J Plant Growth Regul (2010) 29:1–5
DOI 10.1007/s00344-009-9108-5

SHORT COMMUNICATION

Growth-Regulating Activity of Three 4-Hydroxycoumarin Derivatives on Inoculated Soybean Plants

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Received: 22 October 2008 / Accepted: 4 May 2009 / Published online: 17 July 2009

Abstract Three 4-hydroxycoumarin derivatives (ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl)methyl]-3-oxobutanoate (SS-14), ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl)methyl]-3-oxobutanoate (SS-21), and 2-[(3,4,5-trimethoxyphenyl)(4-hydroxy-2-oxo-2*H*-chromen-3-yl)methyl]-3-oxobutanoate (T-2)] were tested for growth-regulating activity on nitrogen-fixing soybean plants in different concentrations: 10^{-5}, 10^{-4}, and 10^{-3} M. They revealed growth-regulating activity in a concentration-dependent manner. The most powerful suppression effect of T-2 on shoot and root fresh and dry biomass accumulation, length of roots, and height of plants was found. Shoot fresh biomass was suppressed in an equal extent at 10^{-3} M of the three compounds but the order of inhibition regarding the three applied concentrations was T2 > SS-14 ≈ SS-21. The compound SS-14 inhibited nodule number and nodule biomass mainly at the highest applied concentration, 10^{-3} M. The highest inhibition of nitrogenase activity was established at the three applied concentrations of the compound SS-14.

Keywords Plant growth inhibition · 4-Hydroxycoumarins · Nitrogenase · Soybean

Introduction

The coumarins are a widely spread group of natural compounds. They have anticoagulant, antibacterial, antiviral (anti-HIV), spasmolytic, and cytotoxic properties (Hayward 1984; Cespedes and others 2006). The influence of different coumarin derivatives (coumarin, 4-hydroxycoumarin, 7-hydroxycoumarin, psoralen, and xanthotoxin) was explored on root tips in onion plants (*Alium cepa*). Segregation of areas of the cytoplasm by sheets of endoplasmic reticulum and invaginations of plasmalemma in the meristematic cells (Podvielkowska and others 1996) was noted. Cytostatic activity of coumarins in plant cells in vitro (Gawron and Glowniak 1987) was discovered. Decreased growth of cells may be caused by lack of energy, and coumarins were found to inhibit and uncouple oxidative phosphorylation (Knypl 1969). Aleksieva and others (1995) showed the inhibiting activity of some synthetic phosphorus-containing coumarin derivatives on the growth of the shoots of pea, wheat, and cucumbers. Most known coumarins possess potent biological properties and strongly influence biosynthetic pathways (Seigler 1997). Coumarins were the most effective against Gram-negative bacteria (Cespedes and others 2006). The role of some synthetic dicoumarols as growth inhibitors on *Mimosa pigra* Linn. was shown by Chavasiri and others (2001). Some of these dicoumarols that contain hydroxy or methoxy groups as substituents on the benzene ring display high herbicidal activity.

The aim of the present study was to evaluate the effects of three 4-hydroxycoumarin derivatives on the plant growth and nodule parameters and nitrogen-fixing activity in soybean plants inoculated with *Bradyrhizobium japonicum*.

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Materials and Methods

Chemicals

The three tested 4-hydroxycoumarin derivatives have been synthesized by Stanchev and others (2008a, b). They are ethyl

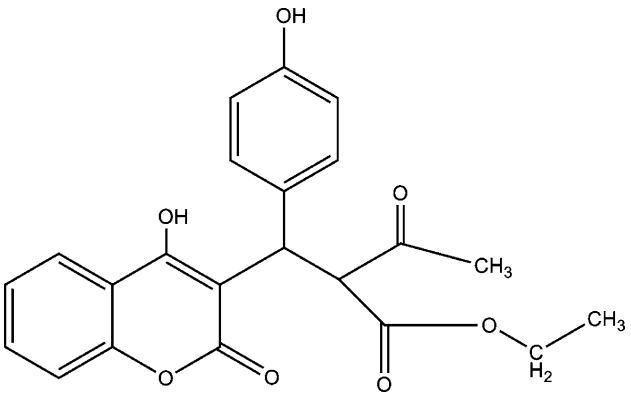

Fig. 1 Ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl) methyl]-3-oxobutanoate (SS-14) (Stanchev and others 2008a)

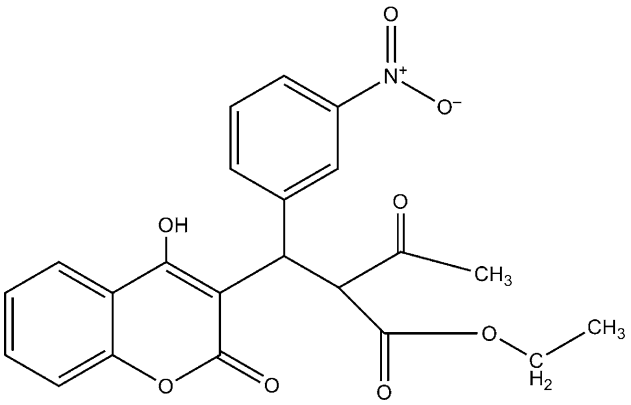

Fig. 2 Ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl) methyl]-3-oxobutanoate (SS-21) (Stanchev and others 2008a)

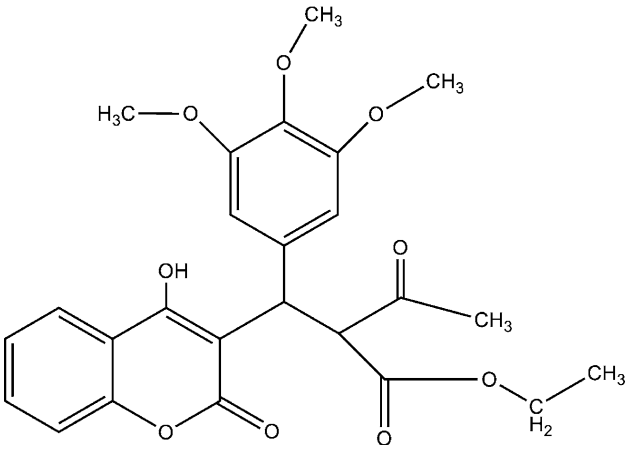

Fig. 3 Ethyl 2-[(3,4,5-trimethoxyphenyl)(4-hydroxy-2-oxo-2H-chromen-3-yl)methyl]-3-oxobutanoate (T-2) (Stanchev and others 2008a)

2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl) methyl]-3-oxobutanoate (SS-14) (Stanchev and others 2008a) (Fig. 1), ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl)methyl]-3-oxobutanoate (SS-21) (Stanchev and others 2008a) (Fig. 2), and ethyl 2-[(3,4,5-trimethoxyphenyl) (4-hydroxy-2-oxo-2H-chromen-3-yl)methyl]-3-oxobutanoate (T-2) (Stanchev and others 2008b) (Fig. 3).

Plant Material and Nitrogenase Activity Determination

Soybean plants (*Glycine max*. [L] Merr.) were grown in a climatic chamber at a 12-h photoperiod, day/night temperature of 25/18°C, and PPFD of 95 μmol m^{-2} s^{-1} until the 32nd day. The seed variety "Pavlikeni 125," supplied from the Soybean Institute, Pavlikeni, was surface sterilized with 4% (v/v) NaOCl for 5 min. Three-day-old seedlings were inoculated with the bacterial suspension of *Bradyrhizobium japonicum* strain 639 at approximately 10^8 cells/cm^3. Plants were grown as a water culture in 1.2-L pots (2 plants per pot) on Hellriegel nutrient solution with 0.5 mM NO_3^- completed with micronutrients after Hoagland and Arnon (1950). The nutrient solution was replaced at three-day intervals and was aerated continuously. The treatments with coumarin derivatives were done once on the 25th day. The three compounds, T-2, SS-14, and SS-21, were dissolved in a small volume of dimethylsulfoxide (DMSO) and d.H_2O was added to the final volume. The treatments were applied at concentrations of 10^{-3}, 10^{-4}, and 10^{-5} M by spraying (5 ml per plant). Control plants were treated with the same volume of DMSO and water. Samples were taken and morphological and enzymatic determinations were performed on the 7th day after chemical treatments. The root length and the height of the shoots were measured from the base to the growing apices. The nitrogen-fixing (nitrogenase) activity of nodules (EC 1.7.99.2) was determined by the acetylene reduction assay according to Hardy and others (1973). Nitrogenase activity was accounted for on the basis of formed ethylene. One gram of nodules was incubated at 28°C for 30 min in 30 ml-plastic bottles containing 10% (v/v) acetylene. C_2H_4 production was analyzed by a Perkin–Elmer 104 gas chromatograph with an Al_2O_3 column and a flame-ionizing detector. Enzyme activity was expressed as μmol C_2H_4 h^{-1} g^{-1} FW of nodules. The dry matter yields of roots, shoots, and nodules were recorded separately after drying them at 80°C for 48 h. The percent of inhibition was calculated using the formula: $\% = [1 - T/C] \times 100\%$, where T is the parameter of the treated variants and C is the parameter of the control variants.

Our results are expressed as mean ± standard error where $n = 4$. Comparison of the means was performed by the Fisher least significant difference (LSD) test ($P \leq 0.05$) after ANOVA analysis.

Results and Discussion

Growth-Inhibiting Activity

The elevated concentrations of 4-hydroxycoumarin derivatives suppressed the fresh and dry biomass of the roots

and shoots in comparison with the control plants (Table 1). The root fresh and dry biomass was significantly suppressed at the highest concentration: 10^{-3} M. At this concentration the most significant reduction of root biomass was observed by the T-2 compound application; the least significant influence was that of SS-21. The substance SS-14 had a middle effect of inhibition among the three compounds at 10^{-3} M. Considering the effects of all the applied concentrations, the following relative order of inhibition was proposed: T2 $\approx$ SS-21 $>$ SS-14 for the root fresh biomass and T2 $>$ SS-21 $>$ SS-14 for the root dry biomass. The fresh and dry biomasses of the shoots decreased to a greater extent compared with those of the roots as a result of the influence of the applied substances (Table 1). Shoot fresh biomass was suppressed to an equal extent at 10^{-3} M of the three compounds but the order of inhibition regarding the three applied concentrations was T2 $>$ SS-14 $\approx$ SS-21. The highest inhibition percent regarding shoot dry biomass was observed at 10^{-3} M of T-2, whereas the lowest shoot dry biomass inhibition was established at 10^{-5} M of SS-14. The strong inhibitory effect of T-2 on the root biomass and especially on the shoot biomass could be due to the presence of three methoxy groups as substituents on the benzene ring. Plant-growth inhibitory activities of five 7-methoxycoumarins and one 5,7-dimethoxycoumarin isolated from *Myrraya paniculata* were described by Jiwajinda and others (2000).

Inhibiting activity of coumarin derivatives was expressed with respect to the length of the roots and the height of the shoots of the soybean plants, but in a lower degree compared with the respective fresh weight (Table 2). The height of the shoots was reduced more significantly compared with the length of the roots as a result of the influence of 4-hydroxycoumarin derivatives. In that case, the strength of the influence of the compounds SS-14 and SS-21 was not distinguishable, but a strong inhibitory effect was observed at the highest applied concentration of methoxy groups containing compound T-2 (T2 $>$ SS-14 $\approx$ SS-21). Jiwajinda and others (2000) reported that the effects of methoxycoumarins are expressed more significantly on the second leaf sheath elongation of the rice seedlings than on the root growth of cucumber. Chavasiri and others (2001) proved the herbicidal activity of some synthetic dicoumarols with respect to the length of the shoots and roots of *Mimosa pigra* Linn.

The number and dry weight of the nodules (Table 3), organs formed as a result of inoculation with nitrogen-fixing bacteria, were also suppressed by application of the compounds. The reduction was greatest after treatment with the highest concentration of SS-14: 10^{-3} M. At this concentration the relative order of nodule dry weight inhibition was SS-14 $>$ T-2 $>$ SS-21. The effect of the other concentrations (10^{-4} and 10^{-5} M) in relation to nodule biomass was not distinguishable among the compounds applied. Application of SS-21 in concentrations of 10^{-4} and 10^{-5} M did not affect nodule number, whereas T-2 inhibited the nodule number equally at the three concentration treatments (Table 3). Treatments with 4-hydroxycoumarin derivatives strongly suppressed the nitrogen fixation process in the nodules. A significantly high percentage of nitrogenase activity inhibition (98%) at the three applied concentrations was established as a result of treatment with SS-14, but the

Table 1 Root and shoot biomass accumulation of soybean plants, treated with 4-hydroxycoumarin derivatives

Variants	Root fresh biomass (g plant^{-1})	% Inhibition	Root dry biomass (g plant^{-1})	% Inhibition	Shoot fresh biomass (g plant^{-1})	% Inhibition	Shoot dry biomass (g plant^{-1})	% Inhibition
Control (C)	5.297^{d}	–	0.326^{f}	–	7.820^{e}	–	1.455^{f}	–
T-2								
10^{-3} M	3.571^{a}	33	0.196^{a}	40	2.564^{ab}	67	0.584^{a}	60
10^{-4} M	3.839^{ab}	28	0.22^{bc}	33	4.263^{bc}	45	0.853^{bc}	41
10^{-5} M	4.435^{c}	16	0.236^{b}	28	4.968^{cd}	36	0.903^{cd}	38
SS-14								
10^{-3} M	3.979^{b}	25	0.211^{ab}	35	2.538^{a}	68	0.626^{a}	57
10^{-4} M	4.695^{c}	11	0.281^{e}	14	5.671^{cd}	27	0.855^{bc}	41
10^{-5}M	5.374^{d}	–	0.285^{e}	13	5.685^{cd}	27	1.213^{e}	17
SS-21								
10^{-3} M	3.539^{a}	33	0.24^{bd}	26	2.646^{ab}	66	0.788^{b}	46
10^{-4} M	3.730^{ab}	30	0.258^{d}	21	5.685^{cd}	27	0.969^{d}	33
10^{-5} M	4.624^{c}	13	0.235^{b}	28	6.104^{d}	22	0.936^{d}	36
LSD ($P \leq 0.05$)	0.375		0.021		1.699		0.081	

Values are mean $\pm$ SE, $n = 4$. Different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA analysis

Table 2 The effects of treatment with 4-hydroxycoumarin derivatives on the root length and shoot height of the soybean

Variants	Root length (cm plant^{-1})	% Inhibition	Shoot height (cm plant^{-1})	% Inhibition
Control (C)	38.33^{b}		19.00^{cd}	
T-2				
10^{-3} M	30.17^{a}	21	13.67^{ab}	28
10^{-4} M	40.83^{b}	–	25.00^{e}	–
10^{-5} M	38.67^{b}	–	17.67^{cd}	7
SS-14				
10^{-3} M	40.00^{b}	–	16.17^{bc}	15
10^{-4} M	33.33^{ab}	13	17.33^{cd}	9
10^{-5} M	39.73^{b}	–	17.67^{cd}	7
SS-21				
10^{-3} M	40.33^{b}	–	15.83^{bc}	17
10^{-4} M	37.00^{ab}	5	17.00^{bc}	11
10^{-5} M	36.93^{ab}	4	20.67^{d}	–
LSD ($P \leq 0.05$)	8.10		3.42	

Values are mean ± SE, $n = 4$. Different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA analysis

Table 3 Nitrogenase activity and nodule parameters of soybean treated with 4-hydroxycoumarin derivatives

Variants	Nodule dry weight (g plant^{-1})	% Inhibition	Nitrogenase activity (μmol C_2H_4 g^{-1} FW h^{-1})	% Inhibition	Nodule number (plant^{-1})	% Inhibition
Control (C)	0.173^{h}		130.45^{i}		30.67^{e}	
T-2						
10^{-3} M	0.060^{b}	65	5.16^{e}	96	21.25^{ab}	30
10^{-4} M	0.084^{d}	51	10.95^{g}	92	21.75^{ef}	30
10^{-5} M	0.130^{g}	25	23.03^{h}	83	21.50^{bc}	30
SS-14						
10^{-3} M	0.050^{a}	71	2.35^{b}	98	19.00^{a}	38
10^{-4} M	0.083^{d}	52	3.01^{c}	98	26.75^{d}	13
10^{-5} M	0.129^{g}	25	2.21^{b}	98	23.75^{c}	23
SS-21						
10^{-3} M	0.073^{c}	58	1.22^{a}	99	21.67^{bc}	29
10^{-4} M	0.096^{e}	45	4.69^{d}	96	34.00^{f}	–
10^{-5} M	0.110^{f}	36	7.52^{f}	94	30.00^{e}	2
LSD ($P \leq 0.05$)	0.009		0.085		2,26	

Values are mean ± SE, $n = 4$. Different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA analysis

inhibition of nodule number was about 25%. There is no correspondence between the number of nodules and nitrogenase activity. It is known that nitrogenase activity is much more sensitive to stress conditions than the number of nodules. The lowest concentration of T-2 (10^{-5} M) suppressed the nitrogen-fixing activity in the smallest degree. The relative order of nitrogen-fixing activity inhibition from the three compounds was SS-14 > SS-21 > T2. Because the process of nitrogen fixation has high energy requirements (conversion of one molecule of nitrogen to ammonia demands 16 molecules of ATP), the inhibitory effect of 4-hydroxycoumarin derivatives could be due to the capability of coumarins to inhibit and uncouple oxidative phosphorylation (Knypl 1969). The strong inhibitory effect of SS-14 on the nitrogenase activity, nodule number, and dry weight probably was a result of the presence of two hydroxyl groups in the structure of the compound. It was reported (Cespedes and others 2006) that dihydroxylated coumarins were most active with antibacterial inhibitory activity.

Testing the three 4-hydroxycoumarin derivatives, T-2, SS-21, and SS-14, in three elevated concentrations showed the most powerful suppression effect of T-2 on the shoot and root fresh and dry biomass accumulation, root length, and plant height. An inhibitory effect of 4-hydroxycoumarin derivatives was observed regarding nodule biomass, nodule number, and especially nitrogen-fixing activity. The compound SS-14 inhibited nodule number and nodule biomass mainly at the highest concentration of 10^{-3} M.

The strongest effect of suppression on nitrogenase activity was established at the three applied concentrations of compound SS-14.

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