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# Antifungal activity of leaf extracts from several buckwheat varieties against plant pathogenic fungi

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**Abstract** Common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*F. tataricum*) is a commonly grown food crop in Japan. The classic Japanese work on agriculture, Nihon Nousho Zenshu, mentions that extracts of buckwheat leaves were used to control rice blast in the Edo Period. Recently, we evaluated whether the characteristics associated with infection and fungal growth of rice blast fungus, *Magnaporthe oryzae,* the causal agent of rice blast disease, could be suppressed by using extracts of buckwheat leaves. However, it remains unclear whether leaf extracts of several buckwheat variety would show similar inhibitory activity against other plant-pathogenic fungi. In the present study, we evaluated the broader fungicidal activity of extracts of leaves of several buckwheat varieties against the pathogens *Ceratocystis fimbriata*, *Cochliobolus miyabeanus*, *Corynespora cassiicola*, *Fusarium buharicum,* and *M. oryzae*. We found that an ethyl acetate extract of common buckwheat leaves (cv. Harunoibuki, cv. Kitawasesoba, and cv. Shinano 1 gou), and tartary buckwheat leaves (*F. tataricum*: Dattansoba) significantly inhibited conidial germination of *C. fimbriata*, *C. miyabeanus*, *C. cassiicola, F. buharicum*, and *M. oryzae*. The ethyl acetate extract of common buckwheat leaves (cv. Harunoibuki, cv. Kitawasesoba, and cv. Shinano 1 gou), and tartary buckwheat leaves (Dattansoba) were shown to have fungicidal activity against *C. fimbriata*, *C. miyabeanus*, *C. cassiicola, F. buharicum*, and *M. oryzae*. These results suggest that fungicidal substances from the leaf extracts of common buckwheat and tartary buckwheat may be a promising source for the development of new chemical fungicides to prevent plant diseases caused by fungal pathogens.

**Keywords** : Buckwheat, Fungicidal activity, Leaf extract, Plant pathogenic fungus

## Introduction

Agricultural chemicals are applied as useful and efficient pest and disease control methods. However, such chemical control methods sometimes have detrimental effects on the ecosystem (Tase *et al.* 1989) and non-target beneficial microorganisms (Channabasava *et al*. 2015). Additionally, due to the continuous acquisition of resistance by the causative fungus, several fungicide chemicals are no longer effective. Therefore, there is a need to identify natural components and develop new agents to control fungal pathogens. The collected works of books on Japanese Agriculture, Nihon Nousho Zenshu, mention that buckwheat straw, stems, and leaves were used to control rice blast during the Edo era (1603 to 1868) in Japan. Recently, we investigated whether the infective behavior and lesion formation by rice blast fungus (*Magnaporthe oryzae*), the causal agent of rice blast disease, could be suppressed using extracts obtained from common buckwheat (*Fagopyrum esculentum* cv. Shinshuoosoba) leaves and straw (Tamura *et al*. 2017a, 2017b). The results provided compelling evidence that the inhibitory substances contained in buckwheat extracts may contribute to the development of new fungicides.

In the present study, we investigated the potentially broader antifungal activity of leaf extracts of common buckwheat (cv. Harunoibuki, cv. Kitawasesoba, and cv. Shinano 1 gou) and tartary buckwheat (*F. tataricum*: Dattansoba) against several plant pathogenic fungi, including *Ceratocystis fimbriata*, *Cochliobolus miyabeanus*, *Corynespora cassiicola*, *Fusarium buharicum*, and *Magnaporthe oryzae*.

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## Materials and Methods

### Pathogen and plant materials

Plant pathogenic fungi *C. fimbriata, C. miyabeanus*, *C. cassiicola, F. buharicum,* and *M. oryzae* were maintained on potato sucrose agar slants until use. *C. fimbriata, C. miyabeanus*, and *F. buharicum* were grown on rice bran agar (50 g/L rice bran, 20 g/L sucrose, 20 g/L agar, and $H_2O$) at 25 ± 2 ℃ for 7 days to induce abundant conidiation, and synchronously formed conidia were used to prepare an inoculum. *C. cassiicola* and *M. oryzae* were grown on rice bran agar at 25 ± 2 ℃ for 10 days, washed with running distilled water to remove aerial hyphae, and maintained at 25 ± 2 ℃ under near-ultraviolet radiation (FL20s BL-B; Panasonic, Osaka, Japan) for 2–4 days to induce conidiation, and synchronously formed conidia were used to prepare an inoculum.

Common buckwheat (cv. Harunoibuki, cv. Kitawasesoba, and cv. Shinano 1 gou) and tartary buckwheat (Dattansoba) were grown in plastic pots (9 cm diameter), containing commercial garden soil (Sun Soil S; Nagata Co., Ltd. Shimane, Japan) for 1 to 3 months.

### Preparation of leaf extracts from buckwheat

Leaf extracts were prepared from buckwheat plants. Well-developed leaves (50 g) were collected and cut into small segments, which were placed into a flask (3 L) containing distilled water. Flasks were boiled at 121 ℃ for 20 min. After cooling, the extract was filtered through gauze and concentrated in a rotary evaporator. The volume of extracted samples was adjusted to 1 mL and extracted twice with 2 mL of ethyl acetate. The ethyl acetate fraction was added to distilled water and evaporated at 50 ℃ under reduced pressure until only the water fraction remained. The aqueous volume was adjusted to 5 mL (10-fold), and these solutions were used as ethyl acetate extracts of buckwheat leaves (referred to as BW leaf extracts hereafter, for brevity).

### Effect of BW leaf extracts on conidia germination of plant pathogenic fungi

*C. fimbriata* conidia ($1.2 \times 10^5$ conidia/mL), *C. miyabeanus* conidia ($1.0 \times 10^5$ conidia/mL), *C. cassiicola* conidia ($1.0 \times 10^5$ conidia/mL), *F. buharicum* conidia ($1.2 \times 10^5$ conidia/mL) and *M. oryzae* conidia ($8.0 \times 10^4$ conidia/mL), suspended in BW leaf extracts, were dropped onto glass slides and maintained in a moist chamber at 25 ± 2 ℃. After 24 h, percentages of conidial germination were determined by assessing 300 conidia under a light microscope, using the following formula:

Percentage conidia germination = (number of conidia germinated/total number of conidia) × 100.

### Investigation of fungicidal activity of BW leaf extracts

*C. fimbriata* conidia ($1.2 \times 10^5$ conidia/mL), *C. miyabeanus* conidia ($1.0 \times 10^5$ conidia/mL), *C. cassiicola* conidia ($1.0 \times 10^5$ conidia/mL), *F. buharicum* conidia ($1.2 \times 10^5$ conidia/mL) and *M. oryzae* conidia ($8.0 \times 10^4$ conidia/mL), were treated with BW leaf extract in a microtube, and then maintained in a cold room at 4 ± 2 ℃. As a control, sterile distilled water was used. After 24 h, the supernatant was removed by centrifugation, and then sterile distilled water (1 mL) was added to the remaining pellet. Aliquots of conidial suspension (30 μL) were inoculated on potato sucrose agar medium containing 20 ppm chloramphenicol. The inoculated Petri dishes were incubated at 25 ± 2 ℃ for 3 days, after which the mycelial growth areas of the five plant pathogenic fungi were measured using LIA 32 software. Experiments were repeated three times, and in each experiment, we examined five Petri dishes.

### Statistical analysis

Data are presented as the means ± standard deviations. Germinated conidia data were normalized using arcsine square root transformation to enhance the homogeneity of variance. Significant differences in the experimental values between groups were determined using Tukey–Kramer tests, using SPSS Statistics ver. 22.0 for Windows (IBM, Armonk, NY, USA). P-values $< 0.05$ were considered to indicate a statistically significant difference.

## Results and Discussion

To determine the direct effects of BW leaf extracts, the conidia germination of plant pathogenic fungi in the presence of the BW leaf extracts was assessed. The leaf extracts of common buckwheat and tartary buckwheat were found to have antifungal activity against *C. fimbriata*, *C. miyabeanus*, *C. cassiicola*,

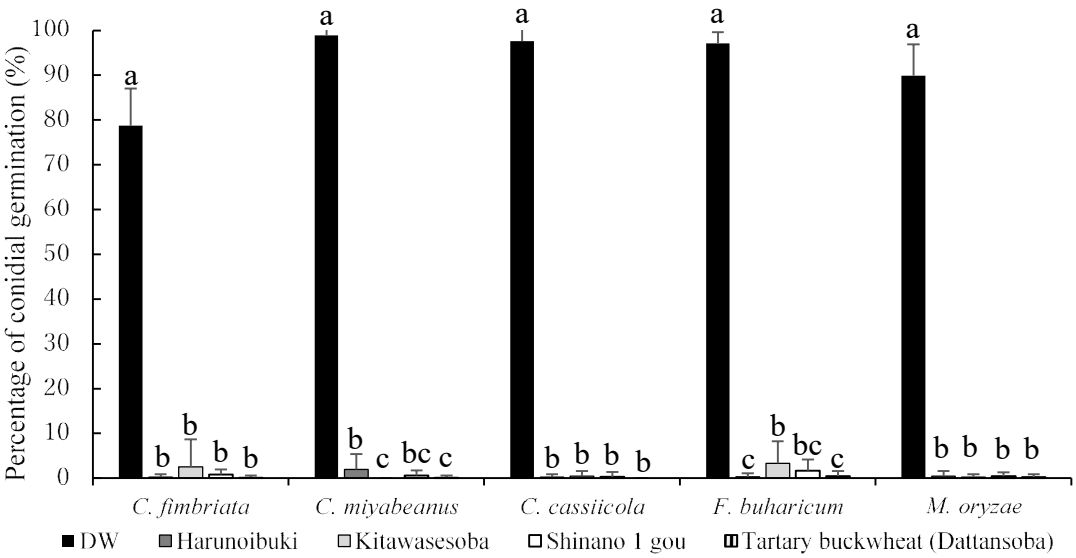


Fig 1. Inhibitory activity of buckwheat (BW) leaf extracts on conidial germinations of plant pathogenic fungi. *Ceratocystis fimbriata* ($1.2 \times 10^5$ conidia/mL), *Cochliobolus miyabeanus* ($1.0 \times 10^5$ conidia/mL), *Corynespora cassiicola*, ($1.0 \times 10^5$ conidia/mL), *Corynespora cassiicola*, ($1.2 \times 10^5$ conidia/mL) and *Magnaporthe oryzae* ($8.0 \times 10^4$ conidia/mL), suspended in BW leaf extracts, were dropped onto glass slides and maintained in a moist chamber at 25 ± 2°C. As a control, sterile distilled water (DW) was used. After 24 h, percentages of conidial germination were determined by assessing 300 conidia under a light microscope. The data presented are the means of the results of three independent performed with six replications. Bars represent the standard deviation of the mean. Mean values denoted by the same lowercase letter are not significantly different at the 5% level, as determined using Turkey's test.

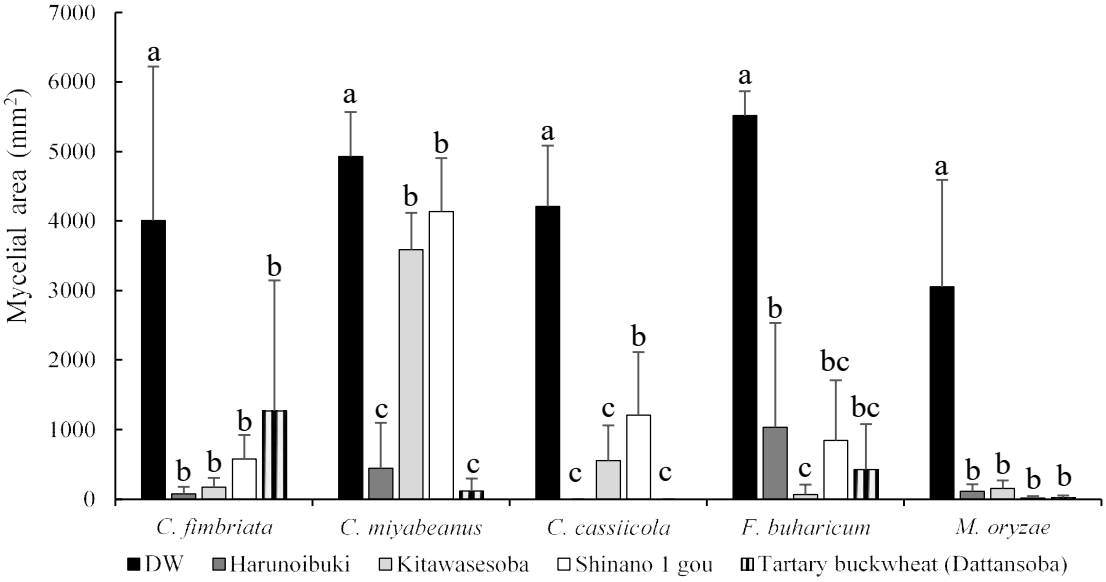


Fig 2. Fungicidal activity of buckwheat (BW) leaf extracts against plant pathogenic fungi. Conidia suspension of *Ceratocystis fimbriata* ($1.2 \times 10^5$ conidia/mL), *Cochliobolus miyabeanus* ($1.0 \times 10^5$ conidia/mL), *Corynespora cassiicola*, ($1.0 \times 10^5$ conidia/mL), *Corynespora cassiicola*, ($1.2 \times 10^5$ conidia/mL) and *Magnaporthe oryzae* ($8.0 \times 10^4$ conidia/mL) were treated with BW leaf extract in a microtube and then maintained in a cold room at 4 ± 2°C. As a control, sterile distilled water (DW) was used. After 24 h, the supernatant was removed by centrifugation and then sterile distilled water (1 mL) was added to the remaining pellet. Aliquots conidial suspension (30 μL) were inoculated on potato sucrose agar medium containing 20 ppm chloramphenicol. The inoculated Petri dishes were incubated at 25 ± 2°C for 3 days, after which, the mycelial growth areas of five plant pathogenic fungi were measured using LIA 32 software. Experiments were repeated three times, and in each experiment, we examined five Petri dishes. Bars represent the standard deviation of the mean. Mean values denoted by the same lowercase letter are not significantly different at the 5% level, as determined using Turkey's test.

*F. buharicum*, and *M. oryzae,* and germination percentages were low, ranging from 0% to 3.3 ± 4.9%. In contrast, in the control treatment, the percentage of conidia germination of each plant pathogenic fungus in distilled water ranged from 78.8 ± 8.2% to 98.9 ± 1.6% (Fig. 1). The results indicate that several buckwheat and tartary buckwheat varieties produce antifungal substances against wide range of plant pathogenic fungi.

To confirm the fungicidal activity of BW leaf extracts, we examined the effects of BW leaf extracts on mycelial growth of *C. fimbriata*, *C. miyabeanus*, *C. cassiicola*, *F. buharicum*, and *M. oryzae* in Petri dishes. In the control treatment, the area of mycelial growth of each plant pathogenic fungus ranged from 3052.1 ± 1539.0 $mm^2$ to 5517.5 ± 348.7 $mm^2$ (Fig. 2). In contrast, mycelial growth of *C. fimbriata* (73.5-1266.9 $mm^2$), *C. cassiicola* (0-1207.4 $mm^2$), *F. buharicum* (67.0-1030.8 $mm^2$), and *M. oryzae* (16.9-150.7 $mm^2$) were significantly inhibited by BW leaf extracts. However, *C. miyabeanus* in buckwheat leaf extracts was found to have a wide range of mycelial growth areas, ranging from 116.5 ± 179.8 $mm^2$ to 4136.9 ± 767.5 $mm^2$ (Fig. 2). These results suggest that leaf extracts of the different common buckwheat varieties and tartary buckwheat produced fungicidal substances against a wide range of plant pathogenic fungi. Common buckwheat contains more rutin than other plants (Dietrych-Szostak and Oleszek, 1999), although Tamura (2017b) found that rutin does not inhibit the conidial germination of plant pathogenic fungi. On the other hands, it has been reported that a low molecular weight protein or a polypeptide with a trypsin inhibitor, obtained from the seeds of buckwheat, inhibits the growth of several fungi (Sakamoto *et al*., 1998; Ruan *et al*. 2011). However, in the present study, BW leaf extracts contained heat-stable and ethyl acetate soluble substances. This suggests that there is a possibility that the antifungal substances in BW leaf extracts differ from previously described substances. Further studies are required to identify the active antifungal substances in BW leaf extracts.

In conclusion, this study on the effects of BW leaf extracts on fungal pathogens may contribute to the development of new fungicides for the control of plant diseases caused by plant pathogenic fungi.

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