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Original article

Asparagus decline: Autotoxicity and autotoxic compounds in asparagus rhizomes

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ABSTRACT

Asparagus (*Asparagus officinalis* L.) is a perennial vegetable, but its crop productivity and quality decrease gradually. One possible reason for "asparagus decline" is thought to be the autotoxicity of asparagus. However, the autotoxic property of asparagus rhizomes remains unknown. The objective of this study was to determine the potential role of rhizomes in the autotoxicity of asparagus. An aqueous methanol extract of asparagus rhizomes inhibited the growth of asparagus seedlings and six other test plants in a concentration-dependent manners: garden cress (*Lepidum sativum* L.), lettuce (*Lactuca sativa* L.), alfalfa (*Medicago sativa* L.), ryegrass (*Lolium multiflorum* Lam.), timothy (*Phleum pratense* L.) and barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.). These results suggest that asparagus rhizomes contain autotoxic compounds. The extract was purified through several chromatographic steps with monitoring the autotoxic activity, and *p*-coumaric acid and iso-agatharesinol were isolated. These compounds inhibited the shoot and root growth of asparagus and two other test plants, garden cress and ryegrass, at concentrations higher than 0.1 mM. The concentrations required for 50% inhibition of the root and shoot growth of these test plants ranged from 0.36 to 0.85 mM and 0.41–1.22 mM for *p*-coumaric acid and iso-agatharesinol, respectively. Therefore, these compounds may contribute to the autotoxicity caused by asparagus rhizomes and may be involved in "asparagus decline".

1. Introduction

Asparagus (*Asparagus officinalis* L.) is a perennial vegetable that is harvested over several years. One major problem of the asparagus cultivation is the "asparagus decline". Crop production and quality of asparagus decrease gradually year by year (Schofield, 1991; Matsubara et al., 2010; Yeasmin et al., 2014). One of the possible reasons for "asparagus decline" is thought to be the infection by soil-borne pathogenic fungi, mainly *Fusarium* spp. (Blok and Bollen, 1996; Yergeau et al., 2006; Asaduzzaman et al., 2013). The autotoxicity of asparagus can also be a reason for "asparagus decline" (Hartung et al., 1990; Yeasmin et al., 2013). Some autotoxic compounds released by asparagus possibly inhibit the growth of asparagus itself (Rice, 1984).

The growth of asparagus is inhibited by asparagus root residues incorporated into the asparagus cultivation soils (Hartung and Stephens, 1983; Young and Chou, 1985; Blok and Bollen, 1993). This finding suggests that asparagus roots may contain some autotoxic compounds. Several phenolic acids have been isolated from the root extracts as possible autotoxic compounds of asparagus (Young and Chou, 1985; Young, 1986; Hartung et al., 1989). Ferulic acid, isoferulic acid, malic acid, citric acid, fumaric acid and methylenedioxycinnamic acid have also been isolated from asparagus roots as potential autotoxic compounds. However, only ferulic and methylenedioxycinnamic acids inhibited the growth of asparagus seedlings (Hartung et al., 1990) and may act as asparagus autotoxic compounds.

Asparagus has large rhizomes, which may be a possible source of autotoxic compounds. However, there is a lack of information available about autotoxic compounds in asparagus rhizomes. Therefore, the objective of this study was to determine the potential role of rhizomes in the autotoxicity of asparagus. The growth inhibitory activity of the rhizomes against asparagus and other six test plants was determined and two autotoxic compounds were isolated from the rhizomes.

Abbreviations: IC_{50}, the concentrations required for 50% growth inhibition.

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2. Materials and methods

2.1. Plant materials

Rhizomes of asparagus (*Asparagus officinalis* L. cv. Welcome) were collected from a 5-year-old asparagus grown in field soil under a plastic house. The plastic house was opened as required, to keep the temperature at 20–30 °C, and the plants were irrigated six times per month at 9 t/ha for each irrigation. Nitrogen, phosphate and potassium were applied at 63, 51 and 38 kg/ha/year, respectively.

Seeds of asparagus (cv. Welcome) were used for bioassay to determine the autotoxic activity of asparagus. Dicotyledonous plant seeds of garden cress (*Lepidum sativum* L.), lettuce (*Lactuca sativa* L.) and alfalfa (*Medicago sativa* L.), and monocotyledonous plant seeds of ryegrass (*Lolium multiflorum* Lam.), timothy (*Phleum pratense* L.) and barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) were also chosen as test plants. Garden cress, lettuce and alfalfa are often used as a test plants in allelopathic research and ryegrass, timothy and barnyardgrass were selected as weed plants.

2.2. Extraction and bioassay

Fresh asparagus rhizomes (100 g dry weight equivalent) were cut into small pieces and extracted with 500 mL of 70% (v/v) aqueous methanol for two days. After filtration using filter paper (No. 2; Toyo, Tokyo, Japan), the residue was extracted again with 500 mL of methanol for two days and filtered, and both filtrates were combined.

The extracts obtained from 0.3, 1, 3, 10, 30 and 100 mg of the asparagus rhizome tissue were evaporated to dryness at 40 °C in vacuo, dissolved in 0.3 mL of methanol, and added to a sheet of filter paper (No. 2) in a 3-cm Petri dish. Methanol was evaporated in a fume hood. The filter paper in the Petri dishes was then moistened with 1 mL of a 0.05% (v/v) aqueous solution of Tween 20 (polyoxyethylene sorbitan monolaurate), which was used as a surfactant and did not have any toxic effects. Ten seeds of asparagus were placed on the Petri dishes after the incubation at 25 °C with an 18-h photoperiod for 120 h to break seed dormancy. The lengths of the roots and shoots of the asparagus seedlings were measured after 48 h of incubation in darkness at 25 °C. The percentage length of the seedlings was determined by referring to the length of control seedlings. For the control treatment, methanol (0.3 mL) was added to the filter paper in the Petri dish and evaporated as described above. Control seeds were then placed on the filter paper moistened with an aqueous solution of Tween 20. The bioassay was repeated separately four times using a completely randomized design with 10 plants for each determination (n = 40). The concentrations required for 50% growth inhibition (IC_{50} value) of the extracts on the test plant roots and shoots were determined using a logistic regression function (sigmoid) based on the concentration response bioassay: $Y = (A_1\text{-}A_2)/\{1 + (x/x_0)^p\} + A_2$, (Y; % of control, X; concentration, A_1; initial value, A_2; final value, x_0; center; p; power).

Ten seeds each of garden cress, lettuce, alfalfa, ryegrass, timothy and barnyardgrass were placed on separate Petri dishes after germination in darkness at 25 °C for 36 h. The lengths of the roots and shoots of these seedlings were measured after 48 h of incubation in darkness at 25 °C as described above. The bioassay was also repeated separately four times using a completely randomized design with 10 plants for each determination.

2.3. Separation of an asparagus rhizome extract

Asparagus rhizomes (1000 g dry weight) were extracted as described above, and the extract was concentrated at 40 °C in vacuo to produce an aqueous residue. The aqueous residue was then adjusted to pH 7.0 with 1 M phosphate buffer and partitioned three times against an equal volume of ethyl acetate. The ethyl acetate fraction had greater inhibitory activity than the aqueous fraction. Thus, the ethyl acetate phase was evaporated to dryness and separated on a column of silica gel (100 g, silica gel 60, 70–230 mesh; Merck), eluted with 20, 30, 40, 50, 60, 70 and 80% ethyl acetate in *n*-hexane (v/v; 100 mL per step), ethyl acetate (100 mL) and methanol (200 mL). The biological activity of all the fractions was determined using an asparagus bioassay as described above. Two active fractions were obtained by elution with 50 (fraction 4) and 80% (fraction 7) ethyl acetate in *n*-hexane.

2.4. Purification of an active compound in fraction 4 obtained from a silica gel column

Active fraction 4 obtained from a silica gel column was evaporated and the residue was then purified using a column of Sephadex LH-20 (100 g; Amersham Pharmacia Biotech, Buckinghamshire, UK), and eluted with 20, 40, 60 and 80% (v/v) aqueous methanol and methanol (200 mL per step). The active fraction was eluted with 20% aqueous methanol and evaporated to dryness. The residue was dissolved in 20% (v/v) aqueous methanol (2 mL) and loaded onto reverse-phase C_{18} cartridges (YMC Ltd., Kyoto, Japan). The cartridge was eluted with 20, 40, 60 and 80% (v/v) aqueous methanol, and methanol (30 mL per step). The active fraction was eluted with 20% aqueous methanol and evaporated to dryness. The residue was finally purified using reverse-phase HPLC (SPC-10A, Shimadzu, Kyoto; column; ODS AQ-325; 10 mm i.d. x 50 cm, YMC Ltd.) eluted at a flow rate of 1.5 mL min^{-1} with 35% aqueous methanol and detected at 220 nm. Inhibitory activity was found in a peak fraction eluted in 70–71 min, yielding active compound **1** as a colorless oil. The compound was characterized by high-resolution ESI mass, H NMR spectra (400 MHz, TMS as internal standard).

2.5. Purification of an active compound in fraction 7 obtained from a silica gel column

Active fraction 7 obtained from a silica gel column was evaporated and the residue was then purified using a column of Sephadex LH-20 as described above. The active fraction was eluted with 80% aqueous methanol and evaporated to dryness. The residue was dissolved and loaded onto reverse-phase C_{18} cartridges as described above. The active fraction was eluted with 80% aqueous methanol. The residue was finally purified using reverse-phase HPLC eluted at a flow rate of 1.5 mL min^{-1} with 45% aqueous methanol. Inhibitory activity was found in a peak fraction eluted in 41–42 min, yielding active compound **2** as a colorless oil. The compound was characterized by high-resolution ESI mass, H NMR spectra (400 MHz, TMS as internal standard) and ^{13}C NMR spectra (100 MHz, TMS as internal standard).

2.6. Bioassay of the isolated compounds

The isolated compounds (final assay concentration was 0.03, 0.01, 0.3, 1 and 3 mM) were separately dissolved in 0.2 mL of methanol and added to a sheet of filter paper (No. 2) in a 3-cm Petri dish. The biological activity was examined with bioassays of asparagus, garden cress and ryegrass as described above. The bioassays were repeated separately five times using a completely randomized design with 10 plants for each determination.

2.7. Statistical analysis

The bioassays were repeated 3–5 times using a completely randomized design with 10 plants for each determination. Significant