Assessing the Effects of the Innovative Fungicide Pyraclostrobin Capsule Formulation on Silver Barb (*Barbonymus gonionotus*) in Rice Paddy: the case of Thailand

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Fish culture in rice paddies offers one of the best means of contemporaneous production of grain and animal protein at the same piece of land (Schuster, 1955). Whenever water is stagnated within bunds for rice culture, fish, which naturally occur in the irrigation canals, nearby tanks and pools, enter the rice paddies and grow there until rice harvest. Thailand is one of the countries that culture fish in rice paddies (Swingle & Shell, 1972). One of the common fish cultured in rice paddies is the silver barb (*Barbonymus gonionotus*) which is distributed throughout the country. This carp species feeds on plant matters (*e.g.* leaves, weeds, *Ipomea reptans*, and Hydrilla), thus, it has been used to prevent excessive weed growth in reservoirs throughout the country (Froese & Pauly, 2021). Also, this fish is famous for food, and it is an important cultural heritage of Thailand. In the old days, the people of Thailand believed that this fish was a symbol of abundance and strength as it could survive in any water sources and normally grew to full size around the same time that rice was ready for harvesting. For such reason, this period is commonly known as *kao mai pla man*, which means "new rice and delicious fish" (Bangkok Post, 2013).

Nowadays, however, this practice of fish culture in rice paddies has drastically reduced because of the introduction of modern technologies such as the use of large amounts of chemicals as fertilizers and pesticides in rice paddies that make it almost impossible for contemporaneous production of rice and fish. Therefore, the researcher, *Mr. Anon Sirisuriyakamonchai*, from the Department of Fisheries of Thailand conducted a study to compare the effects of the standardized and innovative formulations of the fungicide Pyraclostrobin on fish in the rice paddy.

Rice is the most important staple food for the majority of people in the world (Bandumula, 2018). It is mainly grown in subtropical and tropical regions with sufficient rainfall. Accordingly, rice paddies are often connected to important wetland areas and aquatic ecosystems; and rice paddy itself is an aquatic ecosystem (Halwart & Gupta, 2004). However, a number of fungus, bacteria, viruses, nematodes, and mycoplasma-like organisms cause diseases to rice plants. Among the fungal diseases, rice blast (*Pyricularia grisea*), brown spot (*Bipolaris oryzae*), stem rot (*Sclerotium oryzae*), sheath blight (*Rhizoctonia solani*), and sheath rot (*Sarocladium oryzae*) are the most alarming (International Rice Research Institute, 2006) and considered as serious constraints to rice production which can significantly reduce rice yield.

Silver barb, *Barbonymus gonionotus* (Bleeker, 1849)
(*Source:* FishBase)

Fungicides are used to protect plants from diseases and minimize yield losses (Balba, 2007; Smart, 2003). For many crops worldwide, primarily in cereals and maize, the highly efficient strobilurin fungicide used is Pyraclostrobin (Ambrus, 2004) which is also effective against rice fungal diseases (M. Li *et al*., 2018). The Pyraclostrobin standardized formula is an effective fungicide, however, it has adverse effects to the aquatic ecosystem and is highly toxic to aquatic organisms (Cui *et al*., 2017; European Commission, 2004; Hooser *et al*., 2012; Lewis *et al*., 2016; Liu *et al*., 2018; Morrison *et al*., 2013; Zhang *et al*., 2017, 2020). In aquatic ecosystems, fish are the most sensitive organisms to Pyraclostrobin when applied according to label recommendations. Nevertheless, the demand for efficient fungicides for rice crops is still high (Uppala & Zhou, 2018), which is why significant efforts have been carried out to develop an innovative Pyraclostrobin formulation.

In developing the innovative Pyraclostrobin formulation, the basic principle is to encapsulate the active substance (B. Li *et al*., 2017; M. Li *et al*., 2018), so that when the encapsulated formulation is applied, the capsules dry up on the plant surfaces, and subsequently, open and release the active substance. In water, however, the capsules remain intact and sink on the sediments where the active substance is slowly released; and due to its high soil adsorption coefficient (K_{oc}) of 6,000–16,000 ml/g (European Commission, 2004), it easily binds with the sediments where it is degraded. The capsulated formulation reduces the aquatic toxicity of the active ingredient while maintaining biological performance.

This concerned study was aimed at comparing the effects of Seltima and Headline on silver barb in rice paddy. Seltima is a new capsule suspension (CS) formulation of Pyraclostrobin that demonstrates to reduce toxicity on fish in laboratory and semi-field studies (M. Li *et al*., 2018), while Headline is one of the widely used Pyraclostrobin emulsifiable concentrate (EC) standard formulations for dry land crops.

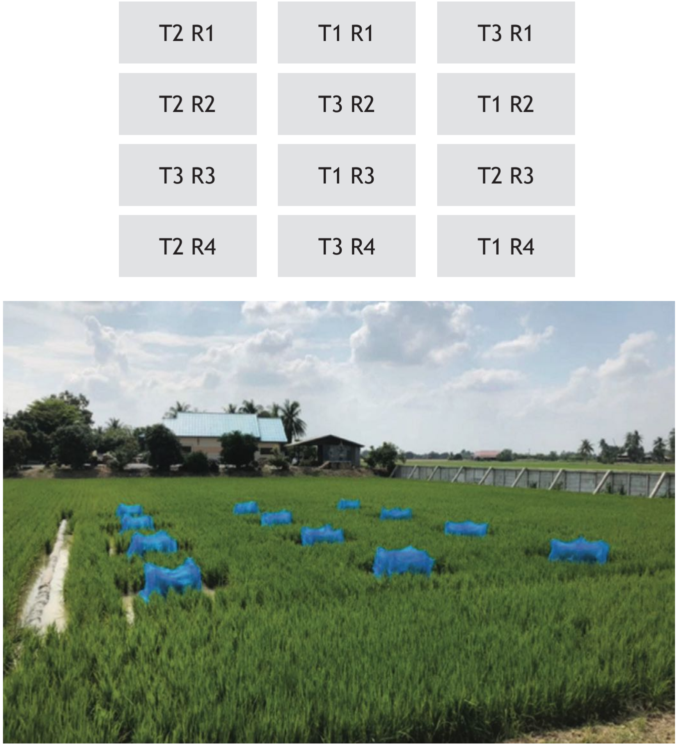
Study area

The study was conducted in a rice paddy in Suphan Buri Province which is located in the central part of Thailand (**Figure 1**) where the alluvial area is intensively cultivated, from August to October 2019. During the rainy season, rice covers the major areas of this region, which accounts for about one-fifth of the total cultivated rice land of the country. The average farm size is large, and a large proportion of the rice land has access to irrigation facilities, allowing many farmers to grow two rice crops during the year. Almost 75 % of the dry-season rice grown under irrigated conditions is located in this region. Farm operations are almost entirely mechanized, and farmers adopt the direct-seeding method of crop establishment to save labor. This region produces mostly long-grain rice and supplies the main rice surplus of the country (Global Rice Science Partnership, 2013).

Figure 1. Study area in Suphan Buri, Thailand

Experimental setup

For the experiment, a total of 12 plots in the rice paddy were used for three treatments (T1, T2, and T3) and each treatment had four replicates (R1, R2, R3, and R4). Each plot was 1.0 m^2 (100 cm × 100 cm) and sealed with mud and plastic sheets to prevent water leakage. The water level in the plots was maintained at 10 cm depth, and the plots that lost water were filled with water from the surrounding fields. **Figure 2** shows the randomized complete block design of the plots with the distance of 3–4 m around each plot to avoid contamination.



T2 R1	T1 R1	T3 R1
T2 R2	T3 R2	T1 R2
T3 R3	T1 R3	T2 R3
T2 R4	T3 R4	T1 R4

Figure 2. Randomized complete block design of experimental plots

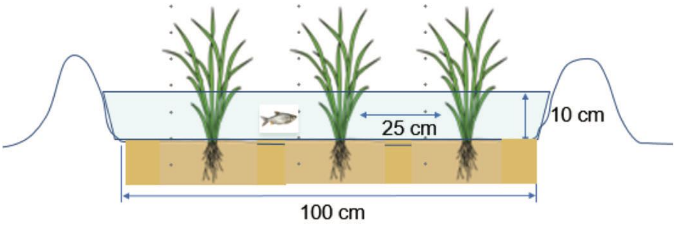


Figure 3. Setup of each experimental plot

On 14 August 2019, seedlings of the rice variety RD41 were transplanted into each plot with 20 cm space between plants and 25 cm between rows (**Figure 3**). The experimental plots were maintained until 56 days after transplant (DAT), the recommended growth stage for the application of Seltima in Thailand. The rice paddy was fertilized twice: the first time was 20 DAT with 16-20-0 (N-P-K) 156.25 kg/ha, and the second time was 50 DAT with 46-0-0 at 62.5 kg/ha. The weeds were removed by hands and insects were controlled using chlorantraniliprole 5.17 % SC at 312.5 ml/ha during 23 DAT and 52 DAT.

Test solutions

Two Pyraclostrobin fungicidal products were used as test solutions for the experiment, namely: Seltima CS (BAS 500 23 F, Batch 0020611003, containing 100 g/l active substance) and Headline EC (25 % EC, BAS 500 13 F, Batch 090918, 0018774134, containing 250 g/l active substance). Each test solution (*i.e.* 10 ml sample of Seltima and 4 ml sample of Headline) was suspended in the electric spray tank filled with 4.0 l water to obtain the 100 g/ha Pyraclostrobin solution. The spray tank was shaken well, then 40 ml of the respective test solutions were sprayed within three seconds directly to the water in the designated plots. T1 plots were the control and sprayed with water. T2 plots were sprayed with Seltima test solution and T3 plots were sprayed with Headline test solution, at the rate of 0.074 ± 0.014 mg/l and 0.086 ± 0.033 mg/l, respectively. A windshield was used in each plot while spraying the test solutions.

To determine the active substance concentrations of the test solutions, 500 ml of water was sampled from each plot at 0 h and 72 h. Only for T1 plots, the water samples collected at 72 h were pooled to one sample. The water samples were collected from the surface down to 8–10 cm of the water column to avoid sediment contamination; and stored in glass bottles which were then sealed and stored in containers with ice and transferred to the analytical laboratory. The water samples were analyzed using UHPLC/MS-MS with 0.002 mg/l limit of detection (LoD) and 0.006 mg/l limit of quantification (LoQ).

Test organisms

Juvenile *B. gonionotus* with a body length of 4–6 cm was used as the test organism. The fish were procured from Nakhon Pathom Province, Thailand approximately 36 h before the exposure. Upon arrival at the study area, the 200 fish were kept in the pond (2 m × 2 m with 30 cm water level) in the rice paddy and not provided with food until exposure. At the start of the experiment, ten fish were impartially introduced to each plot 1–2 h before the application of test solutions. Then, the plots were covered with nets to prevent access of predators which are mainly bird species. Visual inspections of the fish were made at 2 h, 6 h, 24 h, 48 h, and 72 h for signs of toxicity (abnormal appearance and behavior) and mortality. The fish were considered dead if there was no visible movement or respiration. The dead fish were removed from the plots at each observation time. At the end of the experiment (at 72 h), the water was drained and rice plants were completely removed from all plots. Live fish were recovered using a net and their conditions were checked. Missing fish were recorded as missing data.

Physical-chemical parameters

The water quality (level, temperature, dissolved oxygen (DO), and pH) were measured in each plot prior to the application of test solutions and at 24 h, 48 h, and 72 h. The ambient air temperature was measured at 1 h, 24 h, 48 h, and 72 h. To test the differences among water quality of T1, T2, and T3 plots, one-way analysis of variance (one-way ANOVA) was used at a significant level of $\alpha = 0.05$ and the number of dead fish between Seltima and Headline was measured by Fisher's exact test. Moreover, rainfall was measured to examine its influence on the Pyraclostrobin concentration during exposure by placing a measuring cylinder at an elevated position between the plots. Also, the soil composition of the rice paddy was analyzed by the hydrometer method.

Results and Discussion

The water quality in the plots during the course of the experiment is shown in **Table 1**, **Table 2**, and **Table 3**. The average water temperature was 28.8 °C with a range from 28 °C to 30 °C which was high as expected under the local weather condition. The average pH of the water was 7.4 and varied slightly in a range between 7.1 and 7.7. The average DO was 5.4 mg/l which was generally low. However, the plots with low DO levels had lower mortality rates compared to other plots with the same treatment. For statistical analysis, it was found that there was a normal distribution and variance. The water quality measurements were not different in each experimental unit. The ambient air temperature was 32 °C at 1 h, 31°C at 24 h, 30 °C at 48 h, and 31 °C at 72 h. During the experiment, it rained only at 24 h with a rainfall level of 22 mm. The rice paddy soil was composed of 70 % clay, 17 % silt, and 13 % sand. The Fisher's exact test indicated the lack of significant difference between dead fish in T1 and T2 ($p = 0.05$) as shown in **Table 4** and the highly significant difference of dead fish between T1 and T3 ($p < 0.01$) as well

Table 1. Physical-chemical parameters of water quality in T1, T2, and T3 plots

	Observation time (h)	Average water level (cm)	Average temperature (˚C)	Average pH	Average DO (mg/l)
T1	0.0	10.6	28.8	7.4	7.4
	24.0	9.5	28.8	7.3	5.4
	48.0	12.6	28.8	7.4	6.7
	72.0	11.0	28.8	7.3	3.4
	Average	10.9	28.8	7.4	5.7
T2	0.0	10.9	28.8	7.3	4.4
	24.0	8.5	28.3	7.4	5.8
	48.0	10.2	28.9	7.4	5.6
	72.0	9.1	28.7	7.4	3.5
	Average	9.7	28.7	7.4	4.8
T3	0.0	10.6	28.7	7.3	5.4
	24.0	9.0	28.9	7.5	6.1
	48.0	11.8	29.0	7.6	7.0
	72.0	9.5	28.8	7.4	4.7
	Average	10.2	28.8	7.4	5.8
General average		10.3	28.8	7.4	5.4
Range		6.0-14.0	27.3-29.9	7.1-7.7	1.8-10.3

Table 2. Test of homogeneity of variances of water quality in T1, T2, and T3 plots (α=0.05)

Parameters	Levene statistic	df1	df2	Significance
Water level (cm)	0.27	2	45	0.77
Temperature (˚C)	1.61	2	45	0.21
pH	1.87	2	45	0.17
DO (mg/l)	0.62	2	45	0.54

Table 3. ANOVA of water quality in T1, T2, and T3 plots (α=0.05)

		SS	DF	MS	F	Significance
Water level (cm)	Between groups	12.05	2.00	6.02	2.46	0.10
	Within groups	110.14	45.00	2.45		
	Total	122.19	47.00			
Temperature (˚C)	Between groups	0.29	2.00	0.14	0.83	0.44
	Within groups	7.73	45.00	0.17		
	Total	8.01	47.00			
pH	Between groups	0.07	2.00	0.03	1.85	0.17
	Within groups	0.79	45.00	0.02		
	Total	0.86	47.00			
DO (mg/l)	Between groups	9.63	2.00	4.82	1.06	0.36
	Within groups	205.06	45.00	4.56		
	Total	214.69	47.00			

Table 4. The Fisher's exact test on dead fish between T1 and T2

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-square	0.219[a]	1	0.640	0.792	0.421
Continuity correction[b]	0.041	1	0.839		
Likelihood ratio	0.220	1	0.639	0.792	0.421
Fisher's exact test				0.792	0.421
N of valid cases	77				

[a] *0 cells (0.0%) have an expected count less than 5. The minimum expected count is 8.88.*
[b] *Computed only for a 2 × 2 table*

Table 5. The Fisher's exact test on dead fish between T1 and T3

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-square	49.259[a]	1	0.000	0.000	0.000
Continuity correction[b]	45.973	1	0.000		
Likelihood ratio	61.894	1	0.000	0.000	0.000
Fisher's exact test				0.000	0.000
N of valid cases	76				

[a] *0 cells (0.0%) have an expected count less than 5. The minimum expected count is 13.26.*
[b] *Computed only for a 2 × 2 table*

Table 6. The Fisher's exact test on dead fish between T2 and T3

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-square	44.485a	1	0.000	0.000	0.000
Continuity correction[b]	41.402	1	0.000		
Likelihood ratio	56.322	1	0.000	0.000	0.000
Fisher's exact test				0.000	0.000
N of valid cases	79				

[a] *0 cells (0.0%) have an expected count less than 5. The minimum expected count is 13.82.*
[b] *Computed only for a 2 × 2 table*

as between T2 and T3 ($p < 0.01$) are shown in **Table 5** and **Table 6**.

Active substance

The analytical measurements of the water samples from the plots are shown in **Table 7**. Based on the application rate of 100 g/ha active substance of test solution in the plot with the water level of 10 cm, the theoretical concentration would be 0.1 mg/l. However, the rice plants in the plots intercepted the sprayed test solutions. Consequently, the initial data were below the theoretical concentrations without plants but were within the expected ranges under the more realistic conditions of this semi-field study. The initial measurements in T1 plots confirmed the absence of contamination. The data in T2 plots were in the same range as those in T3 plots. On the other hand, the large variations of data in T3 may have been caused by inhomogeneous water sampling during collection and pooling of samples, as the low value in the R1 plot and the

Table 7. Active substance concentration of test solutions in T1, T2, and T3 plots at 0 h and 72 h

Treatment	Replicate	Active substance (mg/l)	
		0 h	72 h
T1	R1	< LoD	-
	R2	< LoD	-
	R3	< LoD	-
	R4	< LoD	-
T2	R1	0.073	< LoD
	R2	0.065	< LoD
	R3	0.065	< LoD
	R4	0.094	< LoD
T3	R1	0.041	< LoD
	R2	0.097	< LoQ
	R3	0.120	0.008
	R4	0.086	< LoQ

high value in the R3 plot were not reflected in the respective fish mortality data.

At the end of the experiment at 72 h, there were no detectable Pyraclostrobin concentrations that could be found in the water samples from T2 plots treated with Seltima. This is in line with the expected behavior of this product that the Pyraclostrobin containing capsules sink to the sediment and the slowly released Pyraclostrobin will bind to the sediment where it is rapidly degraded (European Commission, 2004). In T3 plots which were applied with Headline treatment, Pyraclostrobin concentrations decreased largely to levels below the LoQ due to degradation and dissipation to sediment.

Impacts on fish

Healthy fish were hard to find in the plots due to water turbidity and they tend to hide under the rice plants, whereas the impacted and dead fish were easily spotted. The abnormal behavior and appearance observed in fish over time are shown in **Table 8**. At 2 h, the fish in T3 have shown slow movement and erratic swim. Whereas, from 24 h to 72 h, it was observed that fish in T1 and T2 had a fungal infection. For the cumulative fish mortality, 7.5 % of fish were dead at 24 h and increased over time in both T1 and T2 plots (**Figure 4**). In T3 plots, 85 % were dead at 6 h, 95 % mortality occurred at 24 h, and no fish survived at 48 h. Most of the impacted and dead fish in T1 and T2 plots could be attributed to fungal infection. The harsh condition in the pond where the fish were kept before the experiment was likely responsible for the poor health condition. Nevertheless, the results of this study demonstrated the safety of the fungicidal product Seltima to fish under realistic worst-case conditions, when the condition and behavior of fish in T2 plots did not differ from those in T1 plots.

Table 8. Abnormal appearance and behavior of fish in T1, T2, and T3 plots over time (SM: slow movement; ES: erratic swim; FI: fungal infection; the number in parenthesis is the number of fish)

	Replicate	0 h	2 h	6 h	24 h	48 h	72 h
T1	R1	-	-	-	-	-	FI (1)
	R2	-	-	-	-	-	-
	R3	-	-	-	FI (1)	-	FI (1)
	R4	-	-	-	-	FI (2)	FI (2)
T2	R1	-	-	-	-	-	-
	R2	-	-	-	-	-	-
	R3	-	-	-	FI (1)	-	-
	R4	-	-	-	-	-	FI (3)
T3	R1	-	SM (1)	-	-	-	-
	R2	-	ES (1)	-	-	-	-
	R3	-	-	-	-	-	-
	R4	-	-	-	-	-	-

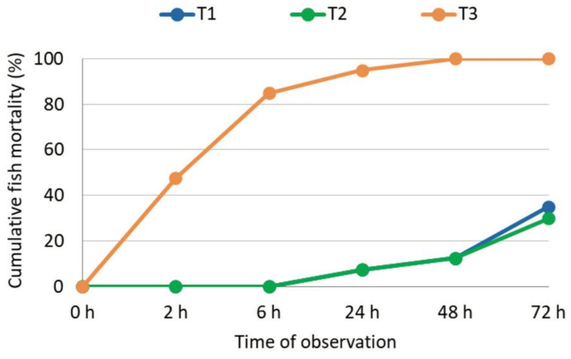


Figure 4. Cumulative fish mortality (%) over time in T1, T2, and T3 plots

For the semi-field study in Thailand under realistic worst-case conditions, the fish were exposed in the rice paddy at earlier rice plant growth stages (BBCH 40 to 49; lesser degree of plant interception) than recommended on the label of Seltima (BBCH 43 to 69; higher degree of plant interception) and lesser Pyraclostrobin, which resulted in higher plant interception and less product, and even decreased the toxicity of the water. Even under these worst-case conditions in China, Indonesia, and Thailand there was only a marginal impact of the Seltima CS formulation on fish, if at all. Considering the reduced exposure to the substance in natural water bodies outside the paddy field (Bullock, 2020), it can be presumed that Seltima applications will be of low risk to fish and other or less sensitive aquatic organisms (European Commission, 2004) in natural ponds and other bodies of water adjacent to rice fields.

Conclusion and Recommendations

The results of this study indicated that even under the worst-case conditions, the Seltima CS formulation caused only a marginal impact on the silver barb. On the other hand, the Headline standard formulation was highly toxic to the fish. Hence, the Pyraclostrobin capsule formula that was developed with the same efficiency as the standard formulation does not severely affect the aquatic ecosystems. Therefore, it is recommended that countries in the Southeast Asian region and other rice-growing countries should apply rice fungicidal products that are harmless to aquatic ecosystems, particularly when safer alternatives are available, and avoid using those highly toxic products.

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