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Assessing the impact of azadirachtin application to soil on urease activity and its kinetic parameters

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Abstract: The kinetic parameters of soil urease have attracted considerable attention; however, little information is available on its kinetic parameters and behaviors in response to azadirachtin application to the soil. A short (14-day) field experiment was conducted using Albic Luvisol soil (loam texture; pH 6.70; electrical conductivity 0.81 dS m^{-1}; $CaCO_3$ content 0.04%; total organic carbon 0.99%) as the experimental soil in the Perm region of the Russian Federation to investigate the effects of different azadirachtin application doses on soil urease activity and its kinetic behaviors. The results showed that the highest urease activity was found under high azadirachtin application doses (0.6 L ha^{-1}) compared to the control (0 L ha^{-1}), recommended (0.3 L ha^{-1}), and low (0.15 L ha^{-1}) azadirachtin application doses at all different incubation times (1, 2, 3, 6, and 12 h). Soil urease enzymes exhibited typical Michaelis–Menten kinetic behaviors; high azadirachtin application doses increased the substrate affinity constant (K_M) and decreased the maximum reaction rates (V_{max}) of soil urease. As compared to the control azadirachtin application dose, the low azadirachtin application dose increased the V_{max} of soil urease; however, with low and recommended application doses, the V_{max} of soil urease decreased. Overall, in this study, all azadirachtin application doses were effective in influencing the kinetic behavior of urease in Albic Luvisol.

Key words: Azadirachtin, kinetic parameters, pesticide, urease, V_{max}, K_M, V_{max}/K_M

1. Introduction

Intensive agriculture has shown spectacular success over the last few decades due to the use of various inputs such as fertilizers and pesticides, along with high-yielding varieties of crops (Chowdhury et al., 2008). Pesticides are the only group of chemicals that are deliberately applied to the environment with the aim of suppressing plant pests and protecting agricultural produce. However, the majority of pesticides do not target only pests, and their application influences nontarget plants as well. Moreover, eventually, there is a build-up of pesticide resistance within the target species (Agyaro et al., 2006). Unfortunately, pesticides belong to the group of xenobiotics, that is, man-made organic chemicals that mimic organic chemicals that are important to sustain life; however, their properties and features are extraneous to living organisms and therefore not recognized by them (Gianfreda and Rao, 2011). The negative effects of pesticides on the environment have prompted the search for alternative means of pest control (Powers et al., 1993; Sarathchandra et al., 1996; Agyaro et al., 2006). An ideal pesticide should be toxic only to the target organism and biodegradable, and its residue should not affect nontarget surfaces (Chowdhury et al., 2008). One such ideal alternative is the use of natural plant products that have pesticidal activity, such as azadirachtin (Akça et al., 2005). Azadirachtin possesses insecticidal activity against many economically important insect pests such as *Helicoverpa armigera*, *Spodoptera litura*, *Plutella xylostella*, *Sitophilus oryzae*, *Sitophilus zeamis*, *Earis vitella*, *Aphis gossypii*, *Bemicia tabaci*, and *Pectiniphora gossypiella*, and nematodes like *Cosmopilitis sordidus*. The belief that such natural insecticides are safe or less damaging to the ecosystem also needs to be further validated, as their effect on nontarget organisms is reportedly very close to threshold chronic toxicity (Schmutterer and Singh, 2002; Gopal et al., 2007).

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In soil, enzyme activities play a predominant role in nutrient cycling and soil fertility. Soil enzymes contribute to the overall biological activity in the soil environment under different states (Dick, 1997) because they are intimately involved in catalyzing reactions necessary for organic matter decomposition, nutrient cycling, energy transfer, and environmental quality (Yang-Fang et al., 2004). Information on soil enzyme activities used to determine soil microbiological characteristics is very important for soil quality and health. Enzymatic activities caused by soil microbial activities are sensitive indicators for detecting the changes occurring in soils (Tabatabai, 1994; Kızılkaya et al., 2004; Khah et al., 2015).

Urease is the commonly used group name for enzymes that catalyze the hydrolysis of urea in aerobic conditions by acting on C–N bonds (nonpeptide) in linear amides. These enzymes are classified as urea amide hydrolases E.C.3.5.1.5 (Riethel, 1971). Urease activity in soil is attributed to extracellular enzymes and the enzymes within proliferating microorganisms (Bremner and Mulvaney, 1978; Kızılkaya and Ekberli, 2008). Moreover, urease activity in soil is affected by the physicochemical properties and agricultural practices of the soil (Kızılkaya and Bayraklı, 2005). Although the hydrolytic efficiency of soil urease enzymes may be strongly influenced by the composition of the surroundings directly or indirectly, insecticides, as extraneous matter to soil component pools, can affect the behavior of soil urease enzymes (Sannino and Gianfreda, 2001; Yao et al., 2006). Therefore, changes in soil urease activities may be indicative of and extremely sensitive to changes in soil health (Bremner and Mulvaney, 1978; Dick and Tabatabai, 1992; Jordan et al., 1995). Kinetic parameters of soil urease enzyme (V_{max} and K_M) imply splitting the velocity of enzyme–substrate complexes into enzyme and reaction products and reflect the conjunction affinity between enzyme and substrate (Paulson and Kurtz, 1970; Aliev et al., 1984). Measuring the kinetic parameters of soil urease activity under pesticide-treated soil will further enable the understanding of changes in the substrate affinity and the catalytic activity (Tabatabai, 1973; Speir et al., 1999; Ekberli et al., 2006). Considerable information is available on the relationship between synthetic pesticide application and soil enzyme activities (Khabirov, 1990; Shaffer, 1993; Sannino and Gianfreda, 2001; Gianfreda and Rao, 2011) in laboratory, greenhouse, and field conditions; however, very few studies have focused on the activities and kinetic properties of urease activities in soil treated with natural plant products, such as azadirachtin-treated soils.

This study aims to evaluate the effects of azadirachtin on urease activity and its kinetic parameters in soil by conducting a field experiment.

2. Materials and methods

2.1. Experimental field and climate

The field experiment was conducted at the Experimental Station of Perm State Agricultural Academy, Perm, Russia (57°56′00″N, 56°14′59″E) at an altitude of 127 m above mean sea level. The experimental area has a typical perhumid climate (R_f = 213.6), with temperatures ranging from –33.1 °C in February to 32.5 °C in July. The annual mean temperature is 2.9 °C, and the annual precipitation is 619.5 mm. The data on climatic parameters such as precipitation and temperature during the experiment are shown in Figure 1.

2.2. Soil

The soil at the experimental site is loam (31.4% sand, 45% silt, and 23.6% clay). A composite surface soil sample from 0–20 cm depth was collected from the experimental site before initiating the experiment and was analyzed for physicochemical properties according to Rowell (1996) and Jones (2001). Soil samples were initially air dried at room temperature and subsequently sieved with a <2-mm screen. The basic physicochemical characteristics of the soil are as follows: pH (1:1, soil:water): 6.70; electrical conductivity (1:1, soil:water): 0.81 dS m^{-1}; $CaCO_3$ content: 0.04%; total organic carbon: 0.99%; total nitrogen (Kjeldahl N): 0.086%; available phosphorus (0.5M $NaHCO_3$ extractable P): 13.34 mg kg^{-1}; and exchangeable potassium (1 N NH_4OAc extractable K): 538.98 mg kg^{-1}. The soils had no history of receiving any pesticide treatment in the 6 months prior to this study. The experimental soil was classified as "Albic Luvisol" according to the FAO (2006).

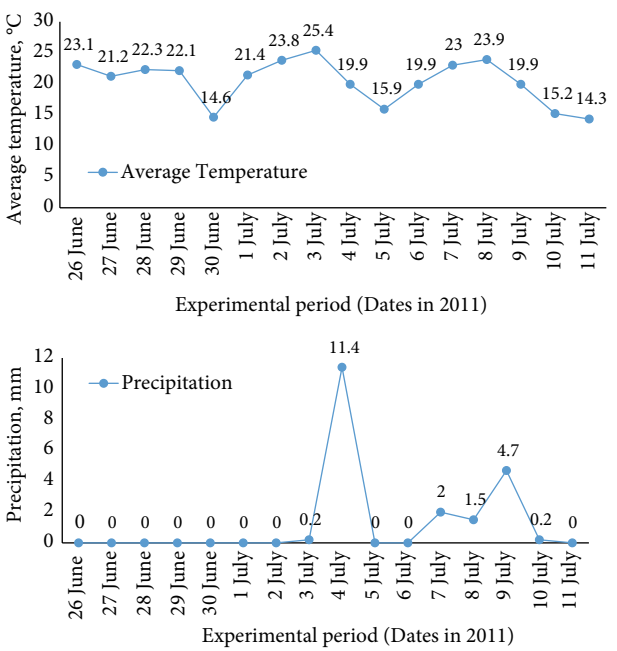

Figure 1. Climatic data in Perm, Russia during the experiment.

2.3. Azadirachtin ($C_{35}H_{44}O_{16}$)

The azadirachtin (NeemAzal–T/S) was imported by VIT-Verim Insaat, Turkey. This insecticide (10 g azadirachtin L^{-1}) was used as a technical-grade chemical and added to the soil. Recommended azadirachtin application dose to control insect pests is 0.25–0.30 L ha^{-1}, according to Akca et al. (2009).

2.4. Field experiment

This experiment was conducted to determine the effects of azadirachtin contamination on soil urease activity and its kinetic parameters under field conditions. The experimental design was a randomized plot design with 3 replications, and was established on 26 June 2011. In the Perm region, it is common to use pesticides to control insect pests during the months of June and July under its conditions of climatic and agricultural practices. The area of each plot was 1 × 1 m. The soil treatments were as follows: (1) control: 0 L azadirachtin ha^{-1}; (2) low application doses: 0.15 L azadirachtin ha^{-1}; (3) recommended application doses: 0.30 L azadirachtin ha^{-1}; and (4) high application doses: 0.60 L azadirachtin ha^{-1}. In order to enable homogeneous application of azadirachtin to the soil, azadirachtin was mixed with 2.5 L of water per m^2. Azadirachtin solution was sprayed on the soil surface with a backpack sprayer (operating pressure 4×10^5 Pa). In addition, no plants were grown in any of the plots. Changes in the urease activities were determined by examining the soil samples taken 14 days after the field experiment was conducted. In soil, extracellular urease enzyme is rapidly adsorbed to a matrix structured by different amounts and types of humic substances as well as clays, and most K_M (Michaelis constant) values therefore vary over a range somewhere between 1.3 and 590 mM, depending on physicochemical properties of the soil and agricultural input such as wastes, fertilizers, and pesticides into the soil. Considering the effect of azadirachtin on soil biological properties (Akca et al., 2005), stability and persistence of soil urease (Bremner and Mulvaney, 1978), and agricultural conditions such as soil properties and application range of pesticides (Cervelle et al., 1976; Martens et al., 1992), the sampling date was established as 14 days.

2.5. Soil sample preparation

Field-moist soil samples were collected only from the surface layer (0–5 cm in depth) and brought to the laboratory in properly labeled and sealed polythene bags. The sieved soil samples (using a <2-mm screen) were homogenized and stored in polyethylene boxes at 4 °C until the analyses were conducted. These acclimatized soil samples were used for analyzing urease activity.

2.6. Assay of urease activity

Urease converts 1 mole of urea to 2 moles of ammonia and 1 mole of CO_2. Urease (EC 3.5.1.5) activity was measured using the method developed by Hoffmann and Teicher (1961). To determine urease activity, 0.25 mL of toluene, 0.75 mL of citrate buffer (pH 6.7), and 1 mL of urea substrate solution were added to 1 g of each of the soil samples; these samples were subsequently incubated at 37 °C. The formation of ammonium was determined spectrophotometrically at 578 nm and the results were expressed as μg N g^{-1} dry soil. All the determinations of urease activities were performed in triplicate, and all the reported values were the averages of the 3 determinations expressed on the basis of oven-dried soil (105 °C).

2.7. Determination of urease kinetics

Kinetic parameters were determined by using 8 different concentrations of the substrate urea, varying from unsaturated to saturated conditions: 0, 0.833, 1.675, 3.330, 6.661, 9.991, 13.211, and 16.651 M (corresponding to 0%, 5%, 10%, 20%, 40%, 60%, 80%, and 100%, respectively) each at different incubation times (1, 2, 3, 6, and 12 h) at 37 °C. The kinetic parameters V_{max} (maximum enzyme velocity) and K_M (substrate affinity constant) were measured using the Michaelis–Menten equation (Cornish-Bowden, 1976; Michaelis and Menten, 1913; Schnell and Maini, 2003), which is expressed as follows:

$$v_0 = \frac{V_{max}[S]_0}{K_M + [S]_0}. \quad (1)$$

The V_{max} and K_M parameters in Eq. (1) were determined by using the STATISTICA 6 software program. In Eq. (1), v_0 is the enzyme initial reaction rate, V_{max} is the maximum enzyme reaction velocity ($V_{max} = k_2 [E]_0$), $[S]_0$ is the initial substrate concentration, $[E]_0$ is the enzyme concentration, k_2 is the maximum substrate molecule number that is converted into substrate in a second by an enzyme molecule and is termed the conversion number (or activity number), and K_M is the Michaelis–Menten constant. K_M indicates the affinity of urease to its specific substrate urea, and provides the substrate concentration at which the reaction rate reaches half of its maximum value ($V_{max}/2$).

The K_M constant is regarded as the substrate concentration, and its reaction velocity is semimaximum. It is also regarded as an affinity measure (inclination, power, and attention) of attachment to enzyme; a small K_M value shows high affinity. In other words, the K_M constant expresses that enzyme reaction velocity reaches V_{max} faster (Lehninger et al., 2005). Moreover, it is also possible to calculate the necessary substrate $[S]_0$ concentration in order to reach maximum V_{max} velocity if the K_M value of any enzymatic reaction is known. V_{max}/K_M rate indicates how productive the enzyme is in converting substrate into product. At the settings in which the V_{max}/K_M rate is high, the enzymes are termed "superproductive".

2.8. Statistical analysis

All data were analyzed using SPSS 11.0 (SPSS Inc.). Analysis of variance (ANOVA) was carried out using 2-factor randomized complete plot design; where significant F-values were obtained, differences between individual means were tested using the LSD (least significant difference) test, with a significance level of $P < 0.01$. All figures presented include standard deviations of the data and F-values. The asterisks *, **, and *** indicate significance at $P < 0.05$, 0.01, and 0.001, respectively.

3. Results and discussion

Pesticides and other xenobiotic substances may present direct (either reversible or irreversible) and indirect effects on enzyme activities in soil (Kızılkaya and Arcak, 1996). Although pesticide molecules are not deliberately synthesized to inhibit enzymes, a direct reversible inhibition of enzyme activities in soil may occur because of reversible interactions of the pesticide with soil enzymes, resulting in possible competitive and/or noncompetitive substrate inhibition or alteration of the protein conformation. If the pesticide molecule is degraded in intermediate metabolites by biotic or abiotic transformation, a similar effect could be shown by its degradation products. On the other hand, indirect effects are the consequence of the influence that pesticides may have on soil microbial populations and their activity. Pesticides may induce detectable changes in size, structure, and functionality of the microbial community, thereby altering life functions, dynamics, and biodiversity of soil organisms (Kızılkaya and Aksoy, 1999; Gianfreda and Rao, 2011).

In this study, azadirachtin was introduced in the soil in increasing doses of 0, 0.15, 0.30, and 0.60 L ha^{-1} in the field experiment. This was done in order to determine the change that the introduction of azadirachtin creates in the urease activity of the soil and in the kinetic parameters of urease enzyme after 14 days. The findings obtained at different substrate concentrations are shown in Figure 2. The results indicated that azadirachtin increases urease activity in the soil according to application, and the highest increase in urease activity was found at the highest application dose (0.60 L ha^{-1}).

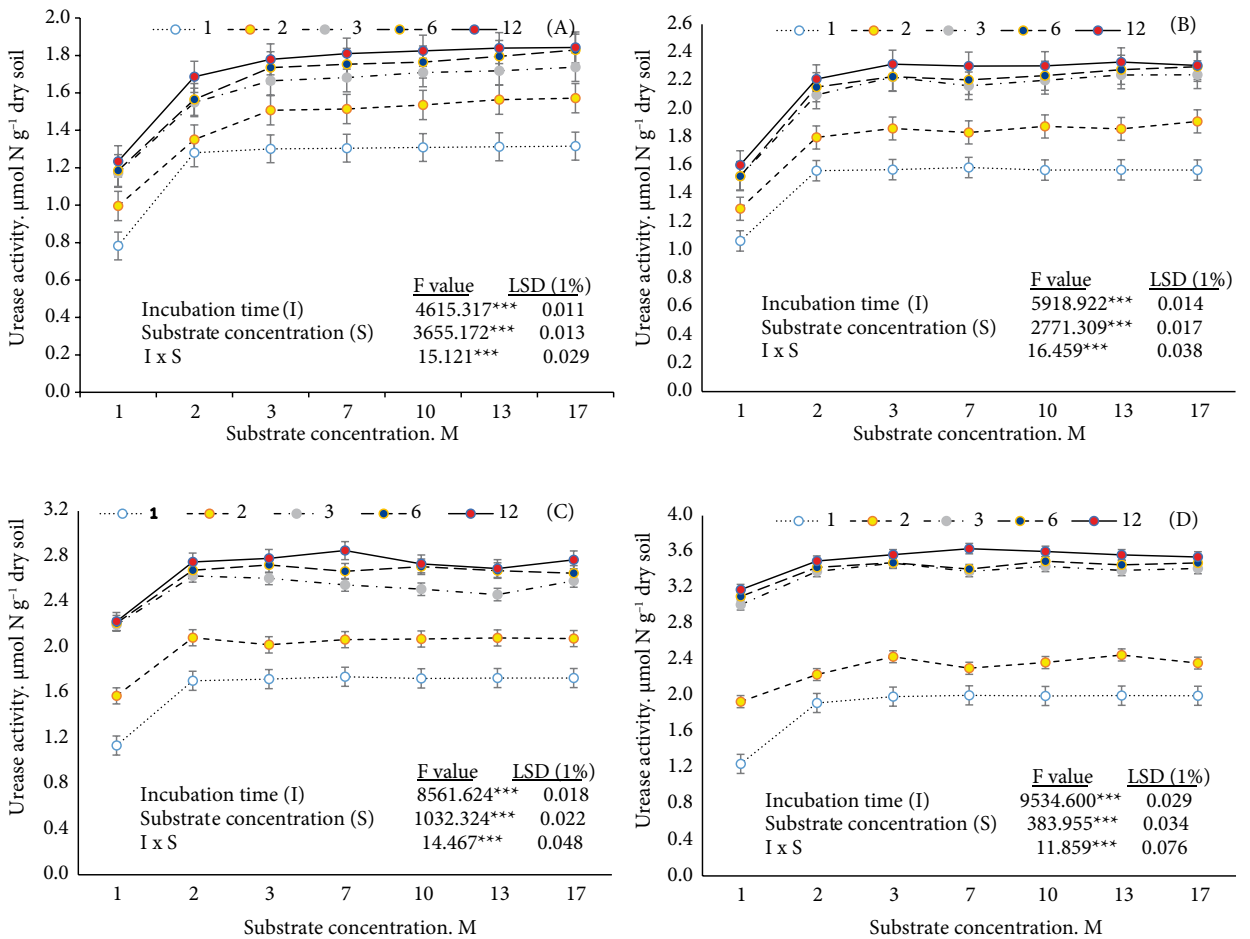

Figure 2. Changes in urease activity in the soil, depending on the incubation time (t = 1, 2, 3, 6, and 12 h), the concentration substrate, and azadirachtin application doses: (A) – control; (B) – 0.15 L ha^{-1}; (C) – 0.30 L ha^{-1}; (D) – 0.60 L ha^{-1}.

Considerable research has been conducted regarding the effects of pesticides on soil enzyme activities (Gianfreda et al., 1995; Megharaj et al., 1999; Sannino and Gianfreda, 2001; Yao et al., 2006), and the findings suggest that the responses of soil enzymes to different pesticides vary. Yang-Fang et al. (2004) found that although mefenacet strongly inhibited soil urease activity in a concentration-dependent manner, it stimulated phosphatase activity. Unfortunately, since azadirachtin is a newer pesticide, there is no extant information regarding its influence on intracellular and/or extracellular enzymes in soil. The present study indicates that increasing doses of azadirachtin stimulated urease activities instead of inhibiting them.

In general, as compared to the control treatment, low doses of azadirachtin application yielded low amounts of urease activity, whereas high doses of azadirachtin application yielded high amounts of urease activity. The reason for the stimulating effects of azadirachtin on urease activity at all application doses can be attributed to the effects of pesticide degradation of azadirachtin, such as di- and triterpenoidal compounds (Sing, 1991), which hinder the use of azadirachtin as a substrate and energy source. A pesticide has 2 effects on soil microflora and their activities. First, because of its physiological activity, a pesticide can influence the microorganisms that are responsible for its degradation. This action may also influence the organisms that are involved in the degradation of other substances. Second, a pesticide also acts as a substrate for the soil microflora. In fact, its degradation may supply certain organisms with the carbon, the energy, and occasionally, the nitrogen that is necessary for their growth. In any case, the most important consequences of these 2 aspects essentially appear in relation to pesticide degradation (Simon-Sylvestre and Fournier, 1979; Akça et al., 2005).

While determining the level of urease activity in the soil, we found that urease activity increased as the incubation time increased. In all azadirachtin applications, the highest urease activity was obtained for the 12-h incubation period. Similarly, we found important increases in the urease activity as the concentration of the substrate solution increased; a slight increase was also registered in urease activity for substrate concentrations above 3.329 M (20% urea).

Urease activity at all incubation times, control applications (Figure 2), substrate concentration of 20% (3.329 M urea), and low, suggested, and high azadirachtin application doses showed a fixation tendency at substrate concentrations of 10% and 20%.

Both effect mechanisms and effectors of enzymes such as inhibitors or activators affected urease enzyme activity. Determination of kinetic parameters such as V_{max} and K_M is important for revealing these effects transparently. For determining these parameters, first, the initial velocity (v_0) of the reaction that the enzyme catalyzed should be determined. One of the basic problems of enzyme kinetics is that the value of initial velocity (v_0) of enzyme is determined according to time. Determining the initial velocity is quite important for preventing various factors from affecting the kinetics of enzyme reaction. The probability that undesirable factors will affect the reaction in the beginning is quite low; however, this problem can be resolved by using 2 different methods, one of which is an empirical (graphic and differential) method and the other an analytical method. The results obtained using the analytical method are more accurate than those obtained using the empirical method. After determining the equation of the kinetic curve by using the experimental data, the value of the initial velocity can be easily found by using the following expression: $v_0 = d\left[P(t)\right]/dt\big|_{t=0}$ (Cornish-Bowden, 1976; Aliev et al., 1984; Mikayilov, 2011).

In order to determine the initial velocity (v_0) of urease enzymes, different doses of azadirachtin were applied to the soil. Subsequently, the kinetic curve, v = [P(t)], was obtained from the urease activity results (Figure 2), which were determined on the basis of soil samples taken from parcels at increasing substrate concentrations and at different incubation periods. Following this, the analytical expression of this kinetic curve for each substrate concentration was determined by using the STATISTICA 6 software program. It was found that the most suitable model was a hyperbolic model, as expressed by Eq. (2):

$$P(t) = \frac{at}{b+t} \quad . \tag{2}$$

a, b parameters and hyperbolic model parameters in Eq. (2) and the initial velocities (v_0) of enzyme reactions were calculated using Eq. (3):

$$v_0 = \left.\frac{dP}{dt}\right|_{t=0} = \frac{a}{b} . \tag{3}$$

After azadirachtin applications, a hyperbolic relationship between substrate concentration and initial value (v_0) was determined at the control level and increasing levels of application. The results indicated that the beginning value increased (Figure 3) according to the increase in substrate concentration, and it stabilized at substrate concentrations greater than 3.3 M (Figure 3).

By using the beginning velocity values in Figure 3, the kinetic parameters of urease enzyme (V_{max}, K_M, and V_{max}/K_M) were determined; the results obtained are presented in the Table.

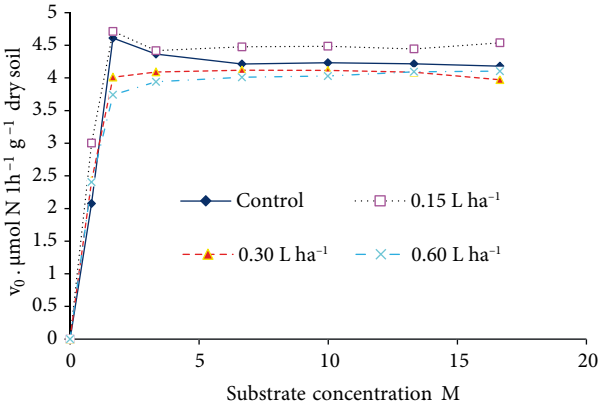

Figure 3. Change in the initial velocity (v_0) of urease enzyme reaction according to substrate concentration (M) and azadirachtin application doses.

Table. Kinetic parameters and kinetic model accuracy.

Azadirachtin application doses	V_{max} $\frac{\mu \text{ mol N}}{\text{h·gram dry soil}}$	K_M M	V_{max}/K_M $\frac{\mu\text{mol N}}{\text{h·gram dry soil·M}}$	η	σ	$\bar{\varepsilon}$ %
Control, 0 L ha^{-1}	4.5567	0.4626	9.8493	0.9418	0.5866	10.82
Low doses, 0.15 L ha^{-1}	4.7058	0.2958	15.9064	0.9782	0.3615	5.13
Recommended doses, 0.30 L ha^{-1}	4.3266	0.4143	10.4429	0.9783	0.3295	6.22
High doses, 0.60 L ha^{-1}	4.3105	0.4780	9.0187	0.9892	0.2291	4.37

η the correlation ratio;
σ standard error of the estimate;
$\bar{\varepsilon}$ the average relative error of approximation.

V_{max} shows the velocity of decomposition of enzyme–substrate complex into enzyme and reaction products. The value of V_{max} is a potential indicator that indicates the speed of the enzymatic processes in the soil; the higher the value, the faster the process and vice versa (Paulson and Kurtz, 1970). The maximum V_{max} value was obtained at the low azadirachtin application dose (0.15 L ha^{-1}). This situation shows that urease activity processes at low doses of azadirachtin application occur more quickly than those at the control dose and other dose applications.

K_M expresses the strength of enzyme–substrate complex. If the K_M value is high, the strength of the enzyme–substrate complex is low. If the K_M value is low, the strength of the enzyme–substrate complex is high (Masciandaro et al., 2000). K_M value was obtained at the low azadirachtin application dose (0.15 L ha^{-1}). This reveals that the strength of the enzyme–substrate complex at low azadirachtin application doses is greater than that at the control dose and other application doses.

V_{max}/K_M expresses the comparison of product constitution from this complex, which occurs when enzyme–substrate complex occurs in the soil. A high V_{max}/K_M rate shows that the dispersion of enzyme–substrate complex is quicker than the constitution of this complex (Masciandaro et al., 2000). In the field experiment, the highest V_{max}/K_M rate was obtained at the low azadirachtin application dose (0.15 L ha^{-1}). This reveals that enzyme–substrate complex occurring at low azadirachtin doses forms products more quickly than at the control dose and the other application doses.

The ratio V_{max}/K_M (so-called ferment efficiency) determines the urease hydrolysis rate

$$CO(NH_2)_2 + H_2O \xrightarrow[+H_2O]{urease} 2NH_3 + CO_2$$

at realistic substrate concentrations. Using a 0.15 L ha^{-1} azadirachtin dose led to the increase of the urease ferment efficiency in soil by about 1.5-fold as compared with the control and with higher doses of azadirachtin (Table).

The variances in kinetic parameters of urease occur as a result of an increase in V_{max}, K_M, and V_{max}/K_M due to variations in physicochemical properties of the soil (Dick et al., 1994), agricultural practices such as fertilizer and pesticide use (Gianfreda et al., 1994), and synthesis of urease enzyme by increasing microbial population. Urease activity showed a positive correlation with N availability, which indicated that this enzyme can be used to make some inferences about the nitrification process in soil and determine if N losses are due to volatilization, nitrification, or denitrification (Kujur and Kumar Patel, 2014). Urease has been widely used for soil quality assessment, since its activity increases with organic fertilization and decreases with synthetic pesticide application on soil (Gianfreda et al., 1994). Further, its stability is affected by organomineral complexes and humic substances (Makoi and Ndakidemi, 2008). Soil urease enzymes exhibited typical Michaelis–Menten kinetic behaviors, and high azadirachtin doses increased the K_M and decreased the maximum reaction rates (V_{max}) of soil urease. As compared to the control, the low azadirachtin application dose increased the V_{max} of soil urease; however, with low and recommended application doses, the V_{max} of soil urease decreased. V_{max}/K_M has been considered as an index of the catalytic capacity of enzyme through enzymatic reactions. The highest V_{max}/K_M rate was obtained at the low azadirachtin application dose, indicating an increase of catalytic ability in the urease enzyme.

We conclude that there exists a strong relationship between soil urease activities and the application of natural plant products such as azadirachtin in short-term field experiments. Moreover, activities of soil urease enzymes that are sensitive to pest control practices may have the potential to be used as indicators of soil quality and sustainability. Additionally, all kinetic parameters that were adaptive in Albic Luvisol (Perm, Russia) can be used as indicators for monitoring soil health and quality. Despite the fact that only one type of soil was studied in this research, the results obtained are of significance for drawing reliable conclusions of general interest, which are presumably extendable to other soils and agricultural management practices.

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