Testing of two microsprodia

by Nuraeni Nuraeni

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Abstract. Pebrine disease is a significant disease attacking silkworm (*Bombyx mori* L.) caused by microsporidia (*Nosema bombycis* Nageli). This research aims to test breeds of silkworm that are resistant to pebrine disease. Microsporidia are isolated from local seeds (PL-614) and imported seeds (PC-614). The resistance of four breeds of the silkworm is tested by applying the topical method to mulberry leaves sized 3x4 cm^2 for the third instar larvae and 5x7 cm^2 for the fourth instar larvae. Each spore concentration of microsporid isolates used is $1x10^5$ per ml. The research result shows that breeds of new seeds, namely SS01, SS02, and SS03 are resistant to infection of the two isolates. This research is useful as a reference for the regional or central government to release breeds of new seeds that can replace or complete the existing seeds.

1. Introduction

Natural silk is one of the non-timber forest products and becomes a national featured product. Silk fiber comes from cocoon woven by silkworm insects (*Bombyx mori* L). Some cocoon grains of which fiber is pulled are combined into yarn as a raw material of the textile industry. Besides being processed as yarn, silk fiber can be used for military needs such as bulletproof vests, medical needs such as operating sewing threads, and nanofiber replacement for human tissue (*tissue engineering*) [1].

National production of cocoon and silk thread continue to decrease. One of the main factors of the decrease of national silk production is a disease attacking silkworm. In 1976, 1998, and 2009, it is reported that silk production failure is caused by pebrine disease. Furthermore, in 2009-2010, some central cultivation carries out a moratorium on caterpillar cultivation, in several regencies such as Enrekang, Majalengka, Bogor, and some central cultivation in West Java since the high intensity of the disease attack. Moreover, until the end of 2013, the intensity of this disease is still high, especially in Wajo Regency. According to [2][3][4], pebrine disease is one of the serious diseases that can thwart sericulture in any country. It is the disease that affects farmer psychology in this business, so many of them turning to other commodities.

Efforts to control pebrine disease are carried out through disease-free parent checks before releasing silkworm egg or seed to the farmer, as a preventive action. Imported seeds are also tested by Agricultural Quarantine staff before making it legal in the domestic market, as a deterrent and regulative action. However, pebrine disease is still often found in farmers' cultivation. According to [5][6][7][8][9][10],

pebrine pathogen (*Nosema bombycis* Naegeli) can also be spread from the mouth of contagious leaves. Farmers do not get a guarantee for seeds, time lost, energy, and mulberry leaves without taking cocoon.

Pathogen detection of disease is an effort for introducing and ensuring the types of pathogen, either through the form of morphology, characteristics, and its pathogen. Meanwhile, the more important thing is how to get seeds that are resistant to pest and disease. One of the efforts is through the utilization of various genes of silkworm to get high-quality strains. The use of various genes becomes the basic component of long-term management strategy [11]. Character choices as crossing material turn to be important while planning breeding program to obtain new strains [12]. Research on resistance test on diseases on silkworm in South Sulawesi has not been conducted because the release of new strains is still limited to be commercial silkworm seeds for the farmers. Thus, this research tries to compare some new strains with the existing ones that the farmers more familiar with.

2. Material and Method

2.1. *Sample Collection and Pathogen Identification*

The collection of silkworm samples that have been infected with pebrine *(Nb)* is conducted in silkworm cultivation in Donri-donri District, Soppeng Regency, South Sulawesi. Samples of infected silkworm larvae are taken from local seeds and imported seeds. Each sample of the infected silkworm is dissected, and its hemolymph is examined under a microscope at a minimum of 600 times of magnification to ensure the presence of microspore spores [8]. The samples that have been ensured to be infected are collected and then crushed in a mortar. Scouring results are dissolved in distilled water and filtered. The filter results are centrifuged at 2000 rpm for 10 minutes. The centrifuged sediment is separated from the turbid solution by replacing the new distillation water and then centrifuging it again. The centrifugation is repeated several times to get pure spores and is ready to be counted on the hemocytometer.

2.2. *The Provision of Pathogen and Oral Infection*

The concentration of *Nb* spores used is $1x10^5$ spore ml^{-1}. Inoculation is conducted by applying a solution of *Nb* spores on fresh mulberry leaves sized 3x4 cm^2 for instar larvae III and 5x7 cm^2 for instar larvae IV. Before giving leaves that have been applied and dried, larvae that will be tested are starved for 30 minutes. Treatment is conducted to larvae that have changed their skin for the second time for 3rd instar and to larvae that have changed their skin for the third time for the initial 4th instar that has not been fed with mulberry leaves. The number of larvae used in each treatment unit is 75, and each treatment is repeated for three times. Cultivation is conducted based on the standard of JICA (1985), and the condition of the environment inside the cultivation room is also noted (temperature and humidity).

2.3. *Research Design and Data Analysis*

The design used in the resistance test of silkworm strains is completer randomized Factorial Design. The first factor is the tested silkworm strains or seeds are BS09, SS01, SS02, and SS03. The second factor is pathogen isolate *Nb* local silkworm seeds (PL-614) and imported seeds (PC-614). Cultivation room is conditioned into the average temperature of 25.9 °C, humidity of 87.4%, and the lighting is set into bright for 12 hours and dark for 12 hours. Data collected in this stage of research is the resistance of tested larva (equation). The data obtained for different parameters were subjected to analysis of variance (ANOVA) to determine if the differences found among isolates and breeds of silkworm were significant. Following ANOVA, Tukey's studentized range test in a complete randomized design was used at $p<0.05$.

Data of silkworm resistance test is analyzed and tested further with Difference Real Honest (α = 0.05%).

D = % not infected by *Nb* - (a/b x 100%)

Where: D: Resistance toward pebrine attack (%), a: The number of caterpillars attacked by *Nb*, b: Total number of caterpillar cultivated/observed.

3. Results and discussion

3.1. Silkworm Strains Resistance

The silkworm resistance test is an observation that is inversely proportional to mortality; the higher the level of mortality, the lower the value of the resistance will be. The resistance of four strains of silkworm toward *Nb* isolate PL-614 infection on 3rd instar, and instar 4th is presented in Figure 1. The resistance of four strains of silkworm toward isolate PL-614 infection on 3rd instar and 4th instar is presented in Figure 2. Breed BS09 is more susceptible to isolate PL-614 infection and isolate PC-614 on 3rd instar and instar 4th compared to other three breeds. The analysis of the diversity of resistance of four breeds toward two isolates on inoculation of 3rd instar shows that breeds give significant influence toward silkworm resistance. To find out the influence of each factor of breeds and pathogen, the further test is conducted using Turkey test, as it is presented in Table 1. Breed SS03 has the higher resistance, which is 96.67 % on inoculation 3rd instar and 98.67 % on inoculation 4th instar. Breeds SS01, SS02, and SS03 relatively has the same resistance toward *Nb* infection on inoculation 3rd instar and 4th instar.

Table 1. Resistance (%) on silkworm breeds and isolate *Nb* on inoculation 3rd and 4th instar.

	Breeds				Mean
	SS01	SS02	SS03	BS09	
	Inoculation to 3rd instar				
PC-614	92.00	81.33	97.33	34.67	76.33
PL-614	100.00	90.67	96.00	65.33	88.00
Mean	96.00[a]	86.00[a]	96.67[a]	50.00[b]	
	Inoculation to 4th instar				
PC-614	90,67	88.00	98.67	36.00	78.33
PL-614	90.67	92.00	98.67	36.00	79.33
Mean	90.67[a]	90.00[a]	98.67[a]	36.00[b]	

*Means in each row followed by the same letters are not significantly different at $p < 0.05$

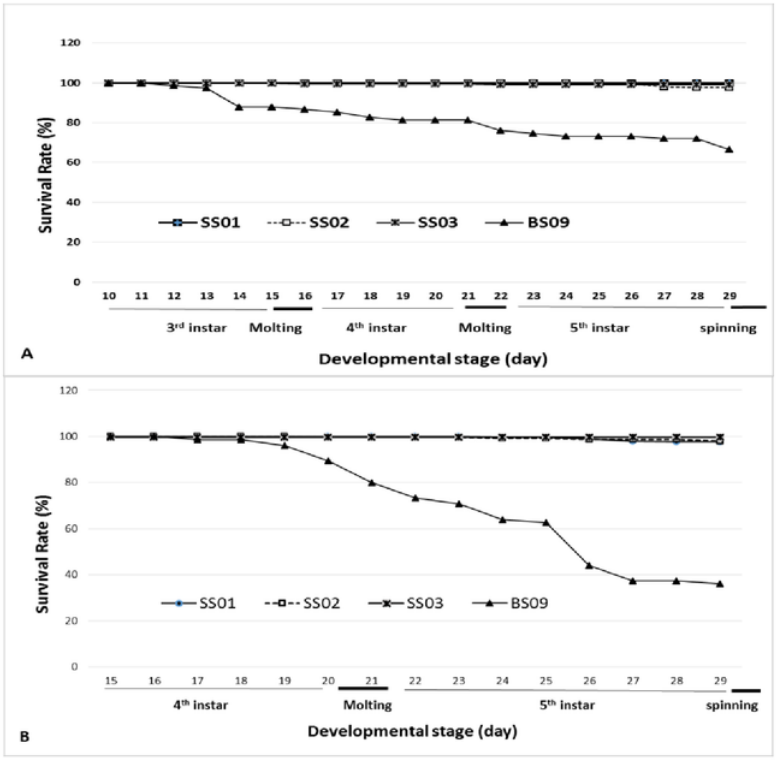

Figure 1. The resistance of silkworm breeds to isolate PL-614 on inoculation 3rd instar (A) and 4th instar (B).

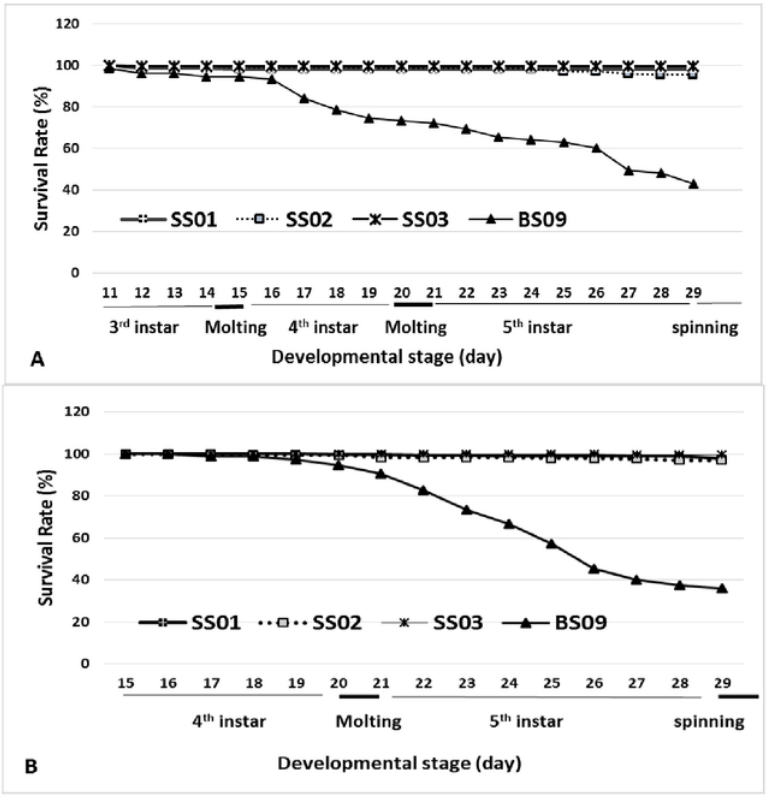

Figure 2. The resistance of silkworm breeds to isolate PC-614 on inoculation 3rd instar (A) and 4th instar (B).

3.2 Silkworm Strains Resistance

One of the purposes of silkworm nursery with crosses of various races that have different characters is to increase the productivity of cocoon and resistance from any disease [13]. Likewise, besides obtaining the productivity quality of cocoon (data is not presented), this research aims to gain strains that are resistant to microsporidia pathogens. Breeds that have ever been released on sericulture have not reached 20 breeds (including breeds that have been tested) with the parent stock of 40 races only. Compared to Japan, it has a collection of 500 races with various characters of the hybrid result of 820 strains [14].

Breeds SS01, SS02, and SS03 are the results of a cross between parent race polyvoltine and bivoltine or race from the tropical region and Chinese race, while strain BS09 comes from parents of bivoltine race. Response to the resistance toward microsporidia of each race is different. As stated by [15], Chinese race is more resistant toward microsporidian, then Japanese race is medium, and Europe race is most nonresistant when being cultivated in the tropical area. According to [16] silkworm race from the multivoltine or polyvoltine race is more resistant to *Nb* infection with the level of mortality is below 50% compared to bivoltine race with the level of mortality above 70%. Multivoltine or polyvoltine is commonly from a tropical area [11]. It is strengthened by [17] stating that silkworm from the bivoltine cross is more susceptible toward disease than multivoltine.

Valuation of silkworm resistance toward disease can be counted directly from its level of mortality, namely race or strain of which its percentage of mortality is the lowest is the most resistant one[16,18–

22]. *Nb* concentration applied is $1x10^5$ spore ml^{-1} can cause mortality as much as 65.33% on BS09, and other strains are only 0% - 18.33%. According to [23], it is stated that *Nb* concentration ($1.52x10^6$ spore ml^{-1}) can cause mortality as much as 25.21% - 90.00%. *Nb* virulence test with concentration $1x10^3$ – $1x10^8$ spore ml^{-1} toward silkworm race can cause mortality as much as 100% [24]. The difference in the resistance toward pathogen infection can be caused by the difference between races or silkworm strains [25]. Silkworm can be said as resistant if it can minimize mortality for 70-80% or larvae mortality only happen ranged 20-30% [26].

Silkworm does not have the same resistance toward all pathogen, depending on the variety/strain and phase/period/growth stadia. According to [27], it is said that silkworm resistance can be inherited by inducing immunity obtained by weakening microbe or inducing certain chemical compound. Meanwhile, according to [28], it is stated that silkworm resistance can be improved through crossing strains with high immunity or resistance.

3.3 Characteristics of Silkworm Strains

Silkworm has more than 450 morphological and biochemical characters that have been written from various parts of the world. Among those characters, 300 characters have been identified on 27 groups of chromosomes of 28 silkworm chromosomes [13]. Characteristics of breeds SS01, SS02, SS03, and BS09 that are tested in this research can be seen in Table 2. Morphologically, the characteristics of silkworm larvae or cocoon of the existing race show the difference. It is the same as each race that has been crossed obtaining new breeds of which morphological characteristics depend on race with dominant genetic. The morphological characters consist of the color of larvae and cocoon, special traits on thoraces of larvae (crescents) and eye spots or special shape of cocoon. According to [29], silkworm characters of Chinese race is commonly plain white, and its cocoon is round to oval of which color is white; while silkworm from Japanese race has a clearer crescent or commonly called spots with cocoon resembling peanuts. [30] states that color and motif of silkworm larvae are determined by factors except for genetic factor, and are influenced by its pigment distribution in hypodermal cell and epiphytic.

Table 2. Characteristics of larval and cocoon.

Breeds	Larval Colour	Larval marking	Cocoon Colour
SS01	Yellowish White	plain	yellow
SS02	Bright white	plain	White
SS03	Bluish white	marked	White
BS09	Blurry white	marked	White

4. Conclusion

Breed BS09 is very susceptible to *N. bombycis* of isolate PC-614 and PL-614. Breeds SS01, SS02, and SS03, are relatively resistant to *N. bombycis* of isolate PC-614 and PL-614. The characteristic of silkworm strains tested is that: the color of larvae SS01 is plain yellow, larvae SS02 is plain white, larvae SS03 is bluish white identified with crescents and clear eyespots, and larvae BS09 is white identified with crescents and clear eyespots. Those four strains of cocoon are oval and white, and except on SS01, it is yellow. Breeds SS01, SS02 and SS03 can become a substitute for the existing strains

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