Science, Engineering and Social Science Series
ISSN/e-ISSN: 2541 – 0369/2613 – 988X
Vol. 4, No. 4, 2020, Printed in the Indonesia

Using soil ATP contents as bioindicator for paddy soil impacted by fluazinam applications

Fuzi Suciati[1,*], Dwindrata Basuki Aviantara[1]

[1]*The Institute for Water and Waste Treatment – BPPT*
Bld. 820 Geostech Puspiptek Serpong, 15314, Province of Banten

Application of fluazinam, a pyridine-based fungicide, and its potential impact on the level of soil microbial biomass of paddy soils as estimated through soil ATP contents has been assessed. Soil ATP has been known to strongly correlated with soil microbial activity as such providing an indication of living soil microorganisms that mediating soil microbially active processes. To evaluate the effects of fluazinam, have on the levels of soil ATP a laboratory experiment was set up. Fluazinam at rate 3000 mg/kg was applied to air dried soils. Then following 1st, 3rd, 7th, and 14th-day of incubation the soil ATP contents were assessed. The levels of soil ATP were evaluated based on the emitted fluorescence intensities of oxyluciferin measured at a wavelength range 550 – 570 nm. Constantly decreasing level of soil ATP was detected throughout two weeks of experimental period, from 0.100 ± 0.006 to 0.043 ± 0.009 nmol/g soil. These finding suggested that fluazinam might induce deleterious effects to soil health and soil quality and might draw concern on environmental problems.

Keywords: xenobiotics, fluazinam, soil ATP, bioindicator, soil health

1. INTRODUCTION

Fungicides as chemical agents to alleviate fungal diseases on crop and plantation still are used world-widely. Fluazinam (IUPAC name: 3-chloro-N-[3-chloro-2,6-dinitro-4-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2-pyridinamine), with chemical structure as shown in Figure 1, is a fungicide commonly used to annihilate soil born fungus [1]. The fungus causes Sclerotinia blight (infected by Sclerotinia minor) on peanuts and late blight (infected by Phytophthora infestans) and white mold (infected by Sclerotinia sclerotiorum) on potatoes and tomatoes [2,3,4,5] (see Figure 1).

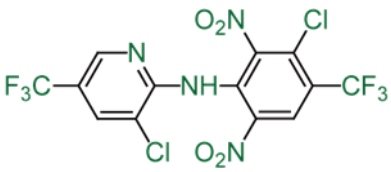

Figure 1. Chemical structure of fluazinam

Fluazinam is considered as a broad-spectrum fungicide classified as a diarylamine compound or more specifically as an anarylaminopyridine compound. The fungicide can be applied as a foliar spray or soil treatment to overcome fungal diseases on crops with symptoms as shown in Figure 2.

Figure 2. Symptoms of fungal disease attacking some crops [3,4]

*Email Address: *fuzi.suciati@gmail.com*

Because fluazinam is a broad-spectrum fungicide then it will not attack specific organisms. As a result, organisms such as soil fungi, soil bacteria, others micro flora and fauna as well as meso fauna all are potentially affected by fluazinam exposure. Therefore, any pesticides, including fluazinam, might have negative impacts to nontarget organisms [6].

Soil is an environmental compartment that is very important in supporting global food sustainability, fiber production, fresh water sustainability, biodiversity as well as the functioning of ecosystem [7]. The functioning of soil is strongly dependent upon soil health and soil quality. Several indicators, such as physical, chemical or biological, have been used to describe soil health [8]. On another hand, soil quality can be defined the fitness of a certain soil to fulfill certain function within its capacity, managed or natural boundary, to sustain plant and animal productivity, maintaining or enhancing water and air quality, supporting human health and habitation [9]. Thus, soil quality might have different meaning and purpose according to different people.

Soil biochemical processes determine the health status of a soil. That processes have been known to be very sensitive to environmental perturbation, particularly as the result of xenobiotics exposure such as pesticides. Soil biochemical processes are mediated by all organisms inhabiting the soil. Certain soil biochemical process, for example soil nutrient cycling, is heavily relied on the activity of soil micro flora such as bacteria and fungi. Bacteria and fungi release extracellular enzymes required for attacking substrates to gain energy from them. However, some studies demonstrated that several soil biochemical processes are driven by extracellular enzymes that were not release directly from soil microbes but from enzymes inhabiting soil for long term and adsorbed to soil constituents. On the other hand, soil biomass measurement and soil respiration have been shown to have a strong relationship to soil microbial population.

Previously fluazinam is considered “suggestive evidence of carcinogenicity, but not sufficient to access human carcinogenic potential” [10,11]. Thus, in order for assessing to what extent the impact of fluazinam application might have on soil health then biological indicator in term of soil microbial biomass was assessed.

There are several methods to measure soil microbial biomass. Some are measuring levels of soil carbon linked to soil microbial biomass. The other are measuring the levels of soil nitrogen linked to soil microbial biomass. Both, soil carbon and soil nitrogen constitute approximately 5% of total organic carbon and nitrogen in soil.

Generally, the determination of soil microbial biomass classifies into two groups, physiological methods and chemical methods [12]. Frequently used physiological methods for determination of soil microbial biomass were soil fumigation and a method developed by Anderson and Domsch. On another hand a chemical method that was frequently used to determine soil microbial biomass was based on measurement of adenosine triphosphate (ATP). In this study, ATP measurement was utilized to determine levels of soil microbial biomass impacted by fluazinam fungicide.

2. METHODOLOGY

Soils for the purpose of this study were taken from experimental lots located in Hyogo Prefecture Technology Center for Agriculture Forestry and Fisheries (HPTCAFF), Hyogo Prefecture, Japan. Before treatments all soil samples were air dried and immediately screened to pass a two-millimeter screener. After that level of soil humidity was adjusted at 50% of water holding capacity by addition of aquadest. Furthermore, soil samples were put into twenty-four wide mouth polycarbonate chambers of size 250 mL each. Soil samples were filled in into each chamber as such filling 3/4 part of the chamber volume.

As sieving usually induced microbial stress [13] it is necessary to recover the microbial activities prior the application of fluazinam. For this purpose, all chambers containing the soil were incubated in dark at 25 centigrade for two weeks. At the end of incubation period of the recovery of soil microbial activity each lid of the chambers was opened and halve of them were spiked with fluazinam at rate 3000 ppm. The other 12 chambers served for control purposes. After manually mixed, then the chambers were continued to incubate for further two weeks. Levels of soil ATP were determined after sampling of triplicate soil at 0-, 1-, 3-, 7-, and 14-day during two weeks incubation period.

The method for determination of soil ATP content followed Jenkinson and Oades procedure [14] but with little modification as described in Aviantara [15]. In the modified method 2 grams of treated soil was taken from the polycarbonate chamber and immediately transferred into erlenmeyer of volume 50 mL. Next 20 mL of extraction reagent that consisted of 0.5 M trichloroacetic acid and 0.25 M Na2HPO4.12H2O was added. Following supersonic shaking for 5 minutes the suspension was filtered and 100 µL aliquot was taken and added with 4900 µL demineralized-sterilized aquadest. From this mixture 100 µL solution was taken and 200 µL luciferinluciferase reagent (Thermo Lab Systems) which was buffered with 0.1 M tris-acetate buffer solution (Thermo Lab Systems) was added. Immediately the intensity of chemiluminescence organic molecule that created was measured by using a luminometer (OPTOCOMP1 MGM USA) at a wavelength range 550 – 570 nm.

3. RESULT AND DISCUSSION

The determination of ATP levels of soil with luciferin reagent based on the formation of bioluminescence compound emitting visible light at wave length in the range 550 – 570 nm. Luciferin is a general term for compounds that capable of providing light in a bioluminescence reaction. Luciferin undergoes enzyme-

catalyzed oxidation by luciferase results in the formation of unstable excited state intermediate the will emit light upon decaying to energy ground state.

During the process luciferase will utilize high energy phosphate bond of ATP to oxidize luciferin gives final products adenosine monophosphate (AMP), phosphate inorganic, carbon dioxide and oxyluciferin. Figure 3 shows steps of bioluminescence reaction linked to ATP determination.

Result of the trial is shown in Figure 4. At the beginning of experiment level of soil ATP for control is 0.122 ± 0.006 nmol/g-soil. A day after, such a level increases significantly to 0.316 ± 0.032 nmol/g-soil. Then at 3-day and 7-day incubation periods the soil ATP levels of controls decrease to 0.206 ± 0.036 and 0.185 ± 0.040 nmol/g-soil. At the end of incubation period (14-day) level of soil ATP of control is 0.234 ± 0.068 nmol/g-soil. The last value is little increment compare to determination at 3-day and 7-day incubation period. However, statistically, determination of soil ATP levels for controls at 3-, 7- and 14-day incubation period do not differ significantly.

Even though measured soil ATP fluctuates along incubation period, soil ATP levels for control are consistently above the levels of soil ATP contents of the treated soil. Such a fluctuation is common as manual mechanical mixing in order for distributing fluazinam evenly throughout soil matrix to some extent will induce stress to soil microbes. It has been known that soil respiration linked well with the level of ATP content [15]. When soil microbes are under threatening conditions usually the level of their respiration will increase. Such an increase is in order to maintain cells of microbes to function properly. Thus, more ATP will be synthesized for that purpose and more energy has to be preserved by soil microbes to keep the soil microbes alive.

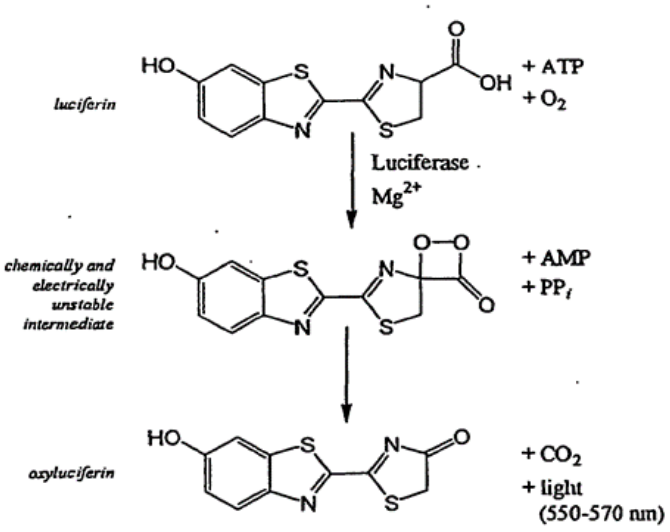

Figure 3. Bioluminescence reaction for ATP determination [17]

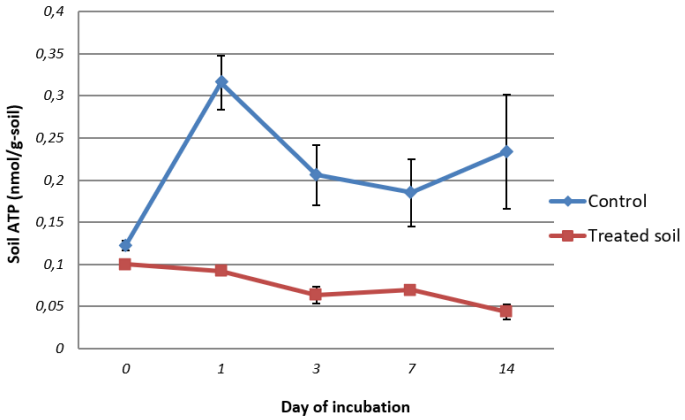

Figure 4. Soil ATP contents at 1st, 3rd, 7th, and 4th-day of incubation after treatment with 3000 ppm of fluazinam (standard deviation is taken from triplicates)

Differ from the control soils treated with fluazinam tend to show decreasing levels of soil ATP contents throughout incubation period. Progressively soil ATP levels of treated soils changed from 0.100 ± 0.006 nmol/g-soil at the beginning of experiment (Day-0) to 0.043 ± 0.009 nmol/g-soil at the end of incubation period (Day-14). Within two weeks of incubation period the soil ATP levels of treated soils has reduced by approximately 57%. It seems that spiking soils with fluazinam at rate 3000 ppm will cause synthesis of ATP by soil microbes inhibited. Fluazinam has been known to interfere with oxidative phosphorylation, a mechanism of energy generation within living organisms. The xenobiotic acts as an uncoupling agent to electron-transport system within membrane cells. As the result the protonmotive force will be destroyed and ATPase loss its capability to synthesize ATP from ADP [18].

To microflora fluazinam also induces unusual uncoupling activity of cells [19]. Metabolic state of targeted cells was interrupted because of lack of functioning of mitochondria [20]. Within mitochondria, it is suspected fluazinam reacts through conjugation mechanisms with glutathione, stopping cell respiration because of inhibition of ATP synthesis. The inhibition of ATP production will definitely because downstream cellular metabolisms is also interrupted.

Another study demonstrated that derivatives of fluazinam have a similar potential to parent molecule fluazinam in inhibiting ATP production of targeted cells [21]. Brandt et al. [21] discovered at least eight fluazinam derivatives capable of inhibiting synthesis of ATP. Even one derivative has uncoupling reactivity higher than molecule fluazinam itself. The findings of their study suggested that derivatives of fluazinam might induce toxicity characteristics more harmful than parent molecule to soil microorganisms.

Fluazinam has been considered as a moderately persistent compound [5]. That was not surprising as most halogenated organic molecules tend to accumulate in the environment. Soils with high content of organic materials and clay have ability to adsorb halogenated organic compounds and protect them from soil microbial attacks. Thus, along with increasing levels of fluazinam in soils gradually the fungicide may show characteristics of persistent organic pollutants (POPs). In order for soil microorganisms able to break fluazinam into simpler molecules then bioavailability must be fulfilled. Otherwise, extracellular enzymes that present in soils will not able to proceed with biodegradation reactions.

Evaluating value of log KOW for fluazinam, as shown in Table 1, it can be predicted that the compound has a tendency to bioaccumulate in lipids. According to that value fluazinam prefer to be present in hydrophobic compartment by at least 3600 factors higher than hydrophilic compartment. As most cells containing lipids then bioaccumulation of fluazinam in biota is not unpredictable.

The solubility of fluazinam in water is also determined by pH of soil solution. More basic the soil solution is more soluble is the fluazinam. Henry's constant for vapor pressure of fluazinam also indicates that the fungicide has little volatility. However, ultraviolet-visible (uv-vis) data indicates that fluazinam may be affected by uv-vis rays and underwent photochemical degradation.

A study on how fluazinam affecting soil enzymes was carried out by Niemi et al. [22]. using microcosms and mesocosms systems. Their findings demonstrated that fluazinam induced toxicity and revealed strong inhibition to luminescence bacteria. Such a strong inhibition in the soil toxicity test and continuing bioavailability of fluazinam were detected throughout their experiments. Therefore, their findings were in agreement with this study (see Table I).

Table I. Physical and chemical properties of fluazinam*

Physical and chemical properties	Value
Partition coefficient octanol/water (log K_{OW})	3.56
Solubility in water at various pH	0.130 mg/L (pH = 5) 0.157 mg/L (pH = 7) 3.380 mg/L (pH = 9)
Henry's constant at various pH	8.11 x 10^{7} atm m^3/mole (pH = 5) 6.73 x 10^{-7} atm m^3/mole (pH = 7) 3.11 x 10^{-8} atm m^3/mole (pH = 9)
Vapour pressure at various temperatures	2.3 x 10^{-5} Pa (T = 25 °C) 1.3 x 10^{-4} Pa (T = 35 °C) 6.7 x 10^{-5} Pa (T = 45 °C)
Ultraviolet – visible spectrum	λ_{max} (nm) = 238 (pH = 2) λ_{max} (nm) = 239; 342 (pH = 7) λ_{max} (nm) = 260; 343; 482 (pH > 10)

*Compiled from [5, 23, 24]

4. CONCLUSION

Form this study it is concluded that fluazinam might have deleterious effects to soil ecosystem. Soil health and soil quality could be reduced progressively resulting from fluazinam toxicity to soil microorganisms. The changes of soil health and soil quality linked to xenobiotics toxicity such as fluazinam could be monitored via soil ATP levels. Soil ATP levels strongly correlated with viable cells of soil microflora or soil microorganisms and thus may serve as s good bioindicator for environmental perturbation.

ACKNOWLEDGEMENT

The authors would like to express sincere gratitude to Japan International Cooperation Agency (JICA) for the financial support to conduct this study in Japan and also to Hyogo Prefecture Technology Center for Agriculture Forestry and Fisheries (HPTCAFF) for laboratory facilities for this research.

References

[1] Smith D.L., M.C. Garrison, J.E. Hollowell, T.G. Isleib & B.B. Shew. (**2008**). *Evaluation of application timing and efficacy of the fungicides fluazinam and boscalid for control of Sclerotinia blight of peanut.* **Journal of Crop Protection** 27: 823–833

[2] Wharton P. & E. Wood. *White mold of potatoes.* (**2013**). *University of Idaho Extention.* 4 pages.

[3] Shew B.B. *Plant disease fact sheets: Sclerotinia blight of peanut https://projects.ncsu.edu/cals/plantpath/extension/clinic/fact_sheets/index.php?do=disease&id=3* (**2011**) [On line, accessed September 3rd 2017]

[4] Maine Department of Agriculture. Late Blight—*Phytophthora infestans http://www.maine.gov/dacf/php/gotpests/diseases/late-blight.htm* (**2005**) *[On line, accessed September 3rd 2017]*

[5] USEPA. *Pesticide fact sheet: Fluazinam*. (**2001**). Office of Prevention, Pesticides Protection and Toxic Substances. 20 pages.

[6] Fishel F.M. *Pesticide effects to nontarget organisms*. (**2017**). IFAS Extension. University of Florida. 6 pages.

[7] McBratney A., D.J. Field & A. Koch. (**2014**). *The dimension of soil security*. Geoderma 213: 203–213.

[8] Brevik E.C. *Soil health and productivity*. (**2009**). **In Soils, Plant Growth and Crop Production (Ed. W. Verheye).** EOLSS Publlisher. UK. 1-26.

[9] Laishram J., K.G. Saxena, R.K. Maikhuri & K.S. Rao. *Soil health and soil quality: a review*. (**2012**). **International Journal of Ecology and Environmental Sciences 38 (1):** 19–37.

[10] Pesticide Action Network (PAN) UK. *The list of lists: A catalogue of lists of pesticides identifying those associated with particularly harmful health or environmental impacts.* London. United Kingdom. 27 pages. (2009)

[11] Neumeister L. & W. Reuter. (**2010**). Die *schwarze liste der pestizide II. Greenpeace.* Hamburg. 188 pages.

[12] Parkinson D. & E.A. Paul. *Microbial biomass*. (**1982**). **In Methods of Soil Analysis 2e. Part 2: Chemical and Microbiological Properties. (Eds. A.L. Page et al).** ASA Inc. & SSSA Inc. Publishers. USA. 821–830.

[13] Ross, D.J., T.W. Speir, K.R. Tate & V.A. Orchard. *Effects of sieving on estimations of microbial biomass, and carbon and nitrogen mineralization, in soil under pasture.* (**1985**). **Australian Journal of Soil Research 2(2):** 319–324.

[14] Jenkinson, D.S. & J.M. Oades. *A method for measuring adenosine triphosphate in soil.* (**1979**). **Journal of Soil Biology and Biochemistry (11)**: 193–199.

[15] Aviantara, D.B. *The effects of fluazinam and flusulfamide on soil ATP microbial biomass.* (**2008**). **Proceeding of the International Seminar on Chemistry 2008.** 269–272.

[16] Kiss S., D. Pasca & M. Dragan-Bularda. *Enzymology of Disturbed Soils. Developments in Soil Science (26).* (**1998**). Elsevier. The Netherlands. 333 pages.

[17] Wood K., R. Hannah & R. Moravec. *Improved method for detection of ATP*. (**2012**). Patent Application EP 2113571 B1.

[18] Pelczar Jr. M.J., E.C.S. Chan & N.R. Krieg. *Microbiology: concepts and applications.* (**1993**). McGraw-Hill, Inc. USA. 896 pages.

[19] Yang C., H. Chantal, V. Vladimir & Y. Gan. *Fungicide: modes of action and possible impact on nontarget microorganisms.* (**2011**). **International Scholarly Research Network Ecology 2011**: 1–8.

[20] Guo H., H. Miyoshi, T. Komyoji, T. Haga & T. Fujita. *Uncoupling activity of a newly developed fungicide, fluazinam [3-chloro-N-(3-chloro-2,6-dinitro-4-trifluoromethylphenyl)-5-trifluoro methyl-2-pyridinamine]*. (**1991**). **Biochimica et Biophysica Acta 1056(1)**: 89–92.

[21] Brandt U., J. Schubert, P. Geck & G. Von Jago. *Uncoupling activity and physicochemical properties of derivatives of fluazinam*. (**1992**). **Biochimica et Biophysica Acta 1101 (1)**: 41–47.

[22] Niemi R.M., I. Heiskanen, J.H. Ahtiainen, A. Rahkonen, K. Mantykoski, L. Welling, P. Laitinen & P. Ruuttunen. *Microbial toxicity and impacts on soil enzyme activities of pesticides used in potato cultivation.* (**2009**). **Journal of Applied Soil Ecology 41**: 293–304.

[23] Anon. *Nebijin (Flusulfamide): Safety data sheet*. (**2006**). Elliot Technologies Ltd. 4 pages.

[24] Alternative Strategies and Regulatory Affairs Division-Pest Management Regulatory Agency. *Fluazinam: Regulatory note.* (**2003**). Ministry of Public Works and Government Services. Canada. 72 pages.

Received: 21 March 2019, Accepted: 25 July 2020