Allelopathic Effects of Endophyte-Infected Achnatherum sibiricum on Stipa grandis (Postprint)

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Date: 2017-03-08T00:00:00+00:00

Abstract

Using Achnatherum sibiricum as experimental material, indoor bioassays were conducted to investigate the allelopathic effects of extracts from A. sibiricum infected with different endophytic fungi on seed germination and seedling growth of Stipa grandis, a dominant species in the community, and gas chromatography-mass spectrometry (GC-MS) was employed to analyze the chemical constituents in the A. sibiricum extracts. The results showed that: (1) Endophytic fungal infection could alleviate the allelopathic effect of the host plant A. sibiricum on S. grandis; (2) The effect of endophytic fungal infection on the allelopathic activity of the host A. sibiricum was dependent on the fungal species; specifically, compared with endophyte-free A. sibiricum, infection with Epichloë gansuensis significantly alleviated the inhibitory effect of the host A. sibiricum on S. grandis, whereas infection with Neotyphodium sibiricum and Neotyphodium gansuensis showed a trend of alleviating the allelopathic effect of A. sibiricum on S. grandis, but the effect was not statistically significant; (3) The A. sibiricum extracts contained potential allelochemicals such as 2,4-di-tert-butylphenol, phthalic acid, dibutyl phthalate, and methyl stearate, and differences in the relative contents of these four substances may be the primary reason for the different allelopathic effects exhibited by A. sibiricum with different endophytic fungal infection statuses; (4) Validation experiments on the allelopathic effects of the four potential allelochemicals in the A. sibiricum extracts revealed that the allelopathic effect of 2,4-di-tert-butylphenol on S. grandis was generally characterized by no effect at low concentrations and inhibition at high concentrations, the allelopathic effect of dibutyl phthalate exhibited a "low-promotion, high-inhibition" trend, whereas both phthalic acid and methyl stearate showed a "dose-effect" allelopathic trend on S. grandis; that is, all four potential allelochemicals could significantly inhibit seed germination and seedling growth of S. grandis at high concentrations.

Full Text

Preamble

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DOI: 10.5846/stxb201604160704

Abstract

Using *Achnatherum sibiricum* as experimental material, we investigated the allelopathic effects of aqueous extracts from plants infected with different endophytic fungi on seed germination and seedling growth of *Stipa grandis* through laboratory bioassays, and analyzed the chemical composition of the extracts using gas chromatography-mass spectrometry (GC-MS). The results demonstrated that endophyte infection could alleviate the allelopathic effect of the host *A. sibiricum* on the dominant community species *S. grandis*, and that this effect was related to endophyte species. Specifically, compared with endophyte-free *A. sibiricum*, infection by *Epichloë gansuensis* (Eg) significantly alleviated the inhibitory effect of the host on *S. grandis*, whereas *Neotyphodium sibiricum* (Ns) and *Neotyphodium gansuensis* (Ng) showed a trend toward alleviating the allelopathic effect but did not reach statistical significance. Four potential allelochemicals were identified in the extracts: 2,4-di-tert-butylphenol, phthalic acid, dibutyl phthalate, and methyl stearate. Differences in the relative content of these four compounds may be the primary reason for the varying allelopathic effects among *A. sibiricum* plants with different endophyte infection statuses. Validation experiments on the allelopathic effects of these four potential allelochemicals revealed that 2,4-di-tert-butylphenol generally showed no effect at low concentrations but was inhibitory at high concentrations. Dibutyl phthalate exhibited a "low-concentration promotion and high-concentration inhibition" effect trend, while phthalic acid and methyl stearate both showed a dose-dependent inhibitory trend on *S. grandis*. All four potential allelochemicals significantly inhibited seed germination and seedling growth of *S. grandis* at high concentrations.

Keywords: endophyte; *Achnatherum sibiricum*; *Stipa grandis*; allelopathy

Introduction

Plant allelopathy refers to the chemical ecological phenomenon where living plants release allelochemicals into the environment through rainwater leaching, root exudation, and decomposition of plant residues, thereby affecting the growth and development of other organisms. Allelopathy is widespread in grassland ecosystems and has become a hot topic in ecological research. Allelochemicals, primarily derived from plant secondary metabolites, can have beneficial or detrimental effects on neighboring plants, influencing processes such as seed germination and seedling growth. In nature, water-soluble allelochemicals mainly enter the soil through rainwater leaching to exert allelopathic effects. Studying the effects of allelochemicals in plant extracts on seed germination and seedling growth of grassland plants can provide theoretical basis for restoration of degraded grasslands and elucidation of plant invasion mechanisms, and plays an important role in understanding plant individual and interspecific interactions and community construction.

Fungal endophytes are defined as fungi that can live most or all of their life cycle within grasses without causing obvious disease symptoms in the host plant, with Poaceae being the largest plant family hosting endophytes. The most studied genus is *Epichloë*, whose asexual forms are named *Neotyphodium*. Numerous studies have shown that endophyte infection can significantly enhance the competitive ability of host grasses, including improved shoot and root growth and greater resistance to biotic and abiotic stresses. However, the role of endophytes in the allelopathic effects of host grasses is less studied.

Achnatherum sibiricum is a perennial sparse bunchgrass that typically occurs as an accompanying species and is highly infected by fungal endophytes. It can be infected by three endophytic fungi: *Neotyphodium sibiricum* (Ns), *Neotyphodium gansuensis* (Ng), and *Epichloë gansuensis* (Eg). Early studies of *A. sibiricum*-endophyte symbionts suggested that endophyte infection could improve the growth and stress tolerance of *A. sibiricum*, allowing the host grass to adapt to stressful or even extreme environmental conditions. However, few studies have examined the effects of endophyte infection on the allelopathic performance of *A. sibiricum*.

In our study, we selected the natural grass *Stipa grandis*, a dominant species in native communities of *A. sibiricum*, as our research subject. The allelopathic effect of aqueous extracts from *A. sibiricum* with different endophyte infection levels on seed germination and seedling growth of *S. grandis* was tested in petri dishes in the laboratory. GC-MS was used to identify chemical components of *A. sibiricum* extracts.

The Inner Mongolia grassland is an important livestock production base and ecological barrier in northern China. Investigations of endophyte infection in native grasses of Inner Mongolia grasslands have found that *A. sibiricum* has the highest infection rate (86%-100%) among 11 grass species and is widely distributed in northeastern and northern China, typically as an accompanying species. *S. grandis* is a perennial dense-clump xerophytic herb and one of the most representative constructive plants in the central Asian grassland subregion. Previous studies by our research group have found that *A. sibiricum* populations from the same geographic region are typically infected with three *Epichloë* endophytes: *Neotyphodium sibiricum*, *Neotyphodium gansuensis*, and

Epichloë gansuensis. Among these, *E. sibiricum* only has an asexual form, while *E. gansuensis* has both asexual (*N. gansuensis*) and sexual (*E. gansuensis*) forms. Therefore, *A. sibiricum* is an ideal material for studying the effects of different endophyte species on host grasses.

This experiment used *A. sibiricum* as material to investigate the allelopathic effects of plants infected with different endophytes on the dominant community species *S. grandis*, and used GC-MS to analyze the chemical composition of *A. sibiricum* extracts, providing theoretical basis for predicting vegetation community dynamics and protecting grassland ecosystems in Inner Mongolia.

Materials and Methods

1. Seed Collection and Endophyte Detection

Achnatherum sibiricum seeds were collected in 2014 from the Hulunber Grassland Ecosystem National Field Scientific Observation and Research Station of the Chinese Academy of Agricultural Sciences (119.67°E, 49.10°N, altitude 629 m). The site has an average annual rainfall of 367 mm and dark chestnut soil. Endophyte detection was performed using aniline blue staining. The results showed 100% endophyte infection rate in seeds from the collection site.

For endophyte isolation and purification, 100 plump seeds from the same spike were selected and surface-sterilized by soaking in 2.5% sodium hypochlorite for 20 minutes, then rinsed 3-5 times with sterile distilled water. Seeds were then soaked in 75% ethanol for 5 minutes, rinsed again, and stored in sterile distilled water. Surface-sterilized seeds were placed on PDA medium (200 g/L potato, 15 g/L agar, 20 g/L glucose) in a sterile laminar flow hood, sealed with parafilm, and cultured in darkness at 25°C. When white hyphae emerged from seed surfaces or edges, hyphal tips were transferred to new PDA medium for single-spore purification. The *tub* sequence of each strain was used as the target sequence for BLAST searches of homologous sequences in GenBank, and phylogenetic analysis combined with morphological methods identified the following endophytes: *N. sibiricum*, *N. gansuensis*, and *E. gansuensis*. Seeds from the same spike were typically infected with the same endophyte species.

To obtain endophyte-free seeds, seeds infected with different endophytes were divided into two portions. One portion was subjected to high-temperature treatment at 60°C for 20 minutes, which can inactivate endophytes in *A. sibiricum* seeds without significantly affecting seed germination, seedling, or adult plant growth.

2. Construction of *A. sibiricum* Populations with Different Endophyte Infection Statuses

Stipa grandis, a perennial dense-clump xerophytic herb and one of the most representative constructive plants in the Inner Mongolia grassland, was provided by Professor Liang Cunzhu of Inner Mongolia University. Seeds with different endophyte infection statuses were soaked overnight and sown in plastic pots (20 cm diameter) filled with vermiculite, placed in a net room at Nankai University with natural light above 70% and temperatures of 10-30°C. After one month, uniform and healthy seedlings were selected and transplanted to 500 mL plastic pots at one seedling per pot, watered with Hoagland nutrient solution (500 mL per pot). After three months of growth, green leaves from *A. sibiricum* plants with the same endophyte infection status were harvested and mixed for later use. Each endophyte infection treatment was replicated 50 times.

3. Preparation of *A. sibiricum* Extracts

Green leaves (20 g fresh weight) from *A. sibiricum* with different endophyte infection statuses were weighed and soaked in 200 mL distilled water for 48 hours at room temperature. The extracts were filtered twice: first through quantitative filter paper, then through 0.45 m filter membranes to prepare microbial-free stock solutions with a concentration of 0.1 g/mL. The stock solution was diluted to obtain final concentrations of 0.01, 0.03, and 0.05 g/mL, which were stored refrigerated for later use.

4. Allelopathic Bioassays

The experiment used indoor bioassay methods. Petri dishes (9 cm diameter) were washed, dried, and lined with two layers of sterilized filter paper. Sterilized *S. grandis* seeds were evenly placed in dishes (30 seeds per dish), and 6 mL of *A. sibiricum* extract at one of three concentrations was added. Distilled water was used as the control (CK). Each treatment had 5 replicates. Dishes were placed in an intelligent incubator at 25°C with a 12-hour photoperiod. During the experiment, extracts were appropriately supplemented to keep filter paper moist. Germinated seeds were counted daily from day 3, with germination defined as radicle or plumule protrusion of 1-2 mm. The experiment continued until no seeds germinated for three consecutive days.

Germination rate and germination potential of *S. grandis* were calculated using standard formulas. At the end of the experiment, 10 healthy seedlings were selected from each dish, dried with absorbent paper, and measured for seedling height and root length with a ruler. Seedlings were then oven-dried at 105°C to constant weight and weighed.

5. Chemical Analysis of *A. sibiricum* Extracts

Extracts from endophyte-free *A. sibiricum* (strong allelopathic activity) and *E. gansuensis*-infected *A. sibiricum* (weak allelopathic activity) were selected for GC-MS analysis. The stock solution (100 mL) was extracted with an equal volume of petroleum ether to obtain petroleum ether extracts, which were concentrated to dryness using a rotary evaporator and dissolved in 1 mL petroleum ether.

GC-MS analysis was performed on an Agilent 7890A-5975C system with an HP-5MS quartz capillary column (30 m × 250 m × 0.25 m). The temperature program started at 70°C for 5 minutes, increased to 180°C at 15°C/min, then to 220°C at 10°C/min, and finally to 280°C at 15°C/min, held for 15 minutes. The injector temperature was 280°C, ion source temperature 230°C, electron energy 70 eV, and scan range 39-450 amu. Data analysis used Agilent software and NIST11 library, with compounds identified by matching coefficients 800 and considering chromatographic retention time. Peak area was used to represent compound content.

6. Validation Tests of Potential Allelochemicals

Based on methods by Zhou Baoli et al., validation tests were conducted on four potential allelochemicals identified in *A. sibiricum* extracts: 2,4-di-tert-butylphenol, phthalic acid, dibutyl phthalate, and methyl stearate. These compounds were first dissolved in anhydrous ethanol, then gradually diluted with distilled water to final concentrations of 0.01, 0.05, and 0.10 mmol/L (final ethanol concentration 1.5%). Ethanol at 1.5% was used as the control (CK). Different concentrations (6 mL each) were added to dried petri dishes lined with two layers of sterilized filter paper for *S. grandis* germination and seedling growth tests, using the same methods as described above.

7. Data Processing

Experimental data were processed using Excel 2010 and SPSS 21.0 software. All data are presented as mean ± standard error. Two-way ANOVA was used to analyze the effects of extract concentration, endophyte infection status, and their interactions on *S. grandis* seed germination and seedling growth. One-way ANOVA with LSD multiple comparisons was used to analyze differences among treatments. Significance level was set at = 0.05.

Results

1. Effects of *A. sibiricum* Extracts on *S. grandis* Seed Germination

The germination rate of *S. grandis* was significantly affected by *A. sibiricum* extract concentration (Table 1). All three endophyte infection statuses showed a concentration-dependent inhibitory effect, with inhibition increasing as extract concentration increased. Germination rate was also significantly affected by endophyte infection status. Specifically, at all three extract concentrations, endophyte-free *A. sibiricum* (E-) showed the strongest inhibition of *S. grandis* seed germination. Endophyte infection alleviated this inhibitory effect, with the degree of alleviation depending on endophyte species. Compared with endophyte-free plants, *E. gansuensis* infection significantly alleviated the inhibitory effect, while *N. sibiricum* and *N. gansuensis* showed a trend toward alleviation but without significant differences.

Germination potential of *S. grandis* was significantly affected by the interaction between extract concentration and endophyte infection status (Table 1). At 0.01 g/mL extract concentration, the inhibitory effect of endophyte-free *A. sibiricum* on germination potential was significantly stronger than that of infected plants. At 0.03 g/mL, the allelopathic effect of endophyte-free extracts was significantly stronger than that of *E. gansuensis*-infected extracts. The 0.05 g/mL treatment showed the strongest inhibition of germination potential, but at this concentration, endophyte infection status had no significant effect.

2. Effects of *A. sibiricum* Extracts on *S. grandis* Seedling Height and Root Length

Seedling height of *S. grandis* was significantly affected by extract concentration (Table 1). All three endophyte infection statuses showed a "low concentration no effect, high concentration inhibition" trend. Compared with the control, 0.01 g/mL extract had no significant effect on seedling height, while 0.03 and 0.05 g/mL concentrations significantly inhibited height. Endophyte infection status had no significant effect on seedling height.

Root length of *S. grandis* seedlings was also significantly affected by extract concentration (Table 1), showing a "low concentration promotion, high concentration inhibition" trend across all endophyte infection statuses. Compared with the control, 0.01 g/mL extract significantly promoted root growth, while 0.05 g/mL significantly inhibited root length. Endophyte infection status had no significant effect on root length.

3. Effects of *A. sibiricum* Extracts on *S. grandis* Shoot and Root Weight

Shoot weight of *S. grandis* seedlings was significantly affected by extract concentration (Table 1), showing a "low concentration no effect, high concentration inhibition" trend for all three endophyte infection statuses. Compared with the control, 0.05 g/mL extract significantly inhibited shoot weight, while 0.01 and 0.03 g/mL had no significant effect. Shoot weight was also significantly affected by endophyte infection status. At all three concentrations, endophyte-free *A. sibiricum* extracts showed the strongest inhibition of shoot weight. Both *N. sibiricum* and *N. gansuensis* showed a trend toward alleviating the allelopathic effect on shoot weight, but only *E. gansuensis* infection significantly alleviated the inhibitory effect compared with endophyte-free plants.

Root weight of *S. grandis* seedlings was significantly affected by extract concentration (Table 1), showing increasing inhibition with increasing concentration across all endophyte infection statuses. Root weight was also significantly affected by endophyte infection status. At all three concentrations, endophyte-free *A. sibiricum* extracts showed the strongest inhibition of root weight. Compared with endophyte-free plants, *E. gansuensis* infection significantly alleviated the allelopathic effect on root weight, while *N. sibiricum* and *N. gansuensis* showed no significant difference.

4. Chemical Composition Analysis of *A. sibiricum* Extracts

GC-MS analysis of petroleum ether extracts from endophyte-free *A. sibiricum* (strong allelopathic activity) and *E. gansuensis*-infected *A. sibiricum* (weak allelopathic activity) identified 20 compounds in the endophyte-free extract and 18 compounds in the infected extract. The main components in the endophyte-free extract were alkanes (18.47%), p-xylene (2.60%), naphthalene (2.36%), 2,3-dimethyldecahydronaphthalene (1.89%), tetradecane (1.27%), and 2,4-di-tert-butylphenol (0.65%). The infected extract mainly contained alkanes (18.39%), tetradecane (1.94%), octacosane (1.74%), and other compounds.

Both extracts contained 2,4-di-tert-butylphenol, phthalic acid, dibutyl phthalate, and methyl stearate, but the content of these four compounds was higher in the endophyte-free extract than in the *E. gansuensis*-infected extract (Table 2). This difference in content may explain the significant difference in allelopathic activity between the two extracts.

5. Validation Tests of Potential Allelochemicals

Compared with the control, 0.01 mmol/L 2,4-di-tert-butylphenol had no significant effect on any measured parameters. At 0.05 mmol/L, it significantly promoted seedling height but inhibited germination rate, germination potential, and root dry weight. At 0.10 mmol/L, it significantly inhibited germination rate, germination potential, seedling height, and root length.

Phthalic acid showed a clear dose-dependent inhibitory effect. At 0.01 mmol/L, it significantly inhibited germination rate, germination potential, and root dry weight. At 0.05 mmol/L, the inhibition of germination rate and potential reached extremely significant levels. At 0.10 mmol/L, it significantly inhibited germination rate, seedling height, and shoot dry weight.

Dibutyl phthalate exhibited a "low-concentration promotion, high-concentration inhibition" effect. At 0.01 mmol/L, it significantly promoted germination rate, seedling height, and shoot dry weight. At 0.05 mmol/L, it significantly inhibited seedling height but had no significant effect on other parameters. At 0.10 mmol/L, it significantly inhibited germination rate, seedling height, and shoot dry weight.

Similar to phthalic acid, methyl stearate showed a dose-dependent inhibitory trend. At 0.01 mmol/L, it significantly inhibited germination rate, germination potential, and seedling height. At 0.05 mmol/L, it showed stronger inhibition, significantly affecting germination rate, seedling height, and shoot dry weight. At 0.10 mmol/L, it significantly inhibited germination rate, seedling height, shoot dry weight, and root length.

Discussion

Current research on the effects of endophyte infection on host plant allelopathic potential has been limited to cultivated grasses such as tall fescue and perennial ryegrass, with inconsistent conclusions. Some studies found that endophyte-infected perennial ryegrass had stronger allelopathic effects on white clover than endophyte-free plants, while others reported that endophyte infection in tall fescue did not significantly affect its inhibition of native grassland plants. Our study using the accompanying species *A. sibiricum* and dominant species *S. grandis* found that endophyte-free *A. sibiricum* showed the strongest allelopathic effect, and endophyte infection alleviated the allelopathic effect on the dominant community species *S. grandis*. These results suggest that endophyte infection does not enhance the interference competition ability of the host plant, and provide experimental evidence for the long-term coexistence of endophyte-infected *A. sibiricum* and *S. grandis* in natural habitats.

Previous studies by our group found that endophyte infection has positive effects on host *A. sibiricum* growth and resistance. Endophyte infection significantly increased green leaf biomass and aboveground total biomass accumulation, promoted host growth and development, and enhanced resistance to insects, pathogens, and abiotic stresses. For *A. sibiricum*, endophyte infection appears to enhance competitiveness by promoting host growth and stress resistance.

Unlike cultivated grasses with relatively simple endophyte communities, native grasses are typically infected with multiple endophyte species. Research on endophyte species effects has focused mainly on plant growth and resistance. Some studies found significant differences in biomass effects between different endophyte species, while others found no significant differences in nutrient growth effects. Our study demonstrates that different endophyte species in *A. sibiricum* cause significantly different allelopathic effects on *S. grandis* seed germination and seedling growth. Compared with endophyte-free plants, *E. gansuensis* infection significantly alleviated the inhibitory effect, while *N. sibiricum* and *N. gansuensis* showed a non-significant alleviating trend.

Plant allelochemicals are often present in trace amounts, making isolation and identification challenging. Modern chemical identification techniques provide powerful support, with GC-MS being commonly used. Our GC-MS analysis identified four potential allelochemicals in *A. sibiricum* extracts: 2,4-di-tert-butylphenol, phthalic acid, dibutyl phthalate, and methyl stearate. All four have been reported as allelochemicals in other plants. The content of these compounds was lower in *E. gansuensis*-infected plants than in endophyte-free plants, which may explain the difference in allelopathic activity.

Validation experiments confirmed that all four compounds significantly inhibited *S. grandis* seed germination and seedling growth at high concentrations, with different concentration-response patterns. However, GC-MS cannot analyze thermally unstable or large molecular weight metabolites, so other allelochemicals may exist in *A. sibiricum*. Further research should investigate other potential allelochemicals and their mechanisms of action.

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Note: Figure translations are in progress. See original paper for figures.

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