Feeding Behavior Analyses Reveal Nicotine Selectively Inhibits Sap Ingestion in Crapemyrtle Bark Scale (*Acanthococcus lagerstroemiae*), an Invasive Insect in the U.S.[1]

Bin Wu[2]* and **Hongmin Qin[2]***

Abstract

Crapemyrtle bark scale (CMBS; *Acanthococcus lagerstroemiae* Kuwana) is an invasive pest that primarily infests crapemyrtles (*Lagerstroemia* spp.), along with a few other economically important plants in the U.S. Current management practices predominantly rely on neonicotinoid insecticides, which are structurally similar to nicotine and act as agonists at nicotinic acetylcholine receptors (nAChRs), disrupting insect nervous systems. However, the specific impact of these insecticides on CMBS herbivory performance remains unclear. This study combines pectinolytic enzyme detection and electrical penetration graph (EPG) techniques to investigate nicotine's impact on CMBS salivation and ingestion. CMBS feeding assays were conducted on agarose plates containing various concentrations of pectin and nicotine. The enzyme detection experiments revealed CMBS salivation involves pectinesterase and polygalacturonase secretion, unaffected by nicotine. Consistent with these findings, EPG analysis confirmed salivation (waveform E1) occurred in nicotine-containing agarose. Unexpectedly, phloem sap ingestion (waveform E2) was absent when nicotine was added to the agarose, indicating selective inhibition of CMBS ingestion. These findings suggest nAChRs specifically regulate sap ingestion in CMBS, but not salivation and other feeding behaviors. These insights into nicotine and neonicotinoid insecticide action on CMBS could inform precision insecticide application in IPM programs, potentially enhancing control efficacy while reducing environmental impact.

Species used in this study: Crapemyrtle bark scale (*Acanthococcus lagerstroemiae* Kuwana).

Chemicals used in this study: Agarose (Bio-Rad Laboratories), pectin (MP Biomedicals), nicotine (Sigma-Aldrich), sucrose (Domino Foods Inc.), glutamine (Thermo Fisher Scientific), ruthenium red (Enzo Life Sciences Inc.).

Index words: Feeding behavior, agarose-mediated plates, pectin, nicotine, enzyme detection, EPG, salivation, sap ingestion, IPM, neonicotinoids.

Significance to the Horticulture Industry

The horticulture industry predominantly relies on neonicotinoids, compounds akin to nicotine, to control crapemyrtle bark scale, a relatively new invasive insect pest spreading across 20 U.S. states and threatening the health and marketability of a wide range of economically and ecologically important plants. Prior to our study, the specific effects of these insecticides on feeding behavior of this insect were not well understood, a crucial gap in knowledge for refining insecticide application. In this study, we utilized an agarose-based artificial diet supplemented with different concentrations of pectin and nicotine to explore its impact on CMBS. Through detailed analyses involving enzyme detection via ruthenium red staining and feeding behavior investigation via EPG techniques, we found that while nicotine does not have a notably negative impact on CMBS salivation, it inhibits sap ingestion at the tested concentrations. This selective inhibition of nutrient uptake indicates that nicotine acts as a neurotoxin, disrupting specific neural pathways related to sap ingestion in CMBS, without affecting other essential feeding processes. The methodologies and findings from this study will inform future research into the nuanced effects of neonicotinoids at both sublethal and lethal concentrations, aiding in the development of more precise and sustainable management practices for CMBS within the horticulture industry.

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[2]Department of Biology, Texas A&M University, College Station TX, USA. H.Q. and B.W. acquired funding; H.Q. and B.W. conceived and designed the study; H.Q. supervised its execution; B.W. carried out the experiments and collected data; B.W. drafted the manuscript; H.Q. performed review and editing. All the authors have read and agreed to the published version of the manuscript.

*Corresponding authors email: hqin@bio.tamu.edu; bin.wu@tamu.edu.

Introduction

In the evolving landscape of invasive species management within the horticulture industry, crapemyrtle bark scale (CMBS; *Acanthococcus lagerstroemiae* Kuwana) has emerged as a significant concern. This polyphagous hemipteran, native to Asia, poses a substantial threat to crapemyrtles (*Lagerstroemia* spp.), and a wide spectrum of economically important and indigenous plants in the U.S. (Gu et al. 2014, Schultz and Szalanski 2019, Wang

et al. 2019, Wang et al. 2016, Wu et al. 2021, Wu et al. 2022c, Xie et al. 2020, Xie et al. 2021a). Since its initial identification in Texas in 2004, CMBS has spread across 20 U.S. states and Washington D.C., propelled by interstate trade and natural vectors such as wind and phoretic dispersal (EDDMapS 2024, Skvarla et al. 2024, Skvarla and Schneider 2022, Wright et al. 2023). Infestation of plants by CMBS compromises the health and ornamental value of its host plants by reducing vigor, causing the development of black sooty mold on foliage and branches as a direct consequence of the honeydew secreted by the insects, and potentially reducing floral abundance. These impacts present an urgent need for strategic management interventions, as the economic burden from control costs and plant losses is estimated to exceed $34 million annually in the U.S. horticulture industry (Chen and Diaz 2022, U.S. Department of Agriculture 2020).

To support the development of integrated pest management (IPM) strategies for curbing CMBS spread, recent efforts have focused on key aspects of CMBS ecology, including host range, susceptibility profiles, and mating and feeding behaviors, while also investigating the potential of natural enemies and insecticides or biopesticides (Franco et al. 2022, Gu et al. 2014, Merchant et al. 2014, Vafaie and Gu 2019, Vafaie et al. 2020, Vafaie and Knight 2017, Wang et al. 2019, Wang et al. 2016, Wu et al. 2022a, Wu et al. 2022b, Wu et al. 2021, Wu et al. 2022c, Xie et al. 2020, Xie et al. 2021b, Xie et al. 2022). One significant finding is the partial resistance to CMBS found in *Lagerstroemia speciosa* Pers. based on greenhouse assays (Wu et al. 2021, Wu et al. 2022c). Studies utilizing electrical penetration graph (EPG) technology show that *L. speciosa* may impede the insect's ability to locate and ingest phloem sap, suggesting an inherent defense mechanism that limits CMBS feeding efficiency, when compared to the feeding parameters on the CMBS preferred host plant, *L. limii* Merr. (Wu et al. 2022a). These findings, coupled with the establishment of a reliable callus-induced regeneration of *L. speciosa* and advances in omics-based investigation of plant defense against sap-sucking insects, open new avenues for research integrating CMBS resistance with enhanced plant resilience to abiotic stress using modern breeding techniques (Aasim et al. 2022, Grover et al. 2022, Jiang et al. 2024, Mondal et al. 2023, Sai Reddy et al. 2022, Wu et al. 2024, Zogli et al. 2020).

The current IPM tools available to the U.S. Green Industry for controlling CMBS are limited and still predominantly rely on soil drench or soil injection of neonicotinoid insecticides (Vafaie and Gu 2019, Vafaie et al. 2020, Vafaie 2019, Vafaie 2021). Neonicotinoids, which are analogs of nicotine - a natural insecticide primarily found in tobacco (*Nicotiana tabacum* L.) - bind selectively to nicotinic acetylcholine receptors in the insect nervous system, acting as neurotoxins that disrupt normal neurotransmission and ultimately lead to insect death (Taillebois and Thany 2022). However, the mechanisms by which these insecticides affect CMBS herbivory performance remain completely unknown.

This study aims to elucidate the mechanism by which nicotine affects CMBS feeding. To achieve this, we used pectin and nicotine at various concentrations in an agarose-based artificial diet to evaluate nicotine's impact on CMBS salivation and sap ingestion through analyses of enzyme detection and feeding behavior. In the presence of pectin- a major polysaccharide in plant cell walls that CMBS must degrade to access plant fluids-pectinolytic enzymes, pectinesterase (PE) and polygalacturonase (PG), were detected in CMBS saliva, reflecting conditions closer to its natural feeding (Voragen et al. 2009, Will et al. 2013). By leveraging the selective binding properties of ruthenium red to pectin, we could visualize and quantify the PE and PG activities and establish a baseline of CMBS feeding-related salivation processes (Steeling 1970). To further dissect CMBS feeding behavior under these conditions, we employed the EPG technique, which records voltage fluctuations as the insect stylet penetrates the substrate and has been widely used to study sap-sucking pests on economically important crops (Cho et al. 2011, Kang et al. 2012, Ma et al. 1990, Maluta et al. 2020, Serikawa et al. 2012, Zhu et al. 2020). Integrating EPG enabled us to correlate the enzymatic activities detected in the pectin-rich artificial diet with the precise behavioral changes triggered by nicotine exposure. The results conclusively demonstrate that nicotine selectively inhibits sap ingestion in CMBS.

Materials and Methods

Insect rearing and plant maintenance. *Lagerstroemia limii* Merr. plants, initially provided by Dr. Gary Knox at the North Florida Research and Education Center (Quincy, FL), were continuously propagated in a greenhouse at the Department of Biology, Texas A&M University (College Station, TX). The CMBS colonies used in this study were established on these *L. limii* plants, building upon our previously developed insect rearing protocols with minor modifications (Wu et al. 2021, Xie et al. 2022). Both the insect colonies and plants were placed in a handmade chiffon mesh-covered (Fabric Wholesale Direct, Farmingdale, NY) cage [75 cm (29.5 in) length, 50 cm (19.7 in) width, 40 cm (15.7 in) height] and maintained in the greenhouse, with a temperature of 25 C (77 F) and a relative humidity (RH) of 65 ± 5%.

Pectinolytic enzymes detection in pectin gels. To validate the probing behavior of CMBS on agarose plates and to ascertain the presence of pectinolytic enzymes in their salivary secretions, we utilized agarose plates as a controllable and reproducible medium supplemented with pectin. By mimicking the pectin content of natural plant tissues, we can ensure accuracy for the subsequent experiment with pectin-nicotine agarose. This step is crucial for establishing a baseline understanding of CMBS enzyme secretion patterns. In this experiment, we detected activities of pectinesterase (PE; indicative of pectin demethylation) and polygalacturonase (PG; correlation with pectin hydrolysis) following CMBS stylet penetration using ruthenium red staining, a method that exploits the selective binding properties of the dye to pectin, resulting in an intense red coloration (Cherqui and Tjallingii 2000, Ma et al. 1990, Steeling 1970). Considering that these enzymes play important roles in degrading the pectin components of plant cell walls, facilitating the insect's

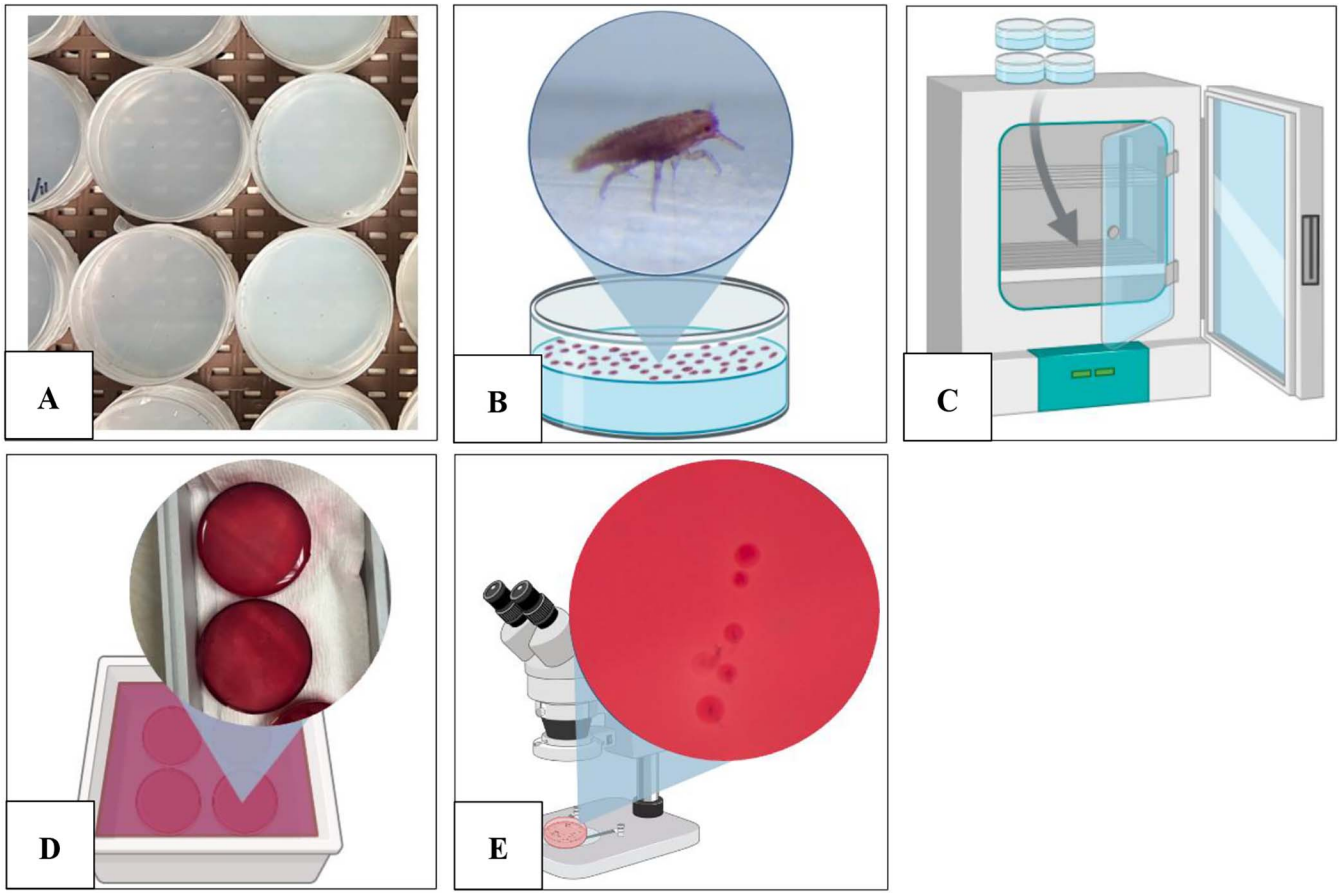

Fig. 1. Schematic illustration of pectinolytic enzyme detection procedure using agarose plates supplemented with different concentrations of pectin. Step 1 (A): Agarose plates were prepared with pectin at 0%, 0.1%, and 1.0%. Step 2 (B): CMBS instars were gently placed onto the pectin-agarose plates and allowed to probe and feed for 24 h. Step 3 (C): After feeding for 24 h, the insects were removed, and the plates were transferred to a 37 C (98.6 F) incubator to promote enzymatic reactions. Step 4 (D): The incubated agarose gels were then stained with 0.02% ruthenium red solution and subsequently rinsed in distilled water. Step 5 (E): The presence of salivary pectinolytic enzymes secreted by CMBS was examined under a stereomicroscope as distinctive red halos. The size and numbers of halos were used to determine the pectinesterase and polygalacturonase activity levels.

access to plant fluids, this experiment also explored whether a correlation exists between enzyme activity levels and pectin concentration, which could provide insights into plant-insect interactions, specifically CMBS feeding strategies and adaptive mechanisms. Agarose plates were prepared by adding 0.0%, 0.1%, or 1.0% (w/v) pectin (MP Biomedicals, Solon, OH) into 1.0% (w/v) microwaved agarose (Bio-Rad Laboratories, Fort Worth, TX) in distilled water. The pH of the pectin-agarose mixture was adjusted to a range from 6.5 to 7.5 using potassium hydroxide before being distributed into 60 mm (2.4 in) Petri dishes (VWR®, Radnor, PA). After ensuring the mixture was free of bubbles, the surface of the agarose in each Petri dish was covered with Parafilm® M (Bemis, Neenah, WI) to prevent contamination and water loss during the experiment.

Fifty CMBS instars were caged on the pectin-agarose gel plates and allowed to feed for 24 h. Subsequently, the instars were gently removed from the plates using a small paintbrush. The plates were then incubated at 37 C (98.6 F) for 1-3 h. Following incubation, the agarose gels were carefully removed intact from the plates and stained with 0.02% ruthenium red (Enzo Life Sciences Inc., Farmingdale, NY) for 30-60 min (Fig. 1). After destaining in distilled water for 3-5 min, the gels were examined under an Olympus SZX7 stereomicroscope with an Olympus LC30 camera (Waltham, MA). Control agarose plates containing 0.0% pectin were utilized for comparison purposes. Each treatment (0.0%, 0.1%, and 1.0% pectin) was replicated four times using newly prepared instars per iteration. To evaluate their enzyme activity levels, regardless of whether they resulted from PE or PG activity, the presence, number, and size of red halos were documented (Ma et al. 1990). Halo quantity was determined by counting the number of halos, and halo size (mm^2) was measured using imaging software cellSens (EVIDENT, Olympus Scientific Solutions Americas DBA, Waltham, MA). This quantification allowed for a direct comparison of enzymatic activity levels across the treatments.

Pectinolytic enzymes detection in pectin-nicotine gels. Nicotine was incorporated with pectin-agarose gels to assess whether nicotine interferes with CMBS salivation and to investigate potential dose-dependent effects. The gel plates were prepared as described previously and incorporated pectin [0.0% or 0.1%, (w/v)] and nicotine [0.0%, 0.1%, or 0.5% (v/v)] (Sigma-Aldrich, St. Louis, MO) into 1.0% (w/v) microwaved agarose solution. Thirty CMBS instars were caged on the pectin-nicotine gel plates and allowed to feed for 24 h

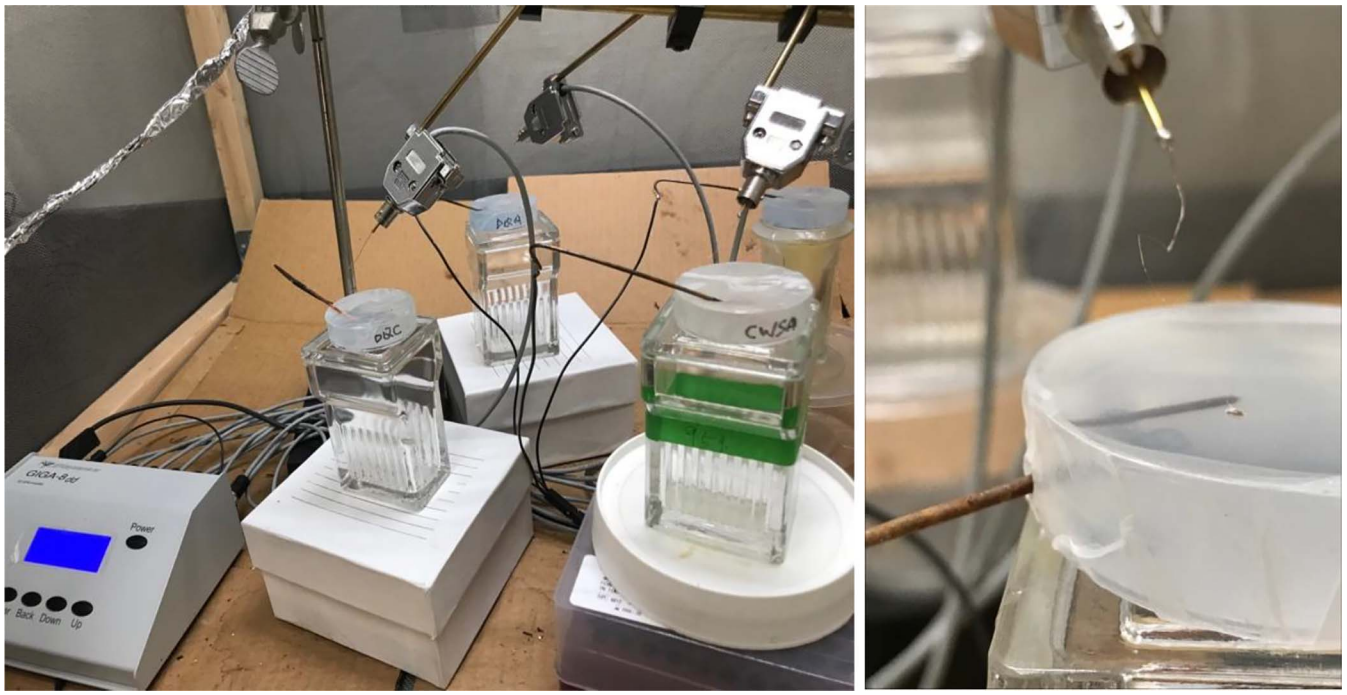

Fig. 2. Probing activities of CMBS on agarose-mediated artificial diet plates were monitored using the EPG-based monitoring system.

and removed. Each treatment was replicated three times using newly prepared instars per replicate. The gel stain and data collection were performed following the same protocol as the pectinolytic enzyme detection in pectin gels mentioned above.

EPG experiment on artificial diet gels. To characterize CMBS feeding behavior on agarose-mediated diets, plain artificial diet gels (without nicotine) were made by preparing an aqueous gel containing 1.25% (w/v) agarose, 30% (w/v) sucrose (Domino Foods, Inc., Yonkers, NY), and 4.8% (w/v) glutamine (Thermo Fisher Scientific, Haverhill, MA). Adding glutamine, one of the main amino acids of the plant phloem sap, in the artificial diet provided phago-stimulatory and nutritive functions for insect feeding behavior in this study (Calatayud et al. 2002, Sandström and Pettersson 1994, Srivastava and Auclair 1975). The solution was microwaved, and the pH was adjusted to 7.5 using potassium hydroxide. Ten mL of the agarose artificial diet was poured into a 60 mm (2.4 in) Petri dish covered with a piece of Parafilm® M membrane. Freshly-made artificial diet gels were offered to CMBS for probing.

Insect probing activities were monitored using a direct current EPG amplifier (Giga-8dd model) with 1-gigaohm input resistance and an analog-digital conversion rate of 100 Hz (EPG Systems, Wageningen, The Netherlands). The recordings were conducted under laboratory conditions [25 C (77 F), 60 ± 5% RH, and a 16:8 h light:dark (L:D) photoperiod] in a custom-built Faraday cage in the Department of Biology at Texas A&M University. Materials for constructing the Faraday cage, including lumber, angle connections, screws, aluminum mesh, and hinges, were procured from a local Home Depot store in 2019 (College Station, TX). After the collected insects [length: 2.1 ± 0.7 mm (0.08 ± 0.03 in); width: 1.2 ± 0.5 mm (0.05 ± 0.02 in)] had been starved for 24 h, a 2-cm (0.79-in) long gold filament [diameter: 18.5 um (0.0007 in)] was attached to the dorsum of each insect using water-based silver glue (EPG Systems, Wageningen, The Netherlands). The opposite end of the gold filament was glued with a copper wire [length: 3 cm (1.18 in); diameter: 0.2 mm (0.008 in)], which was soldered with a brass nail attached to an insect electrode. After a substrate electrode was introduced into the gel, the glued CMBS was placed on the stretched Parafilm® of the agarose-mediated diet (Fig. 2). One insect sample was tested per gel plate for 24 h, and the EPG recordings were acquired from 48 replications.

EPG experiment on nicotine gels. To investigate how nicotine affects CMBS feeding behavior, this experiment incorporated nicotine into the plain artificial diet gels at concentrations of 0.0%, 0.1% or 1.0% (v/v) and monitored CMBS probing activities using EPG techniques. The EPG experiments were conducted following the same protocol described above, with 12, 10, and 14 replications for the 0.0%, 0.1%, and 1.0% nicotine treatments, respectively.

Data visualization and statistical analysis. In the experiment to detect pectinolytic enzymes secreted in the pectin gels, no halos were formed in the gels containing 0.0% pectin; therefore, the control treatment was excluded from the analyses regarding the halo quantity and size. Before conducting a one-way analysis of variance (ANOVA) in JMP® (SAS Institute Inc., Cary, NC), the data's normality of distribution and homogeneity of variance required for the ANOVA were assessed using Shapiro-Wilk's test and Bartlett's test (for normally distributed data) or Levene's Test (for non-normally distributed data), respectively (da Silva-Torres et al. 2013). Data on the halo quantity passed this assessment and was analyzed by ANOVA, followed by Student's t-test ($\alpha = 0.05$) as *post-hoc* to compare the mean values by pectin concentration, subsequently determining whether the halo quantity correlates with

the pectin concentrations in the gels. Data on the halo size was transformed as $\sqrt{(\text{halo size})}$ to improve the normality and homogeneity (Bland and Altman 1996, Wu et al. 2021). The processed data was then subject to ANOVA with t-test ($\alpha = 0.05$) to investigate whether the halo size correlates with the pectin concentrations.

In the experiment to detect pectinolytic enzymes secreted in the pectin-nicotine gels, no halos were formed in the gels containing 0.0% pectin and various concentrations of nicotine (0.0%, 0.1%, and 0.5%); therefore, these three treatments were excluded from the analysis regarding the halo quantity. The assumptions of normality of distribution and homogeneity of variances were assessed using Shapiro-Wilk's test and Bartlett's test, respectively. Following the confirmation of these assumptions, a one-way ANOVA was conducted on the halo quantity data to evaluate the effect of nicotine concentration on enzyme activity levels in CMBS salivary secretion. *Post-hoc* comparisons were subsequently performed using Tukey's Honestly Significant Difference test at a significance level of $\alpha = 0.05$.

In the EPG experiment on artificial diet gels without or with nicotine added, the EPG recordings for all experiments in this study were visualized using Stylet+ software (EPG Systems, Wageningen, The Netherlands). Waveform patterns were identified based on the waveform characteristics of CMBS feeding on the host plant (Wu et al. 2022a), and interpreted according to probing activities of other sap-sucking insects feeding on agarose-mediated artificial diets (Sattar et al. 2019, Sprawka and Goławska 2010). For each treatment, the percentage of CMBS individuals exhibiting a specific waveform was calculated as the ratio of number of individuals displaying the waveform to the total number of replicates per treatment.

Results and Discussion

Detection of pectinesterase and polygalacturonase from salivary secretions when CMBS was probing on pectin gel plates. The stylet sheath forms as phloem-sucking insects insert their stylets into plant tissue, with salivary secretions coating the stylet to protect and lubricate it during feeding (Tjallingii 2006). The sheath facilitates the insect's feeding by creating a pathway for the stylet to move through the plant, often remaining behind after feeding. Figure 3 (green arrows) and Figure 4 (red arrow) show the stylet sheaths on the Parafilm® membranes, confirming CMBS using its stylet to penetrate the agarose. A close observation revealed that some CMBS stylet sheaths were left attached to the Parafilm® membranes after CMBS probing into the pectin-agarose plates (Fig. 4). Staining the agarose gels with the 0.2% ruthenium red revealed distinct enzymatic activities (Ma et al. 1990): PE activity was visualized as maroon halos at the probing sites (white arrows in Fig 3-B, C, and D), while PG activity was denoted by a light-red stain around the stylet sheaths (green arrows in Fig. 3-B and D), forming dark-red rings at their perimeters (black arrows in Fig. 3-B, C, and D). No halos were formed in the pectin-free agarose plates (Fig.3-A). Statistical analysis revealed that, when comparing the mean values of halo quantity between the pectin-supplemented tratements ($F_{(1,7)} = 0.0017$, p-value $= 0.9687$; Table 1), the 0.1% pectin treatment (mean $= 182.3 \pm 23.7$) did not differ from the 1.0% pectin treatment (mean $= 178.8 \pm 82.2$). These findings confirmed the detection of PE and PG activities in CMBS-secreted saliva and suggest that the halo quantities did not exhibit a direct proportion to pectin concentration. Furthermore, the pectin concentration in the agarose gel plates significantly influenced the spatial extent of the halos formed across all concentrations examined ($F_{(1,85)} = 10.2761$, p-value $= 0.0019$; Table 1). In comparisons of pectin-supplemented treatments, the average halo size in 1.0% pectin-supplemented plates (mean $= 0.2293 \pm 0.0017$ mm^2) was significantly larger than that in 0.1% pectin-supplemented plates (mean $= 0.0935 \pm 0.0012$ mm^2). This finding suggests a positive correlation between the halo size and the pectin concentration in the plates.

In this experiment, the positive correlation between halo size and pectin concentration, coupled with the statistically consistent number of halos across treatments, highlights a strategic adaptation in CMBS feeding behavior. Rather than increasing the number of probing sites, CMBS tends to secrete more enzymes at individual sites when faced with higher pectin concentrations, suggesting an adaptive feature that allows the insect to modulate its salivary secretion in response to environmental cues. This specialized secretion strategy is not exclusive to CMBS but is a phenomenon observed across a broad spectrum of economically important crop sap-sucking pests, including aphid (*Myzus persicae* Sulz.), whitefly (*Bemisia tabaci* Genn.), and mealybug (*Phenacoccus manihoti* Mat.-Ferr.), which employ similar enzymatic mechanisms to degrade pectins, the major components of the middle lamellae and primary plant cell walls, and facilitate efficient stylet penetration (Divol et al. 2005, Kempema et al. 2007, Silva-Sanzana et al. 2019). Furthermore, this strategy is not merely about overcoming the plant's physical barriers but also invovles a nuanced interaction with the plant's biochemical pathways, likely affecting the plant's signaling and defensive responses to maintain prolonged access to the phloem tissues for their sustained feeding (Colinet et al. 2024, Pelloux et al. 2007). The ability to vary enzyme secretion according to the environmental conditions underlines a shared adaptive strategy among these insects, aimed at optimzing their feeding efficiency and minimizing energy expenditure. Further research on other specific plant components in this controlled and reproducible environment using agarose plates could establish a sophisticated understanding of these adaptive features in CMBS, which is essential for developing targeted interventions to disrupt its feeding mechanisms. By interfering with its ability to modulate enzyme secretion in response to pectin concentrations, it may be possible to reduce CMBS impact on host plants.

Detection of pectinesterase and polygalacturonase from salivary secretions when CMBS was probing on pectin-nicotine gel plates. In this experiment, we utlized gel plates supplemented with different concentrations of pectin and nicotine- and gained basic knowledge of the enzymatic activities of CMBS salivary secretions. When the gel plates were supplemented with 0.0% pectin, no halo formation was observed in the salivary secretions of CMBS, irrespective of

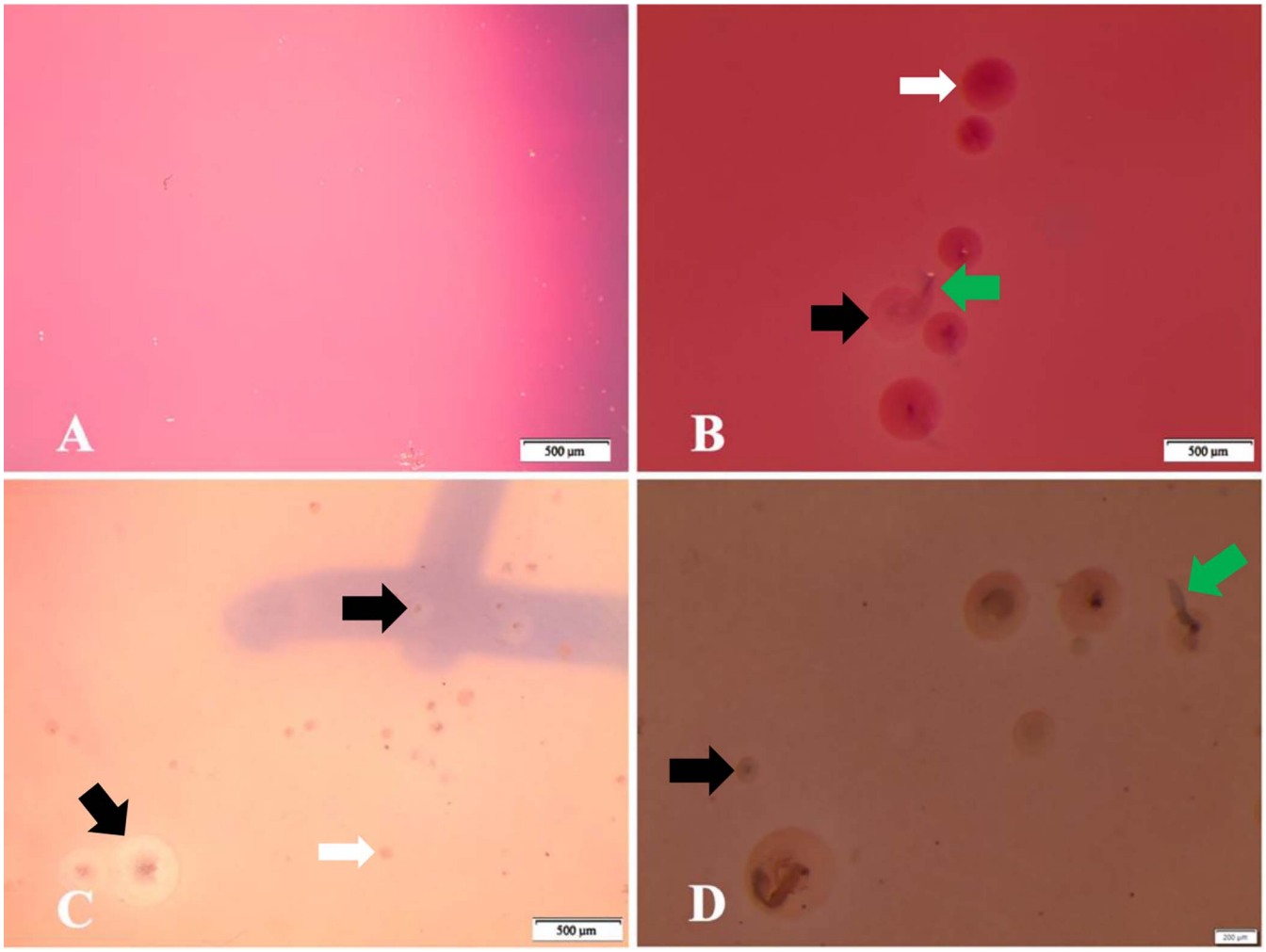

Fig. 3. Visualization of pectinesterase (PE) and polygalacturonase (PG) activity in CMBS salivary secretion in agarose gel plates supplemented with pectin at different concentrations. Through the application of 0.02% ruthenium red staining, it was found that in the absence of pectin (0.0% pectin-added gel plates, Panel A), no detectable enzymatic acivity was observed. Upon the insect's digestion on both 0.1% and 1.0% pectin-added gel plates (Panel B and C, respectively), the PE activity was visualized as maroon-stained halos (white arrows), while the PG activity was demonstrated by the formation of lighter-red halos with a maroon ring at perimeters (black arrows). Addtionally, the residual presence of stylet sheaths (green arrows) in the gel plates (Panels B and D) post-consumption by CMBS provided a physical trace of the probing process.

the nicotine concentrations (0.0%, 0.1%, or 0.5%). However, when the gel plates were supplemented with 0.1% pectin, enzymatic activities of PE and PG were detected under all three nicotine concentrations, as evidenced by the maroon halos and light-red halos surrounded by dark-red rings, respectively. Statistical analysis revealed no significant difference ($F_{(2,17)} = 0.2384$, p-value $= 0.7909$; Table 2) in the mean values of halo quantities among the treatments with 0.0% nicotine (mean $= 15.6 \pm 2.3$), 0.1% nicotine (mean $= 23.7 \pm 8.2$), and 0.5% nicotine (mean $= 14.7 \pm 7.4$). Additionally, no correlation was observed between the halo quantities and nicotine concentrations.

In this experiment, our results demonstrate that nicotine concentrations within the tested range neither stimulate nor inhibit the enzymatic activities. While these results do not support our initial hypothsis that nicotine interferes with CMBS's enzymatic mechanisms to degrade plant cell walls, they nonetheless contribute valuable insights into the potential effects of nicotine on CMBS physiology and behavior. Unlike certain lineages of the green peach aphid (*M. persicae*), which

Table 1. Comparison of halo quantity and size between 0.1% and 1.0% pectin-added agarose plates[z].

Detection parameters	0.0% pectin	0.1% pectin	1.0% pectin	Statistical analysis
Halo quantity	—[y]	182.3 ± 23.7 a	178.8 ± 82.2 a	$F_{1,\ 7} = 0.0017$; $p = 0.9687$
Halo size[w]	—	0.0935 ± 0.0012 b	0.2293 ± 0.0017 a	$F_{1,\ 85} = 10.2761$; $p = 0.0019$

[z]Means with standard error of the mean followed by different letters within a column are different by Student's t-test ($\alpha = 0.05$).

[y]Treatment containing 0.0% pectin was excluded from the analyses regarding the halo quantity and size because no enzymes were detected in the gels.

[w]Data on halo size was transformed as $\sqrt{(\text{halo size})}$ to improve normality of distribution and homegeneity of variance required for one-way analysis of variance. The inverse-transformed means with the inverse-transformed standard error of the mean (mm^2) are presented.

Table 2. **Investigation of nicotine impact on CMBS probing on agarose-mediated plates.**

Treatment	Pectin (%)	Nicotine (%)	N[z]	Halo quantity[y]
A	0	0	90	—[x]
B	0	0.1	90	—
C	0	0.5	90	—
D	0.1	0	90	15.6 ± 2.3 a
E	0.1	0.1	90	23.7 ± 8.2 a
F	0.1	0.5	90	14.7 ± 7.4 a

[z]Total number of CMBS crawlers combined across three replicates for each treatment.

[y]Means with standard error of the mean followed by different letters within a column are different by Tukey's Honestly Significant Differences test ($\alpha = 0.05$).

[x]Treatments A, B, and C were excluded from the analysis regarding the halo quantity because no enzymes were detected in the gels.

can thrive in nicotine-rich environments by inducing detoxification pathways and even enhancing feeding at low nicotine concentrations, CMBS do not appear to employ similar adaptive responses (Zhang et al. 2017). This contrast suggests that, under the conditions tested, CMBS lack the salivary modifications or detoxification mechanisms that might mitigate nicotine's deterrent effects. Whereas nicotine-adapted aphids (*M. persicae*) or tobacco whiteflies (*B. tabaci*) illustrate how some insects evolve complex strategies to overcome nicotine, our findings underscore the importance of managing CMBS populations in a manner that reduces selection pressure for such adaptations (Kliot et al. 2014, Ramsey et al. 2014). Approaches that include the precision application of neonicotinoid insecticides may help delay the evolution of pesticide resistance in CMBS. To further elucidate the enzymatic responses of CMBS to nicotine or neonicotinoids for developing IPM strategies, techniques used in studies of other sap-sucking insects could be applied to research into the adaptative strategies of CMBS, particularly focusing on the salivary glands and feeding behaviors (Morin et al. 2024, Ramsey et al. 2014, Zhang et al. 2017).

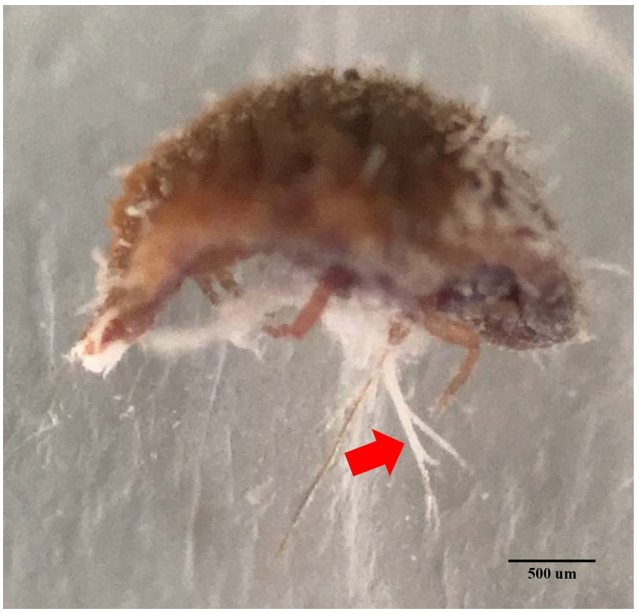

Fig. 4. CMBS stylet coated with white salivary sheaths penetrated an agarose-mediated artificial diet. White salivary sheaths (red arrow) were observed around its stylet bundles when CMBS was probing on the agarose-mediated artificial diets.

Characterization of EPG waveforms when CMBS was feeding on agarose-mediated diet. Four main feeding waveforms were characterized when CMBS was probed on the agarose-mediated artificial diet supplemented with 0.0% nicotine, encompassing waveforms C, E1, E2, and G (Fig. 5-A). Waveform C indicated CMBS puncturing the Parafilm® and penetrating the gel utilizing its stylet tip (Fig. 5-B), similar to the initiation of probing and the activities of the extracellular stylet pathway activities in the epidermis and mesophyll during feeding on the confirmed host crapemyrtle, *L. limii* (Wu et al. 2022a). However, no potential drops were observed during the stylet pathway phase when CMBS fed on the gel plate. Waveform E1, usually detected after waveform C, indicated that CMBS secreted saliva into the agarose gel (Fig. 5-C), comparable to saliva secretion into phloem tissue during feeding on *L. limii*. Waveform E2 suggested CMBS was ingesting nutrients in the gel (Fig. 5-D), similar to ingesting phloem sap during feeding on *L. limii*. Waveform G indicated that CMBS consumed water in the gel, analogous to xylem ingestion during feeding on *L. limii* (Fig. 5-E).

In this experiment, we utlized EPG techniques to successfully characterize CMBS feeding behavior on agarose-mediated plates for the first time. This method revealed four distinct EPG waveforms that correlate with specific feeding activities, suggesting that this agarose-mediated artificial diet can mimic some aspects of natural feeding of CMBS on the host plant, *L. limii*. Unlike rapid, momentary changes in voltage that occur when the stylet penetrates intracellular plant tissues and cause cellular puncturing, no potential drops were noted during the stylet pathway phase when CMBS was probing on the gel plate. This result aligns with research into aphid feeding behavior on agarose gels (Golawska et al. 2014). The absence of potential drops during insect stylet penetration in agarose gels can be primarily attributed to the lack of physiological and chemical complexities, dynamic ionic shifts, or mechanical responses found in living plant cells, which lead to smoother stylet penetration without engaging in intracellular interactions (Tjallingii 1985, Tjallingii and Esch 1993, Tjallingii 2006, Tjallingii and Prado 2001, Walker 2000). Using agarose gels with homogeneous, nonvaried electrical resistance and textural features for studying CMBS feeding behavior allows for controlled environmental conditions, standardizing expeirments and isolating insect-specific behaviors from plant variable effects. This simplification can enhance the sensitivity of EPG techniques, facilitating clearer behavior analysis and enabling targeted pest management strategies by allowing precise manipulation of individual plant compositions and testing of specific hypotheses regarding plant-insect interactions.

EPG experiment on nicotine gels. Our integrated systems identified differences in CMBS feeding waveforms between gel plates supplemented with nicotine and those without nicotine. Among the CMBS that successfully probed the gel plates at all tested nicotine concentrations (0.0%, 0.1%, and 1,0%), 25.0%, 35.0%, and 35.7% of individuals exhibited waveform E1 (salivation into the agarose), respectively. The percentages of individuals having waveform G (water ingestion) were

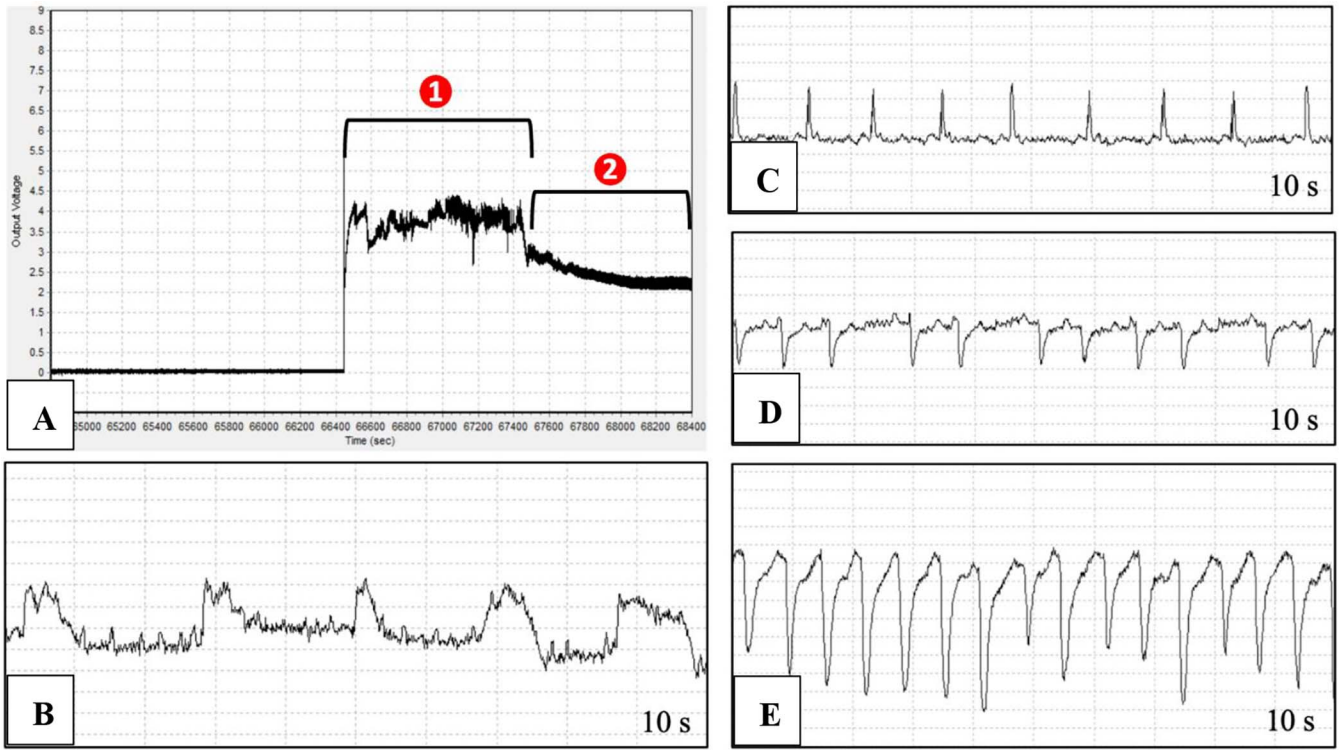

Fig. 5. **Four main EPG waveforms of CMBS probing on agarose-mediated artificial diet. (A) an overview of the general scheme, illustrating both ① the stylet pathway phase and ② the phloem phase. (B) Waveform C denotes the action of piercing the Parafilm® and penetrating the gel using the stylet tip, a process comparable to the stylet pathway phase in a plant. (C) Waveform E1, signifying salivation within the gel, which is analogous to phloem salivation in plant physiology. (D) Waveform E2, depicting the process of nutrient ingestion within the gel, a parallel to phloem ingestion in a plant. (E) Waveform G, indicating the ingestion of water within the gel, an action reminiscent of xylem ingestion within the plant's system.**

16.7%, 10.0%, and 7.1% across the same nicotine concentrations. Interestingly, while 8.3% of the individuals displayed waveform E2 (sap ingestion) on the nicotine-free gel plates, none exhibited E2 on the plates supplemented with 0.1% or 1.0% nicotine (Fig. 6).

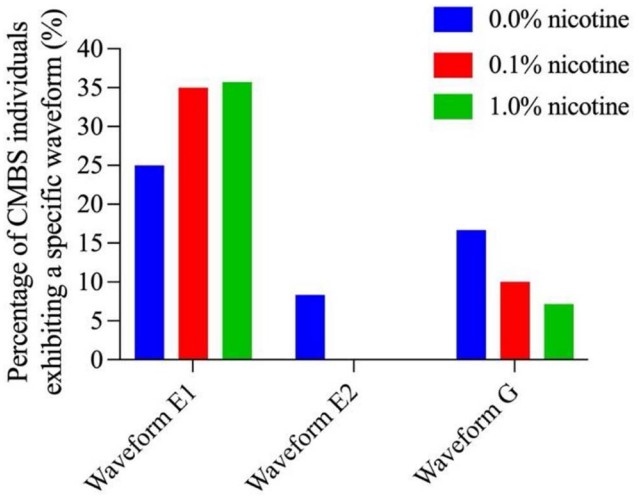

Fig. 6. **Nicotine inhibits the number of CMBS individuals per treatment exhibiting waveform E2. The study showed no significant variation in waveform E1 or waveform G when CMBS fed on an agarose-mediated artificial diet enriched with 0.0%, 0.1%, or 1.0% (v/v) nicotine. However, waveform E2 was absent during feeding sessions on the artificial diet supplemented with either 0.1% or 1.0% nicotine.**

This absence of E2 suggests that nicotine at these concentrations effectively inhibited CMBS from nutrient ingestion in the gels, which supports previous research that points to nicotine's role in disrupting feeding behavior of other sap-sucking insects (He et al. 2013, Montllor 2017). This disruption implies that nicotine possibly interferes with gut absorption processes or neural signals that trigger sap ingestion, likely due to its role as a neurotoxin (Taillebois and Thany 2022, Tomizawa and Casida 2005, Zeng et al. 2020). The findings from this study also strongly suggest that the salivation and sap ingestion phases in CMBS are controlled by separate neural signals. This concept is supported by genetic and electrophysiological evidence from studies on grain aphids (*Sitobion miscanthi* Takahashi) and other sap-sucking insects. For example, targeted silencing of the *SmDSR33* gene in grain aphids through plant-mediated RNA interference clearly altered salivation and ingestion differently (Zhang et al. 2023). The differentiation in neural control implies a specialized adaptation mechanism in sap-sucking insects. Hence, further research should focus on understanding the neural mechanisms involved by integrating molecular and biochemical approaches, exploring alternative insecticides with long-term impact assessment, and investigating precise application of neonicotinoids to exploit this selective inhibition, to enhance the efficacy and sustainability of pest management practices for CMBS (Bantz et al. 2018, Cui et al. 2018, Golawska et al. 2014, Gong et al. 2022, Pavithran et al. 2024, Shi et al. 2011).

In conclusion, this study investigating the effects of nicotine on CMBS feeding behavior through EPG and pectin-nicotine gel assays collectively demonstrates that the effect of nicotine on CMBS is more complex than a simple toxic effect. Specifically, while nicotine does not significantly affect the secretion of pectinolytic enzymes in CMBS saliva (waveform E1) or water ingestion (waveform G), it inhibits the critical sap ingestion phase (waveform E2) at concentrations of 0.1% and 1.0%. This selective inhibition indicates that nicotine acts as a neurotoxin disrupting specific neural pathways associated with sap ingestion in CMBS, without affecting other essential feeding processes. These findings validate the efficacy of using agarose-mediated artificial diets combined with EPG techniques for studying plant-insect interactions and enhance our understanding of nicotine's impact on CMBS feeding behavior. These methods and results have implications for developing IPM strategies for controlling CMBS more efficiently and sustainably, as the insights gained can contribute to shaping future investigations into the effects of neonicotinoids at sublethal and lethal concentrations for precise application.

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