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A Preliminary Study on the Sustainability of Mosquito-killing Effect of *Pythium guiyangense* Su

Liu Ping [1], Yang Xiao [1], Wang Yanhui [2], Su Xiaoqing [1]
1 Department of Biology, Guizhou Medical University, Guiyang 550004, Guizhou, China
2 Yichang Anruipu Biology Science & Technology Company LTD, Yichang 443000, Hubei, China
Corresponding author email: su.xiaoqing@aliyun.com
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Abstract To study the sustainability of mosquito-killing effect of *Pythium guiyangense* Su, so as to develop new types of biological agents for mosquito control. Lab experiments and outdoor artificial simulate mosquito breeding water trials were carried out to observe how long the fungus can survive in water and maintain its capability to control mosquito larvae. Besides, a field experiment was made to test its mosquito control effect in real situation. The infection rate of the fungus increased along with the time of the experiments. In outdoor artificial mosquito breeding water, the observed fungal longest sustaining time was 273 days. In field trail, a 122-day of mosquito control was obtained. *P. guiyangense* can be used as a tool for long-term biological control of mosquitoes, and when combined with relative microbes we can develop new types of agents with high efficiency and sustainability.
Keywords *Pythium guiyangense*; Mosquito biological control; Sustainability; Formulation

Background

Mosquito control becomes an important problem of human society since they not only disturbing people, but they are also vectors of many serious diseases of people, and specially recently when many times of break out of dengue and zika virus caused disease, makes it more serious concern (Meng et al., 2015). But extensive use of chemical insecticides caused environmental pollution and resistance of mosquito to these chemicals (Chen et al., 2011; Dai et al., 2014; Pei et al., 2014; Guan et al., 2015). So, to explore more mosquito control methods, that can reduce or even replace chemical's application is very important task. Biological control is safe to environment, and becomes one of the hot research fields.

Pythium guiyangense (Pg) is a promising biological agent for mosquito biocontrol, and has been researched systematically since 1994, including biological and ecological studies, and its safety to environment and virulence to mosquitoes (Su et al., 2010). The ecological knowledge tells us that Pg is a facultative fungus that can live parasitic life as well as saprophytic life, and that it can easily colonize in water environment. Whether this trait makes it special with long duration for mosquito control? We designed some experiments to verify it, which is reported here.

1 Materials and Methods

1.1 Microbes

The fungus used was Pg strains kept in our laboratory, passed on SFE agar plates, and rejuvenated once a month through mosquito host. An another fungus *Lagenidium giganteum* (Lg) kept in our lab and a mosquito-killing bacteria *Bacillus thuringiensis* var. *Israelensis* (Bti) were used for comparison of capability of controlling mosquitoes with Pg.

1.2 Mosquitoes

Culex quinquefaciatus larvae of 2nd and 3rd instars were from our mosquito rearing room which has temperature of 25C°±1C°, and RH of 70%, light cycle of L:D=14:10.

1.3 Lab bioassay

In each disinfected plastic container, 200 ml of tap water, 3 pieces (each 113 mm^2) of agar with Pg hyphae, 25 mosquito larvae, and 4 drops of 4% liver powder suspension were added. Three pieces of blank agar were added in control instead. Monitor was made twice a day, picking out and checking dead larvae with microscope, recording number of death, and calculating infection rates.

When all of the larvae died or pupated, they were taken out, and another batch of larvae was added in, and monitor was continuing.

Three strains of Pg were studied.

1.4 Observation in artificial outdoor water body

In plastic containers (capacity 6L), disinfected 50 g of soil and 8 g of wood bits, and 4L of tap water were added. Different agents or agent combination were added in experimental containers which were divided into groups of control (C), Pg (P), Pg plus Bti (PB), Pg plus Lg (PL), Lg (L), and Pg plus Bti and Lg (PBL). The doses used were 63.6 cm^2 of agar with mycelia for Pg, 1 ml of culture suspension for Bti and 20 g of wet mycelia for Lg. No agent was added in control group. Mosquito eggs or larvae were added about once a month. The containers were prepared on July 24, 2014, and the agents and mosquito larvae were added on Aug. 8.

For monitoring infectivity of agents, a nylon net was placed in each container with floating foam. The observing method was to place mosquito larvae as sentinel in it, and then taking them back to Lab in 48 hrs later, to check the mortality and infection rate.

1.5 Field observation

In a vegetable field reservoir of 90 m^2, 200 g of wet Pg hyphae and 100 g of wet Lg hyphae were scattered in along the margin, and the mosquito larva density was observed from time to time. When dead mosquito larvae were found, they would be taken back to check for fungal infection with microscope.

1.6 Observation on saprophytic growth of Pg

In container with mosquito larvae killed by Bti wet, Pg hyphae were added, and the dead larvae were taken out in 48 hrs later, and checked with microscope. In other trails, wood bits and particles of liver powder in bioassay containers were taken out and checked microscopically.

2 Results

2.1 Bioassay results in Lab

It was showed in Table 1 that although the virulence of the 3 strains were different, there was a tendency that the general infection rates and the highest and lowest infection in a single container of the second batch of larvae were higher than those of the first batch and the differences were significant.

Table 1 Results of larval infection rates by Pg in successive tests

Tested Pg strains	Tested larval batches	Date of test	Mortalities in control (%)	Mortalities of test groups (%)	Infection rates in test groups (%)	Corrected infection rates (%)	The highest infection rates in a single container (%)	The lowest infection rates in a single container (%)
W41-1	1	2014-4-27	10.4	31.6	31.6	21.2	52.2	20
	2	2017-5-1	35.7	93.6	93.6(1)	57.9(1)	100(1)	86.3(1)
W41-5	1	2014-4-27	10.4	21.6	21.6	11.2	65.4	3.8
	2	2017-5-1	35.7	90.5	90.5(1)	54.8(1)	95.2(1)	83.3(1)
W41-7	1	2014-4-27	10.4	39.8	39.8	29.4	80	16.7
	2	2017-5-1	35.7	77.9	69.5(1)	33.8(1)	90	54.2(1)

Note: When compared with the data of first batch of larvae, $P<0.05$

2.2 Outdoor experiment

On Nov. 25th, 2014 (the 111st day of the experiment), sentinel larvae were added. When recovering, all of the sentinels in control were alive, and those in groups P, PB, L, and PBL were all died. There was Pg hyphae covered

the dead larval body, except those from group L, on which no fungus was found.

On May 1st, 2015, (the 273rd day of the experiment), sentinel larvae were added again, and the recovery results were: all of the sentinel larvae in control group, and groups L, PB, and PBL were alive. Only were those in group P dead and covered by Pg hyphae.

It was showed that when Pg was put into mosquito breeding water, it can keep alive and strong virulence to mosquito for a long time, and the longest duration observed was 273 days.

2.3 Field results

The field trail results showed that mosquito density in 5 days after the application of Pg was significantly lower than that before, and the density dropped sharply thereafter. No larvae were found on the 30th and 57th days. The latest day of observation was the 122nd day of the application when about 80% mosquito larvae died, of which 60% were found infected by Pg. In this trail, Pg actually controlled mosquitoes for about 4 months (Table 2).

Table 2 Observation results of mosquito control in the vegetable field reservoir

Observation time	Larval numbers in 1 ladle	Check microscopically	commentary
Before the experiment	12、7、9、16、6（average 10）		
5 days after the experiment	7、3、4、5、4（average 4.6）		
15 days after the experiment	Only 3 dead larvae were found in total	1 of them was infected by Pg	
30 days after the experiment	No mosquito was found		In control water bodies, mosquito larval density were 10/100ml of water
122 days after the experiment	A lot of dead larvae were found. Alive mosquito density was 1/100ml of water	3 out of 5 dead larvae checked found infected by Pg	There was a period of high temperature before

2.4 The saprophytic growth of Pg

Pg grew from dead mosquito larvae that killed by Bti and produced a big number of sporangia which released a lot of zoospores later. Large amount of Pg hyphae were found by using microscope in wood bits and larval food particles picked out of Pg bioassay containers (Figure 1).

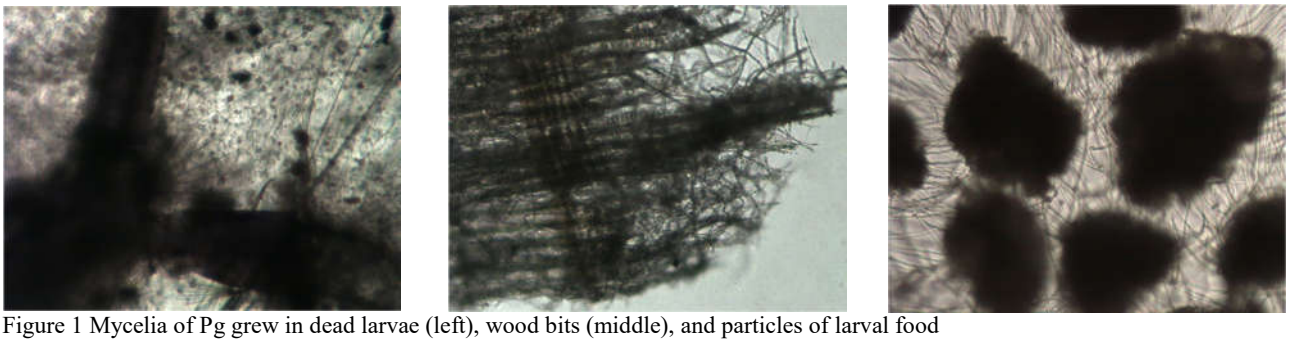

Figure 1 Mycelia of Pg grew in dead larvae (left), wood bits (middle), and particles of larval food

3 Discussion

The effective duration (ED) is a very significant feature of mosquito control agents in fight against mosquitoes and has been causing more and more concern, especially in large water area such as wet land. It is true that when agents with an ED as long as 1 month are used, two thirds of chemicals and labour could be saved than agents with 10-days ED be used. Bti is a widely used mosquito biocontrol agent since it is very effective and is cheap, but its ED is only 7-10 days, and other agents including some chemicals all with relatively short ED. Some chemical insecticides (such as temephos, fenthion, etc.) and biocontrol agent *Bacillus sphaericus* have longer ED (about 1 month), but they were all more expensive than Bti, and would cost more.

Pg is safe to environment, and relatively cheap. If it also has a long ED that will strengthen its value in mosquito biocontrol, which is the point makes us paying strong attention on its ED.

In lab bioassay, the infection rates of second batch of larvae were all higher than those of first batch. The possible explanation could be that after the former cycle of fungus-larvae interaction, Pg grew in dead larvae and organic material in water and produced more mycelia and huge amount viable zoospores which could invade the latter larvae more effectively.

In outdoor containers a proof that Pg could keep alive and infectivity in mosquito breeding water for 200 days was obtained, and in field observation, a 122-days ED of Pg was recorded.

The results showed that, Pg lived a parasitic life when there are mosquito larvae around. It invaded into larval body, decomposed the tissue and organs to get nutrition for growth and reproduction. When mosquito larvae were rare, or when larvae were killed, the fungus lived a saprophytic life. It grew on organic material in the water such as plant bits, or even on dead larvae, which can provide high quality nutrition to increase its virulence to mosquitoes.

As the consequence, Pg can plant in water and become one of the inhibiting factors against mosquito for a long time. This is a significant advantage that makes the fungus very competent member of a mosquito control ecosystem. In the ecosystem, Pg together with other factors such as fish or other natural enemies can reduce the environmental capacity of mosquito, and presses mosquito population to a harmless level. Theoretically, in this way, we can control mosquito population once for ever. But, in fact, there would be a lot of things can influence. When the balance was broken, there could be break out of mosquito population. In that case, other measures including utilization of chemical insecticides and extinguish the pests in a short time. To adopt different methods to resolve different situation is the strategy called IPM (integrated pest management) (Lu, 1978; Richard, 1979).

Since Bti can kill mosquito larvae very fast, but its ED is very short, while Pg takes effect relatively slow, but has a very long ED, a formula has been designed that combines Pg with Bti to obtain a compound (compound PB) which has fast effect as well as long ED to control mosquitoes. Recently, a new formula has been developed that put Pg and mosquito-killing fungus Lg together to get a new compound (compound PL) which has the advantages of PB, but is more effective in use.

Authors' contributions

Liu Ping designed the experiments in lab and outdoor artificial water containers. Yang xiao participated some of the lab and outdoor observation, Wang Yanhui did the field trials. Su Xiaoqing drafted the manuscript. All authors have read and approved the final manuscript.

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