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# PHYTOTOXIC SUBSTANCES IN TWELVE SUBTROPICAL GRASSES I. ADDITIONAL EVIDENCES OF PHYTOTOXICITY IN THE AQUEOUS FRACTIONS OF GRASS EXTRACTS[1,2]

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**Abstract**

Additional phytotoxicity was found in the aqueous fraction of aqueous extracts in twelve subtropical grasses. The degree of phytotoxicity varied with species and the extraction techniques. At 10 milliosmols of osmotic concentration, the aqueous fraction after ether extraction of aqueous grass extracts exhibited phytotoxicity ranging from 38% to 84%. Among them *Plicatulum paspalum* revealed the highest phytotoxicity, being 84% and 78% in the fraction of $A_2$ and $A_3$, respectively, while *Acroceras macrum*, *Eragrostis curvula*, *Panicum maximum*, *Setaria sphacelata*, and *Tripsacum laxum* exhibited above 60% inhibition. The chromatographic bioassay results of ninhydrin positive spots found in the $E_3$ fraction of grass extracts, *Acrocera macrum*, *Brachiaria mutica*, *Chloris gayana*, *Digitaria decumbens*, *Panicum maximum*, *Plicatulum paspalum* and *Setaria sphacelata* exhibited above 78% inhibition. The rechromatographic bioassay results of that revealed 40% inhibition, except species of *Chloris gayana*, and *Plicatulum paspalum*. Furthermore, in the $E_3$ fraction of *Acrocera macrum*, four significant toxic spots were found.

## Introduction

It has been reported that many grasses produced metabolites which are toxic to the growth of other plants (Chou and Chung, 1974; Chou and Young, 1975; Rice, 1964, 1968, 1971, 1972; Rasmussen and Rice, 1971; Rice and Pancholy, 1973; Tannin and Muller, 1972; Tames *et al.*, 1973). Chou and Young (1975) identified six phytotoxins, *p*-coumaric, *p*-hydroxybenzoic, *o*-hydroxyphenylacetic, syringic, vanillic, and ferulic acids, and two unknowns present in the ether fraction of aqueous extract of twelve subtropical grasses. However, it was thought that some phytotoxic substances could be present in the aqueous

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fraction of the extract, and which fraction was much more important than the ether fraction under natural conditions. The aqueous fraction is present in the natural plant leachate which is the key agent in determining the process of a dominant species (Tukey, 1971; Muller and Chou, 1972; Whittaker, 1970; Muller, 1974; Rice, 1974). Thus, it is the purpose of this study to elucidate the phytotoxic nature present in the aqueous fraction of the aqueous extract of twelve subtropical grasses.

## Materials and Methods

### *Materials*

Twelve subtropical grasses were collected from the Taiwan Sugar Corporation Farm Animal Breeding Station at Chunan, Taiwan. The grasses were *Acroceras macrum*, *Andropogon nodosum*, *Brachiaria mutica*, *Chloris gayana*, *Cortaderia selloana*, *Cynodon dactylon*, *Digitaria decumbens*, *Eragrostis curvula*, *Panicum maximum*, *Plicatulum paspalum*, *Setaria sphacelata*, and *Tripsacum laxum*. Several of these species have been introduced for forage. Leaves of grasses were harvested at near mature about 60–100 cm tall. The leaves were brought back to the laboratory and air dried at room temperature.

### *Extract preparation*

The procedures of extracting aqueous extracts of grasses were described by Chou and Young (1975), in which the leaves—water ratio was 1 to 10. After the ether fraction was removed from the aqueous extract, the non-ether soluble fraction was reserved for the following studies and designated as "aqueous fraction" throughout this study. Two subsequent extracts obtained from this aqueous fraction were given as follows. The aqueous fraction (non-ether soluble fraction of the original aqueous extract) was designated as "fraction $A_2$". An aliquot of fraction $A_2$ was allowed to evaporate at room temperature (22–28°C) in hood to get rid of ether residue compeletely. This part was later diluted to 10 milliosmols and reserved for sponge bioassay. Another aliquot part of fraction $A_2$ was evaporated to dry at 40°C, and then re-extracted with ethanol. This extraction made two fractions of extract; one part was ethanol soluble, which was further concentrated to small volume for paper chromatography, designated as "fraction $E_2$"; another part was non-ethanol soluble fraction designated as "fraction $A_3$". The fraction $A_3$ was also dried in a freezer to get rid of ethanol residue. An aliquot part of fraction $A_3$ was reserved and diluted to 10 milliosmols for sponge bioassay, while the rest of aliquot part was added with 5 drops of 2N HCl to acidify the material and further extracted with ethanol. The final ethanol extract

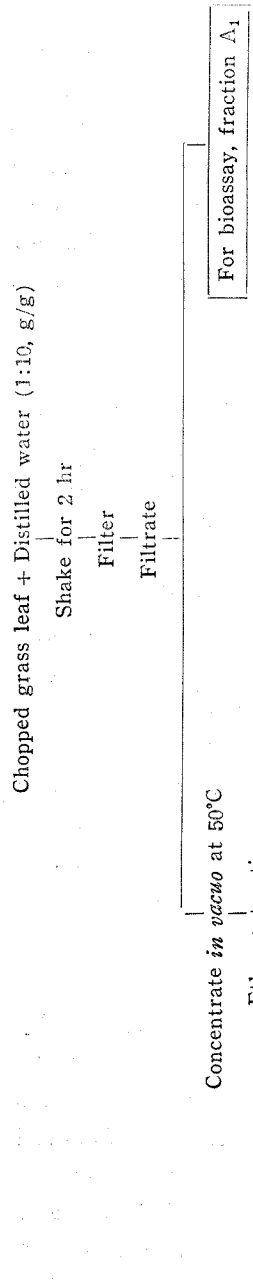


**Fig. 1. A general scheme for extracting phytotoxic substances in grass leaves.**

was used for chromatography and called as "fraction $E_3$". The whole extraction procedures are described in a scheme of Fig. 1.

*Paper chromatography*

To isolate the responsible phytotoxic substances in the aforementioned fractions, paper chromatography was employed. The isolating techniques for phytotoxin from paper chromatogram were described by Chou and Young (1975). One dimentional chromatography was conducted by using paper strip (2 cm × 57 cm) of Whatman 3 MM chromatographic paper. The paper were developed with three solvent systems: (1) 2% glacial acetic acid, (2) BuAW: butanol:acetic acid: water (4:1:5, v/v/v), and (3) phenol saturated with water.

*Bioassay techniques*

Two bioassays were employed to elucidate toxic properties in the aforementioned fractions of extract. The aqueous fractions of $A_1$, $A_2$, and $A_3$ were bioassayed by means of sponge bioassay, a modification of Muller (1966). On the other hand, to locate a toxic spot on chromatogram, the chromatographic bioassay as described by McPherson, Chou and Muller (1971) was used. Thus, the fractions, $E_1$, $E_2$, and $E_3$ of the extract were determined for the responsible phytotoxic spots. The phytotoxicities were revealed by bioassaying relevent chromatographic spots or Rf segments of a whole chromatogram. In some cases of this study, phytotoxic spot was emphasizing on the spot sensitive to ninhydrin spray reagent. The ninhydrin positive spot was eluted from chromatogram, and then rechromatographed with a solvent of 2% acetic acid or BuAW. The bioassay setting was incubated at 25°C for 72 hr, and the results were taken by measuring the length of lettuce radicle in millimeter.

*Identification of toxic spots*

The isolated toxic spots on chromatogram were rechromatographed by using three developing solvents as described above. After developing the spots were detected under short wave U. V. light and the chromatograms sprayed with three reagents: (1) DPNA: diazotized *p*-nitroaniline followed by 10% sodium carbonate (Hais and Macek, 1963), (2) DQC: 2, 6-dichloroquinone chlorimide followed by saturated sodium borate (Vázquez *et al.*, 1968), and (3) 0.3% ethanolic ninhydrin. The first two spray reagents are used to detect phenolic compounds, while the third one is used to detect nitrogen cantaining compounds. Since most of toxic phenolics in grass extracts were identified, the present study has been concentrated on the ninhydrin positive compounds.

## Results

*Phytotoxicity of aqueous extracts of grasses*

The aqueous fraction of the extracts (fraction $A_2$ and $A_3$, see Fig. 1) were bioassayed against lettuce growth, using sponge bioassay techniques. The bioassay results showed that three fractions of extract exhibited tremendous inhibition on the radicle growth of lettuce even the osmotic concentration of the solution was as low as 10 milliosmols (Table 1). The original aqueous extracts revealed phytotoxicity above 46%; some extracts exhibited inhibition even higher than 70% including species of *Acroceras macrum*, *Eragrostis curvula*, *Panicum maximum*, *Plicatulum paspalum*, and *Tripsacum laxum*. After ether extraction of the aqueous extract, the aqueous fractions, fraction $A_2$ and $A_3$, also revealed phytotoxicity higher than 38%, and some revealed 84% inhibition. Among 12 grasses studied, *Plicatulum paspalum* exhibited the highest inhibition in the three fractions of extracts mentioned. The analysis of variance of the bioassay results showed that the phytotoxicity revealed

**Table 1.** *Relative phytotoxicity exhibited by various fractions of aqueous extracts of 12 grasses*

The extracts were made from 1:10 ratio of leaves and distilled water, and diluted to 10 milliosmols.

| Species | % Phytotoxicity | | |
|---|---|---|---|
| | Orginal aqueous extract | Aqueous fraction after ether extraction | |
| | | Fraction $A_2$(1) | Fraction $A_3$ |
| *Acroceras macrum* | 72 | 69 | 66 |
| *Andropogon nodosum* | 46 | 40 | 60 |
| *Brachiaria mutica* | 54 | 45 | 51 |
| *Chloris gayana* | 55 | 51 | 59 |
| *Cortaderia selloana* | 56 | 48 | 53 |
| *Cynodon dactylon* | 58 | 38 | 44 |
| *Digitaria decumbens* | 52 | 56 | 56 |
| *Eragrostis curvula* | 76 | 76 | 68 |
| *Panicum maximum* | 70 | 61 | 65 |
| *Plicatulum paspalum* | 84 | 84 | 79 |
| *Setaria sphacelata* | 69 | 66 | 60 |
| *Tripsacum laxum* | 72 | 66 | 63 |

Analysis of variance (ANOVA)

$F_{\text{between species}}$ $5\%=2.26$, $1\%=3.18$ $F_{\text{between fractions}}$ $5\%=3.44$, $1\%=5.72$

(1) The description of $A_2$ and $A_3$ was given in Fig. 1.

from grass extracts was significantly different between species at 1% level, and was significantly different between each fractions at 5% level.

Furthermore, the aqueous fractions, $A_2$ and $A_3$ were bioassayed repeatly; however, in this bioassay the sample amount was only the half of the bioassay earlier, and the solution used in this bioassay was also diluted to 10 milliosmols. Although the results (Table 2) of bioassay were not fully agreed with the results in Table 1, the phytotoxicity yielded from *Plicatulum paspalum* was still very high among fraction $A_2$. Additionally, the phytotoxicity was sighificantly lower in fraction $A_3$ than fraction $A_2$. Nevertheless, it is evident that phytotoxicity is present in the aqueous fraction of grass extract, which is not extractable by organic solvent as ether.

*Chromatographic bioassay of ninhydrin positive spots*

About 2.5% of the final ethanol solution of fractions $E_2$ and $E_3$ was chromatographed, using a developing solvent as mentioned earlier. The corresponding Rf segment of ninhydrin positive spot was cut out for bioassay against lettuce. It was found that the ninhydrin positive spot exhibited strong phytotoxic effect on the radicle growth of lettuce (Table 3a). The results showed that the phytotoxicity in the $E_2$ fraction was significantly higher than

**Table 2.** *The repeat bioassay results of relative phytotoxicity of aqueous fraction of aqueous extracts of 12 subtropical grasses*(1)

| Species | % Phytotoxicity(2) | |
|---|---|---|
| | Fraction $A_2$ | Fraction $A_3$ |
| *Acroceras macrum* | 89 | 44 |
| *Andropogon nodosum* | 69 | 24 |
| *Brachiaria mutica* | 79 | 33 |
| *Chloris gayana* | 71 | 25 |
| *Cortaderia selloana* | 42 | 24 |
| *Cynodon dactylon* | 61 | 56 |
| *Digitaria decumbens* | 50 | 57 |
| *Eragrostis curvula* | 74 | 48 |
| *Panicum maximum* | 57 | 54 |
| *Plicatulum paspalum* | 86 | 44 |
| *Setaria sphacelata* | 69 | 36 |
| *Tripsacum laxum* | 61 | 42 |

(1) This bioassay was the replication of that in Table 1, but the amount of sample for bioassay was reduced to the half of the original one.

(2) The results of statistical analyses showed that the phytotoxicity was significant different between the fractions at 1% level of confidence, but were insignificant among species.

**Table 3a.** *Relative phytotoxicity exhibited by ninhydrin positive spot on chromatogram of three organic fractions*

Fraction $E_1$: the original fraction, Fraction $E_2$: alcoholic extract of $E_2$ being rewashed with acidic alcohol.(1)

| Species | % Phytotoxicity(2) | | |
|---|---|---|---|
| | Fraction $E_1$ | Fraction $E_2$ | Fraction $E_3$ |
| *Acroceras macrum* | 100 | 75 | 86 |
| *Andropogon nodosum* | 100 | 66 | 70 |
| *Brachiaria mutica* | 100 | 66 | 79 |
| *Chloris gayana* | 100 | 64 | 79 |
| *Cortaderia selloana* | 100 | 23 | 79 |
| *Cynodon dactylon* | 100 | 71 | 69 |
| *Digitaria decumbens* | 100 | 56 | 91 |
| *Eragrostis curvula* | 100 | 66 | 66 |
| *Panicum maximum* | 100 | 47 | 91 |
| *Plicatulum paspalum* | 100 | 61 | 91 |
| *Setaria sphacelata* | 100 | 51 | 78 |
| *Tripsacum laxum* | 100 | 50 | 56 |

(1) About 2.5% (50 μl/2000 μl) of samples was spotted on paper strip developed with 2% acetic acid. The ninhydrin spot (Rf=0.78-0.90) was cut out for bioassay.

(2) Only significant different was found between fractions, being significant at 1% level, ($F=14.012^{**}$).

that in $E_3$ fraction. In another experiment, the solution of $E_2$ and $E_3$ fraction was chromatographed with a solvent of BuAW firstly, and then the ninhydrin spots were eluted and rechromatographed with 2% acetic acid as a solvent. Again, the ninhydrin positive spots were cut out for bioassay by using chromatographic bioassay techniques. The results of bioassay against lettuce growth are given in Table 3b, which shows that the phytotoxicity is present in all listed species in the $E_2$ fraction; the phytotoxicity being ranged between 52–65%. On the other hand, the phytotoxicity was lost in the $E_3$ fraction of several species, such as *Andropogon nodosum*, *Chloris gayana*, *Eragrostis curvula*, and *Plicatulum paspalum*.

Since most phytotoxic substances present in the aforementioned grasses are almost the same, our forcus, thus, has been concentrated on isolation of toxin from *Acroceras macrum*. The ethanol solution of $E_2$ and $E_3$ fractions was chromatographed separately, using the paper strip and developed with BuAW as a solvent The ninhydrin positive spots were generally located at Rf 0.3–0.5 zone of chromatogram, which was cut out for elution. The eluate was then rechromatographed on paper strip with 2% acetic acid. The chromatogram was bioassayed again. The results shown in Table 4 indicated

**Table 3b.** *Relative phytotoxicity exhibited by rechromatographic bioassay of ninhydrin positive spots of two aqueous fractions ($E_2$ and $E_3$) described earlier*

The ninhydrin positive spots were firstly developed with BuAW, then the chromatogram was eluted and rechromatographed with 2% acetic acid.

| Species | % Phytotoxicity[1] | |
|---|---|---|
| | Fraction $E_2$ | Fraction $E_3$ |
| *Acroceras macrum* | 55 | 70 |
| *Andropogon nodosum* | 61 | −40[2] |
| *Brachiaria mutica* | 65 | 40 |
| *Chloris gayana* | 60 | −6 |
| *Cortaderia selloana* | 56 | 33 |
| *Cynodon dactylon* | 62 | 25 |
| *Digitaria decumbens* | 58 | 58 |
| *Eragrostis curvula* | 65 | 1 |
| *Panicum maximum* | 52 | 66 |
| *Plicatulum paspalum* | 58 | 1 |
| *Setaria sphacelata* | 63 | 64 |
| *Tripsacum laxum* | 62 | 61 |

(1) Only significant difference was found between fractions, being significant at 5% level, ($F=6.91^*$).

(2) The negative values of phytotoxicity expressed stimulatory effect.

that one significant toxic spot (Rf 0.11–0.22) was found in the $E_2$ fraction and 4 toxic spots were found in the $E_3$ fraction. It is evident that some toxic substances are present in the aqueous fraction of grass extracts.

## Discussion

Biochemical interactions among plants mediated by detrimental substances have been intensively studied in many woody and shrub vegetations. However, not much information concerning allelopathic influence between grasses was obtained, although several detailed studies have been reported (Rusmessen and Rice, 1971; Rice, 1974; Tinnin and Muller, 1972; Chou and Young, 1975). In these studies the responsible phytotoxic substances present in grasses are almost the same, and can be grouped into phenolic compounds, which are slight water-soluble in nature. In the previous study of 12 subtropical grasses, Chou and Young (1975) found 6 phytotoxic phenolics in the ether fraction of aqueous extract of grass. On the other hand, several phytotoxins are found in the aqueous fraction of aquous extracts in this report. These unidentified toxic compounds are ninhydrin positive and highly polar, which seems more ecologically significant.

**Table 4.** *Relative phytotoxicity of eluates in 2 aqueous fractions of* ***Acroceras macrum***

The solution of $E_2$ and $E_3$ were chromatographed with 2% acetic acid, and than the ninhydrin positive spots were eluted and rechromatographed with BuAW. The chromatogram in segment was bioassayed against lettuce growth, and the phytotoxicity was expressed as % phytotoxicity of distilled water control.

| Rf segment | % Phytotoxicity | |
|---|---|---|
| | Fraction $E_2$ | Fraction $E_3$ |
| 0.11-0.22 | 61(2) | 65(2) |
| 0.22-0.29 | 10 | 43(2) |
| 0.29-0.35 | 2 | 13 |
| 0.35-0.41 | 1 | 1 |
| 0.41-0.47 | — | 6 |
| 0.47-0.55 | 4 | 23(1) |
| 0.55-0.60 | — | 14 |
| 0.61-0.71 | 9 | 40(2) |

$F_{betweens\ egments}$ 5%=3.79, 1%=7.00 $F_{between\ fraction}$ 5%=5.59, 1%=12.25

(1), (2): significant difference at 5% and 1% level, respectively.

As it is well known, the water soluble compounds, which perform allelopathic function, are metabolic waste and accumulated in vacuole, intercelluar space and finally exudated to leaf surface (Tukey, 1971; Muller, 1966; Chou and Muller, 1972). The metabolically active compounds released to the environment are not only phenolics but can be many other group of compounds, such as amino acid, nitrogen containing compound, alkaloid, and terpenoids. It is not surprising that the present unidentified ninhydrin positive compounds could be one of them.

In another aspect of plant interaction, Black *et al.* (1969) indicated that the biochemical basis of plant competition is a predominant factor among many grasses. Many subtropical forages belong to $C_4$ type plants, which are photosynthetically efficient. In this study, grasses of the genus *Andropogon*, *Chloris*, *Cynodon*, *Digitria*, *Eragrostis*, *Panicum* and *Setaria* are of $C_4$-type plant, and these plants also produced phytotoxic substances in a great amount. Nevertheless, the real mechanism, either competition or allelopathy, to be responsible in determining the dominant process of a grass in the field has not yet been clear. Identification of the responsible phytotoxins and the complicated interaction of these compounds in relation to the biochemical competition of efficient plant need to be further investigated.

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# 十二種亞熱帶牧草中植物毒物質的研究

## I. 存在於水溶性牧草萃取液中之植物毒性的進一步證據

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在前一報告中，作者從十二種亞熱帶牧草中以乙醚萃得六種酚酸類的植物毒性物質。本研究進一步地從水溶性部份得到進一步的植物毒性物質。在生態學上，完全水溶性的植物毒物質較以有機溶劑萃得者更爲重要。十二種牧草中以 *Plicatulum paspalum* 之毒性最高，高達84%；另外 *Acroceras macrum, Eragrostis curvula, Panicum maximum, Setaria sphacelata,* 及 *Tripsacum laxum* 具60%毒性。將此水溶性部份再純化分析並從紙色層上淋出毒性斑點，並以淋出之斑點做生物分析得植物毒性高達78%者爲 *Acroceras macrum, Brachiaria mutica, Chloris gayana, Digitaria decumbens Panicum maximum, Plicatulum paspalum* 及 *Setaria sphacelata.* 其中在 *Acroceras macrum* 中發現4個有 ninhydrin 正反應的植物毒物質。