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Optimisation of diallyl disulfide concentration and effect of soil condition on urease inhibition

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Abstract: Diallyl disulfide (DADS) is an organosulfur compound that is expected to exhibit inhibitory property against urease similar to allicin, affirmed through preliminary study. The research aims to optimize DADS's concentration and duration of inhibition and observe the effect of soil moisture, temperature and pH on the inhibitory action of DADS. The calorimetric method was applied to optimize DADS's concentration significant for inhibition. High-performance liquid chromatography was used to quantify DADS present under different parameters relevant to selected soil conditions. The results obtained suggested that 5% of DADS/urea-N (*w/w*) treatment exhibited the highest urea hydrolysis reduction by 27.91% compared to the control sample at the end of 30 days. ANOVA results observed urea hydrolysis is significantly slower by applying 5% DADS/urea-N (*w/w*) treatment compared to the other DADS treatments. DADS also retained its original form longer in soil when the soil conditions were altered to 15% moisture content, 20 °C and pH 4. The findings exhibit the potential of DADS as a natural based inhibitor that is effective at low concentrations, compatible with urea and chemically stable.

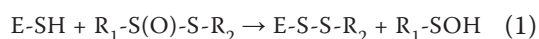
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Urea attributes to 46% of the global nitrogen fertiliser consumption (Sheo Bachan Upadhyay 2012). The growth in the world population caused an increase in fertiliser demand to sustain the increasing food demand (Shaviv and Mikkelsen 1993, Artola et al. 2011). However, the increment in nitrogen use has caused the nitrogen-response efficiency to drop by more than 50% as urea hydrolysis occurs at an estimated rate of 10^{14} times the rate of an uncatalysed reaction due to soil urease (Sheo Bachan Upadhyay 2012, Mathialagan et al. 2017). This results in the production of ammonia (NH₃) and carbon dioxide (CO₂) excessively which causes NH₃ volatilisation and nitrate (NO₃⁻) leaching that has adverse effects on the air and water quality (Gerbens-Leenes et al. 2002, Krajewska 2009, Cameron et al. 2013).

Chemical-based urease inhibitors (UIs) such as N-butyl thiophosphorictriamide (NBPT) have been introduced to enhance nitrogen use efficiency (Halvorson et al. 2014). Garlic is one of many natural extracts that has been identified as an anti-urease agent due to its high thiosulfinate content. Allicin is a primary organosulfur compound in garlic which contributes to its relatively high thiosulfinate content (Olech et al. 2014). However, allicin tends to decompose into other poly-sulfur compounds at elevated temperature hence, affecting its inhibitory property (Iberl et al. 1990). Anti-urease properties of allicin and other organosulfur compounds have been observed in the agricultural and medical fields (Juszkiewicz et al. 2004, Mathialagan et al. 2017).

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Alliin has been proven to inhibit the enzymatic activity of sulfhydryl (-SH) containing enzymes by a thiol-disulfide interaction which leads to the formation of a disulfide compound (Eq. 1) (Rabinkov et al. 1998, Olech et al. 2014):



where: R_1 and R_2 – methyl, propyl, 1-propenyl or 2-propenyl group; E – urease.

Considering the circumstances, it is possible that the presence of the disulfide bond is crucial for inhibition. This hypothesis might not farfetched as the disulfide bond is present in both alliin and DATS, both of which have exhibited inhibitory action against urease.

The incorporation of urease inhibitors is an effective method to control urea hydrolysis. However, most of the commercialised urease inhibitors are chemical-based, which causes other environmental concerns. Considering the structure of DADS is similar to alliin and DATS, also the more stable nature of DADS, it is hypothesised that DADS will show similar inhibitory actions (Naganawa et al. 1996, Olech et al. 2014). The objectives of the research are to optimize DADS's concentration and duration of inhibition and to study the effects of selected soil moisture, temperature, and pH parameters on the inhibitory action of DADS against soil urease.

MATERIAL AND METHODS

Chemicals and equipment. Chemicals: urea prills; diallyl disulfide (DADS); potassium chloride (KCl); phenylmercuric acetate (PMA); diacetyl monoxime (DAM); thiosemicarbazide (TSC); phosphoric acid (H_3PO_4); sulfuric acid (H_2SO_4); methanol (CH_3OH); acetonitrile (CH_3CN); tetrahydrofuran (C_4H_8O); sodium hydroxide (NaOH).

Equipment: UV-Vis spectrophotometer (UV 1800 from Shimadzu, Kyoto, Japan), moisture analyser (HX204 from Mettler Toledo, Switzerland), high-performance liquid chromatography (Agilent 1100 Series from Agilent Technologies, Santa Clara, USA).

Soil sampling. Untreated agricultural soil was acquired from Kompleks Pertanian Titi Gantung in Malaysia (4°36'10.21"N; 100°84'93.8"E) and cleared of plant roots and debris. Then, the soil was grounded and sieved through a 2 mm screen (Dawar et al. 2012). The properties of the soil used are as shown in Table 1.

The soil pH level was tested with a pH meter. The total nitrogen content of the soil was determined

using the micro-Kjeldahl procedure. Organic carbon was identified using the Walkley-Black procedure. The texture of the soil was classified based on the separation of the mineral parts of the soil into various size fractions and by which the proportions of these fractions were determined. The cation exchange capacity (CEC) value was tested using the ammonium acetate method.

Optimization of DADS's concentration and duration of inhibition

Soil sample preparation. The soil moisture level was adjusted corresponding to field conditions. Next, soil samples were prepared in which a standard rate of 1 000 μg urea-N per kg of soil was applied. DADS was applied at different concentrations (2, 5, 10, 15% DADS/urea-N (*w/w*)). A control sample containing no DADS was also prepared. Three replicates were prepared for each treatment.

Inhibition test. Samples on days 1, 3, 5, 7, 10, 14, 25 and 30 were collected. On respective days, the soil samples were mixed with 100 mL of 2 mol/L KCl-PMA solution and put in the mechanical shaker at 150 rpm for an hour and filtered. The filtrate was collected for further analysis.

Analysis to test urea-N presence. The DAM calorimetric method was introduced to test the presence of urea-N in the sample based on the study by Mulvaney and Bremner (1979) and Mohanty et al. (2008) with some modification. To quantify the urea-N present in the samples, the calibration curve was prepared. A calibration curve with regression, $R^2 = 0.9974$, was obtained. The percentage of urea-N hydrolysed over 30 days was calculated with respect to the amount of urea-N retained in the soil. One-

Table 1. Properties of soil used

Parameter	Value
Soil pH	6.59
Total nitrogen	0.53%
Organic carbon	9.27%
Texture	Clay loam
Coarse sand	13.3%
Fine sand	7.2%
Silt	39.3%
Clay	40.2%
Moisture	15.6%
Cation exchange capacity	28.2 cmol_+/kg

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way analysis of variance (ANOVA) was applied and the least significant differences (*LSD*) were observed when the treatment effect was significant at $P < 0.05$ *LSD* using Statistical Analysis System (version 9.1).

Effect of soil moisture, temperature and pH on the inhibitory property of DADS

Soil sample preparation. Soil pH was altered to 4, 6, and 8 by using sulfuric acid and sodium hydroxide. Soil moisture content was altered to 15, 30 and 50% using a Mettler Toledo Moisture Analyser. Soil samples for temperature parameters were placed inside incubators in which the temperature was set to 20, 30 and 40 °C. The standard rate of urea-N and DADS with pre-determined concentration were applied. Three replicates were prepared for each parameter.

Quantification of DADS. Samples on days 1, 3, 6 and 15 were collected. On the respective days, soil samples were mixed with equal volumes of methanol and water. The mixture was put in the mechanical shaker at 150 rpm for an hour and filtered. The filtrate was collected for further analysis. DADS was quantified using HPLC as described in the literature study (Lawson et al. 1991, Wan et al. 2007). The amount of DADS present was calculated using the peak area reading extracted from HPLC with respect to the linear equation obtained from the calibration curve of known DADS concentration with regression, $R^2 = 0.9756$.

RESULTS AND DISCUSSION

Optimization of DADS's concentration and duration of inhibition. Urease inhibitors regulate and control urea hydrolysis to encourage better nitrogenous nutrient uptake (George et al. 2016). The ability of DADS as a soil urease inhibitor is reflected in the amount of urea-N retained in samples amended with DADS in comparison to the control.

Table 2 shows the percentage of urea-N hydrolysed in the respective treatments. Based on Table 2, it is observed that the percentage of urea-N hydrolysed in the control sample is significantly higher in comparison with DADS treatments however no significant difference is observed among the different DADS treatments from day 1 to day 5 which indicates that urea hydrolysis is slower in soil samples amended with DADS. Results on day 7 show that 5% and 15% DADS/urea-N (*w/w*) treatments exhibit the least percentage of urea-N hydrolysis. However, results on day 10 and day 14 further suggest that 5% DADS/urea-N (*w/w*) treatment is the optimum DADS treatment as the percentage of urea-N hydrolysed is significantly smaller compared to the other DADS treatments. From the overall trend, it can be observed that in the control sample, 100% of urea-N is hydrolysed at the end of the 30-day period. However, soil samples amended with DADS experience an average of 73.65% urea-N hydrolysis at the end of 30 days. Furthermore, 5% DADS/urea-N (*w/w*) treatment can reduce urea-N hydrolysis by 27.91% in comparison to the control sample. Hence, this proves that DADS can be reviewed as a modulating agent to control and regulate urease activity through a thiol-disulfide exchange reaction at the active site of urease. A similar deduction was made by researchers in the past for the reaction of allicin and jack bean urease (Juszkiewicz et al. 2004, Olech et al. 2014). Previous research conducted suggested that allicin is a natural based urease inhibitor and the parental component for DADS exhibits optimum urease inhibitory action at 5% allicin/urea-N (*w/w*) (Mathialagan et al. 2017).

Effect of soil moisture, temperature, and pH on the inhibitory property of DADS. The results collected reflect the inhibitory action of DADS but under different soil parameters as it is stated, urease inhibitor should be chemically stable (Modolo et al. 2015). A literature study suggested the retention time of DADS is approximately 10 min using HPLC (Lawson et al. 1991, Wan et al. 2007).

Table 2. Percentage of hydrolysed urea-N over 30 days under different concentration of diallyl disulfide (DADS) treatments at room temperature

Treatment (% DADS)	Day 1	Day 3	Day 5	Day 7	Day 10	Day 14	Day 25	Day 30
0	10.74 ^a	40.70 ^a	60.31 ^a	100 ^a	100 ^a	100 ^a	100 ^a	100 ^a
2	0.51 ^b	5.14 ^b	8.11 ^b	42.61 ^b	48.22 ^b	48.37 ^b	46.35 ^c	72.46 ^{bc}
5	0 ^b	0 ^b	9.72 ^b	11.81 ^c	12.66 ^e	37.31 ^c	39.38 ^c	72.09 ^c
10	0 ^b	0 ^b	3.99 ^b	28.04 ^{bc}	44.34 ^c	48.20 ^b	57.59 ^b	75.67 ^b
15	0 ^b	6.25 ^b	8.88 ^b	16.83 ^c	39.69 ^d	47.27 ^b	46.56 ^c	74.38 ^{bc}

Values in a column followed by different letters are significantly different at $P < 0.05$

Hence, the soil samples are expected to exhibit a peak at an approximately similar retention time to quantify DADS present.

Figure 1A shows the deterioration of the amount of DADS present in the soil throughout intervals of 15 days at 15, 30, and 50% soil moisture content (SMC). 15% SMC is the permanent wilting point for a plant which indicates the minimum soil moisture content at which the plant wilts. 30% SMC corresponds to normal soil moisture content with respect to field capacity taking into account the soil moisture 2 to 3 days after rain or irrigation. 50% SMC is recognized as saturated water content whereby the soil is saturated with water as the pores on the soil particles are estimated to be fully filled with water at this stage (Nair and Mukne 2017). Based on the trend in Figure 1A, the amount of DADS generally decreases

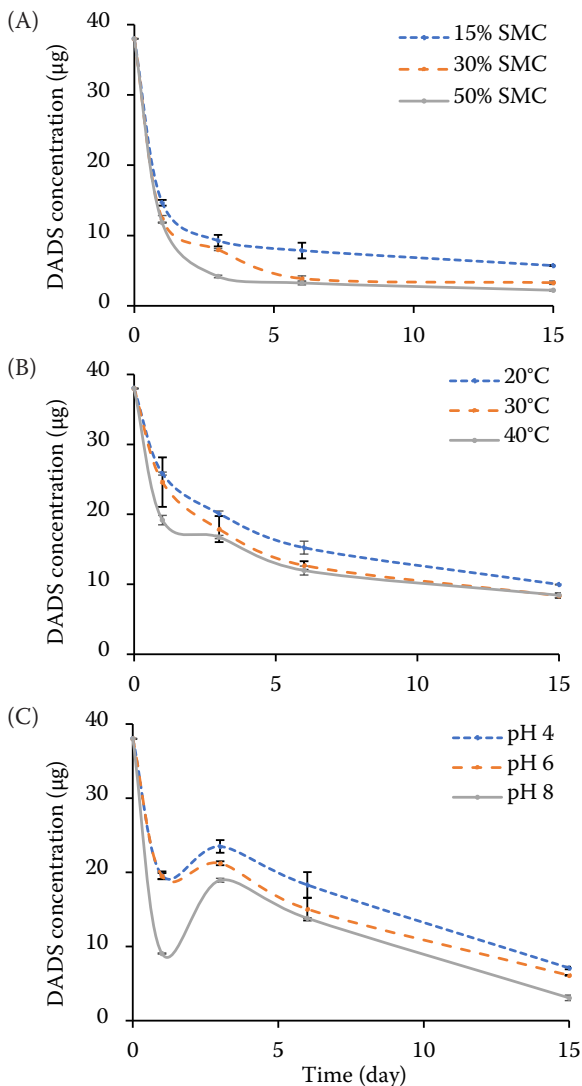


Figure 1. Effect of (A) soil moisture content (SMC) on diallyl disulfide (DADS); (B) temperature on DADS, and (C) effect of soil pH on DADS retained in the soil

with time. It is also observed that higher SMC results in faster DADS deterioration. This is because an increase in soil moisture causes urease activity to increase as well up to field capacity, after which the urease activity remains constant (Sahrawat 1984). Hence, it can be deduced that the thiol-disulfide reaction occurs at the active site of urease to inhibit enzymatic activity hence, resulting in deterioration of DADS. However, from the results, it is observed that DADS exhibits inhibitory action under SMC in the range of 15% to 30%.

Figure 1B shows the decrease in the amount of DADS retained in the soil throughout intervals in a 15-day period in which the temperatures were fixed at 20, 30 and 40 °C. Based on the results, the amount of DADS remaining in the soil generally decreases with time. Research by Moyo et al. (1989) suggests that the urea hydrolysis rate increases as temperature increases from 5 °C to 45 °C and research by Vahed et al. (2011) further supports this theory through experimentation that indicates urease activity increases in higher temperature. Hence, it can be deduced that due to high urease activity, the thiol-disulfide reaction causing deterioration of DADS which was more drastic up to day 3. Research is done by Nair and Mukne (2017) also affirms that the rate of deterioration of DADS increases as the temperature increases. Hence, the thermal stability of DADS might have affected the results yield as well. However, from the results, DADS remained relatively stable and exhibit inhibitory properties under the temperature range of 20 °C to 40 °C for a period of 15 days.

Figure 1C shows the amount of DADS remaining in the soil under different soil pH, which are pH 4, pH 6 and pH 8. According to the global soil survey manual from the US Department of Agriculture, pH 4 is extremely acidic soil, pH 6 is moderately acidic soil and pH 8 is moderately alkaline soil. Based on the results, the concentration of DADS in all the soil samples regardless of the soil pH experiences drastic deterioration, especially soil pH 8 before increasing in concentration and further decreasing steadily until day 15. This might be due to the action of soil urease which is most active at slightly alkaline pH levels (Sekaran et al. 2019). Hence, to control the action of soil urease, the disulfide bond from DADS binds to the active site of urease after which DADS is formed back once more considering increasing pH prompts DADS formation (Yu et al. 1989). However, from the results, DADS does exhibit inhibitory properties under different pH levels common in agricultural grounds.

The efficiency of different DADS treatment varied significantly on the rate of urea hydrolysis. However, 5% DADS/urea-N (*w/w*) treatment was the optimum as

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it reduced urea-N hydrolysis by 27.91% in comparison to the control sample. Different soil parameters had significantly different effects on DADS concentration; however, it was observed that soil at 15% SMC, 20 °C, and pH 4 had the least effect on the concentration of DADS present in the soil samples after the 15-day period.

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