

## Herbicidal potential of rhizosphere soil fungi of passion fruit (*Passiflora edulis Sims.*) and identification of potent herbicidal compounds

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### ABSTRACT

We screened the fermentation broth of fungi from the rhizosphere soil of passion fruit (*Passiflora edulis Sims.*) for their herbicidal potential in bioassays and Pot culture. The potent herbicidal compounds of the fungi fermentation broth were evaluated by bioassay and pot test. The candidate substances in the fungi fermentation broth were analysed and identified. Nine fungi (No. 1, 2, 3, 4, 5, 6, 7, 8 and 9) were isolated from the rhizosphere soil of passion fruit using the plate dilution coating method. In Petriplate Bioassay, the fermentation broth of newly identified fungus *Aspergillus sydowi* 'FJFAFU01' drastically inhibited the growth of lettuce (*Lactuca sativa*) and barnyard grass (*Echinochloa crus-galli*). In Pot Culture, 50- times diluted fermentation broth inhibited the barnyard grass height by 42.73%. The HPLC and GC-MS analysis showed that the fermentation broth contained higher contents of nitrogenous compounds and oxygen compounds, including phenolic acids, terpenoids, flavonoids and alkaloids. The *Aspergillus sydowi* FJFAFU01 screened from the rhizosphere soil of passion fruit contained phenolic acids, terpenoids, flavonoids and alkaloids, these inhibited the growth of barnyard grass. Thus *Aspergillus sydowi* 'FJFAFU01' may be developed as potential mycoherbicide.

**Key words:** *Aspergillus sydowi*, fungus, mycoherbicide, Passion fruit (*Passiflora edulis Sims.*), rhizosphere soil.

### INTRODUCTION

Weeds are one of the major constraints in crop yield reduction, they compete with crops for growth resources (water, fertilizer, light etc.) and are also intermediate hosts for diseases and pests, causing crop diseases and pests (31). Presently chemical herbicides are mainly used to control weeds, but their long-term use has resulted in a several problems viz., herbicide residues, environmental contamination and development of herbicide-resistant weeds (19,28). It is a challenge to develop environmentally friendly substitutes for weed control (4,6). Microbial herbicides are produced by living microorganisms and their metabolites have become a research hotspot due to their pollution-free environment (12,20). Fungal herbicides, because of their high specificity and selectivity to target weeds and no residue in the environment, have been attracted great attention of researchers (20,26).

Passion fruit (*Passiflora edulis Sims.*) is herbaceous vine of *Passiflora* (33). It is an edible, delicious and nutritious fruit called king of natural fruit juice (3). In recent years it has been planted on large-scale (21,37). However, its continuous planting in the same field

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Figure 1. Donor specie passion fruit vine in population (*Passiflora edulis Sims.*) and single plant

caused severe soil toxicity (27). Soil toxicity, known as “soil disease”, has been widely reported in many agricultural crops and is associated with mycotoxins in the soils (9,24). The continuous cropping can lead to the accumulation of harmful substances released by plants and also increases the pathogenic microorganisms in soil (18,39). Screening and selection of specific soil microorganisms with weed suppression capabilities is an important method to select bioherbicides (7,13,23). Our previous studies showed that fungi screened from the rhizosphere soil of *Eucalyptus* and allelopathic rice could be used as mycoherbicides to control paddy weeds (17,34). This study aimed to investigate the herbicidal potential of fungi screened from the rhizosphere soil of passion fruit, identify the weed-inhibitory substances in fungi fermentation broth and evaluate their weed suppression potential.

## MATERIALS AND METHEODS

Mature barnyard grass (*Echinochloa crus-galli*) seeds were collected from rice fields of Fujian Agriculture and Forestry University. Lettuce (*Lactuca sativa*) seeds were purchased from Fuzhou Yongrong Seed Co. Ltd.

### Fungi isolation

Ten plants were chosen randomly and dug the roots up to 40 cm depth. The soils attached to the roots were collected as the rhizosphere soils. The rhizosphere soil was collected from 3-years old passion fruit (*Passiflora edulis Sims.*) in Xinluo town, Longyan City, Fujian Province, China [250.02° N and 116.13° E, altitude : 600 ~ 700 m, annual rainfall : 1700 mm, relative humidity : 80 %, annual average temperature : 16-20 °C]. The

collected soil samples were brought to the laboratory in an ice box for fungal isolation. The fungi were isolated using the standard procedure for soil fungi isolation (1). Briefly, 10 g fresh soil was placed in 90 mL sterile water and shaken at 130 rpm for 30 min to obtain a suspension of  $10^{-1}$  g soil/mL solution. The suspension was further diluted to 3-concentration gradients ( $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$  g soil/mL solution). The 0.5 mL of each diluted suspensions was added to sterilized 15 cm dia Petri dish with Martin's Rose Bengal (MRB) agar medium (containing chloramphenicol 1 g/L and gentamycin 2 mL/L) to inhibit the bacterial growth. The following experiments were repeated thrice. The Petri dishes were incubated at 28 °C in dark for 4 days, thereafter, according to the morphological characteristics of each colony, one strain with different growth characteristics was selected and transferred to MRB agar and was purified thrice. Nine fungal colonies were selected and stored on Potato Dextrose Agar (PDA) medium slants at 4 °C.

Each of the 9 strains were mass fermented in Potato Dextrose Broth (PDB) as per the fermentation conditions (17). Briefly, the strain was inoculated into a medium containing 100 mL of PDB and shaken at 120 rpm for 7 days. The resulting fermentation broths were filtrated with 0.22 µm membrane for the subsequent experiments.

#### Bioassays

(i). **Lettuce** : The fungal fermentation broth's inhibitory potential was first evaluated on lettuce (*Lactuca sativa*) as the receptor as per the method of Zhang *et al.* (34). Briefly, 5-pre-germinated seeds of lettuce (*Lactuca sativa*) were placed in a 250 mL beaker lined with filter paper and added 5 mL fermentation broths (diluted 50 times). The control was 5 mL of distilled water. The beakers were placed in an incubator at  $28 \pm 2$  °C, with 12 h (8:00 - 20:00) light of  $70 \mu\text{mol m}^{-2} \text{s}^{-1}$  intensity. The treatments were replicated thrice in complete randomised design. Three days after treatment, the root length and plant height of lettuce seedlings were measured.

(ii). **Barnyard grass** : The fungal fermentation broths showing > 20 % growth inhibition of lettuce were further evaluated on barnyard grass (*Echinochloa crus-galli*) as the receptor, following the above procedure. The fermentation broth was diluted 20 times. The treatments were replicated thrice in complete randomised design. Then the strains with highest weed suppression capabilities was selected.

#### Pot culture

There were 4 -treatments : TM<sub>1</sub>, TM<sub>2</sub>, TM<sub>3</sub>, TM<sub>4</sub> (undiluted fermentation broth, 5-times diluted fermentation broth, 20-times diluted fermentation broth and 50-times diluted fermentation broth, respectively). Thirty mL of undiluted and diluted fermentation broth were added to the plastic pots (12 cm dia × 6 cm height) with 300 g sterilized soil and 5 germinated seeds of barnyard grass (*Echinochloa crus-galli*) were sown per pot. The control was added 30 mL of sterile PDB. The treatments were replicated thrice in complete randomised design. All the plastic pots were placed in an incubator at  $28 \pm 2$  °C, with 12 h (8:00-20:00) light of  $70 \mu\text{mol m}^{-2} \text{s}^{-1}$  intensity. After 3 days, the plant height was measured and the shoot was dried in oven at 105 °C for 15 min, and thereafter again at 80 °C for 2 days to constant weight.

### Identification of fungal isolate

The morphology of mycelia and conidia of the selected fungal isolate was studied under microscope as per Wei (29). Based on the morphology of mycelium and conidia, the fungus was identified as *Aspergillus sydowii*. Molecular identification of the fungi was based on the sequencing of ITS sequence; the genomic DNA of the selected fungus isolate was extracted and purified by Fungal Genome Extraction Kit (Sangon Biotech, Shanghai, China), based on the specific primers for fungal identification, upstream primer ITS1: 5'-TCCGTAGGTGAACCTGCGG-3', downstream primer ITS4: 5'-TCCTCCGCTTATTGATATGC-3' to amplify the ITS sequences of the strains. The size of the target fragment was approximately 542 bp. The PCR amplification system contained 25  $\mu$ L, containing 2 x TaKaRa Taq TM HS Perfect Mix (Takara, Dalian, China) 3.7  $\mu$ L, 0.5  $\mu$ L of each upstream primer and downstream primer (20  $\mu$ g/mL), DNA 0.5  $\mu$ L and sterile deionized water was added to 25  $\mu$ L. The PCR programme was done as under: Amplification conditions were initial denaturation at 95 °C for 4 min, cycle parameters were 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min. After 30 cycles, extension was performed at 72 °C for 10 min, 5  $\mu$ L of that PCR products were separated on 1.0 % agarose gel, and its purity was determined by the Gel Extraction Kit (Omega Bio-Tek, GA, USA) and then ligated into the pBackZero8-T vector with cloning kit (Takara, Dalian, China). In each case, five positive clones were randomly selected and sequenced by Sangon Biotech (Shanghai) Co. Ltd. The sequence information was analyzed and compared by the National Center for Biotechnology Information (NCBI). A phylogenetic tree, based on the ITS sequence of the fungus No.5, was constructed by implementing neighbor-joining using MEGA 6.05. Bootstrap analysis was performed with 1000 replications.

### Analysis of phenolic acids in fermentation broth

The contents of phenolic acids in the fermentation broth were quantified by HPLC (17,34). Seven phenolic acids (protocatechuic acid, *p*-hydroxybenzoic acid, syringic acid, vanillic acid, salicylic acid, ferulic acid and cinnamic acid) were purchased from Aladdin Industrial Corporation, Shanghai, China and were chosen as standards for the calibration curve. The HPLC instrument was an Agilent 1206 (Agilent Technologies, USA) equipped with a C18 reversed phase column (ZORBAX SB-C18, 150 mm  $\times$  4.6 mm, 5  $\mu$ m). The mobile phase was 0.1 % phosphoric acid (A) and methanol (B) with the following gradient elution programme: A : B = 73:27 (0-10 min), A : B = 50:50 (10-15 min) and A : B = 73:27 (15-20 min). The injection volumes were 10  $\mu$ L. The detection wavelength was 280 nm. The flow rate of mobile phase was 1.6 mL/min. The column temperature was 30 °C. The contents of single phenolic acids in the fermentation broth were determined from the standard references. All tested solutions were repeated thrice.

### Analysis of weed-inhibitory substances in fermentation broth

Fifty mL of fermentation broth was extracted thrice with 150 mL of ethyl acetate, petroleum ether and n-butanol, respectively. The three extracts of each solvent were combined and concentrated to dryness by rotary evaporator, then added into 50 mL

distilled water (containing 0.01 % DMSO). The experiment was repeated thrice. 3-aqueous solutions were evaluated in bioassays on lettuce as the receptor described above.

The ethyl acetate extracts proved most inhibitory to growth of lettuce (Fig. 8), hence, it was further used for analysis of weed-inhibitory substances. The 50 mL fermentation broth (containing 1  $\mu$ L/L dodecane as the internal standard) was extracted with ethyl acetate, then concentrated into dry powder and redissolved in 1 mL of ethyl acetate. The experiment was repeated thrice. These extracts were used for the analysis of weed-inhibiting substances by GC-MS (16). Briefly, the instrument was Agilent 7890-5975A GC-MS spectrometry (Agilent Technologies, USA) equipped with a DB-5 ms column (30 m  $\times$  250  $\mu$ m  $\times$  0.25  $\mu$ m, V Agilent Technologies, USA). The sample was injected at 280  $^{\circ}$ C with a split of 1/30 and a constant helium flow of 1.0 mL/min. The temperature programme started at 60  $^{\circ}$ C for 1 min, followed by 10  $^{\circ}$ C/min to 170  $^{\circ}$ C for 2 min, 5  $^{\circ}$ C/min to 280  $^{\circ}$ C for 5 min, 20  $^{\circ}$ C/min to 300  $^{\circ}$ C for 1.5 min, and 300  $^{\circ}$ C with a hold for 1 min. The mass scan range was set at 40-220 m/z in the 70-eV electron impact ionization mode. The substances were identified and quantified by comparing the spectra with NIST9.0. Quantitative analysis was performed by the internal standard method (16).

#### Evaluation of weed suppression of candidate substances

The 11 candidate substances, identified by GC-MS, were evaluated for weed suppression in laboratory bioassay (36). The 5 candidate substances (protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, salicylic acid and cinnamic acid) chosen from the results of HPLC were also evaluated for the weed suppression in laboratory bioassay (36). Lettuce was used as the receptor. These compounds were tested at 3-different concentrations as under:

Table. Test compounds concentrations used

Concentrations ( $\mu$ mol/L)	Test Compounds
250,500, 1000	Cholicacid, <i>o</i> -coumaric acid, 2,4-decadienal, $\alpha$ -pinene, linoleic acid, protocatechuic acid, <i>p</i> -hydroxybenzoic acid, vanillic acid, salicylic acid, and cinnamic acid
100, 500, 1000	Proline, tyrosine, coumarin, and vanillin
100,110,150	Citral
10,25,125	Piperine

The procedure was same as described above. The treatments were replicated thrice in complete randomised design. The root length and plant height of lettuce were measured after 3 days.

#### Statistical analysis

All experimental data were presented as the mean  $\pm$  standard error (SE). To determine any significant differences among treatments, a one-way ANOVA followed by the least significant difference (LSD) at a 5 % level of probability. Statistical analysis was done using the DPS 7.0 program % inhibition = (1 - treatment/control)  $\times$  100 %.

## RESULTS AND DISCUSSION

### Evaluation of weed suppression potential of fungi to weed

Using plate dilution coating method, 9 fungi were isolated from the rhizosphere soil of passion fruit. The bioassay results showed that 6 fungi (No. 1, 2, 3, 4, 5 and 8) caused  $> 70\%$  inhibition in root length of lettuce (*Lactuca sativa*) and 4 fungi (No. 2, 3, 5 and 8) caused  $> 20\%$  inhibition in plant height of lettuce (Fig. 2). The fungi strain No. 5 caused maximum inhibition (100%) in plant height of lettuce than other fungi ( $p < 0.05$ ).

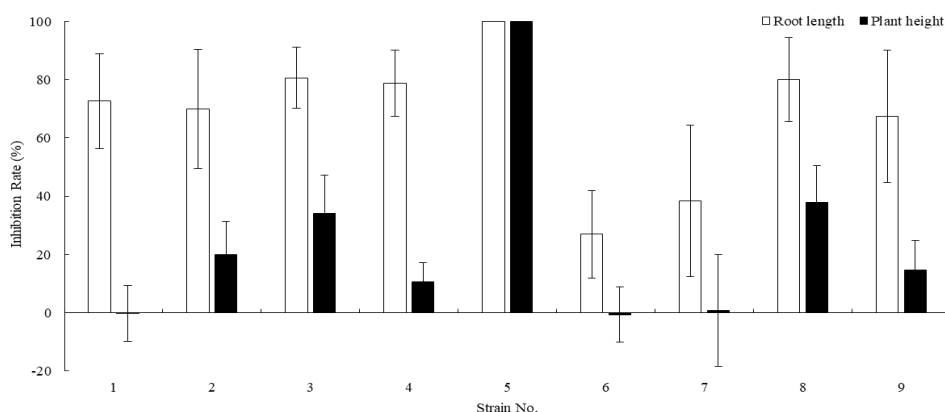


Figure 2. The inhibitory effects of fermentation broths of 9-fungi isolates on seedlings growth of lettuce. The bars represent standard errors of the mean ( $n = 3$ ).

The isolate of fungi No. 3, 5 and 8 significantly ( $p < 0.05$ ) inhibited the root length and plant height of barnyard grass (*Echinochloa crus-galli*) (Fig. 3), but No. 5 proved most inhibitory to the root length and plant height of lettuce and barnyard grass. Hence, the No. 5 isolate was selected for further study.

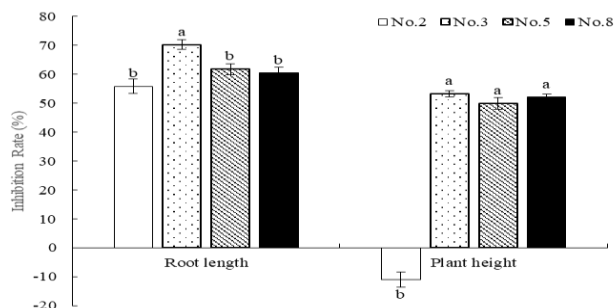


Figure 3. The inhibitory effects of fermentation broths of fungi strains on the growth of barnyard grass. The fermentation broth was diluted 20 times. The bars represent standard errors of the mean ( $n = 3$ ). The different lowercase letters indicated significant difference between treatments and controls at  $p < 0.05$  level.

The pot culture results showed that the fermentation broth of No. 5 fungi strain significantly inhibited the growth of barnyard grass. The inhibition followed the order: TM1 > TM3 > TM2 > TM4 (Fig. 4). The 50-times diluted fermentation broth reduced the barnyard grass height and dry weight 42.73 % and 5.04 %, respectively (Fig. 4).

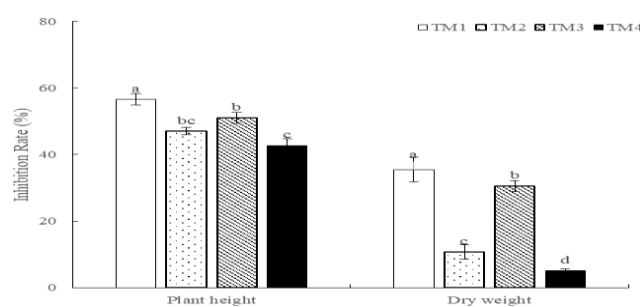


Figure 4. The inhibition (%) of fermentation broth of No. 5 strain on barnyard grass in pot culture with soil. TM1: undiluted fermentation broth, TM2: 5-times diluted fermentation broth, TM3: 20-times diluted fermentation broth, TM4: 50-times diluted fermentation broth. The bars represent standard errors of the mean (n= 3). The different lowercase letters indicated significant difference between treatments and controls at  $p < 0.05$  level.

#### Identification of Fungi No. 5 strain

The colonies of isolated fungi No. 5 strain on PDA medium were light brown in the center, gray-green in the middle layer and white on the edge (Fig. 5A). The surface was dry, velvet and dense with concentric rings. The bottom was milky white and there were no exudates on the surface. The microscopic examination showed septate vegetative hyphae and the sporogenous structure of hyphae was non-septate and thicker than the vegetative hyphae (Fig. 5B). This isolate No. 5 was tentatively identified as *Aspergillus sydowi*, species based on the description by Wei (30).

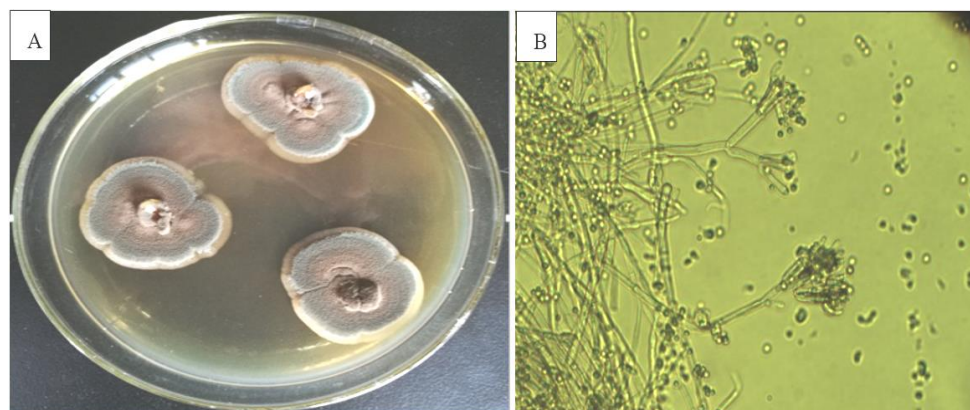


Figure 5. The morphological characteristics of colony and mycelium of No. 5. A: Colony shape.

B: Conidiophore and conidia ( $\times 400$ ).

Further, the ITS sequence of the fungi No. 5 strain (Fig. 6) was performed by BLAST homology alignments online at NCBI (NCBI ID: MT071285). Seven strains of *Aspergillus sydowi*, which had the closest relationship with No. 5 strain were screened from the comparison of results (99 % similarity). Moreover, it belongs to the same clade as the *Aspergillus sydowi* and *Aspergillus versicolor* strain 2-6F (GenBank No. MW077051) was used as out-group (Fig. 7). These results showed that the fungi No. 5 strain is a strain of *Aspergillus sydowi* and was named as *Aspergillus sydowi* strain FJFAFU01.

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TGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAACCTGAAGAAAATGGTTGGAGACGTCGG
CTGGCGCCCGCCGGCCCTAGTCGAGCGGGTGATAAAGCCCCATACGCTCGAGGACCGGACACGGTGCCGCCG
CTGCCCTTCGGGCCCGTCCCCGGGGGGGACGACGCCAACACACAAGCCGGGCTTGATGGCGACCAATGA
CGCTCGGACAGGCATGCCCCCGGAATGCCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCCTGAAT
TCTGCAATTCACATTACTTATCGCAGTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTGTTGAAA
GTTTTGACTGATTTTATATTCAGACTCAGACTGCATCACTCTCAGGCATGAAGTTCAGTAGTCCCCGGCGGCTCG
CCCCGAGGGAGCTCCCCGCCGAAGCAACAGTGTAGGTATTACGGGTGGGAGGTTGGGCGCCCGGAGGCA
GCCCCGACTCAGTAATGATCCTTCCGCA
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Figure 6. The ITS sequence of *Aspergillus sydowii* FJFAFU01.

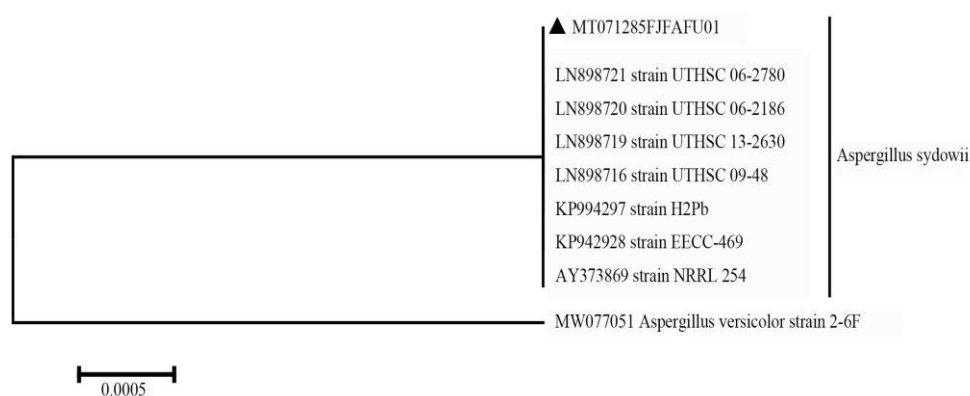


Figure 7. Phylogenetic relationship tree based on the ITS sequence. The tree was generated with MEGA 6.05 software using the neighbor-joining method (bootstrap=1000). The scale bar represents the number of nucleotide substitutions per site. Reference sequences obtained from GenBank are indicated by accession number and strain name. The *Aspergillus sydowii* strain FJFAFU01 was indicated with black triangle (▲). *Aspergillus versicolor* strain 2-6F (GenBank No. MW077051) was used as out-group.

#### Analysis of weed-inhibitory substances in fermentation broth of *A. sydowii* FJFAFU01

Five phenolic acids were detected in fermentation broth of *A. sydowii* FJFAFU01 by HPLC (Table 1). The content of each phenolic acid in the fermentation broth were 0.78

mg/L of protocatechuic acid, 1.05 mg/L of *p*-hydroxybenzoic acid, 4.19 mg/L of vanillic acid, 28.17 mg/L of salicylic acid and 33.39 mg/L of cinnamic acid. The total contents of the 5 phenolic acids were approximately 67.58 mg/L.

Table 1. The contents of phenolic acids in the fermentation broth of *A. sydowii* FJFAFU01

Compound	PA	HA	SY	VA	SA	FA	CA
Content (mg/L)	0.78±0.13	1.05±0.16	ND	4.19±0.71	28.17±2.33	ND	33.39±2.39

Means ± standard error (SE) of three replications. PA: protocatechuic acid, HA: *p*-hydroxybenzoic acid, SY: syringic acid, VA: vanillic acid, SA: salicylic acid, FA: ferulic acid, CA: cinnamic acid, ND : Not detected.

The bioassay results showed that the inhibitory effects of extracts by different organic solvents on the growth of lettuce followed order : ethyl acetate > petroleum ether > n-butanol (Fig. 8). The inhibition rates of ethyl acetate extract on root length and plant height of lettuce were 90.91 % and 64.66 %, respectively and significantly different from other treatments.

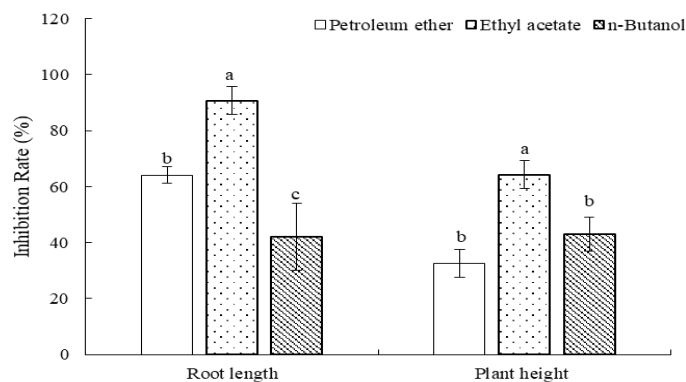
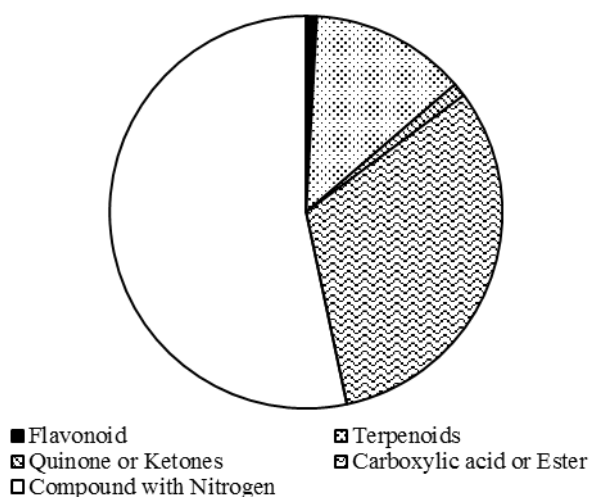


Figure 8. The inhibitory effects of organic extracts from fermentation broth of *A. sydowii* FJFAFU01 on the growth of lettuce. The bars represent standard errors of the mean (n= 3). The different lowercase letters indicated significant difference between treatments and controls at  $p < 0.05$  level.

The GC-MS results showed the presence of 78 compounds in the ethyl acetate extract of fermentation broth (Table 2). Among these, 46 were nitrogenous compounds (859.91 mg/L) including amino acids, amines and amides, and numerous heterocyclic alkaloids. There were 32 oxygen-containing (nitrogen-free) compounds, including 19 carboxylic acids (including phenolic acids) and their esters (513.58 mg/L), 4 ketones (18.26 mg/L) and 7 terpenoids (206.94 mg/L) and 2 of flavonoids (14.13 mg/L). Thus, the main components in the fermentation broth were nitrogenous compounds, carboxylic acids and terpenoids (Fig. 9).

Figure 9. The contents of each substance in the fermentation broth of *A. sydowii* FJFAFU01Table 2. Compounds identified in the fermentation broth of *A. sydowii* FJFAFU01

Compounds	Formula	Content (mg/L)
<b>Nitrogenous compounds</b>		
4,6-Dihydroxybenzofurazan	C <sub>6</sub> H <sub>4</sub> N <sub>2</sub> O <sub>3</sub>	23.71±4.24
4-Methoxyanthranilic acid	C <sub>8</sub> H <sub>9</sub> NO <sub>3</sub>	3.44±0.15
Butyric acid, 4-(2-carbamoylpyrrolidin-1-yl)-4-oxo-	C <sub>9</sub> H <sub>14</sub> N <sub>2</sub> O <sub>4</sub>	31.75±6.91
D-Proline	C <sub>5</sub> H <sub>9</sub> NO <sub>2</sub>	3.22±0.72
DL-Threonine, N-glycyl-	C <sub>6</sub> H <sub>12</sub> N <sub>2</sub> O <sub>4</sub>	5.22±0.48
dl-Alanyl-l-leucine	C <sub>9</sub> H <sub>18</sub> N <sub>2</sub> O <sub>3</sub>	47.90±9.34
Octanoic acid, 2-amino-	C <sub>8</sub> H <sub>17</sub> NO <sub>2</sub>	41.06±5.82
Tyrosine	C <sub>9</sub> H <sub>11</sub> NO <sub>3</sub>	46.40±8.29
dl-Phenylalanine, N-acetyl-	C <sub>11</sub> H <sub>13</sub> NO <sub>3</sub>	9.06±1.33
Pyrrolid-2-one-5-methanol, N-methyl-, acetate	C <sub>8</sub> H <sub>13</sub> NO <sub>3</sub>	3.48±0.85
2-Pyrrolidinone	C <sub>4</sub> H <sub>7</sub> NO	20.74±3.81
2,4-Dimethyl-3-phenyl-isoxazol-5(2H)-one	C <sub>11</sub> H <sub>11</sub> NO <sub>2</sub>	1.99±0.23
(E,S)-2-Pentenoic acid, 4-amino-5-phenyl-, methyl ester	C <sub>12</sub> H <sub>15</sub> NO <sub>2</sub>	10.56±2.74
Fumaric acid, 2-decyl tridecyl ester	C <sub>10</sub> H <sub>17</sub> NO <sub>5</sub>	2.46±0.29
Histamine, N-benzoyl-2-cyano-	C <sub>13</sub> H <sub>12</sub> N <sub>4</sub> O	21.93±3.04
Octopamine	C <sub>8</sub> H <sub>11</sub> NO <sub>2</sub>	3.33±0.67
Benzoic acid, 2-amino-, methyl ester	C <sub>8</sub> H <sub>9</sub> NO <sub>2</sub>	1.15±0.35
Guanidine	CH <sub>5</sub> N <sub>3</sub>	4.49±1.24
L-Prolinamide	C <sub>5</sub> H <sub>10</sub> N <sub>2</sub> O	10.33±3.15
Hexadecanamide	C <sub>16</sub> H <sub>33</sub> NO	2.71±0.23
Piperazine, 1-(2-phenylacetyl)-	C <sub>12</sub> H <sub>16</sub> N <sub>2</sub> O	2.89±0.72
Pyracarbolid	C <sub>13</sub> H <sub>15</sub> NO <sub>2</sub>	16.22±4.82
9-Octadecenamide, (Z)-	C <sub>18</sub> H <sub>35</sub> NO	13.75±4.77
Phensuximide	C <sub>11</sub> H <sub>11</sub> NO <sub>2</sub>	0.66±0.21
2-Pyrrolidinone	C <sub>4</sub> H <sub>7</sub> NO	5.69±1.83
2-Piperidone	C <sub>5</sub> H <sub>9</sub> NO	15.41±4.06

2,4-Imidazolidinedione, 5-methyl-5-(2-methylpropyl)-	C <sub>8</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	4.80±0.71
1H-Indol-5-ol	C <sub>8</sub> H <sub>7</sub> NO	2.97±0.20
Quinoline-4-carbonitrile, 2-methyl-	C <sub>11</sub> H <sub>8</sub> N <sub>2</sub>	8.00±1.07
2,5-Piperazinedione, 3-methyl-6-(1-methylethyl)-	C <sub>8</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	8.51±2.92
1H-1,2,4-Triazole-3-methanol, 5-amino-	C <sub>3</sub> H <sub>6</sub> N <sub>4</sub> O	9.25±1.09
Hydouracil, 1-methyl-	C <sub>5</sub> H <sub>8</sub> N <sub>2</sub> O <sub>2</sub>	5.15±1.35
2-Benzimidazolinethione, hexahydro-	C <sub>7</sub> H <sub>12</sub> N <sub>2</sub> S	7.00±2.73
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	C <sub>11</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	226.15±8.09
5-Azauracil	C <sub>3</sub> H <sub>3</sub> N <sub>3</sub> O <sub>2</sub>	7.76±1.31
1-Benzyl-3-methyl-2,4,5-trioximidazolidine	C <sub>11</sub> O <sub>10</sub> N <sub>2</sub> O <sub>3</sub>	11.20±3.22
2,5-Piperazinedione, 3-benzyl-6-isopropyl-	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	5.17±1.82
2,5-Piperazinedione, 3-(hydroxymethyl)-6-(phenylmethyl)-	C <sub>12</sub> H <sub>14</sub> N <sub>2</sub> O <sub>3</sub>	9.63±2.18
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	C <sub>14</sub> H <sub>16</sub> N <sub>2</sub> O <sub>2</sub>	43.18±10.30
2,5-Piperazinedione, 3-(phenylmethyl)-	C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	2.15±0.92
3-Benzylidene-hexahydro-pyrrolo[1,2-a]pyrazin-1,4-dione	C <sub>14</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	2.06±0.45
1H-Indole, 5-methyl-2-phenyl-	C <sub>15</sub> H <sub>13</sub> N	1.75±0.17
Cyclo-(1-leucyl-1-phenylalanyl)	C <sub>15</sub> H <sub>20</sub> N <sub>2</sub> O <sub>2</sub>	91.21±20.69
1H-Indole, 1-methyl-2-phenyl-	C <sub>15</sub> H <sub>13</sub> N	4.90±1.93
Cyclohexanamine, 5-methyl-2-(1-methylethyl)-, (1 $\alpha$ ,2 $\alpha$ ,5 $\beta$ )-	C <sub>10</sub> H <sub>21</sub> N	49.35±13.92
Menthylamine	C <sub>10</sub> H <sub>21</sub> N	10.97±3.07
<b>Subtotal</b>		<b>859.91</b>
<b>Carboxylic acid or Ester</b>		
Benzoic acid, 4-hydroxy-3-methoxy-	C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>	15.68±5.26
Benzoic acid, 3,5-dihydroxy-	C <sub>7</sub> H <sub>6</sub> O <sub>4</sub>	42.63±10.51
4-Methoxy-3,5-dihydroxybenzoic acid	C <sub>8</sub> H <sub>8</sub> O <sub>5</sub>	36.70±9.83
Benzoic acid, 3,4,5-trihydroxy-	C <sub>10</sub> H <sub>12</sub> O <sub>5</sub>	79.57±16.39
3,4-Dihydroxy-5-methoxybenzaldehyde	C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>	11.09±3.28
trans-Cinnamic acid	C <sub>9</sub> H <sub>8</sub> O <sub>2</sub>	3.84±0.96
Tricarballic acid	C <sub>6</sub> H <sub>8</sub> O <sub>6</sub>	207.29±38.16
5-Phenyl-piperonylic acid	C <sub>14</sub> H <sub>10</sub> O <sub>4</sub>	20.07±8.47
Acetic acid, phenyl-, isopentyl ester	C <sub>13</sub> H <sub>18</sub> O <sub>2</sub>	2.31±1.96
dl-Mevalonic acid lactone	C <sub>6</sub> H <sub>10</sub> O <sub>3</sub>	8.04±2.71
2-Propenoic acid, 3-phenyl-, phenyl ester, (E)-	C <sub>15</sub> H <sub>12</sub> O <sub>2</sub>	3.53±0.88
Benzeneacetic acid, 4-(1,1-dimethylethyl)-, methyl ester	C <sub>13</sub> H <sub>18</sub> O <sub>2</sub>	3.74±0.95
Fumaric acid, 3-methylbut-3-enyl pentadecyl ester	C <sub>24</sub> H <sub>42</sub> O <sub>4</sub>	26.13±5.90
Ethyl citrate	C <sub>12</sub> H <sub>20</sub> O <sub>7</sub>	13.74±3.47
Cyclopentanecarboxylic acid, 3-tetradecyl ester	C <sub>20</sub> H <sub>38</sub> O <sub>2</sub>	26.71±8.36
1-Methyl-2-oxocyclohex-3-enecarboxylic acid, methyl ester	C <sub>9</sub> H <sub>12</sub> O <sub>3</sub>	3.12±0.59
4,8,12,16-Tetramethylheptadecan-4-olide	C <sub>21</sub> H <sub>40</sub> O <sub>2</sub>	1.46±0.31
Valeric acid, undec-2-enyl ester	C <sub>16</sub> H <sub>30</sub> O <sub>2</sub>	1.21±0.25
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester	C <sub>35</sub> H <sub>62</sub> O <sub>3</sub>	6.73±1.06
<b>Subtotal</b>		<b>513.58</b>
<b>Ketones</b>		
2,4-Cyclohexadien-1-one, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-	C <sub>14</sub> H <sub>22</sub> O <sub>2</sub>	1.86±0.26
1,4-Naphthalenedione, 5-hydroxy-	C <sub>10</sub> H <sub>6</sub> O <sub>3</sub>	8.42±1.09
3(2H)-Benzofuranone	C <sub>8</sub> H <sub>6</sub> O <sub>2</sub>	6.16±2.61
2(3H)-Furanone, 5-butyldihydro-4-methyl-	C <sub>9</sub> H <sub>16</sub> O <sub>2</sub>	1.83±0.11
<b>Subtotal</b>		<b>18.26</b>
<b>Terpenoids</b>		
D-Limonene	C <sub>10</sub> H <sub>16</sub>	1.06±0.42
2-Furanmethanol, 5-ethenyltetrahydro- $\alpha,\alpha,5$ -trimethyl-, cis-	C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>	4.80±0.59
(1R)-(-)-Thiocamphor	C <sub>8</sub> H <sub>16</sub> O	41.98±9.55

2-Hydroxy-3,5,5-trimethyl-cyclohex-2-enone	C <sub>9</sub> H <sub>14</sub> O <sub>2</sub>	143.34±38.93
1,6,6-Trimethyl-7-(3-oxobut-1-enyl)-3,8-dioxatricyclo[5.1.0.0(2,4)]octan-5-one	C <sub>13</sub> H <sub>16</sub> O <sub>4</sub>	5.10±1.05
2-Norpinanol, 3,6,6-trimethyl-	C <sub>10</sub> H <sub>18</sub> O	1.71±0.62
3,3,7,11-Tetramethyltricyclo[5.4.0.0(4,11)]undecan-1-ol	C <sub>15</sub> H <sub>26</sub> O	8.94±1.77
<b>Subtotal</b>		<b>206.94</b>
<b>Flavonoids</b>		
Chrysin	C <sub>15</sub> H <sub>10</sub> O <sub>4</sub>	11.62±3.51
4'-Methoxyflavone	C <sub>16</sub> H <sub>12</sub> O <sub>3</sub>	2.51±0.58
<b>Subtotal</b>		<b>14.13</b>

### Evaluation of weed-inhibitory substances of candidate substances

The inhibitory effects of candidate substances on the growth of lettuce varied with concentrations (Table 3). Cholic acid, *o*-coumaric acid, 2,4-decadienal, linoleic acid, citral, vanillin, piperine, vanillic acid, salicylic acid and cinnamic acid inhibited the plant height and root length of lettuce at the highest concentration. Proline, protocatechuic acid, tyrosine and *p*-hydroxybenzoic acid inhibited the root growth of lettuce only at the highest concentration. Contrarily, the root length and plant height of lettuce were promoted by 3-concentrations of  $\alpha$ -pyrene. Overall, the candidate substances were stimulatory at low concentrations and inhibitory at high concentrations. Whereas, the coumarin inhibited the root growth of lettuce at low concentration (100  $\mu$ mol/L) and promoted at high concentration (1000  $\mu$ mol/L).

Table 3. Inhibitory potential (%) of allelochemicals on seedlings growth of lettuce

#	Compound	Plant height			Root length		
		TM1(200)	TM2 (500)	TM3 (1000)	TM1(200)	TM2 (500)	TM3 (1000)
1	Cholic acid	-9.24±0.83b	-4.13±0.47b	13.25±1.05a	-18.23±1.10c	10.56±1.58b	21.28±1.36a
2	Cinnamic acid	-24.34±3.52b	-22.19±2.41b	2.73±0.22a	30.62±6.45c	44.15±5.27b	55.28±3.72a
3	<i>o</i> -Coumaric acid	20.01±3.26b	20.18±1.05b	35.17±4.06a	71.22±5.82a	75.832±8.73a	78.25±6.02a
4	2,4-Decadienal	-28.45±1.07c	-6.13±0.26b	32.74±7.14a	-3.32±0.15c	2.47±0.10b	6.55±0.20a
5	<i>p</i> -Hydroxybenzoic acid	-38.33±7.00b	-34.49±6.93b	-20.12±3.08a	15.27±1.34c	34.81±2.56b	47.28±4.20a
6	Linoleic acid	-8.67±3.27c	11.38±1.46b	30.38±3.74a	-22.32±3.11c	-17.55±2.14b	24.61±4.58a
7	$\alpha$ -Pinene	-31.44±2.06b	-39.56±5.06a	-41.62±7.07a	-8.11±1.29a	-9.27±2.09a	-23.57±0.11b
8	Protocatechuic acid	-26.81±4.22c	-18.17±3.86b	-1.68±0.26a	15.27±1.77c	30.17±2.51b	43.28±4.63a
9	Salicylic acid	-20.17±1.42b	-16.38±1.93b	43.18±9.35a	30.71±5.40b	38.74±4.33b	67.26±12.60a
10	Vanillic acid	-27.55±2.94c	-18.34±3.90b	8.32±1.62a	13.14±2.70c	48.36±5.04b	65.14±3.71a
	Compound	TM1(200)	TM2 (500)	TM3 (1000)	TM1(200)	TM2 (500)	TM3 (1000)
11	Coumarin	39.25±1.01a	26.14±4.52b	-13.37±1.04c	73.18±4.02a	66.44±0.02b	-33.40±4.10c
12	Proline	-45.67±4.24c	-31.02±3.12b	-27.25±3.07a	-21.52±2.10c	-15.05±0.08b	2.18±0.08a
13	Tyrosine	-59.05±9.06c	-30.65±2.85b	-17.70±1.46a	-36.05±4.14c	17.62±2.47b	52.04±7.46a
14	Vanillin	-6.32±0.83c	5.55±1.03b	19.43±4.23a	-42.36±7.06c	41.55±8.12b	72.42±2.15a
	Compound	TM1(100)	TM2 (110)	TM3 (150)	TM1(100)	TM2 (110)	TM3 (150)
15	Citral	6.55±0.41c	54.27±2.14b	85.35±4.23a	5.21±0.31c	25.41±4.06b	98.21±1.62a
	Compound	TM1(10)	TM2 (25)	TM3 (150)	TM1(10)	TM2 (25)	TM3 (150)
16	Piperine	0.40±0.02c	3.14±0.26b	17.28±2.14a	50.58±5.04b	75.56±8.72a	76.52±5.42a

Data in brackets/parenthesis after TM1, TM2, TM3 indicate concentration ( $\mu$ mol/L) of candidate substances.

The reported allelochemicals in fermentation broth were phenolic acids, flavonoids, terpenoids, steroids, amino acids, flavonoids, sulfur compounds, glucosinolates (14,22). Among these substances, phenolic acids are considered the most important allelochemicals (25). The common phenolic acids in the allelopathic rice exudates are protocatechuic acid, *p*-hydroxybenzoic acid, syringic acid, vanillic acid, salicylic acid, ferulic acid and cinnamic acid (5,8,35). Some studies showed that the content of phenolic acids in root exudates of allelopathic rice was significantly higher than in non-allelopathic rice (17,35). Chung *et al.* (2) reported that 7 phenolic acids (syringic acid, vanillic acid, hydroxyphenylacetic acid, ferulic acid, salicylic acid, coumaric acid, and caffeic acid) inhibited the germination of barnyard grass at  $10^{-5}$  mol/L, and the inhibition rate of mixed phenolic acids on the germination of barnyard grass was higher than single phenolic acid.

Yang *et al.* (32) showed that the 50 % inhibition concentration (IC<sub>50</sub>) of *p*-hydroxybenzoic acid, syringic acid, vanillic acid, salicylic acid, ferulic acid and cinnamic acid on root length of lettuce were 1.12 mmol/L, 1.53 mmol/L, 0.65 mmol/L, 0.54 mmol/L, 1.31 mmol/L, 0.72 mmol/L, respectively. The concentration of some phenolic acids and mixed phenolic acids in the fermentation broth of *A. sydowii* FJFAFU01 has reached or exceeded the herbicidal concentration reported previously, indicating that the phenolic acids are the herbicidal components in the fermentation broth. Zhou *et al.* (38) showed that *A. sydowii* affected the amino acids, carbohydrates, flavonoids, and caffeine metabolism of sun-dried green tea leaves and promoted the production of ketoprofen, baclofen and tolbutamide. Besides, in the fermentation broth of *A. sydowii* FJFAFU01, we also detected the metabolites rich in nitrogenous compounds, oxygen compounds, including terpenoids, flavonoids and alkaloids. The inhibitory activity of these substances has been reported as putative herbicides (2,10,15,30,32).

The weeds may be controlled by using living microbial agents directly, such as *Colletotrichum truncatum* to control Hemp sebania (*Sesbania exaltata*) (11). Another approach is to use the active microbial compounds. Li *et al.* (17) reported that a weed-inhibiting fungus, *Penicillium decumbens*, was screened from the rhizosphere soil of allelopathic rice PI312777 and the fermentation broth contained phenolic substances. Zhang *et al.* (34) screened a strain of *Aspergillus niger* from the rhizosphere soil of *Eucalyptus* which inhibits the growth of barnyard grass. The metabolites of *A. sydowii* 'FJFAFU01' contained abundant inhibitory substances with potential as novel herbicides after conducting pot culture experiments.

## CONCLUSIONS

A fungus strain, named *A. sydowii* 'FJFAFU01' was successfully screened and isolated from the rhizosphere soil of passion fruit. Its fermentation broth significantly inhibited the growth of lettuce and barnyard grass. In pot culture experiments, FJFAFU01 had negative effects on the growth of barnyard grass seedlings. Its metabolites contained weed suppressing phenolic acids, terpenoids, flavonoids and alkaloids. These results indicate that *A. sydowii* 'FJFAFU01' could be developed as a potential mycoherbicide.

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## DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

## CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

## ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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