Isolation and Characterization of Allelopathic Substances from Sorghum Stem

Kim, S.Y.*, S.K. De Datta,** R.P. Robles,*** K.U. Kim****
S.C. Lee,**** and D.H. Shin****

수수 줄기에 含有된 他感物質의 分離 및 特性 究明

金相列*·에스 케이 디다타**·알 피이 로블레스***·金吉雄****·李相哲****·申東賢****

ABSTRACT

To better understand the exact nature of the major toxic compound responsible for phytotoxicity of sorghum stem, the most toxic compound from the stem extract was isolated by rapid chromatography and subsequently purified by thin-layer chromatography(TLC) and high pressure liquid chromatography(HPLC). Of the eight fractions isolated by rapid chromatography, the fraction with solvent combinations of butanol (8) : acetic acid (1) : water (1) had the highest toxicity. Further separation of the fraction by TLC in a solvent mixture of butanol (24) : acetic acid (16.4) : water (7) : propanol (1) showed that the spot with an R_f 0.71 had one major peak with retention time of 20.40 minutes. Upon subjecting gas chromatography and the HPLC fraction to the mass spectrometry, the toxic compound is probably one of the four compounds ; 1-methyl-1-(2-propynyl)-hydrazine, 1-aziridineethanol, 5-chloro-2-pentanone, and 2-(methylseleno)-ethanamine.

Key words : allelochemical, sorghum, purification

INTRODUCTION

In an earlier paper, the allelophatic effect of sorghum stem on *Echinochloa colona* and radish was reported[8]. The toxic compound extracted from stem was partially characterized[9].

A number of attempts were made to identify the specific chemicals responsible for allelopathic effects observed for sorghum. Althogh the compounds were not identified, a number of important properties of the compound were deduced. Much of the inhibition obtained has been attributed to the content of phenolic acids[6], although many compounds were implicated as allelopathic agents. However, despite the characterization, isolation and identification of these inhibitory compounds present

* 農業遺傳工學研究所 (Agricultural Biotechnology Institute, RDA, Suweon, 441-701, Korea)
** Crop and Soil Environmental Sciences, Virginia Polytechnic Institute and State University, Blacksburg, USA.
*** College of Agriculture, University of the Philippines at Los Banos, College, Laguna 4031, Philippines.
**** 慶北大學校 農科大學(Collelge of Agriculture, Kyungpook National University, Taegue 702-701, Korea)

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in sorghum plants[4,10] little attention has been given to the exact nature of the major toxic compound responsible for the phytotoxicity.

More chemical isolation and identification work are necessary to confirm most of the observations and to determine the characteristics of the inhibitor. From the isolated compounds, it should also be possible to synthesize related compounds that can produce similar desirable effects.

Therefore, the objective of the study was to isolate and characterize the most phytotoxic compound responsible for phytotoxicty in sorghum stem.

MATERAIALS AND METHODS

Isolaton and Separation of Allelopathic Substances

Since the stem extract showed the greatest inhibitory effect in previous experiments[8,9], the stem extract obtained was freeze-dried. Before doing the rapid column chromatography, the best solvent combinations for the separation of stem extract were obtained through a series of trials using solvents with polarity increasing from nonpolar to more polar. One gram of sample was placed on top of the rapid chromatography column filled with silica gel. The sample was eluted with 200ml of different solvent systems of butanol, acetic acid and water, fraction 1(100 : 0 : 2), 2(90 : 5 : 5), 3(80 : 10 : 10), 4 (70 : 15 : 15), 5(60 : 20 : 20), 6(50 : 25 : 25), 7(40 : 30 : 30), and 8(30 : 35 : 35). Eluents were concentrated through the rotary evaporator and, finally with nitrogen gas to 3ml for the biological activity test. Aliquots(30, 50, 70μl) of each fraction were added to 5.3ml vials with one line of filter paper. The solvent was evaporated and 200μl of distilled water was added to the filter paper. Germination test of *E. colona* and radish were then conducted in same manner as in previous reports[8,9].

Purification of the Allelopathic Substance

The fraction showing the greatest allelopathic activity was further purified by flash flow column chromatography and TLC. A column (30mm in diameter) was filled with 6 inches of dry silica gel 60 [Merk. 7734, particle size 0.0063-0.2000mm(70-230 mesh ASTM)]. The sample was loaded at the top of the flash flow column and eluted with solvent mixtures of butanol(24) : acetic acid(16.4) : water(7) : propanol(1). The eluents collected from column chromatogrphy was run through TLC (Sigma, TLC glass plates, Silica gel Rp-18, 1mm layer thickness, and Merk, TLC plastic sheet, Silica gel 60, 0.2mm layer thickness) and all fractions that show the same R_f were combined. The plates were developed in butanol(24) : acetic acid(16.4) : water(7) : propanol(1). The compound developed was detected by exposing the developed plates to short(254nm) and long(366nm) UV light, iodine and sulfuric acid with vanillin. The spots were scraped off the plate and all scrapings were eluted in methanol and the eluents were filtered and dried. The HPLC was used to purify the compound and to collect enough material for the bioassay and instrumental analysis.

Fifty and 100μl of the individual compounds were then bioassayed for inhibition of germination in *E. colona*. Ten seeds *E. colona* were placed in vials as described in the preceding section. After 5 days, percent germination, lengths of shoots and roots, and percent growth inhibition were determined.

The purified compound was analyzed by HPLC and GC-MS with authentic standards. Separation by HPLC was done by using a reverse phase C_{18} Nova-Pak column. For the

mass spectrometry analysis, E1 direct probe was used.

Statistical Design and Analysis of Data

Experiments were laid out in a completely randomized design with four replications. Data were analyzed using single or two-factor analysis. Treatment means were compared using Least Significant Differences(LSD).

RESULTS AND DISCUSSION

Isolation and Separation of Allelopathic Substances

E. colona germination. At 30μl, germination in *E. colona* was not affected regardless of fractions(Fig. 1). But as the amount of allelopathic substance increased, germination was significantly decreased. At 50μl, fractions 1, 2, and 3 had an inhibitory effect. At 70μl, fractions 2 and 3 had greater inhibitory effect than the other fractions. Of the two fractions, fraction 3 had more inhibitory effect. It inhibited germination 49%, whereas fraction 2 gave only 25% inhibition at 70μl. The other fractions caused slightly inhibited *E. colona* germination.

Radish germination. The germination behavior of radish was similar to that of *E. colona*. At 30μl, there was no reduction in germination (Fig. 1). At 50 and 70μl, fractions 2, 3, and 4 led to significant inhibition. Fraction 3 completely inhibited seed germination of radish. This indicates that fraction 3 contains the most toxic allelopathic compounds.

Germination in both test species showed similar effects. Fraction 3 was more inhibitory to germination of *E. colona* and radish than were the other fraction.

E. colona seedling growth. Shoot and root lengths in *E. colona* were significantly affected by fractions 2, 3, and 4(Figs. 2 and 3). Of the three fractions, fraction 3 had the greatest inhibitory effect although it was not significant(Fig. 4). Generally, root growth was more affected than shoot growth.

Radish seedling growth. There was no seedling growth with fraction 3 at 70μl, but the

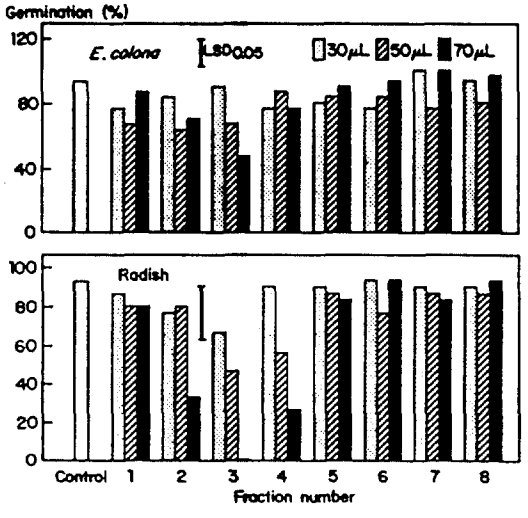

Fig. 1. Percent germination in E. colona and radish as affected by different fractions (1-8) of sorghum stem extracts.

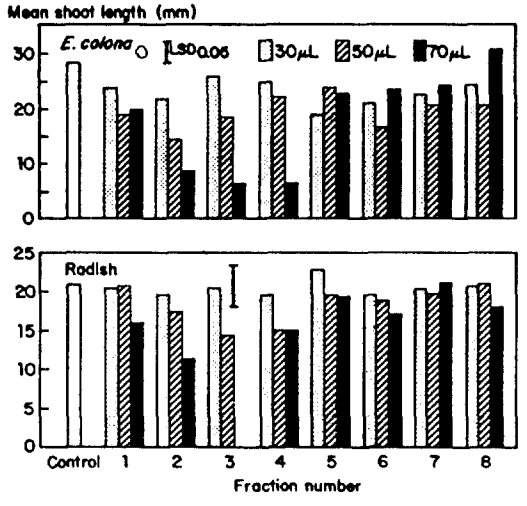

Fig. 2. Shoot elongation in *E. colona* and radish as affected by different fractions(1-8) of sorghum stem extracts.

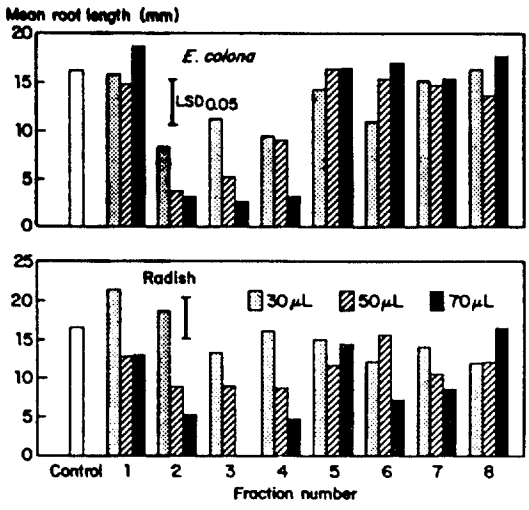

Fig. 3. Root elongation in *E. colona* and radish as affected by different fractions (1-8) of sorghum stem extracts.

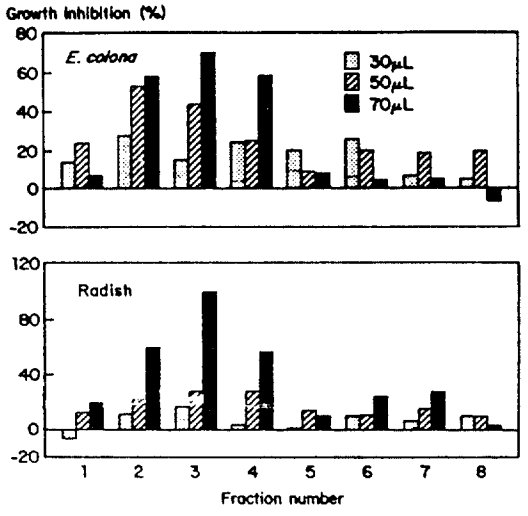

Fig. 4. Percent growth inhibition in *E. colona* and radish as affected by different fractions (1-8) of sorghum stem extracts.

other fractions did not cause significant inhibition (Figs. 2 and 3). Fractions 2 and 4 inhibited seedling growth, but their effect was lower than that of fraction 3 (Fig. 4).

Purification of the Allelopathic Substance

Germination. The extract of fraction 3 yielded four compounds when subjected to TLC separation. When individual spots were removed and bioassayed for *E. colona* germination, only two spots significantly inhibited germination. Germination was not significantly inhibited at 50μl of the Rf 0.39 and 0.71 fractions, but there was significant inhibition at 100μl (Table 1). Of the two inhibitory compounds, the compound with R_f 0.71 was the more inhibitory. It reduced *E. colona* germination by 83%, whereas the compound with R_f 0.39 inhibited germination by 68%. Germination was not affected by the R_f 0.47 and R_f 0.55 fractions, regardless of concentration.

Table 1. Seedling growth in *E. colona* as affected by the phytotoxic compounds isolated form Fraction 3[a].

Rf value	Volume (μl)	Germination (%)	Mean length (mm)	
			Shoot	Root
Control		87.5	17.6	87.5
0.39	50	80.0	22.8	80.0
	100	27.5	2.2	27.5
0.47	50	80.0	8.8	80.0
	100	75.0	10.0	75.0
0.55	50	75.0	12.1	75.0
	100	75.0	6.2	76.0
0.71	50	72.5	5.6	72.5
	100	15.0	0.5	15.0
LSD 0.05		19.8	5.6	26.7

[a] butanol : acetic acid : water (8 : 1 : 1, v/v/v)

The substance at R_f 0.71 was found to be most inhibitory. Differences in concentration of the various components of the fractions may have been partially responsible for the greater inhibition of the test solution containing the compound at R_f 0.71.

Several other scientists have reported that a variety of water soluble substances that can inhibit seed germination and seedling growth are released from different plant species[2,3,5,7,11]. Guenzi and McCalla[6] detected a number of phytotoxic phenolic acids in hydrolysate of sorghum residues, including ferulic, p-coumaric, syringic, vanillic, and p-hydroxybenzoic acids. Chlorogenic acid, p-coumaric acid, and p-hydorxybenzoaldehyde were the principal inhibitors isolated from the herbage and rhizomes of *S. halepense*[1].

Seedling growth. Unlike percent germination, seedling growth in *E. colona* was greatly reduced when 50μl of the R_f 0.71 fraction was used (Table 1). At 100μl, the greatest inhibition was observed in the compound with R_f 0.71. There was slight inhibition of seedling growth with the R_f 0.47 and 0.55 fractions. The greater inhibitory action on root growth than on shoot growth in these chromatographically isolated phytotoxins is consistent with experiments involving crude extracts.

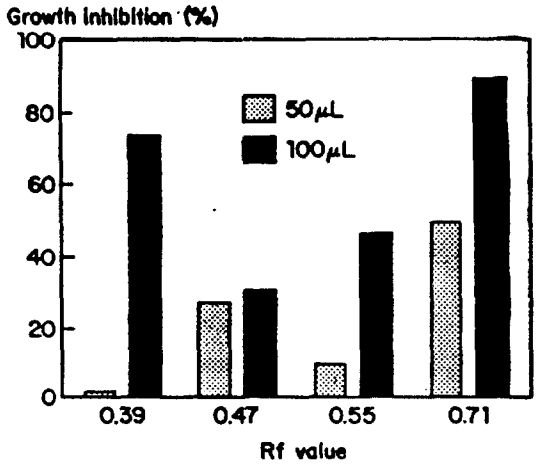

Fig. 5. Percent growth inhibition in *E. colona* by the phytotoxins isolated from fraction 3a.

Based on growth parameters, 89% inhibition was observed at 100μl of the R_f 0.71 fraction, while there was 74% inhibition by the R_f 0.39 fraction at 100μl (Fig. 5).

Characterization of a Toxic Compound

The compound at R_f 0.71 isolated on TLC that had the greatest inhibitory effect of *E. colona* germination was further purified by HPLC. The HPLC chromatogram of the compound, given in Fig. 6, shows the one peak with retention time of 20.40 min, indicating

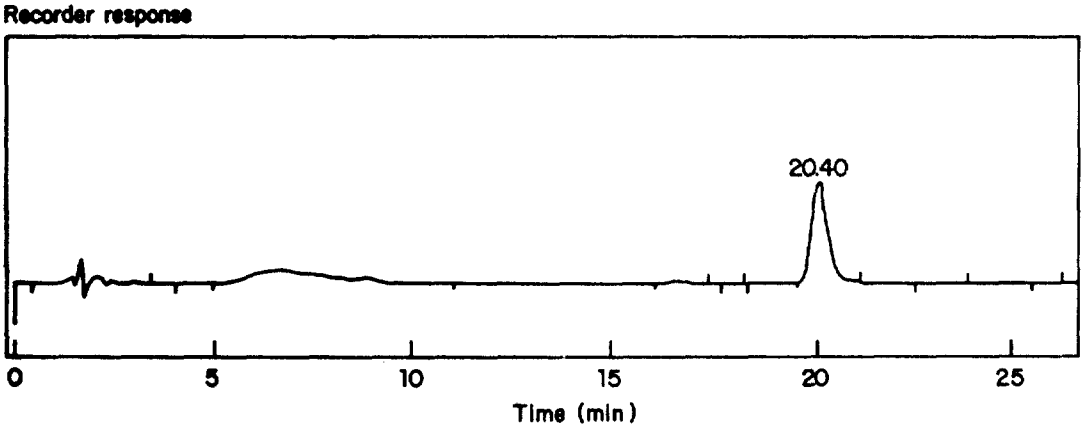

Fig. 6. HPLC chromatogram of the most toxic compound (Rf 0.71) purified with HPLC.

that the peak may have only one compound. The compound did not have a retention time similar to that of any known phenolic compound.

The purified toxic compound was sujected to mass spectrometry. The suggested possible compounds are 1-methyl-1-(2-propynyl)-hydrazine, 1-aziridineethanol, 5-chloro-2-pentanone and 2-(methylseleno)-ethanamine. One of the four compounds may be the toxic compound responsible for the allelopathic activity in the sorghum stem. Since the sample was not enough, IR and NMR analysis were not conducted. Futher analysis is needed to explain the exact nature of the major toxic compound responsible for the allelopathic activity of sorghum stem.

摘 要

수수 줄기에 함유된 他感物質 중 가장 강한 활성을 나타내는 물질을 rapid chromatography, flash flow column chromatography로 분리하고 thin layer chromatography와 HPLC로 정제하여 GC-MS로 분석하였다.

Butanol, acetic acid 및 water의 용매 조합을 달리하여 타감물질을 분리한 결과 butanol (8) : actic acid(1) : water(1) 분획에서 억제효과가 가장 크게 나타났으며, flash flow column chronatography와 TLC로 분리한 결과 가장 활성을 나타내는 물질은 Rf 0.71에서 나타났으며 HPLC로 순수분리한 결과 Rt 20.40min에서 elution되었다. 이 물질은 주황색을 띄며 methanol에 용해성이 있었다.

정제된 물질을 GC-MS로 분석한 결과 예상되는 물질은 1-methyl-1-(2-propinonyl)-hydrazine, 1-aziridineethanol, 5-chloro-2-pentanone, 2-(methylseleno)-ethanamine 중 한 물질일 것으로 추측되었다.

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