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Effects of Artificial Diets on the Rearing of *Pagiophloeus tsushimanus* Larvae

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Abstract **[Objective]** The paper was to investigate the artificial rearing of *Pagiophloeus tsushimanus* larvae and to propose a novel approach for addressing the challenges associated with test insects for pest research experiments. **[Method]** A study was conducted to investigate the effects of three semi-artificial diets on the rearing of *P. tsushimanus* larvae. **[Result]** The study revealed significant differences in the fitness performance of *P. tsushimanus* larvae reared on three distinct semi-artificial diets. Notably, larvae that were fed semi-artificial diets formulated from their natural host plant, *Cinnamomum camphora*, exhibited superior growth and developmental performance. In contrast, *P. tsushimanus* larvae that were provided with semi-artificial diets derived from *Cinnamomum chekiangense* and *Phoebe chekiangensis*, which are closely related species, demonstrated marked fitness costs. These costs were evidenced by a significant prolongation of the larval developmental duration, a substantial decrease in body weight and average daily weight gain, as well as a notable reduction in survival rate, pupation rate, and emergence rate. **[Conclusion]** Semi-artificial diets derived from the natural host plant, *C. camphora*, represent the most effective dietary regimen for the indoor rearing of *P. tsushimanus* larvae. This approach not only ensures a sufficient supply of test insects but also serves as a viable rearing method for research focused on the integrated prevention and control of *P. tsushimanus*.

Keywords Semi-artificial diet; *Cinnamomum chekiangense*; *Phoebe chekiangensis*; Developmental duration; Pupation rate; Emergence rate

Cinnamomum camphora is an evergreen tree that belongs to the genus *Cinnamomum* within the family Lauraceae and the order Laurales[1]. This species is distributed across various regions, including Vietnam, North Korea, Japan, as well as in South and Southwest China. It serves as a significant species for landscaping purposes and is also recognized as a valuable source of timber and an economically important forest species in China[1–2]. *Pagiophloeus tsushimanus* belongs to *Pagiophloeus*, Hylobiini, Molytinae, Curculionidae, Coleptera. This species has been identified as a new record in China and is characterized as a monophagous borer pest that exclusively affects the native species *C. camphora* in the region[3]. The adults primarily harm the epidermis of lateral branches in 1- to 2-year-old shoots within the tree crown. The larvae consume the cambial layer situated between the xylem and phloem, creating a cavity that significantly damages the tree's conducting tissue. This disruption results in a deficiency of water and nutrients in the *C. camphora* tree, leading to diminished vigor and the development of protuberances surrounding the cavity, which adversely impacts the tree's aesthetic appeal[4]. The cavity channels are arranged horizontally and continuously release fine sawdust and insect excrement. Following significant damage, the growth of *C. camphora* is weakened, leading to bark fissures that adversely impact the aesthetic value and pose a substantial threat to the ecological health of *C. camphora* forests. In cases of severe damage, this can result in the mortality of the tree[4]. The resources of *C. camphora* in South China are currently under significant threat from the pest *P. tsushimanus*. Extensive areas of *C. camphora* forests are endangered by this insect, resulting in severe damage. In regions experiencing critical infestations, the damage rate to the plants can exceed 90%. The average population density of the pest is estimated to be 30–40 individuals/plant, with peak infestations recorded at a density of 63 individuals/plant. Furthermore, there is a concerning trend of population spread, which poses potential risks to the ecosystem[4].

Given that *P. tsushimanus* is a re-

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cently identified stem-boring pest, the present study primarily concentrates on several key areas: its taxonomic classification, mating behavior, observation of the microscopic characteristics of antennal sensilla, detoxification mechanisms, chemosensory processes, functional gene analysis, monitoring of attractants, and the management of both larvae and adult populations through perforation injection and microcapsule suspension spraying techniques[4–10]. Currently, the larvae of *P. tsushimanus* are primarily collected by harvesting segments of *C. camphora* wood. However, these wood segments and shoots are highly prone to dehydration and require regular replacement, a process that is both time-consuming and labor-intensive. Furthermore, this method complicates the observation of the developmental stages of the insects. Consequently, the wood segment rearing method is not appropriate for long-term, large-scale artificial rearing, and this method restricts indoor research on this pest. In this study, we selected branches from *C. camphora*, along with two additional species from the Lauraceae family: *Cinnamomum chekiangense*, an evergreen tree belonging to the genus *Cinnamomum*, and *Phoebe chekiangensis*, an evergreen tree classified within the genus *Phoebe*. These plant materials were formulated into semi-artificial diets for the purpose of artificially rearing *P. tsushimanus* larvae, to investigate the effects of artificial rearing on *P. tsushimanus*. This study aims to develop artificial diets and feeding methods for the investigation of the biological and ecological characteristics of the pest, as well as integrated pest management. Additionally, it offers a sufficient supply of test insects for research purposes.

1 Materials and Methods

1.1 Insect collection and adult rearing

Pagiophloeus tsushimanus collected from *C. camphora* plantation forests were transported to the entomological insectary for artificial rearing. Each of the five pairs of male and female *P. tsushimanus* adults was housed in a plastic insect box measuring 30 cm in length, 20 cm in width, and 15 cm in height, which contained branches of *C. camphora* that were 1 to 2 years old. The insect box was designed with ventilation holes measuring 0.5 cm in diameter on both sides. Additionally, the bottom of the box was connected to water-soaked flower clay and featured appropriately sized openings to support the upright positioning and preservation of *C. camphora* branches. Fresh branches were replaced every 5 d, and the egg grains were collected. The eggs were typically laid within the branches and covered with secretions, necessitating the manual removal of the grains. Eggs laid on branches by adults on the same day were collected and transferred to petri dishes paved with skimmed cotton wool. These dishes were periodically misted with sterile water to maintain moisture levels. Subsequently, every 20–30 eggs were placed in a glass petri dish (5 cm in diameter × 1 cm in height) that contained water-saturated skimmed cotton at the base. The eggs were then incubated until hatching occurred. The incubation of eggs was systematically observed and documented at regular intervals on a daily basis. Throughout the entire feeding process, the environmental temperature and humidity were maintained at (28 ± 1) ℃ and (70 ± 5) %, respectively. The photoperiod for the adults was established at 16 : 8 h, while other insects were reared in complete darkness.

1.2 Preparation of semi-artificial diets

In accordance with the method for rearing *Monochamus alternatus* larvae as described by Fan *et al.*[11], branches aged one and two years from three plant species, *C. camphora*, *C. chekiangense*, and *P. chekiangensis*, were collected to serve as the primary components of semi-artificial diets. The leaves were excised, and the remaining branches were subjected to washing and sterilization using a 75% ethanol solution. Subsequently, the branches were rinsed three times with distilled water. Following natural drying, the branches were processed into fine sawdust utilizing a high-speed pulverizer. The components were weighed in accordance with the following formula: 100 g of fresh branch powder, which was passed through a 70 mesh sieve; 4 g of sucrose; 3 g of yeast; 7.5 g of agar; 0.5 g of sodium benzoate; 0.2 g of sorbic acid; 1.5 mL of 0.5 M dilute sulfuric acid; and 150 mL of distilled water. 150 mL of distilled water was initially added to the container and brought to a boil. Subsequently, agar powder was incorporated and the mixture was boiled again until the agar was completely dissolved in the boiling water. Following this, sucrose, yeast powder, sodium benzoate, sorbic acid, and dilute sulfuric acid were added to the container, and the mixture was stirred thoroughly before being boiled once more. Fresh branch powder was then crushed using a pulverizer, added to the container, and mixed well. The resulting mixture was subsequently transferred into Petri dish containers and allowed to cool in preparation for UV sterilization. Finally, the diets were stored in a refrigerator at 4 ℃ for future use.

1.3 Determination of the effect of semi-artificial diets for rearing *P. tsushimanus* larvae

An indoor rearing system for *P. tsushimanus* was developed by adapting the rearing techniques utilized for stem-boring Coleoptera, with specific modifications[12–14]. All three semi-artificial diets were employed for the rearing of *P. tsushimanus* larvae following the outlined method. An adequate quantity of sterilized semi-artificial diet was introduced into each well of a six-well culture plate (with an inner diameter of 3.5 cm and a height of 1.5 cm per well). Subsequently, the newly hatched larvae were transferred to the six-well Petri dish that was filled with semi-artificial diet, facilitating single-well, single-head rearing until the pupation stage. A single hatching larva was placed in a small hole of the diet, allowing the larva to burrow into the diet independently. This method was employed to assess the larva's activity and feeding capability. Only one hatching larva was maintained in each hole of the diet to facilitate observation and data collection. The diet was replaced every 3 d. Following the dietary replacement, the larvae were returned to their original temperature conditions for cultivation. Observations and recordings were made regarding the survival of the larvae, the frequency of molting, the survival rate, and the weight of the insects. These measurements were continuously documented until the occurrence of pupation and emergence. The larvae were weighed every 10 d, and the average daily weight gain was subsequently calculated. Average daily weight gain $\Delta = (a_n - a_0)/n$, where n denotes the number of feeding days, a_n represents the weight of the insect after n days of feeding, and a_0 indicates the weight of the newly hatched larvae. Three replicates were set up, with 20 larvae in each replicate. The developmental duration, larval weight, survival rate, pupation rate, and emergence rate were calculated for each larval instar of *P. tsushimanus* when subjected to three semi-artificial diets. Upon the completion of pupation in the mature larvae, individual pupae were transferred to round-bottomed glass test tubes (20 mL) containing dry skimmed cotton at the base to facilitate incubation until adulthood. The environmental temperature was consistently maintained at (28 ± 1) ℃, with humidity levels kept at (70 ± 5) % throughout the rearing process. The larvae were reared in total darkness.

1.4 Statistical analysis of data The significance of differences in the developmental duration and weight of larvae across all instars was statistically assessed utilizing the Kruskal-Wallis analysis of variance(ANOVA) alongside multiple comparisons in non-parametric tests ($P<0.05$). The significance of differences in survival rate, pupation rate, and emergence rate was evaluated through the *Chi*-square test and Log-likelihood test ($P<0.05$). Raw data were systematically recorded using Excel 2003 software and subsequently analyzed with SPSS 20.0.

2 Results and Analysis

2.1 Effect of semi-artificial diets on the developmental duration of *P. tsushimanus* larvae Following the administration of semi-artificial diets derived from three distinct host plants, the larvae of *P. tsushimanus* exhibited statistically significant differences across various treatments during the first instar larval stage (F=9.072, P<0.001), the second instar larval stage (F=17.444, P<0.001), the third instar larval stage (F=98.843, P<0.001), and the fourth instar larval stage (F = 23.524, P<0.001) as presented in Tab.1. The duration of the first instar stage for larvae feeding a diet of *C. chekiangense* was significantly longer compared to those fed on diets of *C. camphora* and *P. chekiangensis*. However, no significant difference was observed in the first instar duration between larvae fed on *C. camphora* and *P. chekiangensis* diets. In the case of the second instar stage, larvae reared on the *C. camphora* diet exhibited a significantly longer duration than those reared on the *C. chekiangense* and *P. chekiangensis* diets. For the third and fourth instar stages, the developmental duration of larvae fed on the *C. camphora* diet was significantly shorter than that of larvae fed on *C. chekiangense* and *P. chekiangensis* diets. Overall, larvae fed on *C. camphora* demonstrated the shortest total developmental duration from the first to the fourth instar, averaging 46.29 d, followed by those on the *P. chekiangensis* diet at 57.75 d, while larvae on the *C. chekiangense* diet had the longest duration at 60.53 d (Tab.1).

2.2 Effect of semi-artificial diets on the growth of *P. tsushimanus* larvae As illustrated in Tab.2, there was no statistically significant difference in body weight among the newly hatched larvae (first instar larvae) (F=0.233, P=0.793). However, the body weights of the second instar larvae (F=40.173, P<0.001) and the third instar larvae (F=27.555, P<0.001) exhibited significant differences across various treatments following extended rearing on three semi-artificial diets. In contrast,

Tab.1 Effect of semi-artificial diets on the developmental duration of *P. tsushimanus* larvae d

Larva	Duration of larvae in *C. camphora* diet group	Duration of larvae in *C. chekiangense* diet group	Duration of larvae in *P. chekiangensis* diet group
First instar	6.56±0.17 b	7.34±0.18 a	6.39±0.14 b
Second instar	12.35±0.43 a	8.98±0.33 b	9.78±0.44 b
Third instar	11.93±0.39 c	21.90±0.79 a	18.44±0.49 b
Fourth instar	15.45±0.67 b	22.31±0.82 a	23.14±0.71 a

Note: The data presented in the table are mean ± standard error (SE). Different lowercase letters within the same column denote statistically significant differences (P<0.05).

Tab.2 Effect of semi-artificial diets on the weight of *P. tsushimanus* larvae mg

Larva	Weight of larvae in *C. camphora* diet group	Weight of larvae in *C. chekiangense* diet group	Weight of larvae in *P. chekiangensis* diet group
First instar	1.49±0.02 a	1.48±0.12 a	1.47±0.08 a
Second instar	5.48±0.10 a	4.53±0.13 b	4.50±0.08 b
Third instar	10.81±0.26 a	8.72±0.16 b	8.77±0.18 b
Fourth instar	23.59±0.56 a	25.27±0.91 a	25.21±0.76 a

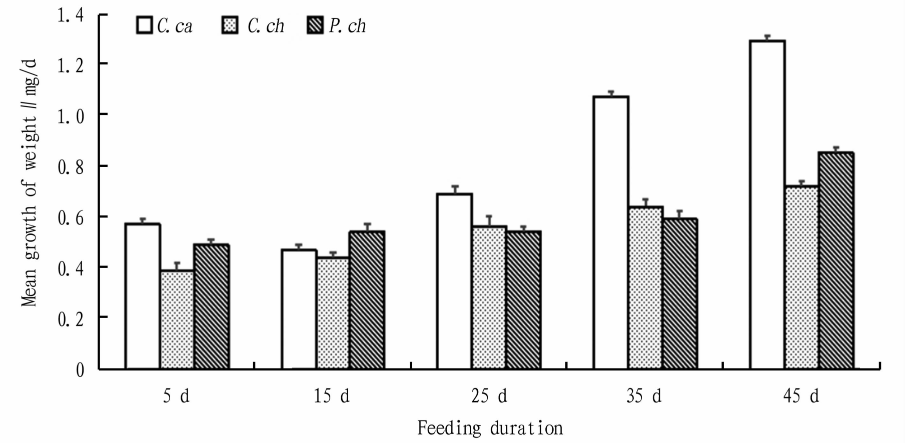
Note: The data presented in the table are mean ± standard error (SE). Different lowercase letters within the same column denote statistically significant differences (P<0.05).

the body weights of the fourth instar larvae (F=2.066, P=0.137) did not show significant variation (Tab.2). Notably, the weights of larvae that were fed semi-artificial diets containing *C. camphora* during the second and third instars were significantly greater than those of larvae fed on semi-artificial diets of *C. chekiangense* and *P. chekiangensis* (P<0.05, Tab.2).

As illustrated in Fig.1, the relationship between the average daily weight gain of *P. tsushimanus* larvae fed on various semi-artificial diets was as follows: *C. camphora* diet > *P. chekiangensis* diet > *C. chekiangense* diet. Analyzing the trend in average daily weight gain over the feeding duration revealed that, starting from day 15, the larvae in the *C. camphora* diet group exhibited the most significant increase in average daily weight gain. This increase was markedly greater than that observed in the *C. chekiangense* and *P. chekiangensis* diet groups. Notably, at the conclusion of the feeding period, specifically at days 35 and 45, the disparity in average daily weight gain between the *C. camphora* diet group and the other two diets was most pronounced (Fig.1).

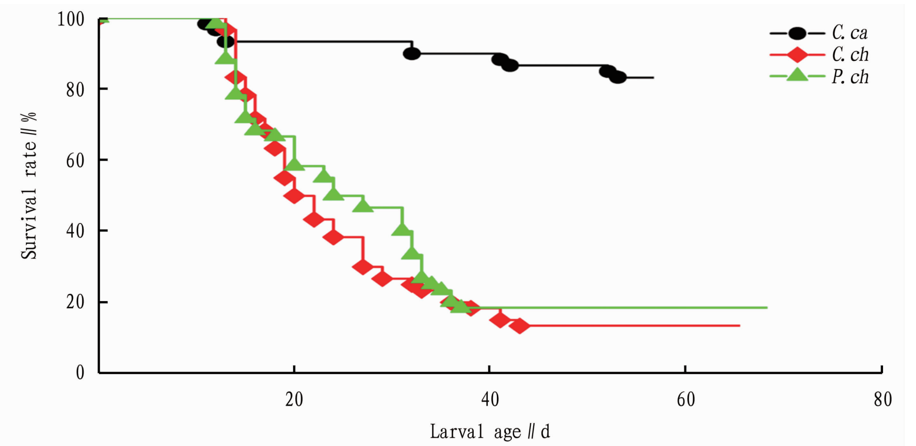
2.3 Effect of semi-artificial diets on the survival rate of *P. tsushimanus* larvae Fig.2 illustrates the survival curves of *P. tsushimanus* larvae that were reared on semi-artificial diets. A statistically significant difference was observed in the survival rates of *P. tsushimanus* larvae across the three semi-artificial diet groups (χ^2= 73.08, P<0.001, Fig.2). The survival rates of *P. tsushimanus* larvae ranked from highest to lowest were as follows: *C. camphora* diet group, *C. chekiangense* diet group, and *P. chekiangensis* diet group. Notably, the larvae in the *C. camphora* diet group exhibited a slight decline in survival rates between days 10 to 20 and days 30 to 50. In contrast, the larvae in the *C. chekiangense* and *P. chekiangensis* diet groups experienced a rapid decline in survival rates between days 10 to 30, ultimately resulting in survival rates falling below 20% for both groups (Fig.2).

2.4 Effect of semi-artificial diets on the pupation rate of *P. tsushimanus* larvae According to Fig.3, the three semi-artificial diets exhibited significantly different effects on the pupation rate of *P. tsushimanus* larvae (χ^2=59.53, P<0.001). The pupation rate for larvae in the *C. cam-*



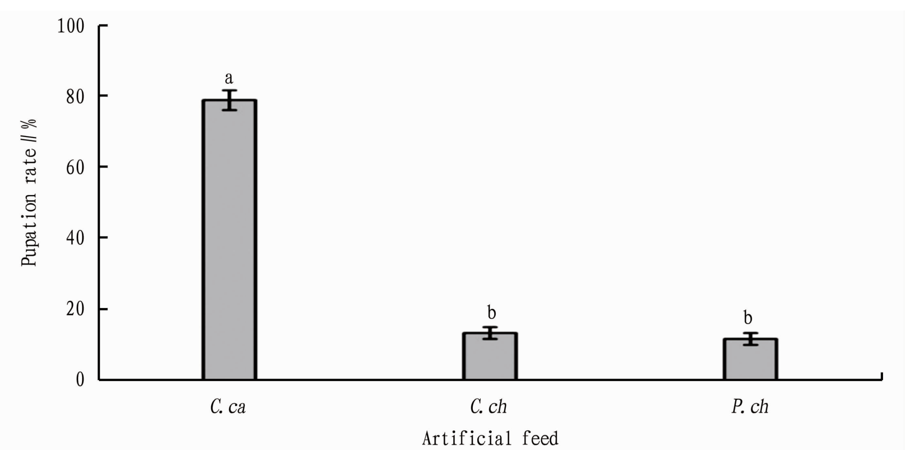
Note: *C. ca*, *C. ch*, and *P. ch* represent the artificial diet of *C. camphora*, *C. chekiangense* and *P. chekiangensis* respectively.

Fig.1 Effect of semi-artificial diets on the average daily weight gain of *P. tsushimanus* larvae



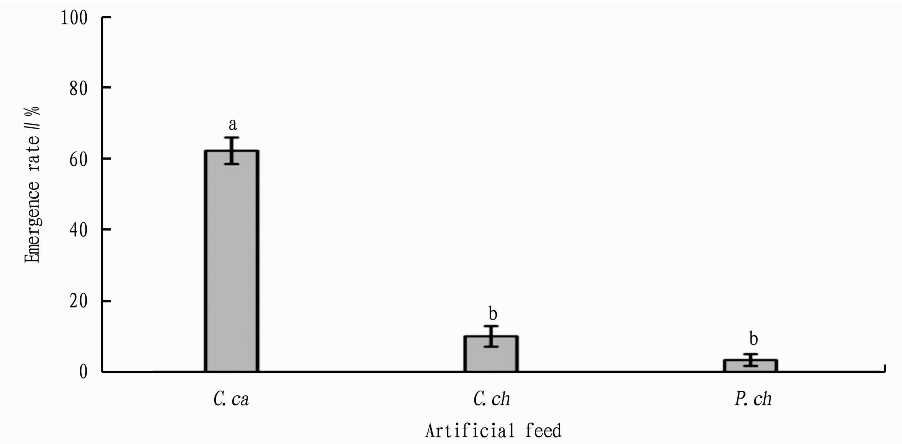
Note: *C. ca*, *C. ch*, and *P. ch* represent the artificial diet of *C. camphora*, *C. chekiangense* and *P. chekiangensis* respectively.

Fig.2 Effect of semi-artificial diets on the survival rate of *P. tsushimanus* larvae



Note: *C. ca*, *C. ch*, and *P. ch* represent the artificial diet of *C. camphora*, *C. chekiangense* and *P. chekiangensis* respectively.

Fig.3 Effect of semi-artificial diets on the pupation rate of *P. tsushimanus* larvae



Note: *C. ca*, *C. ch*, and *P. ch* represent the artificial diet of *C. camphora*, *C. chekiangense* and *P. chekiangensis* respectively.

Fig.4 Effect of semi-artificial diets on the emergence rate of *P. tsushimanus* larvae

phora diet group reached 78.66%, which was significantly higher than that in the *C. chekiangense* and *P. chekiangensis* diet groups. The difference in pupation rates between the larvae fed the *C. che-kiangense* and *P. chekiangensis* diets was not statistically significant, with the latter group exhibiting the lowest pupation rate at 11.67% (Fig.3).

2.5 Effect of semi-artificial diets on the emergence rate of *P. tsushimanus* larvae As illustrated in Fig.4, the emergence rates of *P. tsushimanus* larvae were significantly influenced by the three semi-artificial diets (χ^2=67.13, P<0.001). The final emergence rate for larvae subjected to the *C. camphora* diet group reached 62.38%, which was markedly higher than that observed in the *C. chekiangense* and *P. chekiangensis* diet groups. Furthermore, no significant difference was detected in the final emergence rates between the larvae in the *C. chekiangense* and *P. chekiangensis* diet groups. Notably, the larval emergence rate for the *P. chekiangensis* diet group was the lowest, recorded at 3.33% (Fig.4).

3 Conclusions and Discussion

The biological phenotypes of phytophagous insects are significantly influenced by the diverse array of host plants and their varying characteristics. Additionally, different plant nutrients play a crucial role in determining the biological phenotypes of insect larvae, including growth, development, and reproduction. This variability in nutrients ultimately contributes to a greater range of fitness for insect larvae across different plant species[15]. Specialized feeding insects typically encounter a restricted range of host plants and exhibit a high degree of adaptability to specific host species[16]. However, when a specialist insect is presented with a closely related plant that shares characteristics with its natural host, does this proximity result in significant alterations in the growth and development of the insect larvae? There is a paucity of studies addressing this question. In this study, we investigated the effects of three semi-artificial diets derived from the natural host plant of *P. tsushimanus*, *C. camphora*, as well as its two closely related species, *C. chekiangense* and *P. chekiangensis*, on the growth performance of *P. tsushimanus* larvae. This investigation was conducted despite the similar secondary metabolite compositions observed among these related species, which belong to the Camphoraceae family and are primarily characterized by a variety of terpenes and their derivatives as the predominant secondary metabolites[17]. Our findings revealed significant differences in the fitness performance of *P. tsushimanus* larvae when reared on the three semi-artificial diets. The larvae reared on the semi-artificial diet of *C. camphora* exhibited optimal fitness performance. In contrast, larvae fed the semi-artificial diets of *C. chekiangense* and *P. chekiangensis* demonstrated significant fitness costs, as evidenced by marked reductions in developmental rate, body weight, average daily weight gain, survival rate, pupation rate, and emergence rate. Numerous studies have shown that the composition and ratio of nutrients in plants vary significantly among different plant groups, even those that are closely related. This variation has a direct impact on the growth, development, and fecundity of phytophagous insect larvae[18]. Furthermore, *P. tsushimanus* has been exclusively feeding on a single natural host plant, *C. camphora*, for an extended duration under natural conditions, resulting in a significant degree of adaptation to the nutrient composition, toxicity levels, and specific ratios present in the *C. camphora* tree. Consequently, when rearing the target insect on a semi-artificial diet composed of plant species that are closely related to the natural host, the insect larvae encounter challenges in achieving full adaptation within a brief timeframe, thereby showing a certain fitness cost. The findings suggest that the semi-artificial diet formulated with the natural host plant *C. camphora* represents the most effective approach for rearing *P. tsushimanus* larvae. This rearing method can yield an adequate supply of test insects for research aimed at the prevention and control of the pest *P. tsushimanus*, thereby significantly contributing to the efficient management of this pest.

Insects and plants represent critical components of terrestrial biomes, and the investigation of their interactions can enhance pest management and facilitate plant breeding for increased resistance to insect pests[19]. The current study demonstrated significant differences in developmental duration, insect weight, average daily weight gain, survival rate, pupation rate, and emergence rate among *P. tsushi-*

manus larvae of various ages when fed semi-artificial diets derived from different plant sources. The differences in the duration of the first and second instars of larvae feeding on various semi-artificial diets were minimal. Notably, the duration of the second instar fed on both *C. chekiangense* and *P. chekiangensis* was significantly shorter compared to that observed in the *C. camphora* diet group. This phenomenon can be attributed to the stimulation of larval growth and development induced by the consumption of new plant feeds. Additionally, the feeding amounts during the first and second instars are relatively small, resulting in low nutrient requirements from natural host plants [20]. Subsequently, the larvae progressed to the third and fourth instars. It was observed that the growth and development of larvae feeding on the diets of *C. chekiangense* and *P. chekiangensis* were markedly slowed down, resulting in a significantly prolonged developmental duration compared to the larvae in the *C. camphora* diet group. This phenomenon can be attributed to the fact that the feeding amounts of the third and fourth instar larvae are considerably greater than those of the first and second instars. Furthermore, the diets derived from the non-host plants, *C. chekian-gense* and *P. chekiangensis*, were inadequate to meet the nutritional requirements of the insects during this developmental stage. This finding underscores the pronounced nutritional effects of natural host plants in contrast to non-host plants on insect feeding behavior [20]. Beginning on the 25th day, when the larvae transitioned into the early stage of the third instar, a notable increase in the disparity between the average daily weight gain of larvae fed on *C. camphora* and those fed on the other two host plants was observed. Specifically, the average daily weight gain of larvae consuming semi-artificial diets derived from *C. chekiangense* and *P. chekiangensis* was significantly lower than that of the larvae in the *C. camphora* diet group. Furthermore, the body weights of the second and third instar larvae were markedly greater than those of larvae fed on the semi-artificial diets of *C. chekiangense* and *P. chekiangensis*. This discrepancy can be attributed to the fact that the second and third instar larvae exhibit a greater feeding amount on the natural host plants, which possess higher nutritional value, while the semi-artificial diets from *C. chekiangense* and *P. chekiangensis* are insufficient to meet the nutritional requirements of the second and third instar larvae. *P. tsushimanus* exhibited a rapid mortality rate when fed diets derived from *C. chekiangense* and *P. chekiangensis*, with the peak period of larval deaths occurring between 10 and 30 d. In contrast, the semi-artificial diets based on *C. camphora* resulted in lower mortality rates among *P. tsushimanus* larvae, demonstrating higher survival rates. This observation suggests that the formulation of artificial diets using various host plant species significantly influences the survival rates of the insects[21].

P. tsushimanus larvae that were reared on semi-artificial diets formulated from the natural host plant, *C. camphora*, exhibited superior growth and developmental performance. In contrast, larvae reared on semi-artificial diets derived from the closely related plants *C. chekiangense* and *P. chekiangensis* experienced fitness costs, which included an extended larval duration, a reduction in body weight and average daily weight gain, as well as a significant decline in survival, pupation, and emergence rates. A related study indicated that the developmental duration of *Plagiodera versicolora* larvae feeding on willow leaves was longer compared to those feeding on poplar leaves[22]. Additionally, when adult *Apriona germari* were fed fresh branches of mulberry, conifers, poplar, and willow, the highest feed intake and survival rate were observed in those consuming mulberry, followed by conifers, while the intake and survival rates of those consuming willow and poplar were notably lower. Furthermore, all *A. germari* individuals that were fed poplar leaves died within 3 d[23]. The findings indicate that the variability observed among different plant species, encompassing factors such as nutrient composition and secondary metabolites, can influence phytophagous insects in diverse ways regarding their growth and development. This variability subsequently impacts the host selection behavior exhibited by these insects. Furthermore, it is currently posited that the primary factor constraining the growth, development, and reproductive potential of phytophagous insects is the chemical composition of the host plant, specifically the ratio of protein to carbohydrate content among the principal nutrients [24]. This study aims to investigate whether significant differences in the growth and development of *P. tsushimanus* larvae, when fed semi-artificial diets derived from various plant sources, correlate with the nutritional content, specifically proteins, carbohydrates, and other essential nutrients, of these plants. Furthermore, there is a pressing need to continue exploring improved feed formulations and rearing methods to ensure a reliable and stable supply of test insects for pest control purposes. Consequently, a semi-artificial diet formulated from *C. camphora*, the natural host plant of *P. tsushimanus*, is proposed as the optimal feeding solution for the indoor rearing of *P. tsushimanus* larvae.

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