Revista Brasileira de Entomologia 62 (2018) 19–22

SOCIEDADE BRASILEIRA DE ENTOMOLOGIA FUNDADA EM 1937

REVISTA BRASILEIRA DE
Entomologia
A Journal on Insect Diversity and Evolution

www.rbentomologia.com

Biological Control and Crop Protection

Standard method for detecting *Bombyx mori* nucleopolyhedrovirus disease-resistant silkworm varieties

Yang Qiong*, Xing Dong Xu, Li Qing Rong, Xiao Yang, Ye Ming Qiang

Sericulture & Agri-food Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510610, China

ARTICLE INFO

Article history:
Received 24 March 2017
Accepted 30 November 2017
Available online 8 December 2017
Associate Editor: Mariane A. Nickele

Keywords:
Bombyx mori nucleopolyhedrovirus
Group inoculation method
Individual inoculation method
Resistance detection
Silkworm

ABSTRACT

Bombyx mori nucleopolyhedrovirus (BmNPV) disease is one of the most serious silkworm diseases, and it has caused great economic losses to the sericulture industry. So far, the disease has not been controlled effectively by therapeutic agents. Breeding resistant silkworm varieties breeding may be an effective way to improve resistance to BmNPV and reduce economic losses. A precise resistance-detection method will help to accelerate the breeding process. For this purpose, here we described the individual inoculation method (IIM). Details of the IIM include pathogen BmNPV preparation, mulberry leaf size, pathogen volume, rearing conditions, course of infection, and breeding conditions. Finally, a resistance comparison experiment was performed using the IIM and the traditional group inoculation method (GIM). The incidence of BmNPV infection and the within-group variance results showed that the IIM was more precise and reliable than the GIM.

Introduction

The domesticated silkworm, *Bombyx mori*, is an important economic insect for silk production. There are several types of silkworm diseases, and they cause great economic losses to the sericulture industry. Among them, *B. mori* nucleopolyhedrovirus (BmNPV) disease is the most serious (Jiang and Xia, 2014; Xu et al., 2015). BmNPV disease is acute, and the duration from infection to illness is only approximately 96 h. The body wall of infected silkworms breaks easily and, thus, the pathogen pollutes the mulberry leaves on the silkworm bed, which leads to the rapid spread of the disease. So far, BmNPV has not been controlled effectively by therapeutic agents (Bao et al., 2009).

BmNPV, a member of the *Baculoviridae* family, has two different virion phenotypes: an occlusion-derived virus that is transmitted among hosts, and a budded virus that spreads throughout the host. Occlusion-derived viruses are packaged in polyhedrons and form occlusion bodies (OBs) (Chen et al., 2010; Cheng et al., 2014). BmNPV-resistance is related mainly to silkworm strains (Bao et al., 2009; Lu et al., 2007). The heredity of silkworm resistance to BmNPV is complicated because it is controlled both by major dominant genes and multiple micro-effective genes (Yao et al., 2003). Investigations have shown that most silkworm strains are sensitive to BmNPV infection and only a few strains have high resistance (Bao et al., 2009; Jiang and Xia, 2014). Breeding resistant varieties, which improves silkworm resistance to BmNPV, may help to reduce economic losses (Jiang et al., 2012b). Combining a multi-generation pathogen attack with classical crossbreeding techniques can improve the resistance of silkworm varieties to some extent (Wu et al., 2010). However, traditional crossbreeding methods have some limitations, such as poor accuracy and low efficiency. Since 2000, increasing molecular techniques have been applied widely to breeding disease-resistant silkworms (Isobe et al., 2004; Kanginakudru et al., 2007; Huang et al., 2009; Xu et al., 2013; Zhang et al., 2014; Jiang et al., 2014). Compared with traditional breeding technologies, molecular markers and transgenic technology are faster and more effective ways to select and improve disease resistance (Jiang and Xia, 2014; Subbaiah et al., 2013; Zhang et al., 2014). A few BmNPV-resistant silkworm varieties have been generated using such technologies (Xu et al., 2013). At present, the group inoculation method (GIM) is used widely by breeding units (Lu et al., 2007; Xu et al., 2013; Yang et al., 2013). However, the GIM does not have a standard operation program; thus, different researchers use different operational parameters, such different mulberry size and pathogen volumes. Therefore, the shortcomings of this method, such as its large standard deviation and poor repeatability, lead to unreliable results. In addition to advances in breeding technology, a more scientific and reliable resistance-detection method is needed to guide the breeding of BmNPV-resistant silkworm varieties.

* Corresponding author.
E-mail: serilover@126.com (Q. Yang).

https://doi.org/10.1016/j.rbe.2017.11.007

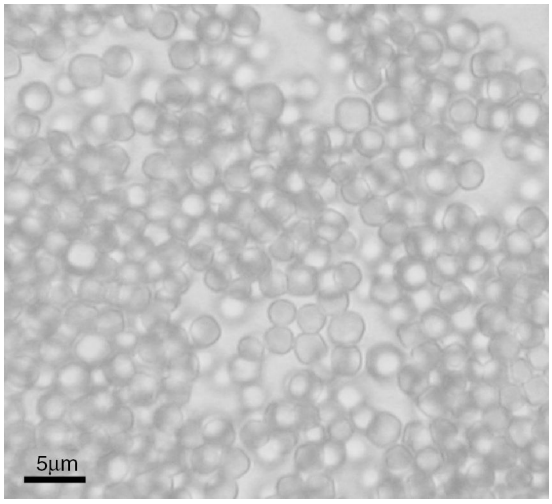

Fig. 1. BmNPV OB.

Materials and methods

Silkworm strains and pathogen

Silkworm strains Dazao, Yangshi, and shi7 were preserved in the laboratory of the Sericulture and Agri-food Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China. The wild-type BmNPV Guangdong strain was also maintained in the same laboratory.

The individual inoculation method (IIM)

Mulberry (*Morus*) leaves were cut into different sizes according to the instars of the tested silkworm larvae as described below. Different volumes of a pathogen suspension were transferred with a pipette and dropped onto the surface of the leaves and gently smeared to dry. The appropriate volume of the pathogen suspension is one that dries easily within 1 h. Subsequently, the pathogen preparation, mulberry cutting, individual inoculation parameters, and rearing conditions were manipulated as described in Sections 'BmNPV preparation' to 'IIM'.

BmNPV preparation

Newly exuviated fifth-instar larvae were inoculated onto mulberry leaves coated with 10^9 OBs/mL for 4 h, and then they were fed normal mulberry leaves. The OBs were obtained from the hemolymph of the infected silkworms, and they were purified by repeated centrifugation (Rahman and Gopinathan, 2004). The OB (Fig. 1) concentration was determined with a hemocytometer. The pathogen suspension was stored at 4 °C before use.

Mulberry leaf size

The favorable size was that at which the leaves are eaten within 4 h by most of the silkworms, because the leaves remained sufficiently fresh for the silkworms to eat during the time. The suitable

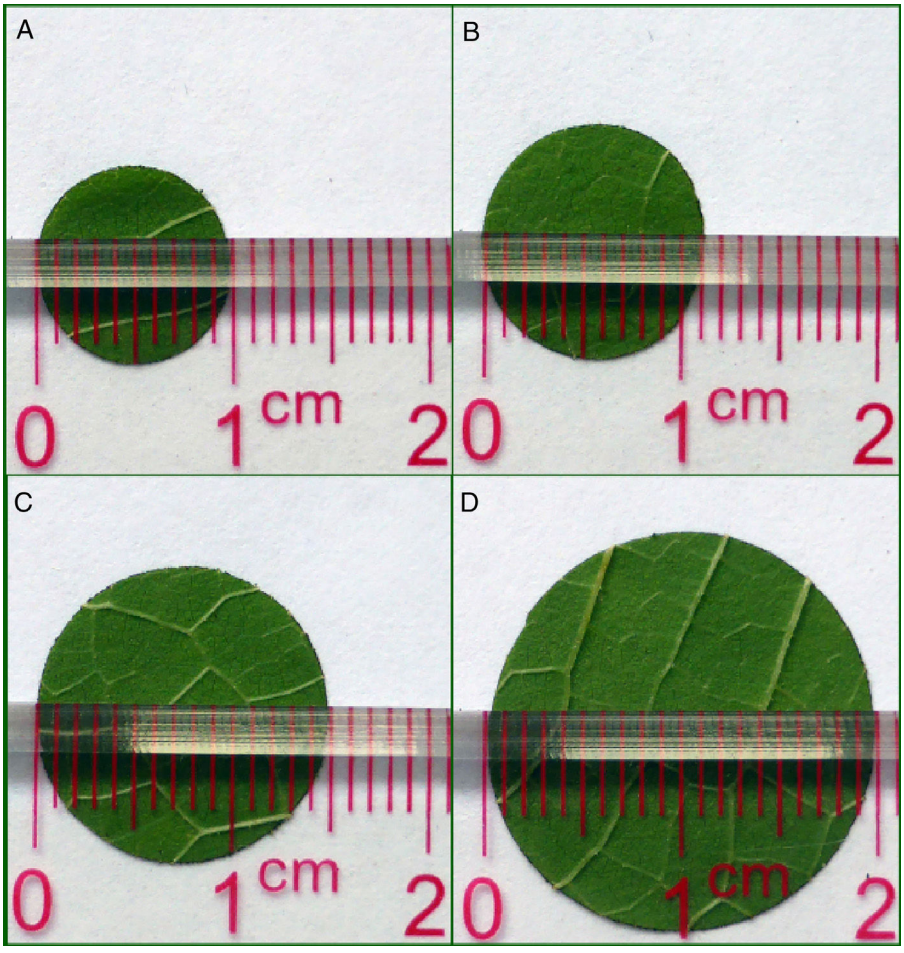

Fig. 2. Mulberry leaf size standards of different larval stages for the IIM. (A) Leaf size for second-instar larvae. (B) Leaf size for third-instar larvae. (C) Leaf size for fourth-instar larvae. (D) Leaf size for fifth-instar larvae.

Table 1
Parameters of the IIM and GIM.

Method	Per leaf size (com)	Larvae number/per leaf (worm)	Pathogen volume/per leaf (μL)	Pathogen dosage/(OBs/mL)
GIM	3.5	5	25	2×10^8
IIM	1.5	1	5	2×10^8

Table 2
The BmNPV infection incidences of the three silkworm strains using the IIM and GIM.

Method	Silkworm strains	Number of larvae inoculated	Incidence (%)/$\bar{x} \pm S$
GIM	Darzhao	180	39.4 ± 20.4
	Yangshi	180	26.1 ± 10.0
	shi7	180	41.1 ± 12.9
IIM	Darzhao	180	54.4 ± 1.9
	Yangshi	180	40.6 ± 4.7
	shi7	180	50.6 ± 2.8

mulberry leaf sizes for second-, third-, fourth-, and fifth-instar silkworms should be 1.0, 1.1, 1.5, and 2.0 cm in diameter (Fig. 2), respectively.

The pathogen inoculation volume

The favorable inoculation pathogen volume is 5 μL for the 1.1- and 1.5-cm diameter leaf disks, and 10 μL for the 2.0 cm leaf disk.

Larval stage

Although silkworm larvae have five instars, younger larvae (first and second instars) were too easily injured during the experiment. The fifth instar was too late for the experiment because it need to eat too much pathogen, but the pathogen concentration was too high and difficult to smear on the leave. Thus, third- and fourth-instar larvae were used.

Silkworm rearing cell

The silkworm rearing cell should be made of plastic, which can be disinfected easily.

Silkworm breeding conditions

First- and second-instar larvae were reared at 27–28 °C at 85–90% relative humidity (RH). Third-instar larvae were reared at 26–27 °C at 85–90% RH. Fourth- and fifth-instar larvae were reared at 25–26 °C at 75–85% RH. A 12 h light:12 h dark photoperiod was used throughout the larval stage.

IIM

Leaves were cut according to the larva instar as described in Section 'Mulberry leaf size'. Single and intact (without any crevices or holes) leaf disk was picked and placed in a plastic rearing cell. Five microliters of the pathogen suspension was transferred with a pipette and dropped onto the surface of the leaf disk, smeared with a stainless steel or plastic stick, and air dried. After exuviation and fasting for 8–12 h, silkworms were placed in the plastic cell and fed the pathogen-contaminated leaf disks. One silkworm should correspond to one mulberry leaf. After feeding for 4 h, the silkworms that ate the mulberry leaves were selected and grouped, and then these silkworms were reared on fresh mulberry leaves under the required breeding conditions. The numbers of the silkworms that exhibited the typical symptoms of BmNPV were recorded daily, and the incidence of BmNPV infection was analyzed using statistical software as described below.

Comparison of the IIM and GIM

Resistance detection was performed by the IIM and GIM using the three silkworm strains mentioned above. Newly exuviated fourth-instar silkworms were used for the test. The sizes of the mulberry leaves, numbers of larvae per leaf, and pathogen volume per leaf are given in Table 1. Infected silkworms were recorded during the process of breeding. Each method was replicated three times and each replicate consisted of 60 larvae. The incidence of BmNPV infection and the standard deviation were analyzed using SPSS for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Comparison of the incidence of BmNPV infection using the IIM and GIM

The incidences of BmNPV infection of silkworms (Table 2) of the same strain differed using the two detection methods (Figs. 3 and 4). The BmNPV infection incidence using the IIM was higher than that of the GIM, and the intra-group standard deviation was lower.

Discussion

The incidences of BmNPV infection using the GIM and IIM were compared. The IIM group exhibited a higher incidence of infection and a lower intra-group standard deviation than the GIM, which can be explained because the amount of BmNPV eaten by each silkworm was not equal using the GIM, as some larva did not eat or ate only a small amount of the BmNPV-treated mulberry leaves. Thus, the greatest weakness of the GIM was its inability to quantify how much each larva ate, which made it difficult to distinguish individual differences of BmNPV resistance. In contrast, the IIM, with individual inoculation at its core, avoids many of the problem caused by different external factors. Thus, the IIM is better than the GIM, and it provides detection results with good reliability and repeatability. In previous studies, we detected and selected some disease-resistant silkworm varieties using the IIM, which were created by transgene overexpression technology or RNA interference (Jiang et al., 2012a, 2012b, 2013a, 2013b).

Fig. 3. GIM for placing silkworms on mulberry leaves. (A) The leaves were arranged in the box after smearing them with BmNPV, and then they were air dried. (B) Five larvae were placed on each leaf. (C) The leaves after being eaten by the silkworms.

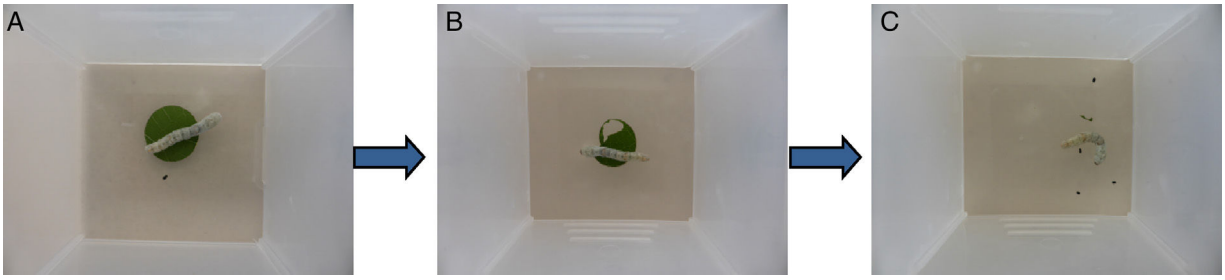

Fig. 4. IIM for placing silkworms on mulberry leaves. (A) One larva was placed on each leaf. (B) The larva eating the leaf. (C) The leaf after being eaten by the silkworm.

Rose Meire Costa Brancalhão's research group used a similar inoculation method to study the cytopathology of the *B. mori* trachea and pylorus infected with BmNPV (Baggio et al., 2014; Senem et al., 2016). They provided the parameters for mulberry leaf disk size and pathogen volume for newly molted fifth-instar larvae. Here, we described more parameters that are suitable for different instars. The method could be used to study other infectious silkworm diseases by oral infection, such as *B. mori* cytoplasmic polyhedrosis virus and *Nosema bombycis*.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by Guangdong Province Research Project Grants 2015A040404032 and 2015A010107009.

References

Baggio, M.P.D., Ribeiro, L.F.C., Vessaro-Silva, S.A., Brancalhão, R.M.C., 2014. *Bombyx mori* pylorus infection by Alphabaculovirus. Genet. Mol. Res. 13, 1–8.

Bao, Y.Y., Tang, X.D., Lv, Z.Y., Wang, X.Y., Tian, C.H., Xu, Y.P., Zhang, C.X., 2009. Gene expression profiling of resistant and susceptible *Bombyx mori* strains reveals nucleopolyhedrovirus-associated variations in host gene transcript levels. Genomics 94, 138–145.

Chen, H., Li, G., Huang, G., Chen, K.P., Yao, Q., Guo, Z., 2010. Characterization of ORF29 of *Bombyx mori* nucleopolyhedrovirus. Acta Virol. 54, 275–280.

Cheng, Y., Wang, X.Y., Hu, H., Killiny, N., Xu, J.P., 2014. A hypothetical model of crossing *Bombyx mori* nucleopolyhedrovirus through its host midgut physical barrier. PLOS ONE 9, e115032.

Huang, K., Li, C., Gan, J., Meng, B., Zhi, L., Zhou, Z., 2009. A preliminary study on utilizing RNA interference to confer enhanced resistance to BmNPV in transgenic silkworm. Sci. Sericult. 35, 308–313.

Isobe, R., Kojima, K., Matsuyama, T., Quan, G.X., Kanda, T., Tamura, T., Sahara, K., Asano, S.I., Bando, H., 2004. Use of RNAi technology to confer enhanced resistance to BmNPV on transgenic silkworms. Arch. Virol. 149, 1931–1940.

Jiang, L., Xia, Q.Y., 2014. The progress and future of enhancing antiviral capacity by transgenic technology in the silkworm *Bombyx mori*. Insect Biochem. Mol. Biol. 48, 1–7.

Jiang, L., Cheng, T., Zhao, P., Yang, Q., Wang, G., Jin, S., Lin, P., Xiao, Y., Xia, Q., 2012a. Resistance to BmNPV via overexpression of an exogenous gene controlled by an inducible promoter and enhancer in transgenic silkworm, *Bombyx mori*. PLOS ONE 7, e41838.

Jiang, L., Wang, G., Cheng, T., Yang, Q., Jin, S., Lu, G., Wu, F., Xiao, Y., Xu, H., Xia, Q., 2012b. Resistance to *Bombyx mori* nucleopolyhedrovirus via overexpression of an endogenous antiviral gene in transgenic silkworms. Arch. Virol. 157, 1323–1328.

Jiang, L., Zhao, P., Wang, G., Cheng, T., Yang, Q., Jin, S., Lin, P., Xiao, Y., Sun, Q., Xia, Q., 2013a. Comparison of factors that may affect the inhibitory efficacy of transgenic RNAi targeting of baculoviral genes in silkworm, *Bombyx mori*. Antiviral Res. 97, 255–263.

Jiang, L., Peng, Z., Dang, Y., Zhou, P., Xiao, Y., Xing, D., Xia, Q., 2013b. Construction and resistance assay of transgenic silkworm expressing RNAi targeting nonessential immediate gene ie-2 of BmNPV. Sci. Sericult. 39, 710–715.

Jiang, L., Zhao, P., Xia, Q.Y., 2014. Research progress and prospect of silkworm molecular breeding for disease resistance. Sci. Sericult. 40, 571–575.

Kanginakudru, S., Royer, C., Edupalli, S.V., Jalabert, A., Mauchamp, B., Prasad, S.V., Chavancy, G., Couble, P., Nagaraju, J., 2007. Targeting ie-1 gene by RNAi induces baculoviral resistance in lepidopteran cell lines and in transgenic silkworms. Insect Mol. Biol. 16, 635–644.

Lu, R., Shi, M., Bi, L., Gu, J., Huang, J., 2007. A preliminary investigation on the resistance of the silkworm variety resources in Guangxi to BmNPV. Sci. Sericult. 33, 117–121.

Rahman, M.M., Gopinathan, K.P., 2004. Systemic and in vitro infection process of *Bombyx mori* nucleopolyhedrovirus. Virus Res. 101, 109–118.

Senem, J.V., Torquato, E.F.B., Ribeiro, L.F.C., Brancalhão, R.M.C., 2016. Cytopathology of the trachea of *Bombyx mori* (Lepidoptera: Bombycidae) to *Bombyx mori* nucleopolyhedrovirus. Micron (Oxford 1993) 80, 39–44.

Subbaiah, E.V., Royer, C., Kanginakudru, S., Satyavathi, V.V., Babu, A.S., Sivaprasad, V., Chavancy, G., DaRocha, M., Jalabert, A., Mauchamp, B., Basha, I., Couble, P., Nagaraju, J., 2013. Engineering silkworms for resistance to baculovirus through multigene RNA interference. Genetics 193, 63–75.

Wu, F., Yang, Q., Xiao, Y., Chen, L., Liu, X., Xing, D., Luo, G., Tang, C., Wang, Z., 2010. Preliminary report of the selective effect of continuous feeding with BmNPV. Guangdong Sericult. 44, 20–23.

Xu, A.Y., Lin, C.Q., Qian, H.Y., Sun, P.J., Zhang, Y.H., Liu, M.Z., Li, L., 2013. Breeding of a new silkworm variety 'Huakang 2' with tolerance to *Bombyx mori* nucleopolyhedrovirus disease. Sci. Sericult. 39, 275–282.

Xu, K., Li, F., Ma, L., Wang, B., Zhang, H., Ni, M., Hong, F., Shen, W., Li, B., 2015. Mechanism of enhanced *Bombyx mori* nucleopolyhedrovirus-resistance by titanium dioxide nanoparticles in silkworm. PLOS ONE 10, e0118222.

Yang, H., Liu, Z.H., Liu, S., Li, T., Tian, M.H., Tang, F.F., Li, M., Xue, A.Y., 2013. Resistance of silkworm variety 871N × 872N in Yunnan Province, China. Sericulture 34, 42–44.

Yao, Q., Li, M.W., Wang, Y., Wang, W.B., Lu, J., Dong, Y., Chen, K.P., 2003. Screen of molecular markers for NPV resistance in *Bombyx mori* L. (Lep., Bombycidae). J. Appl. Ent. 127, 134–136.

Zhang, P., Wang, J., Lu, Y., Hu, Y., Xue, R., Cao, G., Gong, C., 2014. Resistance of transgenic silkworm to BmNPV could be improved by silencing ie-1 and lef-1 genes. Gene Ther. 21, 81–88.