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RESEARCH ARTICLE

INTERFERENCE BY NON-HOST PLANT ROOTS AND ROOT EXUDATES IN THE INFECTION PROCESSES OF *PHYTOPHTHORA NICOTIANAE*

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KEYWORDS

fennel and tobacco rotation, infection behavior, Phytophthora nicotianae, reactive oxygen species, vanillin

HIGHLIGHTS

- The roots of non-host plant interfere infection of *Phytophthora nicotianae.*
- Vanillin and other compounds play key roles in antimicrobial activity of fennel roots.
- ROS accumulation is the potentially mechanism involved in inhibition of antimicrobial compounds on *P. nicotianae*.

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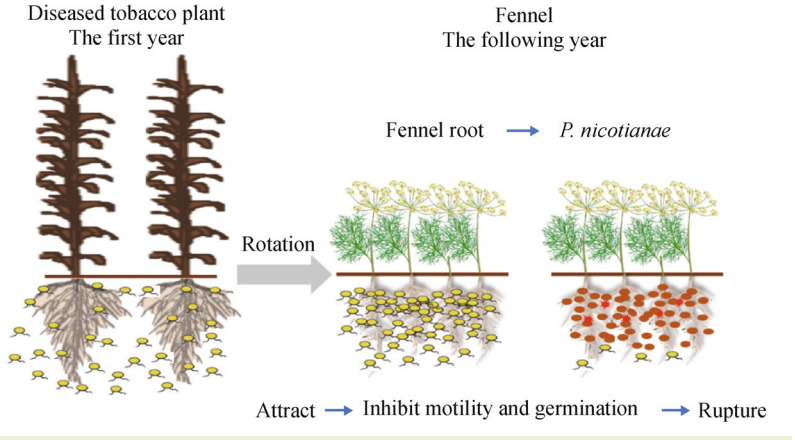
GRAPHICAL ABSTRACT



ABSTRACT

Crop rotations are widely used because they can significantly reduce the incidence of pests and diseases. The interactions between non-host roots and pathogens may be key in the inhibition of soilborne pathogens in crop rotations. Interactions between fennel (*Foeniculum vulgare*) roots/root exudates and *Phytophthora nicotianae* were investigated because of the known allelopathy between fennel and tobacco (*Nicotiana tabacum*). The effects of the key compounds in the fennel rhizosphere on the mycelial growth and zoospore behavior of *P. nicotianae* were assessed. The roots of fennel attracted *P. nicotianae* zoospores and inhibited their motility and the germination of cystospores, with some cystospores rupturing. 4-ethylacetophenone, vanillin and N-formylpiperidine were consistently identified in the fennel rhizosphere and were found to interfere with the infection of *P. nicotianae*, especially vanillin. Hyphae treated with these compounds produced more abnormal

branches and accumulated reactive oxygen species. These interspecific interactions between non-host roots and pathogens were found to be an important factor in the inhibition by fennel of infection by *P. nicotianae*.

1 INTRODUCTION

Biodiversity intercropping or crop rotations are widely used in agroecosystems to reduce pest and disease incidence and severity and increase crop yields[1–3]. Interspecific root interactions can effectively inhibit soilborne pathogens in these systems, for example in the interactions of tomato/chive on tomato bacterial wilt[4], watermelon/rice on watermelon Fusarium wilt[5] and wheat/broad bean on wheat take-all[6]. Knowing the host range of particular pathogens is important in avoiding infections in commercial plant production.

The suppression of soilborne pathogens by antimicrobial compounds secreted from non-host plants has been identified as the main explanation for disease control in intercropping and crop rotation systems[2,7]. Previous studies have found that rapeseed roots can attract *Phytophthora nicotianae* zoospores and simultaneously inhibit zoospore motility and cystospore generation, helping to reduce *Phytophthora* blight in tobacco (*Nicotiana tabacum*) fields[1]. This phenomenon has also been found in the interactions between garlic and *Phytophthora capsica*, maize and *P. capsica*, and maize and *Phytophthora sojae*[2,8,9], and it may be an important factor in the suppression of *Phytophthora* blight in intercropping and crop rotation systems. In addition to rapeseed, previous studies have found that tobacco can be intercropped or rotated with other crops to reduce the incidence of disease in the field and reduce the pathogen population in the soil[10,11]. For example, tobacco rotated with fennel (*Foeniculum vulgare*) can significantly decrease the incidence of black shank[12]. However, interactions between exudates of plant roots and *Phytophthora* zoospores are difficult to observe in the rhizosphere, and they can be affected by microorganisms[13], nutrients[14], environmental conditions[15] and other edaphic factors. The underlying mechanisms therefore remain to be fully elucidated.

Fennel is a typical multipurpose aromatic plant, extracts of which have wide antibacterial activity[16]. Fennel can be cropped with onion[17], pepper[18], tomato[19] and other crops to increase economic benefits and reduce damage by pests and pathogens. For instance, fennel and melon rotation can significantly increase the species richness and diversity of the rhizosphere soil bacterial community, reduce the incidence of melon root rot and alleviate replanting failure[20]. Fennel can also increase the seedling survival rate of *Panax notoginseng* and enrich the abundance of antagonistic bacteria in rotations[21].

Reactive oxygen species (ROS) provide an important signaling mechanism in interactions between plants and pathogens and are important in plant resistance to the infection processes of diseases and pathogens[22]. Natural antimicrobially active substances from root exudates can interfere with infection by soilborne pathogens by inducing ROS accumulation[22,23]. ROS accumulation may therefore be key in the inhibition of *Phytophthora* by non-host plant roots.

Here, we have observed interactions between fennel roots/root exudates and *P. nicotianae*. Antimicrobial compounds in fennel root exudates were identified by gas chromatography-mass spectrometry (GC-MS) and high-pressure liquid chromatography (HPLC), and their antimicrobial activities on the infection processes of *P. nicotianae* were tested. The amounts of ROS in the mycelium were also determined. The data help to explain the mechanisms by which fennel can reduce the incidence of tobacco black shank in the field.

2 MATERIALS AND METHODS

2.1 Plant and pathogen

The fennel (cultivar Siji) used was obtained from a commercial source (Ciba Agricultural Product Market, Kunming, China) and had being growing in field soil for 45 days. *Phytophthora nicotianae* strain YXCJ-1 was provided by the State Key Laboratory for Conservation and Utilization of Bio-Resources at Yunnan Agricultural University and was cultured on V8 medium (150 mL V8 juice with 15 g agar, 0.5 g $CaCO_3$ and 850 mL ddH_2O autoclaved at 121°C for 30 min) at 25°C in an incubator with a 12:12 h L:D photoperiod for 7 days to produce sporangia. The mycelium was scraped, sterilized water was added and the culture refrigerated at 4°C for 30 min before returning to room temperature for 30 min to release zoospores. A zoospore suspension was prepared by filtering through gauze and diluting to 10^5 zoospores mL^{-1} by counting on a hemocytometer.

2.2 Interactions between *P. nicotianae* zoospores and fennel roots

The interaction between fennel roots and zoospores was observed according to Yang et al.[2]. In brief, a U-shaped chamber was formed by placing a bent capillary tube on a glass slide and covering it with a coverslip. The fennel roots (~ 2 cm long) were excised with a sterile razor blade. The root tips were inserted into a zoospore suspension (10^5 mL^{-1}) in the chamber. The behavior of the zoospores in the rhizosphere was recorded every 5 min by taking photographs of the rhizosphere along the root cap zone under a microscope (DM2000, Leica, Wetzler, Germany) and adjusting the magnification (40 or 100 times) according to the requirement for photography. A capillary tube was inserted into a chamber containing the same zoospore suspension concentration as the control. Each treatment was replicated three times. A chemotactic ratio was calculated as the number of the zoospores and cystospores on the test root divided by the number of these spores in the control. The inhibition ratio of the swimming zoospores and the cystospore germination was calculated according to Zhang et al.[8].

2.3 Inhibition of *P. nicotianae* hyphal growth by fennel root exudates

The collection and pretreatment of fennel root exudates was conducted according to Fang et al.[1]. Fennel root exudates were added to carrot agar medium to final concentrations of fennel root exudates of 200, 400, 600 and 1000 $mg \cdot L^{-1}$. Carrot agar medium with methanol of the same concentration was used as a control treatment. Hyphal plugs (7 mm) of *P. nicotianae* were cut and transferred to the amended carrot agar medium which was then incubated at 25°C in the dark. Colony diameter was measured after that of the control reached 60 mm in the 90-mm Petri dishes. The calculation of the inhibition rate followed Fang et al.[1].

2.4 Fennel root exudate compounds identified by GC-MS

GC-MS analysis was conducted using a Thermo Quest TRACE GC2000 with the mass selective detector Thermo Ques TRACEMS (Thermo Fisher Scientific, Waltham, USA). Samples (1 μL) of fennel root exudates were separated through a HP-5MS silica capillary column (30 m × 0.25 mm × 0.25 μm). Helium (99.999% purity) at a 1.0 $mL \cdot min^{-1}$ flow rate was used as the carrier. The parameter settings of the injector temperature, initial column temperature program, electron impact and monitoring wavelength were according to Fang et al.[1]. Mass spectra were obtained in electron impact ionization mode at 70 eV by monitoring the full-scan range (m/z 50–550). The compounds were identified by matching the mass spectra obtained with those of the reference stored in the Wiley 7n.l mass-spectral library.

2.5 Inhibition of *P. nicotianae* hyphal growth by pure compounds

The inhibitory activity of the target compounds on the mycelial growth of *P. nicotianae* was determined as for the root exudates[1]. Standard compounds identified in fennel root exudates by GC-MS were added to carrot agar medium and the final concentrations that gave 10%–90% inhibition were screened along a 10–1000 $mg \cdot L^{-1}$ gradient. Each of the compounds had four gradient concentrations. Each treatment was replicated five times.

2.6 High-performance liquid chromatography of root exudates

The standards of 11 putative compounds identified by GC-MS were purchased from the Guizhou Dida Biological Technology Co. (Guizhou, China) for antimicrobial activity analysis, and then three compounds involved in antimicrobial activity were further selected to determine their concentrations in the fennel root exudates by HPLC using an Agilent 1260 Infinity instrument (Agilent, Santa Clara, USA). The HPLC separations were conducted on a Kinetex-C18 column (4.6 mm × 100 mm, 2.6 μm) (Phenomenex, Guangzhou, China) with the following solvent system: solvent A, methanol (Fisher Scientific, Shanghai, China) and solvent B, 10% methanol and 0.1% phosphoric acid (Sigma-Aldrich Co., Beijing, China) in water (Fisher Scientific, Shanghai, China). A multistep gradient was used for all separations, with initial injection volume of 10 μL and flow rate of 0.5 $mL \cdot min^{-1}$. The multistep solvent gradient was as follows: 0–7 min consisting of 22% to 58% (v/v) solution A, 7–20 min consisting of 58%–95% (v/v) solution A, and 20–25 min consisting of isocratic conditions of 95% solution A. The column temperature was maintained at 30°C. Chromatograms were recorded at 210 and 254 nm and the retention times of the target compounds were established from the standards. The compounds in the samples were identified by comparing the results to authenticated standards. The concentrations of the target compounds in the samples were quantified using standard curves that showed the linear relationships between the peak areas and the concentrations as described by Fang et al.[1].

2.7 Antimicrobial activity of the key compounds in fennel root exudates against *P. nicotianae*

The antimicrobial activity of the key compounds detected in fennel root exudates against the infection stages (chemotaxis, zoospore motility and cystospore germination) of *P. nicotianae* was determined. Chemotaxis of *P. nicotianae* zoospores toward the key compounds was observed at 0, 0.5, 1, 10, 50 and 100 mg·L^{-1} following the methods of Zhang et al.[7]. Antimicrobial activity of the key compounds in fennel root exudates against zoospore motility and cystospore germination was tested at 0, 0.5, 1, 10, 50 and 100 mg·L^{-1} as described by Zhang et al.[8]. Each treatment was replicated three times.

The effect of the key compounds on *P. nicotianae* hyphal growth was evaluated as described by Zhang et al. with several modifications[8]. Specifically, 60 mL of carrot liquid medium in 100 mL flasks and each flask contained 6 dishes of *P. nicotianae*; the samples were cultured for 24 h on a shaker (140 r·min^{-1}) at 28°C. Then 600 μL of the key compounds at 0.5, 1, 10, 50, and 100 mg·L^{-1} were added to separate flasks. 600 μL methanol was used as a positive control and the mycelial weight after culturing for 24 h on a shaking table as a negative control. Each treatment was replicated three times. After continuous shaking for 12 h the liquid medium was removed by filtration; the hyphae were then wrapped in filter paper, dried and weighed to calculate the inhibition rate of the hyphae. The calculation method was as follows: mycelium inhibition rate (%) = (mycelial weight of positive control − treated mycelial weight) / (mycelial weight of control positive − mycelial weight of negative control) × 100.

2.8 Effects of the key compounds in fennel root exudates on *P. nicotianae* hyphal morphology

The effects of the key compounds on hyphal morphology were determined based on the hyphal growth of *P. nicotianae* in sealed plates as previously described[1]. In brief, V8 agar was overlaid with a cellophane membrane (#1650963, Bio-Rad Laboratories, Hercules, USA) and inoculated with a hyphal plug for 5 days at 25°C in the dark. Cellophane cultures with hyphae on top were incubated with or without exposure to 200 mg·L^{-1} 4-ethylacetophenone, 200 mg·L^{-1} vanillin and 600 mg·L^{-1} N-formylpiperidine for 48 h. Under these conditions a sparse hyphal monolayer was produced on the cellophane membrane that could be observed directly under the microscope (DM2000).

2.9 Measurement of reactive oxygen species

Based on the effects of the key compounds on hyphal morphology, the cytoplasmic ROS level was determined using 2,7-dichlorodihydrofluorescein diacetate (DCHF-DA) according to Qin et al.[24]. In brief, the plug was added to a 5 mL polyethylene tube with 1 mL V8 liquid medium and cultured for 12 h on a shaker (220 r·min^{-1}) at 28°C. Then 50 mg·L^{-1} 4-ethylacetophenone, vanillin and N-formylpiperidine, respectively were added to separate flasks. The methanol was added as a positive control as above. Each treatment was replicated three times. After continuous culturing for 1 h the liquid medium was removed. The hyphae were then washed with sterilized deionized water and DCHF-DA was added to the hyphae at 2.5 μmol·L^{-1} and further incubated in the dark for 20 min at 25°C. The plug was then removed. Fluorescence microscopy (DM2000) was used for observations with the following parameters for 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA): EX BP 488, FT 525 and LT 515.

The H_2O_2 content was evaluated using a multimode absorbance microplate reader (Molecular Devices, San Jose, USA) according to the H_2O_2 kit instructions (Grace Biotechnolgy Co. Ltd., Suzhou, China). 50 mg of test material was sampled, acetone was added, the mixture was homogenized in an ice bath and then brought up to 500 μL with acetone. The mixture was centrifuged at 12,000 r·min^{-1} for 10 min at 4°C. The supernatant was kept on ice for later testing. Then the first and second kit reagents were added sequentially according to the instructions. The mixture was centrifuged again at 12,000 r·min^{-1} for 10 min at 25°C. The final kit reagent was added and after the precipitate dissolved it was kept at room temperature for 2 min, and the absorbance (A) was measured at 415 nm. Change in A (ΔA) was calculated as sample A minus control A. The H_2O_2 content (μmol·g^{-1}, FW) was calculated as 3.46 × (ΔA − 0.0006) / the sample fresh weight.

Similarly, oxygen free radicals were evaluated using a multimode absorbance microplate reader (Molecular Devices) in accordance with the oxygen free radical kit instructions (Grace Biotechnolgy Co. Ltd., Suzhou, China). Extracting solution (500 μL) was added to 50 mg of sample tissue. The mixture was crushed with a mortar and pestle and centrifuged at 12,000 r·min^{-1} for 10 min at 4°C. The supernatant was placed on ice for later testing. The three kit reagents were added sequentially according to the instructions. Finally, a 200 μL supernatant was taken and A measured at 540 nm and. ΔA was calculated as above. The oxygen free radical content (nmol·g^{-1}, FW) was calculated as 3361.3 × (ΔA + 0.0022) / the sample fresh weight.

2.10 Data analysis

Statistical analysis was conducted using the SAS software package (SAS Institute Inc, Rockville, USA), and the figures

were drawn using GraphPad Prism 8 for Windows 10 (Microsoft Corporation, Redmond, USA). The statistical significance of the differences between the treatments was analyzed using a single-factor ANOVA or *t*-test. The least significance difference was used for comparison of mean values.

3 RESULTS

3.1 Fennel roots interfere with the behavior and development of zoospores

Zoospores of *P. nicotianae* showed strong chemotaxis toward fennel roots. The dynamic process of chemotaxis (Fig. 1(a)) shows that the zoospores were significantly attracted by fennel roots within 5 min. After approaching the fennel roots, the zoospores rapidly lost their motility and transformed into cystospores, even leading to cystospore rupture (Fig. 1(a,b)), whereas the zoospores in the control remained motile (Fig. 1(b)). Statistical analysis of chemotactic ratios indicates that the fennel roots had significantly positive chemotaxis activity toward zoospores after interacting for 5 min (Fig. 1(c)). After 25 min, the germ tubes that moved toward fennel roots, toward/backward rate and rupture rate were significantly higher in the fennel root treatment than in the control (Fig. 1(d)). However, the germination of the cystospores showed no significant change (Fig. 1(d)).

3.2 Inhibitory activity of fennel root exudates against hyphal growth of *P. nicotianae*

Fennel root exudates showed significant dose-dependent antimicrobial activity against *P. nicotianae* hyphal growth. When the concentration was 1000 mg·L^{-1}, the inhibition rate was 47.6% (Fig. 2).

3.3 Compounds identified in fennel root exudates

Thirty-five putative compounds were identified by GC-MS (similarity > 80%) (Table 1, Fig. S1). These compounds comprised six acids, four esters, two nitrogen-containing compounds, five alkanes, four ketones, four phenols, an amine, five amides and four other compounds (Table 1, Fig. S1)

3.4 Inhibition of *P. nicotianae* by root exudate compounds

The antimicrobial activity of 11 compounds in the fennel root exudates was tested using pure compounds. Propionic acid, chloroiodomethane, butyric acid, 2,2,4,6,6-peatamethyl-heptan, N-formylpiperidine, N-cyclohexyl formamide, 4-ethylacetophenone, vanillin, palmitic acid, benzothiazole, and crotonic acid showed dose inhibition activity toward the mycelial growth of *P. parasitica* (Fig. 3).

3.5 Root exudate compounds showed strong activity against the infection processes of *P. nicotianae*

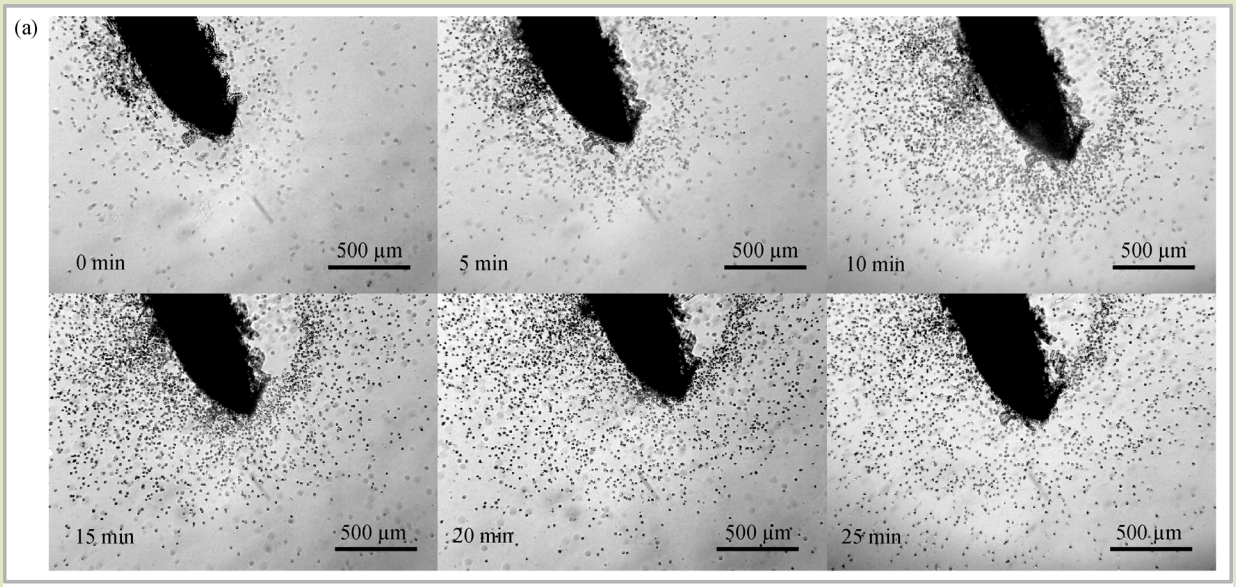
The concentrations of the above antimicrobial compounds in the fennel root exudates were further analyzed by HPLC. Only 4-ethylacetophenone, vanillin and N-formylpiperidine were quantified by HPLC (Table 2). Of the three compounds, N-formylpiperidine showed the strongest positive chemotaxis toward zoospores of *P. nicotianae* and the other two compounds showed no discernible positive chemotaxis (Fig. 4(a)). However, 4-ethylacetophenone and vanillin showed dose-dependent inhibitory effects on the zoospore motility of *P. nicotianae* but N-formylpiperidine did not (Fig. 4(b)). Vanillin showed the strongest activity against zoospore motility which was inhibited by 72.6% after 5 min at 10 mg·L^{-1} (Fig. 4(b)). The three compounds did not show inhibitory effects on cystospore germination (Fig. 4(c)) but had significant dose-dependent activity against *P. nicotianae* hyphal growth (Fig. 4(d)).

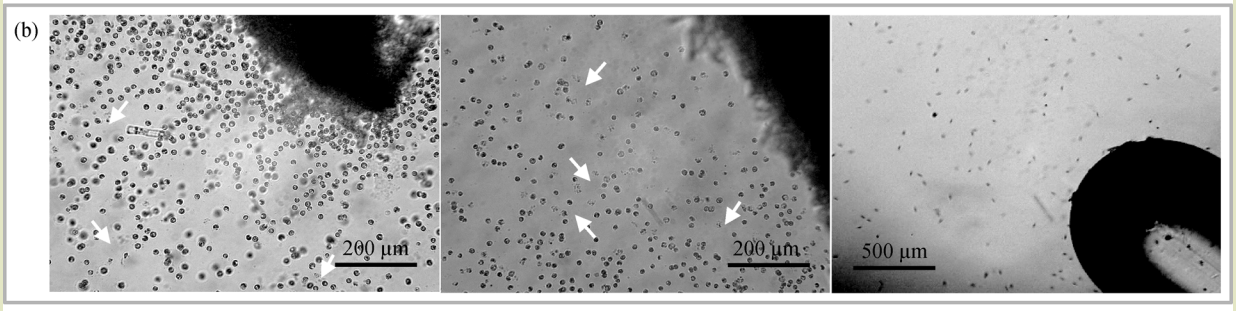
3.6 The key compounds changed the hyphal morphology of *P. nicotianae*

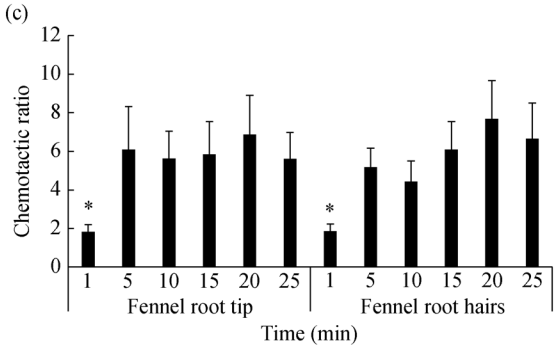
Hyphae without exposure to the key compounds grew normally (Fig. 5(a)) but hyphal morphology showed abnormalities after exposure to these compounds (Fig. 5(b–d)). In the presence of the three key compounds the hyphae at the colony edges were dense and short with more branches. The hyphae treated with 4-ethylacetophenone and vanillin at 200 mg·L^{-1} produced more abnormal branches after 6 days (Fig. 5(c,d)). However, the hyphae treated with N-formylpiperidine did not show any significant changes (Fig. 5(b)).

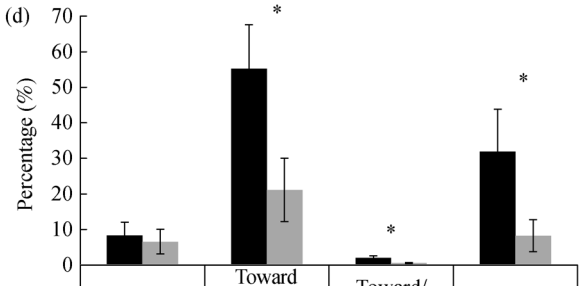
3.7 Qualitative and quantitative assessment of reactive oxygen species

As determined by the signal of DCHF-DA, the three key compounds (4-ethylacetophenone, vanillin and N-formylpiperidine) significantly elevated the intracellular ROS level in the cytosol, and cells associated with a green signal for DCHF-DA were seldom detected in the controls (Fig. 6(a)). In addition, ROS were also determined in hyphae incubated with 200 mg·L^{-1} 4-ethylacetophenone, 200 mg·L^{-1} vanillin, and 800 mg·L^{-1} N-formylpiperidine. The results show that the accumulation of H_2O_2 and oxygen free radicals increased in response to









	Germination rate	Direction of germ tube: Toward fennel root /Capillary tube	Direction of germ tube: Toward/ Backward	Rupture rate
Fennel root	8.28	55.27	2.00	31.88
Capillary tube	6.55	21.11	0.49	8.26

Fig. 1 The interaction between fennel roots and zoospores of *Phytophthora nicotianae*. (a) Dynamic process of *P. nicotianae* zoospore attraction by fennel roots. (b) Zoospores lost their swimming ability and transformed into cystospores after approaching the fennel roots. Some ruptured cystospores (indicated by arrows), while in the control, the zoospores were still motile. (c) Chemotaxis ratios (CR) from 1 to 25 min. After 5 min, fennel root tips and root hairs showed significant attraction to zoospores. (d) Germination rate, direction of germ tubes and rupture rate in fennel root and zoospore interactions. The germination rates of the fennel root treatment and control were very low and showed no significant differences. In the fennel root treatment, the germ tubes of the cystospores were oriented toward the roots, but in the control the orientation was the reverse. The rupture rate in the fennel root treatment of cystospores was significantly higher than that in the control.

Table 1 Compounds identified by GC-MS analysis of fennel root exudates

Group	Peak[a]	Closest compound	Formula	Molecular weight	Characteristic fragments	Spectra similarity (%)[b]
Acids	1	**Propionic acid**	$C_3H_6O_2$	74	39, 42, 45, 57, 74	90
	4	**Butyric acid**	$C_4H_8O_2$	88	42, 55, 60, 73, 88	91
	5	Isocrotomic acid	$C_4H_6O_2$	86	39, 41, 43, 45, 57, 60, 73, 86	90
	6	**Crotonic acid**	$C_4H_6O_2$	86	37, 39, 41, 43, 45, 53, 55, 71, 86	91
	20	Lauric acid	$C_{12}H_{24}O_2$	200	55, 60, 65, 69, 73, 93, 115, 121, 125, 129, 143, 157, 171, 183, 200	95
	28	**Palmitic acid**	$C_{16}H_{32}O_2$	256	43, 60, 73, 83, 97, 129, 157, 185, 199, 213, 227, 239, 256	97
Esters	2	Methyl thiocyanate	C_2H_3NS	73	40, 45, 58, 73	90
	7	Phenyl isocyanate	C_7H_5NO	119	51, 61, 64, 74, 77, 88, 91, 119	94
	27	Methyl hexadec anoate	$C_{17}H_{34}O_2$	270	43, 74, 87, 121, 143, 227, 270	89
	32	Bis (2-ethyl hexyl) adipate	$C_{22}H_{42}O_4$	370	57,101,112,129,147,157,1-99, 212, 223, 241, 313	89
Nitrogen-containing compounds	12	**Benzothiazole**	C_7H_5NS	135	39, 45, 58, 63, 69, 74, 82, 91, 108, 135	80
	22	2-Benzothiazolol	C_7H_5NOS	151	69, 78, 96, 106, 123, 151	91
Alkanes	3	**Chloroiodomethane**	CH_2ClI	176	49, 127, 141, 162, 176	91
	9	**2,2,4,6,6-Pentamethyl-heptan**	$C_{12}H_{26}$	170	41, 57, 71, 85, 99, 112	83
Alkanes	26	Nonadecane	$C_{19}H_{40}$	268	43, 57, 71, 85, 113, 183, 197, 268	96
	29	n-Docosane solution	$C_{22}H_{46}$	310	43, 57, 65, 71, 77, 85, 105, 155, 211, 253, 310	92
	34	n-Pentacosane	$C_{25}H_{52}$	352	43, 57, 71, 85, 99, 113, 127, 141, 155, 169, 183, 197, 239, 253, 295, 352	93
Ketone	11	4-Piperidinone	C_5H_9NO	99	39, 42, 55, 58, 70, 73, 82, 99	87
	15	**4-Ethylacetophenone**	$C_{10}H_{12}O$	148	43, 51, 63, 74, 79, 89, 105, 133, 148	95
	23	Psoralen	$C_{11}H_6O_3$	186	38, 43, 63, 69, 102, 151, 158, 186	91
	24	7-Hydroxy coumarin	$C_9H_6O_3$	162	51, 78, 105, 134, 162	93
Phenols	17	4-Allyl phenol	$C_9H_{10}O$	134	51, 55, 77, 87, 103, 107, 115, 119, 134	93
	18	**Vanillin**	$C_8H_8O_3$	152	39, 43, 77, 81, 93, 105, 109	80
	19	Alpha-methoxy-p-cresol	$C_8H_{10}O_2$	138	39, 51, 55, 65, 73, 77, 81, 91, 95, 107, 121, 138	87
	33	2,2'-Methylene bis (6-tert-butyl-4-methyl phenol)	$C_{23}H_{32}O_2$	340	41, 57, 77, 91, 105, 121, 133, 149, 161, 177, 228, 269, 284, 325, 340	96

(*Continued*)

Group	Peak[a]	Closest compound	Formula	Molecular weight	Characteristic fragments	Spectra similarity (%)[b]
Amines	8	Aniline	C_6H_7N	93	39, 51, 66, 78, 93	91
Amides	13	2-Oxohexa-methyl-enimine	$C_6H_{11}NO$	113	39, 42, 55, 58, 67, 113	94
	14	**N-cyclohexyl formamide**	$C_7H_{13}NO$	127	46, 50, 53, 56, 63, 67, 71, 77, 81, 84, 98, 127	90
	16	N-cyclohexyl acetamide	$C_8H_{15}NO$	141	39, 43, 56, 60, 82, 98, 126, 141	90
	31	Oleamide	$C_{18}H_{35}NO$	281	51, 59, 72, 98, 105, 112, 119, 126, 140, 147, 154, 170, 184, 191, 198, 238, 264, 281	83
	35	Erucylamide	$C_{22}H_{43}NO$	337	59, 72, 83, 97, 126, 137, 165, 193, 240, 249, 263, 277, 294, 320, 337	90
Other compounds	10	**N-formylpiperidine**	$C_6H_{11}NO$	113	39, 42, 53, 56, 59, 67, 70, 73, 84, 98, 113	90
	21	Fenuron	$C_9H_{12}N_2O$	164	39, 44, 51, 65, 72, 77, 91, 106, 119, 147, 164	87
	25	3-Amino-9-ethyl carbazole	$C_{14}H_{14}N_2$	210	51, 55, 77, 85, 91, 127, 195, 210	91
	30	Columbianetin	$C_{14}H_{14}O_4$	246	59, 77, 115, 131, 160, 175, 187, 203, 213, 228, 246	98

Note: [a]See Fig. S1 for peak numbers and retention times. [b]Spectral similarity compared with the mass spectra of the reference compounds stored in Wiley7n.l mass-spectral library, in which the compounds with similarities over 80% have been listed. The compounds marked in bold were chosen to test their inhibition activity toward the hyphal growth of *Phytophthora nicotianae* in Fig. 3.

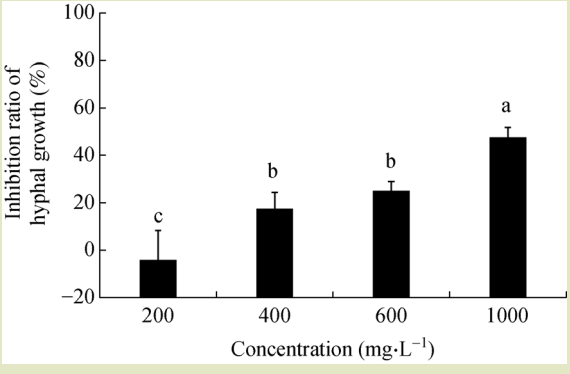


Fig. 2 Effects of fennel root exudates on the hyphal growth of *Phytophthora nicotianae*. The error bars indicate the standard errors of the means (n = 4). Significant differences are based on ANOVA. Means with the same letter are not significantly different at $P < 0.05$.

treatment with vanillin, but not the other compounds, compared to the control (Fig. 6(b,c)).

4 DISCUSSION

Numerous studies have found that interspecific root interactions that occur during intercropping can lead to the suppression of soilborne pathogens[2,25,26]. Previous studies show that rotation between fennel and other plants such as tobacco, melon and *P. notoginseng* can significantly suppress the incidence of soilborne pathogens including tobacco black shank[12], melon wilt[20] and *P. notoginseng* root rot[21]. Here, fennel roots attracted *P. nicotianae* zoospores, suppressed zoospore motility and cystospore germination, and caused the rupture of spores (Fig. 1). Also, root exudates of fennel inhibited the growth of *P. nicotianae* (Fig. 2), causing the pathogen to lose its infection ability. Hence, the interaction between fennel roots and *P. nicotianae* may explain the lower incidence of black shank in fennel-tobacco rotations. Yang[2] found that the maize roots could attract zoospores of *P. capsici* and simultaneously secrete antimicrobial compounds suppressive to zoospore infection. Also, rapeseed roots were reported to inhibit the growth of *P. nicotianae in vitro* and decrease the incidence of *Phytophthora* blight in the field[1]. This attract-and-kill phenomenon of non-host plant roots on zoospores also occurs in the interactions between garlic roots

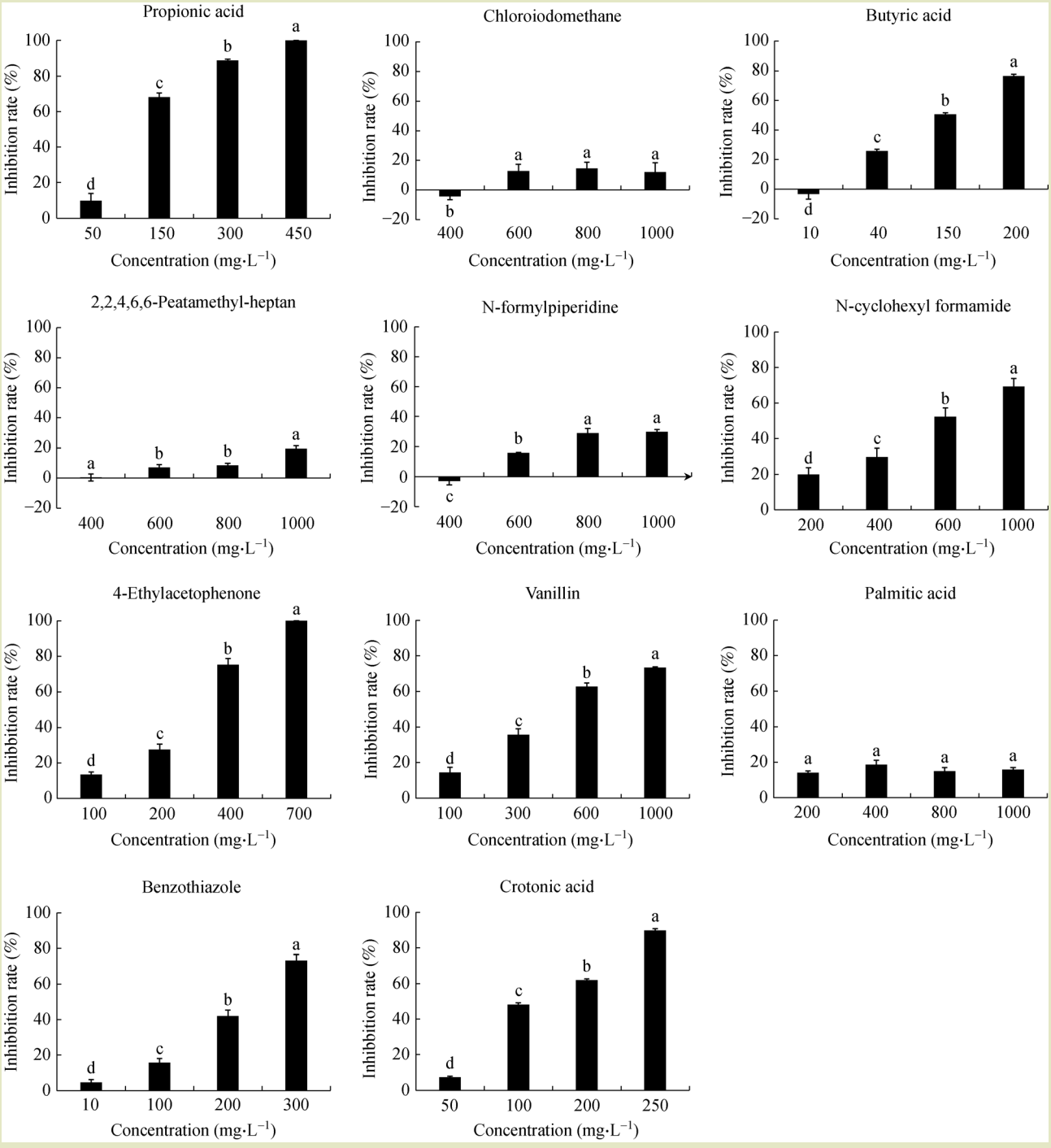


Fig. 3 Effects of fennel compounds identified by GC-MS (as indicated in bold in Table 1) on the hyphal growth of *P. nicotianae*. Error bars indicate the SD of five replicates. Values with the same letter are not significantly different at $P < 0.05$

and *P. capsica*[27] and maize roots and *P. sojae*[8]. Hence, the interference of fennel roots/root exudates with the *P. nicotianae* infection processes may be an important mechanism inhibiting *Phytophthora* blight in tobacco in a fennel-tobacco cropping system.

There are numerous reports that the antimicrobial compounds in root exudates drive interaction between plants and rhizosphere microorganisms[1,2,8,27,28]. Here, 4-ethylacetophenone, vanillin and N-formylpiperidine were identified by both GC-MS (Table 1) and HPLC (Table 2) as key compounds. This is the first

Table 2 Concentrations of the target compounds in fennel root exudates based on HPLC analysis

Compound	RT (min)	Peak area	Molecular weight	Concentration (mean±SE) (mg·L^{-1})
4-Ethylacetophenone	12.87	52.1	148.2	2.48±0.02
Vanillin	5.85	77.4	152.15	7.59±0.75
N-formylpiperidine	4.43	64.9	113.16	3.88±0.19

Note: RT, retention time (n = 3).

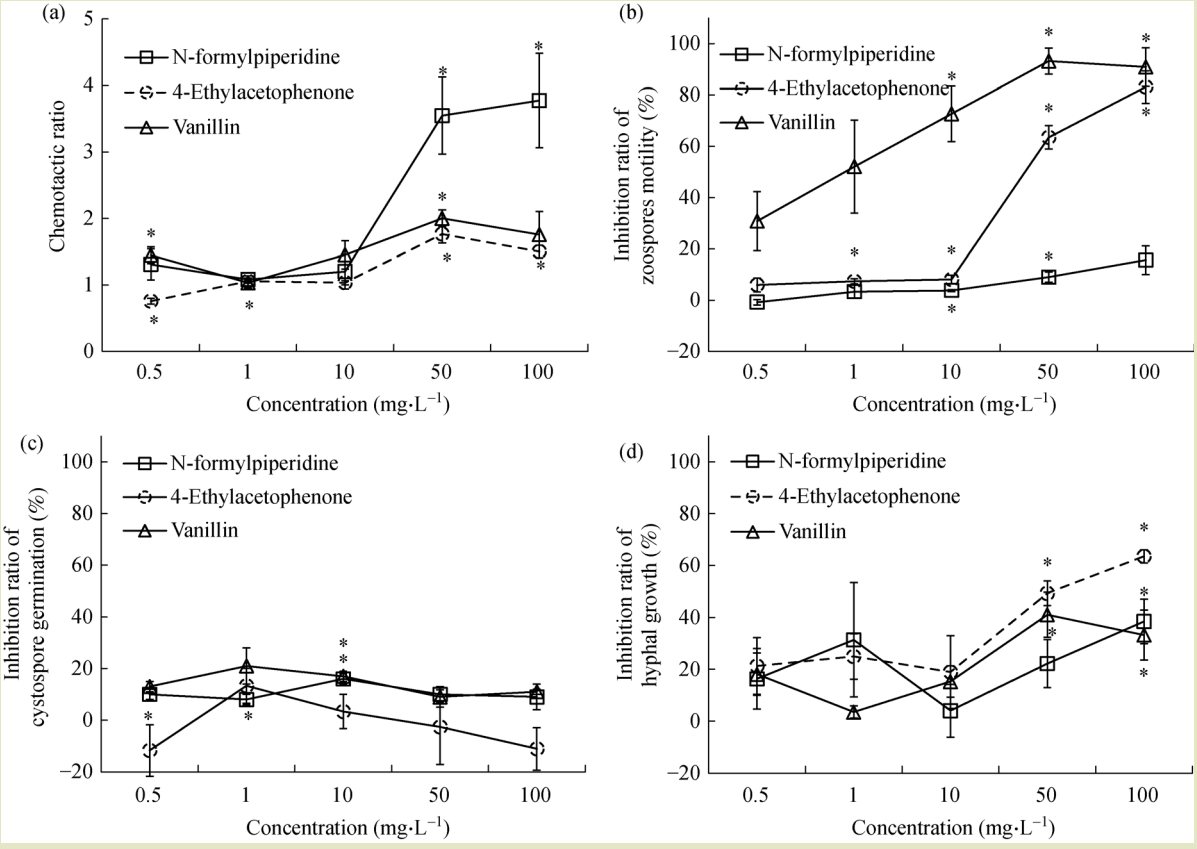


Fig. 4 Effects of compounds on chemotaxis of zoospores (a), zoospore motility (b), cystospore germination (c), and hyphal growth (d) of *Phytophthora nicotianae*. Significant differences are based on an ANOVA test. The error bars indicate standard errors of means (n = 3). *Significant difference from the control at $P < 0.05$.

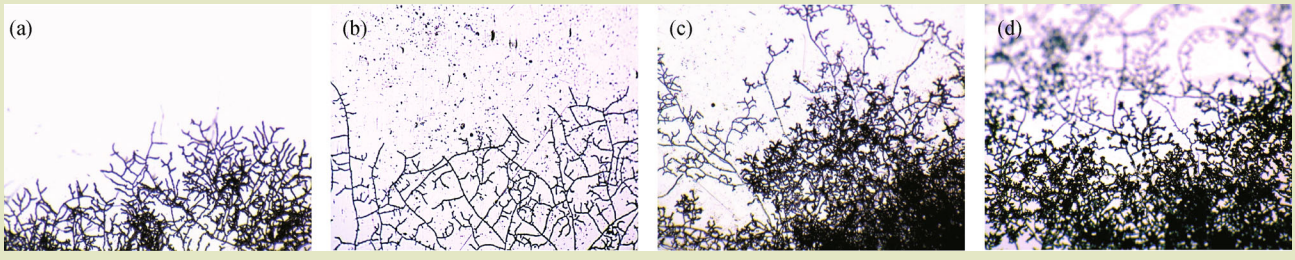


Fig. 5 Colony edges observed under a light microscope. (a) Hyphae treated with 1% methanol. (b) Hyphae treated with 600 mg·L^{-1} N-formylpiperidine. (c) Hyphae treated with 200 mg· L^{-1} 4-ethylacetophenone. (d) Hyphae treated with 200 mg·L^{-1} vanillin.

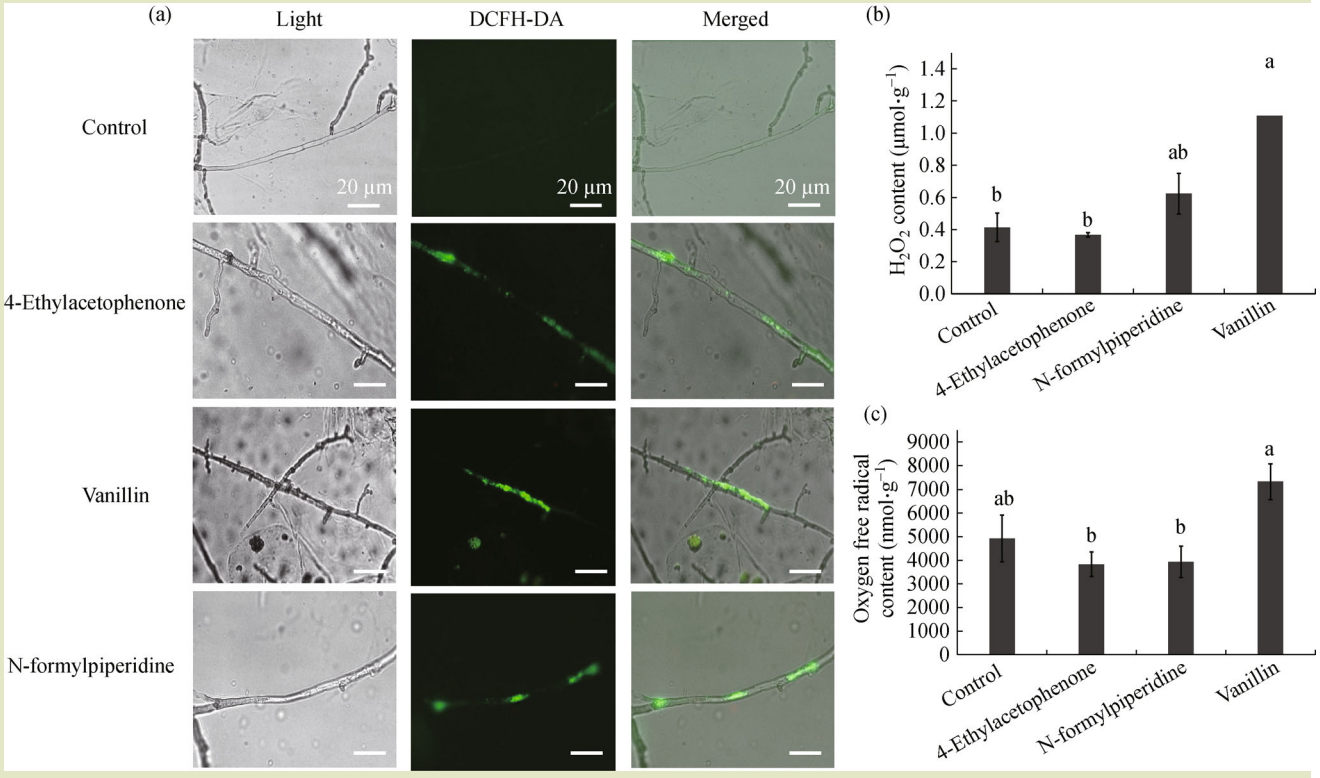


Fig. 6 Effects of three key root exudate compounds on the ROS concentrations in *Phytophthora nicotianae* hyphae. (a) Hyphal ROS visualizations for effective concentrations of 50 mg·L^{-1} 4-ethylacetophenone, 50 mg·L^{-1} vanillin, or 100 mg·L^{-1} N-formylpiperidine. (b) H_2O_2 concentrations. (c) Oxygen free radical concentrations. Significant differences are based on an ANOVA test. The error bars indicate the standard errors of the means (n = 3). Mean values with the same letter are not significantly different at $P<0.05$.

discovery of N-formylpiperidine in plant root exudates. However, vanillin and 4-ethylacetophenone are widely known in the root exudates of many other plant species, for example eggplant[29], peanut[30], *Arabidopsis thaliana*[31] and rapeseed[1]. These three compounds, especially vanillin, significantly inhibited zoospore motility and hyphal growth (Fig. 4(b,d)). A range of studies suggest that vanillin has wide antimicrobial activity toward, for example *Botrytis cinerea*[32], *Colletotrichum* spp.[33]. and *Pseudomonas*[34]. Antimycobacterial activity may also be exhibited by 4-ethylacetophenone[35]. In addition, antimicrobial compounds such as benzoxazinoids and phenolic acids have been demonstrated to interfere with infection processes of soilborne pathogens[2,8,36]. Hence, compounds in root exudates including 4-ethylacetophenone, vanillin and N-formylpiperidine are likely to be important in the inhibition of *P. nicotianae* by fennel roots.

Plant exudates act as chemotaxis signals to attract the zoospores in the rhizosphere[37]. In the present study the zoospores of *P. nicotianae* displayed positive chemotaxis toward vanillin and N-formylpiperidine but showed only a slight response to 4-ethylacetophenone (Fig. 4(a)). Hayakawa also found that vanillin attracted zoospores of actinomycetes in soil[38]. Prunetin (secreted by pea roots)[39] and cinnamic acid (secreted by maize roots)[8] also act as non-host specific attractants for *Phytophthora*. These results show that some general compounds exist in plant root exudates that have the ability to attract zoospores, confirming that some compounds attracting zoospores are not host specific[40]. Vanillin and N-formylpiperidine may therefore be important substances in attracting *Phytophthora* zoospores to the roots of non-host plants.

Induced ROS accumulation is increasingly recognized as involving important molecules that participate in plant and pathogen interactions[41,42]. Here, the compounds, especially vanillin, induced ROS accumulation in the hyphae (Fig. 6(a)), which may have resulted from the increased H_2O_2 and oxygen free radical concentrations (Fig. 6(b,c)). Mathias also found that coumarin in root exudates of *A. thaliana* shifted the microbial community profile in the rhizosphere by an ROS-mediated mechanism[22]. Another aldehyde compound (cinnamaldehyde) can also increase ROS concentrations in *P. nicotianae* hyphae, which inhibited hyphal radial growth and disrupted hyphal morphology[23]. These findings are consistent with our results

indicating that vanillin and 4-ethylacetophenone may also affect hyphal growth at the colony edges and produce more abnormal branches (Fig. 5(b,c)). Hence, ROS accumulation may be one of key mechanisms by which fennel roots inhibit plant pathogens.

5 CONCLUSIONS

We used fennel roots and *P. nicotianae* to investigate the mechanism underlying the inhibition of black shank in fennel-tobacco rotation. The non-host plant (fennel) produced root exudates that attracted zoospores of *P. nicotianae* and secreted a series of antimicrobial compounds that killed the pathogen. Three antimicrobial compounds, namely 4-ethylacetophenone, vanillin and N-formylpiperidine, were identified in the fennel rhizosphere that can inhibit the various infection processes of *P. nicotianae*. In addition, ROS accumulation is potentially the main mechanism of inhibition of *P. nicotianae* by fennel roots.

Supplementary materials

The online version of this article at https://doi.org/10.15302/J-FASE-2021399 contains supplementary material (Fig. S1).

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Compliance with ethics guidelines

Yuxin Yang, He Zhang, Yuting Fang, Ying Li, Xinyue Mei, Huichuan Huang, Fei Du, Shusheng Zhu, Min Yang, and Yixiang Liu declare that they have no conflicts of interest or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

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