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Production of Weak Acid by Anaerobic Fermentation of Soil and Antifungal Effect

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Abstract Acetic acid and butyric acid were produced by the anaerobic fermentation of soil mixed with wheat or rice bran. The concentration of acetic acid produced in the wheat and rice bran-treated soil was 31.2 mM and 8 mM, respectively, whereas the concentration of butyric acid in the wheat and rice bran-treated soil was 25.0 mM and 8 mM, respectively. The minimal fungicidal concentration (MFC) for all the fungal strains was 40–60 mM acetic acid, 20–40 mM butyric acid, and 40–60 mM mixture of acetic acid: butyric acid (1:1, v/v). Consequently, the efficacy of mixing wheat-bran with soil to control soil diseases was demonstrated.

Keywords: Anaerobic fermentation, antifungal, rice bran, weak acid, wheat bran

The soil environment in Korea has been ruined because of soil contagious pathogens and high salt, resulting from successive cultivation of the same crops 4 or 5 times, the excessive application of chemical fertilizers, and use of animal excrement as fertilizer [6, 12, 17].

Fusarium, *Phytopthora*, and *Monosporascus* sp. are known as the main soil contagious pathogens occurring in protected cultivation areas, and reduce the yield [3]. Therefore, crop rotation, soil covering, solar heating, water logging, the cultivation of resistant cultivars, plus chemical and biological methods have all been utilized to control these soil contagious pathogens [5]. However, most methods have drawbacks as regards practical application to fields. For example, sterilization with solar heat must be performed during the summer, as it requires a temperature higher than 60°C, water logging contaminates the surrounding area and consumes an excessive amount of water, and crop rotation or soil covering are not practical, as they involve excessive farming work. Consequently, chemical methods are more commonly used [10], as they only take a short time to apply and their effectiveness has been proven; however, they are not environment-friendly and also have an adverse effect on the soil ecosystem.

Weak acids, such as sorbic acid, succinic acid, benzoic acid, and acetic acid, produced during fermentation or metabolism, have already been used as food preservatives against fungi, and the antifungal effect of these weak acids is known to be due to an increase of acidity inside microorganisms, causing the suppression of hypha growth or their liquidation [1, 4, 7, 9, 15].

Accordingly, since weak acids are not harmful to humans or animals, the present study investigated the use of rice bran or wheat bran to control soil contagious pathogens by measuring the production of weak acids from soil mixed with cereal byproducts, such as rice and wheat bran, and examining their antifungal effect.

The experiments were conducted in a greenhouse in which field fruit vegetables and leaf vegetables had been cultivated for the past 10 years. Wheat bran or rice bran that had not decomposed was mixed with the soil (20,000 kg bran/ha), followed by the addition of enough water to wet a 30-cm depth of soil. To facilitate anaerobic fermentation of the wheat bran and rice bran, the mixed soil was tightly covered by vinyl during 20 days.

The weak acids were then analyzed from soil samples taken at soil depths of 15 cm and 45 cm at 3 different sites and kept at 4°C until analysis to minimize any changes in the microorganism and organic compounds. A 5-fold excess amount of water was added to the soil samples,

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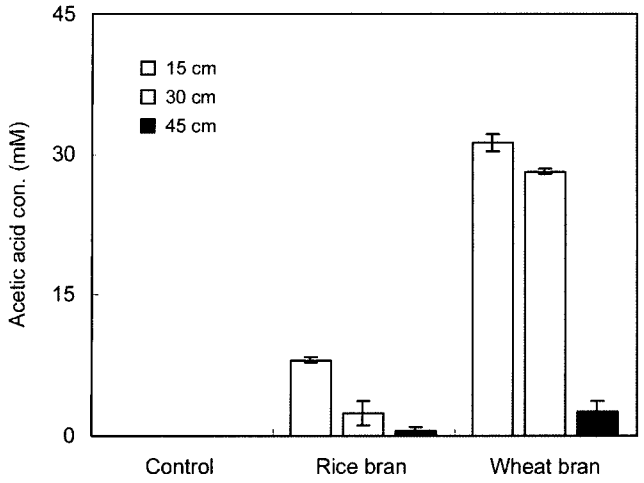

Fig. 1. Concentration of acetic acid affected by anaerobic fermentation of rice bran and wheat bran at different soil depths.

which were then shaken for 30 min, filtered through a 0.45 ul sealed filter, sterilized for 15 min at 121°C, and finally kept at -24°C until analysis.

The analysis was conducted using Agilent HP1100 GC (Refractive index, Hewlett Packard) with an aminex HP-87H column (Bio-rad, Hercules, CA, U.S.A.) and 0.01 M H_2SO_4 as the solvent at a flow rate of 0.6 ml/min.

The soil contagious pathogens used in the present study were provided by KACC (Table 2), and all showed pathogenic activity and caused wilting symptoms in tomatoes and watermelons. The pathogens were used after being cultivated on a Potato Dextrose agar (PDA) for 7 days, and the anti-fungal effect of the weak acids was represented by the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC).

The main weak acids identified in the soil after 20 days of treatment were acetic acid and butyric acid (Fig. 1, 2),

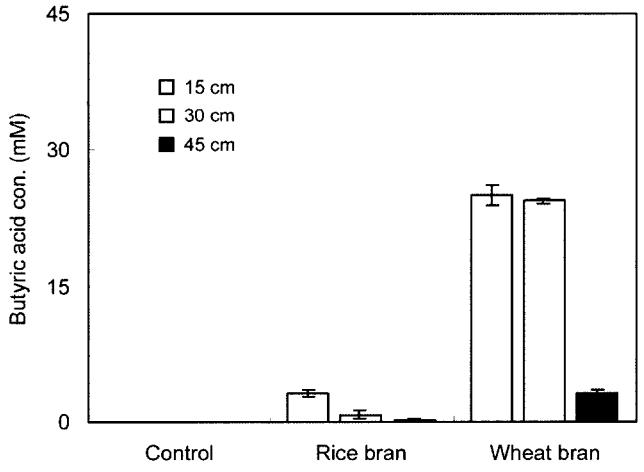

Fig. 2. Concentration of butyric acid affected by anaerobic fermentation of rice bran and wheat bran at different soil depths.

whereas no lactic acid, formic acid, pyruvic acid, or succinic acid was observed (data not shown). The concentrations of acetic acid and butyric acid showed no variation up to a soil depth of 30 cm; however, the concentration decreased dramatically at a soil depth of 45 cm.

The concentration of acetic acid produced in the wheat bran-treated soil was 31.2, 28.2, and 2.6 mM at a soil depth of 15, 30, and 45 cm, respectively, whereas the concentration in the rice bran-treated soil was around 8, 2, and 1 mM, respectively (Fig. 1). The concentration of butyric acid was 25.0, 24.4, and 3.2 mM in the wheat bran-treated soil and 8, 2, and 1 mM in the rice bran-treated soil at a soil depth of 15, 30, and 45 cm, respectively (Fig. 2).

The low weak-acid concentrations in the deeper soil samples may have been because a relatively smaller amount of the wheat or rice bran was mixed in because of plowing limitations, and because soil microorganisms are comparatively scarce in deeper soil [8].

The antifungal activity of the acetic acid and butyric acid was also investigated using ten species of *Fusarium*, *Phytophthora*, *Phythium* sp., and *Monosporascus cannohballus*, where the fungal growth was found to be suppressed as the weak acid concentration increased (Fig. 3) and antifungal activity was exhibited for all the fungi tested (Table 1).

The fungal growth was suppressed in a decreasing order of the *Pythium* sp. *Monosporascus*, *Phytophthora*, and *Fusarium* strains. To determine the MIC of the weak acids, the *Fusarium* strains were cultivated for 7 days, and found not to grow with an acetic acid concentration of 20–40 mM, butyric acid concentration of 10–20 mM, and 1:1 (v/v) mixture of acetic acid and butyric acid. To confirm a fungi plaque, which does not support the growth of hyphae, the fungi plaques were transferred to a medium that did not contain any weak acids and cultivated for 3

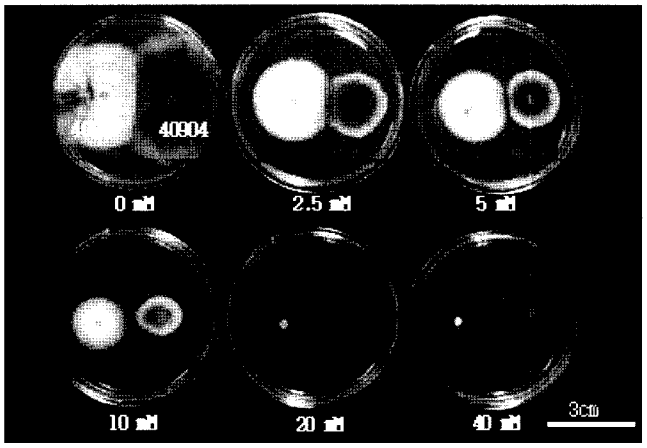

Fig. 3. Mycelial growth of *Fusarium* strains affected by different concentrations of weak acids at 30°C [weak acid: (Acetic acid:Butyric acid=1:1 (v/v)) KACC 40901 (*Fusarium oxysporum* f. sp. *Niveum*), KACC 40904 (*Fusarium oxysporum* f. sp. *melonis*)].

Table 1. MIC and MFC values for fungal strains according to acetic acid and butyric acid tested *in vitro*.

Fungal	Source	No. (KACC)	Acetic acid (mM)		Butyric acid (mM)		A+B[a] (mM)	
			MIC	MFC	MIC	MFC	MIC	MFC
Pythium sp.	Tomato	40581	5	10	2.5	5	2.5	2.5
Monosporascus cannonballus	melon	Wild-type	20	20	10	10	20	20
Phytophthora capsici Leonian	Red pepper	40157	20	20	10	10	20	20
Phytophthora capsici Leonian	Paprika	40158	20	20	5	10	10	20
Phytophthora capsici Leonian	Tomato	40177	20	20	10	10	20	20
Phytophthora capsici Leonian	Pumpkin	40178	20	20	10	10	20	20
Phytophthora capsici Leonian	Watermelon	40179	10	20	10	10	10	20
Phytophthora capsici Leonian	Korean melon	40180	10	20	5	10	10	20
Phytophthora capsici Leonian	Cucumber	40181	10	10	5	10	10	20
Phytophthora capsici Leonian	Tomato	40470	20	20	5	10	20	20
Phytophthora capsici Leonian	Watermelon	40471	20	20	5	10	10	20
Phytophthora capsici Leonian	Korean melon	40472	20	20	10	10	20	20
Fusarium sp.	Tomato	40031	40	60	10	20	40	40
Fusarium sp.	Red pepper	40240	40	40	10	20	20	20
Fusarium sp.	Eggplant	40241	40	40	10	10	20	20
Fusarium oxysporum	Butterfly orchid	41088	40	60	20	40	20	40
Fusarium oxysporum f. sp. Niveum	Watermelon	40901	40	40	10	10	20	40
Fusarium oxysporum f. sp. Niveum	Watermelon	40902	40	40	10	10	20	20
Fusarium oxysporum f. sp. Melonis	Cucumber	40904	40	40	10	20	20	20
Fusarium oxysporum f. sp. Lycopersici	Tomato	40032	40	60	20	40	20	40
Fusarium oxysporum f. sp. Lycopersici	Tomato	40038	40	60	20	40	40	40
Fusarium oxysporum f. sp. Lycopersici	Tomato	40043	40	60	10	40	20	40

[a]A+B: [Acetic acid:Butyric acid=1:1 (v/v)].

days. As a result, the MFC was found to be 40–60 mM acetic acid, 20–40 mM butyric acid, and a 1:1 (v/v) mixture of acetic acid and butyric acid.

The MIC for the *Phytophthora* strains was slightly lower than that for the *Fusrium* strains, as they did not grow at an acetic acid concentration of 10–20 mM, butyric acid concentration of 5–10, and 10–20 mM 1:1 (v/v) mixture of acetic acid and butyric acid. The MFC was also determined to be a similar level. The MIC and MFC values for the *Monosporascus*, *Cannohballus*, and *Pythium* sp. were both very low compared with those for the *Phytophthora* and *Fusarium* strains.

The difference in the MICs for the various weak acids is thought to depend on the molecular weight and pKa values [2, 13], where the pKa values for acetic acid and butyric acid are 4,757 and 4,981, respectively. According to the Henderson-Hasselbach formula, the higher the pKa value, the lower the ionization rate. This theory also supports the idea that butyric acid contains more XCOOH, which allows butyric acid to permeate the plasma membrane of fungal cells more easily than acetic acid. Furthermore, since the two molecular weights are 30% different, 30% more butyric acid was added when the same number of moles was applied. It has already been reported that the antifungal activity of saturated fatty acids depends on the length of the hydrophobic side-chains [11, 14]. As distinct from the antifungal function of other weak acids (acidification of cytoplasm), sorbic acid suppresses the propagation of fungi by removing the electric potential of the membrane. However, since butyric acid has a larger partition coefficient and requires a lower concentration to suppress the fungi than acetic acid [16], this suggests a similar mechanism to sorbic acid. However, more studies are needed to confirm this assumption.

The soil pH is very important for the antifungal function of weak acids, which is only conducted under non-ionic conditions. According to the Henderson-Hasselbach formula ($\log[H_3O][A]/[HA]=pH-pKa$), the lower the pH, the higher the non-ionic acid [2].

Accordingly, the present results demonstrated that wheat-bran treatment would seem to be an effective and environmentally friendly method for controlling soil diseases due to successive cultivation.

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