

Visualization of Leaf and Bracteal Nectaries of Cotton using Digital Microscopy to Improve Scoring Accuracy and Data Preservation

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Abstract

Nectaries are distinct nectar-producing glands, present in many plant species. Nectaries exhibit diverse structures and functions. In cotton, traditional scoring of nectary trait is error-prone, unreliable and has limitations as phenotypes of this trait are often not visible to the naked eye. Nectary trait expressions are controlled by genes, *Ne1* and/or *Ne2*. Additionally, trait expression can be influenced by the environment and growth stages, stressing the need for accurate scoring methods. Particularly, phenotypic scoring by digital images results in a more accurate scoring method of nectaries. This method overcomes the limitations of the traditional scoring by generating high-resolution images. Further, facilitates the identification and differentiation of fine differences of nectary trait expression while preserving these digital images for future reference. This phenotyping scoring method described here can be easily adapted to score other plant traits such as glands, hairs, and color. These scoring methods can be tailored to other plant species. In this article, we explain step-by-step procedure on how to collect samples from the field or greenhouse, dissect to observe them using digital microscopy, and preserve these images for future scoring analysis. For this method, we will use, for example, scoring of leaf and bracteal samples of cotton plants to differentiate the presence of nectaries (fully developed, reduced and vestigial) and the absence of nectaries.

Introduction

Plants have specialized glands called nectaries that synthesize and produce nectar in most angiosperms, some ferns, and some gymnosperms^{1,2,3,4}. Nectaries are classified into three types, namely mesophyllary, trichomatic, and epithelial types, based on the origin of cells that produce nectar⁵. Nectaries in cotton are modified stomata made up of glandular trichomes known as papillae and are ranked as trichomatic type^{5,6}. Most of the *Gossypium* species have nectaries; however, the number of nectaries present in this genus differs from species to species⁷. Floral nectaries (FNs) are more common than extrafloral nectaries (EFNs) in plants⁸. These nectaries can occur anywhere on the plant

except roots^{1,2}. For instance, *Gossypium hirsutum* shows both floral and extrafloral nectaries⁹. Domestic cotton plants show three extra floral and one floral nectary¹⁰. The three extra floral nectaries are foliar, bracteal, and circumbracteal nectaries¹¹. The foliar nectary is vegetative and is typically present on leaves on the lower side of the midrib, while bracteal and circumbracteal nectaries are reproductive and develop at the base of the bract and abaxial calyx surface. Floral nectary is associated with a flower, which develops on the adaxial (upper) surface of the calyx. This nectary trait is controlled by a single gene locus¹². Studies by two independent research groups identified that the nectary trait is controlled by one gene, *Ne1* of the A genome or *Ne2* of the D genome, mapped to chromosomes 12 and 26, respectively^{12,13}. This trait is only

expressed in a double recessive condition, which means that only a homozygous recessive condition will express the nectariless trait.

In addition to these genes, environmental conditions and growth stages play a role in controlling the degree of expression. Therefore, there needs to be an accurate method to score this trait. The current study focuses on the phenotyping of leaf and bracteal nectaries in cotton. Plants with visible nectar-producing nectaries are scored as nectaried, while plants that lack this trait are scored as nectariless^{1,2,3,4}. The main goal of this article is to present accurate scoring methods of the nectary trait using digital microscopy technology. Traditional scoring by direct visual observation cannot easily detect differences in the expression variation of the nectary trait *in situ* with the naked eye. These subtle differences in nectary trait expressions can be visualized using digital microscopy. To illustrate, in cotton leaf nectary, the scoring rubric follows a standard scale of 1-4 in which 1 represents no nectary, 2 represents a bump out on the vein phenotype, 3 represents underdeveloped pads or ridges with no nectar, and 4 represents fully formed/ complete nectaries with clear pads and ridges¹³. This phenotype scoring was generated utilizing digital images of the leaf nectaries [using digital images of the abaxial (lower) side of the leaf midrib]. In general, the absence of nectaries is scored as 0, but for statistical significance, the value 0 cannot be used and replaced by the value 1. Hence, the phenotyping scoring range was modified to 1-4 from the standardized classification of 0-4¹³. The scoring rubric for flowers follows a similar scoring pattern of 1-4 in which, 1 represents nectariless, no prominent glands with no pads or ridges, 2 for obfuscated glands in which nectaries have only subtle pad markings and no nectar, 3 for poorly formed glands with faint or absent ridges and /or pads, and 4 for fully formed nectaries with nectar. This scoring pattern shows 4 for nectaried phenotypes (homozygous/heterozygous dominant for one of the genes), 3, 2 for differential expression of nectary trait as in heterozygous, and 1 for nectariless (homozygous recessive for both genes).

Likewise, flowers are collected and dissected as described step by step in this article to collect digital images for scoring bracteal nectaries. This phenotype can be visualized with the microscope for accurate scoring that can be stored in the form of digital images. In cotton, nectary traits not only attract pollinators but also attract pests that cause yield losses¹⁴. To solve this issue, breeders selected plants with no nectaries (nectariless) traits as an alternative to control pests naturally without the use of chemical pesticides^{9,15}. Nectariless trait was originally introgressed from *Gossypium tomentosum* to *Gossypium hirsutum* (cultivated Upland cotton)⁸. This scoring method is especially useful for identifying the nectariless trait segregation in populations that are generated by crossing nectaried parents with nectariless parents. As a result of these crosses of diverse parents, F₂ (Second Filial Generation) shows different genotypes of

homozygous nectaried, heterozygous nectaried, and homozygous nectariless. Only one dominant gene is necessary for nectary trait expression, which follows the segregation ratio of 15:1 (9:3:3:1). Hence, 1 in 16 will express the nectariless trait in homozygous recessive condition with genotype *ne1ne1ne2ne2*. However, researchers in breeding programs observed more nectariless lines than the expected ratio of 1 in 16. This means that the nectary trait is expressed when the genes are expressed as *Ne1Ne1Ne2Ne2*, *Ne1ne1Ne2ne2*, *ne1ne1Ne2Ne2*, *ne1ne1Ne2ne2*, *Ne1ne1ne2ne2*, and *ne1ne1ne2Ne2*. The diverse pattern of nectary trait expression in such populations of homozygous nectaried (*Ne1Ne1Ne2Ne2*), heterozygous nectaried (*Ne1ne1Ne2ne2*), and homozygous nectariless (*ne1ne1ne2ne2*) plants can be perfectly scored by detecting the changes visualized in the digital images^{12,13}. As heterozygous plants with reduced nectary may not show nectary trait visually and might resemble the nectariless trait with no nectary, the visual phenotyping poses challenges in the reliable selection of this trait. These issues magnify in the late growing season, where nectaries are not present in certain cotton cultivars. Differences between heterozygous plants and homozygous nectariless plants can be detected easily with digital imaging, as heterozygous plants might show small / reduced nectaries while homozygous plants completely lack this trait. Phenotypically, the presence of nectary is classified as nectaried (homozygous/heterozygous with at least one dominant gene), the presence of small or vestigial nectaries as heterozygous, and the absence of nectaries as homozygous nectariless plants. Digital image scoring reduced the inaccurate scoring of heterozygous plants as nectariless plants. Similarly, the mid-flowering stage is preferred when there is maximum trait expression. Hence, leaf and flower samples were collected at this stage to carry out these phenotyping scoring experiments for accurate and reliable scoring of nectary traits. Further, visualization of nectary traits using digital microscopy prevents/ reduces false positives of populations with no nectary traits. This phenotypic scoring of the nectary trait is also being used in mapping studies to identify DNA markers associated with nectariless trait that breeders can use for marker-assisted selection (MAS) of the nectariless trait¹³. This scoring technique can be extended to other plant species in addition to studying other traits such as glands, hairs, and color. Overall, digital image scoring not only resolves the problem of inaccurate nectary trait scoring by providing high resolution images but also identifies subtle expression changes and stores the digital images for future use. Cotton with nectariless trait may be used for biocontrol of pests, in addition to answering research questions on how this trait encourages beneficial insect interactions.

Protocol

1. Greenhouse/field sampling of leaves (Figure 1)

1. Prepare sample zip-lock bags with sample IDs. Store sample bags at room temperature until use.
2. Place the cooler in the refrigerator a day before sample collection. Place an ice pack from the freezer at the bottom of the cooler. Place a plastic tray on the ice pack in the cooler.
NOTE: Any portable cooler can be used for this purpose. Place ice pack in the cooler followed by a plastic tray (this separation prevents freezing damage, which occurs by the direct contact of sample with ice) before transporting cooler to the sample collection site.
3. Transport cooler and labeled zip lock bags to the greenhouse/field for the collection of samples. Regular sprays were scheduled for field plants to collect pest-free samples. Similarly, for greenhouse plants, regular sprays and fertilizer schedules were followed for healthy plants.

4. Collect young leaf tissue from an individual 8 to 12 week old greenhouse or field-grown cotton plants in the labeled sample bags.

1. Select the mid-flowering stage for sample selection because of the highest expression of the nectary trait at this stage. Select young leaves from all plants at this developmental stage. In addition to genetic and environmental influence, the nectary trait is also influenced by the developmental stage.
2. To keep the developmental stage constant for comparison for different genotypes within the F_2 population, collect leaf samples uniformly at one stage to restrict comparison to this stage. Use the leaf size of 5 to 7 cm as a yardstick, but the same size young leaves can be collected from all samples. Old leaves show nectary trait expression, but in some lines, the nectary trait will not be expressed at later stages of development. To avoid this variation and improve consistency of data collection, follow these directions while harvesting samples.

5. Collect the sample leaf tissue and place it in the respective sample bag. Collect at least 2 leaves per plant sample.
6. For leaf tissue, select healthy leaves of 5 to 7 cm across the leaf blade from the top branch of the plant.

NOTE: Young leaves present on the top branches are preferred for all samples. This type of sample collection not only restricts sampling to a specific developmental stage but also reduces the errors in phenotyping by keeping the type of sample constant. Other parameters include easy access and healthy leaves. Consistent data comparison will be made when several plant leaves are collected from the same developmental stage to identify genotype-based phenotypic differences in the population.

7. Place each sealed sample bag with the leaf tissue in the cooler. Transport the cooler to the lab.
8. Transfer individual samples to the refrigerator to maintain cooling conditions of 4 °C. Store samples in the refrigerator until digital imaging of the nectary. Leaves can be stored up to 2 days for digital imaging, but it is preferred to take images on the same day or the following day.
9. For screening a large number of leaf samples, collect samples in batches to finish imaging in the time slot of 1 day after harvesting them. Collect samples in batches of 10-20 or use multiple coolers to prevent tissue damage or folding of the tissue. Care should be taken in the collection of samples for good digital images.

2. Greenhouse/field sampling of flowers (Figure 1)

1. Follow steps 1.1 to 1.6. Collect flowers from a 8 to 12 week old greenhouse or field-grown cotton plant. Collect at least 2 flowers per plant sample.
 1. Collect flowers usually from the top branches when plants are in the mid-flowering stage. Nectary trait shows the highest expression at the mid-flowering stage.
2. Pick healthy flowers. Place each sealed sample bag with at least two flowers in the cooler. Transport the cooler to the lab.
3. Transfer individual samples to the refrigerator to maintain cooling conditions of 4 °C. Store samples in the refrigerator until digital imaging of the bracteal nectaries.
 1. Process samples on the same day or the following day after collection. Collect flower samples, store them at 4 °C and process bracteal nectaries on the same day. Collect flowers that are produced later the following day or later in the week and process for digital imaging of bracteal nectaries the same day.

3. Digital microscope initial setup (Supplementary Figure 1)

NOTE: Other comparable microscopes can be used for digitally capturing and storing the images.

1. Turn on the microscope (VHX 600) and complete the initial steps. Place a half-folded A4 white sheet that matches the size of the microscope stage.
2. Adjust the light on the microscope platform/ stage using the small light knobs present on the console controller.
3. Turn the light switch (small knob on the console) to maximum for maximum light and brightness switch (large knob on the console) to medium high by turning the knob on the console 3/4 of the way.

- The inbuilt VHX 600 software shows options to adjust lens on the computer screen. Adjust the lens to 10x magnification by selecting 10x lens option on the screen. Turn on the monitor power switch on the computer front screen, and the main menu will appear, which shows options for lens and record image options.
- Take a small number of samples (3 to 5) from the refrigerator into the cooler for digital imaging. Place the cooler with samples beside the microscope.
- Keep the cutting board and sterile blade on the bench close to the microscope. Pick one sample from the cooler and take out the sample tissue to proceed with digital imaging (**Figure 1**).

4. Leaf nectary digital imaging and scoring (**Figure 2**)

- Follow steps 1 and 3. Open the sample zip lock bag and place the leaf sample onto the cutting board. Cut the petiole of the leaf using a sterile blade.

NOTE: For safe use of blades, wear gloves and keep the sharp edges of the blade facing away from your fingers, with the tissue being positioned between fingers while making incisions.

- Transfer the leaf tissue onto the precut white sheet placed on the stage. Flip the leaf tissue in the center of the microscope stage with the abaxial side facing up.
- Focus on the midrib of the leaf by gently rotating the coarse and fine adjustments of the microscope knobs. Continue adjusting until there is no blur in the image.
- As the nectary is present on the lower end of the midrib, keep it centered where the midrib and diverging later veins were observed. Keep the leaf centered in focus on the stage.

NOTE: Only one leaf nectary is observed in domestic cotton, while wild types show three nectaries, one on the midrib and one on each lateral vein on either side of the midrib (total nectaries :3).

- Adjust the coarse and fine adjustments of the microscope to improve the clarity of the digital image. Make all image adjustments to capture and save the nectary image of the leaf.
 - To avoid light reflection on the digital image, turn off all lights and record the image on the computer screen. Turn off all lights in the room and use a table lamp to process each sample. After all steps are carried out, turn off the lamp and leave only microscope light on and record the image.
- Save the image once the program prompts a window showing several options to save. Save the image (by labeling each image) with the ID number of the sample. Score the leaf

nectary digital image as 1, 2, 3 and 4 based on the phenotype of the nectary. Update your scoring sheet for each sample ID.

5. Digital imaging and scoring of the bracteal nectary (**Figure 3**)

- Follow steps 2 and 3. Transfer a small number of flower samples (2-3) for imaging. Take the flower sample out of the zip-lock bag.
- Remove bracts from the flower using forceps or manually by hand. Place the flower on the clean cutting board. Use a sterile blade to cut the stalk of the flower. Use a sterile blade to make a straight incision along the edge of the removed bracts.
- Place the tissue upside down (with petiole facing up) on the precut white sheet laid on the microscope stage.
- Use coarse and fine adjustments to improve the clarity of the image displayed on the screen. Make all image adjustments and click the record button on the console controller to capture the image of the leaf.
 - Make Image adjustments by setting the small knob/ light switch to maximum and the large knob/ brightness switch to medium high (by turning the knob to $\frac{3}{4}$ th). Then, improve the resolution of the image by rotating the coarse and fine adjustments of the microscope. Keep these adjustments constant and switch samples until images are recorded for the entire set of samples. To avoid light reflection on the digital image, turn off all lights and record the image on the computer.
- Then, the program prompts a window showing all computer locations to save the image. Save the digital image in the computer with the ID number of the sample.
- Score the bracteal nectaries (**Supplementary Figure 2**) with 1, 2, 3, and 4 based on the phenotype observed in the acquired digital images. Update the scoring sheet with sample ID and the respective score for future analysis

Results

Cotton plants grown in the field for 8 to 12 weeks were selected for this study. At least two technical replicates were collected for each plant per tissue type. Healthy young leaf samples are collected from the top branches with 5 to 7 cm long leaf blades. Healthy flower samples are collected from open flowers or flower buds that will open on the same day. Leaf and flower samples were collected from the field from different plant lines, and digital images were generated for both tissue types in the lab using a microscope (**Figure 1**). All steps were followed as described above in the procedure from sample collection to imaging (as explained in **Figure 2**, and **Figure 3**). The representative results for both leaf and bracteal nectaries typically show the absence of nectary

(1), the presence of nectary with intermediate phenotypes (2, 3), and a fully developed nectaries producing nectar (4). The data generated in **Figure 4** are the digital images acquired from two different cotton plants (nectaried and nectariless). Results of the digital image scoring of the leaf abaxial surface (on the lower side of the midrib) showed two phenotypes with scores 1 (with no nectaries on the midrib) and 4 (with fully developed nectary with nectar; **Figure 4A,B**). Similarly, when flower samples were analyzed for bracteal nectaries, they showed two phenotypes of 1 (with no nectary) and 4 (fully formed nectary producing nectar; **Figure 4C,D**). Ideally, both leaves and flowers collected from the same plant should follow the same pattern, which means that the nectariless leaf and nectariless flower should belong to one plant, while the nectaried leaf and nectaried flower should belong to the same plant. **Figure 5** is produced by collecting digital images of both leaf and bracteal nectaries of nectaried plants at 10x, 20x, and

40x for clearly visualizing nectary traits. Further, to understand how this scoring is given in segregating F_2 populations of nectaried and nectariless cotton parents, leaf tissues were collected from one of these populations, and digital images were produced for each leaf nectary sample. Selected leaf digital images corresponding to the standard format scoring of 1, 2, 3, and 4 are highlighted in **Figure 6**¹³. The common and easy pattern to identify is the absence of nectary and the presence of nectary. The absence of nectary is given the lowest score, while a fully developed nectary is given the highest score of 4. The range of scores between 1 and 4, which are 2, 3, are underdeveloped and smaller than regular nectaries. This pattern can be observed in homozygous nectariless, which is 1 (absent), heterozygous condition as in 2 and 3 scores (reduced nectaries), and 4 (fully developed) nectaries. Additionally, nectaried and nectariless parental lines can be grown along with populations to compare and understand these differences.

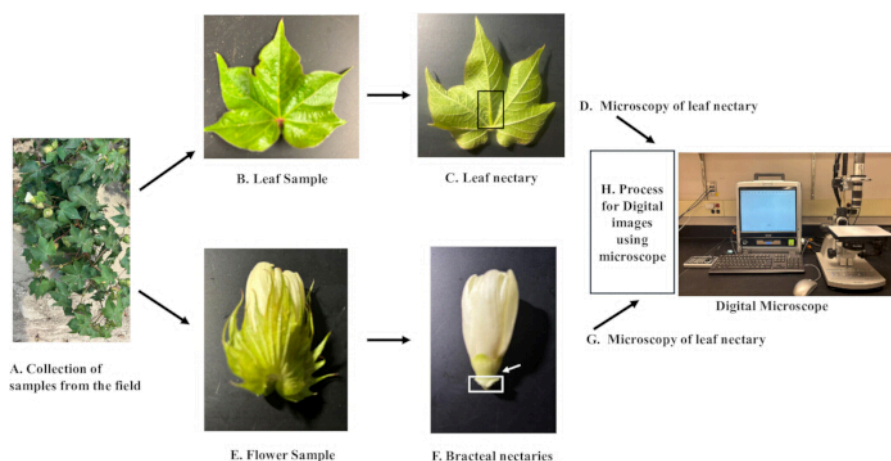


Figure 1: Overview of leaf and bracteal nectaries visualization steps starting from sampling to digital microscopy. (A) Select mid-flowering stage cotton plants for both leaf and flower sample collection. (B) Collect leaf samples from the field for observing nectary traits on the leaf. (C) Flip the leaf so that the abaxial side of the leaf faces up and observe the nectary trait in the highlighted black box region. (D) Place the leaf on the microscope stage, and keep the focus in the highlighted black box region for recording digital images of the leaf nectary. (E) Collect flowers at the mid-flowering stage from the field (A). (F) Make an incision by placing the flower on a cutting board and incise in a straight line in the white box region in the direction of the arrow, separating the base of the flower. (G) Place the incised section on the microscope stage for digital imaging of the bracteal nectaries. [Please click here to view a larger version of this figure.](#)

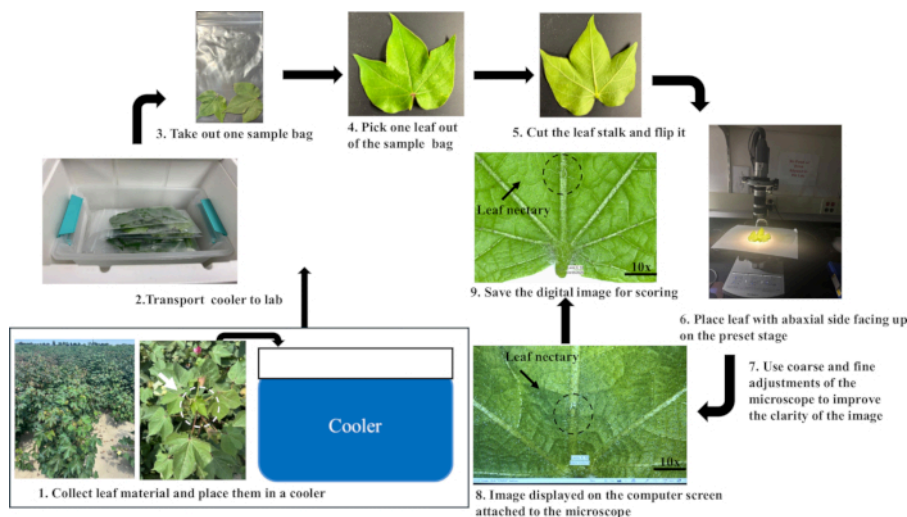


Figure 2: Stepwise procedure for digital imaging and scoring of the leaf nectaries in cotton. 1. Collect leaf material from the field in labeled sample bags and place it in a cooler 2. Transport the cooler with samples to lab 3. Take out individual samples from the cooler 4. Open the individual sample bag and take out leaf 5. Cut the petiole of the leaf manually or use a blade. 6. Place the leaf on the preset microscope stage with the abaxial (lower) side facing up 7. Adjust the zoom to 10x in the computer screen VHX 600 program. Adjust coarse and fine adjustments of the microscope for the best resolution of the image. 8. Look at the computer screen for any adjustments of the image observed on the computer screen (adjust light and brightness controlled by small and large knobs by rotating these knobs, use fine and coarse buttons on the microscope for best image resolution, and turn off other lights to remove reflection, etc.) in the leaf nectary 9. Save the digital image for scoring. Dash circle around the nectary in leaf highlights the region of leaf nectary in images 8 and 9. Observe leaf nectaries under microscope at 10x (100 μ m) magnification. Only one leaf nectary is observed in domestic cotton (as observed in this image). [Please click here to view a larger version of this figure.](#)

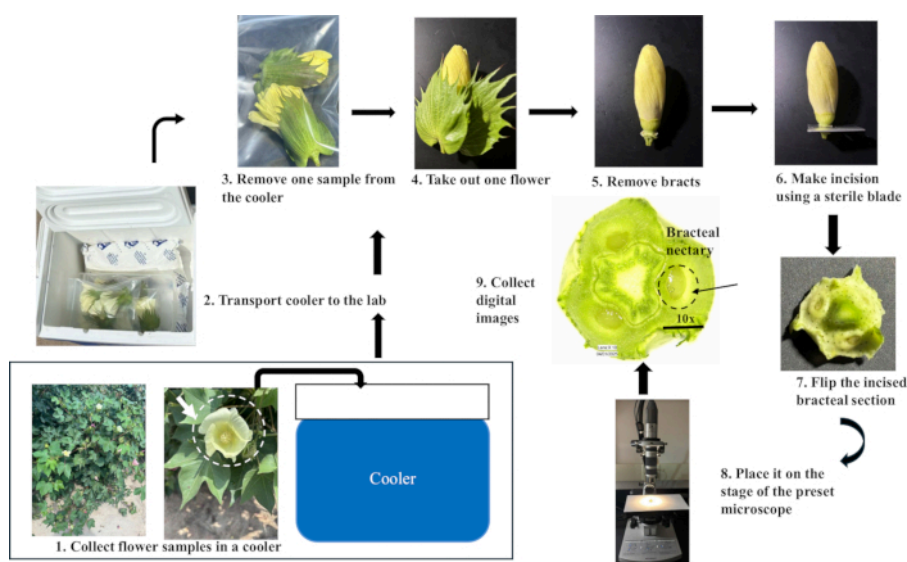


Figure 3: Stepwise procedure for digital imaging and scoring of the bracteal nectaries in cotton. 1. Collect flower samples from the field in the labeled sample bags and keep them in the cooler 2. Remove one sample from the cooler 3. Take out one flower 4. Remove Bracts manually by cutting them off away from the flower 5. Make an incision using a sterile blade by cutting along the bracteal edge in a straight line (white box shown in [Figure 1](#) before proceeding to digital imaging of bracteal nectaries) 6. Flip the incised section 7. Place the incised section with petiole side facing up on the stage of the microscope stage 8. Make light adjustments using the light and brightness switches on the console attached to the microscope. Use coarse and fine adjustments on the microscope for collecting images with good resolution. All images are observed under a microscope at 10x (100 μ m) magnification. Collect digital images for scoring the bracteal nectaries. Circles in the digital images of bracteal nectary highlights the presence of nectary, and there are 3 bracteal nectaries in domestic cotton. [Please click here to view a larger version of this figure.](#)

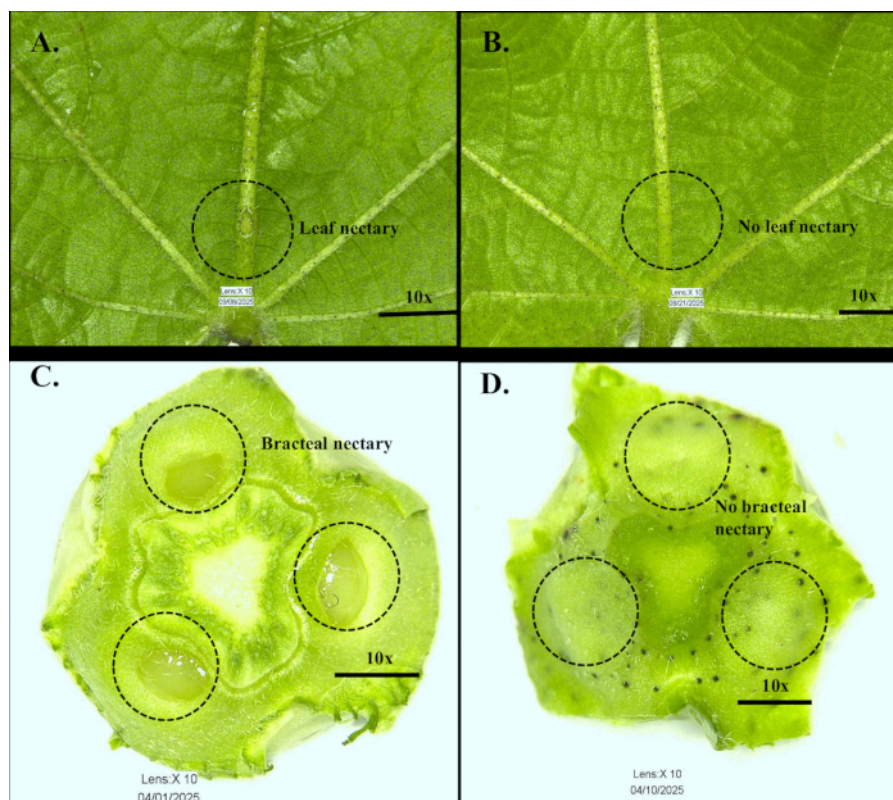


Figure 4: Cotton leaf nectary and bracteal nectary digital images. (A) Leaf with nectary; (B) Leaf without nectary; (C) Flower showing 3 bracteal nectaries, and (D) Flower showing no bracteal nectaries. Observe both leaf and flower nectaries under a microscope at 10x (100 μ m) magnification. Dash circles show the presence and absence of nectary in leaf nectary and bracteal nectaries. [Please click here to view a larger version of this figure.](#)

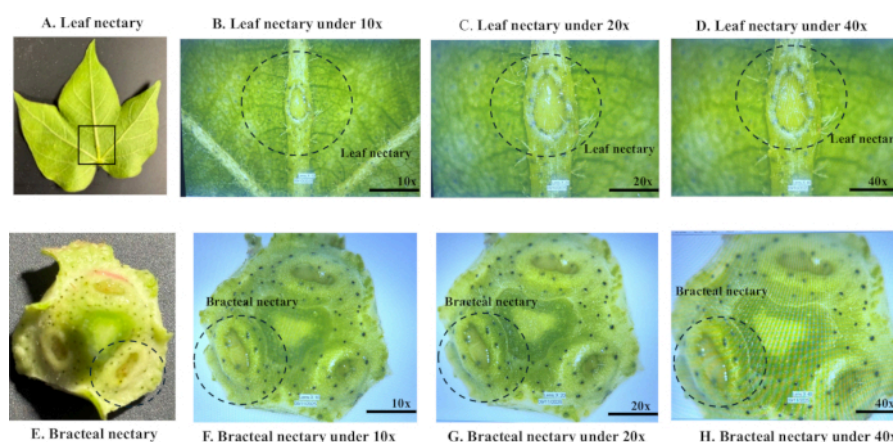


Figure 5: Leaf and bracteal nectaries were zoomed at 10x, 20x, and 40x for clear identification of nectaries in digital images. (A) Collect leaf samples to observe the nectary trait on the midrib. (B) Observe the leaf nectary on the midrib under the microscope at 10x magnification. (C) Observe the leaf nectary on the midrib under the microscope at 20x magnification. (D) Observe the leaf nectary on the midrib under the microscope at 40x magnification. (E) Incise flower section for bracteal nectaries. (F) Observe bracteal nectaries under microscope at 10x magnification. (G) Observe bracteal nectaries under microscope at 20x magnification. (H) Observe bracteal nectaries under microscope at 40x magnification. Scale bars on each image represent the magnification at which the leaf nectary or bracteal nectary images were taken (as shown here as 10x, 20x, and 40x). [Please click here to view a larger version of this figure.](#)

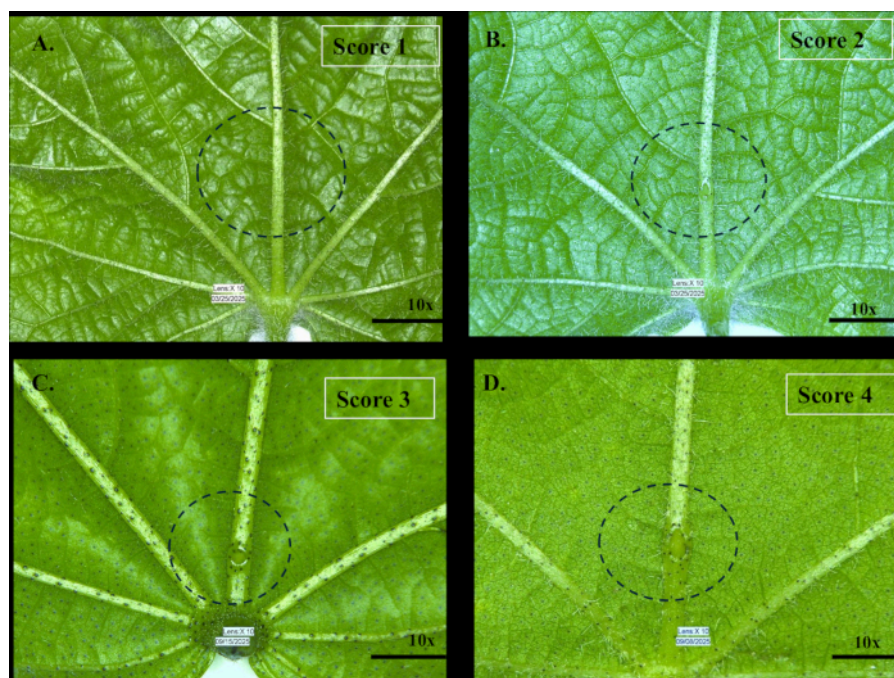


Figure 6: Standard scoring pattern of leaf nectary following patterns of 1, 2, 3, and 4. (A) Leaf sample with no nectary as highlighted in the dashed circles (Score 1 for the absence of nectary). (B) The small leaf nectary observed shows a pattern of one of the heterozygous condition (dashed circle picture of nectary scored as 2). (C) Leaf nectary with score 3, another pattern of the heterozygote. (D) Fully formed nectaries with a score of 4. Scale 10x represents the magnification at which images were taken. [Please click here to view a larger version of this figure.](#)

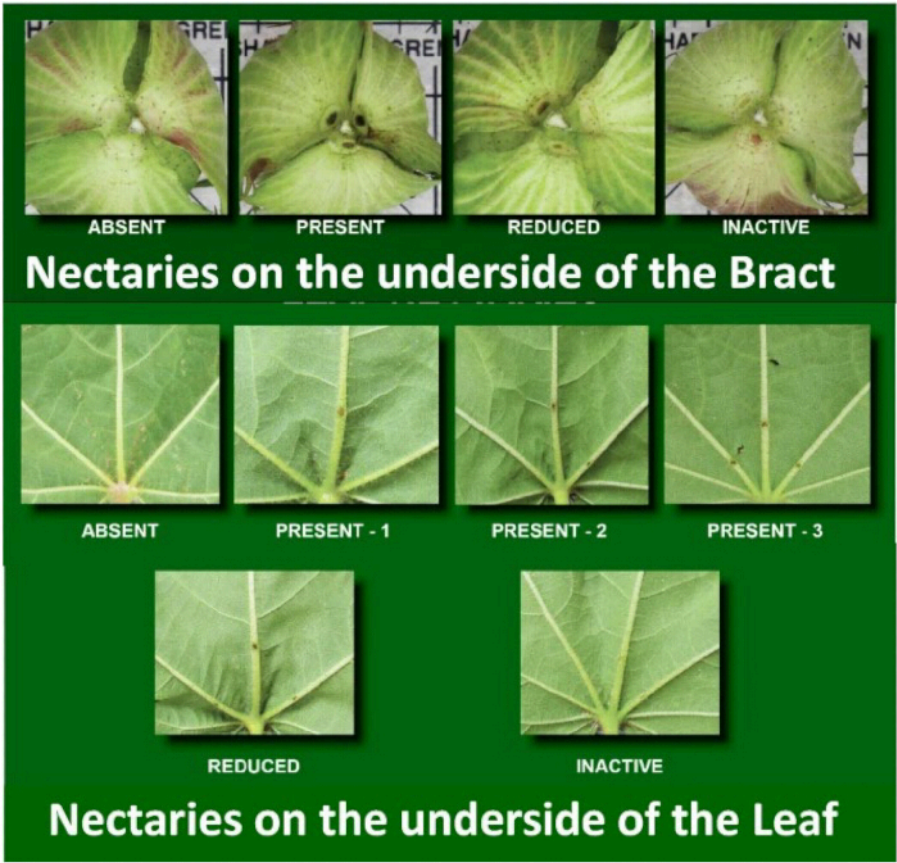


Figure 7: Leaf nectary and bracteal nectaries without using a microscope. The figure depicts how nectaries appear with traditional scoring. This figure has been adapted from¹³. [Please click here to view a larger version of this figure.](#)

Cross				
Ne ₁ Ne ₁ Ne ₂ Ne ₂		x	ne ₁ ne ₁ ne ₂ ne ₂	
F1		Ne ₁ ne ₁ Ne ₂ ne ₂		
		Self Pollinate		
F2				
Possible F2		Genotypes		
Gametes	Ne ₁ Ne ₂	Ne ₁ ne ₂	ne ₁ Ne ₂	ne ₁ ne ₂
Ne ₁ Ne ₂	Ne ₁ Ne ₁ Ne ₂ Ne ₂	Ne ₁ Ne ₁ ne ₂ ne ₂	Ne ₁ ne ₁ Ne ₂ Ne ₂	Ne ₁ ne ₁ ne ₂ ne ₂
Ne ₁ ne ₂	Ne ₁ Ne ₁ ne ₂ ne ₂	Ne ₁ Ne ₁ Ne ₂ ne ₂	Ne ₁ ne ₁ Ne ₂ ne ₂	Ne ₁ ne ₁ ne ₂ Ne ₂
ne ₁ Ne ₂	Ne ₁ ne ₁ Ne ₂ Ne ₂	Ne ₁ ne ₁ ne ₂ Ne ₂	ne ₁ ne ₁ Ne ₂ Ne ₂	ne ₁ ne ₁ Ne ₂ ne ₂
ne ₁ ne ₂	Ne ₁ ne ₁ ne ₂ ne ₂	Ne ₁ ne ₁ Ne ₂ ne ₂	ne ₁ ne ₁ ne ₂ Ne ₂	ne ₁ ne ₁ ne ₂ ne ₂
Possibly Scored as	nectaried	Blue	Possibly Scored as	nectariless
				Green

Figure 8: Possible genotypes of the population The figure shows genotypes of the F₂ populations resulting from a cross of diverse parents, nectaried and nectariless. This table has been adapted from¹³. [Please click here to view a larger version of this figure.](#)

Supplementary Figure 1: Digital microscope setup. [Please click here to download this File.](#)

Supplementary Figure 2: Different number of bracteal nectaries observed. [Please click here to download this File.](#)

Discussion

Nectaries are specialized glandular trichomes that produce nectar in plants for successful cross-pollination. Both vegetative and reproductive nectaries are present in plants. *Gossypium* genus (cotton) has more than 50 species, and the majority of them contain leaf nectaries¹⁶. However, this trait in cotton also attracts pests, leading to additional yield losses¹⁷. Breeders selected naturally existing nectariless traits (absence of nectaries) first discovered in *G. tomentosum* to solve this problem. For this reason, they introgressed this nectariless trait into cultivated upland cotton¹⁷. Eventually, several populations were generated by selecting nectaried and nectariless lines as parents. As homozygous nectariless and heterozygous nectariless populations do not show any differences when observed with the naked eye, there needs to be a special tool to differentiate these plants. Therefore, this scoring method by digital microscopy, as opposed to traditional scoring (as shown in **Figure 7**), not only visualizes reduced nectaries but also prevents inaccurate scoring of heterozygous nectariless plants as homozygous nectariless plants.

The phenotypic scoring using digital images is dependent on several key factors, such as the specific time point of sample collection, selection of the sample, use of a standard scoring scale for leaf and bracteal nectaries, possible genotype, and how the scoring data can be interpreted for downstream applications. Firstly, it is important to collect leaves and flowers during the growing season when the nectary secretion is highest, generally at mid-flower stage in July. Secondly, the selection of leaves or flowers at the right size and stage plays a critical role in phenotype scoring. For leaves, top branches with 5 to 7 cm long leaf blades were preferred for leaf nectaries scoring. Similarly for bracteal nectaries scoring, healthy flowers from top branches were chosen. Sample selection at specific developmental stages will help, even when comparing all plants within a population by excluding developmental stage-dependent trait expressions (as shown in **Figure 8**). To check if the scoring is repeatable, digital images were generated for at least two samples per tissue per plant. Collecting multiple replicates of the same plant helps with consistent data collection.

The possible limitation of the method is maintaining pest-free plants until sample collection. Pest infestation areas can be identified by patches of tissues or eggs laid in the tissues or damage to the nectary regions with black areas with no visual appearance of nectary because of their consumption by aphids or other insects. This was observed during the screening of hundreds of samples. In such cases, healthy leaves and flowers were collected again and analyzed for consistent data from those samples. This can be resolved by maintaining regular pest

control schedules and supplementing fertilizer to achieve healthy plants. Following all the critical steps as described will help in troubleshooting for phenotype scoring.

This technique has several applications, such as mapping to identify DNA markers that help breeders identify genotypes for marker-assisted selection. This phenotype scoring requires confirmation from DNA markers because of the environmental and developmental stage influences, in addition to the genes that control this trait. Hence, phenotyping this trait using digital microscopy will be a starting point to narrow down a large number of plants from several populations to a small number of suspected nectariless lines. Using DNA markers, these lines are further validated for use in breeding programs to develop nectariless trait mediated disease resistance in newly developed varieties. This method can also help researchers understand the role of the nectary trait in plants and beneficial insect interactions.

Disclosures

Authors declare that there are no disclosures.

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