Plant growth inhibitors from the culture broth of fairy ring-forming fungus Lepista sordida

Elsevier Editorial System(tm) for Mycoscience

Manuscript Draft

Manuscript Number: MYC-D-17-00015R4

Title: Plant growth inhibitors from the culture broth of fairy ring-forming fungus Lepista sordida

Article Type: Notes

Corresponding Author: Professor Hirokazu Kawagishi,

Corresponding Author's Institution: Shizuoka University

First Author: Akinobu Ito

Order of Authors: Akinobu Ito; Jae-Hoon Choi, Ph. D.; Jing Wu, Ph. D.; Hidekazu Tanaka ; Hirofumi Hirai, Ph. D.; Hirokazu Kawagishi

Abstract: Four compounds (1-4) were isolated from the culture broth of fairy ring-forming fungus Lepista sordida. In the bioassay examining plant growth regulatory activity using bentgrass, compounds 1, 2 and 4 inhibited the root growth at 100 and 1000 nmol/paper. Among them, compound 1 showed the strongest inhibitory activity against the root.

Note

Plant growth inhibitors from the culture broth of fairy ring-forming fungus *Lepista sordida*

Akinobu Ito [a], Jae-Hoon Choi [a,b], Jing Wu [b], Hidekazu Tanaka [a], Hirofumi Hirai [a,b], Hirokazu Kawagishi [a,b,c,*]

[a] *Graduate School of Integrated Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*
[b] *Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*
[c] *Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*

[*] Corresponding author.
H. Kawagishi
Tel: +81 54 238 4885.
Fax: +81 54 238 4885.
E-mail address: kawagishi.hirokazu@shizuoka.ac.jp

Text: 7 pages; figures: 2

ABSTRACT

Four compounds (1–4) were isolated from the culture broth of fairy ring-forming fungus *Lepista sordida*. In the bioassay examining plant growth regulatory activity using bentgrass, compounds 1, 2 and 4 inhibited the root growth at 100 and 1000 nmol/paper. Among them, compound 1 showed the strongest inhibitory activity against the root.

Keywords:

Plant growth regulator; Structural identification

"Fairy rings" are zones of stimulated grass growth owing to the interaction between a fungus and a plant (Couch 1995). The term "fairy rings" originates from the myths related to this phenomenon in the Middle Ages. Since the first scientific report on fairy rings was published in 1675 and the subsequent research on them was reviewed in *Nature* in 1884 (Evershed 1884), this phenomenon had been attributed to an unknown "fairy".

In the previous reports, we investigated the possibility of specific plant growth regulators produced by one of the fairy ring-forming fungi, *Lepista sordida* (Schumach. : Fr.) Singer, and reported that 2-azahypoxanthine (AHX) and imidazole-4-carboxamide (ICA) were isolated from the culture broth of the fungus (Choi et al. 2010a, b). Subsequently, 2-aza-8-oxohypoxanthine (AOH) was isolated from rice (Choi et al. 2014). We named the three compounds "fairy chemicals" (FCs) after the title of the article in *Nature* (Mitchinson 2014). FCs exhibited growth regulatory activity towards various kinds of plants regardless of families they belong to (Choi et al. 2010a, b). Furthermore, FCs increased seed yields of rice and wheat significantly, suggesting the possibility of its practical use in agriculture (Tobina et al. 2014; Asai et al. 2015). In the course of further search for other plant growth regulators from *L. sordida*, we succeeded in purifying four diketopiperazines (compounds 1 to 4; Fig. 1) from the culture broth of the fungus.

Here, we describe the isolation, structural identification, and growth regulatory activity against bentgrass of compounds 1 to 4.

Extraction and isolation

The mycelia of *Lepista sordida* AKK101010 were collected in Akita Prefecture and provided by Fumio Kobayashi of Nasu Biofarm, Ltd. (Nasu, Japan). Five pieces (5 × 5 × 3 mm) cut from 20-d-old mycelia cultures of *L. sordida* on PYG agar (0.3% polypeptone, Nippon Becton Dickinson Company, Ltd., Minato, Tokyo, Japan; 0.3% yeast extract, Nippon Becton Dickinson Company, Ltd.; 1% glucose, Wako Pure Chemical Industries, Ltd., Chuo, Osaka, Japan; 1.8% agar, Sigma-Aldrich Co., LLC., Meguro, Tokyo, Japan) were inoculated into a 500-mL Erlenmeyer flask containing 300 mL of PYG liquid medium and incubated at 25 °C with 120 rpm for a mo in the dark (Bio-Shaker BR-300LF, Taitec Co., Ltd., Koshigaya, Saitama, Japan). The culture broth (12 L) was evaporated under reduced pressure, and the concentrate was divided into a hexane soluble part, an EtOAc soluble part and a water soluble part. The EtOAc soluble part (1.5 g) of the culture broth was fractionated by silica gel flash column chromatography (Slica gel 60N, Kanto Chemical, Tokyo, Japan); 95% (fractions 1 to 3), 85% (fractions 4 to 7), 70% (fractions 8 to 11), 40% (fractions 12 to 14) CH_2Cl_2/MeOH; MeOH (fractions 15 to 18); 90% (fractions 19 to 20) MeOH/H_2O to obtain 20 fractions (fractions 1 to 20). HPLC separations were performed with a JASCO Gulliver system. Fraction 10 (76.1 mg) was further separated by normal-phase HPLC (Senshupak Aquasil AQ, Senshu Scientific Co., Ltd., Suginami, Tokyo, Japan; $CHCl_3$) to afford compound 1 (8.9 mg) and compound 2 (6.2 mg). Compound 3 (1.2 mg) was purified from fraction 7 (51.0 mg) by normal-phase HPLC (Develosil silica, Nomura Chemical Co., Ltd., Seto, Aichi, Japan; 95% $CHCl_3$/MeOH). Fraction 14 (238 mg) was fractionated by normal-phase HPLC (Senshupak Aquasil AQ; $CHCl_3$) to obtain 18 fractions (fractions 14-1 to 14-18). Subsequently, fraction 14-7 (49.2 mg) was further separated by reverse-phase HPLC (X-Bridge Phenyl, Waters,

Shinagawa, Tokyo, Japan; 33% MeOH) to obtain 14 fractions (fractions 14-7-1 to 14-7-14). Fraction 14-7-9 (17.3 mg) was fractionated by reverse-phase HPLC (Develosil C30-UG-5, Nomura Chemical Co., Ltd., Seto, Aichi, Japan; 40% MeOH) to give compound 4 (13.1 mg).

Identification of active compounds

^{1}H NMR spectra (one- and two-dimensional) were recorded on a JEOL lambda-500 spectrometer (JEOL, Tokyo, Japan) at 500 MHz, and ^{13}C NMR spectra were recorded on the same instrument at 125 MHz. The electrospray ionization-mass spectrometry (ESIMS) spectra were measured on a JMS-T100LC mass spectrometer (JEOL, Tokyo, Japan). The specific rotation value was measured with a JASCO DIP-1000 polarimeter (JASCO, Tokyo, Japan).

We obtained four known compounds from the culture broth of *L. sodida* (Fig. 1).

Compound 1 was purified as white crystals. The structure of compound 1 was identified to be a diketopiperazine that consisted of proline and phenylalanine by interpretation of the NMR spectra including DEPT, COSY, HMQC and HMBC. All the data including the specific rotation were identical with those of (3*S*,9*S*)-3-benzylhexahydropyrrolo[1,2-α]pyrazine-1,4-dione (Christopher et al. 2004). This compound has been isolated from *Lactobacillus plantarum* and showed antibacterial activity (Strom et al. 2002; Wang et al. 2010). LC-MS analysis suggested the existence of this compound in the edible mushroom *Agaricus bisporus* (J. E. Lange) Imbach (Mar Delgado-Povedo et al. 2016).

Compound 2 was purified as white crystals. The structure of compound 2 was identified to be a diketopiperazine of proline and valine by interpretation of the NMR spectra (Furtado et al. 2005). The absolute configurations, L and L, of the two amino acids in compound 2 were identified by hydrolysis of the compound with hydrochloric acid and then HPLC analysis (CROWNPAK CR(+), Daicel Chemical Ind., Ltd., Minato, Tokyo, Japan; H_2O). Compound 2, (3*S*,9*S*)-3-isopropylhexahydropyrrolo[1,2-α]pyrazine-1,4-dione, has been isolated from sponge *Leucophloeus fenestrate*, and its antibacterial and antilarval activity have been reported (Omar et al. 1988; Qi et al. 2009).

Compounds 3 and 4 were also identified to be diketopiperazines of proline and leucine, proline and phenylalanine, respectively, by interpretation of the NMR spectra (Furtado et al. 2005; Wang et al. 2010). Their absolute configurations were identified by the same way as that of compound 2. Compound 3, (3*S*,9*S*)-3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione, has been isolated from *Aspergillus fumigatus* and showed antimicrobial, antifungal and antitumor activity (Furtado et al. 2005; Khedr et al. 2013; Shaala et al. 2016). Compound 4, (3*S*,9*R*)-3-benzylhexahydropyrrolo[1,2-α]pyrazine-1,4-dione, has been isolated from marine *Bacillus subtilis* and showed antimicrobial activity (Wang et al. 2010; Kumar et al. 2013).

Compound 1: white crystals; ESIMS *m/z* 267 $[M+Na]^+$; $[\alpha]^{26}_D$ -30 (*c* 0.22, EtOH); ^{1}H NMR (in $CDCl_3$) δ_H: 1.89 (m), 1.96 (m), 2.01 (m), 2.32 (m), 2.82 (dd), 3.56 (m), 3.63 (m), 3.66 (m), 4.08 (t), 4.29 (dd), 7.24 (d), 7.29 (t), 7.35 (t); ^{13}C NMR δ_C: 22.5, 28.3, 36.8, 45.4, 56.2, 59.1, 127.5, 129.1, 129.2, 135.9, 165.0, 169.3.

Compound 2: white crystals; ESIMS *m/z* 219 $[M+Na]^+$; ^{1}H NMR (in $CDCl_3$) δ_H: 0.87 (d), 1.05 (d), 1.87 (dd), 1.98 (m), 2.00 (m), 2.32 (ddd), 2.58 (d), 3.49 (m), 3.59 (m), 3.90 (s), 4.04

(t), 6.37 (s); ^{13}C NMR δ_C: 16.0, 19.1, 22.3, 28.4, 28.5, 45.1, 58.8, 60.4, 164.9, 170.2.

Compound 3: white crystals; ESIMS *m/z* 233 $[M+Na]^+$; ^{1}H NMR (in $CDCl_3$) δ_H: 0.93 (d), 0.98 (d), 1.50 (ddd), 1.61 (s), 1.72 (m), 1.89 (m), 2.04 (m), 2.31 (m), 3.54 (m), 3.99 (dd), 4.08 (t), 5.86 (s); ^{13}C NMR δ_C: 21.3, 22.8, 23.4, 24.8, 28.2, 38.7, 45.6, 53.5, 59.1, 166.2, 170.2.

Compound 4: white crystals; ESIMS *m/z* 267 $[M+Na]^+$; ^{1}H NMR (in $CDCl_3$) δ_H: 1.63 (m), 1.89 (m), 2.03 (ddd), 2.62 (dd), 2.98 (dd), 3.18 (dd), 3.31 (m), 3.53 (ddd), 4.19 (ddd), 7.18 (dd), 7.29 (dd); ^{13}C NMR δ_C: 22.5, 29.8, 41.0, 46.1, 59.1, 59.8, 128.5, 129.6, 131.3, 136.7, 167.4, 171.3.

Plant growth regulatory activity

Bentgrass seeds (Japanese lawn grass T.T.S noshiba; Takii Co., Ltd., Tokyo, Japan) were sterilized with ethanol for five minutes and with 1% sodium hypochlorite solution for ten minutes, and then washed with sterilized water ten times. Seeds were seeded on 0.8% agar medium (Sigma-Aldrich Co., LLC., Japan) in a Petri dish (ϕ60 × 20 mm) and pre-cultured for 5 d (25 ˚C, 16 h-8 h (L/D) photoperiod). Samples were dissolved in 1 mL of dichloromethane (10, 100 and 1000 nmol/mL) and then poured on filter paper (Advantec No. 2, ϕ55 mm; Toyo Roshi Kaisha, Tokyo, Japan) in a Petri dish (ϕ60 × 20 mm). After the sample-loaded paper had been air-dried, 1 mL of distilled water was poured on the sample-loaded paper or intact filter paper (control). The preincubated bentgrass (n = 16 in each Petri dish) was transferred onto the sample-loaded filter paper or control filter paper and incubated in a growth chamber for one week (25 ˚C, 16 h-8 h (L/D) photoperiod). The lengths of the root and shoot were measured to an accuracy of 0.01 mm with an absolute digimatic caliper (Mitutoyo Co., Kawasaki, Kanagawa, Japan).

Effect of compounds 1, 2 and 4 on the growth of bentgrass was examined (Fig. 2). 2,4-Dichlorophenoxyacetic acid (2,4-D) was used as control. The effect of the isolated compounds was also compared with that of ICA, which is the growth inhibitor produced by the fairy ring-forming fungus *L. sordida* (Choi et al. 2010a). Compounds 1, 2 and 4 inhibited the root growth of bentgrass dose-dependently. These compounds inhibited the root growth at 100 and 1000 nmol/paper. Among them, compound 1 showed the strongest inhibitory activity against the root and the activity was as high as ICA.

Although all the four compounds from *L. sordida* have been reported already, this was the first reported isolation of all the compounds from mushroom-forming fungi. Furthermore, plant growth regulating activity of compounds 1, 2 and 4 was first found in this study. In the previous report, ICA strongly inhibited the growth of bentgrass shoot and root (Choi et al. 2010a). In fairy rings, the grass is occasionally killed or damaged (Couch 1995). In addition to ICA, compounds 1, 2 and 4 might contribute to the growth inhibition by the fungus in the rings.

Disclosure

The authors declare no conflict of interest. All the experiments undertaken in this study comply with the current laws of Japan.

Acknowledgments

This work was partially supported by a Grants-in-Aid for Young Scientists (Start-up; 26892013, and A, 16H06192) from MEXT. We thank F. Kobayashi (Nasu Biofarm, Ltd.) for providing the fungus *L. sordida*.

REFERENCES

Asai T, Choi JH, Ikka T, Fushimi K, Abe N, Tanaka H, Yamakawa Y, Kobori H, Kiriiwa Y, Motohashi R, Deo VP, Asakawa T, Kan T, Morita A, Kawagishi H, 2015. Effect of 2-azahypoxanthine (AHX) produced by the fairy-ring-forming fungus on the growth and the grain yield of rice. *Japan Agricultural Research Quarterly* 49: 45–49; http://dx.doi.org/10.6090/jarq.49.45.

Choi JH, Abe N, Tanaka H, Fushimi K, Nishina Y, Morita A, Kiriiwa Y, Motohashi R, Hashizume D, Koshino H, Kawagishi H, 2010a. Plant-growth regulator, imidazole-4-carboxamide produced by fairy-ring forming fungus *Lepista sordida*. *Journal of Agricultural and Food Chemistry* 58: 9956–9959; http://dx.doi.org/10.1021/jf101619a.

Choi JH, Fushimi K, Abe N, Tanaka H, Maeda S, Morita A, Hara M, Motohashi R, Matsunaga J, Eguchi Y, Ishigaki N, Hashizume D, Koshino H, Kawagishi H, 2010b. Disclosure of the “fairy” of fairy-ring forming fungus *Lepista sordida*. *ChemBioChem* 11: 1373–1377; http://dx.doi.org/10.1002/cbic.201000112.

Choi JH, Ohnishi T, Yamakawa Y, Takeda S, Sekiguchi S, Maruyama W, Yamashita K, Suzuki T, Morita A, Ikka T, Motohashi R, Kiriiwa Y, Tobina H, Asai T, Tokuyama S, Hirai H, Yasuda N, Noguchi K, Asakawa T, Sugiyama S, Kan T, Kawagishi H, 2014. The source of “fairy rings”: 2-azahypoxanthine and its metabolite found in a novel purine metabolic pathway in plants. *Angewandte Chemie International Edition* 53: 1552–1555; http://dx.doi.org/10.1002/anie.201308109.

Christopher JW, Dennis F, Susan CK, Phillip C, 2004. Using a kinase screen to investigate the constituents of the sponge *Stelletta clavosa* obtained from diverse habitats. *Bioorganic & Medicinal Chemistry* 12: 5631–5637; http://dx.doi.org/10.1016/j.bmc.2004.07.061.

Couch HB, 1995. Fairy rings. In: Couch HB (ed), *Diseases of turfgrasses, 3rd edn*. Krieger, Malabar, pp 181–186.

Evershed H, 1884. Fairy rings. *Nature* 29: 384–385; http://dx.doi.org/10.1038/029384e0.

Furtado NAJC, Pupo MT, Carvalho I, Campo VL, Duarte MCT, Bastos JK, 2005. Diketopiperazines produced by an *Aspergillus fumigatus* Brazillian strain. *Journal of the Brazilian Chemical Society* 16: 1448–1453; http://dx.doi.org/10.1590/S0103-50532005000800026.

Khedr AIM, Kouno I, Tanaka T, Yamada K, 2013. New diketopiperazine derivatives from culture broth of *Staphylococcus* sp. isolated from *Corallina officinalis* Linnaeus. *Heterocycles* 87: 1029–1037; http://dx.doi.org/10.3987/COM-13-12691.

Kumar N, Mohandas C, Nambisan B, Kumar DRS, Lankalapalli, 2013. Isolation of proline-based cyclic dipeptides from *Bacillus* sp. N strain associated with rhabitid entomopathogenic nematode and its antimicrobial properties. *World Journal of Microbiology and Biotechnology* 29: 355–364; http://dx.doi.org/10.1007/s11274-012-

1189-9.
Mar Delgado-Povedano M, Medina VS, Bautista J, Priego-Capote F, Castro MDL, 2016. Tentative identification of the composition of *Agaricus bisporus* aqueous enzymatic extracts with antiviral activity against HCV: a study by liquid chromatography-tandem mass spectrometry in high resolution mode. *Journal of Functional Foods* 24: 403–419; http://dx.doi.org/10.1016/j.jff.2016.04.020.
Mitchinson A, 2014. Fairy chemicals. *Nature* 505: 298; http://dx.doi.org/ 10.1038/505298a.
Omar S, Tenenbaum L, Manes LV, Crews P, 1988. Novel marine sponge derived amino acids 7. The fenestins. *Tetrahedron Letters* 29: 5489–5492; http://dx.doi.org/10.1016/S0040-4039(00)80794-7.
Qi SH, Xu Y, Gao J, Qian PY, Zhang S, 2009. Antibacterial and antilarval compounds from marine bacterium *Pseudomonas rhizosphaerae*. *Annals of Microbiology* 59: 229–233; http://dx.doi.org/10.1007/BF03178321.
Shaala LA, Youssef DTA, Badr JM, Harakeh SM, 2016. Bioactive 2(1*H*)-pyrazinones and diketopiperazine alkaloids from a tunicate-derived actinomycete *Streptomyces* sp. *Molecules* 21: 1116–1124; http://dx.doi.org/10.3390/molecules21091116.
Strom K, Sjogren J, Broberg A, Schnurer J, 2002. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-phe-L-pro) and cyclo(L-phe-*trans*-4-OH-L-pro) and 3-phenyllactic acid. *Applied and Environmental Microbiology* 68: 4322–4327; http://dx.doi.org/10.1128/AEM.68.9.4322-4327.
Tobina H, Choi JH, Asai T, Kiriiwa Y, Asakawa T, Kan T, Morita A, Kawagishi H, 2014. 2-Azahypoxanthine and imidazole-4-carboxamide produced by the fairy-ring-forming fungus increase yields of wheat. *Field Crops Research* 162: 6–11.
Wang G, Dai S, Chen M, Wu H, Xie L, Luo X, Li X, 2010. Two diketopiperazine cyclo(pro-phe) isomers from marine bacteria *Bacillus subtilis* sp. 13-2. *Chemistry of Natural Compounds* 46: 583–585; http://dx.doi.org/10.1007/s10600-010-9680-8.

Figure legends

Fig. 1– Structures of compounds 1 to 4.

Fig. 2– Effect of compounds 1, 2 and 4 on the growth of bentgrass. Bentgrass seedlings were treated with compounds 1, 2 and 4. Respective length of growth compared with the control ± standard deviation (*$p < 0.05$, **$p < 0.01$ vs control, n = 13–16).

Dear Dr. Tsutomu Hattori,

Thank you for your e-mail regarding our manuscript entitled “Plant growth inhibitors from the culture broth of fairy ring-forming fungus *Lepista sordida*”. According to your suggestion, we revised the manuscript.

I believe that this manuscript has been improved satisfactorily and is acceptable for publication in *Mycoscience.*

Sincerely yours,

Hirokazu Kawagishi, Ph. D.
Professor
Research Fellow of Shizuoka University

Highlights

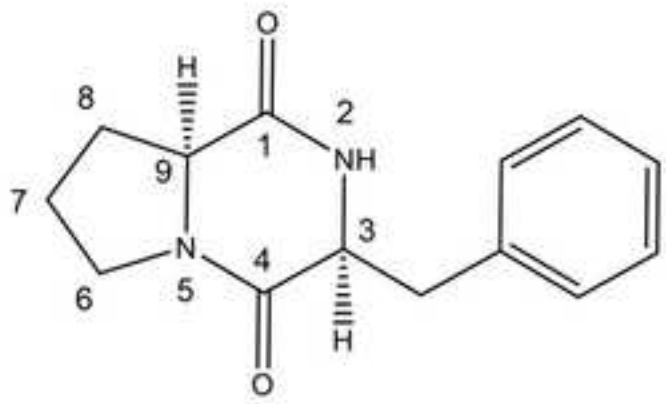
• Four known compounds were isolated from the culture broth of *Lepista sordida*.

• All the compounds were isolated from mushroom-forming fungi for the first time.

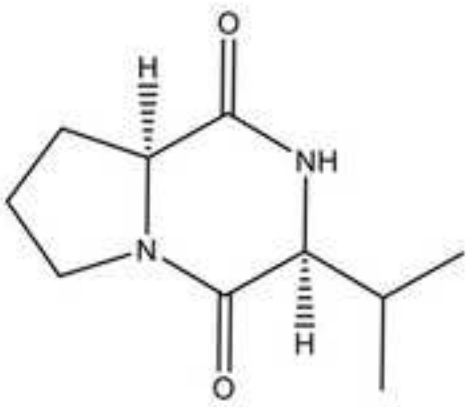
• Three of the four compounds inhibited the root growth of bentgrass at 100 nmol/paper.

• The biological activity of the compounds against plant was first found in this study.

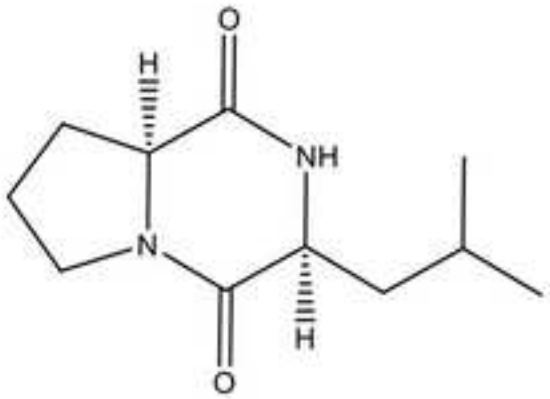
Figure
Click here to download high resolution image



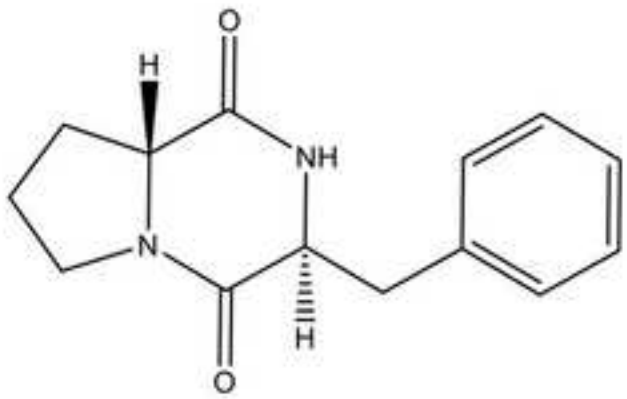
1



2



3



4

Fig. 1 Ito *et al.*

Figure

Click here to download high resolution image

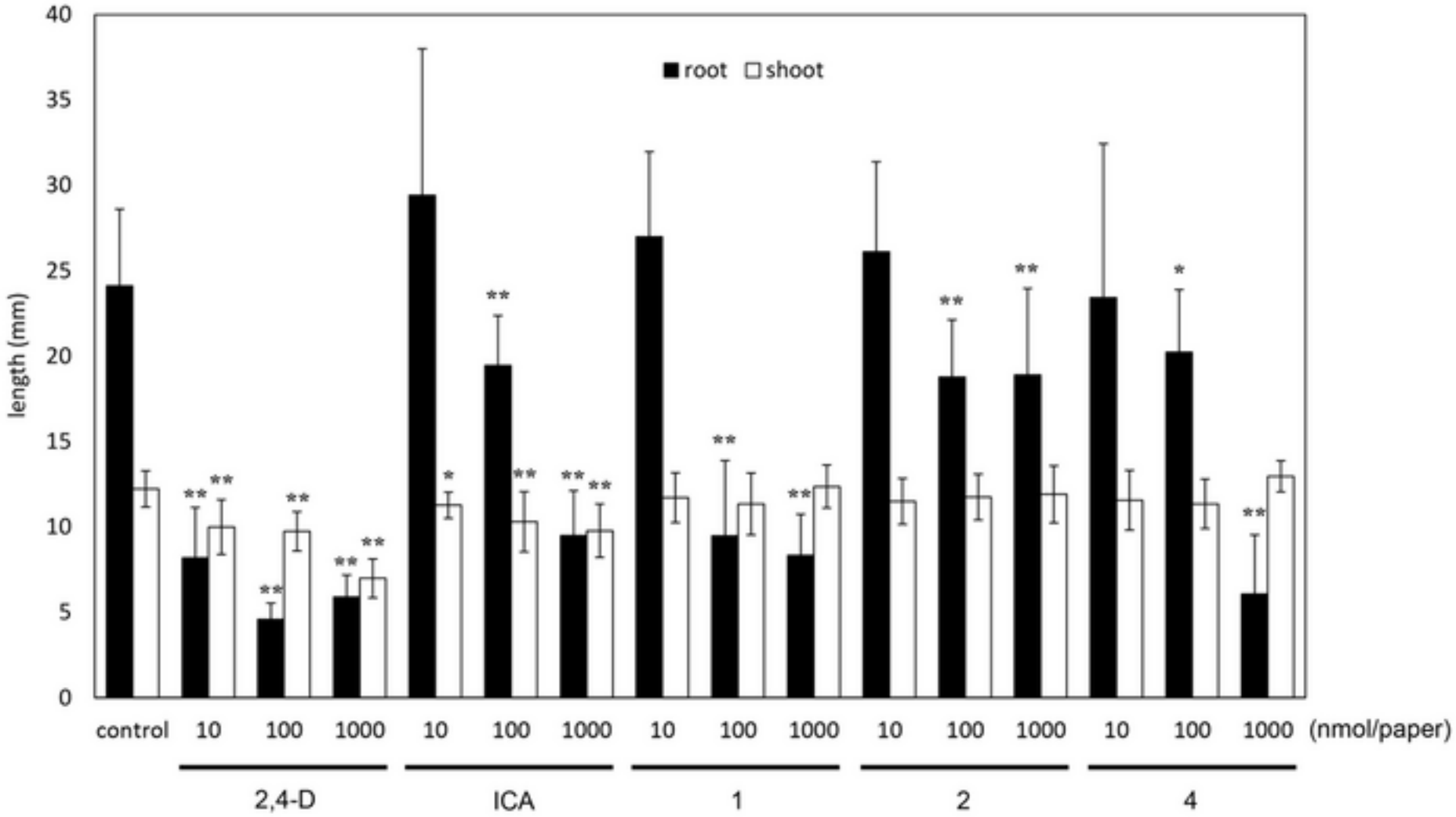


Fig. 2 Ito *et al.*