

FLUORESCEIN DIACETATE HYDROLYSIS AS A METHOD OF DETERMINING SOIL MICROBIAL ACTIVITY IN COLUMBIA BASIN SILTY LOAM SOILS

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Introduction

Carbon is a key element of both soil organic matter (SOM) and crop residues that provides energy for soil microorganisms (Wagner and Wolf 1998). Therefore, a reliable method of determining microbial metabolic activity can provide useful estimation of SOM and associated nutrient turnover. Various techniques for estimating soil microbial activity include the determination of specific enzyme activities, soil respiration, and the measurement of adenosine triphosphate (Fuhrmann 1998). Any technique for measuring total microbial activity must be sensitive, nonselective, and any incubation should be as short as possible.

Fluorescein diacetate (3', 6'-diacetylfluorescein [FDA]) is a nonfluorescent compound that can be hydrolyzed by a number of enzymes including proteases, lipases, and esterases (Guilbault and Kramer 1964). The product of hydrolyzed FDA is fluorescein, which fluoresces and is quantifiable with fluorometry or spectrophotometry. Its nonspecific nature makes it suitable for determining total microbial activity and estimating SOM turnover (Schnürer and Rosswall 1982, Inbar et al. 1991, Yokoyama et al. 1993). There is substantial spontaneous hydrolysis of FDA at temperatures above 30°C and in alkaline conditions (pH above 8.5); consequently, the temperature and pH must be controlled during FDA determinations. Fluorescein

reaches maximum fluorescence near pH 8.0 (Guilbault and Kramer 1964).

Soil organic matter is an important reservoir for soil carbon with chemical, physical, and biological properties fundamental to maintenance of soil quality. Soil organic matter improves nutrient holding capacity (i.e., cation exchange capacity) and acts as a buffer against rapid shifts in soil acidity or alkalinity. As it decomposes, SOM slowly releases organically bound nutrients including N, P, and S. Soil structure, aggregation, water infiltration and soil water-holding capacity are all enhanced as SOM content increases. Soil organic matter provides a slowly available carbon and energy source to support a large, diverse, metabolically active microbial community. It has a high adsorptive capacity for organic compounds, which can reduce the bioavailability of toxic xenobiotics (i.e., compounds foreign to biological systems, materials produced by humans that are resistant to biodegradation and decomposition.). Unfortunately, any adsorption of fluorescein by SOM would reduce its concentration following hydrolysis and subsequently underestimate the metabolic activity of the soil microorganisms.

The purpose of this research was to evaluate the use of FDA hydrolysis in a silt loam soil common to the Columbia Basin and determine if this assay could provide useful and reliable estimates of soil microbiological activities. In addition, the

studies were planned to obtain preliminary data on patterns of metabolic activity in soils under tillage practices common to the Columbia Basin.

Materials and Methods

A FDA working solution was prepared by diluting a FDA stock solution (1.0 mg FDA/mL acetone) with sterile sodium phosphate buffer (60 mM, pH 7.6) to give a final concentration of 5.0-20.0 mg FDA/L acetone. All FDA solutions were stored at -20°C. The working solution was cleared by vacuum filtration through Whatman no. 5 paper. Both the FDA stock and working solutions were prepared fresh prior to the assays.

A single strain of bacteria, isolated from a coarse silty loam soil on the Rowell farm southwest of Prosser, Washington, was used for pure culture studies. The strain, designated HHM2, was isolated by dilution plating on nutrient agar, and checked for purity by repeated streaking on nutrient agar. A fresh culture of strain HHM2 was inoculated into flasks containing 50 mL of nutrient broth and the flasks were incubated in a waterbath shaker (27°C) for 2.5 hours. The bacteria were collected by centrifugation (5 minutes at 3,000 x g), washed twice in fresh sodium phosphate buffer (60 mM, pH 7.6), and resuspended in 10 ml of sterile buffer. Aliquots of the suspension were transferred to centrifuge tubes for the assay. Each tube received 10 mL of FDA working solution, except the control, which received 10 mL buffer. The centrifuge tubes were incubated in a shaking waterbath at 25°C. The reaction was terminated by adding 10 mL acetone. The hydrolyzed FDA was measured at 0, 30, 60, and 90 minutes. The tubes were centrifuged for 6 minutes at 4,000 x g, the supernatant

decanted and filtered through Whatman no. 1 paper, and the fluorescein in the supernatant was determined at A₄₉₀.

To evaluate fluorescein binding to SOM, the FDA working solution was hydrolyzed in boiling water for 30 minutes. Hydrolyzed fluorescein was added to sieved soil and placed in a shaker bath at 25°C for up to 120 minutes. At 30-minute intervals, absorbance readings at 490 nanometers were taken and compared to controls of hydrolyzed FDA. The mean absorbance of the controls were compared to the mean absorbance of the soil and FDA tubes.

Soil cores (15.2 cm x 6.4 cm diameter) were taken from the long-term plots at Pendleton Experiment Station. The treatments included the continuous winter wheat plots (CWW), the manure treatment from the crop residue plots (CR), and the no-till, summer-fallow plots (NTA). Also, cores were taken from an ornamental turf on the station. A minimum of three cores were taken from each treatment. The soil was passed through a 2-mm sieve and a subsample (about 100 g) was taken to determine water content. A total of 200 mg of soil was placed in each 50-ml centrifuge tube (Sorval 03530) and 10 mL FDA working solution was added. Controls, without FDA solution, were prepared by adding 10 mL sodium phosphate buffer (60 mM, pH 7.6) to 200 mg sieved soil in a centrifuge tube. The tubes were incubated in a shaking waterbath (New Brunswick Gyrotory, Scientific Model G76) at 25°C for up to 120 minutes. The soil hydrolytic activity was terminated by adding 10 mL of acetone to each centrifuge tube. The resulting suspension was centrifuged (Sorvall RC-5B with an SS-34 rotor) at 4,000 x g for 6 minutes. The supernatant was decanted and gravity filtered through

Whatman no. 1 filter paper. A Bausch and Lomb Spectronic 100 was used to determine fluorescein concentrations at 490 nm (A_{490}). Controls, also determined at A_{490} , were used to measure background. Beer's Law ($A = bc\epsilon$) was used to calculate an extinction coefficient (ϵ), where A is the absorbance at 490 nanometers, b is the path length of the

spectrophotometer cuvette (1 cm), and c is the concentration of the working solution

= 0.0672 and exhibited a high level of correlation ($R^2 = 0.9999$).

The FDA hydrolytic activity of bacteria increased linearly with time for 90 minutes (Fig. 2). The spontaneous hydrolysis was observed to be low. Other reports have indicated that all fungi and most bacteria investigated show evidence of

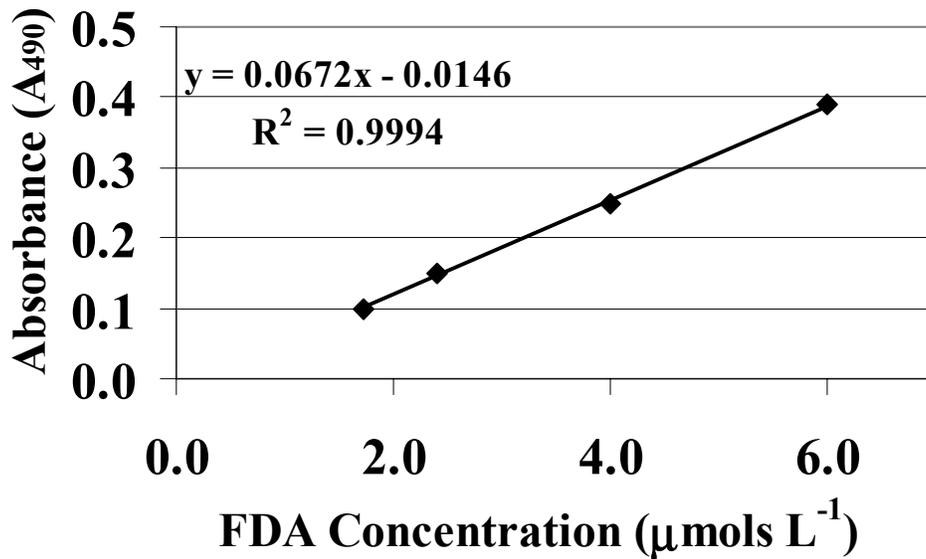


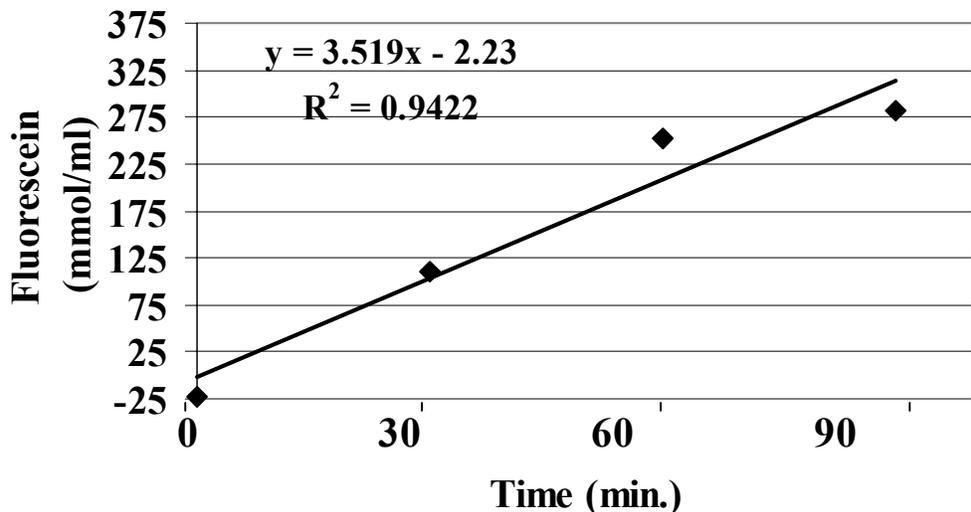
Figure 1. Extinction coefficient derived from experimental data at different fluorescein concentrations plotted against the absorbance at 490 nm. The slope of the fitted line is the extinction coefficient, ϵ_{490} , 0.0672/ $\mu\text{M}/\text{cm}$. The coefficient of determination ($R^2 = 0.9994$) is extremely high.

(Fig. 1). Simple linear regression was used to determine ϵ . This ϵ value was used in later assays to determine the fluorescein product concentration, $c = A_{490}/(\epsilon$

FDA hydrolytic activity (Schnürer and Rosswall 1982). The enzymes in the soil that hydrolyze FDA come from living

Results and Discussion

A graph of fluorescein concentration versus its absorbance at 490 nm is shown in Figure 1. The extinction coefficient was used to calculate the slope of the fitted



showing that adsorbance of hydrolyzed fluorescein by SOM was not a factor.

Fluorescein hydrolysis assays were conducted on soil samples from the unfertilized CR plot (conventional tillage, summer-fallow), from the CR plot with added manure (conventional tillage, summer-fallow), and from the no-till experiment (no-till, summer-fallow, 120 lb/acre N fertilizer). The A_{490} readings of these three assays were converted to the fluorescein concentration by using $\epsilon = 0.672$.

| Time, (min) | A_{490} | Time, (min.) | A_{490} |
|--------------|-------------|--------------|-----------|
| Control | 0.437 | 0 | 0.402 |
| Control | 0.432 | 0 | 0.397 |
| Control | 0.419 | 30 | 0.402 |
| | | 30 | 0.400 |
| | | 60 | 0.389 |
| | | 60 | 0.400 |
| | | 90 | 0.398 |
| | | 90 | 0.366 |
| | | 120 | 0.436 |
| | | 120 | 0.416 |
| Mean | 0.42933 | | 0.4006 |
| Variance | 8.63333E-05 | | 0.000316 |
| Observations | 3 | | 10 |

Table 1. Adsorption of hydrolyzed FDA to soil samples. Means are the average of at least three determinations. Controls were made of hydrolyzed FDA and buffer. Fluorescein was hydrolyzed by boiling.

| | Control Samples | Timed Samples |
|------------------------------|-----------------|---------------|
| Mean | 0.429333 | 0.4006 |
| Variance | 8.63E-05 | 0.000316 |
| Observations | 3 | 10 |
| Pooled Variance | 0.000274 | |
| Hypothesized Mean Difference | 0 | |
| df | 11 | |
| t Stat | 2.634723 | |
| P(T<=t) one-tail | 0.011605 | |
| t Critical one-tail | 1.795884 | |
| P(T<=t) two-tail | 0.023211 | |
| t Critical two-tail | 2.200986 | |

Table 2. A t-test was done to test whether the means in Table 1 were equal. At a 5 percent level of significance (11 degrees of freedom) the calculated t-statistic was greater than the critical t-value.

There was concern that the fluorescein might adsorb to SOM and thus cause an underestimation of microbial activity. To study this possibility, fluorescein adsorption to SOM was followed at 30-minute intervals. The mean of the controls (A_{490}) was compared with the mean of the of hydrolyzed fluorescein incubated with soil (Table 1). The means were tested using the t-test for two samples assuming equal variances (Table 2). The means were found to be equal at a high level of significance,

The soil with the lowest rate came from the unfertilized CR plots (Fig. 3), followed by the CR plot with added manure (Fig. 4); the no-till plot, with 120 lb/acre N (Fig. 5), showed the greatest activity. The increases in FDA hydrolysis may be a result of higher concentrations of enzymes from an elevated level of soil microbial activity;

however, it may be a consequence of elevated enzymatic activity. These results are consistent with suggestions that reduced tillage, fertilization, and improved soil quality support increased soil microbial activity.

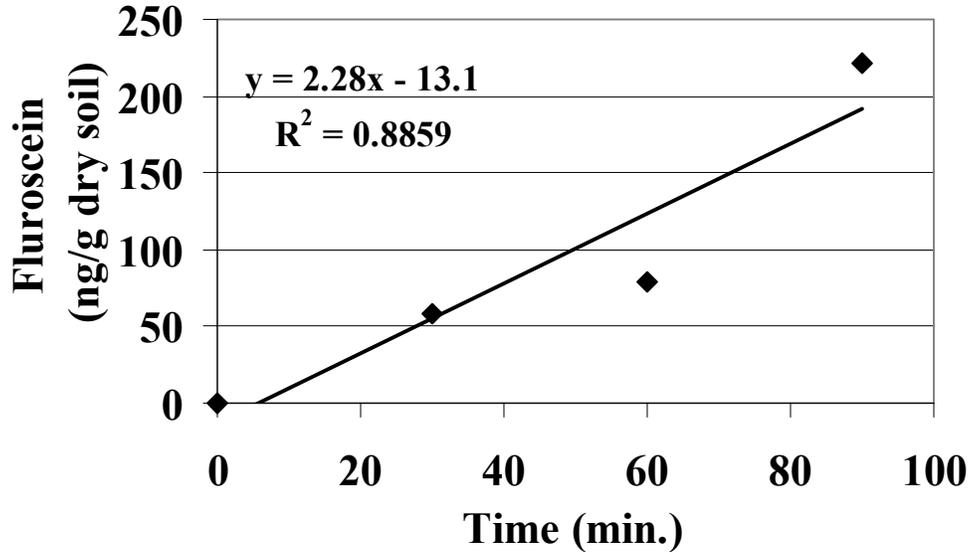


Figure 3. Hydrolysis of FDA by soil from the CR plots at the Pendleton Research Center, Oregon, October 25, 2000. The plot treatment is winter wheat, summer-fallow, with no nitrogen fertilization. The soil sample is an amalgam of at least four cores. Data points are the mean of at least two determinations. The coefficient of determination was $R^2 = 0.8859$.

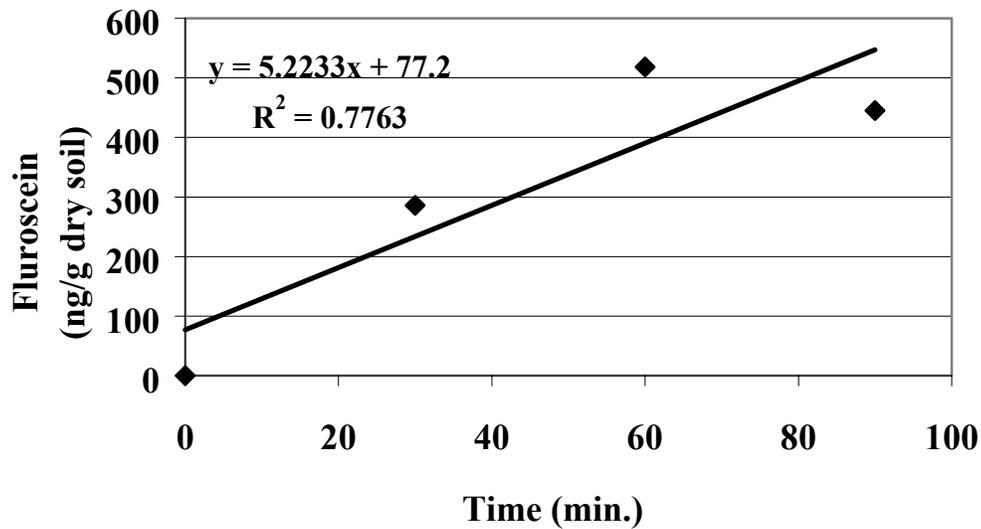


Figure 4. Hydrolysis of FDA by soil from the CR manure plots at the Pendleton Research Center, Oregon, October 25, 2000. The plot treatment is winter wheat, summer-fallow, with 10 tons steer manure added during the fallow year. The soil sample is an amalgam of at least four cores. Data points are the mean of at least two determinations. The coefficient of determination was $R^2 = 0.7763$.

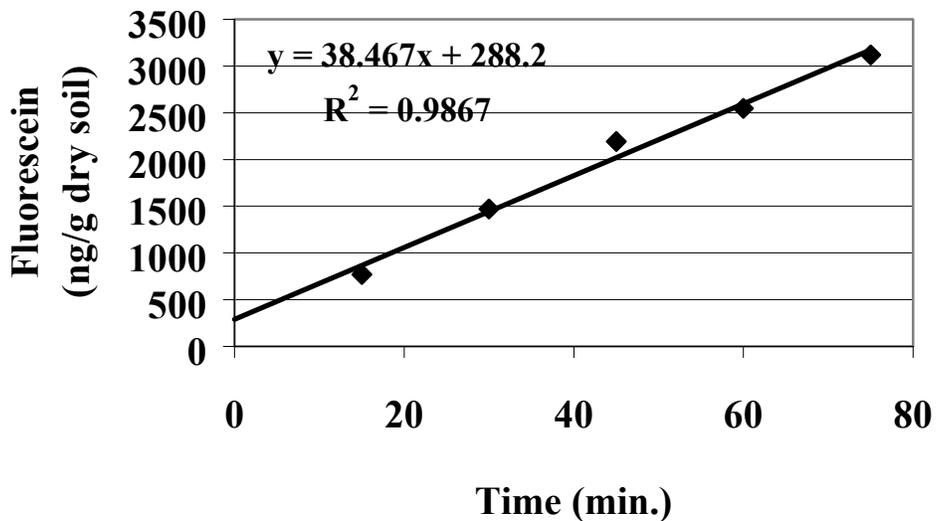


Figure 5. Hydrolysis of FDA by soil from the no-till plot at the Pendleton Research Center, Oregon, October 18, 2000. The plot treatment is winter wheat, summer-fallow, with 120 lb N fertilization. The soil sample is an amalgam of at least four cores. Data points are the mean of at least two determinations. The coefficient of determination was $R^2 = 0.9867$.

Summary

In this preliminary study, the measurement of FDA hydrolysis is an acceptable indicator of soil microbial activity in the silt loam soils of the Columbia Basin. The measurement is a relatively simple procedure that can be accomplished with a basic, inexpensive spectrophotometer. Adsorption of hydrolyzed fluorescein by any soil component appears insignificant. Rates of FDA hydrolysis and, consequently soil microbial activity, appear to be positively correlated to enhanced soil quality.

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