Allelopathic Activity and Determination of Allelochemicals from Sunflower(*Helianthus annuus* L.) Root Exudates

II. Elucidation of Allelochemicals from Sunflower Root Exudates

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해바라기(*Helianthus annuus* L.) 根分泌物質의 他感作用 및 他感物質의 同定

II. 他感性 해바라기 根分泌物質의 同定

朴光鎬* · Keith Moody · 金純哲*** · 金吉雄******

ABSTRACT

Regardless of the test species, germination was adversely affected by the different concentrations of the acidic and neutral fractions of sunflower root exudates while the basic and aqueous fractions had no effect on germination. In both test species, root lengths were inhibited slightly more than shoot lengths. Significant reduction in fresh weights of the test species was observed when the test species were treated with the acidic and neutral fractions but not with the basic and aqueous fractions. Six compounds, hydroquinone, β-resorcyclic acid, vanillic acid, caffeic acid, salicylic acid, and quercetin, were characterized from the acidic fraction. Seven compounds, hydroquinone, gentisic acid, β-resorcyclic acid, vanillic acid, caffeic acid, ferulic acid, and quercetin, were elucidated from the neutral fraction.

Key words : germination, acidic and neutral fractions, sunflower root exudates, basic and aqueous fractions, root lengths, shoot lengths, fresh weights, hydroquinone, β-resorcyclic acid, vanillic acid, caffeic acid, salicylic acid, gentisic acid, ferulic acid, and quercetin.

INTRODUCTION

The allelopathic effects of numerous kinds of chemical compounds released from plants into the environment have been discussed by many investigators (Tukey, 1969 ; Harborne, 1977 ; Horsley, 1977 ; Rice, 1979). The ecological importance of these compounds in natural and manipulated ecosystems has attracted the attention of many scientists. The natural products that cause allelopathy are a subset of the array of secondary compounds synthesized by plants and microorganisms. Most of the currently identified compounds are products of the shikimic acid and acetate pathways(Rice, 1984). Those frequently found include a diverse group of phenolic compounds, including cinnamic and benzoic acids, coumarins, tannins, flavonoids, terpenoids, a few alkaloids, steroids, and quinones. Many that contribute to allelopathic interference have not been

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identified. It is extremely important to dispel the illusion that situations of allelopathic inhibition are caused by a single compound. Generally, interference arises from the combined action of a number of allelochemicals, and this impact is also influenced by other environmental stresses (Einhellig, 1987). Collection of allelopathic chemicals from the undisturbed plant root system is difficult because of their low concentrations and the high level of contaminants in growth media such as soil. A new approach for the continuous trapping of quantities of extracellular chemicals from donor plants is described (Tang and young, 1982). The objectives of these experiments were to isolate the active substances of sunflower root exudates responsible for allelopathic effects and attempt to characterize these allelochemicals.

MATERIALS AND METHODS

Experiment 1. Collection of Allelopathic Compounds from the Root Exudates of Sunflower and their Inhibitory on other species.

Collection of allelopathic Compounds

This trial was conducted using a modified continuous root exudate trapping system (Tang and young, 1982) (Fig. 1). Containers made from 1-gallon brown glass solvent bottles with the bottoms removed were wrapped with aluminum foil to prevent possible photoconversions and then heat-sterilized for 48 h at 100℃. The containers were filled with two layers of glass bead ; the first 50 mm layer had glass beads of 6 mm outer diameter and the next 50mm layer had glass beads of 3 mm outer diameter. These was covered with styrofoam to a depth of 18 mm.

Five sunflower seeds were planted in each container at the IRRI phytotron with the following conditions : temperature (day/night), 28/21℃ ; relative humidity, 70% ; light, natural, and irrigated with Hoagland's solution.

When the plants were 2 cm tall, the circulation attachments and columns containing XAD-4 resin were connected to the bottoms of the containers. The solution was circulated at a rate of 100 ml/day. Distilled water was added as needed. A control without sunfower was treated in the same manner.

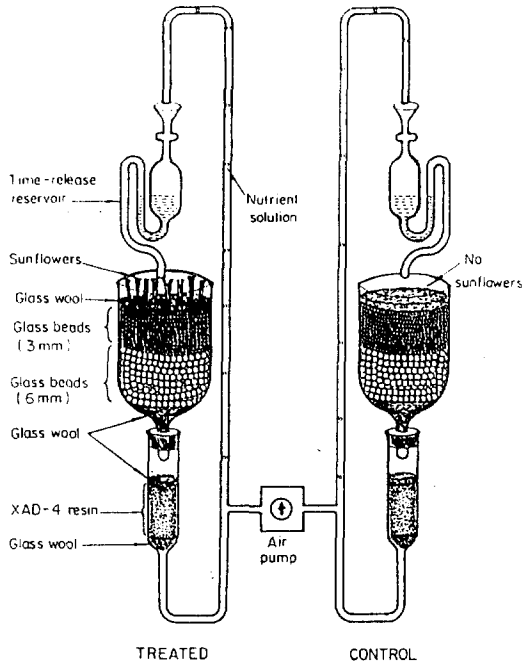

Fig. 1. Method for collection of allelopathic compounds from the root exudates of sunflower.

Because most of the ultraviolet portion of sunlight is removed by the glass in the phytotron, supplementary ultraviolet light was added for 6 h per day using Q-Panel 313 UV-B sunlamps with mylar film. The distance between the lamps and the containers was 100 cm.

Column preparation was the same as in Experiments 2 and 3 of the previous report. The plants were grown for 5 weeks. In order to increase the adsorptive efficiency of the resin, the column was changed every 3 days.

Extraction and bioassay of Allelopathic Compounds

The following procedures, based on Harboner (1973) and Yopp et al. (1986), were used for the extraction and biolassay of allelopathic compounds. *Extraction* (Fig. 2) The columns were detached every 3 days, washed with 2 L of distilled water, and then eluted with 200 ml methanol (MeOH). Elutes from the columns were pooled, and methanol was evaporated under reduced pressure at 60℃. The concentrate was diluted with water to 50 ml (pH 5.5-6.0) and extracted three times in a separatory

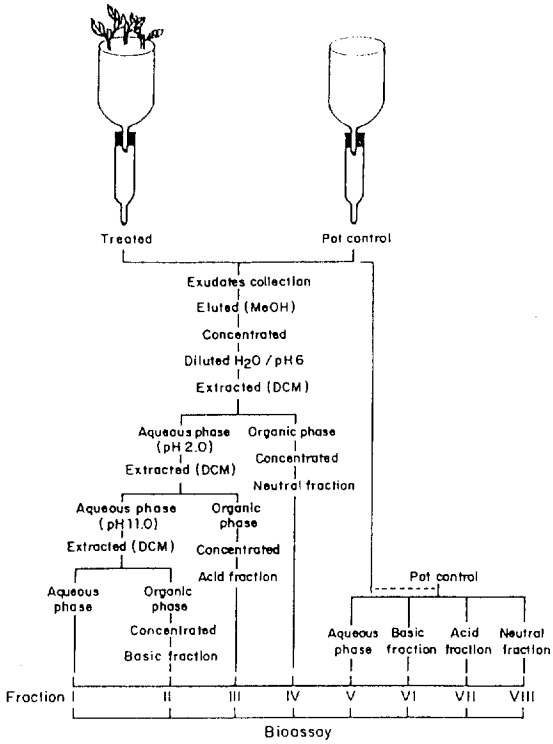

Fig. 2. Method used for fractionation of root exudates of sunflower.

funnel with 100 ml methylenedichloride(DCM). The extracts were combined, dried over anhydrous magnesium sulfate and concentrated to 20 ml *in vacuo*. Final concentration to 3 ml was carried out using a nitrogen jet evaporator(The MEYER). This concentrated material represented as the neutral fraction.

The remaining aqueous fraction was acidified to pH 2.0 with 1 N HCl and again extracted with DCM to give the acidic fraction. The acidified aqueous fraction was then adjusted to pH 11.0 with 1N sodium hydroxide and extracted with DCM to give the basic fraction. The acidic and basic fractions were concentracted to final volumes of 3 ml.

Bioassay Aliquots(10, 30, and 50μl)of the neutral, acidic, basic, and aqueous fractions were added to coarse filter papers(46 cm×57 cm, Edirol, England) which were cut to fit 5.3ml sample vials. The solvent was evaporated and 0.2 ml of distilled water was added to the filter paper.

Ten radish(*Raphanus sativus* L.) seeds and twenty *Echinochloa colona* (L.)Link seeds were placed in

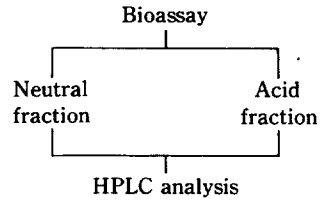

Fig. 3. HPLC identification of allelopathic compounds from root exudates.

each sample vial and the vials were placed inside a incubator in the light at 30℃. After 72 h, the percentage germination, lengths of shoots and roots, fresh weights of shoots and roots, and total fresh weight were determined.

Experiment 2. identification of Allelopathic Compounds from the root Exudates of Sunflower

Liquid chromatographic separations of neutral and acid fractions(Fig. 3) were performed on a Waters(Model RCM-100) Assoc. (Milford, Mass., U.S.A) HPLC equipped with a LC spectrophotometer Lambda-Max Model 481 operationg at 254 nm, two pumps(Model 501 and 510), automated gradient controller, and Nova-Pak C_{18} column

Two solvents, methanol(A) and acetic acid-water (5 : 95%, v/v)(B), were used. The elution profile of the linear gradient was : 0-25 min, 15-40% A : 25-30 min, 40% A ; 30-45 min, 40-63% A ; 45-47 min, 63% A ; 47-51 min, 63-99% A. The flow-rate was 2 ml/min and the column pressure was 70.3g/cm^2

In order to identify the neutral and acid fractions, reference compounds, p-hydroxybenzoic, salicylic, α-resorcylic, vanillic, p-coumaric, ferulic, caffeic, β-resorcylic, gentisic acids, vanillin, umbelliferone, quercetin, orcinol, coumarin, and hydroquinone, were purchased from commercial suppliers and used in the study. The injected volume of the standard solutions and each fraction was 10μl.

RESULTS AND DISCUSSION

Experiment 1. Collection of Allelopathic compounds from Root Exudates of Sunflower and their Inhibitory Effect on other species

Germination

E. colona : The acidic and neutral fractions signif-

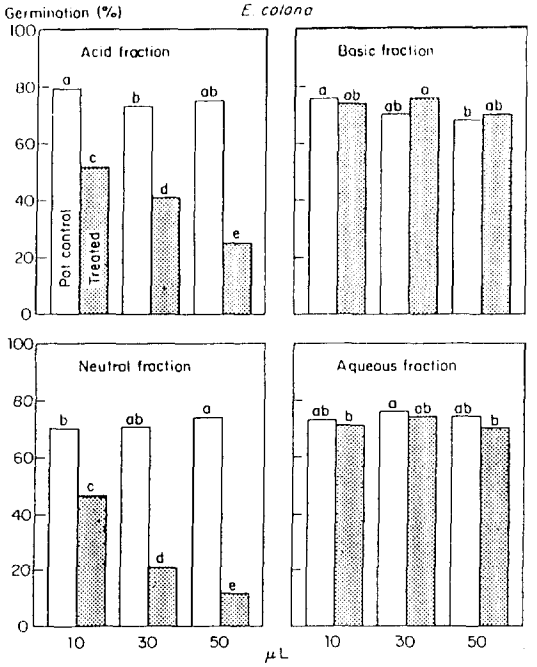

Fig. 4. Effect of different fractions from the control and from root exudates of sunflower on germination of *Echinochloa colona*. (Within each fraction, means followed by the same letter are not significantly different by DMRT at the 5% level).

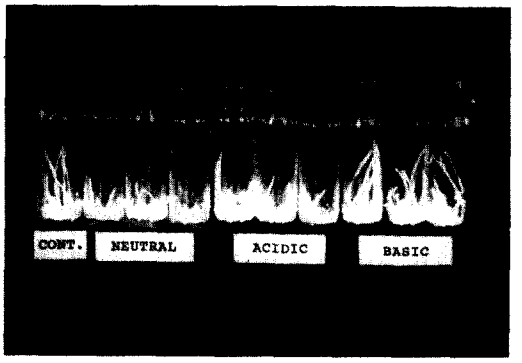

Fig. 5. Growth inhibition of *Echinochloa colona* by 50μl dose of sunflower root exudates.

icantly inhibited *E. colona* germination (Fig. 4 and 5) but there were no inhibitory effects of the basic and aqueous fractions. As the concentration increased, there was more inhibition in the acidic and neutral fractions.

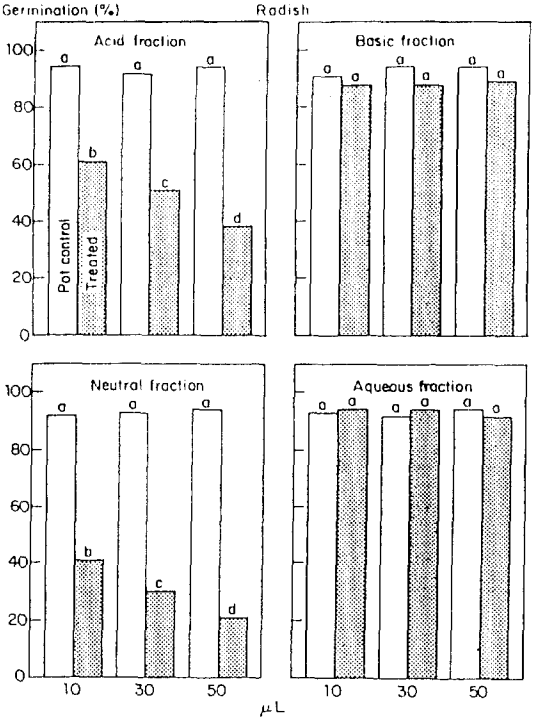

Fig. 6. Effect of different fractions from the control and from root exudates of sunflower on germination of radish. (Within each fraction, means followed by the same letter are not significantly different by DMRT at the 5% level).

Radish : Percent germination of radish was also significantly inhibited by the acidic and neutral fractions. With increasing concentration, there was more inhibition. The basic and aqueous fractions did not have any inhibitory effect on germination of radish (Fig. 6).

Germination was similarly affected in both test species (Fig. 4 and 6). The neutral fraction of sunflower root exudates was the most inhibitory to germination in *E. colona* and radish followed by the acidic fraction and inhibition intensified at increasing concentrations. There was no inhibition in basic and aqueous fractions.

Seedling growth

E. colona : Shoot and root lengths were significantly reduced at 10μl by the acidic and neutral fractions (Fig. 7) and inhibition increased with increasing concentration. The basic and aqueous

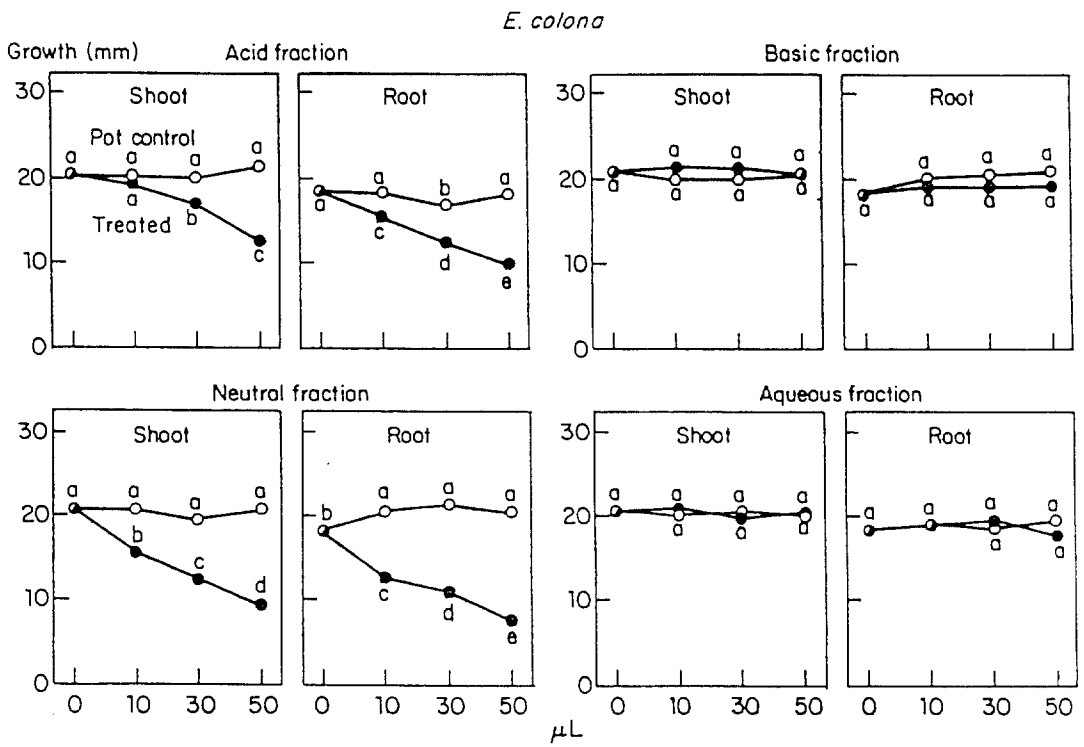

Fig. 7. Effect of different fractions from the control and from root exudates of sunflower on shoot and root growth of *Echinochloa colona*. (Within each parameter, means followed by the same letter are not significantly different by DMRT at the 5% level.)

fractions had no effect on shoot and root lengths. Compared to the inhibition of shoot lengths, that of root lengths was slightly more affected by both the acidic and neutral fractions (Fig. 7). Fresh weight of *E. colona* was similarly affected (Fig. 8 and 9). The acidic and neutral fraction are inhibitory to seedling growth of *E. colona* but the basic and aqueous fractions had no effect.

The neutral fraction was more inhibitory to germination, seedling growth and fresh weight of the test plants than the acidic fraction.

Radish : Regardless of concentration, shoot and root growth were greatly affected by the acidic and neutral fractions of sunflower root exudates (Fig. 10). However, there were no marked differences in shoot and root lengths in both the basic and the aqueous fractions. In the acidic fraction, root elongation was markedly inhibited compared to shoot elongation.

Fresh weight of radish was also significantly affected by the acidic and neutral fractions (Fig. 11). The neutral fraction inhibited fresh weight less than the acidic fraction (Fig. 11 and 12). The basic and aqueous fractions had no effect on the fresh weight of radish. Percent inhibition based on fresh weight of radish was similar in the acidic and the neutral fractions unlike *E. colona* (Fig. 12). There was a slight inhibition in fresh wight at 30 and 50μl in the aqueous fraction.

Regardless of the test species, germination was adversely affected by the different concentrations of the acidic and neutral fractions of sunflower root exudates (Fig. 4 and 6) while the basic and aqueous fractions had no effect on germination. In both test species, root lengths were inhibited slightly more than shoot lengths. This may be attributed to the fact that the emerging root probably absorbed more of the inhibitor than the shoot. Similar findings were reported by Bachie (1988) for sweet potato, rice, and cucumber and Mercardo-Nories (1989) for *E.*

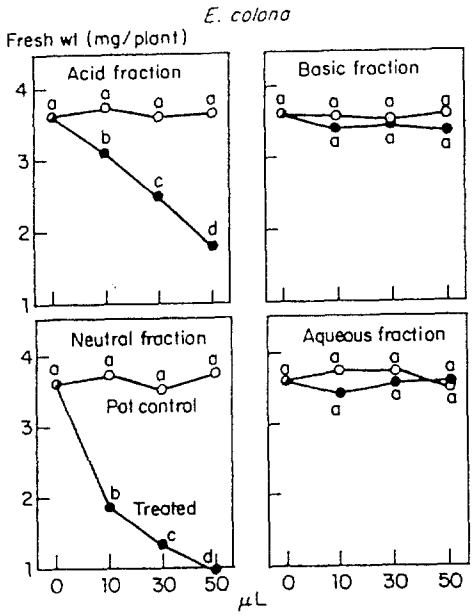

Fig. 8. Effect of different fraction from the control and from root exudates of sunflower on fresh weight of *Echinochloa colona*. (Within each fraction, means followed by the same letter are not significantly different by DMRT at the 5% level.)

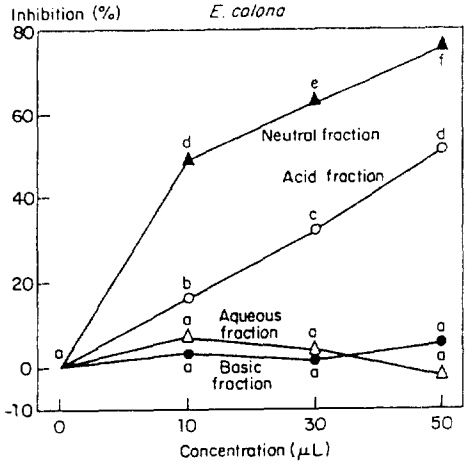

Fig. 9. Percent inhibition of *Echinochloa colona* seedling growth by different fractions of sunflower root exudates. (Within a concentration level, means followed by the same letter are not significantly different by DMRT at the 5% level.)

colona.

Significant reduction in fresh weights of the test species was observed (Fig. 8 and 11) when the test

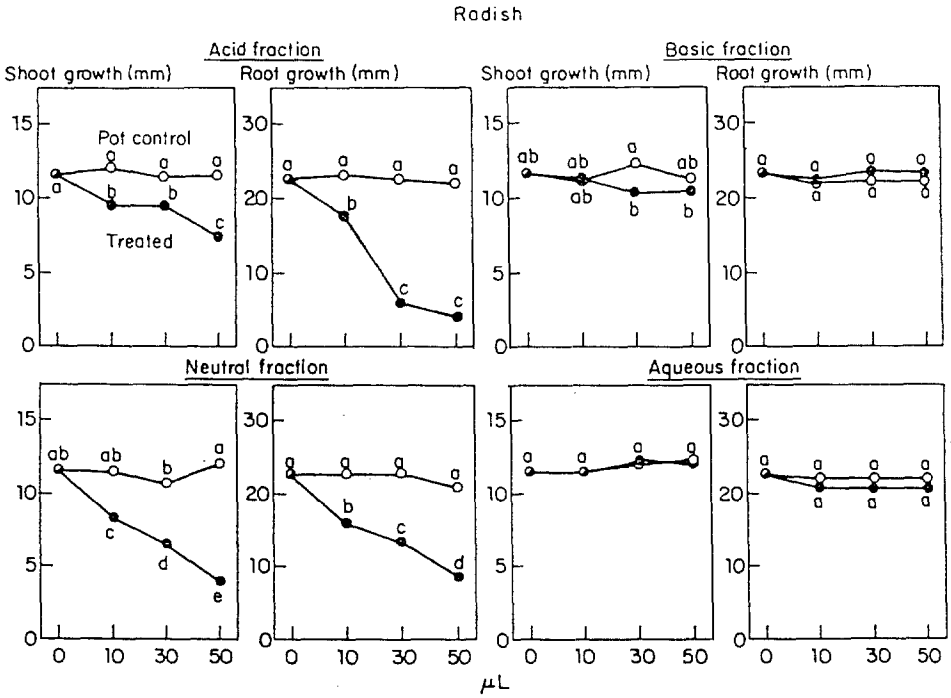

Fig. 10. Effect of different fractions from the control and from root exudates of sunflower on shoot and root growth of radish. (Within each shoot and root growth, means followed by the same letter are not significantly different by DMRT at the 5% level.)

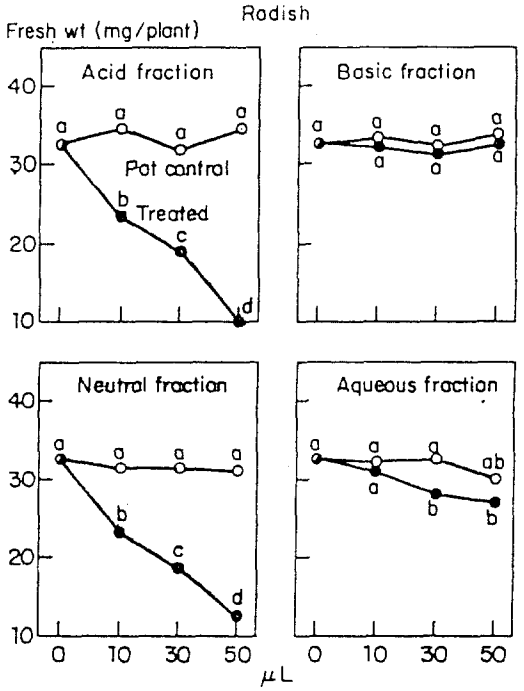

Fig. 11. Effect of different fractions from the control and from root exudates of sunflower on fresh weight of radish. (Within each fraction, means followed by the same letter are not significantly different by DMRT at the 5% level.)

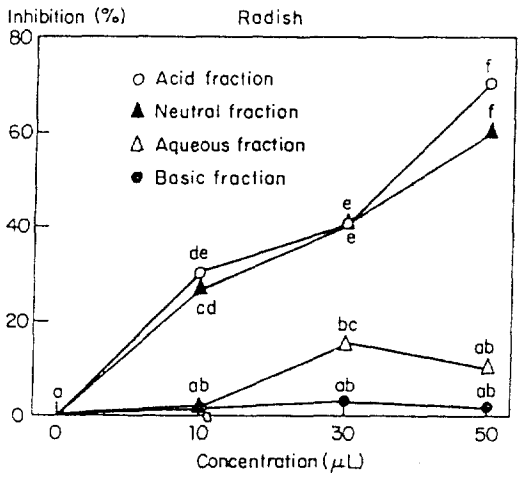

Fig. 12. Percent inhibition of radish seedling growth by different fractions of sunflower root exudates. (Within a concentration level, means followed by the same letter not significantly different by DMRT at the 5% level.)

species were treated with the acidic and neutral fractions but not with the basic and aqueous fractions. These results are in agreement with those of Tang and Young (1982) who observed toxicities in both the neutral and acidic fractions, but not in the basic fraction of the elute of *Hemarthria altissima* (Poir.) Stapf.

Results from the seed bioassays of the test species (Fig. 4 to 12) indicated that the inhibitory substances were effectively trapped by the XAD-4 resin column. Also, sunflower root exudates contain allelochemicals which may be inhibitory to test plants.

Experiment 2. Identification of Alloelopathic Compounds from the Root Exudates of sunflower

The biologically active acid and neutral fractions from Experiment 1 were concentrated *in vacuo* and analyzed by HPLC with a linear gradient system. The HPLC chromatogram of 15 phenolic compounds eluted according to a linear gradient is shown in Fig. 13. A list of the compounds identified, according to their different retention time values, is given in Table 1.

The HPLC showed that the acidic fraction contained more than 30 compounds (Fig. 14) ; among them, six compounds ; Hydroquinone, β-resorcyclic acid, vanillic acid, caffeic acid, salicylic acid, and quercetin, were resolved well enough for characterization, based on the comparison of thier retention times with those of the respective standard compounds. Fig 15 shows the HPLC chromatogram of the neutral fraction from sunflower root exudates. The neutral fraction from the root exudates of sunflower had more than 35 compounds (Fig. 15) ; among them, seven compounds ; Hydroquinone, gentisic acid, β-resorcyclic acid, vanillic acid, caffeic acid, ferulic acid and quercetin, were characterized.

Alsaadawi (1988) indicated that aqueous extracts and residues of roots and shoots of *H. annuus* significantly reduced the nitrification rate in soil. When added to soil, *H. annuus* debris from plants grown under various nutrient stresses in the greenhouse and field had a significant negative effect on *Amaranthus retroflexus* seedling dry weight production (hall et

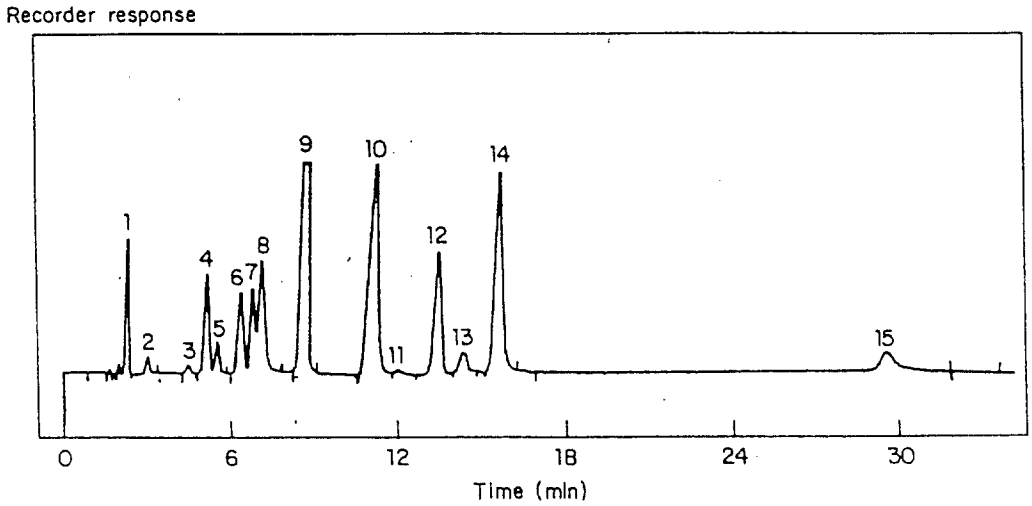

Fig. 13. Chromatogram of the HPLC of phenolic compounds eluted using a linear gradient of a Nove-Pak C_{18} column.

Table 1. Retention times of phenolic compounds eluted using the linear gradient on a Nova-Pak C^{18} column.[a]

PEAK NO	RETENTION TIME(tR) (min)	PHENOLIC COMPOUNDS
1	2.26	Hydroquinone
2	2.91	α-Resorcyclic acid
3	4.44	Gentisic acid
4	4.95	Orcinol
5	5.31	p-Hydroxybenzoic acid
6	6.16	β-Resorcyclic acid
7	6.71	Vanillic acid
8	7.24	Caffeic acid
9	8.37	Vanillin
10	10.92	Umbelliferon
11	11.79	p-Coumaric acid
12	13.49	Ferulicacid
13	13.97	Salicylic acid
14	15.28	Coumarin
15	29.18	Quercetin

[a] Values are averages of six runs.

al., 1983). It was possible to partially simulate inhibition of *A. retroflexus* seedling dry weight production when chlorogenic acid alone was added to the soil instead of *Helianthus* debris.

Annual sunflower, *H. annuus*, has been shown to have marked allelopathic effects as an early component of old-field succession. This species releases ecologically effective toxins from the leaves as leachate and from the roots as exudate. The toxic constituents include chlorogenic and isochlorogenic acid, scopolin, and an α-naphthol derivative(Wilson and Rice, 1968). The inhibition of *Amaranthus* growth by *Helianthus* debris and chlorogenic acid was not evident when nutrient solution was applied to the soil.

With increased UV intensity(Koeppe et al., 1969), an increase in scopolin concentration was found in sunflower leaves. Chlorogenic acid concentration of sunflower leaves was less under low UV radiation than in comparable sunflower plants of control or of higher UV treatments. The differences in results may be explained by differences in weed ecotypes, indicator species, stage and part of weed used, the type of herbicidal compound extracted, the

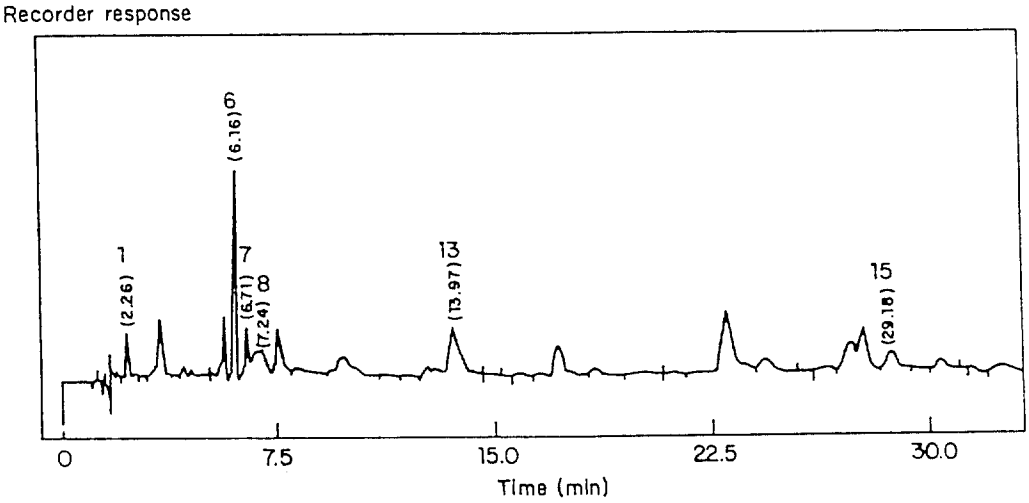

Fig. 14. Chromatogram of the HPLC of the acidic fraction eluted using a linear gradient on a Nova-Pak C_{18} column.

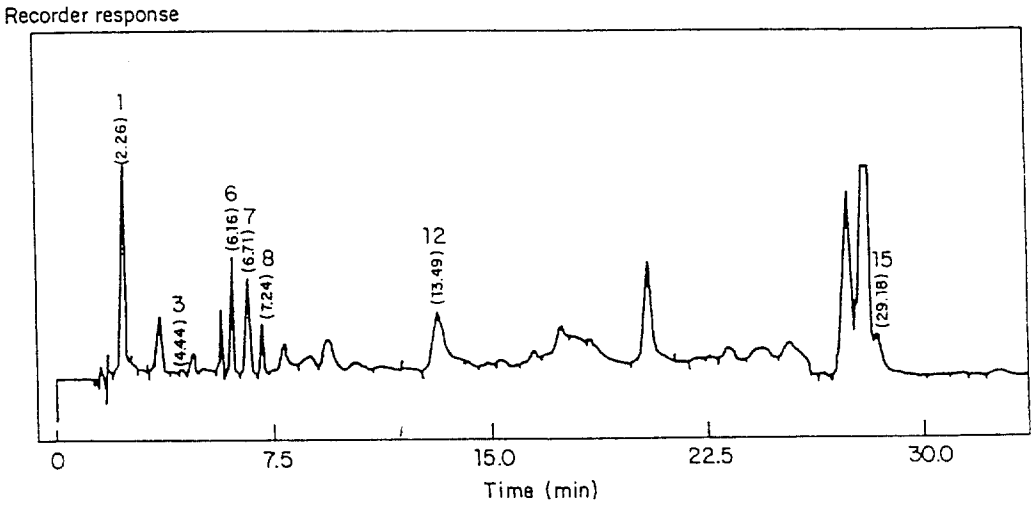

Fig. 15. Chromatogram of the HPLC of the neutral fraction eluted using a linear gradient on a Nova-Pak C_{18} column.

method of extraction and environmental factors (Mercardo-Noriel, 1989). The possibility that the inhibitory effect of the extracts is due to their high osmotic concentration was considered.

摘 要

해바라기의 相對抑制作用(他感作用, Allelopathy) 및 天然除草劑로서의 開發可能性에 관한 試驗을 1988-1991年에 걸쳐 國際米作硏究所 農學, 生化學部 및 國立 필리핀 大學校 化學科 天然物 硏究室에서 遂行하여 얻은 結果中 相對抑制作用性 해바라기 根分泌物質의 生物檢定 및 相對抑制作用物質(Allelochemicals)의 HPLC 分析結果는 다음과 같다.

1. 해바라기 根分泌物質의 酸 및 中性分劃物質을 生物檢定材料로 사용한 피(*Echinochloa colona* (L.) Link 및 무(*Raphanus sativus* L.) 의 發芽가 有意性있는 抑制를 보인 반면 알칼리 및 水溶性分劃에서는 抑制作用이 나타

나지 않았다.

2. 피의 경우 해바라기 根分泌物質의 分劃物質 10μl 濃度에서부터 有意的인 生長抑制가 認定되었으며 地上部 伸張抑制보다 地下部 伸張抑制가 酸 및 中性分劃에서 더 강하게 나타났다.
3. 무의 경우 濃度에 관계없이 地上 및 地下部 伸張抑制자 酸 및 中性分劃에서 각각 높게 나타났다.
4. 生物檢定에서 강한 相對抑制作用을 보인 酸性分劃物質의 HPLC分析結果 hydroquinone, β-resorcyclic acid, vanillic acid, caffeic acid, salicylic acid, quercetin 등의 物質이 確認되었다. 한편, 中性分劃物質에서는 Hydroquinone, gentisic acid, β-resorcyclic acid, vanillic acid, caffeic acid, ferulic acid, quercetin 등이 각각 同定되었다.

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