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Letter

Identification of lotus cultivar-specific rhizome compounds and evaluation of their growth inhibitory activity against *Fusarium commune*

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ABSTRACT — Lotus (*Nelumbo nucifera*) is an aquatic vegetable cultivated in Asia. Lotus rhizome rot is a sever disease that reduces productivity. We extracted and analyzed the compounds contained in the seed rhizomes of 'Bicchu' and 'Lotus,' cultivars that differ in resistance to rhizome rot. Interestingly, triglycerides were identified only in 'Lotus,' which exhibits a stronger disease resistance than 'Bicchu.' However, trilinolein, one of the major triglycerides in rhizomes, did not inhibit the growth of *Fusarium commune* isolated from rhizomes. Therefore, the differences in the rhizome lipid contents were unrelated to their resistance to rhizome rot. Although in this study we analyzed the major compounds in uninfected rhizomes, certain minor anti-fungal phytochemicals in disease-resistant rhizomes might be induced post-infection with pathogenic fungi. This cultivar comparison approach would be useful for further comprehensive studies on these phytochemicals.

Key words: *Fusarium*, Rhizome rot, *Nelumbo nucifera*, Lotus, Growth inhibition

INTRODUCTION

Nelumbo nucifera Gaerth., commonly known as lotus, is a rhizomatous, aquatic, and vegetatively propagated plant belonging to Nelumbonaceae. It is widely distributed throughout Asia, and used for its ornamental flowers, as a medicinal herb, and as a vegetable (Chen *et al*., 2019). In Japan, edible lotus has been cultivated and consumed for more than 1000 years. Lotus rhizome rot is one of the most serious diseases reducing productivity. The usual symptoms of diseased lotuses are discolored rhizomes and wilted leaves. It is mainly caused by infection with *Fusarium* spp such as *F. commune* and *F. oxysporum* (Deng *et al*., 2022; Watanabe *et al*., 2023). Thus, it is vital to suppress the growth and infection by *Fusarium*, but neither effective pesticides nor prevention methods have been developed.

Although breeding of edible lotus is difficult due to the requirements of long periods and large fields for flowering, dozens of cultivars have been bred to improve harvest yield and disease resistance. 'Bicchu' is one of the traditional cultivars that was imported to Japan from China more than a hundred years ago (Fig. 1). It is favored for its rich taste and large rhizome size but has a slow growth phenotype and vulnerability to lotus rhizome rot. On the other hand, 'Lotus White' (abbreviated as 'Lotus') grows faster than 'Bicchu' and shows an enhanced resistance against rhizome rot (Sawada, 2013; Shinohara *et al*., 2016). Therefore, 'Lotus' has been favorably cultivated especially in Tokushima and Aichi prefectures of Japan.

Considering the differences in disease resistance among lotus cultivars, we hypothesized that the rhizomes of resistant cultivars may contain natural prod-

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Fig. 1. The rhizome and flower of 'Bicchu' and 'Lotus'.

ucts with anti-fungal properties. Various phytochemicals such as flavonoids, alkaloids, and lipids have been identified from each part of *N. nucifera* using chromatographic purification or LC-MS-based metabolomics (Paudel and Panth, 2015; Zhao *et al*., 2023). Some of them exhibited beneficial biological activities such as anti-inflammatory, anti-oxidant, and anti-tumor effects. These compounds account for the medicinal effectiveness of lotus. However, although several phenolic compounds were identified in a disease-resistant cultivar (Shimomura *et al*., 1955), anti-fungal natural products responsible for resistance against rhizome rot have not been reported. In this study, we tried to identify natural products specifically contained in the disease-resistant cultivar to search for anti-fungal compounds against *F. commune*.

MATERIALS AND METHODS

General remarks

NMR spectra were recorded in chloroform-*d* on a JNM-ECZ500 (JEOL, Tokyo, Japan). High-resolution electrospray ionization mass spectra (HR-ESI-MS) were recorded on an HCT (Brucker Daltonics, Billerica, MA, USA). Silica gel 60 F_{254} (Merck, Darmstadt, Germany) was used for thin-layer column chromatography. Other chemicals were purchased from the Tokyo Chemical Industry (Tokyo, Japan). *F. commune* was isolated from infected lotus rhizome and maintained on potato dextrose agar (PDA) medium.

Extraction and isolation

Dry seed rhizomes of 'Lotus' (9.4 g) were extracted with methanol (150 mL) at room temperature. The methanol extract (0.48 g) was partitioned between ethyl acetate (EtOAc) and H_2O to obtain an EtOAc extract (22.9 mg). This extract (15.9 mg) was subjected to thin-layer silica gel column chromatography (20% diethyl ether (Et_2O) in *n*-hexane) to obtain a mixture of three triglycerides (2.7 mg). HR-ESI-MS: *m/z* 853.7235 $[M + Na]^+$ (calcd for $C_{53}H_{98}O_6Na$, 853.7261), *m/z* 877.7229 $[M + Na]^+$ (calcd for $C_{55}H_{98}O_6Na$, 877.7261), *m/z* 901.7231 $[M + Na]^+$ (calcd for $C_{57}H_{98}O_6Na$, 901.7261); ^1H NMR, ^{13}C NMR, and MS/MS spectra: see Supplementary material.

Dry seed rhizomes of 'Bicchu' (71.8 g) were extracted with methanol (150 mL) at room temperature. The methanol extract (2.4 g) was partitioned between EtOAc and H_2O to obtain an EtOAc extract (65.4 mg). This extract (47.8 mg) was subjected to thin-layer silica gel column chromatography (20% Et_2O in *n*-hexane) to obtain a mixture of fatty acid methyl esters that mainly contained methyl linoleate (3.0 mg). HR-ESI-MS: *m/z* 317.2441 $[M + Na]^+$ (calcd for $C_{19}H_{34}O_2Na$, 317.2457); ^1H and ^{13}C

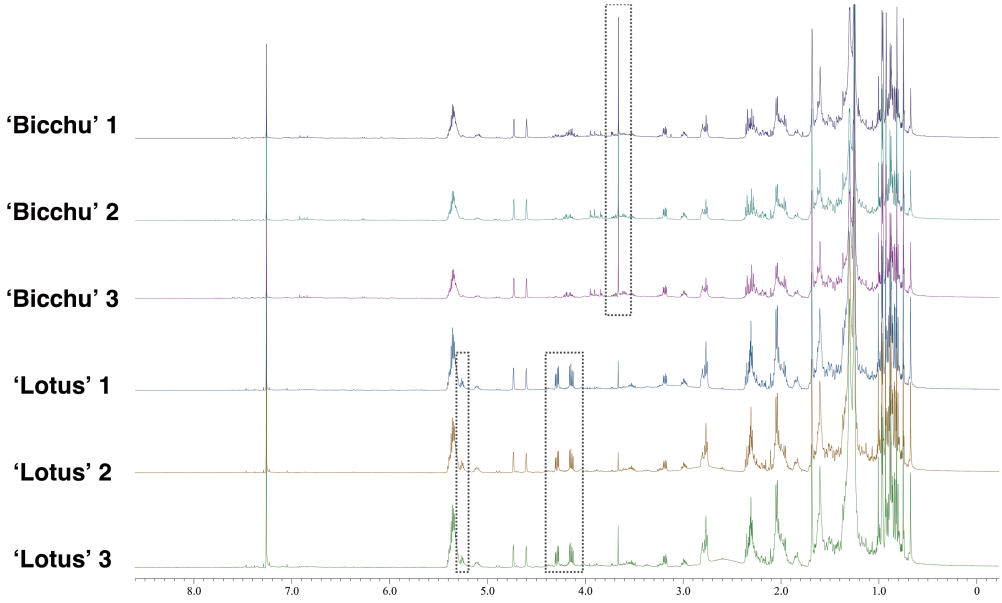

Fig. 2. ^{1}H NMR spectra of extract of 'Bicchu' and 'Lotus' rhizomes.

NMR spectra: see Supplementary material.

Growth inhibition test against *F. commune*

F. commune cells grown in PDA medium were added 0.1% Tween 80 and 0.85% aqueous NaCl solution to obtain the fungal suspension. It was diluted to an OD_{600} of 0.1 in 0.85% aqueous NaCl and further diluted 50-fold with RPMI1640 medium (186-02115: Fujifilm Wako, Osaka, Japan) with resazurin. A 100 µL sample in RPMI 1640 medium was added to100 µL of the fungal suspension and the cultures in 96 well plates were incubated at 27°C for 45 hr. The absorbance at 570 nm was measured using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA).

RESULTS

'Bicchu' and 'Lotus' seed rhizomes, three pieces of each, were extracted with methanol, and each extract was partitioned between EtOAc and H_2O to obtain the phytochemicals and remove the water-soluble nutrients such as sugars and amino acids. The EtOAc layer was concentrated *in vacuo* and subjected to ^{1}H NMR analysis. The extracts gave specific peaks (Fig. 2). The 'Lotus' extract showed double of doublet peaks (4.29 and 4.14 ppm) and a multiplet peak (5.26 ppm), suggesting the presence of compounds that including oxygenated methylene and methine groups. Since a 'Lotus'-specific spot (spot 1,

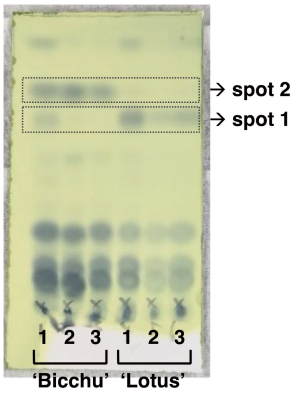

Fig. 3. Thin-layer chromatographic analysis of extract of 'Bicchu' and 'Lotus' rhizomes. The extract of each rhizome was spotted on a silica gel thin-layer chromatography plate and eluted by 20% Et_2O in hexane. The separated compounds were stained with phosphomolybdic acid.

Fig. 3) was detected in the thin layer chromatography (TLC) analysis, this compound was isolated by silica gel preparative TLC with 20% Et_2O in hexane as the eluent. NMR and MS/MS analyses revealed it to be a mixture of three triglycerides, which in the descending order of content were dilinoleyl-palmitoyl-glycerol, trilinolein, and dipalmitoyl-linoleyl-glycerol (Supplementary mate-

rials). On the other hand, the 'Bicchu' extract showed a singlet peak (3.66 ppm) in ^{1}H NMR spectrum, suggesting the presence of a methyl ester group, and showed a specific spot during TLC (spot 2, Fig. 3). This was purified by preparative TLC and identified by NMR and MS analyses to be a mixture of fatty acid methyl esters that mostly contained methyl lineolate. The 'Bicchu' rhizome ethanol extract did not contain methyl lineolate but its ethyl ester. Thus, we concluded that linoleic acid was contained in the 'Bicchu' rhizome, and it was methylated during the extraction process.

Although lotus rhizomes commonly contain fatty acids and triglycerides (Zhao *et al*., 2013), the differences in their contents among cultivars have not been the focus of interest for disease resistance. Some fatty acids were reported to inhibit the growth of some pathogenic fungi (Guimarães and Venâncio, 2022), but the anti-fungal activity of triglycerides has not been reported. Thus, we next evaluated the growth inhibitory activity of triglyceride and fatty acid against *F. commune* isolated from the rhizomes. Since linoleic acid is the most abundant fatty acid in lotus rhizomes (Zhao *et al*., 2013), we evaluated the growth suppression activity of trilinolein, linoleic acid, and methyl linoleate (Fig. 4). Trilinolein and methyl linoleate did not exhibit proliferation inhibitory activity against *F. commune*., while 1000 μM linoleic acid inhibited its growth (Fig. 5).

DISCUSSION

As mentioned above, triglycerides were detected in the rhizomes of the disease-resistant cultivar 'Lotus,' but not in those of 'Bicchu.' However, trilinolein did not inhibit the growth of *F. commune* at 1000 μM or less. These results suggested that triglycerides were not involved

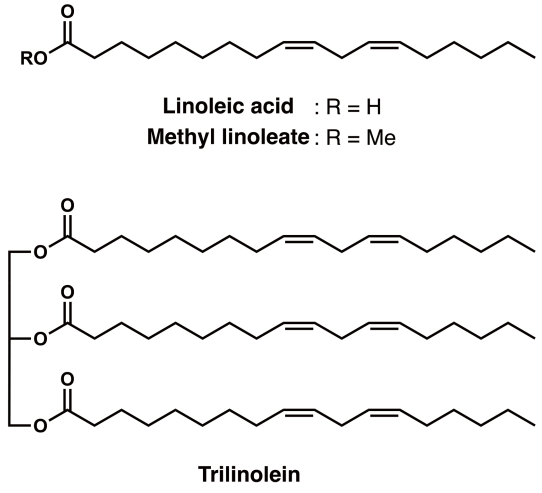

Fig. 4. Structure of linoleic acid, methyl linoleate, and trilinolein

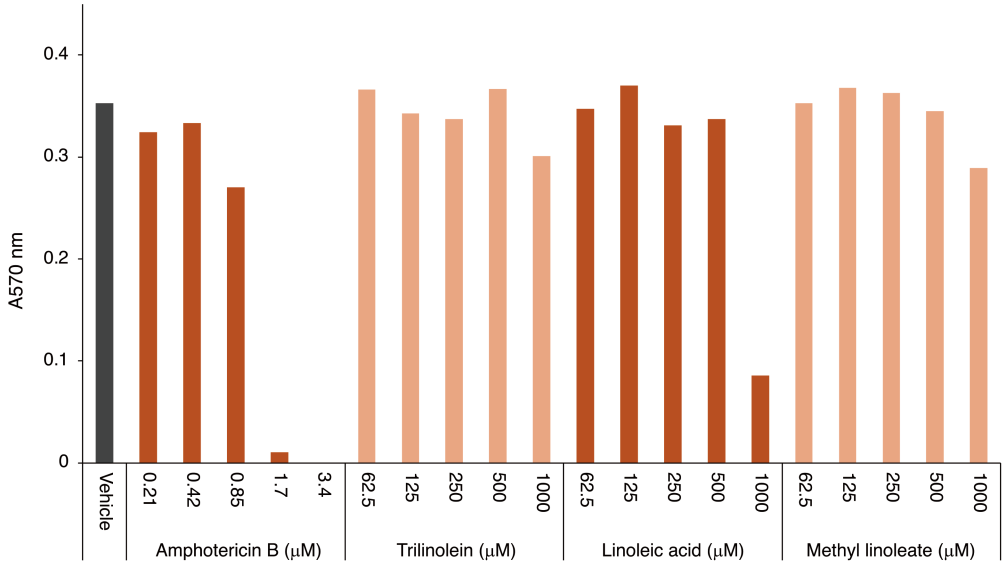

Fig. 5. Growth inhibitory activity of trilinolein, linoleic acid, and methyl linoleate against *Fusarium commune*. *Fusarium commune* cells were treated with the indicated concentration of each compound for 45 hr. Thereafter, cell number was estimated by resazurin assay, and the absorbance was measured at 570 nm. Amphotericin B was used as a positive control.

in the resistance to rhizome rot. Since the rhizomes of 'Lotus' grow faster than those of 'Bicchu,' the variation in lipid storage could affect their growth phenotype. Interestingly, methyl linoleate showed negligible growth inhibitory activity, suggesting that the carboxy group was necessary for the inhibition of *F. commune*. On the contrary, methyl linoleate exhibited a stronger suppression of *F. graminearum* than linoleic acid (Zhao *et al*., 2020). Thus, there might be species-specific growth inhibitory mechanisms.

The expression of genes involved in biosynthesis of some phytochemicals is induced by various environmental stresses (Harborne, 1999). Thus, minor anti-fungal compounds might be induced in 'Lotus' rhizomes after contact or infection with pathogenic fungi. In the next studies, we intend to analyze phytochemicals contained in 'Bicchu' and 'Lotus' rhizomes before and after exposure to *F. commune*. LC-MS or NMR metabolomics may also help to identify trace amounts of anti-fungal phytochemicals (Hanaki *et al*., 2022; Tomita *et al*., 2015). Although we could not identify anti-fungal compounds in this preliminary study, our approach focusing on cultivar-specific phytochemicals would form a basis for future further studies.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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