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ISOLATION AND QUANTIFICATION OF THE PLANT GROWTH REGULATOR 1-TRIACONTANOL FROM MOSO BAMBOO (*Phyllostachys pubescens*) SHOOT SKIN AND ITS COMPOST

SUMMARY

To investigate chemical uses of Moso bamboo (*Phyllostachys pubescens*) shoot skin, we identified the main component of non-polar solvent extracts. To this end, a white precipitate from *n*-hexane extracts was evaluated using silica-gel column chromatography. The fraction with the highest recovery showed a single spot in silica-gel thin-layer chromatography (TLC) analyses. In subsequent nuclear magnetic resonance (NMR) and electron impact-mass spectrometry (EI-MS) analyses, we identified the compound in the fraction as 1-triacontanol, which is a known regulator of plant growth. In addition, gas chromatography-mass spectrometry (GC-MS) experiments showed 1-triacontanol concentrations of 13.3 and 41.7 ppm in fresh and boiled skins, respectively. In boiled skins, 1-triacontanol concentrations reached a maximum of 71.3 ppm after 2 weeks of composting. Although concentrations gradually decreased thereafter, they remained at 19.7 ppm after compost maturation for 6 months. In a further experiment, seeds of Welsh onion were sown on absorbent cotton impregnated with authentic 1-triacontanol solutions, significant increase in hypocotyl length was observed. Due to the presence of 1-triacontanol, Moso bamboo shoot skin has potential as functional compost that promotes plant growth for agricultural uses.

Keywords: 1-triacontanol, compost, Moso bamboo shoot skin, non-polar solvent extract, *Phyllostachys pubescens.*

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INTRODUCTION

Bamboo shoots are a popular delicacy with a crunchy texture and pleasant taste, and are known as the King of Forest Vegetables (Chongtham et al., 2011). Annual consumption of bamboo shoots has been maintained at approximately 200,000 t for the past forty years in Japan (Forestry Agency, Ministry of Agriculture, Forestry and Fisheries, Japan, 2020). Moso bamboo (*Phyllostachys pubescens*) is the highest yield species of bamboo shoots in Japan (Torii and Isagi, 1997). Moso bamboo shoots are mainly available in fresh and canned forms. When harvested during winter (off-season), Moso bamboo shoots are marketed as the fresh form without peeling. Spring (peak season) harvests, however, are often processed by boiling and canning for long term storage. Boiled shoots are generally peeled prior to canning, leading to the accumulation of numerous shoot skins at canning factories during the peak season. Because they generate an unpleasant odor with decay, their disposal demands significant time and cost.

In our previous studies of Moso bamboo shoot skins, components from fresh skins were roughly divided into polar and non-polar compounds by extracting with *n*-hexane and then extracting the insoluble fractions with methanol and dichloromethane (Tanaka et al., 2011, 2013). We then examined the functions of polar solvent-extracts and isolated the compounds stigmasterol and dihydrobrassicasterol from dichloromethane-soluble fractions of methanol extracts. These compounds had antibacterial activity against *Staphylococcus aureus* (Tanaka et al., 2011, 2013).

Moreover, in an *in vitro* immunization system (Iwamoto et al., 2013), these methanol extracts suppressed immunoglobulin E production following stimulation by the cedar pollen antigen (Cry j 1) in human peripheral blood mononuclear cells (Tanaka, 2013). Antioxidant activities of methanol extracts were also described in oxygen radical absorption capacity assays and inhibition of melanin biosynthesis was demonstrated in cultured B-16 melanoma cells (Tanaka, 2013).

These studies collectively indicate that Moso bamboo shoot skins have functional ingredients with promise in cosmetics and other health-related products following extraction with polar solvents, such as methanol. Compounds that were extracted with the non-polar solvent *n*-hexane had limited antibacterial, antiallergic, antioxidant, and antimelanoma activities (Tanaka, 2013).

In this study, we further investigated functions of non-polar solvent extracts from Moso bamboo shoot skins by isolating major components of *n*-hexane extracts and identified the plant growth regulator 1-triacontanol (Ries et al., 1977b; Naeem et al., 2012). We also determined 1-triacontanol concentrations in composted boiled (hot water-extracted) shoot skins. Finally, we report the effects of 1-triacontanol treatments on seed germination and hypocotyl lengths of a plant species.

MATERIAL AND METHODS

Moso bamboo shoot skin and composting

Moso bamboo (*P. pubescens*) shoots were harvested in Miyako or Yame, Fukuoka prefecture, Japan, and non-boiled or boiled skins were provided by Life Design Co., Ltd. (Fukuoka, Japan) or Kazue Bussan Inc. (Fukuoka, Japan). Boiled skins (about 1 m^3) were composted in a pile on a composting platform at Fukuoka Agriculture and Forestry Research Center. The pile was turned manually every 2 weeks to maintain aerobic conditions and was watered when necessary. Samples were taken before boiling (fresh) and after 0, 1, and 2 weeks, and 2, 3, and 6 months after composting. Samples were roughly crushed into small pieces and were lyophilized after storage at −30°C.

Isolation and identification of the main components in n-hexane extracts from Moso bamboo shoot skin

Non-boiled (fresh) skins of Moso bamboo shoots were dried at room temperature and were then ground to powder. The powder (17.8 kg) was extracted with 72.5 l of *n*-hexane for 48 h. The *n*-hexane solution was then evaporated, yielding 40.1 g (dry weight) of extract. A 39 g sample of the extract was dissolved in 500 ml of *n*-hexane by sonicating and heating. The resulting solution was stored overnight at −20°C and was then decanted and the precipitated solute was purified to a yellowish crude powder.

The dissolving and decanting process was repeated until the solute was bleached and 5 g of white precipitate was finally obtained. Subsequently, 4 g of crude precipitate was applied to silica-gel column chromatography (column, φ 8.0 × 32.0 cm) with 800 g of Wakogel C-200 and was eluted with 2000 ml of *n*-hexane:ethyl acetate mixtures at ratios of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 2.5:7.5, and 0:10, and then with methanol (2000 ml). For each elution stage, 500 ml fractions were collected, leading to a total of 36 fractions (fl–f36). Fractions were combined to form 12 fractions as follows: F1 (f1–f4, 8.1 mg), F2 (f5–f8, 7.2 mg), F3 (f9–f11, 473.7 mg), F4 (f12–f13, 641.9 mg), F5 (f14–f16, 325.0 mg), F6 (f17–f19, 290.1 mg), F7 (f20–f21, 32.2 mg), F8 (f22–f24, 63.8 mg), F9 (f25–f28, 50.2 mg), F10 (f29–f32, 22.1 mg), F11 (f33–f34, 2.4 mg), and F12 (f35–f36, 106.1 mg). These fractions were analyzed using silica-gel thin-layer chromatography (TLC) on silica-gel 60 F254 plates (Merck Co., Darmstadt, Germany) with *n*-hexane:ethyl acetate:acetic acid at 9:1:0.1 (F1–F3), 7:3:0.1 (F4–F8), or 6:4:0.1 (F9–F12). Spots were made visible by spraying with 10% sulfuric acid/methanol and then heating on a hot plate to 140°C. Among fractions, F4 was recovered with the highest quantity and contained a pure compound that formed a single spot in TLC analyses.

The chemical structure of the compound was analyzed using nuclear magnetic resonance (NMR) and electron impact-mass spectrometry (EI-MS). ^{1}H and ^{13}C-NMR spectra were generated on a Bruker DRX 400 NMR spectrometer (Bruker Daltonics Inc., MA, USA) using tetramethylsilane (TMS) as an internal standard for chemical shifts. Chemical shifts (δ) were expressed in ppm with

reference to TMS resonance. EI-MS was performed using a JEOL JMS 700 spectrometer (JEOL, Japan). Organic solvents and Wakogel C-200 were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Chloroform extraction from Moso bamboo shoot skin

Lyophilized samples were ground into 1-mm particles using a Wiley mill and were extracted with chloroform, because authentic 1-triacontanol (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was more soluble in chloroform than in *n*-hexane in our preliminary tests. Ground samples (10 g) were extracted three times with 100 ml of chloroform at room temperature with shaking at 160 rpm for 24 h. After dehydration with anhydrous sodium sulfate, solutions were filtered through filter paper (No. 5A, 150 mm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and were concentrated using a rotary evaporator. Dried extracts were redissolved in chloroform to a concentration of 5 mg/ml.

Quantitative gas chromatography-mass spectrometry analysis of 1-triacontanol

Forty-microliter aliquots of *N,O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) were added to each chloroform extract (160 µl), and derivatization was achieved by heating the mixtures at 60°C for 10 min. Gas chromatography-mass spectrometry (GC-MS) analyses were performed using an Agilent 7890A gas chromatograph (Agilent Technologies, Inc., CA, USA) equipped with an Agilent 7693A autosampler (Agilent Technologies, Inc., CA, USA) and a Agilent 5975C inert XL MSD mass selective detector (Agilent Technologies, Inc., CA, USA). Into a DB-5MS capillary column (30 m long, 0.25 mm inner diameter, 0.25 µm film thickness; Agilent Technologies, Inc., CA, USA) in the splitless mode, 1 µl aliquots of derivatized sample were injected. The oven temperature was operated at 40°C for 3 min, and the temperature was then increased to 300°C at 15°C/min and was held at this temperature for 10 min. Helium was used as a carrier gas. Injector and detector temperatures were both 250°C. 1-Triacontanol was identified as a trimethylsilyl derivative by comparing retention times and mass spectra with those from the NIST 08 library and an authentic compound in total ion monitoring (SCAN) mode. Ions at *m/z* 471 and 495 were chosen as targets for identification and quantification of 1-triacontanol in selected ion monitoring (SIM) mode, because they were the most abundant and lacked cross-interferences in mass spectra. In SIM mode, 1-triacontanol concentrations were quantified using an external standard method with a 5-point calibration curve and peak areas of authentic standards ranging from 25 to 400 µg/ml.

Plant growth test

Solutions of authentic 1-triacontanol were adjusted to 1000 ppb in distilled water with 0.1% Tween20, and were then autoclaved at 121°C for 20 min. They were serially diluted to 1, 10, and 100 ppb in distilled water. Thirty Welsh onion

(*Allium fistulosum*) seeds were sown on absorbent cotton impregnated with 40 ml of each solution using a Germination Index Kit (JPec Co., Ltd., Tokyo, Japan). After incubation for 6 days in the dark at 25°C, germination rates and hypocotyl lengths were measured. The test was independently repeated three times.

RESULTS AND DISCUSSION

Isolation and identification of 1-triacontanol from n-hexane soluble fractions of Moso bamboo shoot skins

Silica-gel column chromatography of white precipitates from *n*-hexane extracts of fresh Moso bamboo shoot skins were collected in 12 fractions. A single spot from the fraction (F4) on TLC plates showed the highest recovery (641.9 mg) of all fractions. This spot was also detected in TLC analyses of the other four fractions (F5 to F8), with close polarities to that of F4 (data not shown). Thus, the main component of the white precipitate was isolated from F4 and was considered a pure compound.

The pure compound 1 was isolated as a white powder from F4 (Fig. 1). ^{13}C spectrum signals were discriminated into CH_3 resonance at δ_C 14.3 and a signal due to a primary alcoholic group at δ_C 63.3 (Fig. 2). Other signals were assigned to aliphatic CH_2 groups, which resonated at δ_C 22.9–33.0. These data suggested that compound 1 is an aliphatic straight chain primary alcohol. ^1H-NMR spectrum revealed a signal at δ_H 3.64 (t, J = 6.8 Hz), indicating a CH_2OH group, and a signal at δ_H 0.88 (t, J = 8.0 Hz, 3H) for a terminal CH_3 group (Fig. 3). An upfield broad signal resonated at δ_H 1.25 (54H, m) and was assigned to H3–H29.

The number of protons at δ_H 1.25 was assumed based on the integrated intensity of the terminal methyl at δ_H 0.88 and this was confirmed using EI-MS (Fig. 4). In the splitting pattern, a triplet peak at δ_H 0.88 was integrated for 3H and indicated the presence of CH_3CH_2 as a partial structure of the compound. Another triplet peak with 2H at δ_H 3.64 was considered to be from an α-hydrogen adjacent to a hydroxyl group, indicating the presence of CH_2CH_2OH. A broad peak with 54H at δ_H 1.25 and a multiplet peak with 2H at δ_H 1.57 indicated the presence of $[CH_2]_{27}$ and CH_2, respectively. Correlated spectroscopy spectra showed correlations between protons at δ_H 0.88 and δ_H 1.25, at δ_H 1.25 and δ_H 1.57, and at δ_H 1.57 and δ_H 3.64 (Fig. 5).

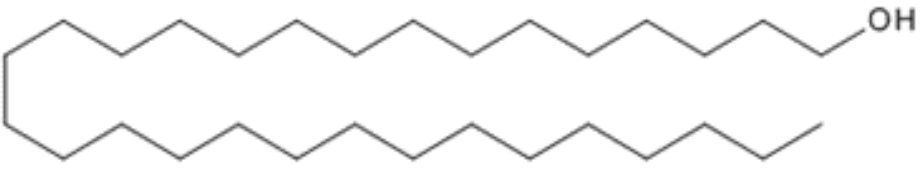

Fig. 1. Chemical structure of 1-triacontanol ($C_{30}H_{62}O$).

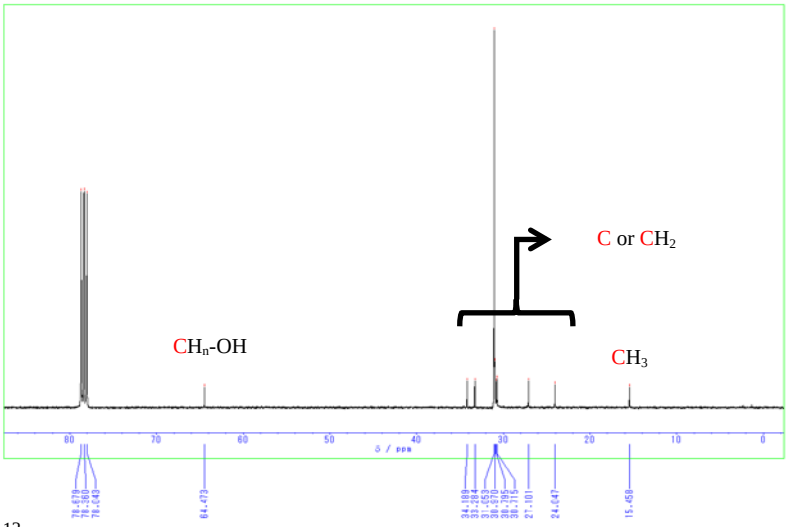

Fig. 2. ^{13}C nuclear magnetic resonance (NMR) spectra of the fraction F4 from *n*-hexane extracts of fresh Moso bamboo shoot skins; herein, chloroform appeared in a triplet peak with a chemical shift at 78.36 ppm. Because this value is usually 77.2 ppm, presented x values were adjusted by −1.16 ppm.

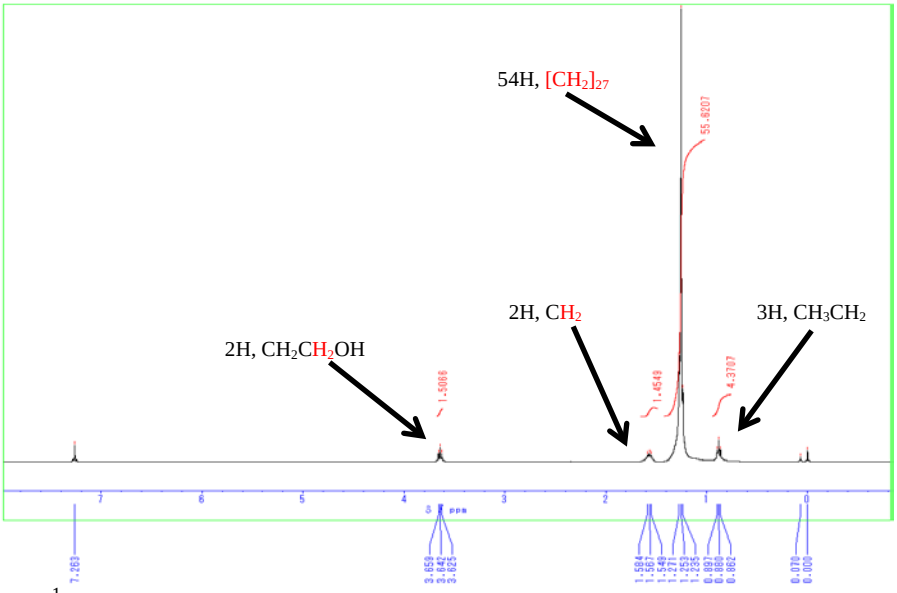

Fig. 3. ^{1}H nuclear magnetic resonance (NMR) spectra of fraction F4 of *n*-hexane extracts from fresh Moso bamboo shoot skins.

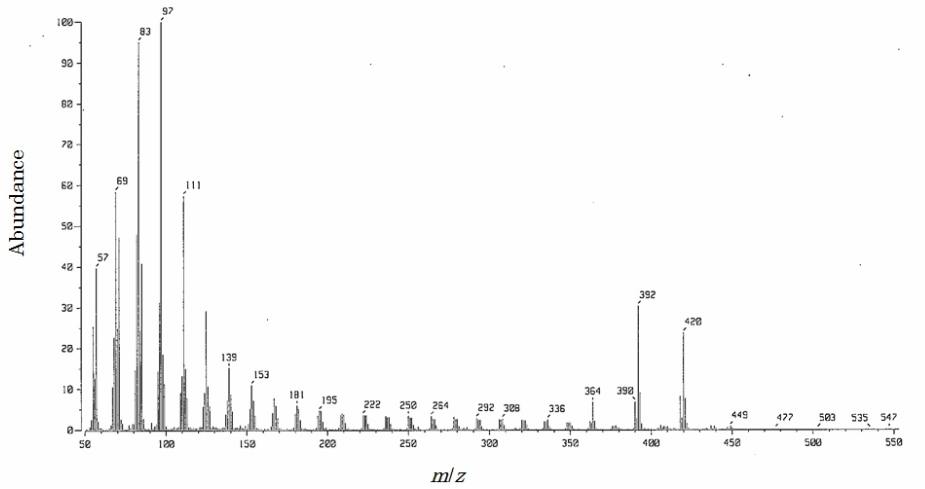

Fig. 4. Electron impact-mass spectrometry (EI-MS) of fraction F4 of *n*-hexane extracts from fresh Moso bamboo shoot skins.

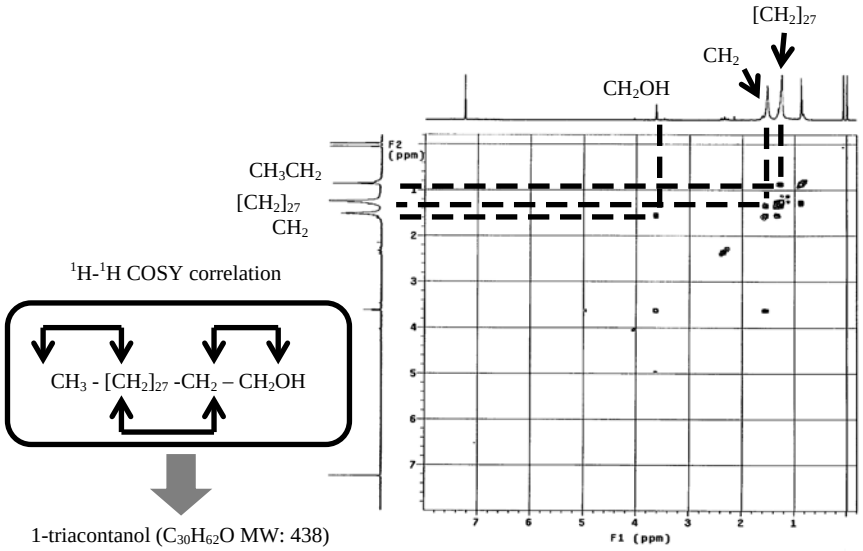

Fig. 5. ^{1}H-^{1}H correlated spectroscopy spectra of fraction F4 of *n*-hexane extracts from fresh Moso bamboo shoot skins.

Connection of the partial structures based on these correlations strongly suggested that the compound in F4 was 1-triacontanol. EI-MS spectra showed a base peak at *m*/*z* 420 $[M\text{-}H_2O]^+$, thus confirming the molecular formula $C_{30}H_{62}O$ and the molecular weight of 438 g/mol (Fig. 4). This base peak also supported the presence of a hydroxyl functional group in the compound. Data from NMR and EI-MS analyses of compound 1 were matched with those reported previously (Du et al., 2009; Upadhyay et al., 2006; Randrianasolo et al., 2015). Chibnall et al.

(1931) was the first to discover 1-triacontanol, and described the compound as a constituent of apple peel wax. Subsequently, this compound was isolated from epicuticular waxes of alfalfa leaves (Chibnall et al., 1933), rice leaves (Uchiyama and Ogasawara, 1981), soybean (*Glycine max*) leaves (Hagedorn et al., 2017), and from sub-epidermal cells of jade plant (*Crassula argentea*) leaves (Kolker, 1978), potato (*Solanum tuberosum*) tubers (Kolker, 1978), and beeswax (Jackson and Eller, 2006). We are the first to report 1-triacontanol in Moso bamboo shoot skins.

Quantification of 1-triacontanol from Moso bamboo shoot skins during composting

Concentrations of 1-triacontanol in dry matter non-boiled (fresh) and boiled Moso bamboo shoot skins during composting are shown in Fig. 6. Before composting, boiled shoot skins contained 1-triacontanol at 41.7 ppm, more than threefold higher than in non-boiled skins (13.3 ppm). With progress of composting, 1-triacontanol concentrations significantly increased and peaked at 71.3 ppm after 2 weeks. Thereafter, 1-triacontanol concentrations gradually decreased to 19.7 ppm after 6 months of composting.

According to quantitative determinations by Kolker (1978), 1-triacontanol concentrations in rice, maize (*Zea mays*), and alfalfa leaves were 481, 234, and 173 ppm, respectively, much higher than in fresh Moso bamboo shoot skins (13.3 ppm) and boiled skins (41.7 ppm) (Fig. 6). According to assessments from the Organization for Economic Co-operation and Development (OECD), alcohols with carbon chain lengths up to C16 are readily biodegradable, with 100% degradation in less than 10 days (OECD, 2006). In addition, alcohols with chain lengths of C16, C18, and over C18 were degraded by 62%, 76%, and 37%, respectively (OECD, 2006). In the present study, 1-triacontanol concentrations in boiled skins increased to 71.3 ppm after 2 weeks of composting (Fig. 6). Hence, we suggest that the long chain length (C30) of 1-triacontanol may be more resistant to degradation than alcohols with shorter chain lengths. Therefore, various low-molecular weight compounds, including those from hot-water extracts, are thought to have been preferentially degraded. We observed gradual decreases in 1-triacontanol concentrations with further composting, with a remaining concentration of 19.7 ppm at 6 months (Fig. 6). In assessments of germination rates of komatsuna (*Brassica rapa* var. *perviridis*) using Germination Index Kits, boiled skins needed 4 months for compost maturation (Tanizaki, unpublished data).

Thus, mature composts of boiled skins could be useful as functional composts, because they do not negatively affect plant growth by limiting nitrogen and contain the plant growth regulator 1-triacontanol.

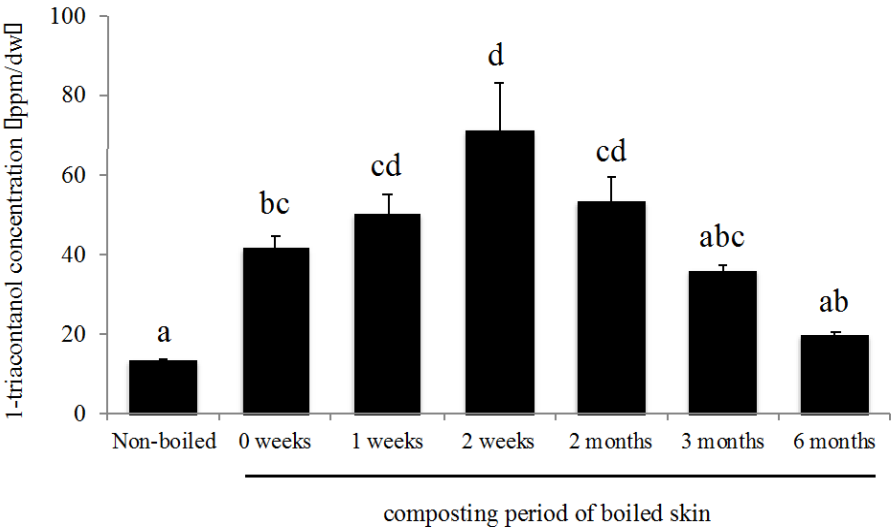

Fig. 6. Concentrations of 1-triacontanol in Moso bamboo shoot skins; bars indicate mean concentrations of 1-triacontanol per dry weight of skins (mean ± SE, *n*=3). Different lower-case letters indicate significant differences in concentrations ($p < 0.05$), as indicated by Tukey's tests.

Effects of 1-triacontanol treatments on plant growth

In this study, we monitored germination rate and hypocotyl length of Welsh onion after treatments with authentic 1-triacontanol solutions (Table 1). Germination rates of Welsh onion were over 90% in all of these treatment conditions except in the presence of 10 ppb 1-triacontanol. Yet hypocotyl lengths of Welsh onions were significantly greater (1.25 times) in 1000 ppb 1-triacontanol solution than in controls. No significant effects of 1-triacontanol treatments on Welsh onions were observed at lower concentrations.

Table 1. Germination rate and hypocotyl length of Welsh onion after treatment with authentic 1-triacontanol solutions at different concentrations

1-triacontanol concentration	Germination rate (%)				Hypocotyl length (mm)			
	mean		SE		mean		SE	
0ppb	96.7	±	0.0	ab	18.3	±	1.0	a
1ppb	98.9	±	1.1	a	20.1	±	0.9	ab
10ppb	88.9	±	2.9	b	17.8	±	1.2	a
100ppb	95.6	±	2.2	ab	20.3	±	0.9	ab
1000ppb	98.9	±	1.1	a	22.9	±	0.8	b

Different lower-case letters indicate significant differences in germination rates or hypocotyl lengths ($p < 0.05$) from Tukey's tests.

1-Triacontanol was originally isolated from chloroform extracts of alfalfa (*Medicago sativa*) as a plant growth regulator that promoted water uptake and dry weight of rice (*Oryza sativa*, Ries et al., 1977b). According to a review article (Naeem et al., 2012), 1-triacontanol enhances growth, yield, photosynthesis, and chlorophyll contents of various plant species. When applied to shoots of rice plants, 1-triacontanol rapidly elicited biosynthesis of 9-β-L(+)-adenosine in the roots (Ries, 1991; Naeem et al., 2012) as a probable source, adenosine monophosphate from adenosine diphosphate and adenosine triphosphate (Olsson and Pearson, 1990; Naeem et al., 2012). Subsequently, apoplastic ion concentrations increased in plant tissues (Ries et al., 1993; Naeem et al., 2012). In published suppression subtractive hybridization and Northern blotting analyses, 1-triacontanol upregulated photosynthetic and photorespiratory genes and downregulated abscisic acid and stress- and wound-related genes (Chen et al., 2002). These events are thought to trigger various metabolic activities, including photosynthesis, nutrient uptake, and enzyme activity, likely leading to accelerated plant growth.

In this study, 1-triacontanol significantly promoted hypocotyl lengths of Welsh onion, but did not affect germination at the tested concentrations (Table 1). When micro propagated balm (*Melissa officinalis*) was planted in medium supplemented with 1-triacontanol at 2–10 ppb, shoot lengths increased significantly (Tantos et al., 1999). Significantly enhanced shoot growth was also observed in herbal plants, including coriander (*Coriandrum sativum*, Idrees et al., 2010), sweet basil (*Ocimum basilicum*, Hashmi et al., 2011), and *Artemisia annua* (Aftab et al., 2010) following treatments with foliar spray containing 1-triacontanol at 439, 4.39, 43.9, and 1.5 ppm, respectively. In another study, seed germination of maize, paddy, and sunflower was significantly promoted by soaking in solution containing 1–10 ppm 1-triacontanol (Niranjana et al., 1999). In contrast, however, germination rates of seeds from 15 species, including soybean, lettuce (*Lactuca sativa*), and purslane (*Portulaca oleracea*), were not significantly enhanced when sown on absorbent paper in petri dishes containing 1-triacontanol at 4.39 ppm (Hoagland, 1980).

Thus, the plant growth promotion effect of 1-triacontanol likely depends on concentration, treatment mode, and species. In this study, we demonstrated that boiled skins of Moso bamboo shoot hold sufficient concentrations of 1-triacontanol to promote hypocotyl lengths of Welsh onion even after compost maturation. Further studies are required to determine volumes of Moso bamboo skins that are required to improve soils and promote growth or improve yields of agricultural plants such as Welsh onion.

More evidence is required to confirm that 1-triacontanol was responsible for increased growth of the present plants. Increased yields of vegetable or crop plants following application of boiled Moso bamboo skins will set a precedent, as shown with alfalfa plants (Ries et al., 1977a). Discarded biomass from bamboo shoot production in a village could be converted to local compost contributing to agricultural production within the same village.

CONCLUSIONS

We isolated the main component of *n*-hexane extracts from fresh Moso bamboo shoot skins using silica-gel column chromatography and identified the plant growth regulator 1-triacontanol in NMR and EI-MS analyses. In our GC-MS analyses, 1-triacontanol concentrations remained 19.7 ppm even after boiled skins were composted for 6 months. Our laboratory experiments show that 1-triacontanol significantly promotes hypocotyl length of germinated Welsh onion. Thus, Moso bamboo shoot skins may provide functional compost that contains a plant growth regulator.

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