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Study on the Allelopathy of Alfalfa (*Medicago sativa* L.)

II. Isolation and identification of allelopathic substances in alfalfa

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Abstract : Allelopathic substances in alfalfa plants (*Medicago sativa* L.) were purified from methanol extracts of alfalfa shoots. The methanol extracts were fractionated into an acidic fraction, and the sample was divided into 10 fractions by thinlayer chromatography (TLC). Growth of radish seedlings was inhibited by the substances obtained from Rf values of 0.5～0.6 and 0.6～0.7.These fractions were eluted with distilled water, and the eluates were analyzed by high-performance liquid chromatography (HPLC). Six peaks were detected on the chromatogram. Growth of radish seedlings was inhibited on three peaks. Of these peaks, two were identified using HPLC, a mass-spectrometer, IR, and ^{1}NMR as follows : peak b, ferulic acid and peak f, salicylic acid. The growth of alfalfa and radish seedlings was inhibited at lower concentrations of these substances.

Key words : Alfalfa, Allelopathy, Ferulic acid, Inhibitory substances, Injury by continuous cropping, Salicylic acid.

アルファルファのアレロパシーに関する研究　第2報　アルファルファのアレロパシー物質の単離と同定 : 中久加菜*・続　栄治・寺尾寛行・小瀬村誠治** (*鹿児島大学連合大学院・宮崎大学農学部・**慶応大学理工学部)

要　旨 : アルファルファのアレロパシーを明らかにするため，アレロパシー物質の単離・同定を行なうことを試みた．アルファルファ地上部のメタノール抽出液の酸性画分を TLC で分画し，各分画の影響を調べた結果，Rf 0.5～0.6 および Rf 0.6～0.7 はダイコンの初期生長を有意に阻害した．これら2つの部分の溶出物から，HPLC によって，6つのピークが得られた．そのうちの3個のピークは，ダイコンの初期生長を阻害し，これらは，HPLC, マス・スペクトロメーター，IR および NMR による分析からフェルラ酸とサリチル酸と同定された．アルファルファおよびダイコンの生長は，標品のフェルラ酸とサリチル酸の低濃度において阻害されたので，フェルラ酸およびサリチル酸はアルファルファのアレロパシーに関係しているものと推察した．

キーワード : アルファルファ，アレロパシー，フェルラ酸，サリチル酸，生長抑制物質，連作障害．

Allelopathy is defined as the biochemical interaction in all plants, including microbes[8]. The effects of allelopathy are important in interactions among plant species in both natural and agricultural ecosystems.

Allelopathy has been considered to connect with injury by continuous cropping. Chemical substances produced by plants have been shown as one of the causes of injury by continuous cropping in red clover (*Trifolium pratense* L.)[6,13,14], peach (*Prunus persica* Sied. et Zucc.)[11], and apple plants (*Malus pumila* Mill.)[1].

Alfalfa, a pasture legume, has also been known to show such a phenomenon[3,5,7,9].

Previously, we suggested the possibility of allelopathy as one of the causes of injury by continuous cropping of alfalfa plants[9].

Here we report the chemical and physiological natures of inhibitory substances in alfalfa.

Materials and Methods

1. Extraction of allelopathic substances from alfalfa shoots

One kilogram of alfalfa shoots before flowering was cut into small pieces and homogenized by a juicer (Hitachi, VA-W 25) with 2000 m*l* of 70% aqueous methanol. After standing overnight at room temperature, the homogenate was filtrated through Toyo filter paper (No. 2) and the resultig filtrate concentrated into small volumes under reduced pres-

sure at 40°C. The extraction procedure is shown in detail in a flow chart (Fig. 1).

2. Purification of allelopathic substances by thinlayer and high-performance liquid chromatographies

The acidic fraction was subjected to thin-layer chromatography (TLC) by the ascending method with toluene : ethyl formic acid : formic acid (5 : 4 : 1) solvent system. TLC plates were coated with a 500 μm layer of silica gel G (Merck). The chromatogram was subjected to bioassay using radish to examine inhibitory activity. The Rfs 0.5～0.6 and 0.6～0.7, which showed activity on the TLC plates were eluted with distilled water. Active substances were also purified by HPLC. Conditions of HPLC were as follows : Column ; TSK gel ODS-80TM (4.6 mm $\phi \times 15$ mm), Temperature in column ; 40°C, Mobility phase ; A solution (0.2% TFA, 99.8% D.M.), B solution (0.2% TFA, 99.8% AcCN), wavelength of ultraviolet absorption of detector ; 240 nm.

3. Identification of allelopathic substances by IR, mass and ^{1}NMR spectra

The active substances (Retention time 10.71 min. and 11.41 min.) purified by HPLC were identified by the interpretation of the IR, mass and ^{1}NMR spectra. Compound b as an amorphous powder : $C_{10}H_{10}O_4$ [m/z 194.0556 (M^+)] ; IR (film) 3400 (br.), 1660, 1585, 1500, and 1260 cm^{-1} ; σ_H ($CDCl_3$) 7.70 (1H, d, J = 16.0 H_z), 7.11 (1H, dd, J = 8.3, 1.9 H_z), 7.06 (1H, d, J = 1.9 H_z), 6.94 (1H, d, J = 1.9 H_z), 6.30 (1H, d, J = 16.0 H_z), 5.89 (1H, br. s), 3.95 (3H, s). Compound f as an amorphous powder : $C_7H_6O_3$ [m/z 138.0332 (M^+)] ; IR (film) 3230-2530 (br.), 1660, 1605, 1575, 1440 and 1295 cm^{-1} ; σ_H ($CDCl_3$) 10.44 (1H, S), 7.92 (1H, dd, J = 8.1, 1.8 H_z), 7.53 (1H, ddd, J = 8.6, 7.0, 1.8 H_z) 7.02 (1H, dd, J = 8.6, 1.0 H_z), 6.94 (1H, ddd, J = 8.1, 7.0, 1.0 H_z).

4. Bioassay

The TLC plate was divided into 10 zones. These were then eluted with 7 m*l*-distilled water. The eluate was used for bioassay using radish seeds. Inhibitory zones, Rfs 0.5～0.6 and 0.6～0.7 on TLC were isolated by HPLC. The active fractions were also bioassayed using radish seedlings. Ten seeds of radish were planted on filter paper in a petri dish which contained the test solution. The petri dishes were then placed in a growth chamber at 20°C under fluorescent light (about 4000 lux). At five days after incubation, the length of the hypocotyl and root was measured.

Ferulic and salicylic acids were used as authentic substances in the growth-inhibition bioassays of alfalfa and radish seedlings. Two substances were dissolved in small volumes of methanol, and water was added to give final concentrations of 5×10^{-4}, 1×10^{-3}, 5×10^{-3} and 1×10^{-2} M.

The synergistic effect of ferulic and salicylic acids was also investigated for growth-inhibition of alfalfa and radish seedlings. Two substances were dissolved in small volumes of methanol, and water was added to give final concentrations of ferulic + salicylic acids of 3.3×10^{-4}, $1.3 \times 10^{-3} + 6.7 \times 10^{-4}$, $2.0 \times 10^{-3} + 1.0 \times 10^{-3}$, $2.7 \times 10^{-3} + 1.3 \times 10^{-3}$, $3.3 \times 10^{-3} + 1.7 \times 10^{-3}$ M. Each test was replicated at least three times.

Results and Discussions

The acidic fraction in the methanol extracts of the alfalfa shoots inhibited the hypocotyl and root elongation in radish plants. As shown in Figs. 2 and 3, the inhibitory zones of the hypocotyl and root elongation of radish seedlings were clearly found at Rf values of 0.5～0.6 and 0.6～0.7 on the TLC plate.

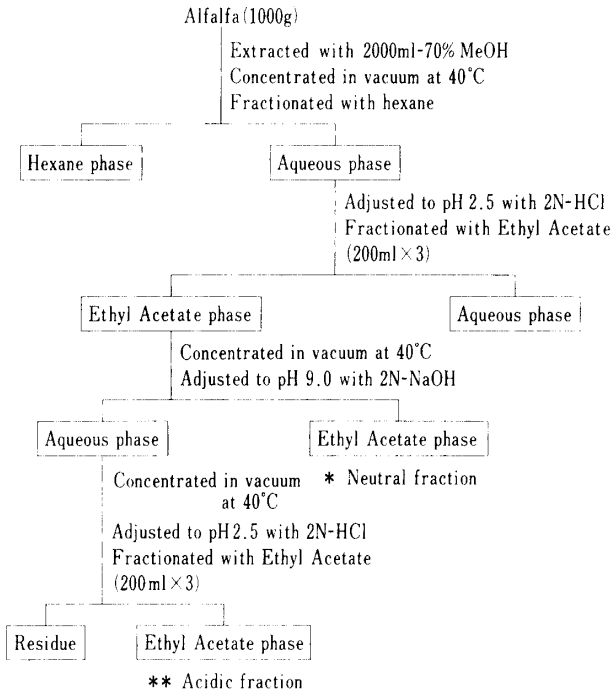

Fig. 1. Extraction procedure for allelopathic substances from alfalfa shoots.

By subsequent HPLC analysis, the zone of Rf 0.5～0.6 was separated into four peaks (Fig. 4-A), and the zone of Rf 0.6～0.7 into two peaks (Fig. 4-B).

Of these peaks, a, b, c and f significantly inhibited the elongation of hypocotyl and root of radish seedlings (Figs. 5 and 6). Peaks d and g were inactive.

The retention times of peaks b and f were 10.7 and 11.4 minutes, respectively. These coincided with Rf values of ferulic and salycilic acids of authentic substances.

Peaks b and f were also investigated using a mass spetrometer, IR, and ^{1}NMR.

Compound b, molecular formula : $C_{10}H_{10}O_4$ [m/z 194.0556 (M^+)] ; exhibited an IR spectrum of 3400, 1660, 1585 cm^{-1}. Its ^{1}NMR spectrum indicated one phenolic hydroxy group [σ 5.89 (1H, br.s)], one methoxy group [σ 3.95 (3H, s)], one conjugated double bond [σ 7.70 (1H, d, J=16.0 H_z), 6.3 (1H, d, J=16.0 H_z)], and one 1, 2, 4, tri-substituted benzene ring. From these data, the structure of compound b can be represented as ferulic acid.

Compound f, molecular formula : $C_7H_6O_3$ [m/z 138.0332 (M^+)] ; exhibited an IR spectrum of 3230-2530 (br.), 1660, 1605, 1575, 1440 and 1295 cm^{-1}. Its ^{1}NMR spectrum indicated one phenolic hydroxy group [σ 10.44 (1H, .s)], and one 1, 2, di-substituted benzene ring [σ 7.92 (1H, dd, J=8.1, 1.8 H_z), 7.53 (1H, ddd, J=8.6, 7.0, 1.8 H_z), 7.02 (1H, dd, J=8.6, 1.0 H_z), and 6.94 (1H, ddd, J=8.1, 7.0, 1.0 H_z)]. From these data, the structure of compound f can be represented as salicylic acid. As shown in Figs. 7-1, 2, ad Fig. 8, peak b was identified as ferulic acid and f as salicylic acid.

Ferulic and salicylic acids were exposed to bioassay using alfalfa and radish seedlings. As shown in Tables 1 and 2, the hypocotyl and root elongation of radish seedlings were significantly inhibited at concentrations of 5×10^{-4} M of ferulic acid and salicylic acid. In the 5×10^{-3} M of salicylic acid, the hypocotyl and root of radish and alfalfa seedlings were not elongated (Tabs. 1 and 4). The root elongation of alfalfa seedlings was significantly promoted in concentrations of 1×10^{-3} M of ferulic acid, but the elongation of hypocotyl and root of alfalfa seedlings was significantly inhibited in a 5×10^{-3} M concentration (Tab. 3). We did not observed elongation of hypocotyl and root of alfalfa seedlings in a 1×10^{-2} M concentration (Tab. 3). Also in the 5×10^{-3} and 1×10^{-2} M concentrations of salicylic acid, we did not observe the elongation of hypocotyl and root of alfalfa seedlings (Tab. 4).

On the synergistic effects of the two authentic substances of ferulic and salicylic acids, we

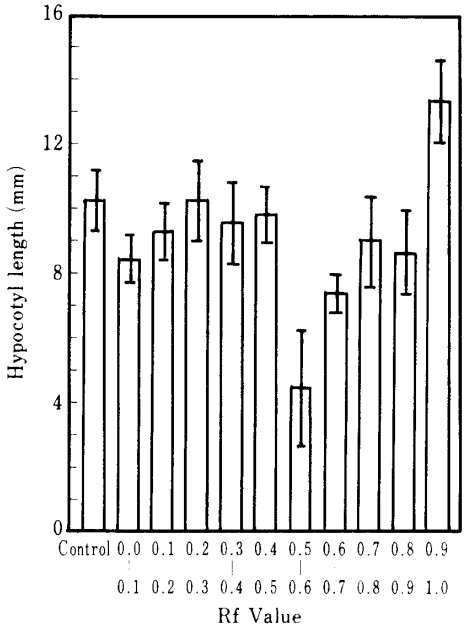

Fig. 2. Hypocotyl length of radish in TLC zones.

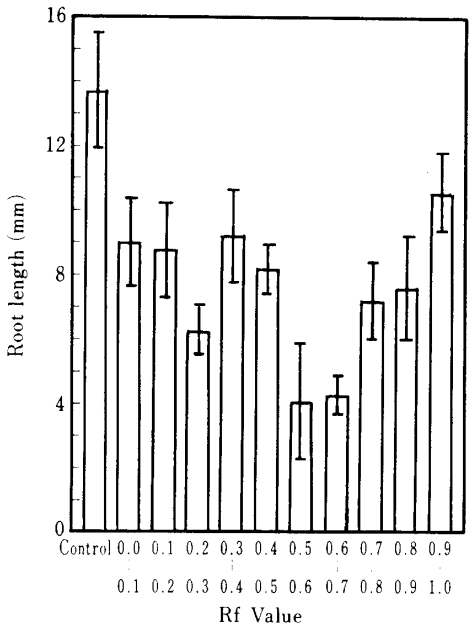

Fig. 3. Root length of radish in TLC zones.

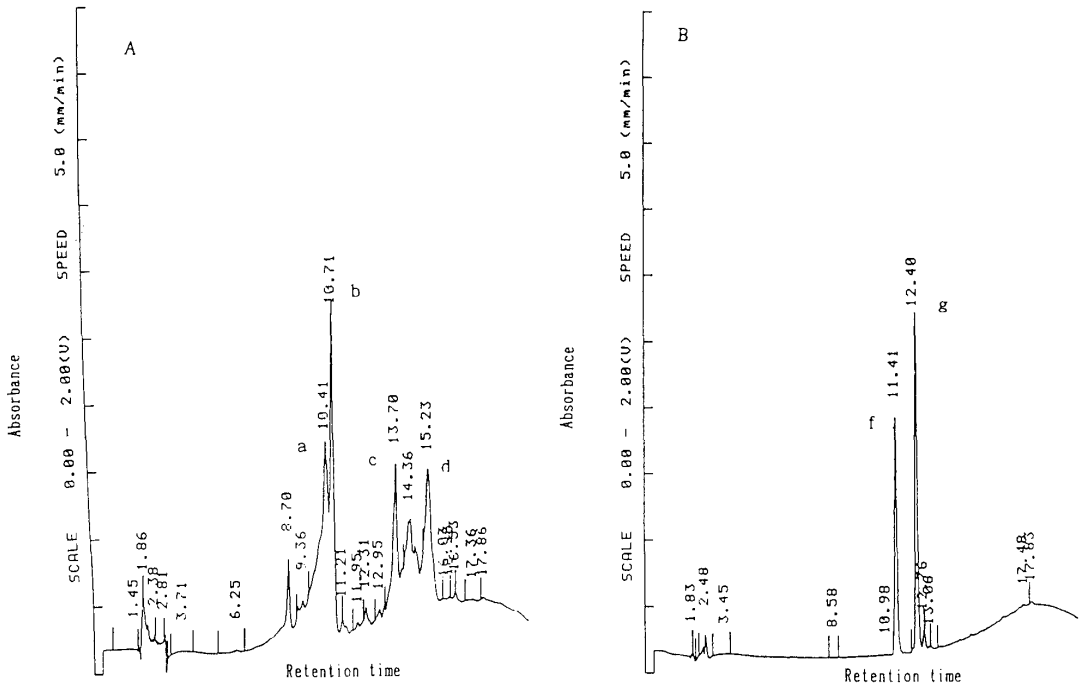

Fig. 4. Separation of allelopathic and authentic substances by HPLC.

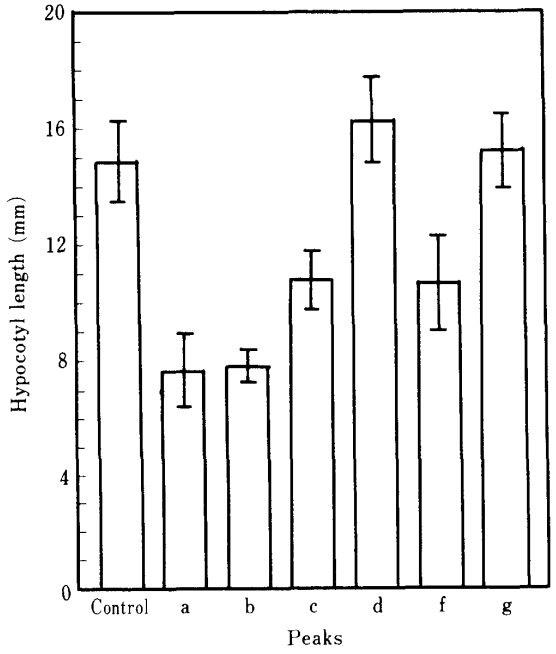

Fig. 5. Hypocotyl length of radish in HPLC fractions.

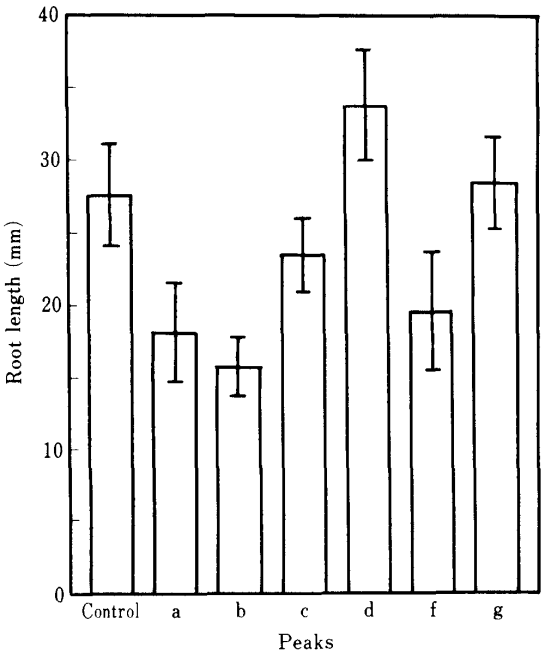

Fig. 6. Root length of radish in HPLC fractions.

did not observe the elongation of hypocotyl and root of alfalfa seedlings in mixed solution of $3.3\times10^{-3}+1.7\times10^{-3}$ M (Tab. 5). On the other hand, hypocotyl and root of radish seedlings were significantly inhibited in mixed solution at $6.7\times10^{-4}+3.3\times10^{-4}$ M of ferulic and salicylic acids as shown in Tab. 6. It was supposed that when ferulic and salicylic acids were used to treat the plants in a mixed solution, the degree of inhibition was greater than when each substance was used independently.

Guenzi et al (1964)[4] reported saponin as an allelochemical of alfalfa. In the present experiment, ferulic and salicylic acids were

identified from methanol extracts of alfalfa shoots. Ferulic acid has been identified from rye (*Secale cereale* L.), bracken fern (*Pteridium aquilinum* Kurn var. *latiusculum* Und.) and yellow nutsedge (*Cyperus esculentus* L.)[10)12)16)]. Chou and Patrick (1976)[2)] reported that the germination of lettuce (*Lactuca sativa* L.) was inhibited by ferulic and salicylic acids. Ferulic acid is considered to be an allelochemical of these plants. Salicylic acid is also considered an allelochemical of rye (*Secale cereale* L.) and maize (*Zea mays* L.)[2)].

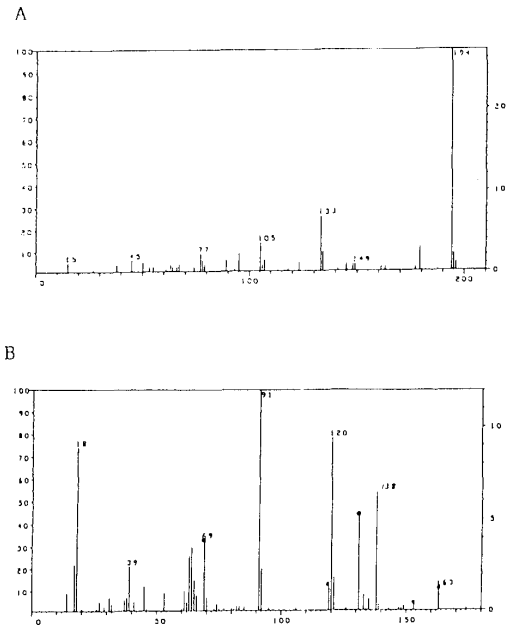

Fig. 7. Mass spectra : (A) peak b from HPLC, (B) peak g from HPLC.

Among plant types containing allelochemicals, there are some types which are allelopathic to the same plants directly adjacent to them. These cause injury by continuous cropping. For example, red clover showed significant inhibition when continuously cropped. Tamura et al.[13)14)] identified some allelopathic substances such as ononin, genistein, biochanin A, biochanin-7-glucoside and formononetin from red clover as chemical substances connected with injury by continuous cropping.

In conclusion, ferulic and salicylic acids isolated from methanol extracts of alfalfa shoots seem to be closely connected with injury by continuous cropping of alfalfa plants.

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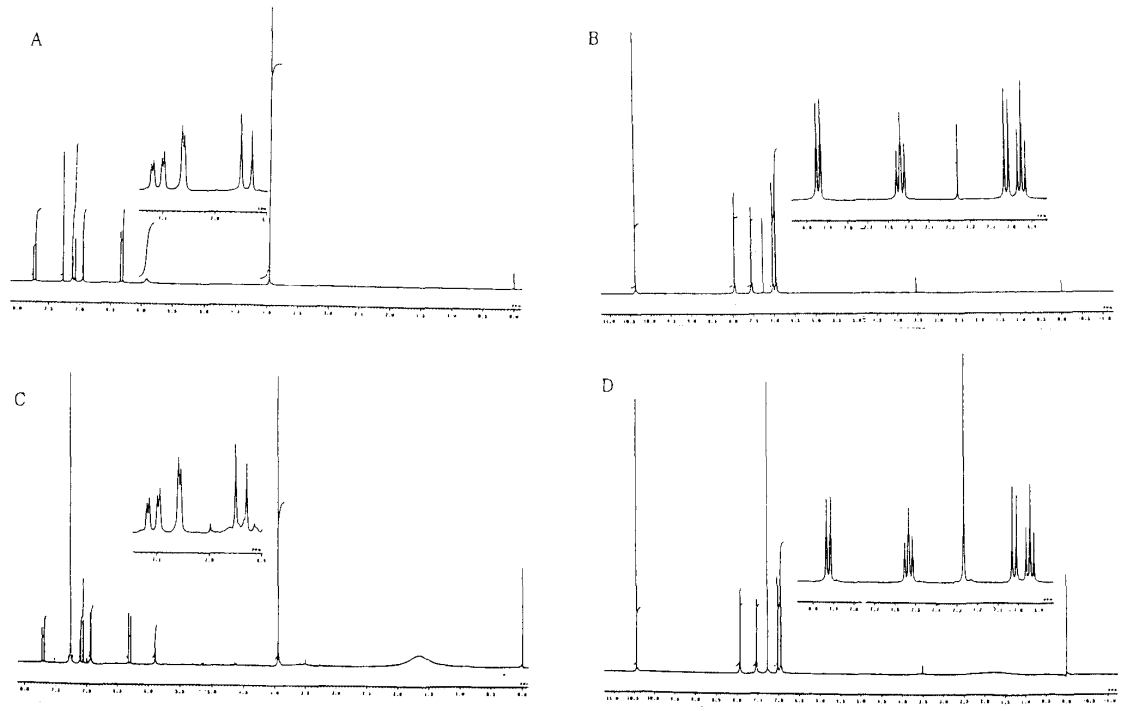

Fig. 8. NMR spectra : (A) ferulic acid, (B) salicylic acid, (C) peak b from HPLC, (D) peak g from HPLC.

Table 1. Effect of salicylic acid on the growth of radish.

Treatment	Hypocotyl length (mm)	Root length (mm)
control	12.5^{a}* ±0.52	18.4^{a} ±2.00
5×10^{-4}M	7.4^{b} ±2.06	8.8^{b} ±3.23
1×10^{-3}M	3.5^{c} ±0.09	1.8^{c} ±0.21
5×10^{-3}M	0^{c}	0^{c}
1×10^{-2}M	0^{c}	0^{c}

* The different letters indicate significant difference at the 5% level.

Table 2. Effect of ferulic acid on the growth of radish.

Treatment	Hypocotyl length (mm)	Root length (mm)
control	12.9^{a}* ±1.13	20.9^{a} ±2.30
5×10^{-4}M	7.5^{b} ±1.02	10.9^{b} ±1.80
1×10^{-3}M	6.9^{b} ±0.33	10.7^{b} ±0.94
5×10^{-3}M	6.5^{bc} ±1.29	7.7^{b} ±4.34
1×10^{-2}M	3.2^{c} ±0.50	1.4^{b} ±0.05

* The different letters indicate significant difference at the 5% level.

Table 3. Effect of ferulic acid on the growth of alfalfa.

Treatment	Hypocotyl length (mm)	Root length (mm)
control	11.4^{a}* ±0.45	18.0^{b} ±0.49
5×10^{-4}M	12.2^{a} ±0.47	22.3^{a} ±0.83
1×10^{-3}M	13.1^{a} ±0.74	23.3^{a} ±1.00
5×10^{-3}M	5.5^{b} ±0.19	2.1^{c} ±0.24
1×10^{-2}M	0^{c}	0^{c}

* The different letters indicate significant difference at the 5% level.

Table 4. Effect of salicylic acid on the growth of alfalfa.

Treatment	Hypocotyl length (mm)	Root length (mm)
control	8.8^{a}* ±0.12	23.5^{a} ±1.12
5×10^{-4}M	8.5^{a} ±0.19	21.7^{a} ±0.07
1×10^{-3}M	8.5^{a} ±0.89	19.2^{a} ±2.90
5×10^{-3}M	0^{b}	0^{b}
1×10^{-2}M	0^{b}	0^{b}

* The different letters indicate significant difference at the 5% level.

Table 5. Synergistic effect of ferulic and salicylic acids on the growth of alfalfa.

Treatment	Hypocotyl length (mm)	Root length (mm)
control	18.1^{ab}* ±0.238	37.1^{a} ±2.011
ferulic + salicylic acids $3.3\times10^{-4}+1.7\times10^{-4}$M	19.2^{ab} ±0.449	38.4^{a} ±2.209
ferulic + salicylic acids $6.7\times10^{-4}+3.3\times10^{-4}$M	20.0^{a} ±0.635	35.7^{a} ±1.916
ferulic + salicylic acids $1.3\times10^{-3}+6.7\times10^{-4}$M	19.8^{a} ±0.4000	37.3^{a} ±1.087
ferulic + salicylic acids $2.0\times10^{-3}+1.0\times10^{-3}$M	17.1^{b} ±0.581	17.1^{b} ±1.205
ferulic + salicylic acids $2.7\times10^{-3}+1.3\times10^{-3}$M	9.9^{c} ±0.635	3.1^{c} ±0.529
ferulic + salicylic acids $3.3\times10^{-3}+1.7\times10^{-3}$M	0^{d}	0^{c}

* The different letters indicate significant difference at the 5% level.

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Table 6. Synergistic effect of ferulic and salicylic acids on the growth of radish.

Treatment	Hypocotyl length (mm)	Root length (mm)
control	$41.8^{a*} \pm 0.944$	$34.0^{a} \pm 2.225$
ferulic + salicylic acids $3.3\times10^{-4}+1.7\times10^{-4}$M	$41.2^{ab} \pm 1.343$	$31.0^{a} \pm 1.920$
ferulic + salicylic acids $6.7\times10^{-4}+3.3\times10^{-4}$M	$35.7^{b} \pm 1.877$	$24.2^{b} \pm 1.831$
ferulic + salicylic acids $1.3\times10^{-3}+6.7\times10^{-4}$M	——**	——
ferulic + salicylic acids $2.0\times10^{-3}+1.0\times10^{-3}$M	——	——
ferulic + salicylic acids $2.7\times10^{-3}+1.3\times10^{-3}$M	——	——
ferulic + salicylic acids $3.3\times10^{-3}+1.7\times10^{-3}$M	$19.9^{c} \pm 0.951$	$7.7^{c} \pm 0.896$

* The different letters indicate significant difference at the 5% level.

** The treatment was not practiced because at $6.7\times10^{-4}+3.3\times10^{-4}$M the growth of radish seedlings was significantly inhibited.

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