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Involvement of an autotoxic compound in asparagus decline

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ABSTRACT

Asparagus (*Asparagus officinalis* L.) is a widely cultivated perennial veritable and can be harvested more than ten years. However, the crop quality and yield decline after a few year's cultivation, which is called "asparagus decline". Even though those asparagus plants were replaced with new young asparagus plants, the productivity and quality of the crop remain relatively low, which is known as a "asparagus replant problem". One of the possible reasons for "asparagus decline" and "asparagus replant problem" is thought to be autotoxicity of asparagus. However, the compounds involved in the autotoxicity is not clear. The objective of this study was therefore to determine the potential role of autotoxicity in the "asparagus decline" and "asparagus replant problem". An aqueous methanol extract of 10-year-asparagus-cultivated soils inhibited the growth of asparagus seedlings and other two test plants with concentration dependent manner. The result confirmed that the asparagus soils have autotoxic activity. The extract was then purified by several chromatographies with monitoring the inhibitory activity and a potent growth inhibitory substance causing the autotoxic effect was isolated. The chemical structures of the compound was determined by spectral data to be *trans*-cinnamic acid. *trans*-Cinnamic acid inhibited the growth of asparagus seedlings at concentrations greater than 10 µM. The concentrations required for 50% growth inhibition of asparagus (IC_{50}) were 24.1–41.6 µM. *trans*-Cinnamic acid accumulated 174 µM in the 10-year-asparagus-cultivated soils, which may be enough levels to cause the growth inhibition on asparagus considering its IC_{50} value. Therefore, *trans*-cinnamic acid may contribute to the autotoxic effect of asparagus soils, and may be in part responsible for "asparagus decline" and "asparagus replant problem".

1. Introduction

Asparagus (*Asparagus officinalis* L.), belonging to Asparagaceae family, is a widely cultivated perennial veritable with a low input and high market value. It can be harvested more than ten years. However, the crop quality and yield decline after a few years, which is called "asparagus decline" (Schofield, 1991; Matsubara et al., 2010; Yeasmin et al., 2014). Even though those old asparagus plants were replaced with new young asparagus plants on the same crop fields, the productivity and quality of the crop remain relatively low, which is known as an "asparagus replant problem" of the cultivations (Blok and Bollen, 1996; Yergeau et al., 2006; Asaduzzaman et al., 2013).

The "asparagus decline" and "asparagus replant problem" are thought to be caused by a combination of biotic and abiotic stresses. The pathogen infection of *Fusarium* species to asparagus was reported to be the main biotic stress (Yergeau et al., 2006; Elmer and Pignatello, 2011; Molinero-Ruiz et al., 2011). However, several fungicide treatments on asparagus fields did not recover significantly the quality and productivity of asparagus (Lacy, 1979).

Autotoxicity of asparagus was reported to be the main biotic stress. Autotoxicity is a particular type of allelopathy and caused by some compounds released from asparagus plants (Rice, 1984; Hartung et al., 1990; Yeasmin et al., 2013). In fact, "asparagus decline" was recovered by the incorporation of charcoal, which absorbs wide range of chemicals, into asparagus soils (Motoki et al., 2008; Elmer and Pignatello, 2011). Root exudates from asparagus had autotoxic activity, which inhibited the growth of asparagus (Young, 1986; Benson, 2002; Watanabe et al., 2011). Those findings suggests that asparagus secrets some autotoxic compounds from the roots into the medium. Those compounds may accumulate in the soils of asparagus fields and be involved in the autotoxicity of asparagus.

The extracts of asparagus roots and rhizomes inhibited the growth

Abbreviations: IC_{50}, the concentrations required for 50% growth inhibition; 4CL, 4-coumarate-CoA ligase; MDCA, phenylpropanoid 3,4-(methylenedioxy)cinnamic acid

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of asparagus and several phenolic acids were isolated from the root extracts (Young and Chou, 1985; Hazebroek et al., 1989; Lake et al., 1993). Some phenolic acids were isolated from asparagus soils (Young, 1986). However, the effects of those phenolic compounds on the growth of asparagus had not been reported. Ferulic, isoferulic, malic, citric and fumaric acids, and methylenedioxycinnamic acid were isolated from asparagus roots (Hartung et al., 1990), but the concentrations of those compounds in asparagus soils had not been reported. Therefore, it is impossible to evaluate if those compounds are involved in the autotoxicity of asparagus. Three growth inhibitory substances, asparagusic acids, dihydroaspragusic acid and (*S*)-acetyldihydroaspragusic acid were isolate from asparagus ferns (Kitahara et al., 1972) and two growth inhibitory substances, *p*-coumaric acid and iso-agatharesinol were isolated from asparagus rhizomes (Kato-Noguchi et al., 2017). However, those compounds has not been reported to be in soils of asparagus fields. Recently, three compounds, oxalic, succinic and tartaric acids were isolated form agar medium of asparagus growth (Yeasmin et al., 2013), but the effects of those compounds on the growth of asparagus were not determined.

As described above, the autotoxicity of asparagus may be one of the reasons for “asparagus decline” and “asparagus replant problem”. However, the compounds involved in the autotoxicity is not clear. The objective of this study was therefore to determine the potential role of autotoxicicity in the “asparagus decline” and “asparagus replant problem”. Thus, autotoxic activity of asparagus soils was determined and an autotoxic compound was isolated and characterized. The growth inhibitory activities of the compound on asparagus and concentrations of the compound in asparagus cultivated soil were also determined.

2. Materials and methods

2.1. Soil samples and plant materials

Asparagus soils were obtained from the soil under 0–50 cm in depth where asparagus (*Asparagus officinalis* L. cv. Welcome) was cultivated for 10 years in a greenhouse condition. The soil type was sandy loam (pH 6.1) and its soil moisture was 29.7 ± 9% (w/w; mean ± SE). The asparagus soil samples were then mixed thoroughly, air-dried and sieved (2 mm mesh) to remove plant residues and stones. Non-asparagus-cultivated soil was collected from the soil beside the greenhouse of asparagus, where no asparagus was grown over 10 years. Non-asparagus-cultivated soil samples were processed as same as asparagus soil. Ten-year-old asparagus plants were also harvested and stored at 80 °C until extraction.

Seeds of asparagus (cv. Welcome) were chosen as a test plant to monitor autotoxicity of asparagus. The seeds of a dicotyledonous plant; garden cress (*Lepidum sativum* L.) and a monocotyledonous plant; *Lolium multiflorum* Lam were also chosen as test plants.

2.2. Extraction and bioassay

Asparagus soil and non-asparagus-cultivated soil samples (each 500 g dry weight) were separately extracted with 1 L of 70% aqueous methanol for two days and filtered with filter paper (No. 2; Toyo, Tokyo, Japan). An aliquot of the extracts (final assay concentration was 10, 30, 100, 300, and 1000 mg dry weight of soil equivalent extract per mL) was evaporated to dryness, dissolved in a 0.3 mL methanol and added to a sheet of filter paper (No. 2) in a 3-cm Petri dish. Methanol was evaporated in a fume hood. Then, the filter paper in the Petri dishes was moistened with 0.8 mL of a 0.05% (v/v) aqueous solution of Tween 20 (polyoxyethylene sorbitan monolaurate), which was used as a surfactant and did not cause any effects.

For asparagus bioassay, after the incubation at 25 °C with a 18-h photoperiod for 120 h for breaking seed dormancy, 10 asparagus seeds were placed on the Petri dishes and incubated for 48 h in darkness at 25 °C. The length of roots and shoots of asparagus seedlings were then measured and the percentage length of the seedlings was determined by reference to the length of control seedlings. Controls for the bioassay were treated exactly as described above, with the exception that 0.3 mL methanol was used instead of the extracts. The bioassay was repeated four times using a randomized design with 10 plants for each determination (n = 40).

For garden cress and *L. multiflorum* bioassay, 10 seeds of those plants were placed on the Petri dishes after germination in darkness at 25 °C for 36 h. The length of roots and shoots of these seedlings were measured after 48 h of incubation in darkness at 25 °C as described above.

2.3. Purification of an active substance in asparagus soil

Asparagus soil samples (5 kg dry weight) were extracted with 10 L of 70% aqueous methanol as described above and the extract was concentrated at 40 °C in vacuo to produce an aqueous residue. The aqueous residue was 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 was evaporated to dryness after drying over anhydrous Na_2SO_4.

The ethyl acetate fraction was then chromatographed 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 separated fractions was determined using an asparagus bioassay as described above. The activity was found in fraction obtained by elution with methanol. After evaporation of the active fraction, the residue was further purified by a column (2.5 cm id × 21 cm) 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 by 80% aqueous methanol and evaporated to dryness. The residue of the active fraction 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 by 80% aqueous methanol and evaporated to dryness. The residue was finally purified by reverse-phase HPLC (10 mm i.d. × 50 cm, ODS AQ-325; 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 between 42 and 43 min, yielding an active compound **1** as a colorless oil. The compound was characterized by high-resolution ESI mass and H NMR spectra (400 MHz, tetramethylsilane as internal standard).

2.4. Bioassay of the isolated compound

The active compound **1** was dissolved in 0.2 mL of methanol, added to a sheet of filter paper (No. 2) in a 3-cm Petri dish, and the methanol was evaporated in a fume hood. The filter paper in the Petri dish was moistened with 0.8 mL of 0.05% (v/v) aqueous solution of Tween 20. Inhibitory activity of compound **1** was determined by asparagus, garden cress and *L. multiflorum* as described above. The bioassay was repeated five times using a randomized design with 10 plants for each determination (n = 50).

2.5. Concentration of the isolated compound in soils and asparagus plants

Asparagus soil samples (100 g dry weight) were extracted and the