

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

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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.

1 **Note**

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

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28 Text: 7 pages; figures: 2

1 ABSTRACT

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

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14 Plant growth regulator; Structural identification
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1 “Fairy rings” are zones of stimulated grass growth owing to the interaction between a fungus
2 and a plant (Couch 1995). The term “fairy rings” originates from the myths related to this
3 phenomenon in the Middle Ages. Since the first scientific report on fairy rings was published
4 in 1675 and the subsequent research on them was reviewed in *Nature* in 1884 (Evershed
5 1884), this phenomenon had been attributed to an unknown “fairy”.

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

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

20 21 ***Extraction and isolation***

22
23 The mycelia of *Lepista sordida* AKK101010 were collected in Akita Prefecture and provided
24 by Fumio Kobayashi of Nasu Biofarm, Ltd. (Nasu, Japan). Five pieces (5 × 5 × 3 mm) cut
25 from 20-d-old mycelia cultures of *L. sordida* on PYG agar (0.3% polypeptone, Nippon
26 Becton Dickinson Company, Ltd., Minato, Tokyo, Japan; 0.3% yeast extract, Nippon Becton
27 Dickinson Company, Ltd.; 1% glucose, Wako Pure Chemical Industries, Ltd., Chuo, Osaka,
28 Japan; 1.8% agar, Sigma-Aldrich Co., LLC., Meguro, Tokyo, Japan) were inoculated into a
29 500-mL Erlenmeyer flask containing 300 mL of PYG liquid medium and incubated at 25 °C
30 with 120 rpm for a mo in the dark (Bio-Shaker BR-300LF, Taitec Co., Ltd., Koshigaya,
31 Saitama, Japan). The culture broth (12 L) was evaporated under reduced pressure, and the
32 concentrate was divided into a hexane soluble part, an EtOAc soluble part and a water soluble
33 part. The EtOAc soluble part (1.5 g) of the culture broth was fractionated by silica gel flash
34 column chromatography (Slica gel 60N, Kanto Chemical, Tokyo, Japan); 95% (fractions 1 to
35 3), 85% (fractions 4 to 7), 70% (fractions 8 to 11), 40% (fractions 12 to 14) CH₂Cl₂/MeOH;
36 MeOH (fractions 15 to 18); 90% (fractions 19 to 20) MeOH/H₂O to obtain 20 fractions
37 (fractions 1 to 20). HPLC separations were performed with a JASCO Gulliver system.
38 Fraction 10 (76.1 mg) was further separated by normal-phase HPLC (Senshupak Aquasil AQ,
39 Senshu Scientific Co., Ltd., Suginami, Tokyo, Japan; CHCl₃) to afford compound 1 (8.9 mg)
40 and compound 2 (6.2 mg). Compound 3 (1.2 mg) was purified from fraction 7 (51.0 mg) by
41 normal-phase HPLC (Develosil silica, Nomura Chemical Co., Ltd., Seto, Aichi, Japan; 95%
42 CHCl₃/MeOH). Fraction 14 (238 mg) was fractionated by normal-phase HPLC (Senshupak
43 Aquasil AQ; CHCl₃) to obtain 18 fractions (fractions 14-1 to 14-18). Subsequently, fraction
44 14-7 (49.2 mg) was further separated by reverse-phase HPLC (X-Bridge Phenyl, Waters,

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

5 *Identification of active compounds*

6
7 ¹H NMR spectra (one- and two-dimensional) were recorded on a JEOL lambda-500
8 spectrometer (JEOL, Tokyo, Japan) at 500 MHz, and ¹³C NMR spectra were recorded on the
9 same instrument at 125 MHz. The electrospray ionization-mass spectrometry (ESIMS)
10 spectra were measured on a JMS-T100LC mass spectrometer (JEOL, Tokyo, Japan). The
11 specific rotation value was measured with a JASCO DIP-1000 polarimeter (JASCO, Tokyo,
12 Japan).

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

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

22 Compound 2 was purified as white crystals. The structure of compound 2 was identified
23 to be a diketopiperazine of proline and valine by interpretation of the NMR spectra (Furtado
24 et al. 2005). The absolute configurations, L and L, of the two amino acids in compound 2 were
25 identified by hydrolysis of the compound with hydrochloric acid and then HPLC analysis
26 (CROWNPAK CR(+), Daicel Chemical Ind., Ltd., Minato, Tokyo, Japan; H₂O). Compound 2,
27 (3*S*,9*S*)-3-isopropylhexahydropyrrolo[1,2- α]pyrazine-1,4-dione, has been isolated from
28 sponge *Leucophloeus fenestrata*, and its antibacterial and antilarval activity have been
29 reported (Omar et al. 1988; Qi et al. 2009).

30 Compounds 3 and 4 were also identified to be diketopiperazines of proline and leucine,
31 proline and phenylalanine, respectively, by interpretation of the NMR spectra (Furtado et al.
32 2005; Wang et al. 2010). Their absolute configurations were identified by the same way as
33 that of compound 2. Compound 3, (3*S*,9*S*)-3-isobutylhexahydropyrrolo[1,2- α]pyrazine-1,4-
34 dione, has been isolated from *Aspergillus fumigatus* and showed antimicrobial, antifungal and
35 antitumor activity (Furtado et al. 2005; Khedr et al. 2013; Shaala et al. 2016). Compound 4,
36 (3*S*,9*R*)-3-benzylhexahydropyrrolo[1,2- α]pyrazine-1,4-dione, has been isolated from marine
37 *Bacillus subtilis* and showed antimicrobial activity (Wang et al. 2010; Kumar et al. 2013).

38 Compound 1: white crystals; ESIMS m/z 267 [M+Na]⁺; [α]_D²⁶ -30 (*c* 0.22, EtOH); ¹H
39 NMR (in CDCl₃) δ _H: 1.89 (m), 1.96 (m), 2.01 (m), 2.32 (m), 2.82 (dd), 3.56 (m), 3.63 (m),
40 3.66 (m), 4.08 (t), 4.29 (dd), 7.24 (d), 7.29 (t), 7.35 (t); ¹³C NMR δ _C: 22.5, 28.3, 36.8, 45.4,
41 56.2, 59.1, 127.5, 129.1, 129.2, 135.9, 165.0, 169.3.

42 Compound 2: white crystals; ESIMS m/z 219 [M+Na]⁺; ¹H NMR (in CDCl₃) δ _H: 0.87 (d),
43 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

1 (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.

2 Compound 3: white crystals; ESIMS m/z 233 $[\text{M}+\text{Na}]^+$; ^1H NMR (in CDCl_3) δ_{H} : 0.93 (d),
3 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
4 (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.

5 Compound 4: white crystals; ESIMS m/z 267 $[\text{M}+\text{Na}]^+$; ^1H NMR (in CDCl_3) δ_{H} : 1.63 (m),
6 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
7 (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,
8 167.4, 171.3.

10 ***Plant growth regulatory activity***

12 Bentgrass seeds (Japanese lawn grass T.T.S noshiiba; Takii Co., Ltd., Tokyo, Japan) were
13 sterilized with ethanol for five minutes and with 1% sodium hypochlorite solution for ten
14 minutes, and then washed with sterilized water ten times. Seeds were seeded on 0.8% agar
15 medium (Sigma-Aldrich Co., LLC., Japan) in a Petri dish ($\phi 60 \times 20$ mm) and pre-cultured
16 for 5 d (25 °C, 16 h-8 h (L/D) photoperiod). Samples were dissolved in 1 mL of
17 dichloromethane (10, 100 and 1000 nmol/mL) and then poured on filter paper (Advantec No.
18 2, $\phi 55$ mm; Toyo Roshi Kaisha, Tokyo, Japan) in a Petri dish ($\phi 60 \times 20$ mm). After the
19 sample-loaded paper had been air-dried, 1 mL of distilled water was poured on the sample-
20 loaded paper or intact filter paper (control). The preincubated bentgrass ($n = 16$ in each Petri
21 dish) was transferred onto the sample-loaded filter paper or control filter paper and incubated
22 in a growth chamber for one week (25 °C, 16 h-8 h (L/D) photoperiod). The lengths of the
23 root and shoot were measured to an accuracy of 0.01 mm with an absolute digimatic caliper
24 (Mitutoyo Co., Kawasaki, Kanagawa, Japan).

25 Effect of compounds 1, 2 and 4 on the growth of bentgrass was examined (Fig. 2). 2,4-
26 Dichlorophenoxyacetic acid (2,4-D) was used as control. The effect of the isolated
27 compounds was also compared with that of ICA, which is the growth inhibitor produced by
28 the fairy ring-forming fungus *L. sordida* (Choi et al. 2010a). Compounds 1, 2 and 4 inhibited
29 the root growth of bentgrass dose-dependently. These compounds inhibited the root growth at
30 100 and 1000 nmol/paper. Among them, compound 1 showed the strongest inhibitory activity
31 against the root and the activity was as high as ICA.

32 Although all the four compounds from *L. sordida* have been reported already, this was
33 the first reported isolation of all the compounds from mushroom-forming fungi. Furthermore,
34 plant growth regulating activity of compounds 1, 2 and 4 was first found in this study. In the
35 previous report, ICA strongly inhibited the growth of bentgrass shoot and root (Choi et al.
36 2010a). In fairy rings, the grass is occasionally killed or damaged (Couch 1995). In addition
37 to ICA, compounds 1, 2 and 4 might contribute to the growth inhibition by the fungus in the
38 rings.

40 **Disclosure**

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

Acknowledgments

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27

28 **Figure legends**

29 Fig. 1– Structures of compounds 1 to 4.

30 Fig. 2– Effect of compounds 1, 2 and 4 on the growth of bentgrass. Bentgrass seedlings were

31 treated with compounds 1, 2 and 4. Respective length of growth compared with the control \pm

32 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,

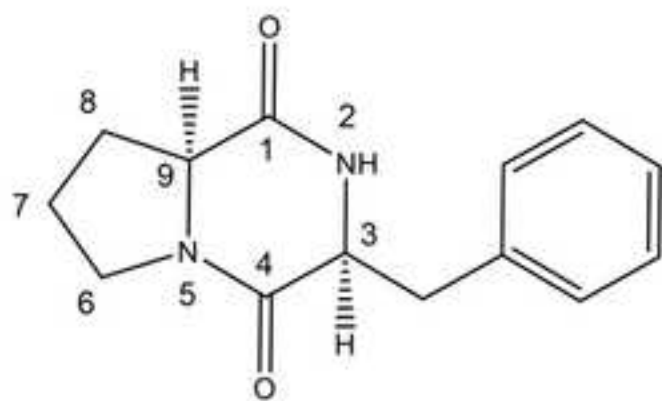
Hirokazu Kawagishi, Ph. D.
Professor
Research Fellow of Shizuoka University

Highlights

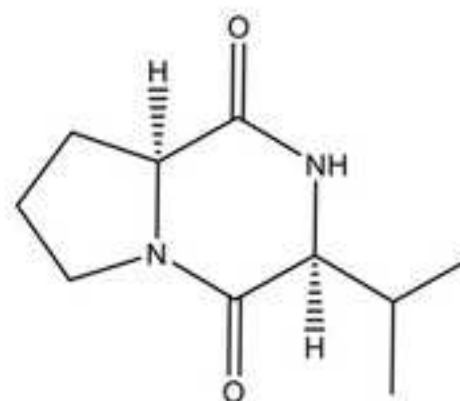
- Four known compounds were isolated from the culture broth of *Lepista sordida*.
- All the compounds were isolated from mushroom-forming fungi for the first time.
- Three of the four compounds inhibited the root growth of bentgrass at 100 nmol/paper.
- The biological activity of the compounds against plant was first found in this study.

Figure

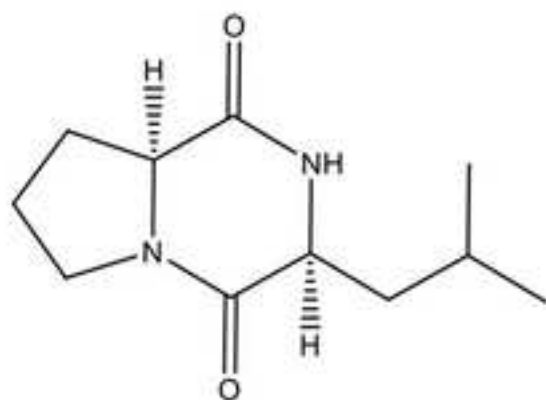
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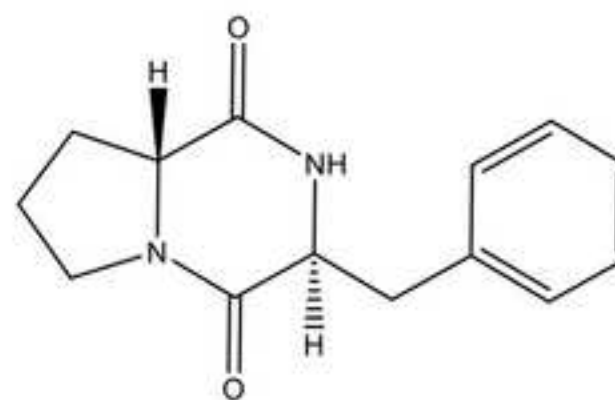
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Fig. 1 Ito *et al.*

Figure

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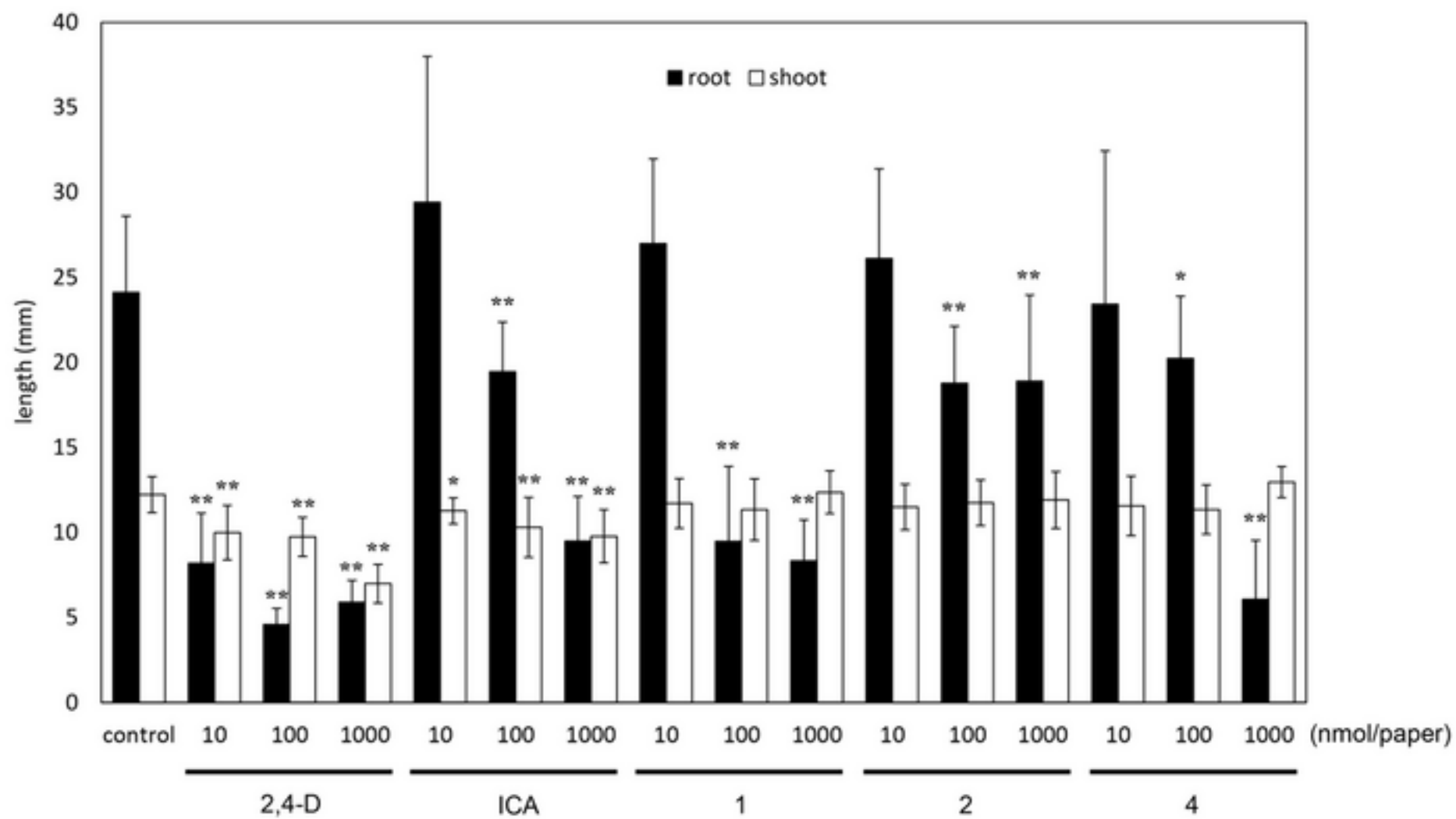


Fig. 2 Ito *et al.*