Annulohypoxyton sp. FPYF3050 produces volatile organic compounds against the pine wood nematode, *Bursaphelenchus xylophilus*

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Summary – Natural volatiles released by the fungus, *Annulohypoxyton* sp. FPYF3050, were evaluated against the pine wood nematode (PWN), *Bursaphelenchus xylophilus*. Our results showed that volatile organic compounds (VOCs) caused 64.1 and 58.4% mortality of second-stage juveniles (J2) and mixed-stages (eggs, J2, third- and fourth-stage juveniles, and adults) of populations of PWN, but no inhibitive effects were detected on nematode eggs in the experiment. Analysis of the gases within the Petri plate by gas chromatography-mass spectrometry (GC-MS) showed a yield of a unique volatile with dominant 1,8-cineole in 77.4% relative area (RA) after 72 h treatment of nematodes with *Annulohypoxyton* sp. FPYF3050 and *Botrytis cinerea*. The commercial 1,8-cineole at concentrations of 2, 5, 10 and 15 $\mu\text{l ml}^{-1}$ was applied to examine nematocidal activity. The results showed that 1,8-cineole had a 40-100% inhibition on the nematode eggs during 48 h treatment, more than 82.9% mortality of J2 after 24 h, 48 h and 96 h, and 18.7-91.9% mortality of the mixed-stage population, depending on the period after exposure. This result indicates that 1,8-cineole in the volatile gas emissions of *Annulohypoxyton* sp. FPYF3050 may play a crucial inhibitory effect on the pine wood nematode. The nematocidal volatile gas from fungi may provide a useful biocontrol agent for controlling *B. xylophilus*.

Keywords – 1,8-cineole, biological control, fungus, nematocidal activity, pine wilt disease.

Pine wilt disease caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, was first reported by Cheng in 1982 in China (Cheng *et al.*, 1983). At that time, the disease was limited to the site of Sun Yat-sen's Mausoleum in Nanjing, where the infestation covered 200 hm^2 and affected 256 trees of *Pinus thunbergii*. However, it has extended to 18 provinces during the last 30 years, damaged much of China's forest resources and caused great economic losses. Currently, more effective means are expected to be found to control *B. xylophilus*.

Fungi are unique candidates for using in an 'inundation' biocontrol strategy (Jackson *et al.*, 2009). Some types of fungi have been widely used as agents in the biocontrol of plant-parasitic nematodes. Nematode-trapping

fungi have evolved complex trapping devices to capture and consume nematodes (Lopez-Llorca *et al.*, 2007; Ulzurrun & Hsueh, 2018). Nematode-endoparasite fungi produce numerous lunate conidia that adhere to nematodes, killing them (Shih & Tzean, 1999). Other fungi produce toxic secondary metabolites that immobilise and kill nematodes (Freire *et al.*, 2012; Yang *et al.*, 2012; Pimenta *et al.*, 2017).

Volatile organic compounds (VOCs) can permeate the air and travel a long distance, which makes fungal VOCs a potential, useful and additional tool in biocontrol strategies (Morath *et al.*, 2012). Some studies suggested that the VOCs of some endophytic fungi were able to inhibit the growth of plant pathogens by augmenting host

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defence responses against pathogens (Macías-Rubalcava *et al.*, 2010). A series of endophytic fungi were recently found to produce VOCs that inhibited nematodes (Freire *et al.*, 2012; Yang *et al.*, 2012; Pimenta *et al.*, 2017). Pimenta *et al.* (2017) reported that VOCs of 28 endophytic fungal isolates immobilised more than 76% of second-stage juveniles (J2) of *Meloidogyne incognita*. The fungal species *Epicoccum nigrum* and *Schizophyllum commune* produced VOCs that immobilised J2 after 6 h exposure. VOCs emitted by 13 fungi isolated from coffee roots caused more than 40% immobility of J2 *M. incognita* (Pimenta *et al.*, 2017). However, there is no information on the action of volatile gases from endophytic fungi on pine wood nematodes.

Volatile terpenoids are a class of VOCs produced by many fungal species (Shaw *et al.*, 2015), and they are usually monoterpene and sesquiterpene. Many monoterpenoids showed suppression of nematodes and were more toxic to some nematodes than the commercial nematocide Oxamyl (Tsao & Yu, 2000). It has been reported that 1,8-cineole, a monoterpene, had 63.5, 47.0 and 46.0% nematocidal action on *M. incognita*, *Pratylenchus vulnus* and *Xiphinema index*, respectively (Avato *et al.*, 2017).

In this study of the endophytic fungal volatiles on important plant pathogens, we found that the volatiles of *Annulohypoxyton* sp. FPYF3050 produced a unique volatile with abundance of 1,8-cineole. We investigated the effects of the VOCs emitted by the fungus, *Annulohypoxyton* sp. FPYF3050, on different life stages of PWN, including egg, J2, and mixed-stage that contained eggs, J2, third- (J3) and fourth-stage (J4) juveniles and adults. We also evaluated the nematocidal activity of different concentrations of commercial 1,8-cineole on PWN. Our results demonstrated that VOCs of *Annulohypoxyton* sp. FPYF3050 had an obvious effect on PWN and could be a potentially useful biocontrol agent against the PWN.

Materials and methods

FUNGAL AND NEMATODE MATERIAL

Annulohypoxyton sp. FPYF3050 was isolated from branches of the shrub plant *Neolitsea pulchella* (Wang *et al.*, 2017). The strain (Nxy61) of PWN in the present study was isolated from *P. massoniana* trees in Ningbo, P.R. China (Liang *et al.*, 2013), in October 1992, and maintained on *Botrytis cinerea* on corn-meal agar (CMA) in the culture collection of the Chinese Academy of Forestry (Beijing, P.R. China). Potato dextrose agar (PDA; potato 200 g, glucose 20 g, agar 25 g, and H₂O 1000

ml) medium in a Petri dish (diam. 90 mm) was used for incubating fungi, *Annulohypoxyton* sp. FPYF3050 and *B. cinerea* in this experiment.

COLLECTING *B. XYLOPHILUS* EGGS AND J2

PWN (Nxy61) were cultured on *B. cinerea* in the dark at 25°C. After 5 days, 20 ml of dd H₂O was added to wash off the nematodes from the PDA plate to obtain mixed stages (eggs, J2, J3, J4 and adults). The nematode suspension was partly used in the test on mixed-stage nematodes and partly for collecting the eggs. To collect eggs, the nematode suspension was transferred to a new and clean glass Petri dish (90 mm diam.) and left for 20 mins at 25°C for eggs to settle on the base of the Petri dish, where they adhered to the glass due to glycoproteins in their surfaces (Shinya *et al.*, 2009). After carefully removing the water and worms, the eggs were rinsed repeatedly with sterile water and transferred to a centrifuge tube to obtain the pure eggs. To obtain synchronous development of J2, the collected eggs were kept in sterile water in an incubator at 25°C for 24 h (Shinya *et al.*, 2009; Zhu *et al.*, 2016).

NEMATOCIDAL ACTIVITY TESTS FOR FUNGAL VOCS

The nematocidal activity of VOCs was determined by the methods previously described (Strobel *et al.*, 2001; Yan *et al.*, 2018). A 2 cm wide central strip was removed from the PDA medium in a Petri dish (90 mm diam.). Then, the PDA medium was divided into two semicircular PDA medium sheets (Fig. 1A).

For the test on eggs, a 2 ml Eppendorf centrifuge tube cover was inverted onto one half-moon of the agar, and *Annulohypoxyton* sp. FPYF3050 was inoculated onto the other half-moon of the agar. The Petri dish was incubated in a growth chamber in the dark at 25°C for 5 days. An egg suspension (100 µl), which contained 100 eggs, was introduced into the tube cover and left in the dark at 25°C for J2 to hatch. The eggs were checked at 6, 12, 18, 24, 30 and 48 h intervals. The same treatment without *Annulohypoxyton* sp. FPYF3050 served as the control (Fig. 1B).

For the synchronously developed J2 and mixed-stage test, before the bioassay experiment, *Annulohypoxyton* sp. FPYF3050 and *B. cinerea* were inoculated onto one half-moon of an agar individually. After 5 days, two halves, one with *B. cinerea* and the other with *Annulohypoxyton* sp. FPYF3050, were recombined into a new agar in a Petri dish on the fifth day and 2000 synchronously developed J2 or mixed-stage nematodes in 0.2 ml suspension were then

Fig. 1. Assay methods for nematicidal volatiles emitted by fungi. A: Experimentation on eggs. *Annulohypoxyton* sp. FPYF3050 was inoculated on the left and the centrifugal tube cover was on the right. Five days later, an egg suspension was injected into the tube cover; B: Experimentation using second-stage juveniles (J2) of *Bursaphelenchus xylophilus*, and mixed-stage nematodes. *Annulohypoxyton* sp. FPYF3050 and *Botrytis cinerea* were cultured for 5 days and combined in the same Petri dish, *Annulohypoxyton* sp. FPYF3050 was positioned on the left and the *B. cinerea* fungus on the right; 2000 nematodes were inoculated in the centre of *B. cinerea*, which caused a pit to form in the centre.

inoculated on the colony of *B. cinerea*. A Petri dish with only *B. cinerea* on the half plate served as control. The method was used for surveying the effect of volatiles on PWN during the whole study (Fig. 1B).

In the previous study, the most abundant 1,8-cineole of *Annulohypoxyton* sp. FPYF3050 occurred in the 3 days between days 5 and 7 after the endophytic fungus inoculation in the PDA of the Petri dish in the dark at 25°C (Wang *et al.*, 2017). Previous work showed reduction in the survival of J2 after 72 h of exposure to VOCs emitted by other fungi (Yang *et al.*, 2012; Pimenta *et al.*, 2017), so we chose 72 h as the treatment time. The colony of *B. cinerea* was established by inoculating from a 0.5 cm plug of *B. cinerea* and maintained for 5 days; our pilot study found that 2000 PWN of mixed-stage or J2 took 72 h to exhaust such a colony. Hence, 2000 J2 were transferred into the *B. cinerea* colony of each treatment group to ensure that *B. cinerea* was digested completely

in 3 days. The same treatment was also used for the mixed-stage experiment. After the 72 h treated with VOCs of *Annulohypoxyton* sp. FPYF3050, the nematodes were washed off from the PDA with 20 ml of sterile water. The total volume of 20 ml nematode suspension was collected in a 50 ml centrifuge tube. A 100 µl suspension was removed from the 50 ml centrifuge tube for counting the nematodes under a microscope (Nikon Eclipse E100), with ten separate counts at a magnification of 4×. The nematodes from the control were processed in the same way. The toxicity level of VOCs was defined by the mean mortality of the nematodes.

$$\text{Mortality} = (1 - a/b) \times 100\%,$$

where *a* is the number of nematodes and eggs in a treatment plate; and *b* is the number of nematodes and eggs in the control plate.

ANALYSIS OF MIXED VOLATILE COMPOUNDS IN CO-CULTURING TWO FUNGI IN PDA AFTER 72 H

For analysing fungal VOCs in the air space above plates, the gases were collected by SPME-GC-MS. This investigation was done on four groups: i) *Annulohypoxylo* sp. FPYF3050 and *B. cinerea* with nematodes; ii) *Annulohypoxylo* sp. FPYF3050 and *B. cinerea*; iii) *Annulohypoxylo* sp. FPYF3050; and iv) *B. cinerea*. The fungus of each group grew a colony for 5 days with the same culture conditions as described above. After treatment for 3 days, the compounds released in the headspace of the plates were checked using the GC-MS protocol as described in detail by Wang *et al.* (2017). In brief, a solid phase microextraction (SPME) syringe (DVB-PDMS-Carboxen 50/30, Supelco) was inserted into the headspace inside the Petri plate, for 40 min, through a small hole (0.5 mm diam.) drilled on the side of the Petri plate. After exposure to the VOCs, the syringe was inserted into the injection port of the TRACE DSQ inlet, a Thermo Finnigan gas chromatograph and an HP-5 column (5% phenyl-95% dimethylsiloxane) with dimensions 30 m $\times$ 0.25 mm $\times$ 0.25 μ m. The MS was scanned at a rate of 5 scans s⁻¹ over a mass range of 41–560 amu. The desorption time was 1 min. The temperature program of the GC oven was: 40°C for 2 min and then the temperature increased to 220°C at a rate of 5°C min⁻¹. PDA Petri plates not inoculated with the strain were used as the control to identify and deduct those compounds contributed by the medium. The tentative identification was made with GC against the NIST compounds library. For comparing mass spectra, we considered only peaks with a spectra similarity greater than 60%.

EFFECT OF COMMERCIAL 1,8-CINEOLE ON *B. XYLOPHILUS*

1,8-cineole was emitted by *Annulohypoxylo* sp. FPYF3050 and accounted for 77% RA in mixing volatiles. Hence, 1,8-cineole could be posited to play a critical role in the reduction of PWN. In order to prove this, appropriate amounts of commercial 1,8-cineole (Macklin) were added to a 0.5% water solution of Triton X-100 (Acros Organics) to prepare 4, 10, 20 and 30 μ l ml⁻¹ 1,8-cineole solutions for PWN inhibition tests. Batches of 100 eggs suspended in 0.05 ml distilled water was placed in 0.2 ml Eppendorf vials. A 0.05 ml volume of each concentration of 1,8-cineole solution was poured into each vial containing the nematode suspension to obtain final concentrations of 2, 5, 10 and 15 μ l ml⁻¹ and mixed immediately by pipetting. After 24, 48 or 96 h of treatment with each con-

centration of the test solutions, the survival nematodes in the Eppendorf vials were checked under the microscope. A 0.5% Triton X-100 solution was included as control. This method also was used to evaluate the nematicidal activity on J2 and mixed-stages with 100 individuals in the test solution. All of the treatments and controls were cultured in the dark at 25°C.

$$\text{Inhibition of hatching} = (1 - (a - c)/b) \times 100\%.$$

Where a is the number of eggs containing unhatched J2 after the treatment; b is the number of J2 in the water control; and c is the number of eggs containing unhatched J2 in the Triton X-100 control.

$$\text{Mortality of J2} = (1 - a/b) \times 100\%.$$

Where a is the number of nematodes after the treatment; and b is the number of nematodes in the water control.

In the experiment on mixed-stage, we compared the initial number of nematodes in each treatment with the nematodes after treatment to calculate the mortality.

$$\text{Mortality of mixed-stage} = (1 - a/b) \times 100\%.$$

Where a is the number of nematodes after treatment; and b is the number of nematodes before treatment.

STATISTICAL ANALYSIS

To achieve greater accuracy, all of the nematodes used in treatment and control came from the same centrifuge tube. The population size of nematodes was counted under a microscope (Nikon Eclipse E100) at a magnification of 4 $\times$ by the same tester. Three replicates were conducted for each treatment and control, and each *in vitro* and Petri dish experiment was repeated three times. SPSS 25 was used to carry out an analysis of variance (ANOVA) of the data. Independent sample *t*-test was applied for evaluation of results; $P < 0.05$ was used as the level of significance. Values shown are mean $\pm$ SD.

Results

TOXICITY OF VOCs EMITTED BY *ANNULOHYPOXYLON* SP. FPYF3050 TO *B. XYLOPHILUS*

In the assay of the volatile gas substances produced by *Annulohypoxylo* sp. FPYF3050 against the PWN eggs, the number of eggs with unhatched J2 remaining in the treated group and control group were significantly different only at 18 h ($P = 0.008$) and 30 h ($P = 0.028$), indicating the inhibitory effect of volatile gases on PWN

Fig. 2. Number of unhatched J2 (number of eggs) of *Bursaphelenchus xylophilus* after exposure for 6–48 h to volatile organic compounds (VOCs) from *Annulohypoxyton* sp. FPYF3050 and control with no VOCs. Error bars = SD of the mean.

eggs in this experiment was limited. At 14 h, the number of eggs with unhatched J2 in the treatment group was slightly greater than that in the control group, indicating minor inhibition (Fig. 2).

In the experiment on synchronously developed J2, more nematodes survived in the control (2526) than in the J2 treatment (913). This 64.1% reduction indicated a strong nematicidal effect of the VOCs released by *Annulohypoxyton* sp. FPYF3050 on J2 of PWN after 3 days of treatment ($P < 0.05$) (Fig. 3A).

For the effect of the fungal VOCs on the mixed-stage nematode population, 2211 nematodes survived in the control, while far fewer (945) nematodes survived in the treatment, a reduction of 58.4% after 72 h treatment (Fig. 3A). The number of J2 decreased the most, with a decrease of 69.5%, which was basically consistent with the result of the experiment on synchronously developed J2 above. The mortality of J3 and adults was 49.5 and 5.03%, respectively. The impact on J4 was the weakest with only 26.5% mortality (Fig. 3B).

ANALYSIS OF THE COMPONENTS OF VOCs IN FOUR TREATMENTS

We analysed the components of VOCs in four treatments after a 3-day period: i) *Annulohypoxyton* sp. FPYF3050 and *B. cinerea* with nematodes; ii) *Annulohypoxyton* sp. FPYF3050 and *B. cinerea*; iii) *Annulohypoxyton* sp. FPYF3050; and iv) *B. cinerea*. The major compound products in treatment i) were identified as 1,8-cineole, (+)-sativene and isocaryophyllene, account-

ing for 77.4, 7.9 and 7.4% RA, respectively. The three compounds were also found in ii) with 70.6, 2.5 and 15.9 RA. Only 1,8-cineole was detected as a major product in iii) with 66.3% RA. The GC/MS analysis results of the four treatments revealed that there were no obvious products released by *B. cinerea*, and 1,8-cineole emitted by *Annulohypoxyton* sp. FPYF3050 was the most abundant compound in the VOCs (Table 1).

EFFECT OF COMMERCIAL 1,8-CINEOLE ON PWN

In order to verify the nematicidal activity of the main product, 1,8-cineole, in VOCs of FPYF3050, a commercial product of 1,8-cineole was used to study its inhibitive effects on hatching, and mortality of J2 and mixed-stage.

After 24 h treatment of eggs, the percentage hatching inhibition in the concentrations of 2, 5, 10 and 15 $\mu\text{l ml}^{-1}$ of the commercial 1,8-cineole were 39.8, 79.1, 87.2 and 92.9%, respectively. The rates were 42.1, 94.5, 98.0 and 100% at 48 h and 0.8, 61.8, 77.0 and 86.7% at 96 h (Fig. 4A).

In the experiment of 1,8-cineole against synchronously developed J2, there was a mortality of more than 82.9% for any test time and all concentrations (Fig. 4B).

In the experiment with 1,8-cineole on the mixed-stage, the overall mortality with treatment time was from 18.7 to 91.9% in 2 $\mu\text{l ml}^{-1}$ 1,8-cineole solution. With 5 $\mu\text{l ml}^{-1}$ 1,8-cineole, nematode mortality was 40.4, 54.0 and 65.4% at 24 h, 48 h and 96 h, respectively. After 24 h, 48 h, 96 h exposure to 10 $\mu\text{l ml}^{-1}$ 1,8-cineole solution mortality was 89.2, 75.4 and 56.1%, respectively. Mortality in the 15 $\mu\text{l ml}^{-1}$ 1,8-cineole solution ranged from 47.3 to 73.1% after 24 to 96 h exposures.

For further analyses of the effects on each development stage in the mixed nematodes experiments, the solution of the commercial 1,8-cineole had a marked effect on PWN J2, resulting in a mortality of almost 100% in all treatments except 2 $\mu\text{l ml}^{-1}$ at 24 h. J3 had almost 22.0% mortality after exposure to 2 $\mu\text{l ml}^{-1}$ of 1,8-cineole for 24 h, increasing to 100% at 96 h. With exposure to 5 $\mu\text{l ml}^{-1}$ solution, J3 had 36.4–63.6% mortality. In 10 $\mu\text{l ml}^{-1}$ solution, J3 had mortalities between 57.7 and 62.8%, increasing with exposure time. Mortalities caused by 15 $\mu\text{l ml}^{-1}$ of 1,8-cineole ranged from 54.7 to 62.8%. In the treatment group and the control group of each time and concentration, about ten J4 survived (Supplementary material, Table S1). The numbers of J4 surviving in most groups after treatment did not differ significantly (Fig. 5). The commercial 1,8-cineole displayed strong

Fig. 3. A: Mortality of *Bursaphelenchus xylophilus* (mean number of individuals) in the experiments with second-stage juveniles (J2) and mixed-stages after exposure to the volatile organic compounds (VOCs) of *Annulohypoxyton* sp. FPYF3050. B: Mortality of different stages of *Bursaphelenchus xylophilus* after exposure to VOCs of *Annulohypoxyton* sp. FPYF3050. Error bars = SD of the mean. Bars with the same letter are not significantly different based on the independent sample *t*-test. J3 = third-stage juveniles; J4 = fourth-stage juveniles.

Table 1. Volatile organic compounds emitted by fungi as identified by SPME-GC-MS after 72 h of cultivation. Four Petri plate treatments were prepared: i) *Annulohypoxyton* sp. FPYF3050 and *Botrytis cinerea* with nematodes (*Bursaphelenchus xylophilus*); ii) *Annulohypoxyton* sp. FPYF3050 and *B. cinerea*; iii) *Annulohypoxyton* sp. FPYF3050; and iv) *B. cinerea*.

	Retention time	Area (%)			
		<i>Annulohypoxyton</i> sp. FPYF3050 and <i>Botrytis cinerea</i> with nematodes	<i>Annulohypoxyton</i> sp. FPYF3050 and <i>B. cinerea</i>	<i>Annulohypoxyton</i> sp. FPYF3050	<i>B. cinerea</i>
CO ₂	1.74	–	–	–	1.1 ± 0.7
Alanine	1.79	0.6 ± 0.1	–	–	–
Ethyne, fluoro-	2.06	–	–	–	–
N ₂ O	2.36	–	–	–	0.6 ± 0.1
3-Heptene,	9.52	–	–	–	8.2 ± 6
2,2,4,6,6-pentamethyl-1,8-cineole	10.2	77.4 ± 4.4	70.6 ± 12.7	66.3 ± 10.3	–
γ-Terpinene	10.93	–	2.3 ± 0.4	1.6 ± 0.3	–
Sulfurous acid, cyclohexylmethyl octadecyl ester	17.37	–	–	–	4.8 ± 2.6
2,4,4,6,6,8,8-Heptamethyl-2-nonene	17.37	1.4 ± 0.4	–	–	5.5 ± 3.3
(+)-Sativene	19.41	7.9 ± 7	2.5 ± 1.2	–	–
Isocaryophyllene	20.12	7.4 ± 3	15.8 ± 5.5	–	–

The values indicate means (%) ± SD of three replicates. The compounds in the PDA have been removed from the table.

activity against adult nematodes with 28.5, 34.4 and 89.6% mortality after 24, 48 and 96 h exposure to 2 μl ml⁻¹ solutions, respectively; equivalent exposure times

of adults to a 5 μl ml⁻¹ solution resulted in 18.5, 46.7 and 76.5% mortality, respectively. In addition, the range of mortality after exposure to 10 μl ml⁻¹ solution was

Fig. 4. Over-time hatch inhibition and mortality of *Bursaphelenchus xylophilus* second-stage juveniles (J2) after immersion in 1,8-cineole solution. A: Hatch inhibition after 24, 48 and 96 h; B: Mortality of J2 after different processing time. Error bars = SD of the mean. Bars with the same letter are not significantly different based on the independent sample *t*-test.

around 38.5–67.0%. After immersion in the 15 µl ml⁻¹ solution, mortality percentages were from 53.9–76.5%. The detailed number of nematodes of various ages is given in Table S1 (Supplementary material). In the control group, there was no significant difference in the number of PWN over time and the stages developed normally (Fig. 6).

Discussion

We found that the VOCs of *Annulohypoxyton* sp. FPYF3050 had significant nematocidal effects on the juveniles and adults of *B. xylophilus*. The inhibition of J2 was particularly prominent. The mortality of synchronously developed J2 was 64.1% and the mortality of J2 in the mixed-stage test was significantly higher than other stages at 69.5%. 1,8-cineole was the dominant component of the volatiles of *Annulohypoxyton* sp. FPYF3050 with nematocidal activity and its RA accounted for 77.0%. Therefore, we examined the effects of commercial 1,8-cineole on PWN. The results showed that the commercial 1,8-cineole also had a significant inhibitory effect on J2 under the tested concentration range. With exposure to 2 µl ml⁻¹ for 24 h, the mortality of synchronously developed J2 was greater than that of J2 in the mixed-stage. The reason may be that the synchronised J2 were in a unified physiological state and were weaker and less resistant to toxic substances, but the J2 in the mixed-stage batches were in different phases of J2 development. All the above results indicated that the J2 was the most sensitive stage to external stimuli, which has been reported for J2 of other plant-

parasitic nematodes (Andrés *et al.*, 2012; Pimenta *et al.*, 2017).

Volatile gases caused a 26.5% mortality of J4, which was significantly lower than that for other stages, while commercial 1,8-cineole also had a weaker effect on J4 than other stages. The external cortical layer of the cuticle of J4 is thicker than other stages, making J4 more resistant to stress (Giblin-Davis, 1993). Studies had also shown that PWN J4 required more *trans*-2-hexenal than other stages to achieve a lethal effect (Cheng *et al.*, 2017). Thus, J4 was the most resistant stage to treatment with VOCs. In addition, natural volatile gases from *Annulohypoxyton* sp. FPYF3050 had a stronger effect than commercial 1,8-cineole on J4, which suggested that there might be other substances in the volatile gases of *Annulohypoxyton* sp. FPYF3050 that affected the PWN. Commercial 1,8-cineole improved the toxicity of other xenobiotics (Rossi & Palacios, 2015). 1,8-cineole/carvone (0.1–0.4 mg ml⁻¹) caused 100% J2 mortality and 87% hatch suppression; however, carvone or 1,8-cineole alone showed no nematocidal activity against *M. javanica* (Andrés *et al.*, 2012). In addition, 1,8-cineole is a key element affecting the *Ditylenchus destructor* that was found in *Ajanía fruticulosa* and *A. potaninii* essential oils (Liang *et al.*, 2018). Therefore, this nematocidal effect of active volatiles from *Annulohypoxyton* sp. FPYF3050 may be caused by 1,8-cineole synergies with other substances.

The inhibitory effect of the commercial 1,8-cineole on the eggs was clear. Avato *et al.* (2017) also reported that 2, 5, 10, 15 µl ml⁻¹ concentrations of 1,8-cineole had clear hatching inhibitory effects on eggs of *M. incogni*-

Fig. 5. The mortality and hatch inhibition of different stages of *Bursaphelenchus xylophilus* after treated with different concentrations (2, 5, 10 and 15 µg ml⁻¹) of 1,8-cineole at different times (24, 48 and 96 h) in the dark at 25°C. Error bars = SD of the mean. Bars with the same letter are not significantly different based on the independent sample *t*-test. J2 = second-stage juveniles; J3 = third-stage juveniles; J4 = fourth-stage juveniles.

ta, *P. vulnus* and *Xiphinema index*. However, eggs exposed to volatile gases of the *Annulohypoxyton* sp. were weakly affected, possibly because 1,8-cineole was almost

insoluble in water (Amrine *et al.*, 1996), so the slight amount of 1,8-cineole in water did not reach the concentration required to inhibit egg development. The effect of

Fig. 6. A 0.5% Triton X-100 solution was included in the experiments as a control. Numbers of *Bursaphelenchus xylophilus* in the control treatments after 14, 48 and 72 h in the dark at 25°C. Nematode populations developed normally. J2 = second-stage juveniles; J3 = third-stage juveniles; J4 = fourth-stage juveniles.

1,8-cineole was not obvious at low concentration but at high concentrations it showed remarkable antifungal effects against all the fungal isolates of chickpea (Shukla *et al.*, 2012).

In summary, the volatile gas produced by *Annulohypoxyton* sp. FPYF3050 was able to cause mortality of PWN and 1,8-cineole may be the main active substance. Using nematicidal VOCs of fungi in this way should reduce the environmental damage caused by applying chemical nematicides. This study also contributed to the screening of volatile gases emitted by fungi that can control nematodes. The *Annulohypoxyton* sp. fungus comes from plants and can grow on a variety of agricultural and forestry residues, which may provide long-term biological control effects after being introduced to the natural environment (Wang *et al.*, 2017). Our results suggested that nematicidal VOCs could be used to develop novel nematicidal agents through further empirical research. However, the nematicidal mechanism of this gas is still unclear, and this needs to be explored in more in-depth future research.

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Supplementary material

Table S1. The experiment with mixed-stages (see Materials and methods); the numbers of *Bursaphelenchus xylophilus* remaining after treated with different concentrations of 1,8-cineole at different times.

Time (h)	Dose ($\mu\text{g ml}^{-1}$)	Eggs	J2	J3	J4	Adults	Total
0	water	25.3 ± 2.8	25 ± 5.3	32.3 ± 5.8	10 ± 2.4	5 ± 0.8	97.7 ± 6.1
24	Triton X-100	12.6 ± 4.2	30.2 ± 4.9	24.2 ± 4.5	11 ± 2.6	4.6 ± 1.3	82.6 ± 6.5
	2	10.8 ± 3.7	33 ± 6.1	25.1 ± 3.1	9.8 ± 1.7	3.7 ± 1.2	82.3 ± 8.6
	5	26.7 ± 5.2	0 ± 0	20.6 ± 3.1	9 ± 2.8	4.1 ± 1.3	60.3 ± 4.9
	10	22.1 ± 5	0 ± 0	14.6 ± 3.2	8.7 ± 1.6	3.1 ± 1.5	48.4 ± 6
	15	26.1 ± 3.4	0 ± 0	14.3 ± 2.5	10.7 ± 3	2.3 ± 0.7	53.4 ± 6.8
48	Triton X-100	14.7 ± 2.7	21.6 ± 3.2	16.7 ± 2.1	11 ± 3.1	5.3 ± 1.2	69.2 ± 4.5
	2	6.3 ± 2.6	0.4 ± 0.5	13.9 ± 4.8	14.2 ± 3.3	3.3 ± 0.9	38.2 ± 3.6
	5	14.7 ± 4.1	0.4 ± 0.7	17.4 ± 2.7	11.3 ± 4	2.7 ± 1.2	46.6 ± 5.5
	10	20.7 ± 5.3	0 ± 0	15.2 ± 4.1	12.9 ± 3	2.6 ± 0.8	51.3 ± 6.7
	15	23.6 ± 3.1	0 ± 0	15.6 ± 3.6	10.9 ± 3.6	2.4 ± 0.8	52.4 ± 4.5
96	Triton X-100	2 ± 0.9	29.2 ± 2.2	20.7 ± 2.6	14.3 ± 2.2	3.4 ± 0.7	69.7 ± 6.3
	2	4 ± 1.5	0 ± 0	0 ± 0	3 ± 1.6	0.6 ± 0.5	8.2 ± 5.2
	5	9 ± 2.8	0.1 ± 0.3	11.9 ± 2.6	12.9 ± 3.2	1.2 ± 1	35.1 ± 4.5
	10	17.6 ± 5.2	0.2 ± 0.4	12.1 ± 3.4	8.1 ± 2.7	1.7 ± 0.8	39.7 ± 3.2
	15	9.6 ± 2.8	0 ± 0	7.7 ± 2.9	8.9 ± 1.3	1.2 ± 0.6	27.3 ± 5.6

Values are means $\pm$ SD of the mean. J2 = second-stage juveniles; J3 = third-stage juveniles; J4 = fourth-stage juveniles.