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Effects of the Fermentation Product of Herbs by Lactic Acid Bacteria against Phytopathogenic Filamentous Fungi and on the Growth of Host Plants

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The fermentation product of herbs by lactic acid bacteria (FHL) was assayed for antifungal activities against *Rosellinia necatrix*, *Helicobasidium mompa*, *Fusarium oxysporum*, *Pythium graminicola* and *Pyricularia oryzae*. FHL completely inhibited the growth of *R. necatrix*, *H. mompa*, *P. graminicola* and *P. oryzae*, and reduced the growth of *F. oxysporum* by 35%. When the seeds of *Medicago sativa* L. (alfalfa), *Asparagus officinalis* L. (asparagus), *Brassica campestris* L. (komatsuna), *Oryza sativa* L. (rice), *Spinacia oleracea* L. (spinach), *Festuca arundinacea* Schreb. (tall fescue), and *Lycopersicum esculentum* Mill. (tomato) were put on plates containing 0.69 mg/ml FHL, their germination rates did not decrease. The root elongation of *A. officinalis*, *B. campestris*, *O. sativa*, and *L. esculentum* seedlings was suppressed on plates containing 6.92 mg/ml FHL, but the root elongation of *M. sativa* was not suppressed on the 6.92 mg/ml FHL plate. When FHL was diluted to less than 1.73 mg/ml, the diluted FHL solution did not suppress the germination of *B. campestris* seeds, but it suppressed the root elongation of *B. campestris* seedlings. An FHL concentration higher than 0.35 mg/ml hastened the growth of seedlings of *B. campestris* in the presence of a chemical fertilizer but delayed the growth of these seedlings in the absence of the chemical fertilizer, suggesting that inorganic elements could affect the efficiency of FHL.

[**Key words:** antifungal activity, *Rosellinia necatrix*, *Helicobasidium mompa*, *Fusarium oxysporum*, *Pythium graminicola*, *Pyricularia oryzae*, phytopathogenic, organic acid]

Phytopathogenic filamentous fungi cause most plant diseases and many effective pesticides for fungi have been developed, commercialized and applied to the field. These pesticides have increased crop production and reduced costs, but they have some potential risks for the health of consumers because traces of the original toxic pesticides or other compounds, to which some original pesticides are decomposed, can still remain in the commercial products. Currently, consumers are concerned about food safety and demand safe food products including crops. Under such circumstances, natural organic matter or manure that has antifungal activity is attracting public attention.

Fungi are classified into Ascomycotina, Basidiomycotina, Deuteromycotina, Mastigomycotina, and Zygomycotina. *Rosellinia necatrix* is an Ascomycotina Pyrenomycete that causes white root rot of vine, apple, mulberry, and asparagus (*Asparagus officinalis* L.), among others (1, 2). *Helicobasidium mompa* Tanaka is a Basidiomycotina Hymenomycete that causes violet root rot of apple, mulberry, alfalfa (*Medicago sativa* L.), and asparagus, among others (3, 4). *Fusarium oxysporum* is a Deuteromycotina Hyphomycete that causes vascular wilt diseases on a wide range of crops (5). *F. oxysporum* f. sp. *tulipae* is a pathogen that causes tulip bulb rot (6) and *F. oxysporum* is also reported to infect mustard (*Brassica campestris* L.) (7), rice (*Oryza sativa* L.) (8), spinach (*Spinacia oleracea* L.) (9), and tomato (*Lycopersicum esculentum* Mill.) (5). *Pythium graminicola* is a Mastigomycotina Oomycete in causing Pythium blight that is a disease of turf grasses and home lawns on golf courses (10). Pythium species are reported to infect tall fescue (*Festuca arundinacea* Schreb.) (11), spinach (12), and rice (13). *Pyricularia oryzae* (syn. *Magnaporthe grisea*) is a Deuteromycotina Hyphomycete that causes rice blast, considerably decreases yield and finally leads to severe hardship and famine in developing countries (14). Komatsuna (*Brassica campestris* L.) that is a popular Japanese green vegetable and a variety of mustard is a model plant for estimating the efficiency and plant-growth suppression of manures or fertilizers in Japan.

In a previous study, we investigated the antifungal activity of the fermentation product of herbs by lactic acid bacteria (FHL) against *Trichophyton rubrum* and *T. mentagrophytes* (15), which are Deuteromycotina Hyphomycetes. Some or-

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ganic acids are used as food additives to prevent the growth and spore germination of bacteria and fungi (16–18), and FHL contains some organic acids (15). Thus, we expected that the fermentation product would be an antifungal agent against phytopathogenic filamentous fungi. There are few reports on lactic acid bacteria or fermentation products having antifungal activities against phytopathogenic filamentous fungi (15, 19). In this study, *R. necatrix*, *H. mompa*, *F. oxysporum*, *P. graminicola*, and *P. oryzae* were assayed as representative phytopathogenic filamentous fungi to determine the broader antifungal activities of FHL *in vitro*. Moreover, the effects of FHL on plant growth were evaluated using *M. sativa*, *A. officinalis*, *B. campestris*, *O. sativa*, *S. oleracea*, *F. arundinacea*, and *L. esculentum* that are host plants for the fungi mentioned above.

MATERIALS AND METHODS

Strains, chemicals and media *R. necatrix* R-9101 was kindly provided by Okayama Prefectural Agricultural Experiment Station (Okayama). *H. mompa* Tanaka IFO31651, *F. oxysporum* f. sp. *tulipae* IFO32203, *P. graminicola* IFO32330, and *P. oryzae* IFO31175 were purchased from the Institute for Fermentation, Osaka (Osaka). Potato dextrose agar medium (PDA; Difco Laboratories, Detroit, MI, USA) was employed as the standard medium for phytopathogenic filamentous fungi. As synthetic fungicides, Tachigare-Ace (Sankyo, Tokyo) and Frowncide (Ishihara Sangyo Kaisha, Osaka) were commercially obtained. Tachigare-Ace that contained 30.0% hydroxyisoxazole (3-hydroxy-5-methylisoxazole) and 4.0% metalaxyl (methyl *N*-(2-methoxyacetyl)-*N*-(2,6-xylyl)-DL-alaninate) was used as a positive control for the antifungal assay of *H. mompa*, *F. oxysporum*, *P. graminicola* and *P. oryzae*. Frowncide that contained 39.5% fluazinam wettable powder was employed as a positive control for the antifungal assay of *R. necatrix* and *P. oryzae*. The fermentation product of herbs by lactic acid bacteria (FHL) was provided by Biobank (Okayama) (15).

Assay for antifungal activities of organic acids, synthetic fungicides and FHL against phytopathogenic filamentous fungi Antifungal activities against phytopathogenic filamentous fungi were assessed by the agar dilution method as described previously (15). Before assaying for antifungal activities, five phytopathogenic filamentous fungi were pre-incubated on standard plates. *R. necatrix*, *H. mompa*, *F. oxysporum*, *P. graminicola*, and *P. oryzae* were then cultured on standard plates at 24°C for 5 d, 20 d, 5 d, 5 d, and 7 d, respectively.

Antifungal activity was calculated using the following equation (15):

$$\text{Antifungal activity (\%)} = \frac{(\text{colony diameter of control} - \text{colony diameter of sample})}{(\text{colony diameter of control} - \text{diameter of mycelial disk})} \times 100$$

Antifungal activity of proteinase-treated FHL Antifungal activities of proteinase-treated FHL were assessed using the method described previously (15).

Effect of FHL on germination rate and root elongation Seeds of *M. sativa* L. (alfalfa), *A. officinalis* L. (asparagus), *B. campestris* L. (komatsuna), *O. sativa* L. (rice), *S. oleracea* L. (spinach), *F. arundinacea* Schreb. (tall fescue), and *L. esculentum* Mill. (tomato) were commercially purchased and were used for germination tests. Seeds were sterilized in 70% (v/v) ethanol for 1 min and then in a solution of 5% (v/v) sodium hypochlorite and 0.05% (v/v) Tween 20 for 1 min. The sterilized seeds were washed three times with sterilized distilled water to remove the germicide.

TABLE 1. Physical and chemical properties of the soil

Type	Andosol
Soil texture	SiL
Alluvial soil or diluvial soil	Diluvial soil
pH (H_2O)	5.8
Exchangeable acidity (Y_1)	0.3
Electric conductivity (mS/cm)	0.08
Cation exchange capacity (cmol/kg dry soil)	28.0
Bulk density (g/ml)	0.69
Maximum water holding capacity (g/kg)	101

Distilled water supplemented with 1.0% (w/v) agar was autoclaved. Diluted FHL was also autoclaved. After autoclaving, the agar solution and FHL were mixed to prepare FHL plates at the concentrations of 0.35, 0.69, 1.38, and 6.92 mg (as dry matter)/ml. The FHL-agar mixture was poured into petri dishes and the dishes were left until the agar gelled. Forty seeds of *M. sativa*, *A. officinalis*, *B. campestris*, *O. sativa*, *S. oleracea*, *F. arundinacea*, or *L. esculentum* were arranged on the agar plates. *A. officinalis* and *L. esculentum* were incubated at 28°C under dark conditions. *M. sativa*, *B. campestris*, *O. sativa*, *S. oleracea*, and *F. arundinacea* were incubated at 25°C. After incubation, seedlings were pulled out from the agar plates and the lengths of roots were measured.

Root elongation determination for *B. campestris* in the medium with FHL or organic acids Seeds of *B. campestris* were sterilized as described above. The washed seeds were soaked with sterilized distilled water and shaken using a reciprocal shaker until root development (for 48 h). Murashige–Skoog (MS) medium supplemented with 2.0% agar, 3.46 mg/ml FHL, 3.0 mM acetic acid, 3.0 mM lactic acid, 3.0 mM malonic acid, and 0.36 mM oxalic acid were separately prepared and autoclaved and then MS medium with 2.0% agar and 3.46 mg/ml FHL, 3.0 mM acetic acid, 3.0 mM lactic acid, 3.0 mM malonic acid, or 0.36 mM oxalic acid were mixed in a ratio of 1 to 1. The mixture was poured into square petri dishes, and the dishes were left until the agar gelled. After root development, the seedlings were transferred to the square petri dishes containing 50% concentrated MS medium with 1.0% agar and organic acids the concentration of which was given. The square petri dishes with aseptically transplanted seedlings were placed vertically at 25°C in a growth chamber. After 72 h of incubation, the length of roots was measured.

Growth of *B. campestris* seedlings Forty seeds of *B. campestris* were sown in a plastic pot (113 mm diameter, 65 mm high) containing volcanic ash soil (andosols, *kurobokudo* in Japanese) collected from Chiba prefecture with or without a chemical fertilizer. The properties of the soil determined by general methods are listed in Table 1. Ammonium sulphate, superphosphate of lime and potassium chloride were used as chemical fertilizers and were added at 25 mg per pot as N, P_2O_5, and K_2O. *B. campestris* seedlings were cultivated for 3 weeks in the incubator. The germination rate, first leaf length and fresh weight of the seedlings were determined.

RESULTS

Antifungal activities of FHL against phytopathogenic filamentous fungi The antifungal activities of FHL against *R. necatrix*, *H. mompa*, *F. oxysporum*, *P. graminicola* and *P. oryzae* and the pHs of the media are summarized in Table 2. Tachigare-Ace and Frowncide, which were used as positive controls, completely inhibited the growth of *R. necatrix*, *H. mompa*, *F. oxysporum*, *P. graminicola* and *P. oryzae* at the concentration used. FHL had antifungal activ-

TABLE 2. Antifungal activity of FHL (%)

Condition	*R. necatrix*	*H. mompa*	*F. oxysporum*	*P. graminicola*	*P. oryzae*	pH
FHL	98	97	65	97	97	4.1
N-FHL[a]	45	88	−27	−8	−12	(7.5)
Mixture[b]	99	98	96	97	97	2.6
30 mM acetic acid	99	100	100	100	99	3.9
30 mM lactic acid	98	35	68	99	70	3.2
30 mM malonic acid	99	69	84	99	99	2.8
3.6 mM oxalic acid	0	36	51	99	−1	3.7
Fungicide I[c]	98	97	98	98	96	(5.1)
Fungicide II[d]	98	–[e]	–	–	87	(5.1)

[a] Neutralized FHL.
[b] 30 mM acetic acid + 30 mM lactic acid + 30 mM malonic acid + 3.6 mM oxalic acid.
[c] Tachigare-Ace.
[d] Frowncide.
[e] –, Not determined.

TABLE 3. Effects of pH on the antifungal activities against phytopathogenic filamentous fungi (%)

pH value	*R. necatrix*	*H. mompa*	*F. oxysporum*	*P. graminicola*	*P. oryzae*
1.8	93	97	95	99	96
2.7	94	52	72	99	94
3.6	−17	21	49	98	54

ity similar to that of the two types of synthetic fungicides against *R. necatrix*, *H. mompa*, *P. graminicola* and *P. oryzae*, but neutralizing FHL with sodium bicarbonate decreased the antifungal activities against *R. necatrix* and *H. mompa*, and accelerated the growth of *F. oxysporum*, *P. graminicola* and *P. oryzae* (Table 2). Since FHL contained 300 mM acetic acid, 266 mM malonic acid, 296 mM lactic acid and 36 mM oxalic acid at final concentrations (15), the antifungal activity of these organic acids was determined individually or in combination. On plates containing 30 mM acetic acid, 30 mM malonic acid, 30 mM lactic acid and 3.6 mM oxalic acid at final concentrations, the growth of all tested fungi was completely inhibited. When plates containing 30 mM acetic acid, 30 mM malonic acid, 30 mM lactic acid or 3.6 mM oxalic acid were assayed, only 30 mM acetic acid showed antifungal activity against all phytopathogenic filamentous fungi. Although the pH of the plate containing 30 mM acetic acid was close to that of the plate containing 3.6 mM oxalic acid, the antifungal activities of 30 mM acetic acid were higher than those of 3.6 mM oxalic acid against all fungi. When the pH of the standard medium was adjusted to 3.6 with HCl, the antifungal activity was similar to that of 3.6 mM oxalic acid (Table 3). When the pH of the medium was 2.7, the growth of *H. mompa* and *F. oxysporum* was not completely inhibited.

Antifungal activity of proteinase-treated FHL The antifungal activity of FHL treated with pepsin or trypsin to digest proteinaceous antifungal compounds according to the method described previously was determined (15). FHL treated with pepsin or trypsin had antifungal activities as high as FHL not treated with either proteinase against *R. necatrix*, *H. mompa*, *P. graminicola* and *P. oryzae*, whereas FHL treated with pepsin or trypsin exhibited decreased antifungal activity against *F. oxysporum* (Table 4).

TH10-broth, which constituted de Man, Rogosa, Sharpe broth (Oxoid, Basingstoke, UK) incubated at 37°C for 2 d after inoculation of *Enterococcus faecalis* TH10, had no antifungal activity against fungi other than *P. graminicola* (Table 4).

Effect of FHL on germination rate and root elongation When the seeds of *M. sativa*, *A. officinalis*, *B. campestris*, *O. sativa*, *S. oleracea*, *F. arundinacea*, and *L. esculentum* were incubated on FHL plates at concentrations of 0.35, 0.69, 1.38, and 6.92 mg/ml, the germination rates and root elongation were determined (Tables 5 and 6). The germination rates of *A. officinalis*, *S. oleracea*, *F. arundinacea*, and *L. esculentum* decreased when these seeds were placed on the plate containing 6.92 mg/ml FHL but the germination rates of *M. sativa*, *B. campestris*, and *O. sativa* did not. The germination rates of all seeds were not reduced on the plates containing 0.69 mg/ml FHL or less. The root elongation of *A. officinalis* and *O. sativa* was suppressed by 0.69, 1.38, and 6.92 mg/ml FHL compared with the root elongation on the control plate (1.0% agar), and the root elongation of *B. campestris* was inhibited by 6.92 mg/ml FHL. The root elongation of *L. esculentum* was decreased at all concentrations up to 6.92 mg/ml. In contrast, 0.35, 0.69, and 1.38 mg/ml FHL in the plates accelerated the root elongation of *M. sativa*. Since the germination rates of *S. oleracea* and *F. arundinacea* were extremely low, root elongation was not determined.

Root elongation of *B. campestris* in the medium with FHL or organic acids FHL consisted of 0.27% (w/w) nitrogen (N), 0.10% phosphoric acid (P_2O_5) and 0.97% potash (K_2O) and the C/N ratio of FHL was 138.5. When FHL was diluted to 17.3 mg/ml and *B. campestris* seeds were

TABLE 4. Antifungal activity of proteinase-treated FHL (%)

Condition	*R. necatrix*	*H. mompa*	*F. oxysporum*	*P. graminicola*	*P. oryzae*	pH
FHL	98	97	65	97	97	4.1
Pepsin-treated	96	98	27	98	96	3.8
Trypsin-treated	97	98	23	98	96	3.7
TH10-broth	20	18	−7	98	13	4.7

TABLE 5. Effect of FHL on the germination rate of plant seeds (%)

	M. sativa	*A. officinalis*	*B. campestris*	*O. sativa*	*S. oleracea*	*F. arundinacea*	*L. esculentum*
Incubation time (d)	2	6	2	5	5	8	2
FHL condition							
Control	83	90	85	98	53	13	93
6.92 mg/ml FHL	88	60	85	90	8	5	5
1.38 mg/ml FHL	85	80	98	90	30	8	95
0.69 mg/ml FHL	85	90	93	93	38	13	95
0.35 mg/ml FHL	75	95	93	93	23	25	98

TABLE 6. Effect of FHL on the root elongation of seedlings (cm)

	M. sativa	*A. officinalis*	*B. campestris*	*O. sativa*	*L. esculentum*
Incubation time (d)	4	14	4	8	4
FHL condition					
Control	2.1±0.8	4.8±1.7	2.0±1.1	7.7±2.8	4.4±1.1
6.92 mg/ml FHL	1.8±0.9	2.5±1.3**	1.0±0.7**	1.8±1.0**	0.6±0.2**
1.38 mg/ml FHL	2.5±0.8*	3.3±1.2**	2.3±0.9	3.8±1.9**	2.1±0.6**
0.69 mg/ml FHL	2.5±1.0*	3.5±1.0**	1.8±1.1	5.6±2.7**	2.8±0.7**
0.35 mg/ml FHL	3.0±0.9**	4.2±1.4	1.6±1.1	7.5±2.9	3.7±1.0**

Differences between mean sensitivity of treated plants and that of control plants were significant at * $P<0.05$ and ** $P<0.01$ by Student's *t*-test.

soaked in this solution, they did not germinate. However, when FHL was diluted to 0.69 and 1.73 mg/ml, the germination rate of *B. campestris* seeds was not decreased. When FHL was diluted to less than 1.73 mg/ml, the diluted FHL suppressed the root elongation of *B. campestris* seedlings (Fig. 1). The effects of organic acids, which are present in FHL, on the root elongation of *B. campestris* seedlings were assessed (Fig. 2). The concentration of each organic acid was almost the same as the concentration of the organic acid in the 1.73 mg/ml FHL solution. The root elongation of *B. campestris* seedlings was significantly inhibited with 1.5 mM acetic acid, 1.5 mM malonic acid or 1.5 mM lactic acid. Acetic acid inhibited the elongation to a greater extent than did organic acids.

Growth of *B. campestris* seedlings The properties of the soil and the nutrient content of FHL, which were used in the nursery test of *B. campestris*, are shown in Tables 1 and 7, respectively. When a chemical fertilizer was applied to the soil in which *B. campestris* seeds were grown with FHL diluted to less than 1.38 mg/ml, the fresh weight of the seedlings was heavier than that of the untreated seedlings after a 19-d incubation (Table 8). When only FHL was supplied without a chemical fertilizer, the fresh weight of the seedlings was less than that of untreated *B. campestris* seedlings because of the lack of inorganic elements.

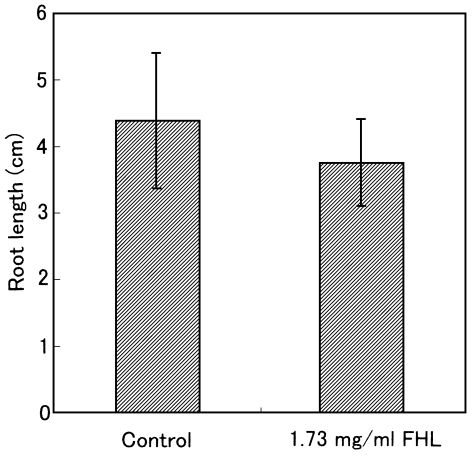

FIG. 1. Effect of FHL on root elongation of *B. campestris* L. seedlings. The seedlings of *B. campestris* L. were grown on plates with 50% concentrated Murashige–Skoog (MS) medium, 1.0% agar and organic acids in square petri dishes at 25°C for 72 h in a growth chamber. The square petri dishes with aseptically transplanted seedlings were placed vertically to measure the root lengths. Experiments were carried out with 20 seedlings.

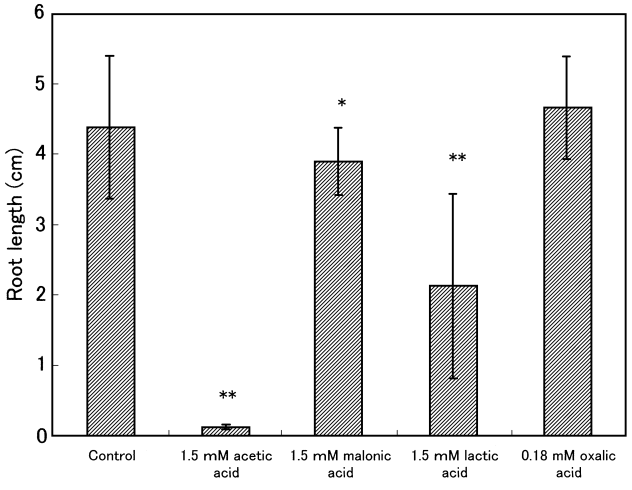

FIG. 2. Effects of organic acids on root elongation of *B. campestris* L. seedlings. The seedlings of *B. campestris* L. were grown on the plates with 50% concentrated MS medium, 1.0% agar and organic acids in square petri dishes at 25°C for 72 h in a growth chamber. The square petri dishes with aseptically transplanted seedlings were placed vertically to measure the root lengths. Experiments were carried out with 20 seedlings. Differences between mean sensitivity of treated plants and that of control plants were significant at * $P<0.05$ and ** $P<0.01$ by Student's *t*-test. Vertical lines represent SD.

TABLE 7. Nutrient content of applied FHL

Treatment		Applied FHL (g/pot)	Content (mg/pot)		
			N	P_2O_5	K_2O
FHL	0.35 mg/ml	0.15	0.4	0.2	1.5
	0.69 mg/ml	0.30	0.8	0.3	2.9
	1.38 mg/ml	0.60	1.6	1.6	5.8
FHL+CF	0.35 mg/ml	0.15	0.4	0.2	1.5
	0.69 mg/ml	0.30	0.8	0.3	2.9
	1.38 mg/ml	0.60	1.6	1.6	5.8
Control (CF)		–	–	–	–

CF, Chemical fertilizer.

DISCUSSION

Antifungal activities of FHL were assayed against *R. necatrix*, *H. mompa*, *F. oxysporum*, *P. graminicola* and *P. oryzae* FHL inhibited more than 97% of the growth of *R. necatrix*, *H. mompa*, *P. graminicola* and *P. oryzae*, and 65% of the growth of *F. oxysporum* (Table 2). The pHs of the FHL plate and the organic acid-mixture plate containing 30 mM acetic acid, 30 mM malonic acid, 30 mM lactic acid and 3.6 mM oxalic acid were 4.1 and 2.6, respectively (Table 2). The antifungal activities of the organic acid-mixture against *R. necatrix*, *H. mompa*, *P. graminicola* and *P. oryzae* were as high as those of FHL, but the antifungal activity of the organic acid-mixture against *F. oxysporum* was higher than that of FHL. As the pH of the organic acid solution decreased, the concentration of undissociated organic acids increased (20). It was suggested that more of the carboxyl groups of the organic acids in organic acid-mixture existed in undissociated forms than in the FHL because the pH of the organic acid-mixture was lower than that of FHL. Undissociated organic acids are more hydrophobic and more cytosol-permeable than dissociated organic acids and this permeation causes the destruction of the proton-motive force with higher frequency to restrict substrate transport (20, 21). These phenomena caused by undissociated organic acids increased the antimicrobial activity. Moreover, when the pH of the organic acid-mixture was adjusted to 4.0, the antifungal activities against five phytopathogenic filamentous fungi were the same as those of the organic acid-mixture without pH adjustment (data not shown). When the antifungal activities of 30 mM acetic acid, 30 mM malonic acid, 30 mM lactic acid or 3.6 mM oxalic acid were assayed, only 30 mM acetic acid had antifungal activities against all fungi. It was suggested that acetic acid in FHL mainly inhibited the growth of phytopathogenic filamentous fungi among the tested organic acids.

When FHL was diluted to 17.3 mg/ml with PDA, the diluted FHL had antifungal activities against *R. necatrix*, *H. mompa*, *P. graminicola* and *P. oryzae*. When FHL was diluted to 6.92 mg/ml with PDA, the diluted FHL had antifungal activities against only *P. graminicola* (data not shown). When the antifungal activities of diluted FHL against phytopathogenic filamentous fungi were compared to those against *T. rubrum* and *T. mentagrophytes*, the antifungal activities of FHL against *R. necatrix*, *H. mompa*, *P. graminicola* and *P. oryzae* were higher than those against *T. rubrum* and *T. mentagrophytes* (15). It was suggested that FHL would need to be applied at less than 6.92 mg/ml when it was used to control the growth of phytopathogenic filamentous fungi.

FHL treated with pepsin or trypsin exhibited lowered antifungal activity against *F. oxysporum* (Table 3), suggesting that some proteinaceous compound(s) in FHL could suppress the growth of *F. oxysporum*. However, since the pH of FHL is not optimum for trypsin, trypsin might not function as a proteinase to digest proteinaceous compound(s) in FHL. Lactic acid bacteria (enterococci) are known to produce antimicrobial peptides called bacteriocins (22). However, there are no reports that enterococci produce antifungal peptides. Tricine SDS–PAGE detected a peptide of which the molecular weight was about 5000 in FHL, but the antifungal activity of the peptide against *T. rubrum* was not detected using the agar dilution method (data not shown). A microassay of the antifungal activity of the peptide would need to be established.

When the germination rates of *M. sativa*, *A. officinalis*, *B. campestris*, *O. sativa*, *S. oleracea*, *F. arundinacea*, and *L. esculentum* were determined on FHL plates at concentrations ranging from 0.35 to 6.92 mg/ml, those of *A. officinalis*, *S. oleracea*, *F. arundinacea*, and *L. esculentum* decreased on the plates containing 6.92 mg/ml FHL while those of *M. sativa*, *B. campestris*, and *O. sativa* did not. When *M. sativa*, *A. officinalis*, *B. campestris*, *O. sativa*, *S. oleracea*, *F. arundinacea*, and *L. esculentum* were seeded on vermiculite and were sprinkled with water or FHL diluents, the germination rates showed a similar tendency to those on the plates containing FHL (data not shown). Therefore, it was concluded that sterilization did not affect the germination rates of crop seeds. The root elongation of *L. esculentum* on the plates containing 6.92 mg/ml FHL was markedly suppressed. With regard to *A. officinalis*, *B. campestris*, and *O. sativa*, the inhibition of developing root hairs was observed when these seeds were placed on the plate containing 6.92 mg/ml FHL. Furthermore, the germination rates of *L. esculentum* on the 6.92 mg/ml FHL plates were 5% on the 2nd day (Table 5), but 93% on the 4th day (data not shown). It was suggested that the 6.92 mg/ml FHL solution could delay the development of seedlings.

When the diluted FHL was applied to *B. campestris* seeds, the FHL solution at less than 1.73 mg/ml did not affect the germination rate. The root elongation of *B. campestris* seedlings treated with an organic acid was significantly inhibited for 1.5 mM acetic acid, 1.5 mM malonic acid or 1.5 mM lactic acid (Fig. 2). FHL diluted to 17.3 mg/ml inhibited the germination of *B. campestris* seeds, while FHL at 1.73 and 17.3 mg/ml suppressed the root elongation of *B. campestris*. Less than 1.21 mg/ml FHL did not affect either the germination rate or the root elongation (data not shown). Moreover, the germination rates of *B. campestris* seeds treated with FHL diluted to less than 1.38 mg/ml were the same as the control after incubation for 72 h. When the seedlings of *B. campestris* were grown in the presence of a sufficient amount of chemical fertilizer, less than 1.38 mg/ml FHL did not inhibit the growth of the seedlings (Table 8). It was revealed that the diluted FHL did not have antifungal activities against phytopathogenic filamentous fungi when it was diluted to the concentration that did not inhibit the germina-

TABLE 8. Effect of FHL on growth of *B. campestris* L.

Treatment		Germination rate (%)			Leaf length (cm)		Fresh weight (g)	
		24 h	48 h	72 h	5 d	19 d	19 d	(%)[b]
FHL	0.35 mg/ml	60	90	100	2.1	5.4	5.0	69
	0.69 mg/ml	55	85	100	2.2	5.5	5.6	78
	1.38 mg/ml	58	85	100	2.0	5.4	5.6	78
FHL+CF[a]	0.35 mg/ml	48	88	100	2.1	8.7	10.6	147
	0.69 mg/ml	55	90	100	2.0	8.5	11.4	158
	1.38 mg/ml	58	88	100	2.0	8.8	11.4	158
Control (CF)		60	93	98	2.0	8.3	7.2	100

[a] CF, Chemical fertilizer.
[b] Values are expressed as the percentage of fresh weight of FHL-treated *B. campestris* L. on 19 d relative to fresh weight of the control on 19 d.

tion rate and growth of *B. campestris*. These data suggested that organic matter or manure with antifungal activity, for example FHL, could be suitable for spreading in the field before cultivation to reduce the harmful effect of organic acids on the germination of plants. FHL containing organic acids could become a natural soil supplement supplied commercially.

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