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SHORT COMMUNICATION

Watercress *(Nasturtium officinale)* as a Novel Plant-based Alternative to Synthetic Soil Urease Inhibitor Sources

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Abstract

In order to achieve growth in agricultural production, nitrogen fertilisers are widely used. The urease enzyme, present in soil, vastly accelerates the pace of nitrogen transformation into plant-available forms. Rapid acceleration causes high nitrogen losses and the products of said reactions (e.g., greenhouse gases) which are released into the environment (Hube et al., Agric Ecosyst Environ: 46–54, 2017). Many countries have imposed regulations and laws regarding the compulsory use of urease inhibitory precautions when fertilising with urea. However, the inhibitors in use involve a high cost and new environmental risks. In this study, watercress (*Nasturium officinale*) is presented and investigated as an alternative. Ultraviolet-visible spectroscopy (UV-Vis) Han's method, high-performance liquid chromatography mass spectrometry (HPLC-MS) and proton nuclear magnetic resonance (^{1}H-NMR). An organosulfur compound dimethyl thiosulfinate (DMTS) was recognised in watercress extract, DMTS concentration was assessed and its inhibitory influence on native and soil ureases confirmed. The urease inhibition was of competitive character. Watercress have potential use as a natural source of urease inhibitor both in agriculture and other branches.

Keywords Thiosulfinates · Urea hydrolysis · Sustainable agriculture · Competitive inhibition · Plant-origin inhibitor

1 Introduction

Urea-based fertilisers are among the most commonly used both as natural and as synthetic fertilisers (Cantarella et al. 2018). The global production capacity of urea was 210 million metric tonnes in 2018 (Matczuk and Siczek 2021). Due to the activity of the urease enzyme (EC 3.5.1.5; urea amidohydrolase) naturally occurring in the soil, urea decomposes up to 10^{14} times faster in comparison to non-enzymatic catalysts (Zaman et al. 2008). Urease is the first to be crystallised and the most efficient enzyme known (Callahan et al. 2005; Mazzei et al. 2019). Due to its activity, the urea hydrolysis proceeds faster than plants can uptake nutrients (Näsholm et al. 2009). In consequence, gaseous and ionic pollutants such as NH_3, CO_2, N_2O, NO, NO_2^-, and NO_3^- penetrate the atmosphere and groundwater (Jadoski et al. 2010; Modolo et al. 2015; Hube et al. 2017). Nitrogen leaching is comparable to manure and mineral fertiliser use (Rashid et al. 2022). Cases of widely observed complex environmental risks have been reported (Guo et al. 2010). In addition to direct pollution generated from the fields, the environmental costs of overproduction and the need for costly additional agrotechnical treatments to substitute the lost fertiliser are a major concern. There are currently many preventative regulations in place and in addition to those, new tools and solutions need to be developed. One type of preventive action is soil urease inhibition by enzyme inhibitors. In temperate climate conditions, the nitrogen loss was substantial enough that urease inhibitors or urea fertiliser coatings became mandatory for use in the EU (Byrne et al. 2020). The solutions developed commercially still cause undesirable consequences. Bio-based sources are a promising solution.

Some organosulfur compounds present in different plants were proven to inhibit urease activity (Modolo et al. 2015; Hassan and Žemlička 2016). The described plant-based sources of urease inhibitors are calorie-dense, and their cultivation requires demanding conditions and heavy fertilisation

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(Sardi and Timár 2005). Such is the case of garlic, onions, and cabbage that, due to the cultural inclinations and in strictly economic terms, create doubts regarding their use for urease inhibition purposes. Some plants from the Brassicaceae family (Klimek-Szczykutowicz et al. 2018) contain thiosulfinates (TS) that are known for their urease-inhibiting properties (Agelopoulos and Keller 1994). In the search for plant-based sources of inhibitors, watercress (*Nasturtium officinale*) appears to be a reasonable alternative. It is edible, although it has low nutritional value. Moreover, watercress can be cultivated on floodplains and wasteland. In temperate climate conditions, watercress flourishes in the wild without any supervision and receives little-to-no attention from the public. It remains green most of the year (Haslam 1987).

2 Materials and Methods

2.1 Materials

2.1.1 Watercress Extract

The watercress plants were sourced from a nature reserve (naturally growing) in southern Poland (50° 16 N, 19° 63 E). The watercress extract was prepared directly before testing, in proportions of 3 g of wet mass of watercress to 10 mL of water.

2.1.2 Soil

A topsoil sample was collected from forested lands in close vicinity to Krakow in southern Poland (50° 02 N 19° 54 E). Soil was air-dried at room temperature and sieved through a 2-mm sieve.

2.2 Identification of Thiosulfinates

2.2.1 High-performance Liquid Chromatography Mass Spectrometry

A Perkin-Elmer Clarus 500 gas chromatography system equipped with a Clarus 600C quadrupole mass detector was used. The separation was performed on an HP-5MS column. The flow rate of carrier gas (99.9999% He) was 1 mL/min; initial inlet temperature, 65°C maintained for 1.5 min, then increased at a rate of 15°C/min to 290°C; column temperature: initial 40°C (2-min hold), 15°C/min, 250°C held 29 min; and injection volume, 250 µL of headspace gas using splitless mode. The transfer line was kept at a temperature of 310°C; ion source and quadrupole temperatures, 170°C. The chromatograms were recorded in electronic impact ionisation mode at 70 eV and full scan mode covering 50–250 m/z mass range. Identification was performed based on the Wiley 8th edition library.

2.2.2 Nuclear Magnetic Resonance Spectroscopy

Proton Nuclear Magnetic Resonance (^{1}H NMR) data was obtained at room temperature with a Bruker 300 MHz spectrometer in $CDCl_3$.

2.3 Thiosulfinates Concentration

TS concentration was determined by spectrophotometric Han's method (Han et al. 1995; Olech et al. 2014). Absorbance measurements were performed using the Marcel Media UV-Vis spectrophotometer.

2.4 Inhibition of Urea Hydrolysis

The hydrolysis of urea catalysed by native jack bean urease was studied in a 50 mM phosphate buffer of pH 7.4 and 2 mM EDTA at 25°C. The initial concentration of urea was 50 mM, while the final concentration of urease was 0.1 mg/mL. The amount of ammonia was determined by the modified Berthelot colorimetric method (Cordero et al. 2019; Krom 1980; Weatherburn 1967). Absorbance was measured at 690 nm. The experiment was carried out followed by a 15-min preincubation of enzyme with different concentrations of inhibitor. The urease activity was determined by measuring the ammonia concentration.

2.5 Determination of the Soil Urease Activity

The soil urease activity was determined spectrophotometrically by concentration of ammonia using a modified Berthelot's method (Kandeler and Gerber 1988; Thongkam et al. 2020).

3 Results and Discussion

3.1 Thiosulfiniates in Watercress Extract

The ^{1}H-NMR spectrum registered for the deutered chloroform extract of watercress (Fig. 1) is typical for complex plant extract (Kim et al. 2010)

In the ^{1}H-NMR ($CDCl_3$, 300 MHz) spectra registered, there are signals observed at $\delta = 2.65$ (3H, s, –S–CH_3) and $\delta = 2.98$ (3H, s, –S(O)–CH_3). Of the methyl methanothiosulfinate isolated from the plant-based extract, Seo et al. (2001) observed in the ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) two singlets belonging to the protons of methyl groups. The signal of the value of the chemical shift (circa 3.00 ppm) is assigned to the methyl group joint with –S=O group. The signal at circa 2.70 ppm is attributed to the protons of the

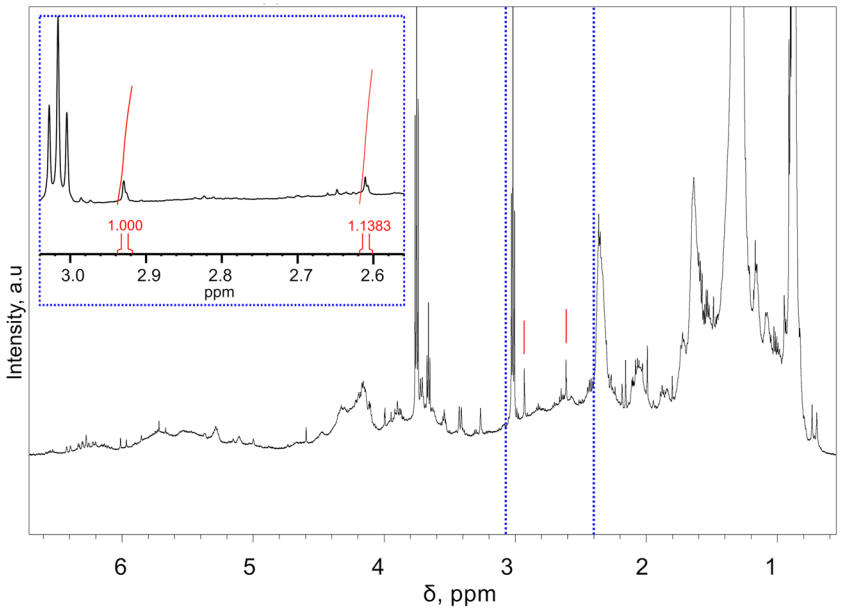

Fig. 1 ^{1}H-NMR spectra of the chloroform extract of watercress

methyl group joint with the sulphur atom (–S–). The difference in chemical shifts between Seo et al. and our study results (Fig. 1) is due to the deshielding effect being more potent in the case of H_3C–S(O)– in comparison (to H_3C–S–).

The watercress extract was additionally analysed with HPLC-MS. The S-dimethyl thiosulfinate (DMTS) was not identified, but products of subsequent reactions that took place after the initial reactions are visible. Those subsequent reactions lead to the production of CH_3–S(O)–S(O)–CH_3, dimethyltiosulfonate and dimethyldisulfide which are the reaction products visible in the spectra acquired (Boyd et al. 2014; Chin and Lindsay 1994; Friedrich et al. 2022). The leading factor for this might be the high pressure applied during the procedure, and thus TS are prone to disintegrate under increased pressures (Small et al. 1947).

The watercress extract was found to have a total TS concentration of (0.96 ± 0.15) mM, which corresponds to (0.35 ± 0.06) mg DMTS/g of the plant fresh mass. In Brassicale genus plants, TS can only originate from S-methylsulfoxide. This is the reason behind the potentially low variety of TS in those plants. The formation of TS is connected to the mechanical tearing of the plant (Freitas et al. 2019). Cysteine sulfoxide lyases are stored in plant vacuoles (Lancaster et al. 2000). They are released as a defensive mechanism, and they decompose S-methylcysteine sulfoxide to pyruvate, ammonia, and methanesulfenic acid. The last product, apart from being the subject of chemical disproportionation, is also the subject of the process of dimerisation and redox, of which the product is DMTS (Friedrich et al. 2022).

The TS concentration of watercress extract (0.96 ± 0.15) mM is comparable to the TS concentration in white cabbage sap (1.2 ± 0.2) mM and is less than measured in brussels sprout sap (3.0 ± 0.2) mM (Olech et al. 2014). The result of the determination of the concentration of TS in the watercress extract in addition to the HPLC-MS and ^{1}H-NMR spectra confirmed the presence of TS in the plant. The TS concentration in the plant's fresh weight, determined to be 0.35 ± 0.06 mg DMTS/g, was found to be sufficient for urease inhibition trials.

3.2 Inhibition of Native Urease by Watercress Extract

Certain bio-based organosulfur compounds are proven to act as urease inhibitors (Juszkiewicz et al. 2004; Olech et al. 2014; Salehuddin et al. 2021) due to the affinity of their structure to the urease active centre structure (Ni^{2+} ions and sulfhydryl group). Organosulfur compounds proven to have an inhibitory effect on urease are allicin, diallyl sulphide, and diallyl disulphide.

To determine the inhibitory effect, the mechanism of urease inhibition, and the possibility of future optimisation of watercress extract use, the effect of different concentrations of substrate (Fig. 2) and inhibitor (Fig. 4) were analysed in time function.

The course of the reaction without the addition of the inhibitor corresponds to the course of the typical enzymatic reaction of a Michaelian enzyme (Goldbeter 2013). Thus, the reaction rate rises logarithmically, per the increase of substrate concentration.

The plot of the reciprocal of initial velocity of the reaction (1/V) versus the reciprocal of the substrate concentration (1/[S]) was used to determine the type of enzyme inhibition

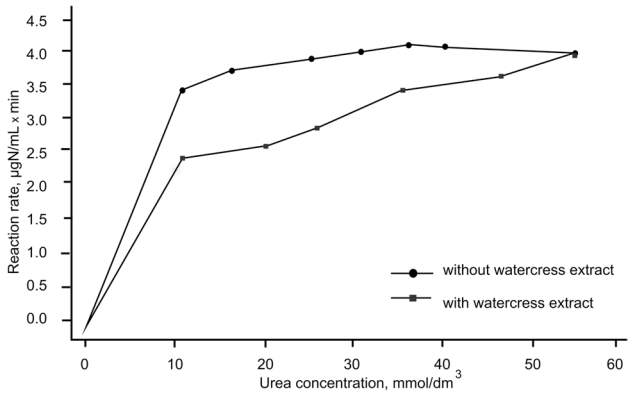

Fig. 2 Michaelis–Menten saturation curves for urea hydrolysis

(Figs. 3 and 4) and to calculate the kinetic parameters. The Lineweaver–Burk equation (Segel 1980; Wang et al. 2020) was used.

For the linear function, the conduction process of the reaction with the use of the inhibitor kinetic parameter was described as follows: $K_{mi} = 7.38$ mM. Similarly, the K_m value was calculated for the reaction without the inhibitor addition: $K_m = 2.70$ mM.

The more inhibitor added, the less active the urease is in comparison to the basic activity (Fig. 5). After 30 min, the activity decreases up to 80%, the decrease in activity is less significant with every dilution of the extract.

The inhibition is of reversible and competitive type ($K_{mi} > K_m$ and $V_{maxi} = V_{max}$) or of a mixed competitive–non-competitive (based L-B) character with the dominance of the competitive component. For the determined K_{mi} and K_m, half of the maximum speed is reached with a concentration 2.73 times lower than in terms of the reaction without the addition of the extract.

By interpreting the Lineweaver–Burk plot, apart from the competitive inhibition, which can be explained by DMTS–urease complex formation, the course of the curves may suggest a mixed (competitive–non-competitive

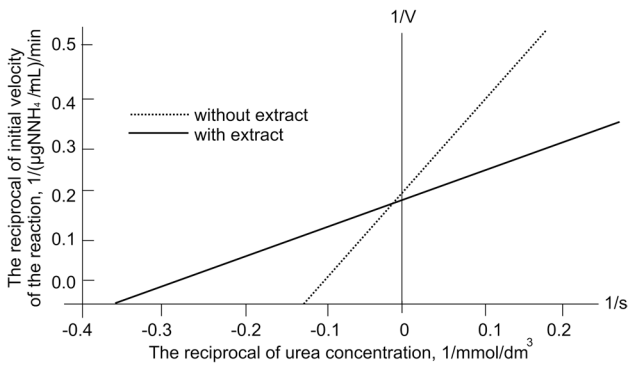

Fig. 3 The Lineweaver-Burk plot for urease inhibition

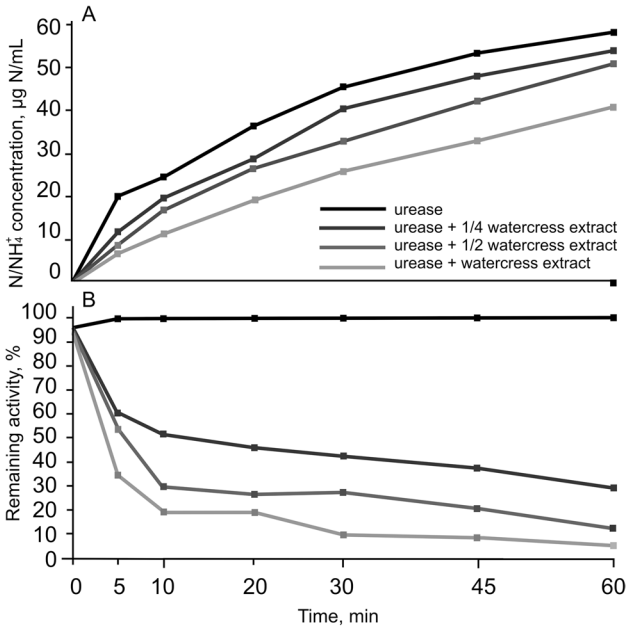

Fig. 4 Watercress extract effect on urease activity: **A** progress curves of urease-catalysed hydrolysis of urea carried out in the presence of watercress extract; **B** change of remaining urease activity for different inhibitor concentration during the reaction

character) inhibition. The mixed inhibition implies that an inhibitor particle can bind to the enzyme both in the active site of the enzyme as well as to the inhibitor–enzyme complex. An argument for a mixed, and not only competitive inhibition hypothesis is the intersection of the curves being slightly shifted towards the *X*-axis. Competitive inhibition is supported by the structure of active site versus the structure of the DMTS molecule; yet, there is still a possibility that another ingredient of the watercress extract functions as an inhibitor due to binding itself to the enzyme. Furthermore, DMTS could interact with the extract resulting with other organosulfur compound formation.

The inhibitory effect of watercress extract was first observed in the study of different herbs used in traditional

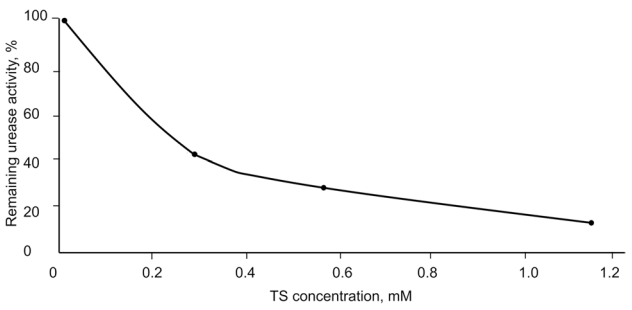

Fig. 5 The effect of inhibitor concentration on urease activity after 30 min of inhibition

medicine for potential *Helicobacter pylori* bacterial infection treatment (Biglar et al. 2012). The authors hypothesised that phenethyl isothiocyanate might be responsible for the inhibitory effect; however, DMTS was not identified in the watercress extract to date, and its presence offers simpler explanation for the inhibitory effect of watercress.

3.3 Watercress Extract as Soil Urea Inhibitors Source

The ammonia generation in soil without the addition of the inhibitor equals $c = 197.59$ mg N_{NH4+}/(kg of soil·24 h), and for the soil incubated with the watercress extract: $c_i = 156.13$ mg N_{NH4+}/(kg of soil·24 h). The watercress extract lowers the initial activity of the soil urease enzyme by 20%.

In cabbage sap, DMTS concentration is only marginally higher than in the water diluted watercress extract—1.2 mmol/dm^3 (Olech et al. 2014). Watercress leaves contain higher levels of DMTS than cabbage leaves and less TS than garlic cloves which have the highest TS concentration—30 times compared to the diluted watercress sap. The TS present in garlic is allicin. Garlic is presented as an alternative source of soil urease inhibitors (Upadhyay 2012), as allicin can reduce the catalytic capacity of soil urease by 40% (Mathialagan et al. 2019).

Allicin is sparingly soluble and therefore more prone to volatilisation with less even distribution in soil fluids. Apart from the energy and resource consumption for garlic production (Samavatean et al. 2011), garlic contains 13.5 times more calories than watercress (U.S. Department of Agriculture), being less optimal from an energy balance perspective. Watercress's phytoremediation potential was examined (Kara 2002; Mustafa and Hayder 2021) and the plant does not accumulate pollutants at a high rate (Kara 2002); therefore, there is a possibility to use floodplains with no value to agricultural activity for cultivation.

4 Conclusions

Using proton nuclear magnetic resonance (^{1}H NMR) and ultraviolet-visible (UV-Vis) spectrophotometry, the presence of dimethyl thiosulfinate (DMTS) with a concentration of 0.96 ± 0.15 mmol/dm^3 has been confirmed in the watercress extract. The native urease inhibition mechanism by thiosulfinates (TS) is competitive and reversible, and the inhibition by watercress water extract is mixed competitive–uncompetitive, with a major dominance of the competitive component. The extract also has an inhibitory effect on urease in complex soil matrix. Plant-based organosulfur compounds offer a promising, cost-effective alternative to optimise nitrogen fertilisation and lower its negative environmental impact.

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Declarations

Conflict of interest The authors declare no competing interests.

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