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Laboratory Rearing Techniques of the Ostrinia furnacalis and Their Application in Bt Toxin Bioassays

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Abstract In this study, an artificial population of Asian corn borer (*Ostrinia furnacalis*) was established under laboratory conditions to provide the insect source required for Bt toxin bioassay. The initial insect source was obtained by collecting larvae and pupae from the field. Under the conditions of constant temperature of 26 °C, relative humidity of 70%~90%, and photoperiod of 10 L: 14 D, the larvae were fed with fresh corn ears every day and their feeding and development processes were observed. It was observed that under laboratory conditions, it takes about 34~37 days for *Ostrinia furnacalis* to complete a generation. The average generation period of females is 36.2 ± 0.72 days, and that of males is 34.8 ± 0.72 days. The larval stage is 17-19 days, the pupal stage is 7-9 days, and the adult lifespan is 5-7 days. The larvae go through 5 instars, and the width of the head shell and the body length of each instar are significantly different. The investigation found that the feeding sites of larvae of different ages on corn plants were different. The second-instar larvae had a wider feeding range, while the older larvae mostly bored into the inside of the corn stalks to cause damage. In addition, the tolerance of larvae to Bt toxins increased with age. The established laboratory rearing method can efficiently cultivate consecutive generations of Asian corn borer, providing a standardized insect source for Bt toxin bioassay and resistance monitoring.
Keywords Asian corn borer; Laboratory rearing; Life history observation; Bt toxin; Bioassay

1 Introduction

The Asian corn borer (*Ostrinia furnacalis*) is one of the major insect pests of corn crops in Asia and the Western Pacific. In most corn-producing areas in China, the corn borer can produce 1 to 7 generations a year, and the larvae feed on a wide range of hosts, including corn, sorghum, cotton and dozens of other crops (Liu et al., 2021; Liu et al., 2023; Wang and Liu, 2023). Young larvae mainly feed on the heart leaves, filaments and tassels of corn, while older larvae bore into the stalks and cobs to feed on the grains, often causing the corn plants to fall over and the ears to mold, resulting in a reduction in corn yield, which can generally be as much as 10% to 30%. Transgenic Bt corn has high resistance to corn borers such as corn borers by expressing Bacillus thuringiensis toxin proteins. It has been widely planted around the world and is regarded as an effective means of controlling corn borers (Li et al., 2024). However, large-scale planting of Bt corn may exert continuous resistance selection pressure on target pests, and corn borer populations are at risk of evolving resistance to Bt toxins. In order to ensure the long-term plant protection effect of Bt corn, it is necessary to establish baseline data for resistance monitoring of target pests before promotion, and to continuously carry out resistance monitoring during the promotion process (Li et al., 2024; Wang et al., 2024).

Laboratory artificial breeding technology is the basis for conducting biological research and resistance monitoring of corn borers. Artificial breeding can continuously provide a large number of developmentally synchronized, healthy and neat insect sources throughout the year for various experiments, such as laboratory bioassays to determine the sensitivity of corn borers to Bt toxins. As early as the 1970s, Chinese scholars began to explore the artificial feed formula and mass breeding technology of Asian corn borers, and made important breakthroughs, making large-scale indoor breeding of corn borers possible (Wang et al., 2000). Typical artificial feeds for corn borers contain nutritional components such as corn or bean powder, bran, yeast powder, vitamins, and some formulas also add agar to solidify the feed (Bernklau and Bjostad, 2008).

However, agar increases feed costs and is prone to breeding bacteria. In recent years, studies have improved the formula of semi-artificial feeds without agar, such as replacing coagulants with corn stalk powder and soluble starch, which not only reduces costs but also is suitable for the growth and reproduction of corn borers, and realizes long-term succession rearing of corn borers (Chen et al., 2018). Countries such as the Philippines and Vietnam have also developed artificial feeds based on raw materials such as red beans and rice bran for large-scale rearing of Asian corn borers, which have been used in pest monitoring since 2009 (Rahayu and Trisyono, 2018). Compared with artificial feeds, direct feeding on host plants often achieves higher feeding rates and survival rates (Da Silva Ramos et al., 2022).

This study was based on the rearing of Asian corn borer on corn ears under laboratory conditions, systematically observing its life history and biological characteristics, analyzing and evaluating the feeding behavior and Bt toxin sensitivity of larvae of different ages, and screening the insect age suitable for Bt toxin bioassay, providing a reference for the standardization of artificial rearing technology of Asian corn borer, standardization of Bt toxin bioassay methods and resistance monitoring.

2 Materials and Methods

2.1 Insect source acquisition

The initial insect source of Asian corn borer was collected from the corn fields in Sanya City, Hainan Province. During the period from tasseling to grain filling, corn plants with borer symptoms were randomly selected in the field, and the corn husks were peeled to check for borers in the corn cobs and stalks. The corn borer larvae found were gently brushed into a clean glass bottle with a clean brush and collected. For individuals that had pupated at the base of the corn cob, the corn borer pupae were carefully removed with tweezers. The collected larvae and pupae were brought back to the laboratory breeding room for separate feeding and management (Figure 1). The field collection time was the autumn corn growing season, and the initial insect source included several larvae and dozens of pupae from multiple natural populations in the field to ensure genetic background diversity (Fang et al., 2021).

Figure 1 Acquisition of Asian corn borer larvae from corn ears

2.2 Rearing equipment and feed

2.2.1 Rearing container

Larvae were cultured in wide-mouth glass bottles (volume 500 mL) with gauze covers or transparent plastic boxes to ensure good ventilation and facilitate observation. Adults were reared in a 40 cm × 40 cm × 40 cm gauze cage with a corn seedling with heart leaves placed inside as an egg-laying substrate (Figure 2).

2.2.2 Feed source

Larvae feed is young corn ears with husks (with milky kernels) freshly picked from the field. Select fresh corn ears that are not infected by borers, remove the outer husks and keep the tender husks and filaments. Provide sufficient fresh corn ears for larvae to feed on every day according to the number of larvae consumed, and replace corn ears that have finished feeding or become dry and deteriorated in time. For adults, prepare 10% honey water solution to soak cotton balls and place them in the cage for adults to feed (Figure 3).

Figure 2 Cloth sarong

Figure 3 Honey solution and cotton

2.3 Laboratory rearing conditions

The environment of the rearing room was kept at a constant temperature, humidity and light cycle. The temperature was set at 26 °C, the relative humidity was 70%~90%, and the light cycle was 10 h of light and 14 h of darkness (10L:14D) per day. The larval culture bottles were placed on the culture racks, arranged in layers, and well ventilated. The feeding and molting of the larvae were checked at a fixed time every day, and the feces were cleaned and fresh corn ears were replaced. The adult cages were placed in the same culture room, and the light was controlled by fluorescent lamps to alternate between light and dark, simulating a circadian rhythm close to nature.

2.4 Experimental methods

2.4.1 Laboratory breeding and observation of corn borer

The corn borer larvae collected from the field were placed in multiple culture bottles for feeding, and one ear of fresh corn was placed in each bottle for about 10 larvae to feed. During the feeding process of the larvae, the conditions of molting, replacement of cobs, etc. were observed and recorded regularly until the larvae left the cobs and quietly stopped on the wall of the bottle to pupate after mating. The mature larvae were collected in a clean culture dish covered with filter paper and allowed to pupate in the dish. The collected corn borer pupae were covered with moist filter paper to maintain a certain humidity and placed in an incubator for further cultivation.

After the adults emerged, a certain number of male and female adults (about 10 to 15 males and females) were placed in an egg-laying cage together, and tender corn plants were placed in the cage for mating and egg-laying. Check the back of the corn leaves in the cage every day to see if there are egg masses, and record the time and number of egg-laying. Cut the egg masses produced and place them in a small culture dish with moist filter paper to continue hatching the next generation of larvae. The above method was used to establish a continuous breeding population of corn borers in the laboratory, and the survival and development of each generation were recorded.

2.4.2 Larval age identification method

In order to establish a standard for judging larval age by morphological indicators, samples of corn borer larvae of various ages were collected during the feeding process (especially after the larvae molted). The head of the larvae was observed under a dissecting microscope, and the width of the head shell was measured as the main basis for age determination. At the same time, the body length of the larvae was measured (the straight length from the head to the tail end). Multiple larvae were measured to obtain the range of head shell width and body length for each age. Since corn borer larvae stop feeding and the head shell color darkens at the moment of molting, behavioral and morphological characteristics can be combined to assist in judging the age. The age boundaries were divided by statistically analyzing the head shell width data, and compared with the standards reported in the literature for verification (Ng et al., 1993; Gardner et al., 2001; Pintilie et al., 2023).

2.4.3 Field feeding site survey

Plots were set up in maize fields in Sanya to observe and record the damaged sites of larvae of different ages under natural field conditions. Field plots without pesticide application were selected to regularly collect several different parts of corn heart leaves, tassels, female ears and stems, and record the age of corn borer larvae found in them. By dissecting the damaged parts of the plants, the feeding location preferences of larvae of different ages were determined, such as heart leaves, leaf sheaths, filaments, grains or stem pith, etc. (Guo et al., 2018; Zastempowski et al., 2024). This field survey is used to assist the laboratory in analyzing the relationship between larval age and feeding behavior, and to provide a basis for screening suitable insect ages for bioassay.

2.4.4 Bt toxin bioassay method

The focus of this study is to screen insect ages suitable for Bt toxin sensitivity determination, so the Bt toxin dose-effect bioassay was not directly implemented in the experiment. In existing studies, the commonly used Bt toxin bioassay uses the artificial feed poisoning method: the purified Bt crystal toxin is added to the semi-artificial feed without agar solidification in a gradient concentration series, and the first-instar larvae of corn borer that have been newly hatched for less than 12 hours are inoculated on the surface of the poisoned feed. After 7 days of feeding under appropriate conditions, the mortality rate is counted to calculate the toxin concentration that mediates 50% mortality (LC50) (Farhan et al., 2023; Smith and Farhan, 2023). This method has been used to monitor the sensitivity baseline of corn borer field populations in different regions of China to toxic proteins such as Cry1Ab and Cry1F (Rahayu and Trisyono, 2018). This study will analyze the selection of appropriate insect age for bioassay in the discussion combined with the experimental results.

3 Results and Analysis

3.1 Laboratory rearing generation record of Asian corn borer

Under constant temperature of 26 °C, it takes about 34~37 days for Asian corn borer to complete a generation cycle (egg-larva-pupa-adult-egg) (Figure 4). There are slight differences in the development period of male and female individuals: females take about 36.2 ± 0.72 days to complete a generation on average, and males take 34.8 ± 0.72 days on average. There is little difference between the two sexes in the length of the larval and pupal stages, but male pupae emerge slightly earlier than female pupae. This data is basically consistent with the results of 27~34 days obtained by Rahayu et al. (2018) under artificial rearing conditions. The width of the head shell of corn borer larvae at different ages is significantly different and there is no overlap, which can be used as a reliable basis for age judgment. The entire feeding period of the larvae is generally about 17~19 days, during which they undergo 4 molting. Molting usually occurs during feeding intervals and is accompanied by behavioral changes (such as stopping feeding, spinning silk to fix the body, etc.).

3.2 Division of larval age by morphological characteristics

By measuring the length of the abdominal tube and the width of the head shell, the experiment divided the larvae of the Asian corn borer into five age stages (Figure 5). The head shell width and body length range of the larvae of each age were measured in this experiment as follows:

Instar larvae: body length is about 3.0~8.2 mm, head shell width is about 0.50~0.82 mm. The body color is milky white, the head is light yellow-brown, and there are scattered small black spots.

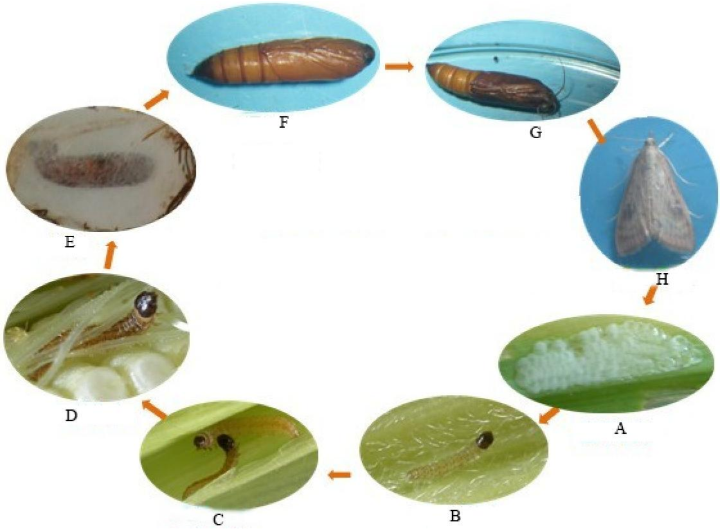

Figure 4 One generation of the Asian corn borer observed in the laboratory

Note: A: corn borer eggs; B: second-instar larvae; C: third-instar larvae; D: fifth-instar larvae; E: pupation; F: corn borer pupa; G: before eclosion; H: corn borer moth

Instar larvae: body length is about 8.2~13.4 mm, head shell width is about 0.82~1.14 mm. The body color is light pink, the spots on the back are clearly visible, and the movement is more active than instar I.

Instar larvae: body length is about 13.4~18.6 mm, head shell width is about 1.14~1.46 mm. The body color gradually deepens, from pink to light brown, and the 4 pairs of oval black spots on the back of the body are more obvious.

Instar larvae: body length is about 18.6~23.8 mm, head shell width is about 1.46~1.78 mm. Body color is pink-brown, the spots on the back are enlarged and blackened, and the feeding amount increases significantly.

Instar larvae: body length is about 23.8~29.0 mm, head shell width is about 1.78~2.10 mm. Body color is light gray or pink-brown, the back spots are relatively lightened, but the spots tend to merge. After entering the mature stage, the body color turns yellow-white, and it stops feeding and prepares to pupate.

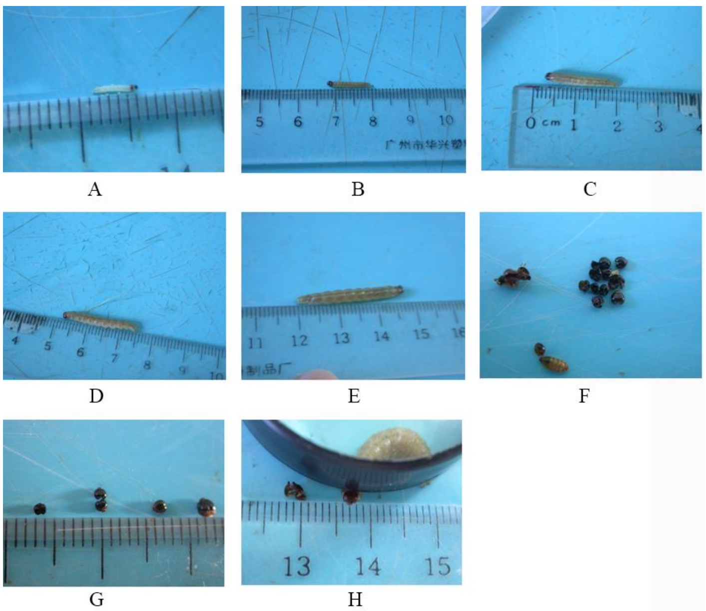

Figure 5 Asian corn borer larvae

Note: A: I instar larvae: 3.5 mm; B: II instar larvae: 12 mm; C: III instar larvae: 18 mm; D: IV instar larvae: 23 mm; E: V instar larvae: 28 mm; F: larval head shell and molting; G: larval head shell width; H: IV instar larvae and head shell

Among them, the length of the abdominal tube and the width of the head shell of each insect age are significantly different, with a small error range and low overlap, with high identification accuracy, and can be used as a morphological standard for insect age division. This is consistent with the view in Mingxia et al. (2013) and Fu et al. (2022) that the width of the head shell is the main criterion for insect age.

3.3 Observation of morphological characteristics of adults, eggs, pupae, etc.

Adult stage: Corn borer adults are medium-sized gray-brown moths, with wingspans of about 30 mm and 25 mm for males and females, respectively. The female moth has yellow forewings with purple-brown wavy patterns, while the male moth is slightly darker and smaller. Adults are mostly active at night, and their lifespan is generally 5~7 days. Mating usually takes place on the second night after emergence. After mating, male moths often become weak and die the next day, while female moths can survive and die after laying eggs. On the corn seedlings provided in the cage, female moths mostly lay egg masses on the back of corn leaves near the midrib, and each female lays an average of about 120 eggs.

Eggs: Milky white, flat and oval, arranged closely like fish scales, and the top of the egg turns black before hatching, which is the "black head stage". After the eggs hatch for about 4~5 days, the new generation of larvae crawl out of the egg mass and begin to feed on the tender parts of the host plant.

Pupa: Mature larvae pupate in the gaps between corn cob husks or in paper rolls provided by humans. The initial pupa is light yellow and turns reddish brown after a few hours. The pupa is about 15~18 mm long, spindle-shaped, and has no cocoon. The pupal period is about 7~9 days at 26 ℃. The difference between male and female in the pupal period can be distinguished by the morphology of the end of the abdomen: the female pupa has a pair of tail spines at the end, while the male pupa has no obvious tail spines but a round reproductive protrusion. It was observed that males often emerge about 0.5~1 day earlier than females. Multiple generations of observation showed that the morphology was stable and no deformation was found. The pupal body color was typed, suggesting that it may be related to pupal age or sexual development, which needs further verification (Figure 6) (Cagáňet al., 2012; Lu, 2014).

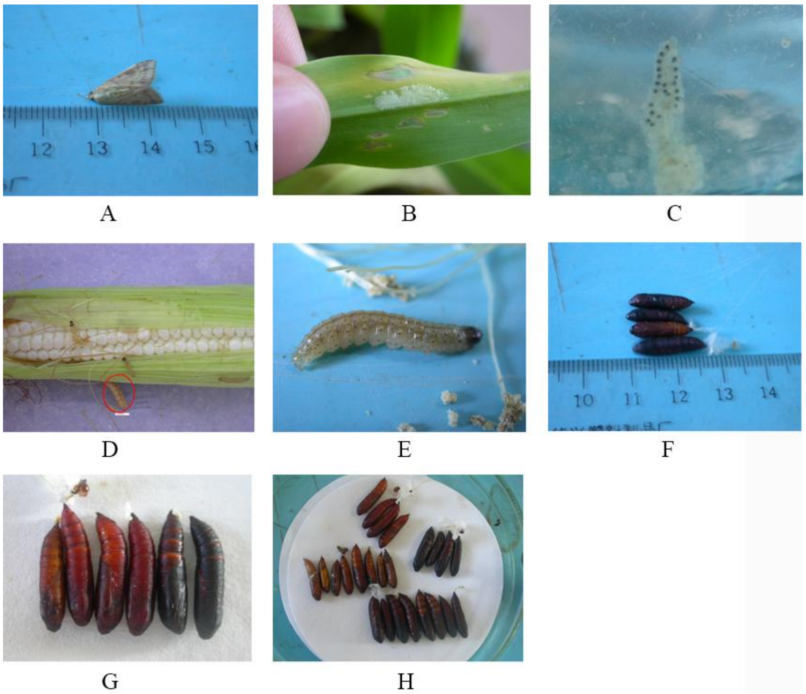

Figure 6 Morphological characteristics of Asian corn borer adults, eggs, and pupae

Note: A: Adults; B: Bot eggs; C: Black-headed Asian corn borer eggs; D: Mature larvae of Asian corn borer; E: Tubers on the back of the abdomen of Asian corn borer; F: Length of Asian corn borer pupae; G: Asian corn borers of different colors; H: Classification of Asian corn borer pupae of various colors

3.4 Screening of larvae suitable for Bt toxin bioassay

Through field plots and indoor observations, it was found that the larvae of the II instar are most suitable for Bt toxin bioassay in terms of damage site, migration ability and body size: the larvae of the II instar have a wide feeding range, feeding on both heart leaves and filaments and the surface of young ears, which is equivalent to a longer "window period" for field damage, which can represent the extensive exposure to Bt toxins; in addition, the adaptability of the larvae of the II instar to the external environment and feed is significantly improved compared with the I instar, and the individuals are larger and easier to handle, but they still maintain a high sensitivity to Bt toxins compared with older larvae (Wen et al., 2005).

As the age increases, corn borer larvae show a gradually increasing tolerance to Bt toxins, which is the phenomenon of "age resistance". Studies have shown that the tolerance of older larvae to Bt toxins can be more than one order of magnitude higher than that of younger larvae (Huynh et al., 2022). For example, the tolerance of older larvae of some lepidopteran insects to Cry1Ab toxin is 12 to 23 times that of younger larvae (Hellmich et al., 2001). Therefore, if larvae of too old an age are used in bioassays, the actual lethal activity of the toxin may be underestimated, which is not conducive to timely detection of resistance signs (Dively et al., 2016). Considering the convenience of operation and test sensitivity, the selection of instar II larvae for Bt toxin bioassay is an ideal choice because it is easy to handle and can sensitively reflect the difference in toxicity.

4 Discussion

This study successfully established a continuous generation artificial population of Asian corn borer in the laboratory by using corn cob feeding method. The results showed that under controlled conditions such as 26℃, high humidity, and suitable light, fresh corn cobs can effectively meet the growth and development needs of larvae, with a larval survival rate of over 91% and stable population reproduction, indicating that this method is easy to operate, has a healthy insect source, and is suitable for bioassays. This is consistent with the effect of using natural feed to feed corn borers in Wang et al. (2023). Compared with artificial feed, cob feeding depends on the corn planting cycle. If it is in the off-season, it is possible to consider introducing improved semi-artificial feed. Recent studies have shown that an optimized formula based on soybean meal, corn meal and vitamins can achieve efficient artificial feeding of corn borers (Rahayu and Trisyono, 2018; Alam et al., 2024). In addition, the agar-free formula reduces feed costs and avoids the problem of Bt toxin adsorption and inactivation (Wang et al., 2023).

In terms of insect age identification, this study uses the width of the head shell as a judgment indicator to clearly divide the five instars, providing a standard for the unification of insect sources for bioassay. The larvae of the second instar are of moderate size, high sensitivity, and easy to operate, making them the ideal insect age for Bt toxin bioassay. Previous studies have shown that as the age increases, the tolerance of corn borers to Bt toxins significantly increases (Hellmich et al., 2001), so the second instar is more convenient to operate and has better sensitivity than the first instar.

In terms of Bt toxin bioassay, the second instar larvae have been widely used in resistance monitoring research in my country. For example, Liu et al. (2022) used the second instar larvae to determine the sensitivity of multiple corn borer populations across the country to Cry1Ab, and no resistance mutations were found. Combined with the results of this study, it is recommended to use the second instar larvae as the standard test insect in the establishment of resistance baselines and field resistance monitoring, with a unified toxin concentration and feeding method to improve data consistency.

In the future, it is possible to consider using transient gene editing without exogenous DNA to transform breeding strains to reduce insect source differences and further standardize the Bt toxin sensitivity assessment process (Dively et al., 2020). It is recommended that monitoring stations in various regions collect field egg masses and artificially raise them to instar II, use a unified bioassay procedure to establish a national sensitivity database, and combine metabolomics and transcriptomics to explore potential resistance mechanisms, providing scientific support for Bt corn resistance management.

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Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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