiana* s.l. and *I. fumosorosea*, respectively; Table 2).

Table 2. Percent germination of *B. bassiana* s.l. or *I. fumosorosea* conidia exposed to different quantities of AITC for 24 h in a chamber.

| Dose of AITC (μg $L^{-1}$ chamber) | Percent germination (mean ± SE) | |
|---|---|---|
| | *B. bassiana* s.l. | *I. fumosorosea* |
| 0 | 100 | 100 |
| 6.3 | 99.0 ± 0.3 | 90.5 ± 2.8 |
| 63 | 12.3 ± 3.6 | 15.1 ± 0.6 |
| 630 | 0 | 0 |

For both fungi, their susceptibility to volatiles from macerated wasabi leaves or AITC was similar, with significant differences between them only occurring at one concentration in each experiment (Tables 1 and 2). This negative effect of plant volatiles on entomopathogenic fungi is more severe than previously observed for fungal plant pathogens of cruciferous plants; for example, the fungal plant pathogens, *Helminthosporium solani* Dur. & Mont. and *Verticillium dahliae* Kleb., were able to grow a little at 7 mg $L^{-1}$ of AITC (Oliver et al. 1999), while in our study, germination of *B. bassiana* s.l. and *I. fumosorosea* was completely inhibited at just 630 μg $L^{-1}$ of AITC (Table 2). This suggests that entomopathogenic fungi may be less well adapted to plant volatiles than plant pathogens.

Inyang et al. (1999) examined toxicity of 11 isothiocyanates, potentially relating to cruciferous plants, on germination of *M. anisopliae* conidia and reported that two isothiocyanates (phenylethyl- and 3-butenyl isothiocyanate) decreased mortality of inoculated adults of the mustard beetle, *Phaedon cochleariae* (Fabricius), when exposed to vapours from 1 μL of each pure compound in a 55-mm diameter glass chamber; its length was not described, but in the figure it appeared to be approximately 200 mm in length (about 500 mL in volume). The inhibitory concentration of these isothiocyanates (1 μL per 500 mL chamber) was apparently higher than the 63 μg $L^{-1}$ or 630 μg $L^{-1}$ of synthetic AITC that we observed resulted in little (12.3% and 15.1% for *B. bassiana* s.l. and *I. fumosorosea*, respectively) or no germination in both fungal species (Table 2). Therefore, such AITC levels may negatively affect the infection process of *B. bassiana* s.l. and *I. fumosorosea* and reduce herbivore mortality. While confirmation of this requires further research beyond the scope of this study, it remains possible that wasabi volatiles may contribute to unsatisfactory biological control of herbivorous pests.

As mentioned previously, AITC plays an important role in the plant's defense strategy against herbivores and disease (Chew 1988; Louda & Mole 1991; Rask et al. 2000; Wittstock et al. 2003). However, in the evolutionary arms race between plants and their herbivores, specialist crucifer herbivores have undergone selection to overcome this defense. For example, in the small white butterfly, *Pieris rapae* L., the hydrolysis reaction is redirected toward the formation of nitriles such as allyl cyanide in the gut, rather than AITC, and this is then excreted in the feces (Wittstock et al. 2004). The diamondback moth, *Plutella xylostella* L., has an enzyme, sulfatase, which desulfates the glucosinolates to metabolites that are not substrates for myrosinase and so do not produce AITC (Ratzka et al. 2002). In addition, these crucifer specialists use volatile AITC as an attractant to detect host plants (Hovanitz & Chang 1963; Hovanitz et al. 1963; Li et al. 2000; Rask et al. 2000), and their parasitoids also use the compound as a cue to detect their herbivore hosts (Geervliet et al. 1994, 1996; Rask et al. 2000; Mattiacci

et al. 2001; Fatouros et al. 2005). Thus, AITC can enhance the fitness of both specialist herbivores and their natural enemies resulting in complex coevolving tritrophic interactions. Negative effects of AITC on entomopathogenic fungi may give an additional advantage to the host herbivores in such interactions.

Cruciferous plants emit AITC and many other isothiocyanates (Kumagai et al. 1994; Oliver et al. 1999); some of these other isothiocyanates have fungicidal activity against entomopathogenic fungi (Inyang et al. 1999) and the plant pathogens *H. solani* and *V. dahlia* (Oliver et al. 1999). In our study, other compounds in addition to AITC were detected in the volatiles from macerated wasabi leaves (data not shown) and it is possible that these compounds may also have negative effects on conidial germination of *B. bassiana* s.l. and *I. fumosorosea*; further studies are required to examine the effects of these other compounds on entomopathogenic fungi.

In conclusion, we have provided evidence that volatiles (including AITC) from wasabi had negative effects on conidial germination of the entomopathogenic fungi, *B. bassiana* s.l. and *I. fumosorosea*. Successful development of biological control strategies for herbivores of wasabi, and other crucifers, must, therefore, be developed under a conceptual framework that accounts for plant volatile-mediated tritrophic interactions between plants, herbivores, and their natural enemies including fungal pathogens, parasites, and predators (Agrawal 2000; Dicke & Van Loon 2000; Elliot et al. 2000; Cory & Hoover 2006). AITC may play a key role in such interactions on crucifers such as wasabi, and its effects on biological control agents should be considered specifically, though this speculation needs to be confirmed by further laboratory and field studies.

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