Sublethal fluazaindolizine doses inhibit development of the cyst nematode *Heterodera schachtii* during sedentary parasitism

Christiane Matera, Florian MW Grundler and A Sylvia S Schleker

Abstract

**BACKGROUND:** Fluazaindolizine is a new compound for the control of plant-parasitic nematodes (PPNs) with an unknown and novel mode-of-action. This compound is very effective against important PPNs. However, investigations elucidating the impact of sublethal fluazaindolizine doses on early nematode virulence and plant–nematode interaction parameters are lacking.

**RESULTS:** The effect of direct exposure of *Heterodera schachtii* juveniles to 50 ppm fluazaindolizine was negligible. Infection assays revealed a 57% reduction in adult females at 1.25 ppm and a 46% reduction in offspring at 40 ppm when juveniles were soaked in the compound for 48 h and subsequently inoculated onto *Arabidopsis thaliana*. Pre-incubation of *A. thaliana* roots with fluazaindolizine was not effective against *H. schachtii*. Conversely, supplementing the plant growth medium with fluazaindolizine led to a significant reduction of adults (−35%), females (−75%) and female size at 1.25 ppm and nearly completely inhibited nematode parasitism at 5 ppm. The impact of fluazaindolizine on *A. thaliana* was dependent on plant age, compound concentration and duration of contact. Very low sublethal fluazaindolizine concentrations, 5 or 10 ppm, did not interfere with nematode mobility, host finding, penetration, and induction of the feeding site, but specifically inhibited sedentary nematode development inside the root in a concentration-dependent manner.

**CONCLUSION:** Fluazaindolizine does not have direct toxicity against PPN infective juveniles, but has a clear effect on nematodes during sedentary development. The formation of females and the development of offspring are strongly reduced. It will be interesting to identify the underlying mechanism in the future.

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**Keywords:** fluazaindolizine; *Heterodera schachtii*; nematode; plant; mode-of-action

1 INTRODUCTION

Plant-parasitic nematodes (PPNs) are a major threat to crop production worldwide. Nematode management is based on crop rotation, resistant and tolerant host varieties, and nematicides as part of an integrated programme. Although nematicides are very efficient, their use is highly controversial and banned in many countries, as many of them have proven to be toxic for humans and animals, and to have a negative impact on the environment. For reduced-toxicity nematicides the avoidance of resistance formation is essential. For this reason, there is a need for new nematicides which differ in their mode-of-action from the commercial products, are specific to the target pests and are environmentally friendly.

One of the new nematicides considered to have desirable toxicological and ecotoxicological characteristics is fluazaindolizine, which belongs to the group of imidazopyridines. Fluazaindolizine (Salibro™) has been shown to be selectively active against PPNs with no or little adverse effects towards non-PPNs, *Drosophila melanogaster*, and a variety of soil inhabitants including beneficial bacteria, fungi and nematodes. The efficacy against a wide range of PPNs including *Globodera* spp., *Heterodera schachtii*, *Meloidogyne* spp., *Mesocriconema xenoplax*, *Paratylenchus* spp., *Pratylenchus* spp., *Rotylenchulus reniformis*, *Tylenchulus semipenetrans*, and *Xiphinema* spp. has been tested in laboratory, glasshouse and field trials on vine, soybean, potato, tomato, carrots, radish, cucumber and cotton. Comparative studies report variations in sensitivity of PPN species and populations towards fluazaindolizine, a phenomenon also documented for other nematicides. Additionally, activity of fluazaindolizine against targets of commercial nematicides, such as acetylcholinesterase, the target of carbamate and organophosphate compounds, has not been observed. Therefore, a novel mode-of-action can be assumed.

* Correspondence to: ASS Schleker, INRES—Department of Molecular Phytomedicine, Rheinische Friedrich-Wilhelms-University of Bonn, D-53115 Bonn, Germany. E-mail: sylvia.schleker@uni-bonn.de

INRES—Department of Molecular Phytomedicine, Rheinische Friedrich-Wilhelms-University of Bonn, Bonn, Germany

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One economically important PPN species is the beet cyst nematode *H. schachtii*, a severe pest in sugar beet production.\(^1\)\(^4\) *H. schachtii* survives as cysts containing hundreds of infective juveniles (J2s) in soil. Once released, the J2s search for the root, invade and develop to adults within two to four weeks by continuous uptake of nutrients from the root. The fertilized females become cysts and eggs can remain viable for a couple of years.\(^1\)\(^5\) *H. schachtii* is capable of infecting >200 plant species of 20 different families with species in the Amaranthaceae and Brassicaceae being particularly affected; its host range includes the model plant *Arabidopsis thaliana*.*\(^1\)\(^6\)\(^,\(^1\)\(^7\)* This facilitates in-depth studies on *H. schachtii*—plant interplay by enabling microscopic observation of the nematode throughout its whole lifecycle, and to investigate pathogen and host at the molecular level at specific stages during parasitism. Because of this, aspects of *H. schachtii*’s complex biology have been elucidated.\(^1\)\(^8\)\^-\(^2\)\(^3\)

Towards contributing to unravel the mode of action of fluazaindolizine, we utilized the pathosystem *H. schachtii*—*A. thaliana* as a model system with the aim of obtaining comprehensive and very detailed information on the impact of fluazaindolizine on *H. schachtii* J2s and particularly at each step during interaction with the host plant. We achieved a highly resolved view on the impact of very low sublethal fluazaindolizine concentrations on *H. schachtii* virulence parameters and parasitism. This not only validates fluazaindolizine to be a very efficacious *H. schachtii* control agent, but also paves the way for directed analyses to decipher the compound’s mode-of-action at the molecular level.

## 2 MATERIAL AND METHODS

### 2.1 Chemical

A chemical marker substance of fluazaindolizine was provided by Bayer AG (Monheim, Germany). Fluazaindolizine concentrations are given in ‘ppm’ which equals ‘mg L\(^{-1}\).’

### 2.2 Plant material and growth conditions

Seeds of *A. thaliana* Col-0 were surface-sterilized for 5 min in 1% sodium hypochlorite, cleaned for 5 min in 70% ethanol and washed three times in sterile d\(_2\)H\(_2\)O. Seeds were kept at 4 °C before use. Plants were cultivated on modified Knop medium supplemented with 2% sucrose and 0.1% Gamborg’s vitamin solution (modified Knop medium).\(^1\)\(^7\) Depending on the experiment, sterilized seeds were placed into Petri dishes (9.6 cm in diameter) on modified Knop medium without or with different concentrations of fluazaindolizine (0–10 ppm). Incubation was performed in a growth chamber at 25 °C with a 16 h:8 h light:dark cycle.

### 2.3 Preparation of nematode inoculum

Cysts of *H. schachtii* were harvested from *in vitro* stock cultures on mustard (*Sinapis alba* cv. Albatros) roots growing on modified Knop medium.\(^1\)\(^7\) Hatching of *H. schachtii* J2s was stimulated by soaking cysts in 3 mM ZnCl\(_2\) for 7 days at 25 °C in the dark. *H. schachtii* J2s were then washed four times in sterile H\(_2\)O before immediate use in the assays.

### 2.4 Effect of fluazaindolizine on nematode viability

In order to determine the sensitivity of *H. schachtii* to fluazaindolizine, J2s were incubated for 6 days in a DMSO (dimethyl sulfoxide)/water solution containing 50, 40, 30, 20, 10, 5, 2.5, 1.25, 0.625, 0.313 or 0 ppm fluazaindolizine. As the chemical is relatively insoluble in water, a stock solution of 20 000 ppm fluazaindolizine in DMSO was prepared and diluted to the appropriate concentrations. Control treatments received the same amount of DMSO as the 50 ppm treatment. The experiment was performed in flat-bottom 96-well plates and five wells for each treatment were analyzed. Each well received 180 µL of a two-fold concentration and then 180 µL of water containing ≈40 *H. schachtii* J2s. To assess mortality, 10 µL of 1 M NaOH were added to each well after the 6-day incubation period and analyzed with a Leica DM4000 microscope (Leica Microsystems, Wetzlar, Germany) equipped with an Olympus C-5050 digital camera (OM Digital Solutions GmbH, Hamburg, Germany). The addition of NaOH allows to clearly distinguish between dead and live nematodes as the living ones curl upon contact. Straight and immobile nematodes were defined as dead. The experiment was repeated four times at room temperature. Some concentrations were only included in three of the four experiments (0, 0.313, 0.625, 1.25, 40 and 50 ppm).

### 2.5 Effect of fluazaindolizine on plant growth

In order to investigate the effect of fluazaindolizine on *A. thaliana* growth and development, three different treatments were evaluated. In the first treatment, seeds were sown directly onto the fluazaindolizine-supplemented medium (0, 0.0625, 0.313, 1.25, 5 and 10 ppm) and the plants were grown on this medium for 14 days. In the second treatment, plants were germinated on the fluazaindolizine-containing medium (0, 0.0625, 0.313, 1.25, 5, and 10 ppm), but the plants were transferred to medium without fluazaindolizine after 7 days. For the third treatment, seeds were sown on medium without fluazaindolizine and transferred to fluazaindolizine-supplemented medium (0, 0.313, 1.25, 5 and 20 ppm) after 7 days. Length of the primary root was measured with a ruler seven and 14 days after sowing. The fresh weight of shoots and roots was determined after 14 days. The experiment was repeated three times with ten plants for each treatment.

### 2.6 Effect of fluazaindolizine on *H. schachtii* infection and development

In order to identify whether fluazaindolizine impacts the ability of *H. schachtii* to infect and develop on *A. thaliana*, three treatments were evaluated. In Treatment 1, *H. schachtii* J2s were soaked in fluazaindolizine (0, 0.0625, 0.313, 1.25, 5, 10, 20 and 40 ppm) for 24 or 48 h. After these incubation periods, nematodes were rinsed three times with sterile water, and then 30–40 J2s were inoculated per plant. In Treatment 2, *A. thaliana* seeds were sown onto medium without fluazaindolizine and then transferred to fluazaindolizine-supplemented medium (0, 0.313, 1.25 and 5 ppm), whereas in Treatment 3, seeds were sown onto fluazaindolizine-containing medium (0, 0.0625, 0.313, 1.25, 5, 10 and 15 ppm) and then were transferred to medium without fluazaindolizine. In Treatments 2 and 3, the plants were transferred 7 days after seeding and inoculated with 30–40 *H. schachtii* J2s 14 days after seeding. For each biological replicate, 16 seeds were sown for each treatment. In all treatments, the numbers of males and females were counted after two weeks. Additionally, 32 females per treatment—or all available—were photographed 14 and 28 days post inoculation (dpi) with a Leica DM2000 dissection microscope and their size was calculated using LAS software (Leica Microsystems). To evaluate the offspring per cyst at 35 dpi, 30 cysts per concentration—or all available—were crushed manually one by one in 50 µL water each. Three times 5 µL were utilized to count the number of eggs and juveniles for each cyst and the average was calculated. All experiments were repeated three times.
2.7 Effect of fluazaindolizine on *H. schachtii* mobility

In order to evaluate the impact of fluazaindolizine on *H. schachtii* mobility, approximately 30 J2s were inoculated in the middle of a Petri dish containing fluazaindolizine-amended agar (0, 5 or 10 ppm). The distance which J2s moved from the inoculation point was marked and pictured after 30 and 60 min, and measured with ImageJ. Five Petri dishes were analyzed for each concentration and the experiment was repeated three times at room temperature.

2.8 Effect of fluazaindolizine on *H. schachtii* stylet thrusting at the root

In order to evaluate whether fluazaindolizine influences the stylet thrusting of *H. schachtii* at the root, plants grown on medium with different fluazaindolizine concentrations (0, 5 or 10 ppm) were inoculated with 30–40 J2s. Within a time span of 4–8 hpi (h post inoculation), nematodes that penetrated the epidermis of the root were marked. The amount of stylet movements was counted for ≥10 min under a Leica DM4000 microscope (Leica Microsystems). Ten *H. schachtii* J2s for each concentration were analyzed and the experiment was repeated three times.

2.9 Effect of fluazaindolizine on *H. schachtii* host finding

In order to observe whether fluazaindolizine influences the ability of *H. schachtii* to find its host, 12 plants per treatment were grown on medium with different fluazaindolizine concentrations (0, 5 or 10 ppm) and inoculated with 30–40 J2s placed at 1 cm distance to the most peripheral root of the root system. After 24, 48 and 72 h the nematodes that successfully reached the root and established there were marked and counted. Nematodes that penetrated the root and only show minimal movement of the stylet were defined as ‘established’. The experiment was repeated three times.

2.10 Effect of fluazaindolizine on *H. schachtii* early development at the root

In order to determine the impact of fluazaindolizine on the development of *H. schachtii*, nematodes were soaked in 0, 5 or 10 ppm fluazaindolizine for 24 or 48 h. After incubation, nematodes were washed five times with sterile water and then inoculated onto 12-day-old *A. thaliana* plants. Additionally, freshly hatched *H. schachtii* J2s were used to inoculate *A. thaliana* plants growing on fluazaindolizine-supplemented medium (0, 5 and 10 ppm) for 12 days, and plants that were transferred from fluazaindolizine-containing medium (0, 5 and 10 ppm) to fluazaindolizine-free medium after 7 days. Each plant was inoculated with 30–40 J2s and ten plants per treatment were used. After 24 h, nematodes that established an interaction with the root were marked and pictured daily with a Leica DM2000 dissection microscope. Nematode size was calculated using LAS software (Leica Microsystems). The experiment was repeated three times.

2.11 Data analysis

Data from all repeated experiments were combined for statistical analysis by one-way ANOVA. Significant differences were determined utilizing post hoc Holm–Sidak or Dunn’s method (*P* < 0.05). The experiment was repeated three times.

3 RESULTS

3.1 Effect of fluazaindolizine on *H. schachtii* J2 viability

In order to determine whether fluazaindolizine was directly nematocidal, freshly hatched *H. schachtii* J2s were incubated in different concentrations of the compound. The nematodes appeared to be very insensitive to fluazaindolizine. Significant differences to the control could be observed starting from 30 ppm (*P* < 0.05). Only 17% of the *H. schachtii* J2s were straight and immotile after 6 days of continuous incubation in 50 ppm (Fig. 1).

3.2 Effect of fluazaindolizine on *A. thaliana* phenotype

The plant development assays indicated that fluazaindolizine influenced *A. thaliana* depending on the time and duration of exposure as well as concentration (Supporting Information, Fig. S1). The strongest phytotoxic effect was observed on plants sown and developed on fluazaindolizine-containing medium (Fig. S1(A), (B)). Primary root length of the 7- and 14-day old plants was significantly affected at 5 and 10 ppm with a reduction by ≥25% and 50% compared to the control, respectively. Concentrations ≥10 ppm caused root tip damage (root tips were brownish). Compared to the control, 10 ppm led to a reduction of shoot weight by 88% and root weight by 93% in the 14-day-old plants.

When 7-day-old plants were transferred from fluazaindolizine-containing to fluazaindolizine-free medium, the plants were able to partially recover from the treatment with fluazaindolizine (Fig. S1(C), (D)). No differences in primary root length were observed between control plants and plants grown on ≤10 ppm fluazaindolizine, although plants transferred from medium with 5 and 10 ppm had shoot weights decreased to 58% (5 ppm) and 43% (10 ppm) and root weights decreased to 42% (5 ppm) and 36% (10 ppm), respectively.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effect of fluazaindolizine on *Heterodera schachtii*. Average percentage of dead *H. schachtii* second-stage juveniles (J2s) after exposure to different concentrations of fluazaindolizine for 6 days. Data represented as mean ± SD of three or four independent biological replicates (*n* = 12–20). Asterisk indicates significant difference to control (5.34 ± 4.13% mortality) (*P* < 0.05).
and 32% (10 ppm) of the shoot and root weights of control plants. When the experiment was reversed so that the plants were grown on a fluazaindolizine-free medium for 7 days and then transferred to a medium containing ≤20 ppm fluazaindolizine, the root of plants on concentrations ≤5 ppm was comparable to the control plants [Fig. S1(E), (F)]. Plants exposed to 20 ppm had roots 62% shorter than control plants, and the root and shoot weight were significantly reduced by 92% and 79%, respectively, on day 14.

### 3.3 Effect of fluazaindolizine on nematode parasitism and development

Although we observed that direct exposure of H. schachtii J2s to fluazaindolizine did not cause nematode mortality up to 20 ppm (Fig. 1), we investigated whether those juveniles were still able to infect a host plant. Therefore, freshly hatched H. schachtii J2s were incubated in different concentrations of fluazaindolizine for 24 or 48 h, the compound was removed by washing and A. thaliana plants were inoculated with the pre-incubated J2s. The 24 h fluazaindolizine treatment did not reduce H. schachtii infection of A. thaliana roots [Fig. S2]. Increasing the incubation time to 48 h enhanced the efficacy of fluazaindolizine. Concentrations ≥1.25 ppm significantly reduced the number of females and ≥10 ppm reduced the number of males [Fig. 2(A)]. Compared to the control, concentrations of 10, 20, and 40 ppm resulted in a significant decline of parasitizing adult nematodes by 46%, 59% and 74%, respectively [Fig. 2(A)]. Concentrations ≥1.25 ppm fluazaindolizine led to a significant shift from females to males without changing the total number of adult nematodes at the root up to 5 ppm [Figs 2(A) and S4]. The female: male ratio of the control was 49:51. This ratio decreased with higher concentrations of fluazaindolizine. Compared to the control at 1.25 ppm fluazaindolizine the female: male ratio was significantly reduced to 26:76 [Fig. S4]. Additionally, pre-treatment of H. schachtii J2s with fluazaindolizine for 48 h significantly reduced offspring by 23% (20 ppm) and 46% (40 ppm) [Fig. 2(C)].

As our results revealed that the duration of contact between H. schachtii and fluazaindolizine is crucial to reduce nematode parasitism of the host, therefore, we tested the efficacy of fluazaindolizine when the nematode was permanently exposed. To do so, we utilized the above-described experimental treatment where the 7-day-old plants were transferred from fluazaindolizine-free medium to fluazaindolizine-containing medium. This treatment revealed a very strong effect of fluazaindolizine on H. schachtii infection and development (Fig. 3). On plants grown in 1.25 ppm fluazaindolizine, H. schachtii females were reduced by ≈75% compared to the control plants [Fig. 3(A)] and female size was reduced by 32% [Fig. 3(B)]. Moreover, 5 ppm reduced the established nematodes to ≈5% compared to the control plants and in three experiments only one female was detected; therefore, female size and offspring were not considered.

In order to investigate whether pre-incubation of A. thaliana influences nematode parasitism and thereby obtain an indication if the induction of plant defence mechanisms could be involved, the experimental set-up in which the plants were sown on fluazaindolizine-containing medium and transferred to fluazaindolizine-free medium was applied. This treatment did not have any significant effect on any of the measured parasitism parameters analyzed except at 0.313 ppm fluazaindolizine where significantly fewer male nematodes were observed [Fig. S3].

### 3.4 Effect of fluazaindolizine on nematode fitness

Towards elucidating the mode-of-action, we investigated whether nematode mobility was affected by fluazaindolizine by examining each phase of nematode parasitism–plant interaction, host finding, stylet thrusting and nematode development. The mobility of H. schachtii J2s was not influenced by fluazaindolizine within 48 h at concentrations ≤10 ppm in the medium [Fig. 4(A)]. Fluazaindolizine-exposed nematodes covered the same distance as control nematodes. Nematodes that were penetrating plant root tissue while being exposed to 5 or 10 ppm fluazaindolizine had the same number of stylet movements per minute as nematodes on the control plates. Thus, fluazaindolizine had no impact on the frequency of stylet thrusting of the invading nematodes [Fig. 4(B)].

Neither pre-incubation of H. schachtii J2s in 5 or 10 ppm fluazaindolizine for 24 or 48 h, nor permanent exposure of the J2s to fluazaindolizine when plants were grown on ≤10 ppm fluazaindolizine-containing medium affected the capability of the nematode to find and penetrate the host root [Fig. 5(A), (C), (E)]. The number of nematodes that were established on the root was equal in treated and untreated plants at 24, 48, and 72 hours post inoculation (hpi).

### 3.5 Effect of fluazaindolizine on nematode development at the root

As we observed that (i) the number of adult individuals was significantly decreased and that nematode development was considerably impaired when pre-incubating the H. schachtii J2s for 48 h in fluazaindolizine [Fig. 2], (ii) this effect was even much stronger when incorporating the compound in the plant growth medium (Fig. 3), and (iii) the compound did not affect the initial establishment of nematode–plant interaction [Figs 4 and 5(A), (C), (E)], we investigated the early nematode developmental stages in the host [Fig. 5(B), (D), (F)].

Analyzing the development of H. schachtii in the root over time showed that growth of the nematodes was significantly inhibited by fluazaindolizine. When the compound was added to the medium, a significant decrease in nematode size of ≥15% was already detectable at 2 dpi for 5 and 10 ppm [Fig. 5(F)]. Ten days postinoculation, nematodes were only about one third or a sixth of the size of untreated nematodes at 5 or 10 ppm fluazaindolizine-containing plant growth medium, respectively [Fig. 5(F)]. In accordance, a pre-incubation of H. schachtii J2s for 24 h did not impair nematode development [Fig. 5(B)], whereas nematode treatment with 5 or 10 ppm fluazaindolizine for 48 h significantly inhibited early development resulting in a size reduction of ≥25% or 58% compared to the untreated control, respectively [Fig. 5(D)]. Microscopic observation revealed that fluazaindolizine did not hinder the induction of a syncytial feeding site and, although nematode development was completely inhibited at 10 ppm, the syncytium increased in size (Fig. 6). This indicates that the fluazaindolizine-treated nematode was able to inject effectors into the initial syncytial cell which mediate the establishment of a feeding structure, but was not able to develop. Thus, fluazaindolizine seems to interfere with nematode feeding or its metabolism rendering the nematode unable to take up or make use of nutrients from the syncytium.

### 4 DISCUSSION

Fluazaindolizine is a novel nematicide with a so far unknown mode-of-action that has been shown to efficiently control a broad range of PPNs.2, 3–12, 25 The compound is reported to not influence the lifecycle of Drosophila melanogaster, the activity of acetylcholinesterase (Diatroicica undecimpunctata), mitochondrial electron transport (Caenorhabditis elegans), nicotinic acetylcholine...
receptors (C. elegans), glutamate-gated chloride channels (Periplaneta americana) nor various nontarget and beneficial soil organisms such as the bacteriophagous nematode Acrobeles buetschii, demonstrating its specificity to PPNs. However, most data are based on root-knot nematodes (Meloidogyne spp.) and investigations with cyst nematodes (Heteroderidae) are rare. Furthermore, studies investigating the effect of fluazaindolizine on PPNs particularly during the very early events of plant–nematode interaction on a highly time-resolved basis are missing. We therefore evaluated the impact of fluazaindolizine on H. schachtii J2 viability as well as infection of, and development and reproduction on, its host plant Arabidopsis thaliana in detail. We demonstrate that fluazaindolizine, although not directly nematicidal against H. schachtii at the tested concentrations, did efficiently control the nematode. We provide evidence that the compound exerts its function at the early stage of the nematode’s sedentary phase interfering with the parasite’s development on its host.

The in vitro vitality assay revealed that exposure of fluazaindolizine to H. schachtii for 6 days did not affect nematode vitality at concentrations ≤20 ppm and only caused death of ≈17% of the

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**Figure 2.** Effect of 48 h nematode pre-incubation with fluazaindolizine on Heterodera schachtii development at Arabidopsis thaliana. H. schachtii second-stage juveniles (J2s) were soaked in different concentrations of fluazaindolizine for 48 h, washed and used to inoculate plants. (A) Developed H. schachtii per plant at 14 dpi (days post inoculation), (B) size of female H. schachtii at 14 and 28 dpi, and (C) number of offspring per cyst. Bars represent mean ± SD of three independent biological replicates [n = 36–43 (A), 20–96 (B), 20–90 (C)]. Different letters indicate significant differences between treatments (P < 0.05).
nematodes at a concentration of 50 ppm. Thus, we concluded that fluazaindolizine is not directly nematicidal for *H. schachtii* J2s at reasonable concentrations. Our observations are in line with a recent report that documented a *H. schachtii* J2 mortality of <20% after 72 h of exposure to 100 mg L\(^{-1}\) fluazaindolizine and a 72 h LC\(_{50}\) (lethal concentration causing 50% of the J2s to die) of 1019.3 mg L\(^{-1}\).\(^{11}\) The same study observed a 72 h LC\(_{50}\) of 365.9 mg L\(^{-1}\) fluazaindolizine for *M. incognita* J2s. Wram and Zasada\(^{26}\) performed experiments with *M. incognita* which revealed that the effective dose needed to cause 50% of the population to become inactive after 24 h was 230 ppm. Lahm *et al.*\(^2\) reported that *M. incognita* J2s exposed to 5–50 ppm fluazaindolizine were paralyzed within 24–96 h of exposure in a concentration-dependent manner and eventually died. Another

**Figure 3.** Effect of fluazaindolizine in the plant growth medium on *Heterodera schachtii* development. Seven-day-old seedlings were transferred from fluazaindolizine-free to fluazaindolizine-supplemented medium and inoculated with *H. schachtii* second-stage juveniles (J2s). (A) Developed nematodes per plant at 14 dpi (days postinoculation), (B) size of female nematodes at 14 and 28 dpi, and (C) number of offspring per cyst. Bars represent mean ± SD of three independent biological replicates (n = 37–42 (A), 68–90 (B), 66–90 (C)). Different letters indicate significant differences between treatments (P < 0.05).
study documented that *M. incognita* J2s treated with 50–250 ppm fluazaindolizine for 7 days were all dead or at least toxicologically affected with this effect being irreversible.\(^8\) Further, Thoden and Wiles\(^4\) observed that root-knot nematodes (*M. incognita* and *Meloidogyne hapla*) exhibited clearly visible symptoms of toxicological effects when incubated in 1–50 ppm fluazaindolizine for 24–48 h. This treatment irreversibly affected J2 motility and infectivity negatively. We did not observe indications for direct compound toxicity in our assays with *H. schachtii*. In accordance with Wu et al.\(^1\), we observed that a pre-incubation of

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**Figure 5.** Effect of fluazaindolizine on *Heterodera schachtii* host finding and early development at *Arabidopsis thaliana*. *H. schachtii* second-stage juveniles (J2s) were soaked in fluazaindolizine for 24 h (A, B) or 48 h (C, D), washed and used to inoculate plants growing on fluazaindolizine-free medium, or untreated J2s were used to inoculate plants growing on fluazaindolizine-supplemented medium (E, F). Average number of *H. schachtii* that penetrated into the root tissue at 24, 48 and 72 hpi (h post inoculation) (A, C, E). Average size of nematodes at 1–10 dpi (days post inoculation) (B, D, F). Data represented as mean ± SE (A, C, E) or SD (B, D, F) of two to three independent biological replicates \(n = 23–36\) (A, C, E), \(9–26\) (B, D, F). Different letters indicate significant differences between treatments at each evaluation time point \(P < 0.05\).
H. schachtii J2s with fluazaindolizine resulted in reduced infection of the host plant, proving that the compound is taken up by the nematode. Therefore, as also mentioned earlier, M. incognita and H. schachtii possess different sensitivity towards fluazaindolizine as a different target and thus mode-of-action seems unlikely. Variable responses of PPNs to fluazaindolizine might be caused, first, by difference in the thickness and permeability of the cuticle, and/or the fastness of cuticle surface turnover. In support of this, the cuticles of Meloidogyne spp. and Globodera rostochiensis J2s are reported to be 0.3–0.4 μm and ≈0.53 μm thick, respectively.27, 28 Secondly, nematodes are able of exchanging cuticle surface components; the period of time for replacing surface components is reported to vary between nematode species. For instance, turnover of certain cuticle surface components of Globodera pallida J2s takes 1–2 h and thus is much faster compared to that of Meloidogyne javanica J2s which require ≈24 h.29, 30

In order to determine whether fluazaindolizine has any effect on the infection and development of H. schachtii on A. thaliana, infection assays were performed with nematodes soaked in 0.06–40 ppm fluazaindolizine for 24 or 48 h and extensively washed before inoculation. No change in infection rate compared to the solvent control could be detected for juveniles exposed to fluazaindolizine for 24 h, whereas incubation for 48 h reduced adult nematodes by ≈46% (10 ppm fluazaindolizine) to ≈74% (40 ppm fluazaindolizine). Wu et al.11 observed that a pre-incubation of H. schachtii J2s with Salibro™ (100 or 200 mg L⁻¹ fluazaindolizine) for 24 h did not significantly alter the number of nematodes that infected radish roots 4 dpi. However, exposing H. schachtii J2s to 200 mg L⁻¹ fluazaindolizine for 48 h pre-inoculation caused an infection decline of 73.5% at 4 dpi compared to the water control. Wu et al.11 and our findings are consistent in that sublethal concentrations of fluazaindolizine efficiently control H. schachtii dependent on the duration of compound contact. One aspect that seems to be conflicting is that Wu et al.11 applied far higher concentrations of fluazaindolizine to obtain an effect on nematode infestation. Moreover, from their observations at 4 dpi they concluded that fluazaindolizine reduces J2 root invasion ability. We did not observe an impact of fluazaindolizine on H. schachtii J2 invasion, but our results do reveal that fluazaindolizine specifically blocks nematode development inside the root after induction of the feeding site. The explanation could be the time point of evaluation used in this and other studies. Wu et al.11 may have observed an impact of 100 mg L⁻¹ fluazaindolizine (48 h variant) when determining the number of adult nematodes at a later time point and we might have seen an effect of fluazaindolizine on H. schachtii J2 activity if much higher fluazaindolizine concentrations were used. Therefore, the impact of the very low sublethal concentrations of fluazaindolizine that we applied in the present study becomes obvious only when H. schachtii physically interacts with the host plant.

The mode-of-action of fluazaindolizine is not known. To approach this and identify which stage during the nematode’s lifecycle the compound’s activity becomes evident, different assays were performed using sublethal fluazaindolizine doses. Host-finding experiments revealed that the compound does not affect the capability of H. schachtii to find and penetrate host root tissue or to establish an interaction, even in media containing fluazaindolizine. The number of H. schachtii that established on the root was equal in treated and untreated plants at 24, 48 and 72 hpi. Furthermore, fluazaindolizine had no negative impact on infection decline of 73.5% at 4 dpi compared to the water control.

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Fluazaindolizine inhibits nematode development

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Stylecthrusting and the penetration rate of the nematode. Ultimately, the developmental assays uncovered that the growth of *H. schachtii* on the plant was significantly reduced or even completely blocked 2 days after inoculation even though the initiation of the syncytium was not impaired. These findings imply that fluazaindolizine, when applied at sublethal concentrations, becomes effective during the sedentary stage of the *H. schachtii* cyst nematode by blocking nematode development and thus likely interfering with the nematode’s feeding behaviour/capability or metabolism. To the best of our knowledge, we are the first to document the effect of fluazaindolizine on a PPN at this high resolution and on nematode development. As outlined above, by contrast with a recent report we did not observe an interference of fluazaindolizine on nematode invasion or any other virulence/activity parameter analyzed that likely can be explained by the differences in applied fluazaindolizine concentrations, evaluation time points, monitoring intensity and resolution. We postulate the observed impact on *H. schachtii* development to be the core function of fluazaindolizine as this effect becomes obvious at only 5 ppm fluazaindolizine.

Lahm et al. mentioned that fluazaindolizine is not considered systemic in plants by soil application. Our infection assays with transferred plants confirmed these results. Although *A. thaliana* plants that were transferred from fluazaindolizine-free medium to fluazaindolizine-containing medium were significantly less infected by *H. schachtii*, plants that were transferred from fluazaindolizine-supplemented medium to fluazaindolizine-free medium exhibited an infection rate comparable to control plants. In accordance to these findings, it was shown that the residues of fluazaindolizine in tomato were below the detection limit 7 days after treatment with a suspension concentrate containing 500 g L⁻¹ fluazaindolizine. Plants grown in *in vitro* culture are generally more sensitive to diverse treatments including plant protection agents. Therefore, we first analyzed how *A. thaliana* reacts towards different procedures of fluazaindolizine exposure in order to identify a suitable concentration range and assay protocols for the infection experiments. Plants sown and developed on fluazaindolizine-containing medium had considerably shorter roots and lower shoot and root weights. The root tips were damaged and shoots did not grow in medium containing >5 ppm of fluazaindolizine. This was not observed when plants were first grown on fluazaindolizine-free medium and the seedlings were subsequently exposed to the compound, thus revealing the necessity to transfer the plants. This phytotoxic effect of fluazaindolizine on *A. thaliana* grown in *in vitro* seems to be a major difference to field or glasshouse applications. Recent studies document no impact on tomato plants or even a positive effect on carrots when fluazaindolizine was applied before seeding.

In summary, we demonstrated that fluazaindolizine was highly effective against *H. schachtii* when the nematode was exposed long enough or permanently to very low fluazaindolizine concentrations. This most likely facilitated uptake of concentrations necessary to achieve impairment of *H. schachtii* development on the host. Our results provide valuable information for practical implementation in PPN management strategies and advance the knowledge of how fluazaindolizine impacts *H. schachtii* parasitism towards unravelling the compound’s mode-of-action.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**SUPPORTING INFORMATION**

Supporting information may be found in the online version of this article.

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