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Research Article

Bioassay Method Development to Test *Sitophilus oryzae* Sensitivity against Phosphine

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ABSTRACT

Sitophilus oryzae is a cereal product pest found in warehouses. The presence of this pest negatively impacts the quality and quantity of stored rice. The common method used to control warehouse pests are fumigation. However, frequent use of insecticides will trigger resistance within target pest populations. This study aimed to develop a bioassay method based on the Food Agriculture Organization (FAO) protocols and modify bioassay protocols to determine S. oryzae susceptibility of populations collected from traditional markets across Yogyakarta. Field populations were collected from eight traditional markets in Yogyakarta (Lempuyangan Market [Kapanewon Danurejan], Kranggan Market [Kapanewon Jetis], Caturtunggal Market [Kapanewon Depok], Godean Market [Kapanewon Godean], Imogiri Market [Kapanewon Imogiri], Pleret Market [Kapanewon Pleret], Kasihan Market [Kapanewon Lendah], and Brosot Market [Kapanewon Galur]). The reference population was obtained from the Southeast Asian Regional Centre for Tropical Biology (SEAMEO BIOTROP). The bioassay was carried out following FAO protocols by using a glass jar fumigation chamber (volume 2 L) which was a modification of the desiccator of FAO recommended method number 16. This test used the lowest testable dose for this method: 0.01×10^{-2} mg/2 L or 0.5×10^{-2} μg/L tested on 100 imagoes which were divided into five replications and obtained 100% mortality on the second day (48 hours). Results showed that the FAO fumigation chamber method could not be used in the test, so modifications were carried out to determine tested doses by changing the volume of the fumigation container. The test used three container volumes, including 20, 60, and 80 L. Result from 20 L container showed 82–100% mortality, 60 L containers showed 69–100% mortality, and 80 L containers showed 24–79% mortality. Results from 80 L container was suitable for the bioassay because it was able to test the lowest dose on reference insects. Eight doses ranging from 0.125 to 1.625×10^{-2} μg/L, and an untreated control were used to determine the LD_{50} of phosphine on each population. Insects were fumigated for 48 hours and then removed to observe mortality. The LD_{50} of the reference population was 0.27×10^{-2} μg/L while 0.29–0.54×10^{-2} μg/L for field populations. These findings indicate that *S. oryzae* populations collected from traditional markets in Yogyakarta were still susceptible to phosphine.

Keywords: bioassay method development; phosphine; rice; *Sitophilus oryzae*; susceptibility

INTRODUCTION

Rice is one of the main staple foods of Indonesian and becomes and parameter for national economic and social stability (Rohman & Maharani, 2017). Rice weevils, *Sitophilus oryzae* (L.) is a main pest of stored cereal products (Park *et al.*, 2003). Tropical climates support the development of this pest (Batta, 2004). *S. oryzae* larvae damage rice grains by consuming endosperm and reduce contained nutrition and vitamins (Bello *et al.*, 2000) and cause rice grains to be easily broken (Isnaini *et al.*, 2015). Moses *et al.* (2020) stated that damage due to this pest can reach 53.30% if grains are stored for too long or even reach 100% if this pest is not controlled.

Fumigation is the main management strategy of this insect in stored grains (Holloway *et al.*, 2016). Fumigations are done using methyl bromide, sulfuryl fluoride, dan phosphine. The use of methyl bromide has been restricted based on Montreal

Protocols in 1995 due to its ability to damage ozone (ACIAR, 1998). Meanwhile, application of sulfuryl fluoride requires high concentrations and can potentially leave residue on commodity causing its use to be not recommended (Jagadeesan *et al.*, 2018). Phosphine is an alternative that is currently widely used. Phosphine enters through insect's spiracles (aspiration toxin) and later spreads to the entire body. Fumigant absorption depends on the target organism's respiration rate, and therefore factors that increase respiration rate also increase fumigant absorption (Kashi & Bond, 1975). Aspiration toxins disrupt cell respiration by inhibiting the function of cytochrome c oxidase enzymes on electron transport chains within the mitochondria that cause phosphorylase oxidative and decrease of ATP production. Decrease of ATP reduces energy within cells that later damage tissue and cause mortality (Prijono *et al.*, 2006). Insects that are exposed to fumigant show early symptoms of agitation and hyperactive action followed by lethargic movement, reduce of metabolic rates, and increase of oxidative pressure (Nath, 2011).

Reliance on phosphine increases resistance probabilities against aluminum phosphide in stored products. First *S. oryzae* resistant strains were reported in the 1970s in a study that showed that 8 from 135 tested strains demonstrated high resistances. Resistant strains from India were 2.5 time resistant against phosphine compared to susceptible strains (Thangaraj *et al.*, 2019). *S. oryzae* resistant against fumigants were found in India, China, Vietnam, Morocco, Brazil, and Australia (Kim *et al.*, 2019). Another survey in Greece showed that *Oryzaephilus surinamensis*, *S. oryzae*, *Tribolium confusum*, *Cryptolestes ferrugineus*, *T. castaneum*, and *Rhyzopertha dominica* were resistant against phosphine (Agrafioti *et al.*, 2019).

S. oryzae susceptibility against phosphine were only done if bioassay methods were available and could be done in target areas. *S. oryzae* susceptibility tests are an important step to manage resistance. Initially, testing was performed using FAO methods (Busvine, 1980), but the lowest dose still caused 100% mortality in tests. Therefore, modifications were done by increasing volume of used containers to obtain relevant mortality based on tested doses. *S. oryzae* susceptibility against phosphine were also done in South Korea (Kim *et al.*, 2019). Australia later developed a bioassay method to test cereal pest resistances (Jagadeesan *et al.*, 2018). This study aimed to test *S. oryzae* susceptibility of populations collected from traditional markets across Yogyakarta against phosphine by following modified FAO methods.

MATERIALS AND METHODS

Test Insects

S. oryzae were collected from traditional market around Yogyakarta. Two hundred imagoes were collected using a fine brush from the surface of rice bags and reared in the Laboratory of Management Technology Sub Pesticide Toxicology, Faculty of Agriculture, Universitas Gadjah Mada to obtain consistent aged imagoes. *S. oryzae* were reared in containers (diameter 12.5 cm and height 12 cm) and were fed with 250 g Ciherang rices with maintained 25^{o}C and 70% humidity. Rearing was done until sufficient number of 7-day-old imagoes were obtained for testing.

Development of Bioassay Method

At first, bioassay followed methods from FAO with 2 L glass fumigation containers (a modification of the desiccator recommended in FAO Number 16) (Busvine, 1980). Figure 1 showed the modified FAO bioassay method. Wire gauze were hanged in the middle of containers to place test insects. Seven days old imagoes were placed into PVC ring (diameter 2.5 cm, height 2.5 cm) that were given bases and closed with cloths. PVC rings were filled with test insects and placed on wire gauze. Containers were sealed using clay to prevent gas leak. Test were only performed on LD_{50} to demonstrate insecticide dosses that cause mortality of 50% of the population. The lowest doses used was 0.01×10^{-2} mg/2L or 0.5×10^{-2} μg/L. Total number of insects used were 100 imagoes that were divided into five replications and resulted in 100% mortality on the second day (48 hours) after the beginning on the test. Fumigations were done for 48 hours. Results showed that insect were lethargic to dead. Exposure effects are affected by gas concentration, exposure duration, and exposure frequency. Thus, modified testing containers were obtained different levels of mortality and analysed using probit to determine *S. oryzae* susceptibility. To obtain accurate results, an additional container size was added while using the similar amount of phosphine to obtain lower concentrations.

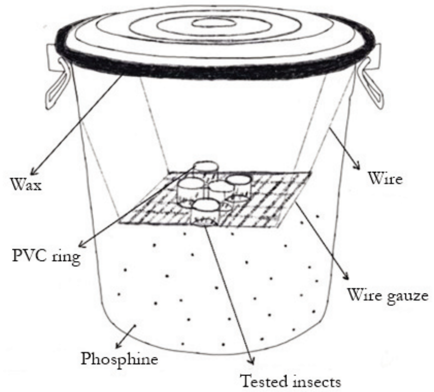

Figure 1. Phosphine testing method using modified method from standard FAO method by using 80 L plastic container (upper diameter 50 cm, lower diameter 40 cm, and height 55 cm)

Fumigation. Phosphine using were in the form of capsules (SHENPOS, aluminium phosphide 56%, PT Biotek Saranatama, Jakarta). Phosphine were weight to obtained tested dose and placed in containers. Containers were sealed to prevent gas leaks.

Container volumes used were 20, 60, and 80 L that were modified from FAO standard of 2 L resulting in final doses of 0.13×10^{-2}, 0.17×10^{-2}, 0.38×10^{-2}, 0.50×10^{-2}, 0.63×10^{-2}, 0.83×10^{-2}, 0.88×10^{-2}, 1.50×10^{-2} µg/L with five replications. Each replications used twenty imagoes and fumigations were done for 48 hours.

Observation. Mortality observation was done by removing all insects from containers and counting dead and living imagoes.

Data Analysis. Data were tested using Analyses of Variance (ANOVA). If significant differences occurred Least Significant Difference (LSD) post-hoc test was done at $\alpha = 5\%$ determine differences between phosphine doses using R Studio.

Sitophilus oryzae from Yogyakarta against Phosphine

Tested Populations. *S. oryzae* were collected from eight traditional market around Yogyakarta (Lempuyangan Market [Kapanewon Danurejan], Kranggan Market [Kapanewon Jetis], Caturtunggal Market [Kapanewon Depok], Godean Market [Kapanewon Godean], Imogiri Market [Kapanewon Imogiri], Pleret Market [Kapanewon Pleret], Kasihan Market [Kapanewon Lendah], and Brosot Market [Kapanewon Galur]) (Figure 2). Reference insects were obtained from SEAMEO BIOTROP Bogor and were never exposed to phosphine.

Rearing Method. *S. oryzae* imagoes were reared Laboratory of Management Technology Sub Pesticide Toxicology, Faculty of Agriculture, Universitas Gadjah Mada. *S. oryzae* were reared in containers (diameter 12.5 cm and height 12 cm) closed with cloth, fed with 250 g of Ciherang rice, and stored at 25°C.

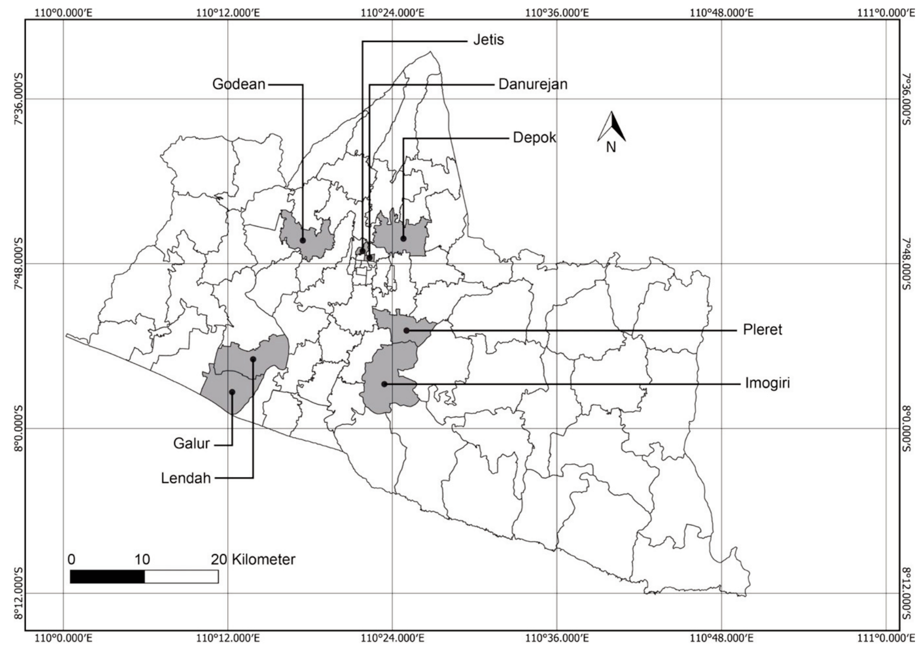

Figure 2. Locations of *Sitophilus oryzae* population collected from traditional market around Yogyakarta between July to October 2021 (two hundred imagoes were collected from each population)

Imagoes used were seven days old F_1. Reference insect used were F_6 *S. oryzae* imagoes obtained from Southeast Asian Regional Centre for Tropical Biology (SEAMEO BIOTROP).

Bioassay Method. Based on bioassay method development, fumigation containers used to test *S. oryzae* was 80 L (upper diameter 50 cm, lower diameter 40 cm, and height of 55 cm). Phosphine doses used were (0 as control, 0.125 × 10^{-2}, 0.375 × 10^{-2}, 0.625 × 10^{-2}, 0.875 × 10^{-2}, 1.125 × 10^{-2}, 1.375 × 10^{-2}, and 1.625 × 10^{-2} μg/L). Twenty 7-days-old imagoes were used on each replicate and five replications were used. Containers were sealed after insects were placed in containers and fumigation were done for 48 hours, except the untreated control.

Observation. *S. oryzae* mortality was observed 48 hours after fumigation by removing all insects from the container and counting dead and alive insects. Mortality was obtained from combined acute effected on imagoes.

Data Analysis. Probit analyses were done using Polo Suite *version* 2.1 to obtain LD_{50} and LD_{95} from each population and Resistance Factor (RF) compared to reference population.

RESULTS AND DISCUSSION

Bioassay Method Development

Modification to FAO methods were done by increasing containers' volume to variate phosphine doses to result in low to high mortality (2–99%) to fulfill probit requirements. Results showed that mortality in 20 L containers reached 82–100% while mortality in 60 L containers reached 69–100%. These results indicated that mortality at the lowest concentration was still >50%. Therefore, the next test used 80 L containers and caused mortality between 24–79% implying that 80 L containers to be selected choice for bioassays (Table 1).

Modification to bioassay have also been done in other countries. Containers with volume of 12 L and dose of 100,000 × 10^{-2} μg/L was used to determine *S. oryzae* susceptibility against phosphine in South Korea (Kim *et al.*, 2019). Australia used 4–6 L containers at doses of 100 × 10^{-2} μg/L to compare mortality and determine *S. oryzae* susceptibility against phosphine (Jagadeesan & Nayak, 2017). The right container volumes for bioassay are determined by insect toxicological susceptibility and the toxicity of tested fumigant. Results from the method development was then used to test the susceptibility of different *S. oryzae* populations against phosphine.

Sitophilus oryzae Susceptibility

Relations of phosphine and *S. oryzae* mortality of populations collected from different traditional markets around Yogyakarta showed low to high variation. Lower mortality was observed from Kapanewon Depok population which showed 1–89% compared to mortality from other populations. Kapanewon Lendah, Jetis, Galur, Danurejan, Godean, and Imogiri populations showed moderate mortality between 1–93% while high mortality was observed in *Kapanewon* Pleret population that were between 5–94% (Figure 3). This implies that low number of daed *S. oryzae* shows low mortality while high number of dead *S. oryzae* imply high mortality against phosphine. In Australia, *S. oryzae* susceptibility against phosphine at doses 100–1,000 × 10^{-2} μg/L for 48 hours of exposure caused 1–99% mortality (Nguyen *et al.*, 2015). Mortality of 100 × 10^{-2} μg/L exposure of 48 hours used in this study was 80–95% while the research in Australia using the same exposure time and doses showed 0–10%. In South Korea, 1,000–400,000 × 10^{-2} μg/L phosphine tested

Table 1. Average mortality of bioassay method development from standard FAO method for phosphine toxicity testing against *Sitophilus oryzae* by modifying container volumes

Container volume (L)	Doses (× 10^{-2} μg/L)							
	1.50	0.88	0.83	0.63	0.50	0.38	0.17	0.13
20	100.00 ± 0.0 a				82.00 ± 0.0 c			
60			100.00 ± 0.0 a		92.00 ± 0.0 b		69.00 ± 0.0 f	
80		79.00 ± 0.0 d		74.00 ± 0.0 e		50.00 ± 0.0 g		24.00 ± 0.0 h

Annotation: Empty column means no tests at the variable combination. Average mortality followed by the same letter showed significant difference compared to other treatments based on LSD post-hoc test at α = 5%

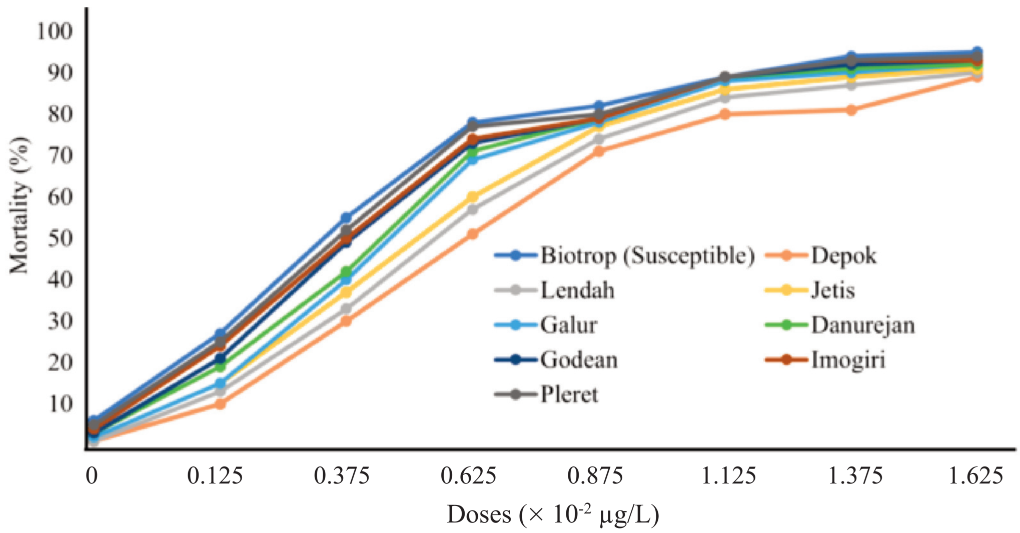

Figure 3. Relation between phosphine dose and *Sitophilus oryzae* mortality collected from traditional market around Yogyakarta and BIOTROP; each testing used 20 imagoes, replicated for five times, and fumigated for 48 hours

on different life stages of *S. oryzae* demonstrated different results. Phosphine at dose of 1,000 × 10^{-2} µg/L caused 100% on eggs, early larvae, and imagoes. However, the same dose on caused 85.56% mortality of older larvae. Pupa was the most less susceptible stage; 400,000 × 10^{-2} µg/L (400 × higher) caused <100% mortality (Kim *et al.*, 2019). The previous study showed that life stage choice is important and uniformity of individual in are important to compare between population in bioassays.

This study showed that *S. oryzae* was still susceptible against phosphine and susceptibility levels of populations from various traditional markets around Yogyakarta were relatively susceptible compared to the BIOTROP population at RF_{50} = 1.07–2 folds (Table 2). The LD_{50} and LD_{95} phosphine of *S. oryzae* from the reference population was 0.27 × 10^{-2} and 1.79 × 10^{-2} µg/L while LD_{50} and LD_{95} phosphine of *S. oryzae* field populations varied between 0.29–0.54 × 10^{-2} and 1.89–2.74 × 10^{-2} µg/L. If LD_{50} of test populations were higher than LD_{50} of reference populations and (RF) >1 implies that test insects have low susceptibility. Kapanewon Depok population had low susceptibility (RF = 2-fold) compared to populations from other traditional markets (Table 2).

Decrease of *S. oryzae* susceptibility against phosphine have been reported in South Korea (Kim *et al.*, 2019). Susceptibility levels of *S. oryzae* that were collected from traditional market across Yogyakarta against phosphine were significantly different based on bioassays (Table 2). Same life stages were used in this study and ones used in the study from Australia. Populations used in this study were more susceptible than ones from Australia with LD_{50} and LD_{99} susceptible phosphine strains of 507 × 10^{-2} and 2,100 × 10^{-2} µg/L while resistant strains were 30,200 × 10^{-2} and 147,000 × 10^{-2} µg/L (Jagadeesan & Nayak, 2017). Phosphine resistances have been reported on other insect species, such as *T. castaneum* in China that showed RF of 2.8–983.4 folds (Huang *et al.*, 2019). This demonstrates that selection toward phosphine resistances may occur simultaneously among different stored produce pests and its rate is affected by several factors, such as phosphine application frequency.

S. oryzae populations collected from rice sold in various traditional market across Yogyakarta were still susceptible against phosphine. Rice sold came from BULOG and farmers. Phosphine application in BULOG is the second management strategy (personal communication, 21 September 2020). The first choice is pirimiphos-methyl 250 g/L. Phosphine is used only if second management actions are required. Insecticide rotation can inhibit the development of insecticide resistance. Although phosphine has been registered in Indonesia since 1998 (National Pesticide Information Center [NPIC], 2009),

Table 2. Susceptibility of *Sitophilus oryzae* populations collected from traditional market across Yogyakarta against phosphine and fumigated for 48 hours

Population	Slope (± SE)	LD_{50} (× 10^{-2} μg/L) (SK 95%)	LD_{95} (× 10^{-2} μg/L) (SK 95%)	RF_{50}	χ^2
Biotrop*)	2.03 (± 0.16)	0.27 (0.22 – 0.32) a	1.79 (1.46 – 2.32)		2.99
Depok	2.33 (± 0.17)	0.54 (0.46 – 0.62) b	2.74 (2.12 – 3.94)	2	5.18
Lendah	2.32 (± 0.17)	0.47 (0.39 – 0.55) b	2.42 (1.86 – 3.51)	1.74	5.97
Jetis	2.29 (± 0.17)	0.43 (0.35 – 0.50) b	2.25 (1.75 – 3.22)	1.59	5.75
Galur	2.33 (± 0.17)	0.39 (0.33 – 0.45) b	2.02 (1.65 – 2.64)	1.44	3.85
Danurejan	2.21 (± 0.16)	0.36 (0.29 – 0.43) a	2.02 (1.58 – 2.89)	1.33	5.44
Godean	2.13 (± 0.16)	0.33 (0.28 – 0.37) a	1.96 (1.63 – 2.49)	1.22	2.73
Imogiri	2.05 (± 0.16)	0.31 (0.25 – 0.36) a	1.98 (1.58 – 2.69)	1.14	3.92
Pleret	2.04 (± 0.16)	0.29 (0.24 – 0.34) a	1.89 (1.52 – 2.52)	1.07	3.55

Annotation: *) SEAMEO BIOTROP reference populations. One hundred imagoes were done at each dose and divided into five replicates. X^2, df = 5, χ^2 table = 11.1 (a = 0.05). RF_{50} value were obtained from LD_{50} of tested population divided by LD_{50} of reference population. Numbers followed by the same letters in a column indicated no significant differences.

its low use frequency can cause low selection pressure. Another explanation on the low *S. oryzae* insecticide resistances can be caused due to cross mating of population from BULOG with ones from farmers. If phosphine resistance is recessive can imply that BULOG populations have experienced resistance dilution due to cross mating. *S. oryzae* phosphine resistance monitoring should be done to ensure its effectiveness. Besides that, continuous monitoring can inform resistance shifts to further manage and inhibit resistance to occur and later cause detrimental loss.

CONCLUSION

Phosphine bioassay method for *S. oryzae* on susceptible populations can be done using 80 L containers. *S. oryzae* population collected from markets around Yogyakarta were still susceptible to phosphine with RF of 1.07–2-fold compared to reference populations with RF 1.07 folds. Population from Kapanewon Depok had the lowest susceptibility compared to other populations (Kapanewon Lendah, Jetis, Galur, Danurejan, Godean, Imogiri, and Pleret) with LD_{50} and LD_{95} of 0.54 × 10^{-2} and 2.74 × 10^{-2} μg/L respectively. Kapanewon Lendah, Jetis, Galur, Danurejan, Godean, and Imogiri populations had LD_{50} and LD_{95} of 0.31–0.47 × 10^{-2} μg/L and 1.98–2.42 × 10^{-2} μg/L while Kapanewon Pleret populations had the lowest LD_{50} and LD_{95} of 0.29 × 10^{-2} and 1.89 × 10^{-2} μg/L.

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