Comparison of Different Methods for Measuring Soil Acid Phosphatase Activity: Postprint

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Abstract

Soil acid phosphatase is most closely related to the mineralization of organic phosphorus and plant phosphorus nutrition. Currently, domestic scholars mainly refer to the method using disodium phenyl phosphate as substrate in Guan Songyin' s "Soil Enzymes and Their Research Methods" when measuring acid phosphatase activity, while foreign scholars mainly refer to the method using p-nitrophenyl phosphate disodium (PNPP) as substrate in Dick' s "Methods of Soil Enzymology" . However, in the process of measuring reaction products using disodium phenyl phosphate as substrate, the problem of indistinct color development often occurs; additionally, the use of different substrates for measuring acid phosphatase activity also creates difficulties in method selection. To rationally select a method for determining soil acid phosphatase activity, this study selected 10 soil samples each of acidic, neutral, and alkaline soils, and measured soil acid phosphatase activity using disodium phenyl phosphate as substrate with pH 5.0 acetate buffer (DPP 1) and pH 9.4 borate buffer (DPP 2) added at the color development stage, respectively, as well as the PNPP method. The effects of different pH buffers and phenol concentrations on the color development reaction of reaction products were also investigated. The results showed that when using disodium phenyl phosphate as substrate and adding buffer with pH $\leq$ 6 at the color development stage, phenol and 2,6-dibromoquinone chlorimide did not develop color; when buffer with pH $\geq$ 8 was added, color developed between them and the Pearson correlation coefficient between phenol concentration and absorbance value was extremely significant. This indicates that low pH is a major reason for the poor color development effect of high phenol concentration and 2,6-dibromoquinone chlorimide. Furthermore, when using the PNPP method, the coefficient of variation of acid phosphatase activity for the 10 samples in acidic, neutral, and alkaline soils increased by 70.04%, 42.44%, and 21.17%, respectively, compared with DPP 2; the ranges were 27.18 times, 26.85 times, and 39.43 times those of DPP 2, respectively. In conclusion, if disodium phenyl phosphate is selected

as substrate for measuring soil acid phosphatase activity, alkaline borate buffer should be added at the color development stage; selecting p-nitrophenyl phosphate disodium as substrate is a simpler and more sensitive method.

Full Text

Introduction

Soil phosphorus is an essential nutrient element for plant growth, with organic phosphorus accounting for 15%–80% of total soil phosphorus [1]. Since organic phosphorus cannot be directly absorbed and utilized by plants, it becomes a major limiting nutrient in terrestrial ecosystems [2]. Phosphatases catalyze the hydrolysis of phosphate esters or anhydrides, and their activity directly affects the decomposition, transformation, and bioavailability of organic phosphorus. Among these, acid phosphomonoesterase is most closely related to the mineralization of organic phosphorus and plant phosphorus nutrition [3–4], and predominates in acidic soils [5].

Enzyme extraction is difficult, so phosphatase activity is primarily measured indirectly based on the amount of product generated or substrate remaining during enzymatic reactions [6–7]. Since Kroll et al. first proposed using phenyl phosphate as a substrate and measuring phenol release to represent phosphatase activity [6], methodological improvements have focused primarily on substrate and buffer types. Reported substrates include natural substrates (mainly nucleic acids), β-naphthyl phosphate sodium, β-glycerophosphate, phenyl phosphate disodium, p-nitrophenyl phosphate sodium, and phenolphthalein phosphate [8–10]. Since traditional spectrophotometric methods are cost-effective and more universally applicable than fluorometric methods, this study focuses on phosphatase substrates suitable for spectrophotometric analysis.

In spectrophotometric methods, most international scholars currently use p-nitrophenyl phosphate disodium as the substrate for phosphatase activity determination, with studies indicating this method is the fastest and most accurate [9,11]. One foreign literature survey found that 706 data points in phosphatase kinetic studies used p-nitrophenyl phosphate disodium as substrate, while only 140 used other substrates [12]. Additionally, Chinese researchers frequently use phenyl phosphate disodium as substrate [13]. Although early studies showed that using phenyl phosphate disodium at pH 6.5 resulted in unstable color development [11,14], this method has been included in several reference books [15–18] and is cited in numerous Chinese literature sources [19–20] as well as occasional Science Direct publications [21–22].

The principle of measuring phosphatase activity using phenyl phosphate disodium as substrate is based on phenol formed after enzymatic hydrolysis reacting with the color developer 2,6-dibromoquinone-4-chloroimide under alkaline conditions. This introduces a quinone imine chromophore at the para position of the phenolic hydroxyl group to generate blue indophenol [23–24]. Some domestic

reference books describe soil acid phosphatase activity determination methods that add acetate buffer during both incubation and color development, causing phenol to react with 2,6-dibromoquinone-4-chloroimide under acidic conditions [16–18]. However, *Methods in Soil Biology* mentions adding acetate buffer during incubation but borate buffer during color development, creating an alkaline environment for the final color reaction [25]. In Guan Songyin's *Soil Enzyme and Its Research Methods*, which most Chinese scholars reference, the description states that acetate buffer should be used for acid phosphatase activity determination during incubation, but only mentions "adding buffer" during color development without specifying whether it should be borate or acetate buffer [15]. Furthermore, the authors' preliminary work and that of other researchers have shown that using phenyl phosphate disodium with acetate buffer for color development results in no color formation, affecting enzyme activity determination.

Therefore, this study hypothesized that acidic conditions are unfavorable for color development between phenol and 2,6-dibromoquinone-4-chloroimide during acid phosphatase activity determination. We selected acidic, neutral, and alkaline soils to compare color reactions based on phenyl phosphate disodium substrate under different pH buffer conditions, while simultaneously comparing the sensitivity of phenyl phosphate disodium-based methods with p-nitrophenyl phosphate disodium-based methods. The results will provide a basis for selecting appropriate methods for soil acid phosphatase activity determination and are significant for improving soil phosphorus nutrition.

1. Materials and Methods

1.1 Soil Samples

In soil biological indicator research, acid phosphatase activity can serve as an indicator of microbial activity and function even in non-acidic soils; selection should not be based solely on soil pH. To investigate the sensitivity of different methods across soil types, this study selected 10 soil samples each from three pH ranges (4–10). Acidic soils (pH 4.32–4.46) were collected from Dagangshan, Fenyi County, Jiangxi Province, representing red soils under natural evergreen broadleaf forest. Neutral soils (pH 6.26–7.35) were collected from Badaling Forest Farm, Beijing, representing brown soils primarily under *Pinus tabulaeformis* Carr. Alkaline soils (pH 9.59–10.05) were collected from Maduo County, Guoluo Tibetan Autonomous Prefecture, Qinghai Province at altitudes above 4,200 m, representing meadow soils under grassland vegetation.

1.2 Determination of Soil Acid Phosphatase Activity

The phenyl phosphate disodium ($C_6H_5Na_2O_4P$, CAS No.: 3279-54-7) substrate method was based on the widely referenced Chinese method in *Soil Enzyme and Its Research Methods* [15] with slight modifications. Two approaches were used: 1) adding acidic acetate buffer during color development (DPP 1), consistent with the method described in the reference; and 2) adding alkaline borate buffer

during color development (DPP 2). Buffer preparation and operational steps followed the reference [15].

The p-nitrophenyl phosphate disodium ($C_6H_4NNa_2O_6P \cdot 6H_2O$, CAS No.: 4264-83-9) substrate method followed the procedure described in *Methods of Soil Enzymology* (PNPP) [11]. One gram of soil was weighed, toluene was added as an inhibitor, followed by 4 mL of pH 6.5 modified universal buffer [containing 12.1 g Tris ($C_4H_{11}NO_3$), 11.6 g maleic acid ($C_4H_4O_4$), 14.0 g citric acid ($C_6H_8O_7$), 6.3 g boric acid (H_3BO_3), and 19.52 g NaOH per liter of buffer] and 1 mL p-nitrophenyl phosphate substrate solution, then incubated at 37°C for 1 h. After incubation, $CaCl_2$ and NaOH solutions were added, and the filtrate was measured at 400 nm.

Control experiments differed among literature sources: DPP 1 and DPP 2 established substrate-free and soil-free controls, while PNPP established post-added substrate controls. To eliminate blank differences, this study uniformly established both substrate-free and soil-free controls. Measured absorbance values for soil-free controls were 0.070, 0.112, and 0.108 for DPP 1, DPP 2, and PNPP, respectively.

1.3 Effect of Buffer pH and Phenol Concentration on Color Development

To understand how different pH buffers affect color formation between phenol and 2,6-dibromoquinone-4-chloroimide to produce blue indophenol, acetate buffers at pH 4.0, 6.0, and 8.0 and borate buffer at pH 10.0 were used to measure absorbance of phenol concentrations at 0, 1, 2, 3, 4, and 6 $\mu g \cdot mL^{-1}$. Each treatment had three replicates.

1.4 Statistical Analysis

Absorbance data are presented as mean ± standard deviation. Differences in absorbance among soil samples were analyzed using LSD tests in SPSS 18.0. Two-way interaction analysis was used for absorbance values under different pH buffers and phenol concentrations. Pearson correlation analysis was used for relationships between phenol/p-nitrophenol concentrations and absorbance. Figures were generated using Matlab R2010b.

2. Results

2.1 Standard Curves for Different Methods

Using DPP 1, absorbance reached a maximum of 0.002 at 1.8 $\mu g \cdot mL^{-1}$ phenol concentration and a minimum of -0.003 at 0.6 $\mu g \cdot mL^{-1}$ phenol concentration . Pearson correlation analysis showed a correlation coefficient of 0.284 between six phenol concentrations and measured absorbance values, which was not significant.

As shown in [Figure 1: see original paper], absorbance gradually increased with phenol concentration. The Pearson correlation coefficient between phenol concentration and absorbance was 0.997 for DPP 2 and 1.000 for PNPP, both significant at the 0.01 level.

2.2.1 Absorbance Values of Acid Phosphatase Activity in Acidic Soils

Since the Pearson correlation coefficient between phenol concentration and absorbance was not significant for DPP 1, a satisfactory standard curve could not be obtained, making phenol concentration calculation impossible. This created difficulties in calculating acid phosphatase activity, which requires phenol concentration. Therefore, this study used absorbance values directly to compare differences among soil samples.

As shown in , DPP 1 showed little variation among the 10 soil samples, with a mean absorbance of 0 and a range of only 0.008 between the maximum (sample 4) and minimum (sample 3). DPP 2 absorbance values ranged between 0.089 and 0.045. PNPP absorbance values ranged between 1.887 and 0.691, with a range of 1.196–27.18 times greater than DPP 2. Additionally, the coefficient of variation among the 10 soil samples was 39.67% for PNPP, a 70.04% increase compared to DPP 2.

When considering only substrate-free controls, DPP 1 produced negative absorbance values for 4 of 10 soil samples, while DPP 2 and PNPP produced no negative values. When considering both substrate-free and soil-free controls, DPP 1 and DPP 2 produced negative values for all soil samples, while PNPP produced no negative values.

2.2.2 Absorbance Values of Acid Phosphatase Activity in Neutral Soils

As shown in , DPP 1 showed significant differences among the 10 soil samples at the 0.05 level, but with a range of only 0.007. DPP 2 and PNPP showed consistent trends in absorbance values among the 10 soil samples, but with different magnitudes. DPP 2 absorbance values ranged between 0.061 and 0.041 (range = 0.020), while PNPP values ranged between 1.012 and 0.475 (range = 0.537)–26.85 times greater than DPP 2. The coefficient of variation among the 10 soil samples was 23.56% for PNPP, a 42.44% increase compared to DPP 2.

2.2.3 Absorbance Values of Acid Phosphatase Activity in Alkaline Soils

Absorbance values for alkaline soils are shown in . DPP 1 showed little variation and some negative values. DPP 2 and PNPP showed consistent trends, but DPP 2 showed significance variation from a to b at the 0.05 level, while PNPP showed variation from a to h. PNPP had a range of 0.276–39.43 times greater than DPP 2–and a coefficient of variation of 35.66%, a 21.17% increase compared to DPP 2.

2.3 Effects of Buffer pH and Phenol Concentration on Color Development

When using DPP 1 to determine acid phosphatase activity, satisfactory results were not obtained even in acidic soils where acid phosphatase predominates. DPP 2 produced blue color from the reaction between phenol and 2,6-dibromoquinone-4-chloroimide. Whether this was related to buffer pH or low phenol concentration from low enzyme activity required further investigation. To minimize soil interference in phenol measurement, this study measured absorbance of different phenol concentrations under various pH buffers. Results showed that pH and phenol concentration had highly significant effects on absorbance, with pH having the greatest effect, followed by phenol concentration .

Furthermore, [Figure 2: see original paper] shows that at buffer pH 4.0 and 6.0, absorbance did not increase with phenol concentration and showed little variation. At pH 8.0 and 10.0, absorbance increased with phenol concentration. At pH 8.0, the Pearson correlation coefficient between phenol concentration and absorbance was 0.969, with absorbance increasing by 0.007 per 1 $\mu g \cdot mL^{-1}$ phenol increase. At pH 10.0, the correlation coefficient was 0.998, with absorbance increasing by 0.048 per 1 $\mu g \cdot mL^{-1}$ phenol increase. Both correlations were significant at the 0.01 level.

Under acidic conditions, color development differences among phenol concentrations were not obvious, while under alkaline conditions, absorbance increased with phenol concentration. Higher pH resulted in greater slope and higher phenol sensitivity. Thus, acidic environments are unfavorable for color development between phenol and 2,6-dibromoquinone-4-chloroimide, while alkaline conditions produce satisfactory color. Low pH is a key factor causing poor color development even at high phenol concentrations, consistent with our initial hypothesis.

Low pH as a factor affecting phenol measurement is supported by other studies. Xia et al. [26] showed that buffers below pH 8 are unfavorable for phenol and 2,6-dibromoquinone-4-chloroimide color development, with slow or absent color formation; the optimal pH range is 8–10, with 9.4 being most suitable [24]. Halstead [27] studied pH effects on phosphatase activity using alkaline conditions for phenol and 2,6-dibromoquinone-4-chloroimide color development. Some researchers following *Methods in Soil Biology* add alkaline borate buffer during color development [25], supporting the DPP 2 approach. Since the reaction is terminated after incubation by adding aluminum sulfate solution and filtration, subsequent addition of alkaline buffer only provides an alkaline environment for the color reaction between phenol and 2,6-dibromoquinone-4-chloroimide to generate blue indophenol, and does not participate in the enzymatic reaction. Therefore, adding alkaline borate buffer during color development does not affect acid phosphatase activity determination.

Although DPP 2 can produce color, considering both soil-free and substrate-

free controls results in negative absorbance values, possibly due to low soil acid phosphatase activity. Furthermore, across acidic, neutral, and alkaline soils, the coefficient of variation among the 10 soil samples was always lower for DPP 2 than for PNPP, indicating lower sensitivity. The PNPP method using p-nitrophenyl phosphate disodium produces p-nitrophenol through enzymatic reaction, enabling rapid and accurate color development after adding NaOH solution, with incubation time proportional to p-nitrophenol production [14]. Satisfactory results were obtained even in alkaline soils with low acid phosphatase activity.

This study only compared different pH soils without considering other soil properties. Future research should conduct more systematic and in-depth comparisons across major soil types in China.

Conclusion

When determining acid phosphatase activity using phenyl phosphate disodium as substrate, adding acetate buffer (acidic conditions) is unfavorable for phenol and 2,6-dibromoquinone-4-chloroimide color development, yielding unsatisfactory absorbance even at high phenol concentrations. Adding borate buffer (alkaline conditions) enables color development, but with lower sensitivity than the p-nitrophenyl phosphate disodium method. The p-nitrophenyl phosphate disodium-based method produces satisfactory results across acidic, neutral, and alkaline mineral soils. In conclusion, when using phenyl phosphate disodium as substrate, alkaline borate buffer should be added during color development; however, the p-nitrophenyl phosphate disodium method is simpler and more sensitive for determining soil acid phosphatase activity.

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Note: Figure translations are in progress. See original paper for figures.