DISCOVERY OF COUMARIN AS THE PREDOMINANT ALLELOCHEMICAL IN *GLIRICIDIA SEPIUM*

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Received May 2012

TAKEMURA T, KAMO T, SAKUNO E, HIRADATE S & FUJII Y. 2013. Discovery of coumarin as the predominant allelochemical in *Gliricidia sepium*. A crude methanol extract of *Gliricidia sepium* (Fabaceae) leaves inhibited the growth of lettuce (*Lactuca sativa*) radicles. To isolate and characterise the inhibitory compound, the extract was fractionated based on the total activity on lettuce radicle elongation. The n-hexane-soluble fraction obtained by liquid–liquid partitioning of the crude methanol extract showed strong inhibitory activity. A compound corresponding to the major peak in high performance liquid chromatography was isolated from the fraction and identified as coumarin. The EC_{50} of coumarin for the growth of lettuce radicles was 23.3 µmol L^{-1}. On the basis of the coumarin content (11.6 mmol kg^{-1} fresh weight) and the total activity of coumarin (500) in *G. sepium*, we concluded that the inhibitory activity of *G. sepium* was primarily due to coumarin.

Keywords: *Lactuca sativa*, specific activity, total activity, dry forest, leguminous tree

TAKEMURA T, KAMO T, SAKUNO E, HIRADATE S & FUJII Y. 2013. Penemuan kumarin sebagai alelokimia utama dalam *Gliricidia sepium*. Ekstrak metanol mentah bagi daun *Gliricidia sepium* (Fabaceae) merencat pertumbuhan radikel salad (*Lactuca sativa*). Untuk mengasing dan mencirikan sebatian perencat ini, ekstrak menjalani pemeringkatan berdasarkan aktiviti pemanjangan radikel salad. Pecahan yang larut dalam n-heksana yang diperoleh daripada pemeringkatan cecair–cecair ekstrak metanol mentah menunjukkan aktiviti perencatan yang kuat. Sebatian yang sepadan dengan puncak utama dalam kromatografi cecair prestasi tinggi diasingkan daripada pecahan dan dikenal pasti sebagai kumarin. Nilai EC_{50} kumarin bagi pertumbuhan radikel salad ialah 23.3 µmol L^{-1}. Berdasarkan kandungan kumarin (11.6 mmol kg^{-1} berat basah) dan aktiviti kumarin (500) dalam *G. sepium*, kami membuat keputusan yang aktiviti perencatan *G. sepium* disebabkan terutamanya oleh kumarin.

INTRODUCTION

Synthetic herbicides play an important role in weed suppression in agricultural fields, gardens and roadsides (Mazur & Falco 1989). However, they can have detrimental effects on crops, groundwater, soil and human health and are not effective for the control of herbicide-resistant weeds (Macías et al. 2001). Natural compounds have the potential to partially replace synthetic herbicides or to serve as starting materials for the chemical synthesis of biodegradable herbicides. In this context, the use of compounds obtained from allelopathic plants has attracted interest. These are plants that release organic chemicals which influence the growth and development of other plants (Putnam & Tang 1986). The exploitation of the allelopathic potential of plants for weed control in a variety of agricultural settings has been investigated (Duke et al. 2000). It is believed that allelopathic plants or products derived from them will be less harmful to the environment compared with synthetic herbicides because the former are expected to readily undergo degradation in the environment (Petroski & Stanley 2009).

Extracts of more than 100 plant species have been tested to source for potentially allelopathic plants and compounds (Takemura et al. 2009). An extract from *Gliricidia sepium* showed the strongest growth inhibitory activity against lettuce (*Lactuca sativa*) seedlings.

Gliricidia sepium, a leguminous tree belonging to the family Fabaceae, is

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distributed throughout seasonally dry forest of Mexico and other Central American countries (Chadhokar 1982). It is used as living fences, fodder for ruminants, green manure, shade, firewood and a source of rodenticide (Csurhes & Edwards 1998).

Fifteen allelochemicals were found in the leaves of *G. sepium*: gallic acid, protocatechuic acid, p-hydroxybenzoic acid, gentisic acid, β-resorcylic acid, vanillic acid, syringic acid, p-coumaric acid, m-coumaric acid, o-coumaric acid, ferulic acid, cis- and trans-sinapinic acid, coumarin and myricetin (Ramamoorthy & Paliwal 1993). These compounds inhibit the elongation of *Sorghum vulgare*, but the contribution of each compound to the allelopathic activity was not determined.

In the present paper, we describe the isolation and characterisation of the compound responsible for the inhibitory activity of *G. sepium*. Our goals were to determine the predominant contributor to the activity and evaluate its environmental behaviour. This information could lead to the development of an effective method of using *G. sepium* in sustainable agriculture.

MATERIALS AND METHODS

Materials

Leaves of 12-year-old *G. sepium* trees were collected from a greenhouse at the National Institute for Agro-environmental Sciences, Tsukuba, Japan.

Analytical methods

High performance liquid chromatography (HPLC) was performed on a system equipped with a Waters 626 pump, Waters 996 photodiode array detector and reversed-phase column at 40 °C. The flow rate was 1.0 mL min^{-1}. Gas chromatography–mass spectrometry (GC–MS) was carried out with a spectrometer and an Equity-5 column. The GC–MS operating conditions were inlet temperature 200 °C, column oven temperature 40 °C for 6 s, ramp at 40 °C min^{-1} to 220 °C and hold for 5 min. ^{1}H- and ^{13}C-NMR spectra were recorded with a spectrometer.

Bioassay

Lettuce (*L. sativa*) seedlings were used for the bioassay. An appropriate concentration of the test solution was poured onto a filter paper (27 mm diameter) in a 27-mm glass Petri dish. After the solvent was completely dried *in vacuo*, 0.7 mL of distilled water was added. Five lettuce seedlings (pre-germinated for 20 hours at 20 °C in the dark) were placed in each Petri dish and incubated for 52 hours at 20 °C in the dark. In control experiments, only 0.7 mL of distilled water was used. Inhibition of radicle elongation was determined by measuring radicle lengths and comparing the values with the control value. The effective concentrations required to induce half the maximum inhibition (EC_{50}) and 95% confidence intervals were calculated by the probit method using SPSS for Windows statistical software.

Liquid–liquid partitioning of the crude methanol extract and isolation of compound

Fresh leaves of *G. sepium* (100 g) were soaked in methanol (1.5 L) for 1 month at room temperature. After evaporation of the solvent *in vacuo*, the resulting water suspension of the extract was subjected to liquid–liquid partitioning. The residue was diluted with 100 mL of distilled water and the aqueous solution was extracted with three portions of n-hexane (approximately 80 mL each), three portions of ethyl acetate (approximately 80 mL each) and three portions of hydrated n-butanol (approximately 80 mL each). The combined n-hexane extracts were purified by preparative HPLC with linear gradient from 20 to 60% methanol in water for 30 min. Collection of the peak with a retention time of 23.2 min (detection at 254 nm) and subsequent evaporation *in vacuo* yielded a compound in the form of a colourless oil.

Quantification of coumarin in crude methanol extract

Fresh leaves of *G. sepium* (30 g) were soaked in methanol for 14 days at room temperature

and concentrated to dryness. The residue was dissolved in 300 mL methanol. A portion (5 µL) of the crude methanol extract (0.1 mg fresh weight equivalent mL^{-1}) was analysed by HPLC (linear gradient from 20 to 60% methanol in water for 30 min, detection at 254 nm). The concentration of coumarin in the extract was determined by comparison of the peak area of the sample with that of an authentic coumarin (Tokyo Chemical Industry).

RESULTS AND DISCUSSION

Isolation and identification of compound

The inhibitory activity of the fractions on lettuce radicle growth revealed that most of the activity of crude methanol extract was retained in the n-hexane fraction (Figure 1). Fifty per cent inhibition of radicle elongation was observed at concentration of 2.3 mg fresh weight equivalent mL^{-1} in this fraction. Three subfractions were obtained through HPLC: one corresponding to the major peak and two fractions that eluted before and after the major peak. The fraction corresponding to the major peak showed the strongest inhibitory activity in the lettuce bioassay.

The ^{1}H-NMR spectrum of the compound showed the presence of a 1,2-substituted benzene ring and a cis-1,2-substituted ethylene moiety. The ^{13}C-NMR spectrum indicated that the compound possessed nine carbon atoms, all of which appeared at lower magnetic field than 115 ppm. The chemical structure of this compound was identified as that of coumarin. The structure was confirmed by comparing the ^{1}H- and ^{13}C-NMR spectra, GC retention time and mass spectrum of the isolated compound with those of authentic coumarin.

Contribution of coumarin to the inhibitory activity of *G. sepium* crude methanol extract

The coumarin content in the methanol extract of the leaves of *G. sepium* was 11.6 mmol kg^{-1} fresh weight (Figure 2). The plot of the inhibitory activity of the crude extract closely matched that of authentic coumarin at the same concentration as calculated for the crude extract. The EC_{50} value for authentic coumarin was 23.3 µmol L^{-1} (95% confidence interval, 17.9–30.4 µmol L^{-1}) and the coumarin concentration in the crude extract that showed 50% inhibition of radicle growth was 9.7 µmol L^{-1} (95% confidence interval, 5.1–15.7 µmol L^{-1}). The result indicated that coumarin accounted for a major part of the inhibitory activity of leaves of *G. sepium*.

Total activity of coumarin in *G. sepium*

Specific activity and total activity are used as concepts for the isolation of bioactive compounds. Specific activity is expressed in general by EC_{50}, which is defined as the effective concentration of the compound to induce half of the maximum inhibition. A small EC_{50} value indicates a compound with high specific activity. Total activity is the biological activity per unit weight of the organism containing the bioactive compound and is defined as: total activity = concentration or content of a compound in a plant/specific activity (EC_{50}).

A compound with high total activity does not necessarily have high specific activity (low EC_{50}) because the value of total activity is a function of both the content of the compound and its specific activity. For this reason, total activity is useful for evaluating the

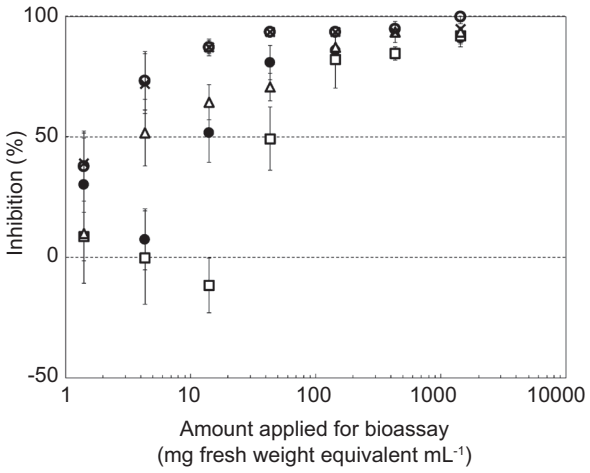

Figure 1 Inhibition of lettuce radicle growth by crude methanol extract of the leaves of *G. sepium* (○), and n-hexane (×), ethyl acetate (△), n-butanol (□) and water (●) fractions obtained from the crude extract; values are means ± standard deviations (n = 5)

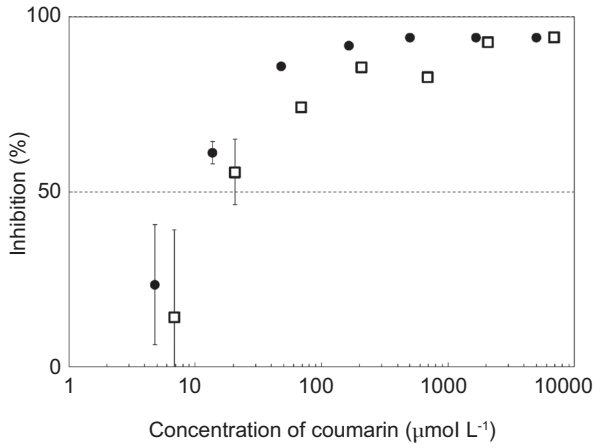

Figure 2 Comparison of inhibition of lettuce radicle growth by authentic coumarin (□) and crude methanol extract of *G. sepium* (●) on the basis of coumarin concentration; values are means ± standard deviations (n = 5)

allelopathic potential of plants and the active compounds they contain (Hiradate 2006).

Several plants have been reported to possess high total activities for inhibition of radicle growth in various plants (Table 1). The present study has demonstrated *G. sepium* to be among them. Some well-investigated plants that are reportedly allelopathic to other species display low total activities. For example, the value of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) in quackgrass (*Agropyron repens*) is 0.1 (Pérez 1990, Friebe et al. 1995), and that of juglone and its glycosides in black walnut (*Juglans nigra*) ranges from 25 to 75 (Coder 1983). In contrast, the total activity of 1-*O*-cis-cinnamoyl-β-D-glucopyranose (cis-CG) and 6-*O*-(4-hydroxy-2-methylenebutyroyl)-1-*O*-cis-cinnamoyl-β-D-glucopyranose (cis-BCG) in the highly allelopathic plant *Spiraea thunbergii* is reported to be 1000 (Hiradate et al. 2004). In the present paper, the total activity of coumarin in *G. sepium* is 500 (Table 1). Thus, the allelopathic potential of the coumarin in *G. sepium* is approximately half that of the cis-CG and cis-BCG in *S. thunbergii*, per unit weight of plant material.

Coumarin belongs to a group of secondary metabolites. These bioactivities have been extensively investigated. This compound is widely distributed in a variety of plant species (Putnam 1988). It inhibits the germination of *Medicago sativa*, *Lolium multiforum* and *Abutilon theophrasti* (Dornbos & Spencer 1990). Coumarin was identified as an allelochemical in *Anthoxanthum odoratum* (Yamamoto 1995). Our results may provide a scientific basis for the use of *G. sepium* in various applications such as living fences and green manure, which are already in practice in Central America. *Gliricidia sepium* could also be useful for suppressing the growth of weeds. Plants with high coumarin content have been used in intercropping systems to inhibit germination and growth of *Bidens pilosa* (Chon et al. 2003, Chon & Kim 2004, Khanh et al. 2006).

CONCLUSIONS

A methanol extract of the leaves of *G. sepium* inhibited radicle growth of lettuce seedlings. An inhibitory compound was isolated from the methanol extract and identified as coumarin by comparing its spectroscopic data with those of an authentic sample. Authentic coumarin had an EC_{50} of 23.3 μmol L^{-1} and the crude extract that showed 50% inhibition of radicle growth contained coumarin at a concentration of 9.7 μmol L^{-1}. This finding indicated that coumarin accounted for a major part of the inhibitory activity of *G. sepium*. The total activity of coumarin in *G. sepium* was among the highest total activity ever reported for an allelopathic plant.

ACKNOWLEDGEMENTS

This research was supported by the Programme for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN), Tokyo, Japan.

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Table 1 Inhibitory activity of allelochemicals expressed as specific activity and total activity in potent allelopathic plants

Compound	Donor plant	Specific activity (M)	Total activity (no unit)	Recipient plant
Coumarin	*Anthoxanthum odoratum*[a]	1×10^{-5}	2000	Lettuce
Juglone	*Juglans alianthifolia*[b]	1×10^{-5}	2000	Lettuce
cis-CG + cis-BCG	*Spiraea thunbergii*[c]	3×10^{-6}	1000	Lettuce
Coumarin	*Gliricidia sepium*	2×10^{-5}	500	Lettuce
L-DOPA	*Mucuna pruriens*[d]	2×10^{-4}	200	Lettuce
Durantanins I–III	*Duranta repens*[e]	5×10^{-5}	200	Mustard

[a]Yamamoto (1995), [b]Jung et al. (2010), [c]Hiradate et al. (2004), [d]Fujii (1999), [e]Hiradate et al. (1999)

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