Chlorophyll Fluorescence Applied in the Analysis on Vertical Movement of Herbicides in Soil

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Five photosynthesis inhibiting herbicides were compared for their mobility in soil columns used as the penetration model. The vertical movement of these compounds in soil was analyzed by a newly developed technique in which herbicide distribution was determined by the intensity of chlorophyll fluorescence in *Chlorella* cells suspended in aqueous extracts of soil samples from various depths. With the aid of a fluorescence microplate scanner, measurement of chlorophyll fluorescence was performed with great efficiency. Photosynthesis inhibiting activity of tested herbicides was determined beforehand by the same method and served in calculating herbicide concentrations in sample extracts. Among tested compounds, hexazinone showed the greatest mobility and diuron the smallest, suggesting water solubility is one of the factors which govern the soil mobility of herbicides.

Introduction

The availability of a herbicide at the target site determines how much of the potential phytotoxicity would develop into the actual herbicidal performance and therefore is of no less importance than its *in vitro* activity. In the process of herbicide molecules applied in the field to reach the target sites in plants operate many factors such as adsorption to soil, chemical and biological degradation, penetration through plant epidermis, and translocation in plant bodies. To study these complex aspects of herbicide behaviour, we must have a sensitive and efficient technique to detect herbicide molecules in various loci.

Fortunately, there is a sensitive indicator for photosynthesis inhibiting herbicides, namely chlorophyll fluorescence. This fluorescence, reemittance of once absorbed light energy by chloroplasts whose photosynthetic electron transport is interfered with [1], clearly reveals distribution and quantity of photosynthesis inhibitors in plant tissues and therefore has been utilized extensively in various areas of herbicide science [2–5]. We already have reported a technique to analyze behaviour of photosynthesis inhibiting herbicides in plant leaves by chlorophyll fluorescence imaging [6]. This report describes another application of chlorophyll fluorescence to analyse vertical movement of these herbicides in soil. In this scheme, a fluorescence microplate scanner was incorporated to facilitate measurement of chlorophyll fluorescence in *Chlorella* cells.

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Materials and Methods

Herbicides

All herbicides tested in the present study were of analytical grade (purity greater than 99%). Water solubility and 1-octanol-water partition coefficients (K_{ow}) for some compounds were quoted from "The Pesticide Manual, 9th edition" by the British Crop Protection Council [7].

Fluorometric measurement of photosynthesis inhibition

Photosynthesis inhibiting activity of herbicides was estimated from the increase of chlorophyll fluorescence. Stock culture of *Chlorella vulgaris* IAM C-30 was maintained in the modified Bristol medi-

um at 25 °C under a 16 h photoperiod of approximately 100 μE/m² and used as the plant material [8]. A 96-well plate containing 10^6 *Chlorella* cells/ml and herbicides at various concentrations was loaded into a fluorescence multi-well plate scanner (Millipore, CytoFluor 2300) in which the fluorescence was excited at 420 ± 20 nm and measured at 690 ± 15 nm. Before the measurement, the prepared plates were illuminated by a table light (*ca.* 150 μE/m²) for about 10 min so that the Kautsky effect would subside and the terminal state of chlorophyll fluorescence would establish. I_{50} for a herbicide was determined by probit analysis from photosynthesis inhibition rates calculated as

$$\text{photosynthesis inhibition (\%)} = 100 \times \frac{\text{observed FI} - \text{FI in untreated cells}}{\text{saturated FI} - \text{FI in untreated cells}}$$

(FI: fluorescence intensity).

The saturated fluorescence was represented by the fluorescence observed in cells treated with hexazinone at 2 μM or higher concentrations as explained later. In a preliminary experiment, the fluorescence intensity in hexazinone treatments required 2 h to reach the plateau, suggesting slow penetration of this herbicide with low lipophilicity into *Chlorella* cells. So, this preincubation time was kept in later experiments.

Evaluation of herbicides mobility in soil columns

Soil columns, stacks of 20 plastic rings measuring 16.5 cm in diameter and 1 cm in height, were filled with sandy loam (pH 6.6, organic matter content 1.93%) whose water content was adjusted to 50% of the water holding capacity (0.378 l/kg). Two such columns were prepared for each of 5 photosynthesis-inhibiting herbicides: diuron, tebuthiuron, thiazafluron, bromacil, and hexazinone. Aqueous suspensions of these herbicides at 0.27% were dripped uniformly onto the top surfaces of the soil columns giving the final application rate of 5 kg^{ai}/ha. After several rounds of artificial precipitation given intermittently in 5 h totaling up to 30 mm, the columns were stood still for one night to allow soil water movement. Then the columns were disassembled and the soil was sliced at every 1 cm. The whole amount of each soil layer was homogenized with a spatula and dried in a greenhouse. Five grams of air-dried soil samples was mixed thoroughly with 5 ml of deionized water and extracted for 2 h, then centrifuged at 1000 × *g* for 5 min. The supernatant was decanted, filtered through Toyo No. 1 filter paper, and stored at 5 °C. Herbicide content in each soil layer was estimated from the dilution of its aqueous extract required to inhibit photosynthesis by 50% as described in Results.

Results

pI_{50}(FL), a new index for photosynthesis inhibition

For all compounds tested, the intensity of chlorophyll fluorescence in *Chlorella* cells increased in proportion to the concentration (Fig. 1). In hexazinone treatments, fluorescence intensity almost reached its maximum at 1 μM and little increase was observed at higher concentrations. Since this fluorescence saturation suggests that the photosynthetic electron transport was almost completely blocked at these concentrations, the fluorescence intensity in *Chlorella* cells treated with hexazinone at 2 or 5 μM was used as the reference for 100% inhibition in the routine procedure. Fifty percent inhibition concentrations and its negative logarithms, pI_{50}, for 8 photosynthesis inhibiting herbicides were determined from respective dose-fluorescence curves (Table I). Among all, diuron showed the highest pI_{50} of 7.38 and fenuron the lowest. Thus obtained pI_{50} value was tentatively termed as pI_{50}(FL) in this article.

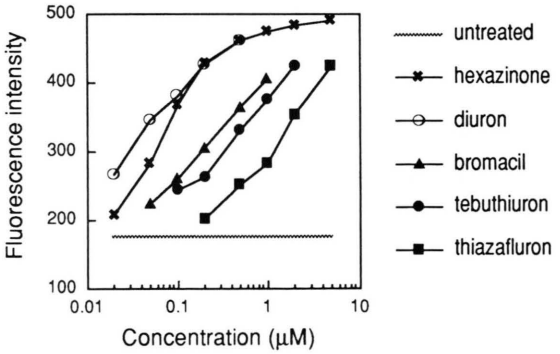

Fig. 1. The dose-fluorescence curves for hexazinone and other photosynthesis inhibiting herbicides. Intensity of chlorophyll fluorescence in *Chlorella* cells treated with various concentrations of herbicides was measured by a fluorescence microplate scanner in which the fluorescence was excited at 420 ± 20 nm and measured at 690 ± 15 nm.

Table I. pI_{50}(FL) and pI_{50}(PQ) for 8 photosynthesis inhibiting herbicides.

Herbicides	pI_{50}(FL)	pI_{50}(PQ)
Diuron	7.38	6.85
Hexazinone	7.03	6.54
Bromacil	6.70	6.48
Monuron	6.47	5.24
Tebuthiuron	6.42	6.07
Thiazafluron	5.96	5.92
Cycluron	5.75	4.90
Fenuron	5.35	4.36

To examine sensitivity and accuracy of the present method, these pI_{50}(FL) values were compared with pI_{50}(PQ) for respective compounds (Table I). The pI_{50}(PQ), defined as negative logarithm of molar concentration required to halve the electrolyte leakage from paraquat-treated cucumber cotyledon discs, is an index for photosynthesis inhibition based on the principle that photosynthesis inhibitors reduce the electron supply from the photosynthetic electron-transport system to paraquat molecules and consequently decrease destructive superoxide radical production in plant tissues (for detail, see ref. [9]). In this comparison, pI_{50}(FL) tends to be a little greater than pI_{50}(PQ), while a good correlation (R = 0.97) was found between two indices.

Determination of herbicides' concentrations in aqueous solutions

Concentration of a herbicide in an aqueous solution can be calculated from I_{50} for the compound and the dilution of the solution to inhibit photosynthesis by 50%, as

$$\text{concentration} = \frac{I_{50}}{\text{dilution for 50\% inhibition}}.$$

The quantitative accuracy of this method was examined using data obtained by gas-liquid chromatography as the reference, and a good correlation (R = 0.98) was found between data by both methods within a range from 0.5 to 50 ppm (data not shown). Herbicide concentrations in aqueous extracts of soil layers were determined by this method.

Vertical movement of photosynthesis inhibiting herbicides in soil columns

The vertical distribution of diuron, tebuthiuron, thiazafluron, bromacil, and hexazinone in soil columns was shown in Fig. 2. The herbicide content of soil layers was expressed not by absolute amount but by its percentage against the total amount recovered from the same column because our extraction procedure does not guarantee 100% recovery due to factors such as strong adsorption to soil. Results from 2 replicas were shown in the same chart.

The greatest mobility was found for hexazinone which penetrated down to 10 cm from the surface with its distribution peak around the depth of 4–7 cm. Bromacil, thiazafluron, and tebuthiuron proved less soil mobile but their distribution patterns are similar to that of hexazinone: these herbicides were washed away from surface layers by soil water movement and their distribution peaks

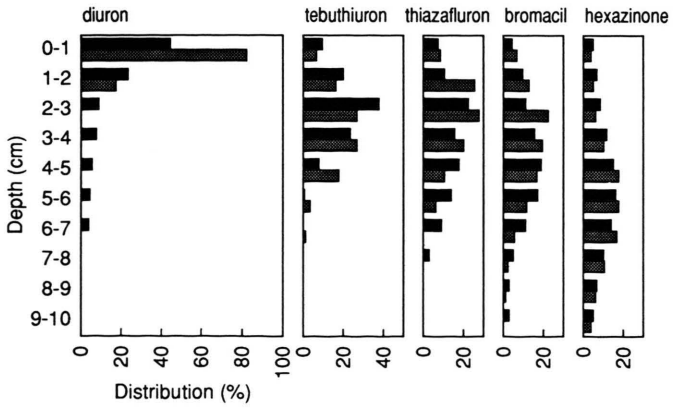

Fig. 2. The vertical distribution of 5 photosynthesis inhibiting herbicides in soil columns. The soil in columns was sliced into 1 cm layers whose herbicide contents were expressed by its percentage against the total amount recovered from the same column. Paired bars (black and gray) stand for data from 2 replicated columns.

moved down to layers of several cm from the surface. On the contrary, more than half of diuron in a column was found in the surface layer of 1 cm, showing poor soil mobility of this herbicide. In one column diuron stayed within 2 cm from the surface while in the other a portion of this herbicide reached the depth of 6 cm, suggesting existence of macropores in this column [10].

Discussion

Already there are several attempts to utilize chlorophyll fluorescence as an indicator for photosynthesis inhibition. In the present study, a fluorescence microplate scanner was employed to measure activity and quantity of photosynthesis inhibiting herbicides. There are yet some disadvantages in fluorescence measurement by microplate scanners. First, wave lengths for excitation and detection are to be chosen from limited number of options. Second, care must be taken to measure fluorescence at the terminal state in which the fluorescence increase due to photosynthesis inhibition becomes most notable. Despite these problems, our present system showed higher sensitivity than our earlier paraquat method.

An advantage of employing a microplate scanner is its efficiency. With a 96-well plate, our system measures photosynthesis inhibiting activity of 6 to 8 compounds simultaneously. This efficiency realized a quantitative technique to measure concentration of herbicides in aqueous solutions by a bioassay of photosynthesis inhibition. This technique showed practical accuracy comparable to that of gas-liquid chromatography and at the same time, time and labour to extract sample solutions by organic solvents can be saved since aqueous sample solutions can be measured directly in this system.

As how herbicide molecules travel to their sites of action is of our main interest, the vertical movement of several photosynthesis inhibiting herbicides was assessed using soil columns as the penetration model. Herbicides were applied at the top surfaces of the soil columns and forced to move downward by artificial precipitation. Then, the columns were sliced into 1 cm layers whose herbicide content was determined by aforementioned measuring system. Thus obtained distribution profiles clearly reflect behaviour of soil applied herbicides and agree well with the empirical knowledge about soil mobility of tested compounds. We already reported that systemicity of photosynthesis inhibiting herbicides in plant bodies is correlated to their water solubility [6]. The present results again suggest that water solubility influences soil mobility of herbicides since the most water soluble compound, hexazinone, showed the greatest mobility and the least water soluble diuron showed the smallest (Table II). Among many preceding attempts to predict the environmental behaviour of pesticides in physicochemical terms [11–15], some are in favour of this speculation [13, 15]. Nonetheless comprehensive studies on relationships between soil mobility and various molecular properties are still to be done to construct a general model to describe behaviour of pesticides in soil.

Table II. Water solubility and log K_{ow} of 5 compounds tested for their soil mobility.

Herbicides	log K_{ow}	Water solubility [mM]
Diuron	2.77	0.2
Tebuthiuron	1.79	11.0
Thiazafluron	1.82	8.7
Bromacil	2.11	3.1
Hexazinone	1.85	130.8

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