

TECHNIQUES FOR DIFFERENTIALLY STAINING *LIRIOMYZA TRIFOLII* (DIPTERA: AGROMYZIDAE) EGGS AND STIPPLES WITHIN COS LETTUCE LEAVES

GREGG S. NUSSLY, RUSSELL T. NAGATA, ERIK S. SKILES, JOLENE R. CHRISTENSON
AND CURTIS ELLIOTT
Everglades Research and Education Center,
University of Florida, IFAS,
Belle Glade, Florida 33430

ABSTRACT

Different staining procedures were evaluated for their time requirements and effectiveness to differentially stain *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae) eggs and wounds (i.e., stipples) made by the female flies ovipositing within cos lettuce (*Lactuca sativa* L.) leaves. The best method for staining eggs within leaves was a lactophenol acid fuchsin solution that cleared the leaves of chlorophyll and stained the eggs pink to dark red. Modification of this established procedure reduced the total staining time to 7 min per leaf. Stipples were counterstained blue by immersing the leaves in a lactophenol cotton blue solution for 1 min immediately following the egg staining procedure. Larvae were not stained by either procedure. A new technique was devised for highlighting stipples using solutions of silver nitrate, sodium carbonate, formaldehyde and citric acid. The procedure resulted in blackened stipples that

were highly visible against the green leaves and did not kill developing larvae within the leaves. Eggs could later be stained using the lactophenol acid fuchsin technique. Sufficient contrast and color differences were produced by these procedures to successfully quantify stipples, eggs and larvae within stained and counterstained leaves from digitized microscopic video images using computer image analysis software.

Key Words: Serpentine leafminer, *Lactuca sativa*, lactophenol, acid fuchsin, cotton blue, silver nitrate.

RESUMEN

Fueron evaluados varios procedimientos para la tinción diferencial de huevos y heridas de *Liriomyza trifolii* (Burgess) producidos por la hembra en hojas de lechuga de la variedad Cos (*Lactuca sativa* L.). Los criterios evaluados fueron el tiempo que duró el procedimiento y la capacidad de teñir solamente los huevos y las heridas. Hasta ahora el mejor método para teñir los huevos dentro de las hojas es usar una solución de lactofenol y fuchsin ácida para aclarar la clorofila y teñir los huevos de rosado a rojo intenso. La modificación de este proceso establecido redujo el tiempo total de teñido a 7 min por hoja. Las punteaduras pueden ser contrateñidas mediante la inmersión de las hojas en una solución de lactofenol y azul de algodón por un minuto inmediatamente después del proceso de tinción del huevo. Las larvas no fueron teñidas mediante ningún proceso. Fué elaborada una nueva técnica para destacar las punteaduras usando soluciones de nitrato de plata, carbonato de sodio, formaldehído y ácido cítrico. El proceso dió como resultado punteaduras negras muy visibles contra el verde de las hojas, y no mató las larvas en desarrollo dentro de las hojas. Los huevos pueden luego ser teñidos usando la técnica del lactofenol con la fuchsin ácida. Hubo suficiente contraste y diferencias producidas por estos procedimientos para cuantificar exitosamente las punteaduras, huevos y larvas dentro de las hojas teñidas y contrateñidas a partir de imágenes de video usando software para análisis de imágenes de computadora.

Serpentine leafminers, *Liriomyza trifolii* (Burgess), are a major pest of lettuce (*Lactuca sativa* L.) and other crops, particularly in Florida (Genung & Janes 1975, Leibe 1981, Foster 1986). Damage to lettuce is in the form of stipples (i.e., scars from feeding and oviposition punctures), leafmines, and pupae within the lettuce heads. Hundreds of acres of lettuce are disked under annually before harvest, or left in the field during harvest because of leafminers. Costs to the industry, including lost production costs and pesticide applications, probably exceed \$1 million annually in south Florida alone.

Host plant resistance is one method under examination to reduce leafminer pressure in lettuce. Field and laboratory studies indicated significant differences in stipple rates among commercial head and cos (i.e., romaine) lettuce cultivars (Nuessly & Nagata 1993, 1994). However, oviposition rates (based on counts of 2-d-old larvae within leaves) varied much less among romaine cultivars than was expected, based on overall stipple counts (unpublished data). This suggested that variation among cultivars mediated the feeding puncture to oviposition puncture ratio, or affected egg or early instar mortality. In order to identify the source of the observed variation, it was necessary to quantify both stipples and eggs.

Stipples on the distal areas of lettuce leaves can be observed using a 10X stereomicroscope with either direct or transmitted light. However, callous tissue and exudates that form over leaf punctures make it difficult to identify eggs, particularly in culti-

vars with thick leaves or with strong wound responses. Eggs and stipples in the proximal area of lettuce leaves are difficult to quantify accurately even under microscopic examination.

Many techniques are available for staining insects, nematodes, and fungal hyphae within plant tissue. However, these techniques may require several hours to days to complete the entire staining, destaining and counterstaining steps (e.g., Carlson & Hibbs 1962, Gilstrap & Oatman 1976, Simonet & Pienkowski 1977). Also, extensive variation in stippling and oviposition wounds within treatments on cos lettuce necessitates large sample sizes to determine meaningful differences among cultivars. Leaves are similar in size and shape, and stipple rates vary significantly with their location on the leaves and plants (Nuessly & Nagata 1994), thus test leaves need to remain separated to preserve their identity. This requires large holding spaces for test leaves if the staining procedures require hours or days to complete. Additionally, stipples on individual leaves or plants, which can number in the thousands, take considerable time to quantify. Magnified images of stipples on leaves acquired with a video camera and microscope, and then digitized into a computer, could be counted quickly if the stipples and eggs could be enumerated based on color. The purpose of this study was to compare several established staining techniques and to evaluate leaf-clearing and staining solutions and dyes, with the goal of finding techniques that would rapidly differentiate (< 1/2 h) stained eggs and stipples on cos lettuce.

MATERIALS AND METHODS

Four cos lettuce cultivars were used in the evaluation: 'Floricos 83', 'Valmaine', 'Tall Guzmaine', and 'Paris Island Cos'. Plants were grown in a greenhouse using methods reported by Nuessly & Nagata (1994). The following methods were used for exposing plants to flies, handling after exposure and evaluating *L. trifolii* mortality and development in cos lettuce. Groups of four plants of the same cultivar were exposed to eight pairs of 48-h-old *L. trifolii* for 24 h within screened cages. After 72 h, all flies were removed, and then the plants were carefully re-caged for another 72 h to insure that no further oviposition occurred. Since larvae emerge from eggs within 70 h, any eggs detected in plants after this time could be assumed to be infertile. Time was allowed also for callous formation at the leaf punctures and for larvae to emerge from their eggs and start mining within the leaves. In addition, other leaves were processed within 24 h of oviposition to verify that the staining technique worked on both viable and inviable eggs. The leaves were excised near the leaf axil and processed individually. Stained leaves were examined with a stereomicroscope at 10 to 30 X using direct and transmitted light.

Most staining techniques for insects, nematodes, or fungal hyphae involve boiling the plant tissues in a solution to clear and stain them. This is followed by a destaining process that may require additional boiling. Tissue disruption can occur during this process. As a result, Parrella & Robb (1982) modified the lactophenol acid fuchsin procedure of Simonet & Pienkowski (1977) to reduce the boiling time. This reduced tissue disruption and improved staining of *L. trifolii* eggs in chrysanthemum, tomato and celery leaves. However, their technique still required the leaves to steep in the solution for ≥ 3 hr after boiling.

Beyond the time concerns, materials used in lactophenol staining procedures are relatively expensive when used in the volumes required for our studies. For example, the lactophenol acid fuchsin stock solution was made as follows: one part water; one part lactic acid; one part phenol; two parts glycerin; and 0.5 g acid fuchsin per 0.5 liter of solution. To address the cost concern, we evaluated other leaf-clearing and de-stain-

ing agents (acetic acid, ethanol, glycerin, lactic acid, phenol, polyethylene glycol and combinations) as well as insect and plant stains (cotton blue, methylene blue, acid fuchsin, methyl red, scarlet red, saffarin, and gentian violet) to find a technique suitable for our studies with lettuce. We also evaluated modifications of the following techniques developed for staining insect eggs and plant parasitic nematodes in plant tissue: lactophenol acid fuchsin (Parrella & Robb 1982), lactophenol cotton blue (Franklin & Goodey 1959), water methyl red (Curtis 1942), and ethanol scarlet red (Sugimoto 1976).

In addition, we evaluated a technique for highlighting stipples that involved excising leaves, washing them in distilled water to remove any surface contaminants, and then immersing them for 5 min in a 0.2% aqueous solution of silver nitrate. The leaves were then washed in distilled water and immersed in a 7% aqueous solution of sodium carbonate (with 0.375 ml 37% formaldehyde added per 100 ml solution) to develop the stain. After 3 min in the developer, the leaves were removed and immersed in a 20% aqueous solution of citric acid to stop the staining. A final distilled water wash completed the procedure. Care was taken not to handle the leaves without gloves, or to abrade or tear the leaves prior to staining, as these areas would also become stained and mask the stipples.

RESULTS AND DISCUSSION

We found that the shape of the container used for the staining procedure ultimately affected the quality of the stain. For example, bending or folding the leaves into beakers resulted in tissue destruction and too much dye infusion. Thus, to maintain the structural integrity of the leaves, it was necessary to use a container large enough for the entire leaf to lay horizontally in the solution. We found that a glass loaf dish, 24 × 14 × 7.5 cm (L × W × D), was large enough for the *cos* lettuce leaves, and small enough for the entire bottom surface of the dish to remain in contact with the heating surface of a hot plate. One-half liter of stain solution provided adequate volume for immersing the leaves during staining without touching the bottom of the dish. The leaves were held immersed approximately 1.0 cm below the surface of the test solutions with a strainer made from 5 mm diam glass rods.

Our goal was to develop procedures by which all staining, destaining and counterstaining could be completed in 15 min. We found it was necessary to boil the leaves in order to achieve leaf-clearing and egg-staining within this time limit, even though this might result in some tissue disruption. Without boiling, the eggs were not sufficiently stained to discern them from the background color of the leaves.

Our preliminary tests with *cos* lettuce leaves indicated that boiling moderate to older aged leaves >1 min, or young leaves >30 s, caused partial separation and tearing of the abaxial and adaxial epidermal surfaces. This resulted in either excessive or limited staining of the compromised tissue, depending on the staining solution.

Leaves boiled in 50, 60, and 70% ethanol produced irregular clearing and the solution evaporated quickly. A boiling aqueous solution of 10% acetic acid and 50% ethanol cleared the leaves well, but again this solution evaporated quickly and was potentially dangerous because of alcohol's flammability. Adding stains to the acetic acid-ethanol solution produced various results. Methylene blue stained the leaf tissues blue, but not the stipples or eggs. Methyl red heavily stained the leaf tissues and stipples, but not the eggs. There was not enough contrast between eggs and stained leaves to be able to easily identify the eggs. Gentian violet and saffarin poorly stained the leaf tissues, stipples and eggs.

The egg staining methods of Curtis (1942) and Sugimoto (1976), who used final staining solutions of water saturated with methyl red and 70% ethanol saturated

with scarlet red, respectively, did not stain *L. trifolii* eggs in lettuce. Lettuce leaves were nearly destroyed by the three separate boiling steps used by Curtis (1942). Other published methods of insect staining were not tested because they involved two separate boiling procedures or long (i.e., > 24 h) staining times.

The lactophenol acid fuchsin solution of Simonet & Pienkowski (1977) provided the best staining of *L. trifolii* eggs and the best overall clearing of leaves. Liquid phenol produced the most consistent results and was easier to work with than phenol crystals. Modifications of their technique allowed us to successfully prepare the relatively soft lettuce tissue. The leaves were cleared and stained in a slow boiling solution to minimize tissue disruption. Total staining time was ultimately reduced to 7 min per leaf.

The exact procedure we used for staining eggs in lettuce leaves was as follows: leaves were placed in the boiling solution and held immersed for 1 min; young leaves with soft tissue required boiling for only 30 s; the solution was removed from the hot plate and the leaves allowed to steep in the stain for an additional 3 min; leaves were removed from the stain and rinsed in warm water for 3 min to remove excess acid fuchsin; stained leaves were then placed in cold water within 15 cm diam glass petri dishes for microscopic examination.

This modified procedure stained the eggs pink to deep red and made them easy to locate within the leaves. The 4 min staining and clearing procedure did not completely clear the leaves of chlorophyll, but the degree of clearing was sufficient to make it easy to locate the eggs. Leaves cleared better if they were boiled >1 min or steeped >3.5 min, but this resulted in greater destruction and darker staining of leaf tissues, respectively. Both of these conditions proved unsuitable for our purposes.

Efforts to remove or replace phenol from this procedure were unproductive. When phenol was left out and the volume of water in the solution was doubled, or when phenol and water were replaced with an equal volume of polyethylene glycol, the leaves became stained too heavily to be de-stained within several hours and the eggs were poorly stained. The addition of 0.1 part acetic acid to either of these solutions without phenol improved leaf-clearing and egg staining, but the eggs were not stained as well as when phenol was in the solution.

While this rapid lactophenol acid fuchsin technique produced well-stained eggs, it did not stain all of the stipples. Many became indistinguishable once the leaves were cleared. Parrella & Robb (1982) found that longer staining periods (≥ 3 h) with acid fuchsin stained stipples pink to red on chrysanthemum, celery, and tomato leaves. However, lettuce leaves steeped 10 min in lactophenol acid fuchsin became darkly stained, while the stipples still could not be differentiated from normal leaf tissue. Increasing steeping times made the eggs difficult to locate, so an alternative to longer staining with acid fuchsin was desired. It was determined that stipples could be quickly counterstained using a lactophenol cotton blue solution (Franklin & Goodey 1959) immediately following the egg staining procedure. The counterstain solution was identical to the lactophenol egg staining solution except the acid fuchsin was replaced with 0.4 g cotton blue per 0.5 liter of solution. The solution was initially boiled and then allowed to cool to room temperature ($25 \pm 1^\circ\text{C}$) before use. Following the 3 min water rinse after the lactophenol acid fuchsin egg stain, leaves were placed in the lactophenol cotton blue solution for 1 min. Leaves were then washed in warm water for 1 min to remove excess stain. This procedure successfully stained the stipples blue which provided a good contrast against the pink leaf tissue. Leaves left in the cotton blue solution for >1 min absorbed excessive stain that blurred the boundaries of individual stipples. Although stipples could still be manually counted, the loss of independent boundaries around each stipple compromised efforts to use computer assisted

image analysis for stipple counts. Efforts to combine the egg and stipple staining steps failed. Leaves boiled in lactophenol acid fuchsin-cotton blue solution resulted in the eggs being stained purple, while the stipples were unstained.

Larvae were not stained by acid fuchsin or cotton blue in lactophenol. However, transmitted light made them appear yellow to greenish brown against the pink background of the leaves following the egg and stipple staining steps. Tunnels within the leaves were occasionally stained pink and blue.

While the lactophenol-cotton blue treatment was satisfactory for intact leaves, it poorly stained stipples in areas of tissue disruption and in areas closely adjoining primary leaf veins at the proximal region of leaves. Thus, the best overall technique for highlighting stipples turned out to be the silver nitrate method. Black silver particles adhered to all damaged portions of leaves and revealed all stipples, even if there was no visible wound response. The blackened stipples provided enough contrast against the green leaves for them to be successfully counted with computer image analysis software. Using this technique offers several benefits. None of the solutions used in this procedure masked or removed markings made on the leaves with felt tipped indelible ink pens, so several leaves could be processed in the same container without loss of leaf identity. These chemicals did not adversely alter leaf or egg tissues, so they could be later stained using the lactophenol acid fuchsin solution to locate eggs. Since there was no boiling involved, the procedure also allowed larvae within the leaves to successfully complete development.

There was no obvious difference in leaf-clearing or egg and stipple staining among the four cos lettuce cultivars examined. Inviabile eggs were stained the same color by lactophenol acid fuchsin as viable eggs. Chorion of empty eggs absorbed little of the acid fuchsin stain.

In spite of the costs of the lactophenol procedures, they worked the best with the dyes to clear leaves and stain target tissues. The modified lactophenol acid fuchsin, lactophenol cotton blue, and silver staining procedures all met our objectives of rapid techniques that would differentially stain eggs and stipples. They produced adequate contrast for counting eggs, stipples, and larvae in one step, and proved suitable for use with computer image analysis software, e.g., Optimas (Optimas Corp., Edmonds, Washington). They will be used to further our understanding of mechanisms involved with host plant resistance to serpentine leafminer in lettuce.

ACKNOWLEDGMENTS

Research was facilitated with support from the Wedgworth Family, Belle Glade, FL, and from South Bay Growers Inc., South Bay, FL. Critical reviews of the manuscript were provided by R. Cherry, L. Datnoff, and J. Dusky (University of Florida). This report published as Univ. of Florida Agricultural Experiment Station, Journal Series no. R-03966.

REFERENCES CITED

- CARLSON, O. V., AND E. T. HIBBS. 1962. Direct counts of potato leafhopper, *Empoasca fabae*, eggs in *Solanum* leaves. *Ann. Entomol. Soc. America* 55: 512-515.
- CURTIS, W. E. 1942. Method of locating insect eggs in plant tissue. *J. Econ. Entomol.* 35: 286.
- FOSTER, R. E. 1986. Monitoring populations of *Liriomyza trifolii* (Diptera: Agromyzidae) in celery with pupal counts. *Florida Entomol.* 69: 292-298.
- FRANKLIN, M. T., AND J. B. GOODEY. 1959. A cotton-blue lactophenol technique for mounting plant parasitic nematodes. *J. Helminthol.* 23: 175-178.

- GENUNG, W. G., AND M. J. JANES. 1975. Host range, wild host significance, and in-field spread of *Liriomyza trifolii* and population build-up and effects of its parasites in relation to Fall and Winter celery (Diptera: Agromyzidae). Belle Glade AREC Res. Rpt. EV-1975-5, 18 p.
- GILSTRAP, F. E., AND E. R. OATMAN. 1976. The bionomics of *Scolothrips sexmaculatus* (Pergande) (Thysanoptera: Thripidae) an insect predator of spider mites. Hilgardia 44: 27-59.
- LEIBEE, G. L. 1981. Insecticidal control of *Liriomyza* spp. on vegetables, pp. 216-220. in D. J. Schuster [ed.], Proceedings of the IFAS-Ind. Conference on the Biology and Control of *Liriomyza* Leafminers. IFAS, Univ. of Florida.
- NUESSLY, G. S., AND R. T. NAGATA. 1993. Evaluation of damage by serpentine leafminer and banded cucumber beetles to cos lettuce. Everglades Res. and Ed. Center Res. Rpt., EV-1993-2:76-77.
- NUESSLY, G. S., AND R. T. NAGATA. 1994. Differential probing response of serpentine leafminer, *Liriomyza trifolii* (Burgess), on cos lettuce. J. Entomol. Sci. 29: 330-338.
- PARRELLA, M. P., AND K. L. ROBB. 1982. Technique for staining eggs of *Liriomyza trifolii* within chrysanthemum, celery, and tomato leaves. J. Econ. Entomol. 75: 383-384.
- SIMONET, E. E., AND R. L. PIENKOWSKI. 1977. Sampling and distribution of potato leafhopper eggs in alfalfa stems. Ann. Entomol. Soc. America 51: 557-566.
- SUGIMOTO, T. 1976. On distribution of egg population of a leafmining fly, *Phytomyza ranunculi* Schrank (Diptera, Agromyzidae) among leaves and in a leaf. Fac. Agric. Kinki Univ. 9: 11-19.

