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Protocol & Techniques

A protocol for the visualization and quantification of *Dalbulus maidis* (DeLong & Wolcott, 1923) (Hemiptera: Cicadellidae) endophytic eggs in maize leaf tissues

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Abstract. This study aimed to develop a simple and efficient method for quantifying eggs of *Dalbulus maidis* (DeLong & Wolcott, 1923) (Hemiptera: Cicadellidae), the corn leafhopper. This insect exhibits endophytic oviposition, inserting eggs into maize leaf tissues, which hinders direct visualization and complicates fertility assessments and life-table studies. Traditional egg detection techniques often rely on labor-intensive clarification procedures, specialized reagents, or extended staining periods, limiting their practicality for large-scale experiments. Here, we present a rapid, low-cost protocol for the clarification and staining of maize tissues containing *D. maidis* eggs. The method enabled clear detection of endophytic eggs while preserving tissue integrity and allowed samples to be stored in the staining solution for up to three weeks prior to analysis. This protocol provides a practical and scalable approach for egg quantification in *D. maidis* and can be readily adapted to different plant developmental stages, tissue characteristics, or insect species exhibiting endophytic oviposition. Overall, this method facilitates efficient reproductive and ecological studies by simplifying egg visualization and enabling high-throughput sample processing.

Keywords: Corn leafhopper, Endophytic oviposition, Insect–Plant interaction, Tissue Clearing.

Dalbulus maidis (DeLong & Wolcott, 1923), the corn leafhopper, is an insect that exhibits an endophytic oviposition pattern. This species has been extensively studied due to its major importance to maize crops, as it is the main vector of several pathogenic microorganisms. These include the phytoplasma associated with maize bushy stunt disease, the spiroplasma *Spiroplasma kunkelii*, the causal agent of corn stunt disease, the maize rayado fino virus (MRFV), and the maize striate mosaic virus (MSMV). The transmission of these pathogens by *D. maidis* leads to severe yield losses and represent a major constraint to maize production, particularly in tropical and subtropical regions of the Americas (Nault 1990; Oliveira & Frizzas 2022; Pozebon et al. 2022; Vilanova et al. 2022).

Most studies include investigations of the biological aspects and reproductive capacity of the corn leafhopper; therefore, egg counting represents a major component of the experimental methodology. Corn leafhopper females insert their eggs with the ovipositor, typically into the mesophyll on the upper leaf surface and along the midrib. Several eggs may be deposited in a row (Heady et al. 1985). Ovipositing females are often found in the whorls of maize seedlings, where they prefer to lay their eggs. The eggs are oval, less than 1 mm long and 0.2 mm wide, almost colourless when first laid and therefore difficult to detect. After 7–10 days, they turn white and develop red eye spots, which makes them easier to observe. A few days after oviposition, a tuft of characteristic microfilaments forms and protrudes from the proximal end of the egg (Heady & Nault 1984).

Oviposition in plant tissue is a common behavior among insect groups, where females lay eggs either on the plant surface or within plant tissues (Cury et al. 2019; Feng et al. 2022). Endophytic oviposition offers advantages for some insects because the eggs remain protected from external biotic and abiotic factors, such as predation, desiccation, and extreme temperatures (Hilker & Meiners 2011; Romero-Lebrón et al. 2022). However, this behavior creates methodological challenges for researchers, as counting eggs is vital for fertility assessments and life table studies, but this behavior makes visualization and data collection difficult with the naked eye. Traditional methods for locating endophytic eggs often require extensive clarification procedures, additional reagents, or prolonged dye fixation periods (Carlson & Hibbs 1962; Khan & Saxena 1986). Therefore, developing faster and more practical methods for visualizing and quantifying eggs is necessary, and this is what we propose here.

In the present method (Fig. 1), maize leaves and stalks containing *D. maidis* eggs, collected at least 48 hours after oviposition, were cut using sterilized scissors and placed in 50 mL conical Falcon® tubes. Samples were immersed in a 2.5% (v/v) sodium hypochlorite solution (Ciclo Farma, Serrana, Brazil), ensuring complete submersion of the plant tissue, and incubated in a static water bath at 85 ± 2 °C for 25 minutes. Following clarification, the Falcon® tubes containing the plant tissues were cooled in water at room temperature (~25 °C) for five minutes. The tissues were then transferred to a new Falcon® tube containing a 0.1% methylene blue (Êxodo Científica, Sumaré, Brazil) solution prepared in absolute ethanol (Labsynth, Diadema, Brazil) and incubated for 30 minutes to allow staining and fixation. Subsequently, the samples were rinsed in 70% ethanol, placed in Petri dishes, and examined under a stereomicroscope at 10× magnification for egg counting (Fig 2). After the clarification and cooling steps, plant tissues may be stored in the methylene blue solution for at least three weeks before evaluation.

For method consolidation and validation, approximately 3,000 maize plants at different phenological stages (V1 to V4) were processed using this protocol, with each plant considered a replicate. Egg densities varied considerably among samples, typically ranging from 5 to 80 eggs per plant during a 48 h oviposition period. The entire procedure, from plant collection to egg counting, requires approximately 1–1.5 h per batch of samples. After clarification and staining, eggs were easily detectable under a stereomicroscope, allowing rapid counting even at low magnification. Compared with traditional clearing methods that often require prolonged fixation or multiple reagents, this protocol reduces preparation steps and allows egg visualization within a short

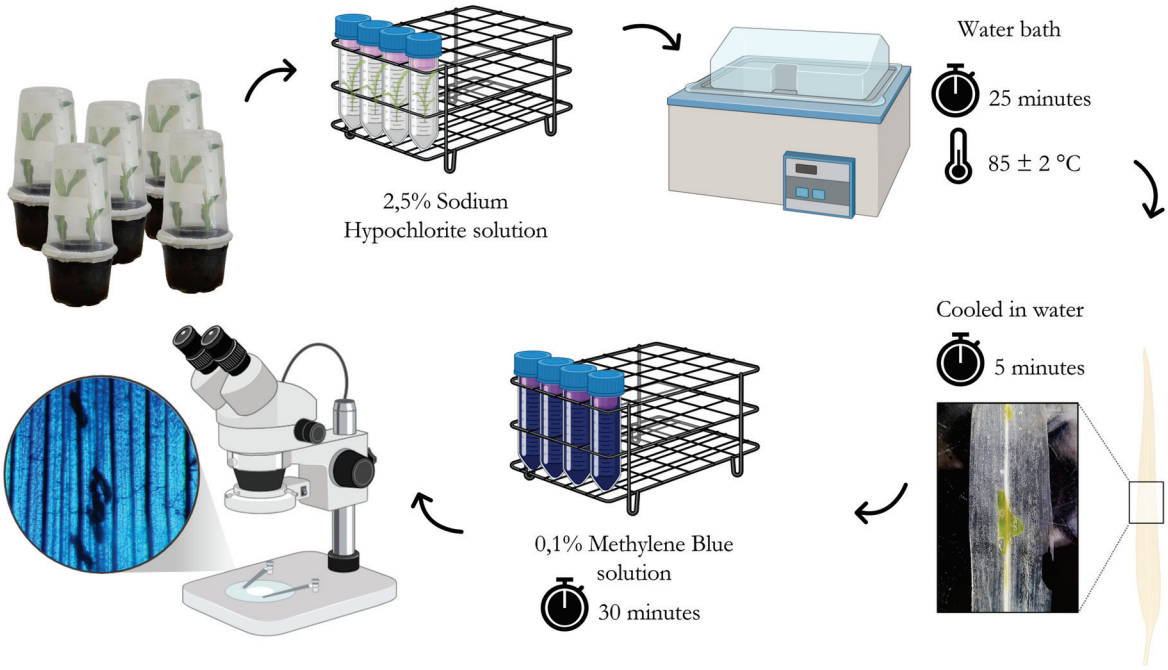

Figure 1. Protocol for clearing and staining maize leaves containing *Dalbulus maidis* (DeLong & Wolcott, 1923) (Hemiptera: Cicadellidae) eggs. Figure created using Biorender (https://biorender.com/). (G.S. Dias).

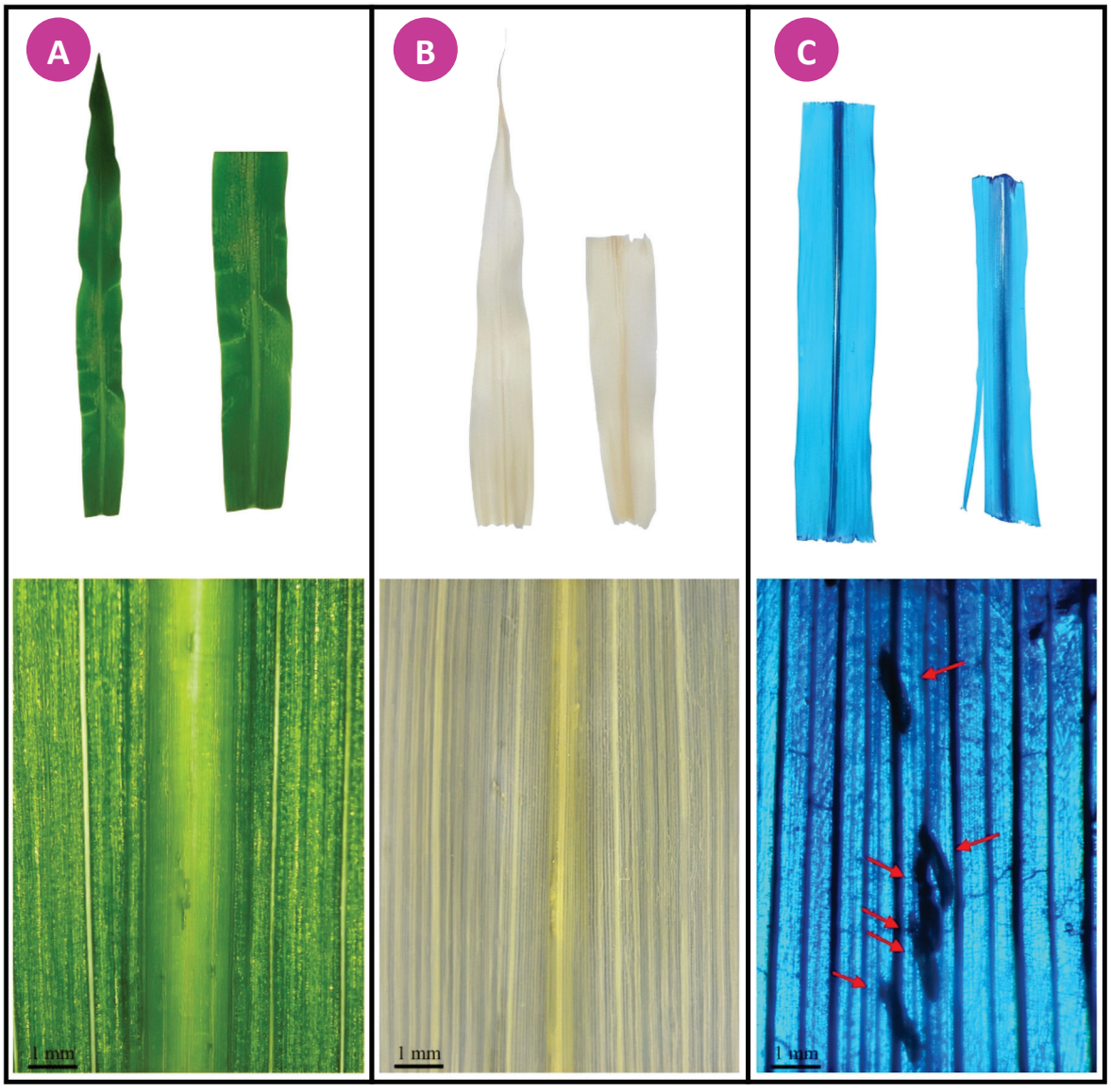

Figure 2. A. Corn leaves before starting the clarification and staining process. B. Corn leaves after removal from the sodium hypochlorite solution. C. Corn leaves after staining and visualization of *Dalbulus maidis* (DeLong & Wolcott, 1923) (Hemiptera: Cicadellidae) eggs. Red arrows indicate *D. maidis* eggs localization; The images were taken using a stereomicroscope with 10× magnification. (Photo: G.S. Dias).

time frame. Moreover, the method can be adapted to accommodate different experimental time frames or applied to other insect species exhibiting endophytic oviposition behavior. The interval between oviposition and the collection of plant tissues, before proceeding to the sodium hypochlorite clarification step, was chosen to allow the eggs to mature sufficiently, which facilitates visualization; however, this timeframe can be adjusted according to experimental requirements.

Minor adjustments to the protocol should consider the characteristics of insect eggs, such as egg size and developmental (maturation) time, and the preferred oviposition site within the leaf tissue, as well as plant characteristics, such as the physical and chemical properties of the leaves. For example, here, plants at phenological stage V2/V3 were used, with the entire plant inserted into the Falcon® tube. If plants at a more advanced phenological stage or with larger leaves are used, they should be segmented into more parts to facilitate contact with the sodium hypochlorite solution. If plants with thinner or younger leaves are used, the immersion time in the sodium hypochlorite solution in the water bath should be reduced to prevent the leaves from disintegrating. In cases where the leaf has more trichomes or is thicker, it may be necessary to increase the immersion time.

In conclusion, accurate assessment of the number of *Dalbulus maidis* eggs on plants requires a careful approach to avoid underestimation due to difficulties in egg visualization. Methodological adjustments, such as optimizing tissue-clearing procedures to preserve leaf integrity, are essential for reliable egg quantification and ultimately contribute to a better understanding of corn leafhopper–maize interactions.

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Authors' Contributions

GSD: Conceptualization, Methodology, Investigation, Writing – original draft; ASG: Methodology, Supervision, Writing – review & editing.

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The authors declare no competing interests.

Ethical Approval

Not applicable.

Data Availability

All relevant data are contained within the article.

Generative AI Statement

The authors declare that no generative AI tools were used in the preparation of this manuscript.

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