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PHYTOTOXIC PROPERTY OF THE INVASIVE PLANT *TITHONIA DIVERSIFOLIA* AND A PHYTOTOXIC SUBSTANCE

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Tithonia diversifolia (Hermsl.) A. Gray is a perennial invasive plant and spreads quickly in the invasive areas. The extracts of *T. diversifolia* were found to be toxic to several crop plant species such as rice, maize, sorghum, lettuce and cowpea, and several putative allelopathic substances were identified. However, there is limited information available for the effects of *T. diversifolia* on wild plants including weed plant species. We investigated the allelopathic potential of *T. diversifolia* extracts on weed plants, and searched for phytotoxic substances with allelopathic activity. An aqueous methanol extract of *T. diversifolia* leaves inhibited the growth of weed plants, *Lolium multiflorum* Lam., *Phleum pretense* L., *Echinochloa crus-galli* (L.) Beauv. The extract was then purified by several chromatographic runs and a phytotoxic substance with allelopathic activity was isolated and identified by spectral analysis as tagitinin C. The substance inhibited the growth of *Lolium multiflorum*, *Phleum pratense* and *Echinochloa crus-galli* at concentrations greater than 0.1 – 0.3 mM. The present results suggest that *T. diversifolia* may possess allelopathic potential on weed plants and tagitinin C may be responsible for the allelopathic effects of *T. diversifolia*. The allelopathic potential of *T. diversifolia* may contribute to its invasive characteristics.

Keywords: Tithonia diversifolia – allelopathy – bioactive substance – phytotoxicity – tagitinin C

INTRODUCTION

Tithonia diversifolia (Hermsl.) A. Gray is a bushy perennial plant, 3–5 m in height, belonging to Asteraceae family. The species originated in Mexico and Central America, and was introduced into several countries as an ornamental plant. However, once introduced *T. diversifolia* to new habitats, the plant spread and dominated quickly in the areas. *T. diversifolia* is very invasive and threatening the local plant communities [23, 26, 29].

Abbreviations: IC_{50} – concentrations required for 50% growth inhibition; HRESI-MS – high-resolution electrospray ionization mass.

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High reproduction and high growth rates, and phenotypic plasticity of the invasive plants may be important for the domination in the new habitats [8, 20, 30]. High defense capacity of the plants against pathogens and herbivores may be necessary in the new habitats [7, 14, 24]. The interaction of the invasive plants with native plants may also be crucial. Some invasive plants have many substances with allelopathic activities, which are highly toxic to native plants [6, 9, 22].

When several test plants were grown in the soil collected from *T. diversifolia* dominated fields, the growth of those plants was suppressed [31, 32]. The incorporation of *T. diversifolia* leaves into the soil also inhibited the growth of rice [31]. The extracts of *T. diversifolia* were highly toxic to several crop plant species such as rice, maize, sorghum, lettuce and cowpea, and several putative allelopathic substances were isolated from the extracts [23, 25, 26, 29, 32]. Therefore, the allelopathic substances of *T. diversifolia* may be important in the domination of *T. diversifolia*. It is also necessary to determine allelopathic effects of invasive plants on not only crop plants but also wild plants for the evaluation of the involvement of allelopathic substances in the invasive characteristics of *T. diversifolia*. However, the information about the effects of *T. diversifolia* on wild plants including weed plants is limited. The objective of this study was the evaluation of allelopathic potential of *T. diversifolia* extracts on weed plants, and the isolation and identification of phytotoxic substances with allelopathic activity.

MATERIALS AND METHODS

Plant materials

Leaves of *Tithonia diversifolia* (Hermsl.) A. Gray were collected from Mae Hong Son province, Thailand, washed with tap water and dried under sunlight. Dry materials were then packed with plastic bags and stored at 3 °C until extraction. Weed plant species, *Lolium multiflorum* Lam., *Phleum pretense* L., *Echinochloa crus-galli* (L.) Beauv were chosen for bioassay. Crop plant, *Lepidium sativum* L. was also chosen because *L. sativum* is sensitive to various plant extracts and phytotoxic substances [21, 28].

Extraction and bioassay

Dried leaves of *T. diversifolia* (200 g) were soaked in 1.5 L of 70% (v/v) aqueous methanol for 48 h. After filtration using filter paper (No. 2; Toyo, Tokyo, Japan), an aliquot of the extract (final assay concentration of tested samples corresponded to the extracts obtained from 3, 10, 30 and 100 mg dry weight of *T. diversifolia* per mL) was evaporated, dissolved in a small volume of methanol and added onto a sheet of filter paper (No. 2) in a 3 cm Petri dish. Methanol was evaporated in a fume hood and the filter paper in the Petri dishes was moistened with 0.6 mL of a 0.05% (v/v) aqueous solution of Tween 20. Ten germinated seeds of *L. multiflorum*, *P. pratense* or *E. crus-*

galli after germination in the darkness at 25 °C for 48 h or 10 seeds of *L. sativum* were placed onto the Petri dishes. The length of roots and shoots of those seedlings were measured after 48 h of incubation in the darkness at 25 °C, and compared to control seedlings. Controls were treated exactly as described above, with the exception that methanol was used instead of the extracts. The bioassay was repeated twice using a randomized design with 10 plants for each determination. Significant differences between control and treatment were examined by Welch's *t*-test. The concentrations of the extracts required for 50% growth inhibition (defined as IC_{50}) of the bioassay plants in the assay were determined by a logistic regression analysis.

Separation and isolation of an active substance

Dried leaves of *T. diversifolia* were extracted as described above. The extract was concentrated at 40 °C in vacuo to produce an aqueous residue. The aqueous residue was then adjusted to pH 7.0 with 1 M phosphate buffer, partitioned three times against an equal volume of ethyl acetate, and separated ethyl acetate and aqueous phase. The ethyl acetate phase was evaporated to dryness and separated on a column of silica gel (50 g, silica gel 60, 70–230 mesh; Nacalai Inc., Kyoto, Japan), eluted with 20, 30, 40, 50, 60, 70 and 80% ethyl acetate in *n*-hexane (120 mL per step), ethyl acetate (120 mL) and methanol (240 mL). The biological activity of all separated fractions was determined using the test plants as described above, and the active fraction was obtained by elution with 40% ethyl acetate in *n*-hexane. After evaporation of the active fraction, the residue was purified by a column of Sephadex LH-20 (50 g, Amersham Pharmacia Biotech, Buckinghamshire, UK), and eluted with 20, 30, 40, 50, 60, 70, 80 and 90% (v/v) aqueous methanol (100 mL per step), and methanol (200 mL). The active fraction was eluted by 60% aqueous methanol. After evaporation, the residue was dissolved in 40% (v/v) aqueous methanol and loaded onto reverse-phase ODS cartridges (YMC-Dispo Pack AT ODS-25; YMC Ltd., Kyoto). The cartridge was eluted with 40, 50, 55, 60, 65, 70 and 80% (v/v) aqueous methanol (180 mL per step), and methanol (360 mL). The active fraction was eluted by 50% aqueous methanol and evaporated to dryness. The residue was finally purified by reverse-phase HPLC (19 mm ID × 150 mm; μBondasphere 5 μ C18-100Å; Waters) eluted at flow rate of 1.5 mL min^{-1} with 50% aqueous methanol and detected at 220 nm. The inhibitory activity was detected at a peak of 101–113 min, yielding active substance as colorless oil. The substance was characterized by HRESI-MS, ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectra (TMS as internal standard), and optical rotation.

Bioassay for an isolated substance

An isolated substance was dissolved in 0.2 mL of methanol, and added to a sheet of filter paper (No. 2) in a 3-cm Petri dish. After evaporation of the methanol, the filter paper in the Petri dish was moistened with 0.6 mL of 0.05% (v/v) aqueous solution

of Tween 20. Inhibitory activity of substance was determined with *L. multiflorum*, *P. pratense, E. crus-galli* and *L. sativum* as described above. The bioassay was repeated twice using a randomized design with 10 plants for each determination. Significant difference between treatment and control plants were by Welch's *t*-test. IC_{50} values of the substance on the test plants were determined by a logistic regression analysis.

RESULTS

Growth inhibitory activity of the extract of T. diversifolia *leaves*

Aqueous methanol extracts of *T. diversifolia* leaves inhibited root and shoot growth of three weed plant species, *L. multiflorum*, *P. pratense* and *E. crus-galli* and *L. sativum* seedlings. Increasing the extract concentration resulted in an increase in the inhibition (Fig. 1). The extract obtained from 10 mg of *T. diversifolia* inhibited the root growth of *L. multiflorum*, *P. pratense* and *E. crus-galli* and *L. sativum* to 17.2, 1.3, 23.1 and 8.2% that of the controls, respectively, and the shoot growth of *L. multiflorum*, *P. pratense, E. crus-galli* and *L. sativum* to 48.7, 61.7, 83.1 and 1.4% that of the controls, respectively. Comparing IC_{50} values, *P. pratense* roots were the most sensitive to the extracts in all roots, and *L. sativum* shoots were the most sensitive to the extracts in all shoots (Table 1).

Identification of a growth inhibitory substance

An aqueous methanol extract of *T. diversifolia* leaves was separated on a silica gel column, Sephadex LH-20, reverse-phase ODS cartridges. The biological activity of all fractions was determined and the most active fraction in each separation step was further purified. An active substance was finally isolated by HPLC. The molecular formula of the active substance was determined to $C_{19}H_{24}O_6$ by as suggested by HRESI-MS at *m/z* 349.1647 $[M+H]^+$ (calcd for $C_{19}H_{25}O_6$, 349.1651,

Table 1

IC_{50} values (mg dry weight equivalent extract per mL) of aqueous methanol extracts of *T. diversifolia* leaves on root and shoot growth of test plants

Test plant	Root	Shoot
L. multiflorum	8.96	11.2
P. pratense	3.17	18.7
E. crus-galli	9.41	22.8
L. sativum	6.37	4.32

The values were determined by a logistic regression analysis after bioassay as described in the text.

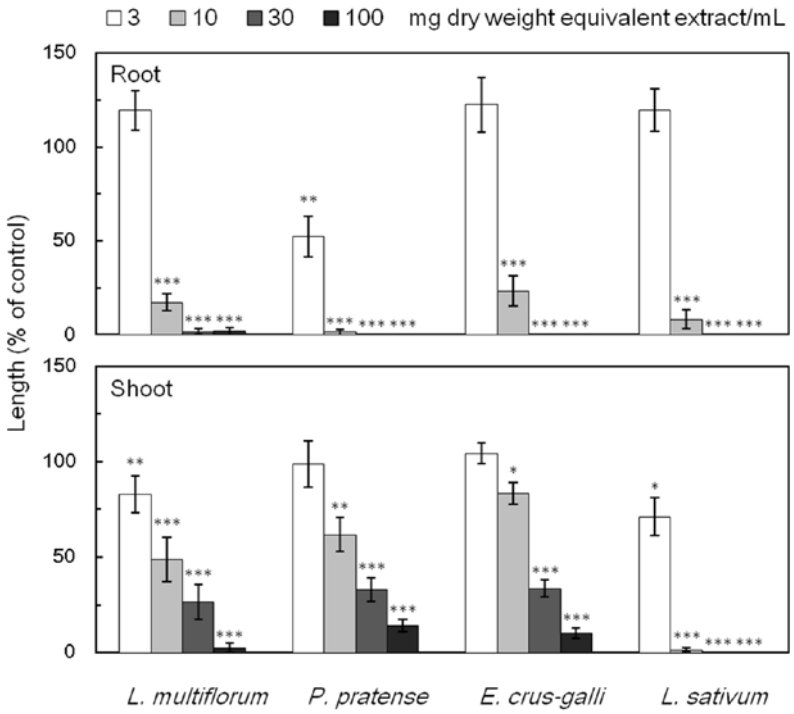

Fig. 1. Effects of aqueous methanol extracts of *T. diversifolia* leaves on root and shoot growth of *L. multiflorum*, *P. pratense*, *E. crus-galli* and *L. sativum*. Concentrations of tested samples corresponded to the extracts obtained from 3, 10, 30 and 100 mg dry weight of *T. diversifolia* leaves per mL. Means±SE from two independent experiments with 10 plants for each determination are shown. Asterisks indicate significant difference between control and treatment: *P<0.05, **P<0.01, ***P<0.001

Δ = –0.4 mmu). The ^{1}H spectrum (400 MHz, $CDCl_3$, TMS as internal standard) of the substance showed δ_H 6.92 (d, J = 17 Hz, 1 H, H-1), 6.36 (d, J = 1.9 Hz, 1 H, H-13a), 6.24 (d, J = 17 Hz, 1 H, H-2), 5.86 (dq, J = 9.1, 1.5 Hz, 1 H, H-5), 5.81 (d, 1.9 Hz, 1 H, H-13b), 5.39 (d br, J = 9.1 Hz, 1 H, H-6), 5.35 (m, 1 H, H-8), 3.54 (m, 1 H, H-7), 2.45 (m, 1 H, H-2'), 2.43 (m, 1 H, H-9a), 1.99 (dd, J = 15, 10 Hz, 1 H, H-9b), 1.96 (d, J = 1.5 Hz, 3 H, H-15), 1.54 (s, 3 H, H-14), 1.07 (d, J = 5.8 Hz, 3 H, H-3'), 1.06 (d, J = 5.8 Hz, 3 H, H-4'). The ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of the substance showed δ_C 196.8 (C-3), 176.3 (C-1'), 169.8 (C-12), 160.0 (C-1), 139.1 (C-4), 137.3 (C-5), 136.1 (C-11), 129.8 (C-2), 124.7 (C-13), 76.1 (C-6), 73.9 (C-8), 72.3 (C-10), 48.6 (C-9), 47.2 (C-7), 34.2 (C-2'), 29.3 (C-14), 19.9 (C-15), 19.0 (C-3'), 18.8 (C-4'). The optical rotation of the substance was $[\alpha]_D^{23}$ –220 (*c* 0.08, $CHCl_3$). The substance was identified as a sesquiterpene lactone, tagitinin C (Fig. 2) by the comparison of those spectrum data with published data in the literature [2, 15, 27].

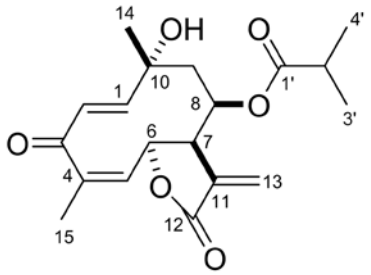

Fig. 2. Chemical structure of tagitinin C

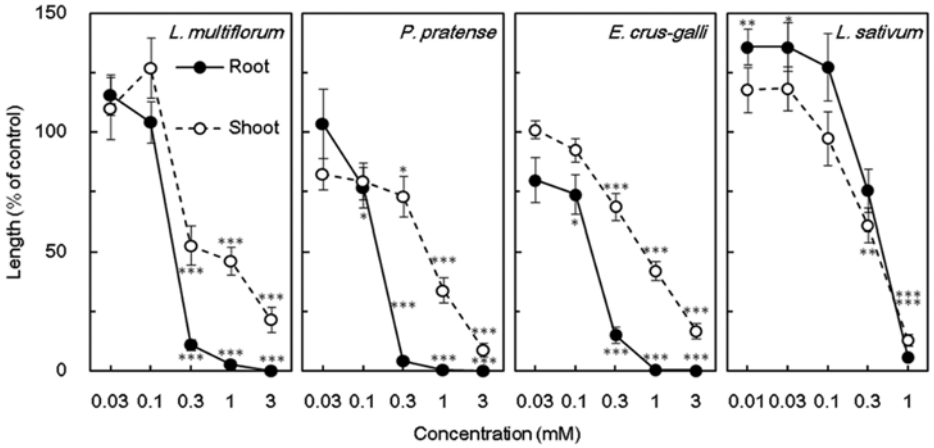

Fig. 3. Effects of tagitinin C on root and shoot growth of *L. multiflorum*, *P. pratense*, *E. crus-galli* and *L. sativum*. Means ± SE from two independent experiments with 10 plants for each determination are shown. Asterisks indicate significant difference between control and treatment: *P < 0.05, **P < 0.01, ***P < 0.001

Growth inhibitory activity of tagitinin C on weed plants

Tagitinin C inhibited root and shoot growth of weed plant species, *L. multiflorum*, *P. pratense* and *E. crus-galli,* and *L. sativum* at concentrations greater than 0.1–0.3 mM (Fig. 3). However, *L. sativum* root growth was significantly increased by tagitinin C at concentration of 0.01 and 0.03 mM, of which phenomenon is known as eustress [18].

Comparing IC_{50} values, *P. pratense* roots were the most sensitive to tagitinin C in all roots, and *L. sativum* shoots were the most sensitive to tagitinin C in all shoots (Table 2), which is consistent with the results of inhibitory activity of the extracts of *T. diversifolia* (Table 1). The results indicate that tagitinin C has growth inhibitory activity on the roots and shoots of weed plant species.

Table 2
IC_{50} values (mM) of tagitinin C on root and shoot growth of test plants

Test plant	Root	Shoot
L. multiflorum	0.217	0.834
P. pratense	0.126	0.634
E. crus-galli	0.128	0.650
L. sativum	0.448	0.363

The values were determined by a logistic regression analysis after bioassay as described in the text.

DISCUSSION

Aqueous methanol extracts of an invasive species, *T. diversifolia* inhibited growth of three weed species, *L. multiflorum*, *P. pratense* and *E. crus-galli*, and a crop species, *L. sativum* (Fig. 1, Table 1). It was reported that *T. diversifolia* extracts had growth inhibitory activity on several crop plant species [23, 25, 26, 29, 32]. The present results suggest that *T. diversifolia* extracts also have inhibitory activity on the weed plants.

After purification of phytotoxic substance in the extracts of *T. diversifolia*, a potent phytotoxic substance was isolated and identified as tagitinin C. Tagitinin C has so far been isolated only from *T. diversifolia* [2, 27] and the substance was reported to have antiplasmodial, antiproliferative, antiglioblastoma cytotoxic and insect feeding deterrent activities [10, 12, 13, 15, 17]. Tagitinin C was also reported to have growth inhibitory activity against radish, cucumber, tomato and onion [3, 23]. However, its inhibitory activity on wild plants including weed plant species was not clear.

The phytotoxic activity of tagitinin C on *L. multiflorum*, *P. pratense*, *E. crus-galli* and *L. sativum* was evaluated. Tagitinin C was active at concentrations greater than 0.1–0.3 mM and IC_{50} values of tagitinin C were 0.126–0.834 mM. These data indicate that tagitinin C, for the first time, had the concentration-dependent growth inhibitory activity on the roots and shoots of the weed species. The phytotoxic substances of invasive plants often have multiple functions such as anti-microbial, anti-fungal and anti-herbivore activities [8, 22]. It was also observed that many of invasive plant species were allelopathic and their phytotoxic substances with allelopathic activity were more toxic to other plants in the invasive areas than in the original areas of the invasive plants [9, 19, 22]. These phytotoxic substances may contribute to the invasive characteristics of the invasive plants. In addition, phytotoxic active substances can be released into the environment by decomposition of plant residues and/or by exudation from living plant tissues [1, 4, 5]. The decomposition rate of invasive plant residues was much higher than that of native vegetation residues [11, 16].

Information on the allelopathic potential of *T. diversifolia* has been accumulated, but most information concerns weed management potentiality of the plant species [23, 25, 26]. The present study discussed the invasive characteristics of *T. diversifolia*

with a potent growth inhibitory substance tagitinin C. Tagitinin C may contribute to the growth inhibitory effects of *T. diversifolia*, and may be in part responsible for the invasive characteristics of *T. diversifolia*.

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