Environ Sci Pollut Res (2015) 22:4759–4762
DOI 10.1007/s11356-014-3922-4

SHORT RESEARCH AND DISCUSSION ARTICLE

Rapid cost-effective analysis of microbial activity in soils using modified fluorescein diacetate method

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Received: 20 October 2014 / Accepted: 25 November 2014 / Published online: 4 December 2014

Abstract Fluorescein diacetate (FDA) is commonly used to determine the hydrolyzing activity of microbial organisms in the soil. However, the costs of chemical reagents and time required to perform routine analysis of large number of samples by soil testing laboratories are limiting. Moreover, existing methods generate significant volumes of hazardous waste. In this context, this study was designed to determine the minimum amount of terminating chemical reagent needed to evaluate microbial hydrolyzing activity. The results showed that 0.2 mL of chloroform was enough to effectively stop the hydrolyzing activity in soil. This proposed terminating chemical reagent (0.2 mL chloroform) was also evaluated by comparing with the 10 mL of chloroform and 5 mL of methanol used in the Adam and Duncan method.

Keywords FDA · Hydrolyzing activity · Extracellular enzymes · Soil health

Introduction

Unsustainable soil management practices based on intensive tillage systems and utilization of higher agricultural inputs deteriorate soil health (soil quality) and cause soil degradation (Dick 1997; Kumar et al. 2014). The physical, chemical, and biological properties define the health and productivity of soil (Yakovchenko et al. 1996; Chintala et al. 2014). Microbial-mediated biochemical properties are more sensitive to changes in management practices of agro-ecosystems compared to other soil properties (Nannipieri et al. 1990; Filip 2002). The microbial population plays a critical role in the release of several hydrolytic enzymes which catalyze soil processes involving decomposition (mineralization) of organic resources and cycling of plant nutrients (Kandeler and Eder 1993; Bandick and Dick 1999; Gil-Sotres et al. 2005). The measurement of total microbial hydrolytic activity determines the overall ability of soil to accommodate these biochemical reactions essential for mineralization of nutrients (Heal and McClean 1975; Chintala et al. 2014). Fluorescein diacetate (3′,6′-diacetylfluorescein (FDA)) is commonly used to measure the total microbial activity in soils (Schnürer and Rosswall 1982; Chintala et al. 2014). Fluorescein diacetate (FDA) can be easily hydrolyzed by several enzymes including proteases, lipases, and esterases released by microbial functional groups to form fluorescein (Lundgren 1981). The measurement of fluorescein using spectrophotometry provides an overall index of total microbial activity about the total microbial activity and soil function (Swisher and Carroll 1980).

Chloroform-methanol mixture (Adam and Duncan 2001) and acetone (Green et al. 2006) have been the common solvents to terminate the hydrolyzing activity of FDA. However, the quantities of these solvents used by these popular FDA methods (Adam-Duncan and Green et al. methods) were found to be high especially for intensively cultivated soils in which the hydrolyzing activity could be low. Adam and Duncan used 15 mL of chloroform/methanol mixture (2:1) for 2 g of soil (Adam and Duncan 2001). Whereas Green et al. used 5 mL of acetone (for 2 g soil) to terminate hydrolyzing reaction in soil (Green et al. 2006). There is a possibility of reducing quantities of these solvents in FDA methods as well as the costs of analysis and hazardous waste disposal. In this context, this study was conducted with an objective of determining the optimum quantity of solvents including chloroform and methanol to terminate FDA hydrolyzing activity—in the standard FDA method (Adam and Duncan 2001).

Responsible editor: Philippe Garrigues

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Materials and methods

Collection of soils

Eleven surface soil samples (0–15-cm depth) were collected from cropland and grasslands in five locations including Morris in Minnesota (45.58 N, 95.91 W) and Brookings (44.30 N, 96.78 W), Colman (43.98 N, 96.81 W), and Warner (45.32 N, 98.49 W) in South Dakota, USA. Soil classification of the soils based on the US Soil Taxonomy system is given in Table 1 (NRCS 2005). The croplands were intensively cultivated for more than 80 years. The grasslands were never tilled and consisted of perennial grasses. These soils were air-dried, crushed, and screened using a 2-mm sieve. Soils were stored at room temperature until they were used to determine the microbial activity using FDA.

Determination of microbial activity using fluorescein diacetate

Total microbial activity was initially determined using fluorescein diacetate method (FDA method) in 11 soil surface samples (Adam and Duncan 2001). In this method, chemical reagents including potassium phosphate buffer (60 mM, pH 7.6), fluorescein diacetate (FDA) stock solution (1000 μg mL^{-1}), and 2:1 chloroform/methanol mixtures were used. Two grams of soil was taken into a 100-mL conical flask and added with 15 mL of 60 mM potassium phosphate buffer (pH 7.6). Then, 0.2 mL of FDA stock solution (1000 μg mL^{-1}) was added to conical flask to initiate the hydrolyzing reaction. Control soil samples were also prepared without adding FDA stock solution. All these samples with FDA and controls in conical flasks were closed with stoppers and shaken with hand for few minutes. These conical flasks of four replications were placed in an incubator with orbital shaker (with 100 rpm) at 30 °C for 20 min. After incubation, 15 mL of chloroform/methanol mixture (2:1) were immediately added to the flask to stop the hydrolyzing of FDA by enzymes released by microbial functional groups in the soils. The contents of conical flasks were then thoroughly shaken before transferring into 50-mL centrifuge tubes. These centrifuge tubes with samples were centrifuged at 2000 rpm for 5 min (using Eppendorf Centrifuge 5810). The clear supernatant solutions were filtered, and the yellow color intensity measured at 490 nm using a UV-Vis spectrophotometer (Thermo Scientific GENESYS 10S UV-Vis spectrophotometer). The concentration of fluorescein in supernatant solutions of samples was determined using a calibration curve of 0–5 μg mL^{-1} FDA standards.

Trials to optimize the quantity of solvents to terminate FDA hydrolyzing activity

Different combinations of chloroform and methanol quantities and of timing addition were tested to optimize their quantities. Chloroform and methanol solvents were added to samples separately and also as mixtures. Chloroform/methanol mixtures were tested at the ratios of 1:0, 1:1, 1:5, 2:0, 2:5, 5:0, and 10:0 against the 2:1 ratio which was proposed by the Adam and Duncan method. Chloroform without methanol was added at the rates of 0.2, 0.02, and 1 mL. These preliminary trials with different combination of chloroform and methanol suggested that 0.2 mL chloroform would be an effective alternative hydrolyzing activity terminator to the 2:1 chloroform/methanol mixture proposed by the Adam and Duncan method.

Table 1 Taxonomic classification of soils used in this study (NRCS 2005)

Soil ID	Land use type	Soil series	Soil classification	Texture	Location (state)
CL1	Crop land	Kranzburg	Fine-silty, mixed, superactive, frigid Calcic Hapludolls	Silty clay loam	Brookings, SD
CL2	Crop land	Dempster	Fine-silty over sandy or sandy skeletal, mixed, superactive, mesic Udic Haplustolls	Silt loam	Colman, SD
CL3	Crop land	Dempster	Fine-silty over sandy or sandy skeletal, mixed, superactive, mesic Udic Haplustolls	Silt loam	Colman, SD
CL4	Crop land	Wentworth	Fine-silty, mixed, superactive, mesic Udic Haplustolls	Silty clay loam	Brookings, SD
CL5	Crop land	Barnes	Fine-loamy, mixed, superactive, frigid Calcic Hapludolls	Loam	Morris, MN
CL6	Crop land	Buse	Fine-loamy, mixed, superactive, frigid Typic Calciudolls	Loam	Brookings, SD
CL7	Crop land	Buse	Fine-loamy, mixed, superactive, frigid Typic Calciudolls	Loam	Brookings, SD
CL8	Crop land	Buse	Fine-loamy, mixed, superactive, frigid Typic Calciudolls	Loam	Brookings, SD
CL9	Crop land	Tonka	Fine, smectic, frigid Argiaquic Argialbolls	Silt loam	Brookings, SD
GL10	Grassland	No name	Coarse-loamy, mixed, Superactive, frigid Typic Udifluvents	Sandy loam	Warner, SD
GL11	Grassland	Harriet	Fine, smectic, frigid Typic Natraquolls	loam	Warner, SD

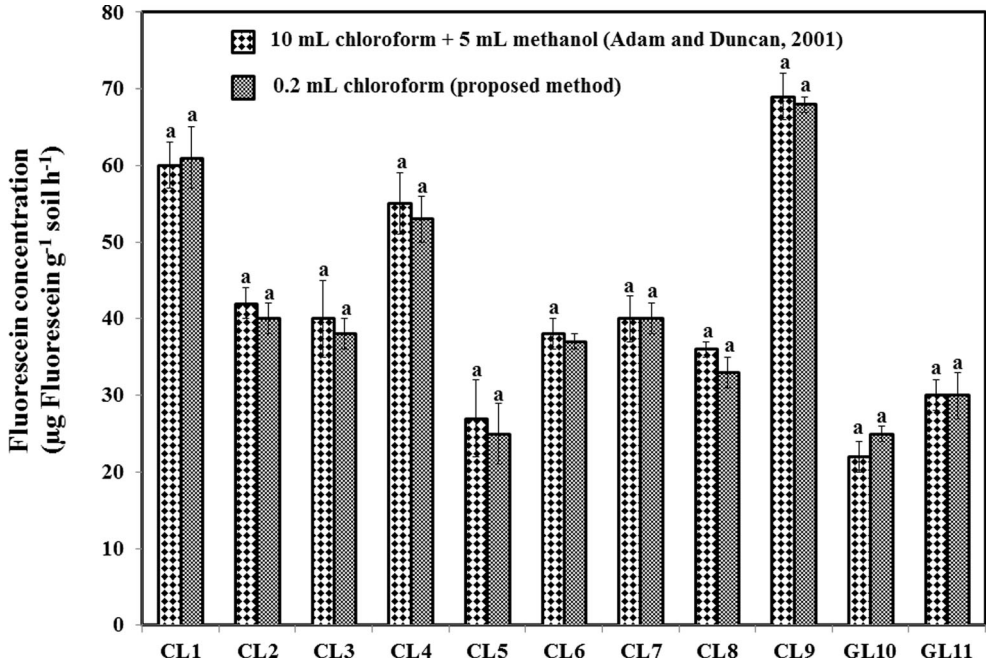

Fig. 1 Total microbial activity (fluorescein concentration) in soils of cropland (*CL*) and grassland (*GL*) from different locations. Each value is mean of four replications with standard error. Significant differences by Holm adjusted Fisher's LSD test at α=0.05. No significant differences were observed between methods with the same letters

Evaluation of alternative solvent for terminating hydrolyzing activity

The microbial activity was determined in 11 surface soil samples using FDA method either with 15 mL of chloroform/methanol mixture (2:1) (Adam and Duncan method) or 0.2 mL chloroform (proposed method). Apart from two different solvents to terminate hydrolyzing activity, the steps in procedure were the same as used in the Adam and Duncan method. The influence of these two methods on the concentration of fluorescein in samples was evaluated by performing ANOVA using SAS Statistical Package, version 9.2. The significance of treatments was assessed at α=0.05. The separation of means was calculated between treatments based on Holm adjusted Fisher's LSD test (Steel and Torrie 1980).

Results and discussion

Total microbial activity (fluorescein concentration) was analyzed in 11 surface soils using two chemical reagents (10 mL chloroform + 5 mL methanol and 0.2 mL chloroform) for terminating hydrolyzing activity of microbial organisms (Fig. 1). Hydrolyzing activity varied widely in these soils and different land use systems. Soil environments in the chosen soils varied widely in their ability to support the microbial growth. For example, the lower microbial activity in GL10 and GL11 is consistent with the poor drainage (flooded system) and sodicity, respectively, of these soils. Measurements of hydrolyzing activity were not significantly different between methods. Especially, an addition of 0.2 mL of chloroform performed well in the soils with low

Table 2 Evaluation and validation of the proposed method (using 0.2 mL of chloroform) by comparing with the Adam and Duncan method (using 10 mL of chloroform and 5 mL of methanol) for terminating hydrolyzing activity

Soil	Terminating reagent	
	10 mL of chloroform + 5 mL of methanol Fluorescein concentration (μg fluorescein g^{-1} soil h^{-1})	0.2 mL chloroform
Maddock (crest soil)	34±6a	31±4a
Brookings (footslope soil)	51±4a	47±2a
Maddock+corn stover biochar	24±5a	22±3a
Brookings+corn stover biochar	36±2a	35±5a

Each value is mean of four replications with standard error. Significant differences by Holm adjusted Fisher's LSD test at α=0.05. No significant differences were observed between methods with the same letters in a row. Corn stover biochar was applied at the rate of 10 g kg^{-1} soil

hydrolyzing activity where there was no need for higher volumes of terminating chemical reagents.

Methanol was observed to decrease the fluorescein concentration in low hydrolyzing activity soils and the sensitivity of spectrophotometer. Whereas chloroform (even added at low volume) did not affect the absorbance of fluorescein for low hydrolyzing activity soils and spectrophotometer readings were stable at least for 90 min. Chloroform (0.2 mL) was also evaluated by measuring the hydrolyzing activity in soils applied with corn stover biochar (at 10 g kg^{-1} soil) (Table 2). There was no significant difference between the two methods of using different terminating chemical reagents.

Chloroform was very effective in stopping the hydrolyzing activity and also did not affect the values of fluorescein concentration. This study found that 0.2 mL of chloroform was an effective chemical reagent for terminating hydrolyzing activity. This is a much reduced volume (1/50) compared to current methods. As a result, there is an opportunity to reduce the cost and time for routine analysis of microbial hydrolytic activity in large number of soil samples by soil testing laboratories.

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