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Chemotaxis of *Caenorhabditis elegans* Toward Volatile Organic Compounds from *Stropharia rugosoannulata* Induced by Amino Acids

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Abstract

A variety of natural substances including both volatile organic compounds and water-soluble compounds play a significant role in the chemotactic behaviors of the model nematode *Caenorhabditis elegans*. We observed chemotactic behaviors of *C. elegans* with respect to response to attractants produced by nematode parasitic fungus *Stropharia rugosoannulata*, which were partially induced by specific amino acids. The results of gas chromatography-mass spectrometer analysis suggested that 1-octen-3-ol was produced and benzaldehyde concentrations increased when L-phenylalanine was added to water agar plate. Similarly, the addition of L-tryptophan to the medium induced the production of benzaldehyde, 1-octen-3-ol and indole. The presence of L-phenylalanine and L-tryptophan increased the attraction of *C. elegans* to *S. rugosoannulata*. With attraction increased, nematode mortality increased more than 6 times higher.

Key words

Caenorhabditis elegans, *Stropharia rugosoannulata*, chemotaxis, attractants

Caenorhabditis elegans elegans has long been known to display chemotactic behavior to small water-soluble compounds like cAMP and NaCl, and neurons and genes required for these responses have been described (Croll, 1970; Ward, 1973; Dusenbery, 1974; Bargmann et al., 1993; Mori, 1999).

The organic compounds are generally more attractive than the ions. Studies also showed that many volatile organic compounds (VOCs) including alcohols, ketones, esters, aldehydes and aromatics are attractive for nematodes (Bargmann et al., 1990; Sengupta et al., 1994). In the soil environment, microorganisms such as bacteria can produce diacetyl (butabediol) as attractants for nematodes (Zhang et al., 1997). Some bacteria such as the *Bacillus nematocida* B16 is capable of releasing a variety of food-like odors as attractants, including benzaldehyde, 2-heptanone, indole, naphthalene, 2, 5-dimethy-lanisole, benzyl benzoate and acetophenone, and these VOCs have shown potent nematode-attracting abilities (Niu et al., 2010).

Monoson and Ranieri (1972) found that attractants for the nematode *Aphelenchus avenae* were related to trap formation of *Arthrobotrys oligospora*. Balan et al. (1974) found that the trapping devices of *Arthrobotrys dactyloides, Arthrobotrys oligospora, Arthrobotrys conoides* and *Monacrosporium rutgeriense* were all capable of attracting *Panagrellus redivivus*. They also provided evidence that the attractants differed from the nematode-killing substances. Volatile methyl 3-methyl-2-butenoate produced by *A. oligospora* additionally triggered strong sex- and stage-specific attraction in several *Caenorhabditis* species. It might mimic sex pheromone in *Caenorhabditis* species (Hsueh et al., 2017). *Stropharia* spp. can produce

unique stellate cells, which resemble a sharp sword and can damage the nematode cuticle, resulting in leakage of nematode inner materials (Luo et al., 2006).

In this study, chemotaxis of the nematode *C. elegans* toward *Stropharia rugosoannulata* was increased, when the fungus was cultured on water agar (WA) plates supplemented with two of ten different amino acids tested. VOCs produced in these cultures were analyzed by using solid-phase microextraction (SPME) in combination with gas chromatography-mass spectrometer (GC-MS). Meanwhile, we also investigated contribution of attractants produced by *S. rugosoannulata* on nematode mortality.

Materials and methods

Strain and nematode cultivation

S. rugosoannulata stain 1.2052 was isolated from a forest in Yunnan Province, P. R. China, and stored in Yunnan University. The free-living nematode *C. elegans* var. Bristol strain N2 was cultured on semi-liquid oat medium (10 g of oats, 6 ml of distilled water) at 28°C for 6 to 8 days. The cultured nematodes were grown to adulthood separated using the Baerman funnel technique, and distilled water suspension of the nematode was prepared as a working stock (Niu et al., 2010).

Chemotactic assay

S. rugosoannulata stain 1.2052 was cultured on WA plates (10 g agar, 1,000 ml distilled water) supplemented with one of 10 different amino acids (0.4 g/liter), including L-Phenylalanine, L-Tryptophan, L-Lysine, L-Proline, L-Asparagine, L-Threonine, L-Valine, L-Leucine, L-Histidine and L-Tyrosine. These plates were incubated at 28°C for 7 days. The inoculated dish was inverted and placed on top of another dish without medium but containing 10 µl nematode suspension in the center (~500 nematodes). The two dishes were sealed with parafilm and were incubated in a dark chamber at 28°C. After 48 hr, the number of nematodes on the upper dishes and total nematodes was counted by using a stereomicroscope. The attraction rate was calculated the percentage of number of nematodes on the upper dishes in total. As controls, we tested WA plates inoculated *S. rugosoannulata* but without amino acid and WA plated containing the amino acid but without *S. rugosoannulata* at the same time. All experiments included five parallel assay plates and repeated three times.

SPME/GC-MS analysis

Cultures used in the chemotactic assay were analyzed by GC-MS. Five samples were analyzed: strain 1.2052 cultured on WA media supplemented with L-Phenylalanine and L-Tryptophan, non-inoculated WA medium supplemented with L-Phenylalanine and L-Tryptophan, and strain 1.2052 inoculated on WA medium without an amino acid. VOCs produced by these cultures were analyzed using SPME in combination with GC-MS. The column temperature was programmed to stay at 50°C for 2 min, next increased to 180°C at the rate of 4°C/min, then raised to 280°C for 10 min at the rate of 6°C/min. The temperature of the injector and detector was 250°C. The split ratio was 10:1 (v/v), and the ionization voltage was 70 eV. The VOCs were collected by 75-µm CAS/PDMS silica fibers (Supelco, 57318), and the SPME fibers were inserted into the front inlet of Agilent 7890A gas chromatograph connected to an HP 5975 mass spectrometer. After the VOCs desorption at 250°C for 2 min, individual VOCs were identified by comparing the mass spectrum of the substance with GC/MS system data banks (NIST05, NIST98, and Wiley 275).

The mortality of attracted nematodes

Strain *S. rugosoannulata* was inoculated on the center of the petri dishes (diameter, 9 cm). When the diameter of the fungal lawn grew to 2 cm, 150 nematodes (*C. elegans*) were placed on five spots 3 cm from the edge of fungi. The plates were sealed with parafilm and incubated in a dark chamber at 28°C. After 48 hr, the attraction rates were assessed by counting the number of nematodes climbed over the edge of the fungal colony and total nematodes using a stereomicroscope. The mortality was calculated the percentage of dead worms in attracted nematodes climbed over the edge of the fungal colony. All experiments included five parallel assay plates and repeated three times.

Statistical analysis

Results were tested with SPSS version 13.0 for Windows by one-way analysis of variance (ANOVA).

Results

Chemotactic assay

Two treatments produced a significant chemotaxis (Fig. 1). Specifically, 6.5% *C. elegans* adults

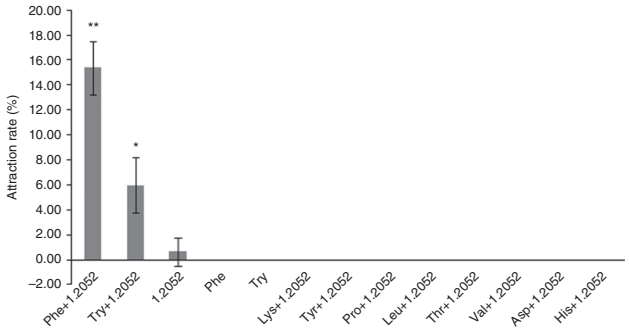

Figure 1: Chemotaxis (percent attracted) of *Caenorhabditis elegans* toward *Stropharia rugosoannulata* stain 1.2052 cultured on water agar supplemented with amino acids. Controls are phenylalanine or tyrosine alone and strain 1.2052 alone. The error bars indicate standard deviation. The statistical differences were analyzed using one-way ANOVA, **P*<0.05, ***P*<0.01.

migrated up to the plate of the *S. rugosoannulata* mycelial lawn cultured on WA supplemented with L-Tryptophan and 15.8% moved up to the lawn containing L-Phenylalanine. There was no significant chemotaxis toward the non-inoculated WA medium supplemented with phenylalanine or tryptophan, or toward strain 1.2052 cultured on WA without amino acids (Fig. 1).

SPME/GC-MS analysis

By comparing VOCs produced by the five cultures with chemical databases, we observed that 1-octen-3-ol was produced and benzaldehyde increased when L-phenylalanine was added to the WA medium. Similarly, when L-tryptophan was added to the medium, benzaldehyde, 1-octen-3-ol and indole were all induced (Table 1 and Fig. 2).

The mortality of nematodes after attracted

Stropharia spp. can produce unique stellate cells which resemble a sharp sword causing damage to the nematode cuticle, resulting in leakage of nematode inner materials (Luo et al., 2006). In this experiment, we tested whether nematode attractants contribute to fungal nematicidal activity. After 48 hr, more than 6 times *C. elegans* was killed compared to control (Figure 3).

Table 1. The attractants produced in the groups supplemented amino acids.

Tested group	Compound name	% area relative content (SD)	Quality (%)
Phe+1.2052	Benzaldehyde	0.90 (0.2)	95
	1-Octen-3-ol	0.51 (0.1)	90
1.2052	–	–	—[a]
Phe	Benzaldehyde	0.61 (0.2)	91
Try+1.2052	Benzaldehyde	0.09 (0.05)	91
	1-Octen-3-ol	1.01 (0.3)	90
	Indole	0.66 (0.2)	97
1.2052	–	–	–
Try	–	–	–

[a]"—" none detected. SD: standard deviation of three replicates. Tested group included: the cultures of stain 1.2052 supplemented with L-phenylalanine or L-tryptophan; the control without amino acids, L-phenylalanine or L-tryptophan alone.

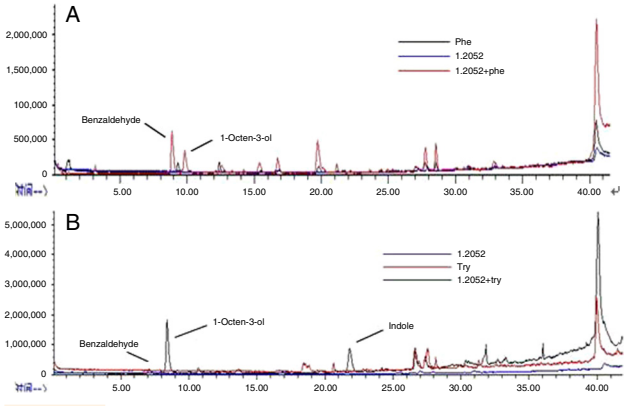

Figure 2: GC-MS total ion chromatography of different samples. A: L-phenylalanine alone, strain 1.202 alone and strain cultured on water agar plus L-phenylalanine, benzaldehyde was increased and 1-Octen-3-ol was newly produced from strain 1.2052 cultures added L-phenylalanine; B: strain 1.202 alone, L-tyrosine alone and strain cultured on water agar plus L-tyrosine, benzaldehyde was decreased and 1-Octen-3-ol and indole were newly produced were produced from strain 1.2052 cultures added L-tyrosine.

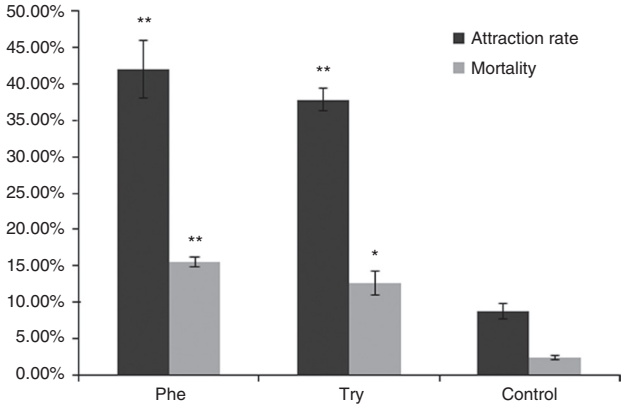

Figure 3: Chemotaxis (percent attracted) and mortality (percent of attracted worms dead) in the groups supplemented with L-phenylalanine or L-tryptophan and the control without amino acids. The error bars indicate standard deviation. The statistical differences were analyzed using one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$.

We also measured the relationship between attraction and nematicidal activity. Our result showed that the mortality of nematode increased following an increase in attraction ratio. Mainly because of the greater attraction in the presence of these attractants induced by amino acids, a greater overall mortality resulted. The attractive ability of *S. rugosoannulata* is stronger when it was incubated on L-phenylalanine-supplemented medium than when it was incubated on L-Tryptophan-supplemented medium.

Discussion

When we supplied specific amino acids in WA, the basidiomycete *S. rugosoannulata* would produce some VOCs, such as indole, benzaldehyde and 1-octen-3-ol. All these compounds are known attractants for *C. elegans* (Matsumori et al., 1989; Bargmann et al., 1993; Niu et al., 2010).

Indole attracts *C. elegans* with an AC_{50} (the concentration of the pure tested compound at which the attractive ability achieved 50% within 30 min) equal to 1 mM (Niu et al., 2010). In our study, we detected indole and benzaldehyde from cultures supplemented with L-Tryptophan. Indole can be produced by bacteria as a degradation product of the amino acid tryptophan. Pig fecal slurries have been shown to convert L-Tryptophan to indole without detectable intermediates or to 3-methylindole (skatole) via indole-3-acetate by a mixed bacterial populations from the large intestine of pigs (Jensen et al., 1995). There is no reports about eukaryotes degrading tryptophan into indole (Lee and Lee, 2010).

Benzaldehyde is well known as an attractant to *C. elegans* with an AC_{50} of 46.7 ppm (Bargmann et al., 1993). When amino acids are absorbed by microorganisms, the rate of protein synthesis can change significantly, thereby affecting the activity and regulation of metabolic pathways carried out by the relevant proteins (Zhao, 1997; Hu et al., 2004; Lü et al., 2006; Song, 2007; Zheng et al., 2007). Many studies have shown that L-phenylalanine can be converted to benzaldehyde by microbes (Berger et al., 1987; Kawabe and Morita, 1993; Krings et al., 1996; Lapadatescu et al., 1997; Lomascolo et al., 2001). In our study, when L-phenylalanine was added to WA, the yield of benzaldehyde increased by 30%, which was consistent with a previous report (Kawabe and Morita, 1993). L-phenylalanine can be a precursor of benzaldehyde, and L-tryptophan is not. At present, no microbe is known to convert L-tryptophan to benzaldehyde and L-tryptophan might play a different role in the formation of benzaldehyde and indole.

1-Octen-3-ol has been identified in many mushroom species. It is the product of an enzymatic breakdown of linoleic acid in mushrooms by lipoxygenase and hydroperoxide lyase (Tressl *et al.*, 1982). This hormone-like compound was isolated from *Botrytis cinerea* and shown to play roles in attraction, development and reproduction in *Bursaphelenchus xylophilus* (Matsumori et al., 1989). 1-Octen-3-ol has been identified as an attractant to the tsetse flies *Glossina palidipes* and *Glossina moristans* (Hall et al., 1984). Grove and Blight (1983) also suggested that 1-octen-3-ol is an attractant to the gravid female phorid fly *Megaselia halterata*. In our study, when L-phenylalanine and L-tryptophan were added to the WA medium, 1-octen-3-ol was detected in cultures of *S. rugosoannulata*. Whether lipoxygenase and hydroperoxidelyase produce 1-octen-3-ol from linoleic acid activated by L-phenylalanine and L-tryptophan in *S. rugosoannulata* needs further study.

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