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RESEARCH ARTICLE

Evaluation of allelopathic competency of *Lamium amplexicaule* and identification of its allelopathic active substance

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ABSTRACT

The weedy annual grass *Lamium amplexicaule* (L.) originated from the Mediterranean region, spreads quickly, and becomes naturalized in areas it invades. It was assumed that volatile phytotoxic substances are one of the invasive characteristics of *L. amplexicaule*. However, no volatile compound has been found. Therefore, we re-evaluated the allelopathic potential of *L. amplexicaule* and investigated the phytotoxic substances with allelopathic activity. An extract of *L. amplexicaule* inhibited the root and shoot growth of *Lepidium sativum*, *Lactuca sativa*, *Arctium lappa*, *Lolium multiflorum* Lam., *Echinochloa crus-galli*, and *Vulpia myuros*, which indicates that the extract has an allelopathic effect. The extract was purified using bioassay-guided chromatographic separations, and a phytotoxic substance with allelopathic activity was isolated and characterized as methyl caffeate. The compound significantly inhibited the root and shoot growth of *L. sativum* and *L. multiflorum*. The present results suggest that methyl caffeate may contribute to the allelopathic effect of the *L. amplexicaule* extracts and consequently, may be partly responsible for the invasive characteristics of the species.

Keywords: Allelopathy; Invasive weed; Growth inhibition; *Lamium amplexicaule*; Phytotoxicity

INTRODUCTION

Lamium amplexicaule (L.) is a weedy annual plant species of the Lamiaceae family that originated from the Mediterranean region and spread in Europe, Asia, and northern Africa. The species has adapted to quickly grow in crop fields, gardens, and pastures (USDA-NRCS, 2015; Stojanova et al., 2016). It competes with crops and other plant species and is categorized as an invasive alien weed in the USA and Canada (CABA, 2018).

Invasive plants possibly have physiological and ecological characteristics, such as high growth and reproduction rates, and phenotypic plasticity (Cappuccino and Arnason, 2006). The interactions of the invasive plants with natural enemies are also important. A strong defense capability against herbivores and pathogens may contribute to invasion (Keane and Crawley, 2002; Cappuccino and Carpenter, 2005). The interaction between invasive plants and native plants is also crucial. Several invasive plants contain unique compounds that are highly toxic to native plants (Callaway and Ridenour, 2004; Chengxu et al., 2011). Therefore, phytotoxicity or allelopathy or both are considered to be important for invasive plants to become naturalized in new habitats (Meiners et al., 2012).

The allelopathic activity of *L. amplexicaule* has been reported (Fujii et al., 1992). The species spreads quickly throughout wheat fields and suppresses crop production (Conley and Bradley, 2005). Jones et al. (2012) evaluated the volatile compounds in *L. amplexicaule* to determine if they contribute to its allelopathy. However, they could not find any volatile compounds that were responsible for the allelopathy. The objective of this study was to re-evaluate the allelopathic potential of *L. amplexicaule* and to isolate and identify potential phytotoxic compounds with allelopathic activity.

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MATERIALS AND METHODS

Plant material

Whole plants of *Lamium amplexicaule* (L.) were collected at Kumagaya, Japan in 2017 and kept at −20°C until extraction. Dicotyledonous *Lepidium sativum* L., *Lactuca sativa* L., and *Arctium lappa* L. and monocotyledonous *Lolium multiflorum* Lam., *Echinochloa crus-galli* (L.) Beauv, and *Vulpia myuros* (L.) C. C. Gmel. were used as test plants for bioassay.

Extraction and bioassay

Whole plants of *L. amplexicaule* (170 g fresh weight) were cut into small pieces and extracted with 850 mL of 80% (v/v) aqueous methanol for two days. After filtration using filter paper (No. 2; Toyo Ltd., Tokyo, Japan), the residue was extracted again with 500 mL of methanol for two days and filtered. The two filtrates were combined and concentrated in vacuo at 40°C.

An aliquot of the extract was added to a sheet of filter paper in a 2.8 cm Petri dish and dried in a fume hood. The filter paper was moistened with 0.6 mL of 0.05% (v/v) aqueous solution of Tween 20 (polyoxyethylene sorbitan monolaurate; Nacalai, Kyoto, Japan). Ten seeds each of *L. sativum*, *L. sativa*, and *A. lappa*, and 10 pre-germinated seedlings each of *V. myuros*, *E. crus-galli*, and *L. multiflorum* were placed onto filter paper in Petri dishes. *L. sativum*, *L. sativa*, *L. multiflorum*, *E. crus-galli*, and *V. myuros* were incubated in the dark at 25°C for 48 h, and *A. lappa* was incubated in the dark at 25°C for 120 h. After incubation, the root and shoot length of the test plants were measured. The bioassay concentrations of the extracts were 1, 3, 10, 30, 100, 300, and 1000 mg fresh weight equivalent extract mL^{-1}. Control test plants were incubated without the extracts. The bioassay was repeated two times using a randomized design with 10 plants for each determination. The concentrations required for 50% growth inhibition (IC_{50} values) of the test plant roots and shoots were determined using a logistic regression function with Microsoft Excel based on the bioassay.

Separation of the extract

Whole plants of *L. amplexicaule* (5 kg fresh weight) were extracted with 18 L of 80% (v/v) aqueous methanol as described above. The extract was concentrated at 40°C to produce an aqueous solution. The aqueous solution was adjusted to pH 7.0 with 1 M phosphate buffer and partitioned three times with an equal volume of ethyl acetate. The ethyl acetate fraction was evaporated to dryness after drying over anhydrous Na_2SO_4, and then separated on a column of silica gel (40 g, silica gel 60, 70-230 mesh; Nacalai) with elution by 20 (fraction 1), 30 (fraction 2), 40 (fraction 3), 50 (fraction 4), 60 (fraction 5), 70 (fraction 6), and 80% (fraction 7) ethyl acetate in *n*-hexane (v/v, 100 mL per step), ethyl acetate (fraction 8; 100 mL), and methanol (fraction 9; 200 mL). The biological activity of all the separated fractions was determined using a *L. sativum* bioassay as described above.

Isolation of an active compound

An active fraction obtained from the silica gel column was evaporated, and the residue was purified using a column of Sephadex LH-20 (40 g; GE Healthcare, Uppsala, Sweden) eluted with 20, 30, 40, 50, 60, 70, 80, and 90% aqueous methanol (v/v, 100 mL per step) and methanol (200 mL). The fraction eluted with 60% aqueous methanol was active, and that active fraction was evaporated to dryness. The residue of the fraction was separated using flash chromatography (KeyChem-Flash, YMC-DospoPack AT ODS-25; YMC Co., Ltd.) eluted with 20, 30, 40, 50, 60, 70, and 80% aqueous methanol (v/v, 150 mL per step) and methanol (300 mL). The active fraction was eluted with 40% aqueous methanol and evaporated to dryness. The residue of the fraction was finally separated using reverse-phase HPLC (*μ*Bondshere 5 μ, 19×150 mm; Waters) eluted at a flow rate of 1.5 mL min^{-1} with 55% (v/v) aqueous methanol and detected at 220 nm. Activity was found in a peak fraction at 55 min. The active compound was characterized using HRESI-MS, ^{1}H-NMR (400 MHz, $CDCl_3$), and ^{13}C NMR (100 MHz, $CDCl_3$) spectra (TMS as internal standard). An active compound of 2.8 mg was isolated from the extracts obtained from an extracted amount of 0.5 kg of *L. amplexicaule*.

Bioassay for the isolated compound

The isolated compound was dissolved in methanol and added to a sheet of filter paper (No. 2) in a 2.8 cm Petri dish. The methanol was evaporated in a fume hood. The biological activity of the compound was determined by bioassay using *L. sativum* and *L. multiflorum* as described above. *L. sativum* and *L. multiflorum* were used as the test plants because those plants had relatively high sensitivity to the extracts of *L. amplexicaule* among the dicotyledonous and the monocotyledonous test plants, respectively. The bioassay concentrations were 0.1, 0.3, 1, 3, and 10 mM. The bioassay was repeated two times using a completely randomized design with 10 plants for each determination.

Statistical analysis

Significant differences between treatment and control were examined using Welch's t-test (two sided) at $p<0.05$, 0.01, or 0.001.

RESULTS AND DISCUSSION

Growth inhibitory activity of the extracts of *L. amplexicaule*

The extracts of *L. amplexicaule* inhibited the root and shoot growth of *L. sativum*, *L. sativa*, *A. lappa*, *L. multiflorum*, *E. crus-galli*, and *V. myuros*. The level of inhibition increased

with increasing concentrations of the extracts (Fig 1). The extracts of *L. amplexicaule* therefore had an allelopathic effect on both the dicotyledonous and monocotyledonous plants. These results suggest that *L. amplexicaule* may contain phytotoxic substances with allelopathic activity.

The IC_{50} values of the extracts on the test plants were in the range of 38.5–137 mg fresh weight equivalent extract mL^{-1} for *L. sativum*, *L. sativa*, and *A. lappa* (dicotyledonous plants) and in the range of 126–937 mg fresh weight equivalent extract mL^{-1} for *L. multiflorum*, *E. crus-galli*, and *V. myuros* (monocotyledonous plants). Thus, the dicotyledonous plants were more sensitive to the extracts than the monocotyledonous plants (Table 1).

Identification of the growth inhibitory substances in *L. amplexicaule*

The extracts of *L. amplexicaule* were separated using a silica gel column, and the most active fraction was eluted with 60% ethyl acetate in *n*-hexane (Fig 2). The active fraction inhibited the *L. sativum* roots and shoots resulting in growth of 12.4 and 13.2%, respectively, compared with the growth of the control plants. Therefore, the active fraction was further purified through chromatographic separation with the inhibitory activity determined by a bioassay using *L. sativum*, which is highly sensitive to the extracts of *L. amplexicaule*, and an active compound was isolated.

The active compound has the molecular formula of $C_{10}H_8O_4$ as suggested by HRESI-MS at m/z 192.9761 $[M-H]^-$ (calcd for $C_{10}H_9O_4$ 193.0501, Δ= −383 ppm). ^1H NMR (400 MHz, CD_3OD) δ_H 7.54 (d, J = 16.2 Hz, 1 H, H-7), 7.03 (d, J = 2.1 Hz, 1 H, H-2), 6.94 (dd, J = 8.4, 2.1 Hz, 1 H, H-6), 6.77 (d, J = 8.4 Hz, 1 H, H-5), 6.26 (d, J = 16.2 Hz, 1 H, H-8), 3.76 (s, 3 H, H-10); ^{13}C NMR (100 MHz, (CD_3OD) δ_C 169.8 (C-9), 149.6 (C-4), 147.0

Table 1. IC_{50} values (concentrations required for 50% growth inhibition) of the extracts of *L. amplexicaule* on *L. sativum*, *L. sativa*, *A. lappa*, *L. multiflorum*, *E. crus-galli*, and *V. myuros*

Test plant	IC_{50} (mg fresh weight equivalent extract mL^{-1})	
	Shoot	Root
Dicotyledonous		
L. sativum	46.9	52.6
L. sativa	67.9	38.5
A. lappa	137	93.3
Monocotyledonous		
L. multiflorum	366	126
E. crus-galli	937	323
V. myuros	506	131

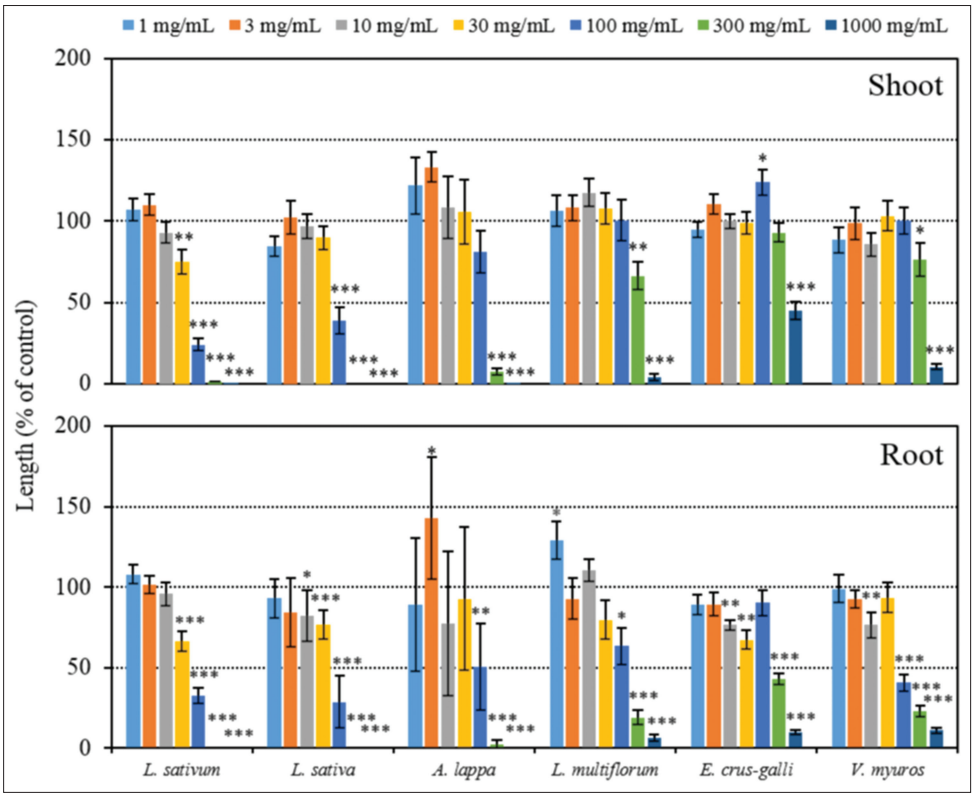

Fig 1. Effect of the extracts of *L. amplexicaule* on the root and shoot growth of *L. sativum*, *L. sativa*, *A. lappa*, *L. multiflorum*, *E. crus-galli*, and *V. myuros*. Concentrations of the tested samples corresponded to the extracts obtained from 1, 3, 10, 30, 100, 300, and 1000 mg fresh weight of *L. amplexicaule* per mL. Means ± SE from 2 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.

(C-3), 146.8 (C-7), 127.6 (C-1), 122.9 (C-6), 116.5 (C-5), 115.1 (C-8), 114.8 (C-2), 52.0 (C-10). The active compound was identified as methyl caffeate (Fig 3) by comparing with previous data (Zhu et al., 2010; Prevost et al., 2013). Although methyl caffeate has been isolated from several plant species (Xiang et al., 2011; Balachadran et al., 2015; Wang et al., 2015), the present report is the first on methyl caffeate as a phytotoxic substance in *L. amplexicaule*.

Biological activity of the isolated substances

Methyl caffeate significantly inhibited the root and shoot growth of *L. sativum* and *L. multiflorum* at concentrations greater than 1 mM (Fig 4 and 5). The IC_{50} values of methyl caffeate on the roots and shoots of *L. sativum* were 2.0 and 1.4 mM, respectively, and on the roots and shoots of *L. multiflorum* were 1.1 and 2.5 mM, respectively. The compound has also been reported to possess anticancer activity against human breast cancer cells (Bailly et al., 2013), antimalarial activity against a *Plasmodium berghei* strain (Alson et al., 2018), antihyperglycemic activity in diabetic rats (Gandhi et al., 2011), and antioxidant activities under *in vitro* conditions (Wang et al., 2015). Methyl caffeate is a methyl ester of caffeic acid, which has also been reported to have biological activity (Fernandez et al., 2009; Scognamiglio et al., 2012). However, the activity of methyl caffeate was 2.9-fold greater than that of caffeic acid (Alson et al., 2018), which suggests that the methoxy group at the C-10 position in caffeic acid may be important for the activity.

Phytotoxic active substances in plants are released into the soil by decomposition of plant residues and exudates from living plant tissues, and act as allelopathic substances. Those substances can inhibit seed germination and plant growth (Bais et al., 2006; Bonanomi et al., 2006; Belz, 2007). Methyl caffeate in *L. amplexicaule* may also be released into the soil in the same manner and act as an allelopathic substance.

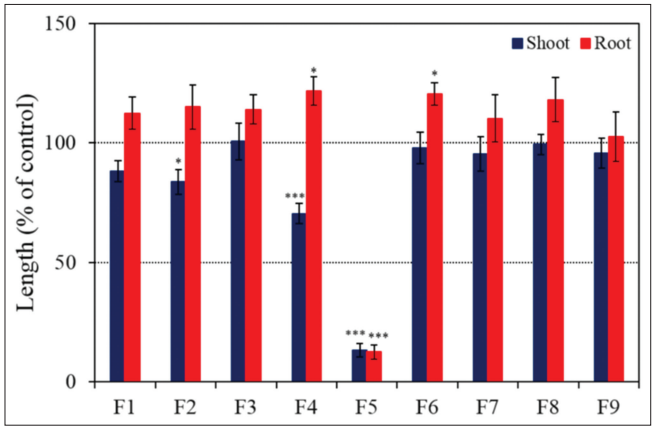

Fig 2. Effect of the fractions obtained from silica gel column chromatography of the *L. amplexicaule* extracts on the root and shoot growth of *L. sativum*. The length of the roots and shoots of the *L. sativum* seedlings was determined after 48 h of incubation in the dark at 25°C. The concentration of the tested samples corresponded to the extract obtained from 1000 mg fresh weight of *L. amplexicaule* per mL. Means ± SE from 2 independent experiments with 10 plants for each determination are shown. Asterisks indicate significant difference between control and treatment: *, $p<0.05$, ***, $p<0.001$.

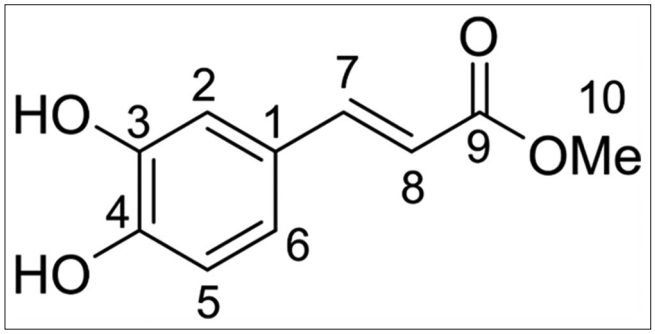

Fig 3. Chemical structure of methyl caffeate.

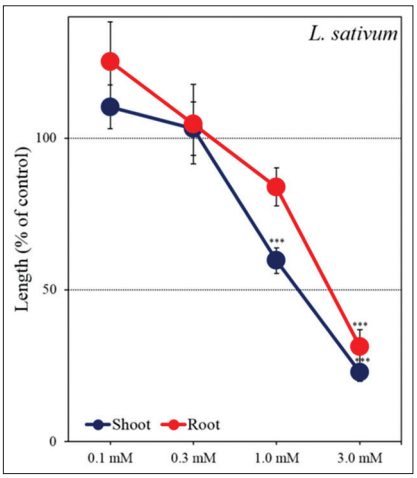

Fig 4. Effect of methyl caffeate on the root and shoot growth of *L. sativum*. Means ± SE from 2 independent experiments with 10 seedlings for each determination are shown. ***, $p<0.001$.

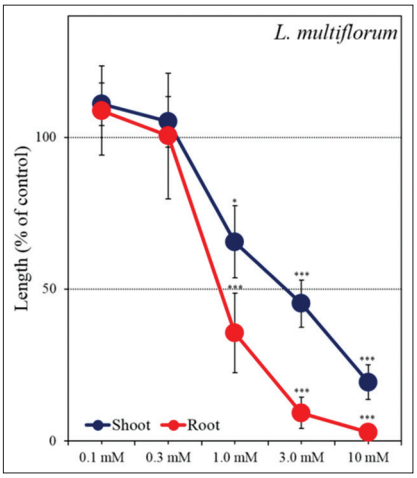

Fig 5. Effect of methyl caffeate on the root and shoot growth of *L. multiflorum*. Means ± SE from 2 independent experiments with 10 seedlings for each determination are shown. *, $p<0.05$, ***, $p<0.001$.

The weedy grass *L. amplexicaule* strongly competes with other plants and is categorized as an invasive alien weed (CABA, 2018). Phytotoxicity and allelopathy are considered to be important for invasion of new habitats (Meiners et al., 2012). It has been reported that *L. amplexicaule* possesses allelopathic potential (Fujii et al., 1992). However, volatile compounds involved in the allelopathy could not be found (Jones et al., 2012). The present research re-evaluated the allelopathic activity of *L. amplexicaule* and isolated methyl caffeate, which showed phytotoxic activity. The phytotoxic compound may contribute to the invasive characteristics of *L. amplexicaule*.

CONCLUSION

The extracts of *L. amplexicaule* show allelopathic activity. The extract was purified using bioassay-guided chromatographic separations, and a phytotoxic substance with allelopathic activity was isolated and characterized as methyl caffeate. The compound significantly inhibited the growth of *L. sativum* and *L. multiflorum*. These results suggest that methyl caffeate may contribute to the allelopathic activity of the *L. amplexicaule* extracts and consequently, may be partly responsible for the invasive characteristics of *L. amplexicaule*. However, further experiments are required to clarify the release route of this compound into the environment.

Authors' contributions

Chisato Sakamoto: ran the experiments and data analysis. Masahiko Suzuki: technical support for the experiments. Arihiro Iwasakiand Kiyotake Suenaga: determination of chemical structures of the compounds. Hisashi Kato-Noguchi: responsible for experimental design and results interpretation coordinated data analysis and wrote the paper.

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