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PLANT GROWTH INHIBITION BY *cis*-CINNAMOYL GLUCOSIDES AND *cis*-CINNAMIC ACID

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Abstract—*Spiraea thunbergii* Sieb. contains 1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (CG) and 6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (BCG) as major plant growth inhibiting constituents. In the present study, we determined the inhibitory activity of CG and BCG on root elongation of germinated seedlings of lettuce (*Lactuca sativa*), pigweed (*Amaranthus retroflexus*), red clover (*Trifolium pratense*), timothy (*Phleum pratense*), and bok choy (*Brassica rapa* var *chinensis*) in comparison with that of two well-known growth inhibitors, 2,4-dichlorophenoxyacetic acid (2,4-D) and (+)-2-*cis*-4-*trans*-abscisic acid (*cis*-ABA), as well as two related chemicals of CG and BCG, *cis*-cinnamic acid (*cis*-CA) and *trans*-cinnamic acid (*trans*-CA). The EC_{50} values for CG and BCG on lettuce were roughly one-half to one-quarter of the value for *cis*-ABA. *cis*-Cinnamic acid, which is a component of CG and BCG, possessed almost the same inhibitory activity of CG and BCG, suggesting that the essential chemical structure responsible for the inhibitory activity of CG and BCG is *cis*-CA. The *cis*-stereochemistry of the methylene moiety is apparently needed for high inhibitory activity, as *trans*-CA had an EC_{50} value roughly 100 times that of CG, BCG, and *cis*-CA. Growth inhibition by CG, BCG, and *cis*-CA was influenced by the nature of the soil in the growing medium: alluvial soil preserved the bioactivity, whereas volcanic ash and calcareous soils inhibited bioactivity. These findings indicate a potential role of *cis*-CA and its glucosides as allelochemicals for use as plant growth regulators in agricultural fields.

Key Words—*Spiraea thunbergii* Sieb, Rosaceae, Thunberg Spirea, allelopathy, plant growth inhibitors, *cis*-cinnamic acid, *cis*-cinnamoyl glucosides.

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INTRODUCTION

Environmental safety is a major concern in many countries. The use of natural products would be a helpful way to reduce the risk from synthetic agrochemicals because, in general, the ecosystem can decompose and recycle natural products. Compounds that regulate plant growth have been sought in plants and microorganisms, and plants contain growth-regulating compounds that control their own growth (phytohormones) or that of other plants (allelochemicals).

In recent decades, research aimed at identifying natural chemicals that might serve as commercial herbicides or as prototypes for the development of synthetic analogs has intensified. The most widely used herbicide in the world, 2,4-dichlorophenoxyacetic acid (2,4-D; Figure 1), is an auxin analog. Some

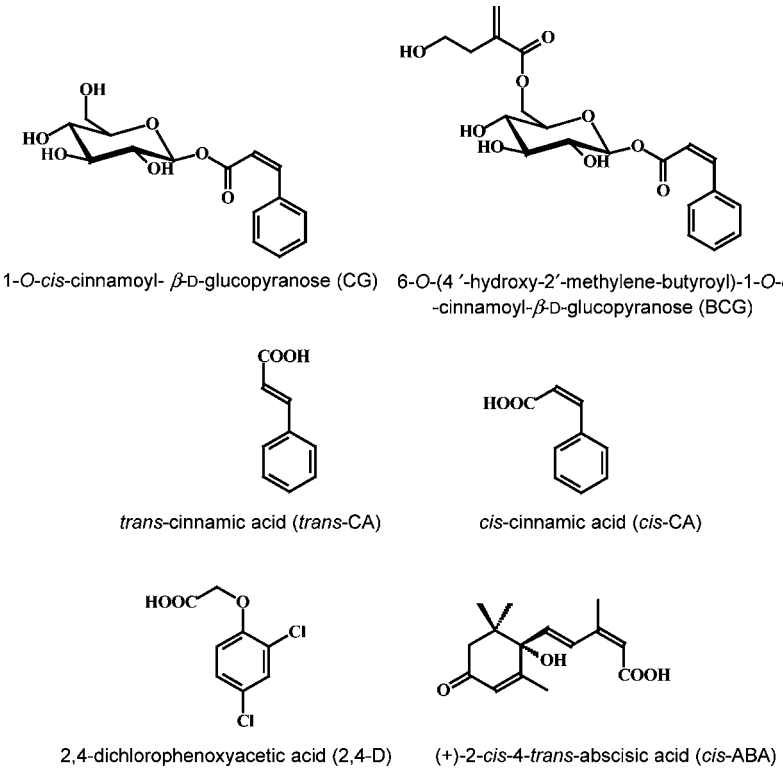

FIG. 1. Chemical structures of 1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (CG), 6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (BCG), *trans*-cinnamic acid (*trans*-CA), *cis*-cannamic acid (*cis*-CA), 2,4-dichlorophenoxyacetic acid (2,4-D), and (+)-2-*cis*-4-*trans*-abscisic acid (*cis*-ABA).

phytohormones, such as (+)-2-*cis*-4-*trans*-abscisic acid (*cis*-ABA; Figure 1), gibberellins, and brassinosteroids, are used as commercial plant growth regulators. The commercial herbicide phosphino-thricylalanylalanin (bialaphos) has also been found among the fermentation products of *Streptomyces hygroscopicus*.

Morita et al. (2001) clarified the growth-inhibition activity of leachates from the leaves of 56 species of woody plants grown in Japan, and found that three Rosaceae plants (*Spiraea thunbergii* Sieb., *S. cantoniensis*, and *S. pruniflora*) had the highest inhibitory activity when assessed with lettuce roots. Hiradate et al. (2004) isolated the inhibitory compounds from *S. thunbergii* using the concept of "*total activity*," which means biological activity per unit weight of the organism containing the bioactive compound, and elucidated the chemical structures from spectroscopic evidence. The compounds were novel *cis*-cinnamoyl glucosides: 1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (CG; Figure 1) and 6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (BCG; Figure 1).

In the present study, we clarified the inhibitory activity (*specific activity*, biological activity per unit weight of the compound) of CG and BCG on the root elongation of five plant species by comparing the two compounds with two well-known plant growth inhibitors (2,4-D and *cis*-ABA). The inhibitory activity of CG and BCG was also compared with that of *cis*-cinnamic acid (*cis*-CA; Figure 1) and *trans*-cinnamic acid (*trans*-CA; Figure 1) to reveal the portion of the chemical structure that is essential for the inhibitory activity of CG and BCG. The activities of CG, BCG, *cis*-CA, and *trans*-CA were also tested in various soil types (alluvial soil, volcanic ash soil, and calcareous soil) to assess the effects of soils on the inhibitory activities.

METHODS AND MATERIALS

Chemicals. 2,4-D (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and *cis*-ABA (Toray Industries, Inc., Tokyo, Japan) were used as reference compounds for the bioassay. Two natural plant growth inhibitors, CG and BCG, were isolated and purified from the leaves of *S. thunbergii* according to the method described by Hiradate et al. (2004). To prepare *cis*-CA, we transformed *trans*-CA (Wako Pure Chemical Industries, Ltd.) into an isomeric mixture of *cis*- and *trans*-CA by irradiating it with an ultraviolet lamp (254 nm) overnight. Pure *cis*-CA was isolated by separating the isomeric mixture using preparative HPLC as described by Sun et al. (2002). To determine the concentrations of CG, BCG, and *cis*-CA, we dissolved 60 μg of *trans*-CA in 0.60 ml of CD_3OD-d_4 in an NMR tube (5-mm i.d.). A solution of each of the purified compounds (hydrated) was also prepared by dissolving an unweighed quantity of the compounds in 0.60 ml of CD_3OD-d_4 in the NMR tube (concentration unknown). Hydrogen-1 (^1H) NMR signals of these samples were recorded quantitatively (acquisition time: 5.46 sec) using an NMR spectrometer (^1H: 600 MHz, JNM α-600, JEOL, Tokyo). We compared the

integrated peak area of the methylene ^{1}H of *trans*-CA with that of the methylene ^{1}H of the cinnamic acid moiety of each compound, and the concentration of each compound was calculated thereby.

Phytotoxic Activities in the Absence of Soil. A filter paper (27 mm diam, Type 1, Toyo Roshi Kaisha, Ltd., Tokyo) was placed in a glass petri dish (27 mm diam). Test compounds were dissolved in water at various concentrations and a 0.7-ml portion of each test solution was added to the filter paper in the petri dishes of each treatment. Five to six pre-germinated (20°C in the dark) seedlings of lettuce (*Lactuca sativa* cv. Great Lakes 366), pigweed (*Amaranthus retroflexus*), red clover (*Trifolium pratense*), timothy (*Phleum pratense*), and bok choy (*Brassica rapa* var. *chinensis*) were used as a single replicate for each treatment, with different species placed on different filter papers to avoid any potential allelopathic effects. Seedlings were incubated for 48 hr at 20°C in the dark, and the inhibitory activity of each test solution on root elongation was detected by measuring the length of each root and comparing it with that of the untreated control (with only distilled water provided). The effective concentration required to induce half of the maximum inhibition (EC_{50}) and its 95% confidence interval were calculated by using the probit method from SPSS for Windows ver. 11.0.1J statistical software (SPSS Japan Inc., Tokyo).

Phytotoxic Activities in the Presence of Soil. An Alluvial soil (Aquept, Soil Survey Stuff, 1999; Anthrosol, FAO, 1998) and a volcanic ash soil (Melanudand, Soil Survey Stuff, 1999; Silandic Andosol, FAO, 1998) were collected from the A_p horizons of paddy and upland agricultural fields in Tsukuba, Japan. A calcareous soil (Hapludalf, Soil Survey Stuff, 1999; Chromic Luvisol, FAO, 1998) was collected from the A_p horizon of an upland agricultural field in Yomitan, Okinawa, Japan. The soils were air-dried and finely sieved (<0.5 mm), and a 500-mg portion (oven-dry basis) of each soil was placed in each well (15.6-mm diam, 17-mm height, 1.9-cm^2 growing area) of a 24-well plate (Nunclon, NalgeNunc, Denmark). We then added 1 ml of a solution containing 10^{-8} to 10^{-2} M of plant growth inhibitor and 0.75% of agar (gelling temperature, 30 to 31°C; Nakalai Tesque, Inc., Kyoto, Japan) at 40°C to each soil-containing well and mixed the components. After incubating the mixture at 30°C for 24 hr in the dark, we placed four lettuce seeds on the gelled agar-soil mixture in the well. The lettuce seeds were grown for 72 hr at 20°C in the dark, and the inhibitory activity was detected by measuring root elongation and comparing it with that of the control (with no plant growth inhibitor, but in the presence of the corresponding soil). This experiment was replicated three times.

RESULTS AND DISCUSSION

Figure 2 shows the inhibitory activities of CG, BCG, 2,4-D, *cis*-ABA, *cis*-CA, and *trans*-CA on the root growth of lettuce seedlings in the absence of soil. In this

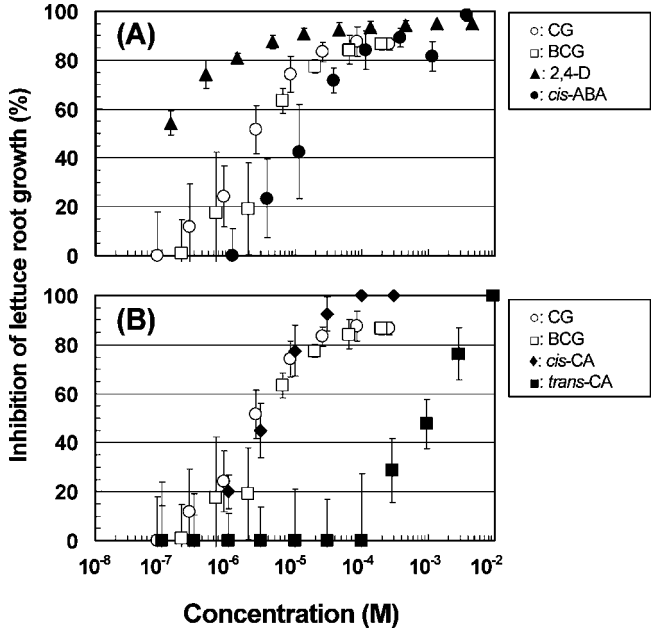

FIG. 2. Inhibitory activities of *cis*-cinnamoyl glucosides (○; CG, □; BCG) in comparison with those of the reference compounds (A: ▲; 2,4-D, ●; *cis*-ABA) and cinnamic acids (B: ♦; *cis*-CA, ■; *trans*-CA) in the absence of soil. Inhibition of root elongation was detected by comparing the root lengths with those of untreated controls after incubation for 48 hr at 20°C in the dark. Bars indicate standard deviations ($N = 5$–6).

experiment, the growth inhibition in the presence of CG, BCG, 2,4-D, *cis*-ABA, and *cis*-CA was attributable to the effects of the compounds themselves, and not to effects of pH or salts, because single treatment with 3×10^{-4} M HCl or 10^{-4} M $CaCl_2$ alone did not affect the growth of lettuce roots (data not shown). As shown in Figure 2, CG, BCG, 2,4-D, *cis*-ABA, and *cis*-CA inhibited the root growth of lettuce seedlings at concentrations below 10^{-4} M.

cis-ABA is one of the most active natural growth inhibitors. Koshimizu et al. (1966) reported that the effective concentration of *cis*-ABA required to induce half the maximum inhibitory action (EC_{50}) on the leaf sheath of rice seedlings was around the 10^{-6} M level. Structural modification of this compound has been attempted to strengthen its specific activity and to make it resistant against biodegradation into inactive forms (Nakano et al., 1995; Todoroki et al., 1997; Arai et al., 1999). In this study, the EC_{50} of *cis*-ABA for root elongation of lettuce seedlings was observed at the 10^{-5} M level (14.9 μM, Table 1).

TABLE 1. INHIBITORY ACTIVITIES OF REFERENCE COMPOUNDS (2,4-D AND *cis*-ABA), CINNAMOYL GLUOSIDES (CG AND BCG), AND CINNAMIC ACIDS (*cis*-CA AND *trans*-CA) ON ROOT GROWTH OF LETTUCE (*Lactuca sativa* CV. GREAT LAKES 366) SEEDLINGS IN FILTER PAPER AND AGAR BIOASSAY SYSTEMS IN THE ABSENCE OF SOIL, AS INDICATED BY THE EFFECTIVE CONCENTRATION AT 50% INHIBITION (EC_{50}) AND 95% CONFIDENCE INTERVAL VALUES (μM) DETERMINED BY PROBIT ANALYSIS

	On filter paper	On agar
2,4-D	0.34 (0.07–1.21)[a]	0.24 (0.19–0.30)
cis-ABA	14.94 (6.39–32.81)	—[b]
CG	3.98 (1.66–9.05)	2.61 (1.12–6.02)
BCG	6.88 (3.39–14.21)	8.68 (2.97–22.76)
cis-CA	3.67 (3.07–4.38)	1.62 (0.17–4.39)
trans-CA	770.92 (549.47–1120.47)	883.40 (379.72–2252.94)

[a] Data in parentheses indicate the 95% confidence interval.
[b] No data available.

The inhibitory activities of CG and BCG on root elongation of lettuce seedlings both occurred at the 10^{-6} M level (Figure 2, Table 1). The EC_{50} values for CG and BCG on lettuce were roughly one-half to one-quarter of the value for *cis*-ABA, meaning that CG and BCG had considerably stronger inhibitory activity than *cis*-ABA. Although the inhibitory activities of CG and BCG were almost one tenth that of 2,4-D, a synthetic commercial herbicide (Figure 2A, Table 1), their inhibitory potential is still among the highest yet reported for natural products.

To establish the portion of the chemical structure that is essential for the high inhibitory activity of CG and BCG, we compared their inhibitory activity with that of *cis*- and *trans*-CA. CG, BCG, and *cis*-CA had comparable levels of inhibitory activity (Figure 2B, Table 1). This suggests that *cis*-CA represents the chemical moiety that is essential for the high inhibitory activity of CG and BCG. We obtained additional confirmation that the stereochemistry of the methylene moiety of CA should be *cis* to provide high inhibitory activity by observing that the *trans*-isomer (*trans*-CA) showed an activity that was two orders of magnitude weaker than that of CG, BCG, and *cis*-CA (Figure 2B, Table 1).

Inhibitory activities of 2,4-D, *cis*-ABA, CG, BCG, *cis*-CA, and *trans*-CA on root elongation of pigweed, red clover, timothy, and bok choy were also established (Figure 3). In all plant species that we tested, the inhibitory activities of CG, BCG, and *cis*-CA were equally high, whereas that of *trans*-CA was significantly weaker. This result supports the suggestion that the portion of the chemical structure that is essential for high inhibitory activity is *cis*-CA and that the stereochemistry of the methylene moiety of CA needs to be *cis* to provide high inhibitory activity. This result is consistent with the observation that the *trans*-isomers of CG and BCG

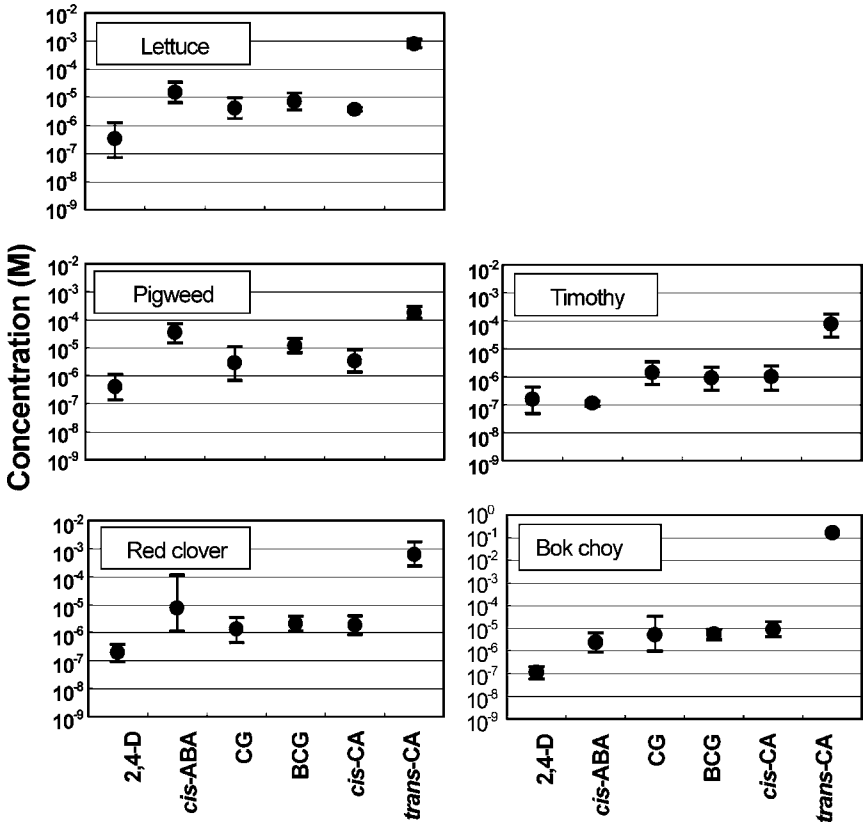

FIG. 3. Inhibitory activities of reference compounds (2,4-D and *cis*-ABA), *cis*-cinnamoyl glucosides (CG and BCG), and cinnamic acids (*cis*-CA and *trans*-CA) on root elongation of germinated seedlings of lettuce (*Lactuca sativa* cv. Great Lakes 366), pigweed (*Amaranthus retroflexus*), red clover (*Trifolium pratense*), timothy (*Phleum pratense*), and bok choy (*Brassica rapa* var. *chinensis*). Inhibition of root growth was detected by comparing root lengths with those of untreated control seedlings after incubation for 48 hr at 20°C in the dark. Data were subjected to probit analyses, and estimated EC_{50} values (•) and 95% confidence intervals (bars) are shown.

produce almost no inhibition of root elongation in lettuce seedlings (Hiradate et al., 2004).

trans-Cinnamic acid is a common phenylpropanoid synthesized from phenylalanine by L-phenylalanine ammonia-lyase (PAL). Bonner and Galston (1944) showed that *trans*-CA is secreted from the roots of guayule (*Parthenium argentatum*). Since then, the allelopathic potential of *trans*-CA has been frequently

reported (e.g., Baziramakenga et al., 1994; Chon et al., 2003). Since *trans*-CA can be converted into *cis*-CA by sunlight and by the presence of an electron-transfer facilitator, it is possible that the conversion of *trans*-CA into *cis*-CA is involved in the allelopathic phenomenon. This transformation might explain the reported synergism between *trans*-CA and polygodial (Fujita and Kubo, 2003) and ABA (Li et al., 1993).

The presence of natural *cis*-CA in plants has recently been reported in *Brassica parachinensis* (Yin et al., 2003). Its biosynthesis, however, is not well understood, but possible pathways have been proposed: (1) sunlight-mediated conversion from *trans*-CA, (2) spontaneous conversion from *trans*-CA in the presence of an electron-transfer facilitator, (3) isomerase-mediated conversion from *trans*-CA, and (4) direct enzymatic biosynthesis from L-phenylalanine (Yin et al., 2003). Further study is necessary.

The inhibitory activity of CG, BCG, and *cis*-CA was higher than that of *cis*-ABA for lettuce, pigweed, and red clover, but the reverse was observed for timothy and bok choy (Figure 3). This suggests that the mechanisms of growth inhibition might differ between *cis*-ABA and *cis*-CA derivatives. In any case, the inhibitory activity of CG, BCG, and *cis*-CA was generally comparable with that of *cis*-ABA.

The effects of soil on the inhibitory activities of these compounds is shown in Figure 4. In this bioassay system, we added agar to the soils to permit stable growth of the lettuce. This step was necessary because the addition of agar had been shown to decrease the coefficient of variation (CV) for the root growth of lettuce in our preliminary research (data not shown). The inhibitory activities of all compounds in the absence of soil (Figure 4, •) were almost identical to those in the filter paper bioassay system (Table 1, Figure 2), indicating that the adsorption of these compounds on the agar was negligible.

The inhibitory activity of CG, BCG, and *cis*-CA decreased in the presence of volcanic ash and calcareous soils (Figure 4). This was clear in comparisons made at 10^{-5} and at 3×10^{-5} M for each treatment. The alluvial soil had minimal effects on growth inhibition. In volcanic ash and calcareous soils, the amount of active surface hydroxyls, which adsorb and inactivate carboxylic acids by ligand exchange reactions, is larger than in alluvial soil (Hiradate and Uchida, 2004). Therefore, an alluvial soil would be more advantageous for the bioactivity of CG, BCG, and *cis*-CA than would volcanic ash or calcareous soils. It is possible that CG and BCG released from *S. thunbergii* work as effective allelochemicals in soil environments.

The inhibitory activity of CG, BCG, and *cis*-CA was lower than that of the synthetic commercial herbicide 2,4-D. The latter has been listed as a possible endocrine disrupter, along with its possible metabolite 2,4-dichlorophenol (Crosby, 1998). Both are frequently found in soils and in ground water (Hall et al., 1993;

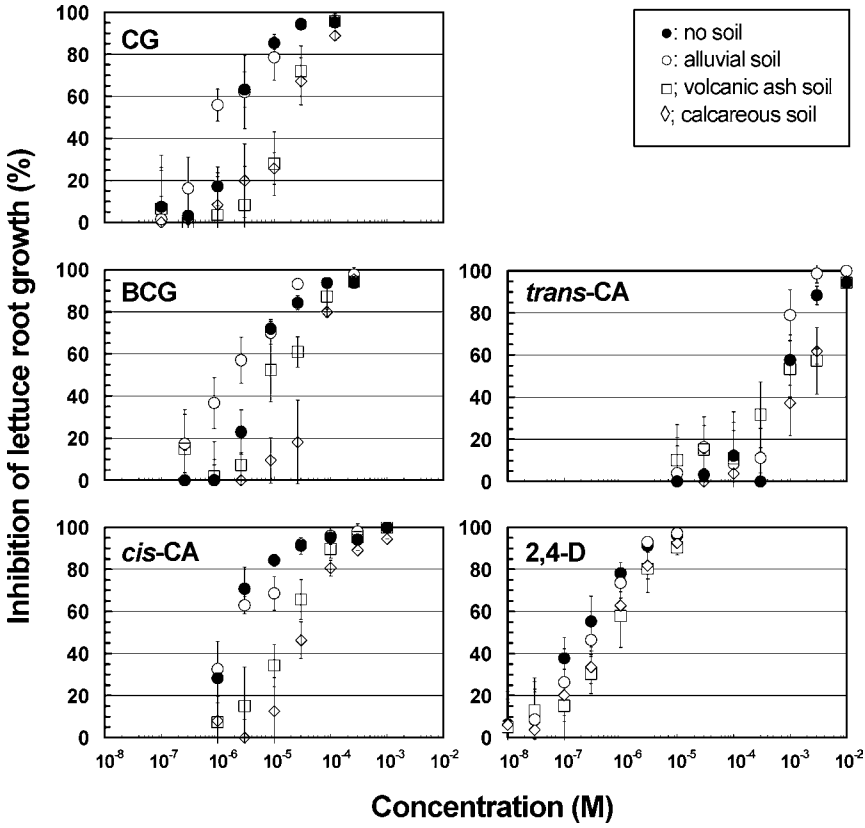

FIG. 4. Inhibitory activities of *cis*-cinnamoyl glucosides (CG and BCG), cinnamic acids (*cis*-CA and *trans*-CA), and the reference compound (2,4-D) on root elongation of lettuce (*Lactuca sativa* cv. Great Lakes 366) in agar media in the absence (●) or presence of soil (○; alluvial soil, □; volcanic ash soil, ◇; calcareous soil). Inhibition of root growth was detected by comparing the root lengths with those of untreated controls for each soil after incubating for 72 hr at 20°C in the dark. Bars indicate standard deviation ($N = 12$).

Wood and Anthony, 1997; Balinova and Mondesky, 1999; Pierzynski et al., 2000). Synthetic products such as 2,4-D often undergo little decomposition in soil environments because the enzymatic and other systems required to degrade them may not be present in nature. In contrast, both *cis*-CA and its glucosides are natural products that could be utilized by various indigenous soil organisms, because cinnamic acid is a common secondary metabolite in most organisms. It is, thus, likely that *cis*-CA and its glycosides are worth considering as plant growth regulators,

as they are inexpensive to synthesize and possess a low risk of environmental toxicity.

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