SUPPLEMENTARY MATERIALS

Antibacterial and herbicidal properties of secondary metabolites from fungi

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Abstract

Twenty eight compounds were isolated from endophytic, soil and marine fungi and their structures were elucidated through spectroscopic methods. The isolated compounds were tested for their antibacterial and herbicidal activities against phytopathogenic bacteria and barnyard grass weed for the first time. Methyleurotinone (**14**) was the most potent compound against *Pectobacterium carotovorum* subsp. *carotovorum*, *Pseudomonas syringae* pv. *syringae*, *Rhizobium radiobacter* and *Ralstonia solanacearum* with minimum inhibitory concentration (MIC) values of 31.3, 125, 31.3 and 125 mg/L, respectively. Compounds **13-15** were highly effective in reducing the development of potato tuber soft rot disease caused by *P. carotovorum* subsp. *carotovorum*. Furthermore, twelve of the tested compounds induced significant reduction in seed germination of *Echinochloa crus-galli* at 2mM with compounds **8** and **26** causing complete inhibition of seed germination. Also, compounds **4**, **22**, **5**, **8, 18** and **25-27** induced remarkable reduction of root and shoot growth of *E. crus-galli* at 2mM.

Keywords: Fungi; Bioactive compounds; Antibacterial effects; Herbicidal activity

3. Experimental

3.1. Test bacteria

Four phytopathogenic bacterial strains, *Pectobacterium carotovorum* subsp. *carotovorum* (Jones, 1901) Hauben et al. 1999 (EMCC 1687), *Pseudomonas syringae* pv. *syringae* Van Hall, 1904 (EMCC 1739), *Rhizobium radiobacter* (Beijerinck & van Delden 1902) Young et al. 2001 (ATCC 19358) and *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1996 (EMCC 1274) were obtained from Microbiological Resource Centre (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The bacterial strains were grown and maintained on nutrient agar (NA) medium (NA: peptone 10 g, meat extract 5 g, sodium chloride 2.5 g and agar 10 g in one liter of distilled water).

3.2. Test Weed

Field biotype seeds of barnyard grass, *Echinochloa crus-galli* (L.) Beauv. (Poaceae), were collected from Faculty of Agriculture Farm, Alexandria, Egypt. Uniform and undamaged seeds were used for the germination and seedling growth tests. Seeds were examined for their germination before experiments. The germination was 60% after 12 days of sowing.

3.3. Fungal strains, isolation and structure elucidation of secondary metabolites

Strains of different endophytic fungi were isolated from different sources, identified by BEX Co. Ltd., Japan, using a DNA analysis of the 18S rDNA regions and had been deposited at our laboratory in the Faculty of Agriculture of Yamagata University. The fungal strains isolation, identification and fermentation procedures were described in previous reports as indicated in Table S1. Twenty eight compounds have been isolated from different endophytic, soil and marine fungi by using combination of chromatographic separation techniques, including column chromatography (CC), preparative high performance liquid chromatography (HPLC) and preparative thin layer chromatography (PTLC). The chemical structures of the isolated compounds were elucidated by using UV, IR, MS, ^{1}H NMR and ^{13}C

NMR spectra as well as 2D NMR spectra of COSY, HMQC, HMBC, DEPT and NOESY. The structures of isolated compounds are shown in Figure 1.

3.4. Preparation of bacterial inoculums

A loopful of bacterial colonies was taken from bacterial strains grown on slant nutrient agar and transferred to a tube containing 5 ml of nutrient broth. The suspension was incubated at 30^{o}C for 18 h to give approximately 1.0×10^{8} CFU/ml.

3.5. Determination of minimum inhibitory concentrations (MIC) of compounds

The antibacterial activity of isolated compounds was determined by using a microdilution method. Nutrient broth was used as culture media for bacterial strains. The inoculums of bacteria were prepared as described previously. Stock solutions of isolated compounds were first prepared in dimethylsulfoxide (DMSO). Appropriate volumes from stock solutions were transferred to 96-well plates containing culture broth ranged between 164 and 178 µL. The highest concentration of DMSO in microplate wells was 8% which had no effect on growth of bacteria. Each well was inoculated with 20 µL of a bacterial suspension. The total volume of each well was 200 µL. Control wells received culture broth, inoculum suspension and DMSO without test compounds. The isolated compounds were tested at final concentrations of 1000, 500, 250, 125, 62.5, 31.3 and 15.6 µg/mL. The microdilution trays were incubated at 30^{o}C for 24 h. The growth of bacteria was detected by adding 20 µl (5 mg/ml) of 2,3,5-triphenyltetrazolium chloride (TTC) and incubating for 30 min under appropriate cultivation conditions in the dark (Ellof 1998). The viable bacterial cells changed the colorless TTC to pink. MIC value was taken as the lowest concentration of isolated compound, which caused complete inhibition of bacterial growth.

3.6. Evaluation the effect of selected compounds on potato soft rot disease control

Three compounds (**13**-**15**), which showed the highest antibacterial activity *in vitro*, were tested *in vivo* for their effect on the development and control soft rot disease caused by *P. carotovorum* subsp. *carotovorum* in potato tubers. Potato tubers were thoroughly washed

with water then with chloride solution (1%), followed by sterilized distilled water. The tubers were cut into 1-Cm cubes. The cubes were placed in in 90-mm petri dishes with moist sterile filter paper (Whatman No. 2). Two concentrations (31.3 and 62.5 µg/mL) of the tested compounds were prepared in distilled water containing 0.05% Triton-X 100. Potato cubes were dipped in the solutions of compounds for 60 min. After dry of compound solutions, each cube was inoculated by spray 20 µl (1.0×10^8 CFU/ml) of bacterial suspension. Three replicates with 8 cubes in each one were used for each concentration. Control cubes were treated with 0.05% Triton-X 100 only. Ampicillin was used as reference antibiotic at similar concentrations. The treated cubes were kept in sealed petri dishes at 30°C for 4 days. Observations on the visible development of soft rot were recorded and expressed as disease severity. The disease severity was estimated based on a 0 - 5 ranking scale, where 0 = no decay (0%), 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80%, 5 = 81-100%. The severity percent was calculated using the following formula:

Severity percent = $(\Sigma nv/5N) \times 100$

where n = number of cubes in each replicate, v = numerical values of each ranking scale, N = total number of the tuber cubes in each treatment, and 5 = highest score on the severity scale. Also, percent soft rot control was calculated using the formula:

Percent of soft rot control = $[(C-T)/C] \times 100$

where C= soft rot severity percent in control and T = soft rot severity percent in treatment.

3.7. Germination and seedling growth inhibition bioassay

The inhibitory effects of isolated compounds on the germination and subsequent seedling growth of *Echinochloa crus-galli* were evaluated using a method described by Abdelgaleil et al. (2009). The compounds were dissolved first in DMSO and diluted with distilled water containing Triton-X 100 (0.02%) as an emulsifying agent. The test compounds were first evaluated at concentration of 2mM. Each treatment was replicated three times with 20 seeds in each replicate. Six milliliters of compound solution were added in each Petri dish (9 cm) lined with Whatman No. 2 filter paper. Then, Petri dishes were put in the bottom of polyethylene bags (0.1mm thick). The bags were expanded to contain air and closed with

rubber bands to avoid the moisture loss. Control seeds were treated with distilled water containing DMSO (0.5% v/v) and Triton-X 100 (0.02%) only and these concentrations of DMSO and Triton-X 100 had no effect on germination or seedling growth of weed. The dishes were kept in a growth cabinet at 26 ± 2 ºC with a 12-h photoperiod. The seed germination was determined by counting the number of germinated seeds and the lengths of root and shoot were recorded after 12 days of sowing. The growth reduction percentages (RP) of root and shoot lengths were calculated from the following equation:

$$RP\ (\%) = [1 - T/C] \times 100$$

where T is the root or shoot length of treatment (cm) and C is the root or shoot length of control (cm). Moreover, four compounds (**8**, **18**, **22** and **26**) which showed the highest phytotoxic effect were further evaluated on germination and seeding growth at a series of concentrations, 1.0, 0.5, 0.25 and 0.125 mM.

4. Statistical analysis

Germination percentages, and root and shoot lengths were subjected to one-way analysis of variance followed by Student–Newman– Keuls test (SPSS 21.0 (SPSS, Chicago, IL, USA) to determine significant differences among mean values at the probability level of 0.05.

Acknowledgments:

The work was supported by Alexandria University Research Fund (ALEX-REP, 2010- 2013) and Japan Society for Promotion of Science (JSPS).

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226 **Table S1.** Names, chemical class and source of tested compounds

Name	Chemical group	Fungus	Reference
Nodulisporone A (**1**)	Phenylisobenzofuranones	*Nodulisporium* sp. SH-1	Hayasaka et al. 2011
Nodulisporone B (**2**)	Phenylisobenzofuranones	*Nodulisporium* sp. SH-1	Hayasaka et al. 2011
Phomaxanthone A (**3**)	Dimeric xanthones	*Phomopsis* sp.	Elsaesser et al. 2005
Deacetylphomaxanthone A (**4**)	Dimeric xanthones	*Phomopsis longicolla*	Ronsberg et al. 2013
Eremoxylarin B (**5**)	Eremophilane sesquiterpenes	Xylariaceous endophytic fungus (YUA-026)	Shiono and Murayama 2005
8α-Acetoxyphomadecalin C (**6**)	Eremophilane sesquiterpenes	*Microdiplodia* sp. KS 75-1	Shiono and Murayama 2005
Phomadecalin D (**7**)	Eremophilane sesquiterpenes	*Microdiplodia* sp. KS 75-1	Hatakeyama et al. 2010
Integric acid A (**8**)	Eremophilane sesquiterpenoids	*Xylaria sp.* (MF6254)	Singh et al. 1999
Myrocin E (**9**)	Isopimarane diterpenoids	*Xylaria polymorpha*	Shiono et al. 2013
Spiropolin A (**10**)	Isopimarane diterpenoids	*Xylaria polymorpha*	Shiono et al. 2013
19-(α-D-glucopyranosyloxy)isopimara-7,15-dien-3β-ol (**11**)	Isopimarane diterpenoids	*Paraconiothyrium* sp. MY-42	Shiono et al. 2011
Citreohybridonol (**12**)	Meroterpenoids	*Penicillium atrovenetum*	Ozkaya et al. 2018
Eurotinone (**13**)	Polyketides	*Eurotium rubrum* IM-26	Wang et al. 2007; Shibuya and Shiono 2016
Methyleurotinone (**14**)	Polyketides	*Eurotium rubrum* IM-26	Wang et al. 2007; Shibuya and Shiono 2016
Dehydroxyeurotinone (**15**)	Polyketides	*Eurotium rubrum* IM-26	Shibuya and Shiono 2016
Anthracobic acid A (**16**)	Polyketides	*Anthracobia* sp.	Shiono 2006
Pyrrocidine A (**17**)	Alkaloids	*Acremonium zeae*	Wicklow and Poling 2009
6-eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one (**18**)	Cyclohexenones	Xylariaceous endophytic fungus (YUA 026)	Shiono et al. 2005
Fasciculol A (**19**)	Lanostane triterpenoids	*Neamatoloma fasciculare*	Ikeda et al. 1977a
Fasciculol B (**20**)	Lanostane triterpenoids	*Neamatoloma fasciculare*	Ikeda et al. 1977b; Kim et al. 2013
Fasciculol C (**21**)	Lanostane triterpenoids	*Neamatoloma fasciculare*	Ikeda et al. 1977c; Kim et al. 2013
Secalonic acid A (**22**)	Dimeric xanthones	*Claviceps purpurea*	Masters and Bräse, 2002
Dehydroaustin (**23**)	Meroterpenoids	*Penicillium brasilianum*	Schürmann et al. 2010
Verruculogen (**24**)	Indole alkaloids	*Penicillium verruculosum*	Uramoto et al. 1982
Equisetin (**25**)	Tetramic acid derivatives	*Fusarium equiseti*	Wheeler et al. 1999
Brifeldin A (**26**)	Macrolides	*Penicillium brefeldianum*	Hutchinson et al. 1983
Pencolide (**27**)	Maleimides	*Penicillium sclerotiorum*	Lucas et al. 2007
YM-202204 (**28**)	Polyketides	*Phoma* sp.	Nagai et al. 2002

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232 **Table S2.** Antibacterial activity of secondary metabolites isolated from endophytic fungi
233 on plant pathogenic bacteria using microdilution assay

Compound	Minimum inhibitory concentration (MIC) µg/mL			
	Rhizobium radiobacter	*Ralstonia solanacearum*	*Pectobacterium carotovorum* subsp. *carotovorum*	*Pseudmonas syringae* pv. *syringae*
Nodulisporone A (**1**)	500	250	500	500
Nodulisporone B (**2**)	>1000	>1000	>1000	>1000
Phomaxanthone A (**3**)	1000	1000	125	500
Deacetylphomaxanthone A (**4**)	125	125	125	125
Eremoxylarin B (**5**)	125	500	500	1000
8α-Acetoxyphomadecalin C (**6**)	>1000	1000	>1000	1000
Myrocin E (**9**)	>1000	500	>1000	1000
Spiropolin A (**10**)	>1000	>1000	>1000	>1000
19-(α-D-glucopyranosyloxy)isopimara -7,15-dien-3β-ol (**11**)	1000	125	1000	500
Citreohybridonol (**12**)	>1000	>1000	>1000	>1000
Eurotinone (**13**)	125	125	31.3	250
Methyleurotinone (**14**)	31.3	125	31.3	125
Dehydroxyeurotinone (**15**)	250	250	62.5	250
Anthracobic acid A (**16**)	1000	1000	1000	1000
Pyrrocidine A (**17**)	1000	1000	1000	1000
6-Eopxy-4-hydroxy-3-methoxy-5-met hyl-cyclohex-2-en-1-one (**18**)	125	125	250	500
Fasciculol A (**19**)	>1000	>1000	>1000	>1000
Fasciculol B (**20**)	>1000	>1000	>1000	>1000
Fasciculol C (**21**)	>1000	>1000	>1000	>1000
Dehydroaustin (**23**)	>1000	>1000	>1000	>1000
Verruculogen (**24**)	>1000	>1000	>1000	>1000
Equisetin (**25**)	500	500	500	1000
Brifeldin A (**26**)	>1000	>1000	>1000	>1000
Pencolide (**27**)	500	500	500	500
YM-202204 (**28**)	>1000	>1000	1000	1000
Ampicillin	31.3	15.7	15.7	62.5

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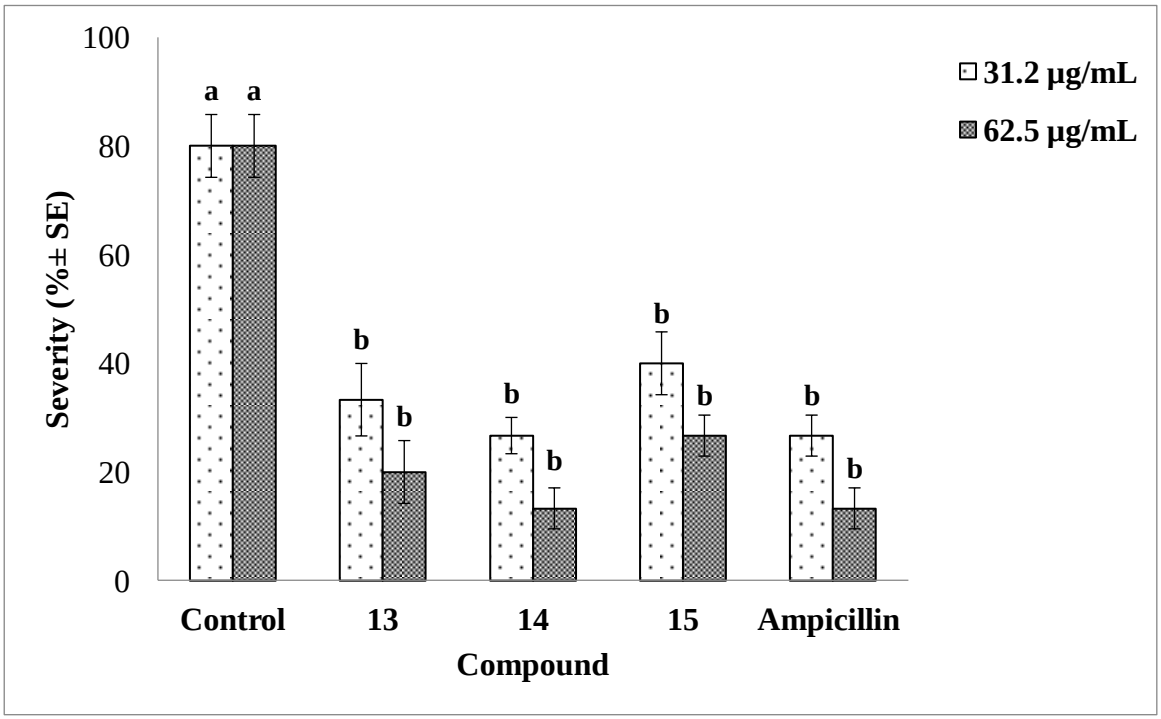

Figure S1. Effect of secondary metabolites isolated from endophytic fungi on soft rot severity percent after 4 days of inoculation with *P. carotovorum* subsp. *carotovorum*. Mean values within a concentration sharing the same letter are not significantly different at the 0.05 probability level.

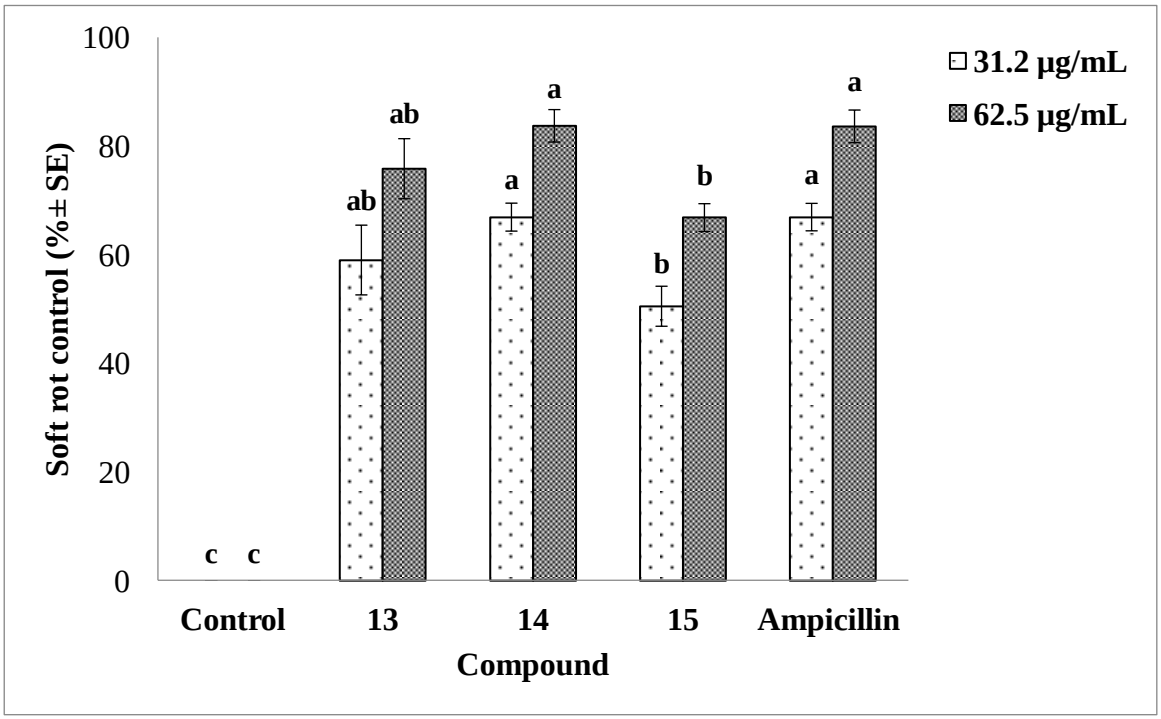

Figure S2. Effect of secondary metabolites isolated from endophytic fungi on soft rot control (%) on potato after 4 days of inoculation with *P. carotovorum* subsp. *carotovorum*. Mean values within a concentration sharing the same letter are not significantly different at the 0.05 probability level.

Table S3. Effect secondary metabolites isolated from endophytic fungi on germination and seedling growth of *Echinochloa crus-galli* seeds after 12 days of sowing at 2mM[a]

Compound	Germination[b] (% ± SE)	Root		Shoot	
		Length (cm) (Mean ± SE)	I[c] (%)	Length (cm) (Mean ± SE)	I (%)
Control	60.0±2.89abc	4.07±0.07bc	0.0	4.0±0.12a	0.0
Nodulisporone A (**1**)	61.7±1.67ab	2.33±0.32fg	42.8	3.07±0.12cdefg	23.3
Nodulisporone B (**2**)	51.7±1.67cde	1.90±0.35gh	53.3	3.43±0.09bc	14.3
Phomaxanthone A (**3**)	41.7±1.67fg	1.53±0.07hi	62.4	2.70±0.15fghi	32.5
Deacetylphomaxanthone A (**4**)	61.7±3.34ab	1.53±0.09hi	62.4	2.33±0.12ijk	41.8
Eremoxylarin B (**5**)	45.0±2.89efg	0.33±0.03j	91.9	2.03±0.12k	49.3
8α-Acetoxyphomadecalin C (**6**)	56.7±1.67abcd	1.43±0.07hi	64.9	2.53±0.12hij	36.8
Phomadecalin D (**7**)	46.7±1.67ef	4.33±0.24ab	-6.4	2.57±0.09ghij	35.8
Integric acid A (**8**)	0.0±0.0i	0.0±0.0j	100.0	0.0±0.0m	100.0
Myrocin E (**9**)	46.7±1.67ef	1.90±0.06gh	53.3	2.83±0.03defgh	29.3
Spiropolin A (**10**)	58.3±3.34abc	3.73±0.17bcd	8.4	3.03±0.09cdefgh	24.3
19-(α-D-glucopyranosyloxy)isopimara-7,15-dien-3β-ol (**11**)	45.0±2.89efg	2.27±0.09fg	44.2	3.77±0.32ab	5.8
Citreohybridonol (**12**)	46.7±3.34ef	3.60±0.17cd	11.5	2.97±0.09cdefgh	25.8
Dehydroxyeurotinone (**15**)	46.7±1.67ef	2.03±0.0gh	50.1	2.71±0.09fghi	32.3
Anthracobic acid A (**16**)	63.3±1.67a	2.37±0.18fg	41.8	3.33±0.23bcd	16.8
Pyrrocidine A (**17**)	51.7±3.34cde	2.83±0.62ef	30.5	3.27±0.15def	18.3
6-Eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one (**18**)	20.0±2.89h	1.17±0.21i	71.3	1.23±0.24l	69.3
Fasciculol A (**19**)	60.0±2.89abc	3.67±0.03cd	9.8	2.93±0.13cdefgh	26.8
Fasciculol B (**20**)	63.3±1.67a	2.87±0.20ef	29.5	3.01±0.15cdefg	24.8
Fasciculol C (**21**)	48.3±1.67def	2.63±0.32ef	35.4	3.40±0.20bc	15.0
Secalonic acid A (**22**)	36.7±3.34g	0.0±0.0j	100.0	2.13±0.24jk	46.8
Dehydroaustin (**23**)	45.0±2.89efg	3.20±0.20de	21.4	3.13±0.23cdef	21.8
Verruculogen (**24**)	53.3±3.34bcde	3.73±0.07bcd	8.4	3.10±0.06cdef	22.5
Equisetin (**25**)	40.0±2.89fg	0.23±0.03j	94.3	2.07±0.03k	48.3
Brifeldin A (**26**)	0.0±0.0i	0.0±0.0j	100.0	0.0±0.0m	100.0
Pencolide (**27**)	51.7±1.67cde	1.53±0.09hi	62.4	1.40±0.23l	65.0
YM-202204 (**28**)	61.7±3.34ab	3.67±0.19cd	9.8	2.80±0.06efghi	30.0

[a] Data are expressed as means ± SE from experiments with three replicates of 20 seeds each.

[b] Mean values within a column sharing the same letter are not significantly different at the 0.05 probability level.

[c] I = Inhibition.

299 **Table S4.** Effect of selected secondary metabolites isolated from endophytic fungi on
300 germination and seedling growth of *Echinochloa crus-galli* seeds after 10 days of sowing[a]

Compound/ Concentration (mM)	Germination[b] (% ± SE)	Root		Shoot	
		Length (cm) (Mean ± SE)	I[c] (%)	Length (cm) (Mean ± SE)	I (%)
Integric acid A (**8**)					
Control	53.3±3.34a	3.67±0.20a	0.0	3.33±0.20a	0.0
0.125	41.7±1.66b	0.47±0.29b	87.2	2.60±0.20b	21.9
0.25	38.3±1.66b	0.00±0.00b	100.0	3.37±0.17a	-1.2
0.5	48.3±1.66a	0.00±0.00b	100.0	2.77±0.15b	16.8
1.0	51.7±1.66a	0.00±0.00b	100.0	2.93±0.07b	12.0
6-Eopxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one (**18**)					
Control	53.3±3.34a	3.67±0.20a	0.0	3.33±0.20bc	0.0
0.125	51.7±1.66a	1.57±0.16b	57.2	3.67±0.13ab	-10.2
0.25	48.3±1.66a	0.5±0.20c	86.4	3.73±0.03a	-12.0
0.5	46.7±1.66a	0.00±0.00d	100.0	3.03±0.03c	9.0
1.0	13.3±3.34b	0.00±0.00d	100.0	0.83±0.09d	75.1
Secalonic acid A (**22**)					
Control	53.3±3.34a	3.67±0.20a	0.0	3.33±0.20a	0.0
0.125	53.3±1.66a	0.50±0.26b	86.4	3.30±0.15ab	0.9
0.25	33.3±3.34c	0.33±0.20b	91.0	2.93±0.07abc	12.0
0.5	43.3±3.34b	0.00±0.00b	100.0	2.50±0.17c	24.9
1.0	36.7±1.66bc	0.00±0.00b	100.0	2.77±0.19bc	16.8
Brifeldin A (**26**)					
Control	53.3±3.34a	3.67±0.20a	0.0	3.33±0.20a	0.0
0.125	35.0±2.89b	0.00±0.00b	100.0	2.37±0.17b	28.8
0.25	28.3±1.66b	0.00±0.00b	100.0	1.83±0.13cd	45.0
0.5	31.7±3.34b	0.00±0.00b	100.0	2.10±0.12bc	36.9
1.0	31.7±3.34b	0.00±0.00b	100.0	1.40±0.17d	58.0

301 [a] Data are expressed as means ± SE from experiments with three replicates of 20 seeds each.
302 [b] Mean values within a column for each compound sharing the same letter are not
303 significantly different at the 0.05 probability level.
304 [c] I = Inhibition.

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