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Inhibitory activity of Shikuwasa peel extract against the Fusarium wilt disease caused by *Fusarium buharicum*

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Abstract: Fusarium wilt, caused by *Fusarium buharicum*, is an emerging disease of okra in Japan, first reported in 2018. It causes severe damage to seedlings. Current control strategies rely on chemical fungicides, but repeated use may reduce pathogen sensitivity. Plant secondary metabolites are promising sources of natural fungicidal compounds. In this study, we evaluated the inhibitory activity of Shikuwasa peel extracts on *F. buharicum*. High-pressure extracts of Shikuwasa peel prepared at 200 °C for 1–3 h showed a time-dependent inhibition of conidial germination. Furthermore, volatile compounds from these extracts, tested using a two-compartment Petri dish method, significantly inhibited conidial germination and showed fungicidal activity. A GC–MS analysis identified 16 volatile compounds that increased after 3 h, with eucalyptol showing the largest rise. These findings indicate that Shikuwasa peel extract could serve as a promising natural control agent against Fusarium wilt in okra.

Keywords: *Fusarium buharicum*, Fusarium wilt disease, Inhibitory activity, Shikuwasa peel extract

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Introduction

Okra (*Abelmoschus esculentus*), a member of the *Malvaceae* family, is an important vegetable crop cultivated worldwide. In Japan, okra production reached 11,100 t in 2022 (MAFF, 2022). Production is concentrated in Kagoshima, Kochi, and Okinawa Prefectures, which together account for 74% of the national output. In Okinawa, annual production reaches about 985 t, underscoring its regional significance. According to the List of Plant Diseases in Japan (Phytopathological Society of Japan, 2025), 18 diseases have been reported on okra. In Okinawa, damping-off during the seedling stage is primarily caused by *Pythium* spp. and *Rhizoctonia* spp., while *Phytophthora* spp. is associated with post-pruning decline (Hokama, 1983, 1990). More recently, Fusarium wilt caused by *Fusarium buharicum* was added to the disease list following surveys in 2015–2016 (Kotani et al., 2018). Although several fungicides were previously shown to be effective (Hokama, 1983), some are no longer registered, and excessive chemical use may lead to resistance, highlighting the need for alternative control strategies.

Plants naturally produce an estimated 200,000 to 1,000,000 secondary metabolites, many of which exhibit antimicrobial properties and serve as potential leads for novel agrochemicals (Ahn et al., 1991; Dixon & Strack, 2003; Serit et al., 1991a, 1991b; Ueno & Yoshikiyo, 2014). Shikuwasa (*Citrus depressa*), a specialty citrus fruit of Okinawa, is produced at approximately 3,270 tons annually (MAFF, 2022), with nearly 50% of the biomass discarded as waste (Okamoto & Okamoto 2025). Its peel contains bioactive compounds such as nobiletin, a polymethoxylated flavonoid (Wada et al., 2017), and aroma constituents including limonene and γ-terpinene, which exhibit antibacterial activity against *Escherichia coli* (Okamoto & Okamoto, 2025). However, antifungal activity against plant pathogens remains unexplored. Previous studies have demonstrated that extracts obtained using high-pressure reaction and decomposition systems can be utilized for plant disease control (Okido et al., 2022). This study aimed to evaluate the inhibitory and fungicidal effects of high-pressure extracts from shikuwasa peel against *F. buharicum*, the causal agent of okra Fusarium wilt.

Materials and Methods

Shikuwasa peel extract

Segments of Shikuwasa peel were cut with a sterile blade. One

gram of peel was placed on gauze suspended above 5 mL of sterile distilled water in a high-pressure reaction vessel (HU-25; SAN-AI Kagaku Co. Ltd., Aichi, Japan) to avoid direct contact with water. The vessel was sealed and heated at 200 ℃ for 1, 2, or 3 h. The resulting extract was filtered through a 0.22-μm nylon syringe filter (AS ONE Corp., Osaka, Japan) and used for subsequent assays.

Pathogen

Fusarium buharicum strain OKI-1, isolated from diseased okra roots in Okinawa Prefecture, was maintained on potato sucrose agar (PSA: 200 g/L potato, 2% sucrose, 2% agar) slants at 25 ± 2 ℃. For inoculation tests, the pathogen was cultured on PSA or rice bran agar (RBA: 50 g/L rice bran, 2% sucrose, 2% agar) for 14 days to induce sclerotia formation.

Inhibitory activity assay

Conidial suspensions (4.5–5.0 × 10^4 conidia/mL) were mixed with Shikuwasa peel extracts prepared under different conditions (200 ℃ for 1, 2, or 3 h) and placed on glass slides in a moist chamber at 25 ± 2 ℃. After 24 h, conidial germination was assessed microscopically. Sterile distilled water served as a control. Three hundred conidia per treatment were examined. Data represent means from three independent experiments, each with three replicates. Germination percentage was calculated as: Percentage of conidial germination = (number of germinated conidia /total number of conidia) × 100.

***A*ntagonistic assay of volatile compounds**

Conidia of *F. buharicum* (1.5-2.0 × 10^4 conidia/mL) suspended in phosphate buffer (pH 7.4) were placed onto glass slides and positioned on one side of a divided Petri dish. Then, 2 mL of Shikuwasa peel extract was added to the other side. As a control, 2 mL of distilled water was added instead of the peel extract. The Petri dish was sealed with parafilm and maintained in a moist chamber at 25 ± 2 ℃ for 24 h after which conidial germination was observed by light microscopy. Two hundred conidia per experiment were assessed for germination in each treatment. Data are the means of results from three independent experiments. The percentage of conidial germination was calculated using the following formula: Percentage of conidial germination = (number of conidia germinated/total number of conidia) × 100.

***In vitro* fungicidal assay of volatile compounds**

Conidia of *F. buharicum* (1.5-2.0 × 10^4 conidia/mL) suspended in phosphate buffer (pH 7.4) were placed onto glass slides and positioned on one side of a divided Petri dish. Then, 2 mL of Shikuwasa peel extract was added to the other side. As a control, 2 mL of distilled water was added instead of the peel extract. The Petri dish was sealed with parafilm and maintained in a moist chamber at 25 ± 2 ℃. After 24 h incubation, the glass slide was transferred into a plastic case containing a moist chamber without Shikuwasa extract and re-incubated at 25 ± 2 ℃. After 24 h, conidial germination was observed by light microscopy. Two hundred conidia per experiment were assessed for germination in each treatment. Data are the means of results from three independent experiments. The percentage of conidial germination was calculated using the following formula: Percentage of conidial germination = (number of conidia germinated/total number of conidia) × 100.

GC-MS analysis

Volatile compounds were analyzed using HS-SPME coupled with GC–MS. An SPME fiber (50/30 μm DVB/Carboxen/PDMS) was exposed to the headspace at 60 ℃ for 40 min, then desorbed at 250 ℃ onto a DB-Heavy WAX column (60 m × 0.25 mm, 0.25 μm). The oven program was: 40 ℃ (5 min), ramp to 135 ℃ at 4 ℃/min, then to 270 ℃ at 12 ℃/min (hold 5 min). MS was operated in scan mode (33–500 m/z). Compounds were identified using the NIST17 library.

Statistical analysis

Data are expressed as mean ± SD. Differences among treatments were analyzed using Tukey–Kramer test (SPSS Statistics v22.0; IBM, Armonk, NY, USA). Significance was set at $p < 0.05$.

Results

Inhibitory activity of Shikuwasa peel extract on conidial germination of *F. buharicum*

To evaluate the direct effect of Shikuwasa peel extract, conidial germination was examined under different extraction conditions (200 ℃ for 1, 2, and 3 h). Morphological deformation and disintegration of conidia were observed in sample treated with extracts obtained after 2 and 3 h (Fig. 1). Shikuwasa peel extract inhibited conidial germination in a time-dependent manner. In

the extract conditions at 200 ℃ 1 h, 2 h, and 3 h, conidial germination rates were 14.4 ± 21.9%, 0%, and 0%, respectively. In the control (distilled water), germination was 48.4 ± 28.7%.

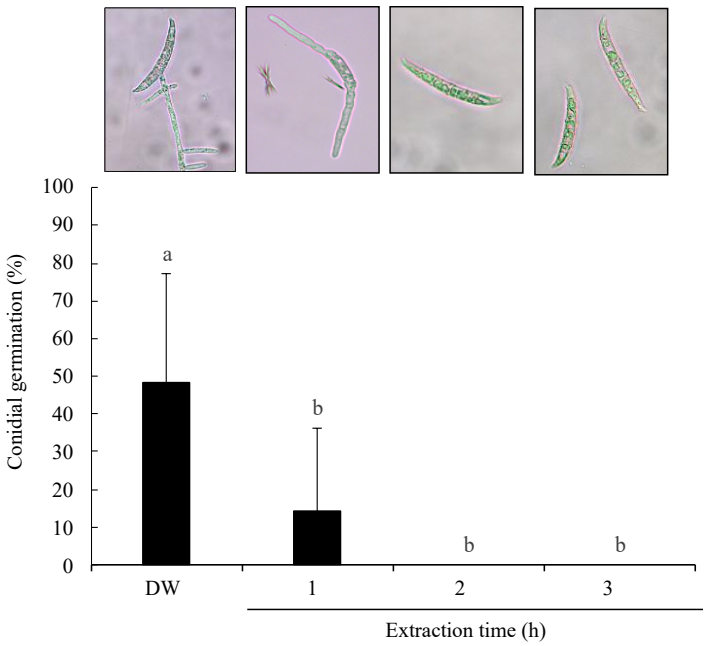

Fig. 1. The effect of Shikuwasa peel extract on conidial germination of *Fusarium buharicum*. Conidial suspensions (4.5–5.0 × 10^4 conidia/mL) were treated with Shikuwasa peel extracts prepared at 200 °C for 1, 2, or 3 h, incubated at 25 ± 2 °C for 24 h, and examined for germination by light microscopy. Distilled water served as the control. Values represent means of three independent experiments with three replicates each. Error bars indicate standard deviation. Means followed by the same letter are not significantly different (Tukey-Kramer test, $p < 0.05$).

The effect of volatile compounds from Shikuwasa peel extract on the conidial germination rate of *F. buharicum*

To evaluate the inhibitory activity of volatile compounds from Shikuwasa peel extract, conidial germination of *F. buharicum* was assessed. Germination was markedly reduced compared to the control (distilled water) (Fig. 2). Germination rates were 78.3 ± 22.1% for the control and 47.7 ± 21.6%, 0.6 ± 1.5%, and 0% for extracts obtained after 1, 2, and 3 h, respectively (Fig. 2)

Fungicidal effect of volatile compounds from Shikuwasa peel extract on the conidial germination rate of *F. buharicum*

To evaluate the fungicidal activity of volatile compounds from Shikuwasa peel extract, conidial germination of *F. buharicum* was assessed. Germination was markedly reduced compared to the control (distilled water) (Fig. 3). Germination rates were 76.1 ± 15.3% for the control and 55.7 ± 23.2%, 3.3 ± 5.4%, and 0% for extracts obtained after 1, 2, and 3 h, respectively (Fig. 3).

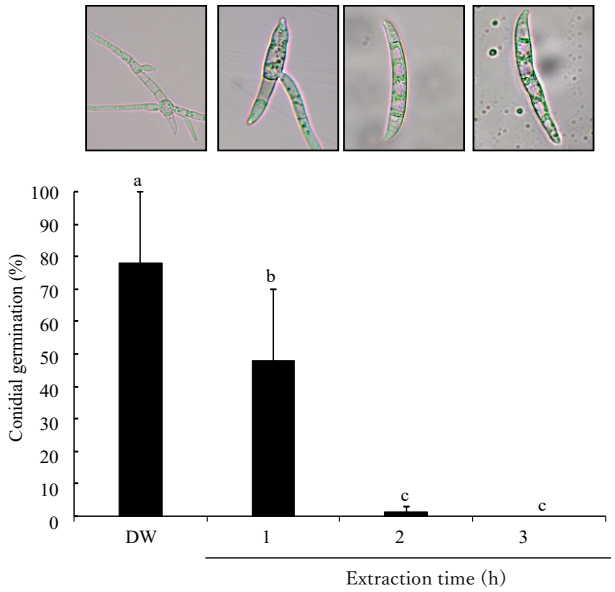

Fig. 2. The effect of volatile compounds from Shikuwasa peel extract on conidial germination of *Fusarium buharicum*. Conidial suspensions (1.5–2.0 × 10^4 conidia/mL) were placed on one side of a divided Petri dish, and 2 mL of Shikuwasa peel extract (prepared at 200 °C for 1, 2, or 3 h) was added to the opposite side. Distilled water served as the control. Plates were sealed and incubated at 25 ± 2 °C for 24 h, after which germination was assessed by light microscopy. Values represent means of three independent experiments with three replicates each. Error bars indicate standard deviation. Means followed by the same letter are not significantly different (Tukey-Kramer test, $p < 0.05$).

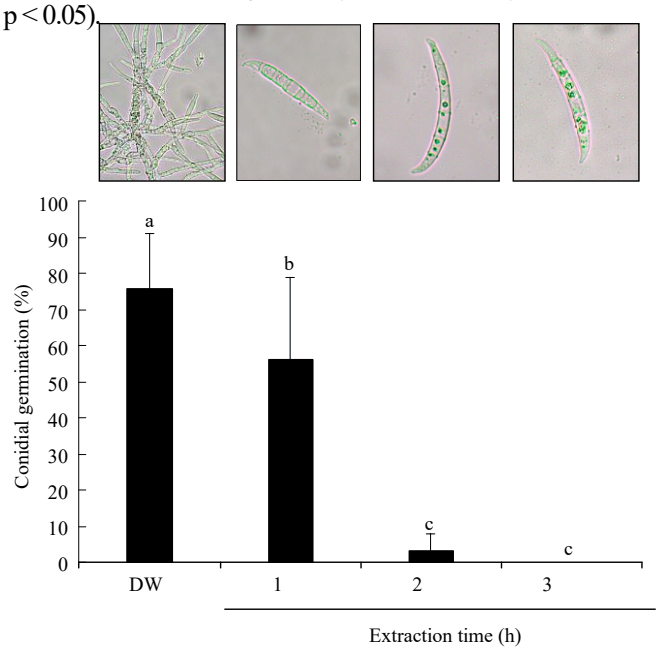

Fig. 3. Fungicidal effect of volatile compounds from Shikuwasa peel extract on conidial germination of *Fusarium buharicum*. Conidial suspensions (1.5–2.0 × 10^4 conidia/mL) were placed on one side of a divided Petri dish, with 2 mL of Shikuwasa peel extract (200 °C for 1, 2, or 3 h) on the opposite side. Distilled water served as the control. Plates were incubated at 25 ± 2 °C for 24 h, then slides were transferred to a fresh moist chamber without extract for another 24 h. Values are means of three experiments with three replicates. Error bars indicate SD. Means with the same letter are not significantly different (Tukey-Kramer test, $p < 0.05$).

Table 1. The effect of extraction time on the production of volatile compounds from Shikuwasa peel

Chemical compounds	Extraxtion time		Fold-change (vs 1h)
	1h	3h	
Eucalyptol	203086	3784998	18.6
Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl-, (1R-endo)	54557	708919	13
Ethanone, 1-(2-furanyl)	41243	500387	12.1
Phenol, 4-ethyl-2-methoxy	20634	233618	11.3
7-Oxabicyclo[2.2.1]heptane, 1-methyl-4-(1-methylethyl)	202722	1916896	9.5
2,4-Cycloheptadien-1-one, 2,6,6-trimethyl	214956	1731236	8.1
Cyclohexanol, 1-methyl-4-(1-methylethenyl)	96430	479937	5
Furfural	2673718	13000000	4.9
Isoborneol	193036	797464	4.1
Benzaldehyde, 4-(1-methylethyl)	65091	197343	3
Phenol, 2-methyl-5-(1-methylethyl)	190256	526364	2.8
Phenol, 2-methyl-5-(1-methylethyl)	76048	208232	2.7
1H-Pyrrole-2-carboxaldehyde	60706	155894	2.6
Cyclohexanol, 1-methyl-4-(1-methylethenyl)	555217	1118070	2
Phenol, 2-methyl-5-(1-methylethyl)	597342	1081581	1.8
Phenol, 3,5-bis(1,1-dimethylethyl)	633481	831617	1.3

Effect of extraction time on the production of volatile compounds from Shikuwasa peel

Volatile compounds in Shikuwasa peel extracts after 1 and 3 hours of extraction were analyzed by headspace GC-MS. Sixteen compounds detected an increase in peak area at 3 h compared to 1 h, with fold changes ranging from 1.3 to 18.6. The compound exhibiting the greatest increase was Eucalyptol, which increased by 18.6-fold (Table 1).

Discussion

The use of synthetic pesticides has long been the primary method for preventing crop losses caused by plant diseases. However, excessive use has led to resistant strains, complicating sustainable disease management (FRAC, 2018). Therefore, compounds with different modes of action are needed to develop alternative strategies. While microbial metabolites have traditionally been explored, plants also produce diverse secondary metabolites with antimicrobial properties. For example, ethanol extracts of bamboo containing 2,6-dimethoxy-1,4-benzoquinone inhibited rice blast infection (Ueno & Yoshikiyo, 2014), and high-pressure extracts from Sasa leaves suppressed rice blast disease (Okido et al., 2022).

This study evaluated the inhibitory effect of high-pressure extracts from *Citrus depressa* (Shikuwasa) peel against *F. buharicum*, the causal agent of okra wilt. Extracts obtained at 200 °C suppressed conidia germination, with longer extraction times (2–3 h) showing stronger activity. These findings indicate that extraction time is critical for obtaining antifungal compounds, consistent with previous reports that time, temperature, and pressure influence antimicrobial yield (Li et al., 2009; Rodrigues et al., 2003).

A notable feature of high-pressure extracts is the retention of volatile compounds. Volatile organic compounds from essential oils of *Origanum vulgare* and *Thymus vulgaris* inhibit fungal growth (Álvarez-García et al., 2023), and our results similarly showed that volatile organic compounds in Shikuwasa peel extracts suppressed conidia germination and exhibited fungicidal activity. GC–MS analysis revealed 16 compounds that increased with extraction time, with Eucalyptol showing the greatest increase. Previous studies reported that Shikuwasa peel contains d-limonene, γ-terpinene, and Eucalyptol (Teramoto et al., 2010), and essential oils from Shikuwasa residues inhibited *Escherichia coli* (Okamoto & Okamoto, 2025). These findings suggest that Eucalyptol and other volatile organic compounds may contribute to antifungal activity, warranting further investigation.

In conclusion, Shikuwasa peel extracts prepared under high-pressure conditions suppressed *F. buharicum*, highlighting their potential for soil disinfection and sustainable disease management.

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References

Ahn, YJ., Kawamura, T., Kim, M., Yamamoto, T., Mitsuoka, T. (1991) Tea polyphenols: selective growth inhibitors of

Clostridium spp. Agricultural and Biological Chemistry, **55**: 1425–1426.

Álvarez-García, S., Moumni, M., Romanazzi, G. (2023) Antifungal activity of volatile organic compounds from essential oils against the postharvest pathogens *Botrytis cinerea*, *Monilinia fructicola*, *Monilinia fructigena*, and *Monilinia laxa*. Frontiers in Plant Science, **14**: 1274770.

Common names of plant diseases in Japan (2025) Phytopathological Society of Japan.

Dixon, RA., Strack, D. (2003) Phytochemistry meets genome analysis, and beyond. Phytochemistry, **62**: 815–816.

FRAC (2018) Publications. Fungicide Resistance Action Committee.

Hokama, K. (1983) Occurrence of okura damping-off and its chemical control on Okinawa Prefecture. Proceeding of the Association for Plant Protection of Kyushu, **29**: 36–39.

Hokama, K. (1990) On the root rot disease of okura by *Phytophthora* spp. Proceeding of the Association for Plant Protection of Kyushu, **36**: 52–55.

Kotani,Y., Yamamoto, K., Ooshiro, A., Ajitomi, A., Yamashiro, M., Hirooka, Y. (2018) Fusarium wilt of okra caused by *Fusarium buharicum*. Japanese Journal of Phytopathology, **84:** 201.

Li, Z., Lin, H., Yamasaki, N., Kamitakahara, M., Ioku, K. (2009) Extraction of organic compounds from Sugi (*Cryptomeria japonica*) leaves using superheated steam and evaluation of the antimicrobial activity of the extracts. Journal of Material Cycles and Waste Management, **20**: 361–370.

MAFF (2022). Statistics on specialty vegetables. Ministry of Agriculture, Forestry and Fisheries of Japan.

Okamoto, T., Okamoto, T. (2025) Bactericidal effect of essential oil of Shiikuwasha squeezed juice residue and estimation of its mechanism. Bulletin of the Center for Research in Science Education Faculty of Education Ehime University, **4**: 44-48.

Okido, T., Ino, M., Kamada, I., Kihara, J., Ueno, M. (2022) Biological activity of Kuma bamboo grass (*Sasa veitchii*) extract against the fungal causal agent (*Pyricularia oryzae*) of blast disease. Advances in Bamboo Science, **1**: 100004.

Rodrigues, MRA., Caramao, EB., Dos Santos, JG., Dariva, C., Oliveira, JV., (2003). The effects of temperature and pressure on the characteristics of the extracts from highpressure CO2 extraction of *Majorana hortensis* Moench. Journal of Agricultural and Food Chemistry, **51**: 453-456.

Serit, M., Okubo, T., Hagiwara, N., Kim, M., Nonaka, GI., Nishioka, I., Yamamoto, T. (1991a) Comparative antibacterial activity of quercitol gallates. Agricultural and Biological Chemistry, **55**: 1893–1894.

Serit, M., Okubo, T., Su, R. H., Hagiwara, N., Kim, M., Iwagawa, T., Yamamoto, T. (1991b) Antibacterial compounds from Oak, *Quercus acuta* Thunb. Agricultural and Biological Chemistry, 55: 19–23.

Teramoto-Inafuku, S., Yamamoto, M., Kinjyo, H., Kitajima, A., Wada, K., Kawamitsu, Y. (2010) Local citrus genetic resources and their polymethoxyflavones content in northern part of Okinawa island. Horticultural Research (Japan), **9**: 263–271.

Ueno, M., Yoshikiyo, K., (2014) 2,6-Dimethoxy-1,4-benzoquinone enhances resistance against the rice blast fungus *Magnaporthe oryzae*. Journal of Phytopathology, **162**: 731–736.

Wada, K, (2017) Shiikuwasha (Citrus depressa Hayata): Biological function and aroma. Journal of Analytical Bio-Science, 40: 271–278.