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ORIGINAL ARTICLE

Nymphstar: An accurate high-throughput quantitative method for whitefly (*Aleurotrachelus socialis* Bondar) resistance phenotyping in cassava

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Abstract

Whitefly (*Aleurotrachelus socialis* Bondar) is a major pest causing significant economic losses in cassava production systems in North South America. It diminishes cassava's photosynthesis by colonizing leaves, directly feeding on phloem sap, or excreting substances that foster sooty mold growth, reducing the photosynthetic area. The most effective pest management approach is deploying natural resistance in the crop. Identifying germplasm with superior whitefly-resistance (WFR) through phenotypic evaluation distinguishing it from whitefly-susceptible responses requires an accurate, high-throughput, quantitative phenotyping method. We developed Nymphstar, an image-based phenotyping tool, as an ImageJ plugin, quantifying third- and fourth-instar nymphs and their leaf area they occupy through red, green, and blue color space analysis. Using Nymphstar, we tested 19 cassava genotypes and classified their resistance to *A. socialis*. The plugin proved efficient, completing the analysis in 25.56 min on average for the entire dataset. In contrast, manual counting for the same set of images took 425.23 min on average averaging around 6.29 min/image. Nymphstar was ~17 times faster showcasing its efficiency. To assess WFR in cassava germplasm, we conducted a full-bench caging free-choice assay. This approach enhanced whitefly colonization on each cassava genotype, providing an accurate representation of resistance/susceptible while reducing operator bias. Nymphstar is a rapid, precise tool for automated nymphs counting and leaf area quantification. It facilitates the large-scale assessment of cassava resistance to whitefly, eliminating bias associated with field assessment and manual counting.

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1 | INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a basic staple food for over 800 million people (FAO, 2019). Known for its adaptability to adverse climatic conditions and harsh soil, cassava thrives where most other crops struggle (El-Sharkawy, 2004; Raphael, 2008). This resilience makes cassava a vital contributor to food security and a promising candidate for climate change adaptation in tropical areas (Burns et al., 2010; Jarvis et al., 2012).

However, cassava production faces significant challenges due to various pests and diseases, with cassava whiteflies (Hemiptera: Aleyrodidae) being particularly problematic across the Neotropics, Africa, and parts of Asia. These whiteflies not only act as vectors but also directly damage cassava by feeding on its phloem, causing chlorosis and premature leave drop (Herrera-Campo et al., 2011). Moreover, whitefly excretes sticky, sugary substances on foliage, fostering the growth of sooty mold fungi (Nelson, 2008).

The Americas exhibit the highest diversity of cassava whiteflies, with 11 reported species (Ovalle et al., 2014; Vásquez-Ordóñez et al., 2015). In South America, *Aleurotrachelus socialis* Bondar stands out as a major whitefly species that can cause significant yield losses when unchecked (Bellotti et al., 2012). Fortunately, resistance to *A. socialis* has been identified in several cassava genotypes (Bellotti & Arias, 2001; Parsa et al., 2015).

Resistance to insect attack in plants, stemming from a coevolutionary process, is vital for their survival (Berlinger, 2008). However, the introduction of novel un-adapted, exotic germplasm can compromise plant defense mechanisms. This can lead to genetic uniformity and increased vulnerability to new pests and diseases, as seen with cassava introduction to Africa over five centuries ago (Jones, 1957). In Africa, endemic organisms like *Bemisia tabaci* and the African cassava mosaic virus have adapted, posing new threats to cassava (Herren, 1994; Maruthi et al., 2019; Omongo et al., 2012). After years of research, scientists are concluding that increases in *B. tabaci* population abundance play a key role not only in the yield losses caused by physical damage but also in the incidence and spread of both Begomoviruses (cassava mosaic disease [CMD] and Ipomoviruses [cassava brown streak disease [CBSD]) in this region (Macfadyen et al., 2018; Milenovic et al., 2019).

Whitefly population control is essential for managing viral diseases and preventing yield losses. Achieving this involves monitoring cassava-growing regions to detect infestation patterns, enabling the deployment of integrated pest management measures. Additionally, the screening of novel germplasm can reveal new sources of whitefly resistance (WFR) for crop improvement. However, both of these processes often rely on manual counting which is labor-intensive, time-consuming, and prone to errors, making it unsuitable for large-scale field monitoring or breeding programs (Bellotti & Arias, 2001; Sseruwagi et al., 2004).

Recent advancements in digital and electronic imaging have introduced image analysis and machine learning processing methods for pest monitoring and phenotyping resistance to insects (Bereciartua-Pérez et al., 2022; Berger et al., 2012; Mundada & Gohokar, 2013). These technologies enable the development of early detection systems based on image or video analysis, effectively counting and identifying various pests (Bechar & Moisan, 2010; Bereciartua-Pérez et al., 2023; Cho et al., 2008; Mundada & Gohokar, 2013; Zayas et al., 1989) and estimating the damage (Bhadane et al., 2013). However, there is a need for image-based identification methods specific to whitefly nymphs, which offer a more precise assessment of the damage caused by whitefly activity on cassava plants (Baldin & Beneduzzi, 2010; Sulystio & Inayati, 2016).

Efforts have been made to develop image-based counting methods for whitefly nymph stages. This method leverages digital image processing and artificial intelligence techniques

Core Ideas

- Investigating whitefly resistance in Latin American cassava germplasm involves a comprehensive characterization searching into the genetic factors influencing resistance to better understand the plant's natural defenses against this pervasive pest.
- Employing high-throughput methods for assessing whitefly resistance responses allows for efficient and rapid data collection, enabling a more thorough analysis of cassava germplasm and its potential sources of the resistance traits.
- Streamlining the identification and selection of whitefly-resistant cassava varieties within breeding programs is crucial for developing resilient crops. By accelerating the breeding process, we can enhance food security and reduce the impact of whitefly infestations on cassava yields.
- Harnessing the power of machine learning in phenotyping plant-pest responses enhances the precision and speed of data analysis. Integrating advanced algorithms enables us to uncover complex patterns in cassava's interaction with whiteflies, providing a better understanding of cassava's defense mechanisms and facilitating more informed decisions in breeding programs and contributing to sustainable agriculture practices.

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to enhance the precision of nymph identification (Barbedo, 2014). For instance, a density map-based algorithm has been developed to count *B. tabaci* nymphs in leaf disks, providing a user-friendly tool for field technicians (Bereciartua-Perez et al., 2022, 2023).

In conclusion, cassava plays a pivotal role in tropical regions as a staple food, but it faces challenges from pests such as cassava whiteflies. To combat these threats, efficient and accurate monitoring methods are essential. Traditional manual counting methods are laborious and prone to errors, necessitating the adoption of digital imaging and machine learning technologies. These innovative approaches offer the potential to streamline monitoring efforts and enhance our ability to assess varietal resistance, ultimately contributing to cassava's resilience and food security in tropical regions.

2 | MATERIALS AND METHODS

2.1 | Mass rearing of *A. socialis* colony

The process for mass rearing of *A. socialis* comprises three phases. First, field production of standard cassava plant material (genotype COL1468) is carried out to obtain a regular supply of seed stakes for host plantlet propagation. Second, screen house host plantlet production (COL1468) is done for controlled glasshouse infestations. Finally, massive adult whitefly production was achieved through a mass-rearing approach using the permanent *A. socialis* colony glasshouse at the International Center for Tropical Agriculture (CIAT). The method described here facilitates the production of an average of 7600 whitefly adults per plant, by adapting previous cassava whitefly studies (Bellotti & Arias, 2001).

In the field production of seed stakes, 300 stakes of the genotype COL1468 were vertically planted every 3 months, with a separation of 1 m between plants and rows. The optimal length of each seed stake was ~20 cm or 5 axillary buds. The plants were fertilized 1 month after planting with N:P:K (15:15:15), and micronutrients were supplemented during the rest of the growing cycle as needed (such as iron and zinc). Pests and diseases were controlled with pesticides, but pesticide use was immediately interrupted 7 months after planting to avoid any effect on whitefly colony development.

In the growing of host plantlets in the screen house, we collected 100 seed stakes (aged 8–10 months) from the field on a weekly basis. The seed stakes were then planted in 2-L pots containing sterile substrate (1:3 sand to black soil; no clay topsoil) and maintained in a whitefly-free screen house for 6 weeks (Figure 1A). Fertilization was applied 15 days after planting with N:P: K (15:15:15) and watered when needed. Pests were manually controlled with continuous monitoring. We avoided using agrochemicals for mites, thrips, and other organisms at this stage, as traces of pesticides could significantly affect whitefly development and population reproduction fitness.

In the whitefly colony phase, the *A. socialis* colony was permanently maintained in a glasshouse with a daily average temperature of 27.5 ± 0.1°C and relative humidity of 66 ± 0.3% (mean ± SEM). The glasshouse was separated into two spaces: the infestation chamber and the development chamber. In the infestation chamber, two groups of plants were permanently kept: infested COL1468 plants with fourth-instar nymphs and COL1468 plants for oviposition. Two times each week, 30 six-week-old COL1468 host plantlets were moved from the screen house into the infestation chamber, where whiteflies were allowed to oviposit for 72–96 h (Figure 1B). Then, another group of 30 six-week-old COL1468 host plantlets was introduced to the infestation chamber, and the previously infested group of plants was shaken to remove the adults. The group of adult-free plants infested with eggs was then transferred to the development chamber (Figure 1C). Overall, this method allows the production of large quantities of *A. socialis* adults to ensure good infestation pressure in a controlled and efficient manner.

Once the *A. socialis* nymphs reach the fourth-instar stage in the development chamber, which occurs ~30 days after infestation (Figure 1D), we carefully spray water on each leaf of the plants. This helps to remove exuviae, honeydew, sooty mold, and most of the white wax that this species produces in their immature stages. This procedure does not disturb the nymph development cycle, and it allows us to inspect each leaf for opportunistic undesirable pests, which can then be manually removed just before the plants are placed into the infestation chamber where whitefly adults will emerge. As adult whiteflies prefer the youngest leaves, we cut the shoot apices of the plants with fourth-instar nymphs to encourage the oviposition on the new, uninfected plants (Figure 1E,F).

2.2 | Phenotypic assay to measure cassava defense responses against whitefly infestation

We developed an easy-to-use and robust assay for whitefly infestation to evaluate cassava's defense responses (resistance vs. susceptible). We designed a free-choice experiment that could be performed under practical glasshouse-based conditions. We refer to this experiment as a glasshouse WFR assay.

We evaluated two full-sib cassava families (240 and 198 individuals) that were segregated for whitefly resistance in eight infestation trials. We conducted 176 replications across four years (2013, 2016, 2017, and 2018). We included 19 cassava genotypes, of which 10 genotypes had a known resistance response to *A. socialis* infestation based on previous studies (Parsa et al., 2015). The other nine genotypes were selected

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FIGURE 1 Graphical representation of *A. socialis* mass rearing including the field production of COL1468 seed-stakes. (A) Genotype COL1468 stakes planted in 2-L pots containing sterile substrate, (B) COL1468 plants at the infestation chamber with whitefly adults, (C) infested COL1468 in the development chamber, (D) *A. socialis* life cycle on COL1468, (E) shoot apices cut to force adult whiteflies to oviposit on new un-infested plants to start a new infestation cycle, and (F) plants are introduced into the infestation chamber, where new-born adults emerge.

for traits of economic importance to the CIAT cassava program stakeholders, although their resistant response to high whitefly infestation levels was unknown (Table 1). We used the data gathered from these 19 *M. esculenta* to validate the effectiveness of the standardized WFR glasshouse assay using high levels of whitefly infestation (Supporting Information 1).

To produce clean cassava planting material, we re-propagated 8-week-old in vitro plantlets of the genotypes listed in Table 1 at CIAT's cassava program tissue culture lab. Once these materials showed four expanded leaves, we transferred them to a screen house for tissue hardening in soil, where they were transplanted into black plastic bags (10 cm W × 15 cm H) filled with sterile soil substrate (1:2 sand: black soil).

Approximately 2 months after soil transfer, we moved the cassava plantlets displaying at least new five fully expanded leaves to the phenotyping infestation glasshouse to conduct the WFR assay. We placed the plants on a table (18 m L × 3 m W), with each plant separated by 20 cm, on an experimental table with a total capacity of 100 plants (Figure 2B). For each replicate, we placed one COL1468 plantlet produced in the second phase of the mass-rearing process as an infestation control. We covered each table with a large white mesh tent (18 m L × 3 m W × 3 m H) to confine the whitefly adults after infestation (Figure 2E).

Before the whitefly infestation, we identified and marked the leaves preferred by *A. socialis* adults as Leaf-1 and Leaf-2. Leaf-1 corresponded to the youngest expanding leaf and Leaf-2 to the next fully expanded leaf (Figure 2C,D). We marked the stem with a permanent ink marker below Leaf-2 to monitor the position of this leaf at the time of the evaluation when whiteflies reached the fourth-instar stage. Six COL1468 plantlets that were kept in the infestation chamber of the whitefly colony glasshouse for 72–96 h (Figure 2A) are transferred using a cage to the phenotyping infestation glasshouse to avoid the scape of perched adults. Once there, these plants are introduced into the mesh tent and shaken above the experimental plants, releasing ~22,000 adults (Figure 2B).

Seven days after infestation, we moved the plants to another screen house to facilitate the development of immature whiteflies while also avoiding unwanted infestations by other pests. At 40 days post-infestation, when most nymphs had reached the fourth instar, we marked Leaf-2 on the upper side of the

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TABLE 1 List of the cassava genotype checks used in bioassays of resistance to whitefly *A. socialis* carried out during 2013–2018. The table includes information on the whitefly responses and other biotic stress responses, author references, and the type of variety and origin.

Genotype	Whitefly response and other biotic stress responses	Author reference	Type of variety, origin
COL1468	WFS is the host for *A. socialis* mass rearing	Bellotti and Arias (2001)	Landrace, Colombia
COL2182	WF response unknown, CBSD resistant	Sheat et al. (2019)	Landrace, Colombia
COL2246	WFS is parental of segregant family	Parsa et al. (2015)	Landrace, Colombia
ECU19	WF response unknown, CBSD resistant	Sheat et al. (2019)	Landrace, Ecuador
ECU41	WF response unknown, CBSD resistant	Sheat et al. (2019)	Landrace, Ecuador
ECU72	WFR is parental of segregant family	Bellotti and Arias (2001)	Landrace, Ecuador
ECU183	WFS	Parsa et al. (2015)	Landrace, Ecuador
PAR41	WF response unknown, CBSD resistant	Sheat et al. (2019)	Landrace, Paraguay
PER183	WFS	Parsa et al. (2015)	Landrace, Peru
PER226	WF response unknown, CBSD resistant	Sheat et al. (2019)	Landrace, Peru
PER317	WFR	Parsa et al. (2015)	Landrace, Peru
PER335	WFR	Parsa et al. (2015)	Landrace, Peru
PER368	WFR	Parsa et al. (2015)	Landrace, Peru
PER415	WFR	Parsa et al. (2015)	Landrace, Peru
PER556	WF response unknown, CBSD resistant	Sheat et al. (2019)	Landrace, Peru
PER597	WF response unknown, CBSD resistant	Sheat et al. (2019)	Landrace, Peru
PER608	WFR	Parsa et al. (2015)	Landrace, Peru
TMS60444	WFS, parental of segregant family	Irigoyen et al. (2020)	African improved variety
TME3	WF response unknown, CMD resistant	Akano et al. (2002)	African improved variety

Abbreviations: CBSD, cassava brown streak disease; CMD, cassava mosaic disease; WFR, resistant to whitefly; WFS, susceptible to whitefly.

petiole with a permanent ink marker for easy recognition during image capturing. We water-sprayed Leaf-1 and Leaf-2 according to the whitefly colony methodology (Figure 2F). We collected clean infested leaves, placed them outspread between two reusable paper towels, and stored them in a plastic box containing all these paper towels on top of each other at 4°C until the image could be captured. This method allowed us to store the leaves for several weeks until the image was captured.

2.3 | Image acquisition to develop the Nymphstar plugin

We developed a new tool called Nymphstar, which uses image-based nymph counts to assess whitefly infestation levels in planta. To ensure high-quality images for accurate data analysis, we pre-treated the leaves with 50% ethanol to remove any unwanted residues such as white wax and honeydew that could introduce noise into the analysis. This process increases the contrast between the black color of the third- and fourth-instar nymphs of *A. socialis*, and the green color of the leaves (Figure 3 (1A, 1B, and 1C)).

To capture the images, we placed each leaf into the ORTech Photo-e-Box Bio using a piece of black fabric as a background to increase the contrast and ensure even lighting of the leaf. We used a Nikon D300s with an AF-S DX Micro-NIKKOR 40 mm f/2.8G lens fixed onto a Copy-Stand (Kaiser Reproduction Stand RS1/RA1 5510) at 70 cm from the black background (Figure 3 (3)). For large leaves that exceeded the visual field, we divided them into two or three pieces [Figure 3 (2)]. We used the Nikon Control Pro 2 software to standardize the image-capture settings in the red, green, and blue (RGB) color model with a resolution of 4228 × 2848 pixels and stored them in JPG format.

With the Nymphstar tool, we can reduce labor and accelerate the data acquisition necessary for whitefly infestation assessments in cassava plants. By pre-treating the leaves

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FIGURE 2 Graphical representation of phenotyping glasshouse-based assay. The COL1468 plants, which serve as the primary material for rearing large numbers of whiteflies, are transferred from the mass-rearing glasshouse into the infestation glasshouse prior to the emergence of adult whiteflies. Once the adults emerge, the COL1468 plants are gently shaken above the experimental plants to encourage the adults to select a new host within the experimental lines. (A and B) The stem marked with a permanent ink marker under the oldest leaf (Leaf-2) next to the youngest one with at least one lobe completely opened (Leaf-1) (C and D), the experimental cassava plants covered with a large white mesh tent (E), and harvesting at 38–40 days post-infestation of Leaf-1 and Leaf-2, marked on the day of infestation, of all the genotypes under study (F).

and optimizing the image-capture process, we can obtain high-quality images for accurate nymph count analysis.

2.4 | Nymphstar: Image analysis for nymphs counting and nymphs density estimation

We developed a Java-based image analysis application, Nymphstar, which functions as a plugin for the ImageJ software (National Institute of Health, USA). Nymphstar was created to analyze leaf images and count whitefly nymphs, as well as estimate nymphs density. The development of Nymphstar involved three main steps: (1) pre-processing, which includes performing operations on the images to suppress undesired objects that distort the nymphs detection; (2) processing, which involves the application of different methods to extract the desired information from the image; and (3) post-processing, which involves analyzing the nymphs data extracted and interpreting the results to obtain the total number and density of nymphs. The overall image processing flow is depicted in Figure 4, and further details about the process can be found in Section 3.

2.5 | Accuracy and efficiency evaluation of Nymphstar image analysis application

We evaluated the accuracy and efficiency of the Nymphstar image analysis application by comparing its performance with the manual counting of ground-truth images. We randomly selected 2% of the total images obtained from the 19 *M. esculenta* checks and classified them into one of three infestation levels adapted from the population scale of six levels proposed by previous studies (Bellotti & Arias, 2001).

Three evaluators counted the number of nymphs including an expert entomologist, a person with an intermediate level of experience in nymphs counting, and a beginner who performed manual counting using the selected images taken with the protocol of image acquisition. For manual counting, the time was recorded with a digital stopwatch, while for digital counting, running Nymphstar on a computer with an Intel Core I7-7500U processor with a speed of 2.7 GHz and 16 GB of RAM, we estimated the time extracting the creation

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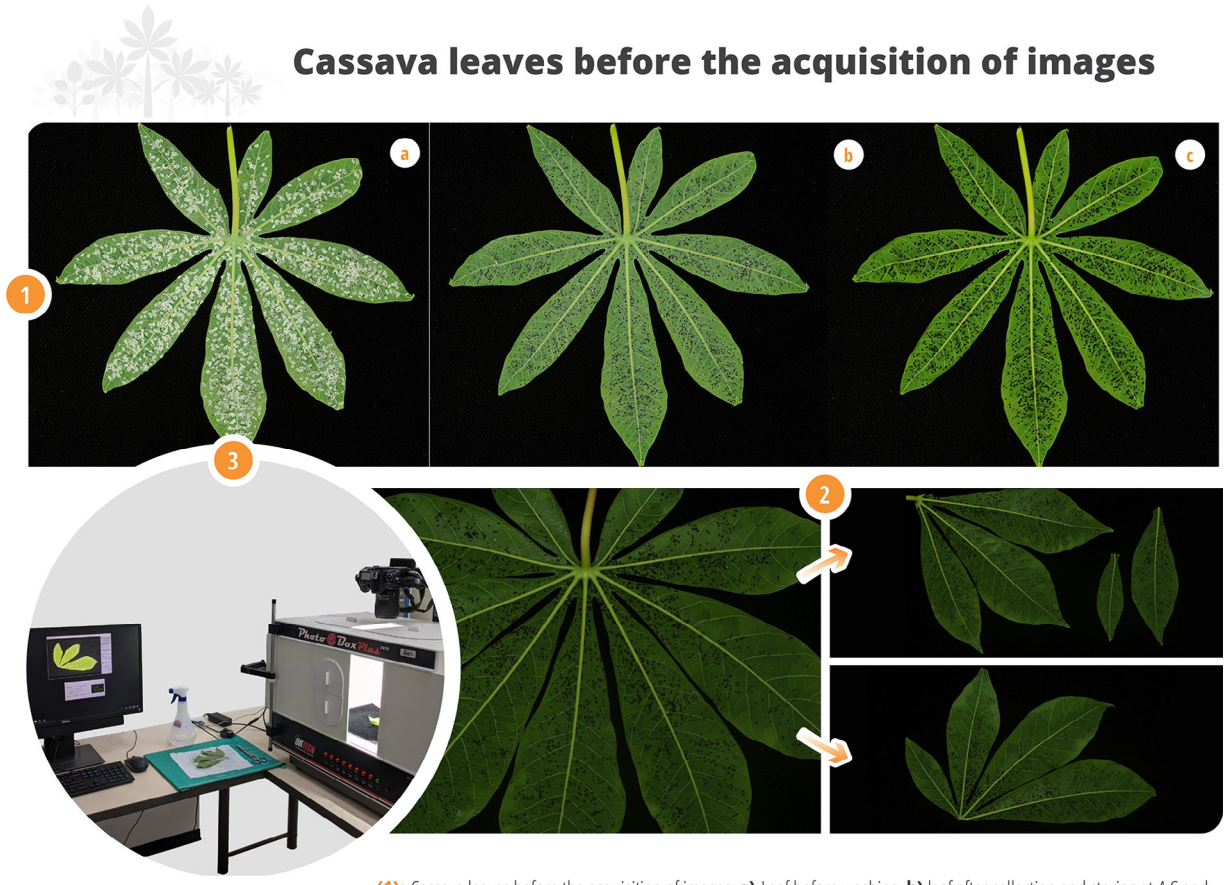

FIGURE 3 Illustration of the steps taken for leaf image capturing: (1) Cassava leaf before image acquisition: (A) third- and fourth-instar whitefly nymphs covered with white wax before washing and collection of the leaf, (B) leaf completely dry after the wax and empty pupal cases (EPC) removing by water spraying and collection between reusable paper towels, and (C) leaf after being moistened with 50% ethanol to increase the contrast between the leaf and nymphs before the image capturing. (2) Some leaves are larger and do not fit into the visual range. In these cases, the lobes of the leaf are separated, and two or three pictures are taken. (3) The system used for image acquisition, including the ORTech Photo-e-Box Bio, a black fabric background to increase contrast and even lighting, a Nikon D300s camera with an AF-S DX Micro-NIKKOR 40 mm f/2.8G lens fixed onto a Copy-Stand at 70 cm from the background, and the Nikon Control Pro 2 software was used to standardize image-capture settings.

time recorded in the file properties (hh:mm:ss) of each post-processing image. Finally, we contrasted the original images with the output images produced by Nymphstar to verify the segmentation between the background, leaf, and nymphs.

2.6 | Statistical analysis

To perform statistical analysis, we used the SAS software 9.3 for Linux with the PROC GLM procedure. We estimated the effect of whitefly (*A. socialis*) infestation on 19 cassava clones (Table 1) by averaging the number of nymphs found in leaves 1 and 2 per plant, obtained from Nymphstar, across the experiments performed in 2013, 2016, 2017, and 2018 for mean nymphs number and in 2017 and 2018 for the nymphs density. Our preliminary descriptive analysis of the data showed that the distribution of the nymphs number variable corresponded to a negative binomial distribution. Therefore, we used a generalized linear model for this type of distribution before establishing differences between means of genotypes using independent-sample least significant difference (LSD) *t*-test. We considered $p < 0.0001$ as significant in detecting statistical differences. We used the same model and test of comparison of means to evaluate the insects density, but the model was adjusted to a binomial distribution.

For the accuracy test of Nymphstar, we used the concordance correlation method to evaluate the agreement between manual counting and Nymphstar plugin counting. We used the epiR R package to calculate the concordance correlation coefficient and the respective confidence interval at 95%. We determine the bias for each pair of comparisons by computing the average difference of both measurements. We produced correlation and Bland–Altman plots using the ggplot2 R package.

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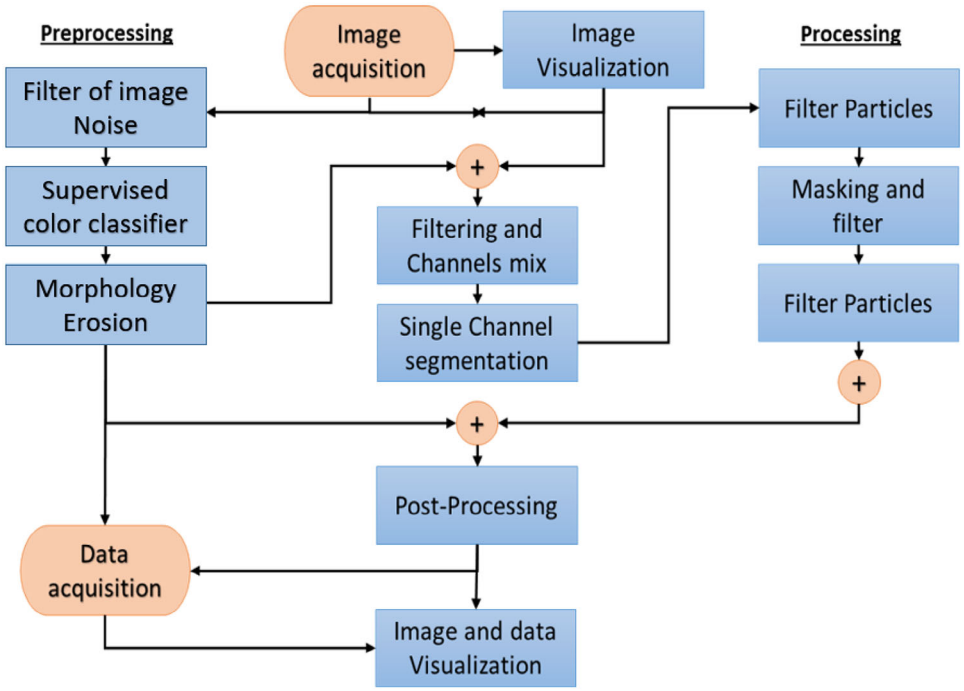

FIGURE 4 Flow chart showing the Nymphstar image processing steps from image acquisition to data acquisition.

3 | RESULTS

3.1 | Nymphstar: Image analysis for nymph counting and density estimation

3.1.1 | Pre-processing and background removal

To extract nymphs count data from the leaf images, we separated the leaf information from the image background by using Bayesian learning, a machine learning technique that leverages color as the training feature. We first applied a Gaussian blur filter to the original image (Figure 5A) to reduce details and noise, including that of the nymphs (Figure 5B). We then used the trained Bayesian learning method to segment the image into two categories: (i) background (black = zero value pixels) and (ii) leaf (white = 255 value pixels). We removed all particles in the background (Figures 5C,D) and calculated the total leaf area to estimate the nymphs density. We then used the resulting image (Figure 5D) as a mask for the original image (Figure 5A) to produce a new RGB image for further processing (Figure 5E).

3.1.2 | Processing: Image segmentation and object detection

Given the green channel contained more information on the leaf, while the blue channel contained more information on the nymphs, we decomposed the image into its red, green, and blue (RGB) channels and subtracted the blue channel from the green channel, which highlighted the black regions corresponding to the nymphs, and facilitated segmentation (Figure 5E). To correct for smoothness and loss of edges, we applied the "Unsharp Mask" filter (Figure 5F). We then filtered the pixels by color using a low-pass filter set at that zero value of the nymphs color. Pixels with intensity levels higher than zero value (grays and whites) were set to zero value and eliminated, while those with the same zero value as the nymphs were set to 255 (white) and considered information. This process produced a binary image with white-colored nymphs on a black background (Figure 5G).

To remove any remaining undesirable object in the binary image, we used the ImageJ plugin "Analyze Particles" with a minimum and maximum pixel size of 20 and 700, respectively, and a circularity range between 0 and 1. We then combined the original image with the binary image with an "AND" logical operator but observed that some nymphs crowded together were considered one, leading to data loss. To account for each individual nymph within the cluster, we used the ImageJ "Watershed Segmentation" plugin. We again used the "Analyze Particles" plugin to filter the remaining undesired objects based on shape and size, setting the new range to a minimum of 30 and infinite for the maximum.

3.1.3 | Post-processing and data analysis

Once each image had been segmented and all informative objects had been detected, we quantified the nymphs by applying the Euler number from the MorphoLibJ package

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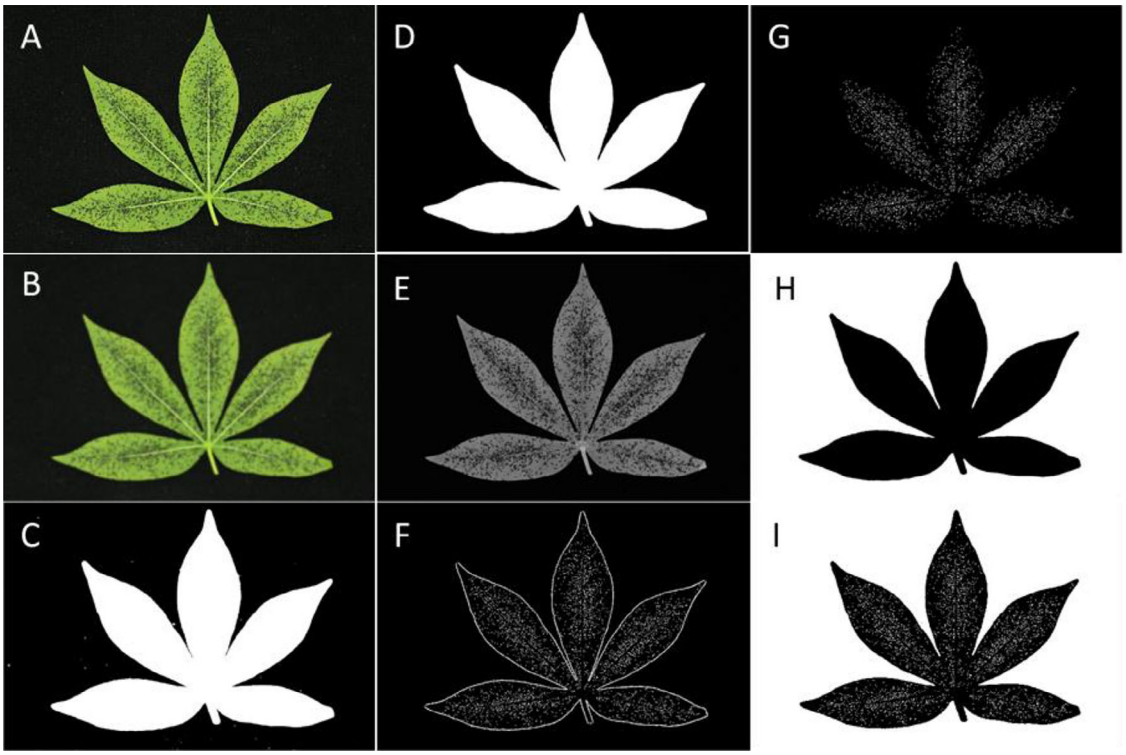

FIGURE 5 Steps of the image analysis process on a cassava leaf. (A) Original image; (B) Gaussian blur filter; (C) image segmented by the supervised classifier; (D) mask for subtracting the background from the figure (A); (E) red, green, and blue (RGB) image for decomposition on channels (channel green–channel blue image); (F) filtered image with "Unsharp Mask" for edge sharpening; (G) filtered pixels by color, detection of objects touching each other, filtered particles by shape and size, to obtain a binary image with nymphs colored in black; (H) Figure 5G is combined with Figure 5C inverted; (I) final JPG image.

of ImageJ. This algorithm quantifies the number of objects by computing the Euler number measurement (E), which is the result of the number of white particles (N) minus the number of holes in those objects (H), $E = N - H$. Using eight-connectivity among pixels, it computes the Euler number measurement. Since nymphs do not have holes inside them, the result was the number of nymphs on the leaf. To estimate the nymphs density, we used the image histogram to extract the number of white pixels. For a better visual appreciation of the results, we combined the processed image (Figure 5G) with the segmented image of the leaf generated in the pre-processing step with colors inverted (Figure 5H). Hence, the full data acquisition package (Nymphstar) provides a processed grayscale JPG image that includes the total nymphs number estimation, and the leaf area and nymphs density (percentage) (Figure 5I).

Our newly developed ImageJ plugin, Nymphstar, can be used to acquire single image data, as well as perform batch analysis on a group of images. For a group of images, traits are analyzed and exported to a CSV file, and processed images are stored in a target folder chosen by the user (see Supporting Information 2, Batch-processing section). For a single image, data processing results are immediately displayed in a log window; in both cases, the resulting image(s) is saved in a JPG format.

3.1.4 | Nymphstar's accuracy and efficiency test

To test the accuracy of the Nymphstar application, we contrasted the manual nymph counts from ground-truth images with the results given by Nymphstar (Table 2). We randomly selected 57 images with a resolution of 12 MPX (4228 by 2848 pixels; Lavalle et al., 2023) from the set of 3871 images obtained from the 1464 plants evaluated (two leaves from each plant and, depending on size, one or several pictures from each leaf), with a range of infestation between 57 and 4107 nymphs per leaf (Figure 6A and Supporting Information 3).

We performed Lin's concordance index for the accuracy test (see statistical results in Supporting Information 4), obtaining the result $r = 0.98$ between the number of nymphs counted by the Nymphstar plugin and the manual count of nymphs. We also used a Bland and Altman plot to compare two measurement techniques based on the differences between their measurements, with differences plotted to observe the dispersion. Ideally, the difference would be zero, and all points should lie on the horizontal line $y = 0$ (dotted line). The solid black line represents the average of the differences obtained, which should be a horizontal line at $y = 0$ in an ideal situation, and if the differences are not zero, this value would represent the bias.

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TABLE 2 Comparison of the average number of nymphs and time spent obtained with manual counting and using the Nymphstar plugin.

		Manual		Nymphstar plugin	
Infestation level	**No. of images**	**No. of nymphs (Mean)**	**Time mean (s)**	**No. of nymphs (mean)**	**Time mean (s)**
Low (0–200)	16	120.6	64.3	136.1	19
		(22–199)	(23.8–93.3)	(57–206)	(18–22)
Medium (201–2000)	20	955.5	339.76	959.7	19.5
		(201–2021)	(97.5–902)	(237–1670)	(18–24)
High (>2001)	21	3044.6	651.87	2648.04	19.33
		(1702–4941)	(196–1929.7)	(1954–4107)	(18–20)

Note: For all variables, we show the range in parentheses. We adapted the infestation levels based on the population scale described before (Bellotti & Arias, 2001). This scale has values from 1 to 6; therefore, the low level would be equivalent to 1 (no whitefly stages present) and 2 (1–200 individuals per leaf). The medium level would be equivalent to 3 (201–500 individuals per leaf) and 4 (501–2000 individuals per leaf). The high level would be equivalent to 5 (2001–4000 individuals per leaf) and 6 (>4000 individuals per leaf).

Here, the difference is calculated based on Nymphstar, so a positive bias would indicate that the Nymphstar count yielded higher values on average than the counts made by the person. A negative bias would indicate that the person counted more nymphs than Nymphstar (Figure 7). Panel B shows a Bland–Altman plot, which displays the difference between manual counts and Nymphstar counts on the *y*-axis and the mean of the two methods on the *x*-axis. The plot allows us to observe the dispersion of the data when comparing manual counting versus counting using Nymphstar. In this case, we can see that most of the data points lie within the limits of agreement, which are represented by the dotted lines. These lines show that 95% of the data fall within ±1.96 standard deviations of the mean difference. The plot indicates that Nymphstar counts tend to be slightly higher than manual counts, but the difference is small and does not affect the accuracy of the results, indicating that Nymphstar is a reliable tool for counting nymphs in this context.

Analysis efficiency: The ImageJ plugin Nymphstar enables us to analyze the 57 images for nymphs quantification with a total time of 1534 s (25.56 minutes) at an average of 19.175 s/image (Table 2). In contrast, the manual counting of the same images took 25,513.7 s (425.23 min) with an average of 6.29 min/image.

3.1.5 | Glasshouse-based whitefly-resistance bioassay

We conducted the whitefly-resistance (WFR) bioassay to assess the relative resistance levels of 19 cassava genotypes to whitefly infestation under glasshouse conditions (Table 1).

Cassava genotypes were tested for whitefly infestation, as measured by the mean number of nymphs found per leaf (F [18, 3451] = 22.32, $p < 0.0001$) (Table 3, Supporting Information 5). The most susceptible genotype was PER556, which had a previously unknown response to *A. socialis* and had significantly more nymphs per leaf than all other genotypes (1634.8 nymphs per leaf). The next most susceptible genotypes were TMS60444, PAR41, ECU183, PER226, and PER415, which had between 1150 and 1300 nymphs per leaf.

The intermediate group consisted of genotypes with a mean number of nymphs per leaf between 913 and 1100, including COL2246, ECU19, COL1468, PER597, TME3, COL2182, PER335, ECU41, and PER183. We categorized this group as "Intermediate" because they fell between two statistically different genotypes PER451 (group "BC") and PER608 (group "DEF").

The resistant genotypes PER608, PER317, and PER368 did not differ significantly from each other, with nymph counts ranging from 635.1 to 841.4. Notably, ECU72, a genotype categorized in previous studies as resistant to *A. socialis*, showed exceptional resistance to whitefly attack (521.3 nymphs per leaf) with a nymphs population lower than all the other genotypes except PER317.

4 | DISCUSSION

4.1 | Automated identification and counts of pests in agriculture

The development of automated methods for identifying and counting pests in agriculture has become increasingly important in recent years because of the advancement of image digitization and data processing automation (Maharlooei et al., 2017; Wang et al., 2018). The focus of most studies in this has been on the development of software or algorithms for the identification of different pests (Barbedo, 2014; Deng et al., 2018; Espinoza et al., 2016; Maharlooei et al., 2017; Solis-Sanchez et al., 2009; Xia et al., 2015) with particular emphasis on adult insects capture in most cases on sticky traps (Deng et al., 2018; Sun et al., 2017; Wang et al., 2018). However, there have been very few attempts to identify insects at early stages of development such as eggs or nymphs (Barbedo, 2014; Bereciartua-Pérez et al., 2023; Bhadane et al., 2013; Chen et al., 2018).

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TABLE 3 Mean ± standard error of the total number of nymphs per leaf in checks analyzed for 4 years (2013, 2016, 2017, and 2018) in eight different experiments (number of plants per experiment shown in Supporting Information 1).

Clone	Mean ± SE (nymphs per leaf)	No. of plants	Whitefly responses reported in previous studies	Whitefly response using Nymphstar
PER556	1634.8 ± 213.5 A	4	Unknown	High WFS
TMS60444	1277.6 ± 38 B	134	WFS	WFS
PAR41	1163.7 ± 130.7 BC	20	Unknown	WFS
ECU183	1122.8 ± 110.4 BC	30	WFS	WFS
PER226	1156.8 ± 84.3 BC	20	Unknown	WFS
PER415	1154.9 ± 80.5 BC	36	WFR	WFS
COL2246	1102.4 ± 41.6 BCD	183	WFS	Intermediate
ECU19	1060.7 ± 127.2 BCDE	20	Unknown	Intermediate
COL1468	1041.1 ± 113 BCDE	185	Unknown	Intermediate
PER597	1046.1 ± 38.5 BCDE	18	Unknown	Intermediate
TME3	1037.9 ± 58.7 BCDE	81	WFS	Intermediate
COL2182	991.1 ± 105.2 CDE	19	Unknown	Intermediate
PER335	946 ± 75.7 CDE	61	WFR	Intermediate
ECU41	931.5 ± 104.2 CDE	20	Unknown	Intermediate
PER183	913.2 ± 65 CDEF	44	WFS	Intermediate
PER608	841.4 ± 46.1 DEF	156	WFR	WFR
PER317	800.6 ± 51.5 EF	89	WFR	WFR
PER368	635.1 ± 62.4 FG	60	WFR	WFR
ECU72	521.3 ± 18.3 G	284	WFR	High WFR

Note: We made these measurements using WFR bioassays. For each clone, the means within a column followed by the same letter are not significantly different (independent-samples least square difference t-tests, $DF = 3433$, $p < 0.0001$).
Abbreviations: WFR, resistant to whitefly; WFS, susceptible to whitefly.

To the best of our knowledge, three studies developed methodologies to count whitefly *B. tabaci* nymphs. The first study developed an algorithm to count whitefly third- and fourth-instar nymphs (Barbedo, 2014), and the other two studies used deep learning-based object detection and density maps for all nymph stages (Bereciartua-Pérez et al., 2023; de Castro et al., 2022).

Developing a precise, quick phenotyping method based on digitized image analysis for *A. socialis* whiteflies is important not just because the early identification of plants carrying resistance to whitefly attack can prevent these species from becoming superabundant and widespread reducing the risk of yield loss or the spread of other viral diseases, but also because *A. socialis* can be a model insect to test plant resistance in Latin America where *B. tabaci* sub-Saharan cryptic species are not present. We developed a tool called Nymphstar, which is a plugin for the open-access software ImageJ, for the identification and quantification of *A. socialis* third- and fourth-instar nymphs and the estimation of the leaf area occupied by nymphs.

We have tested Nymphstar across eight independent glasshouse-based WFR phenotyping trials, analyzing 19 *M. esculenta* checks with their replicas, for a total of 1937 plants, which corresponded to 3874 leaves bearing 775,050 nymphs. The results have been extremely promising with Nymphstar vastly improving data acquisition time. We found that while counting the nymphs manually on these ground-truth images could take an average of 39,471 min (82 standard 8-h labor days), analysis of all images using the automatic batch image analysis of Nymphstar would be completed in 20.63 h, turning a multi-day task into one accomplished in just hours. Nymphstar conclusively proved to reduce the burden of routine *A. socialis* monitoring offering to be a powerful tool for agricultural scientists and extensionists controlling whitefly outbreaks. Moreover, a practical application of this tool is in the field of breeding offering to potentially reduce the WFR selection time in the absence of advanced molecular markers tools.

Nymphstar not only offers a solution to the limitations of the manual assessment of whitefly populations in the field and the glasshouse but also presents the opportunity to undertake large epidemiological surveys or to innovate in the phenotypic characterization of WFR. Although this tool was tested on cassava plants, it could be used for other plant types and other whitefly species, with some modifications in the processing of the images.

Our findings demonstrate the potential of such tools to significantly reduce the burden of routine pest monitoring faced

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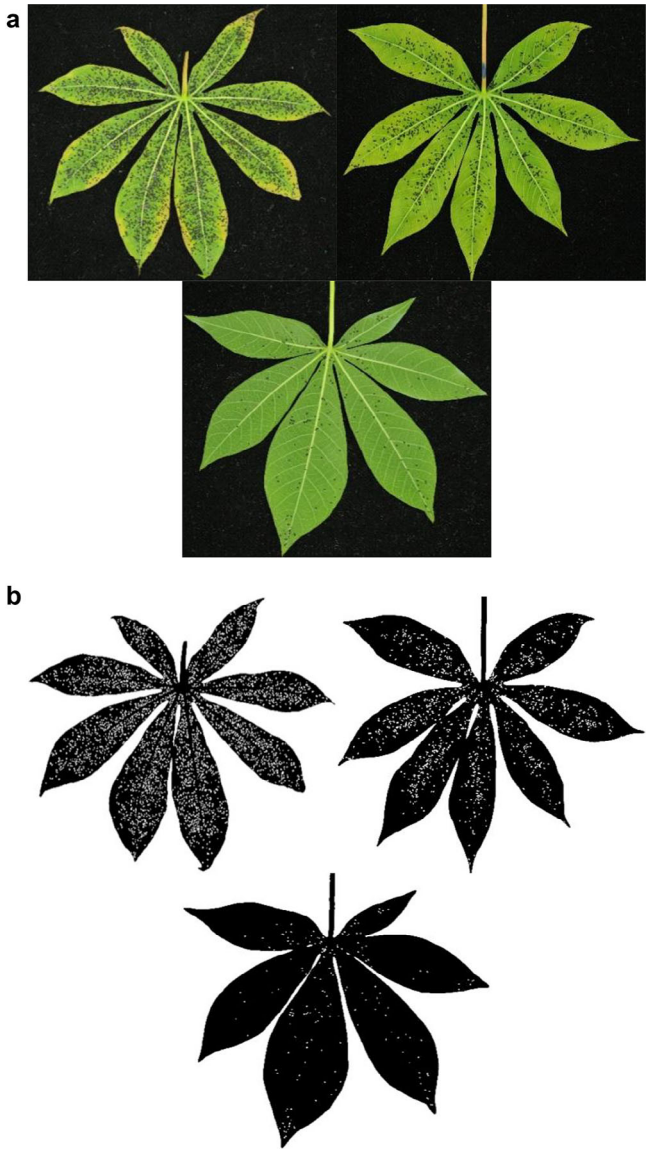

FIGURE 6 (A) Cassava leaves with different levels of infestation of *A. socialis* nymphs high (left), medium (center), and low (right), with populations between $n = 22$ and $n = 4941$ nymphs per leaf. (B) Images of the same leaves were obtained after the processing by the plugin Nymphstar.

by agricultural scientists and extensionists and to improve the accuracy and speed of phenotyping efforts. Further research and development in this area could have important implications for global food security and sustainable agriculture.

4.2 | Image analysis

To develop the Nymphstar plugin of ImageJ for counting *A. socialis* nymphs, we used some techniques to improve its efficiency and accuracy. To identify the pixels corresponding to the leaf and background, we used the naive Bayes approach. This technique is a probabilistic classifier based on the Bayes theorem, which computes the class of each observation using a likelihood-trained model (Hsu et al., 2017). Here, we trained the program with different color samples of leaves and backgrounds to obtain a binary image output where the image was labeled as (i) background (black = zero value pixels) and (ii) leaf (white = 255 value pixels). In this way, we were able to separate the leaf from the background and measure the area corresponding to the leaf.

Image reproducibility is key to the accuracy and efficiency of data acquisition performance (Espinoza et al., 2016). The PhotoBox allows for the use of the same light levels for each image to avoid changes in the characteristics of the image in subsequent analysis. The control of the light conditions and camera height improves image acquisition, avoiding the presence of shadows that could generate unhelpful data and maintaining the same height to compare leaf areas between samples. Nymphs count and measuring of the leaf area are usually performed manually or semi-automatically. The high-throughput method described here is an automated image-based phenotyping system, which can be easily adapted to other whiteflies and plants.

4.3 | Measuring resistance to whitefly in cassava: Phenotyping assay

The identification of effective sources of resistance is central to the development of a whitefly-resistant variety. A natural infestation (choice bioassay) of insect pests in cassava has been an effective but time-consuming process developed over nearly a quarter of a century (Bellotti & Arias, 2001; Parsa et al., 2015). In these choice bioassays, both antibiosis and antixenosis are plant response strategies deployed during host infestation. However, natural infestations are not stable, whitefly populations fluctuate with weather changes, competition, natural enemies presence, and other causes. To control the infestation level and measure the resistance of plants, we proposed a glasshouse-based WFR bioassay (Figure 2).

Our phenotyping methodology allows screening for WFR of many cassava genotypes and plants per trial. We have applied this method to plants propagated in pots or bags and on different substrates, from in vitro, micro, or regular-size stakes (data not shown). With this glasshouse-based methodology, it is possible to have results 3 months after planting, leading to the classification of plants in various resistant/susceptible categories without infestation from other organisms or the effects of changing weather in field experiments.

We were able to differentiate and identify with statistically significant power the WFR (WFR vs. WFS) of each of the cassava genotypes evaluated. Our results are consistent with those based on measurements using damage and

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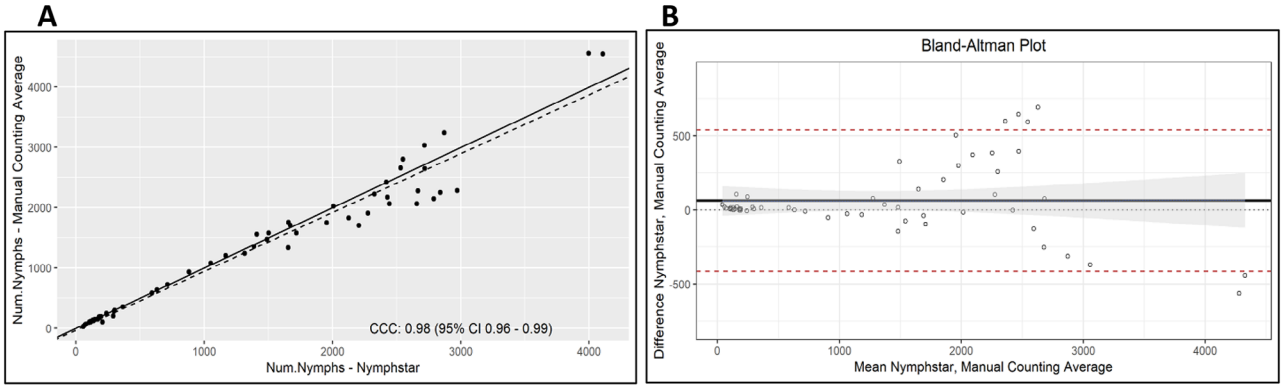

FIGURE 7 Accuracy of Nymphstar in counting the number of nymphs compared to manual observation is shown. Lin's concordance index results on manual nymph count by visual observation (A) and nymph counts obtained from red, green, and blue (RGB) images using the Nymphstar tool (B). The index ranges from 0 to 1, with 1 indicating perfect agreement. The high value of 0.96 indicates that Nymphstar provides accurate counts of the number of nymphs. Plane B shows the Bland–Altman plot where 95% of the data fall within ±1.96 standard deviations of the mean difference. CCC, concordance correlation coefficient; CI, confidence interval.

population scales in previous studies (Bellotti & Arias, 2001; Parsa et al., 2015) in which ECU72 showed high levels of resistance to *A. socialis*. Interestingly, this genotype also has been reported as resistant to *B. tabaci* Sub-Saharan cryptic species in Africa (Omongo et al., 2012) and *Bemisia tuberculata* in Brazil (Barilli et al., 2019), which means that *A. socialis* potentially can be a good model for test cassava resistance to whiteflies. In contrast, with our methodology, we observed that genotypes PER368, PER317, and PER608 previously characterized as highly resistant (Bellotti & Arias, 2001) did not display the high levels of resistance observed in ECU72. Other important genotypes in cassava for breeding CBSD in Africa (Sheat et al., 2019), such as ECU19, ECU41, COL2182, and TME3, have shown intermediate resistance to whitefly as well. Additionally, TME3 is an African genotype that is resistant to the whiteflyborne viruses of the genus *Begomovirus* (family Geminiviridae) that causes CMD (Colvin et al., 2004). Using Nymphstar, we were able to identify the susceptibility of a wide variety of important cassava genotypes used for different purposes over several years, which included PER556, TMS60444, PAR41, PER226, PER415, and ECU183. For instance, TMS60444 was used as a model plant for cassava genetic transformation and served as a male-susceptible parent in establishing a WFR mapping population (Becerra Lopez-Lavalle, personal communication, 2010). The glasshouse-based whitefly bioassay revealed that genotypes previously considered susceptible were intermediate into the group of evaluated genotypes. For cassava breeding programs, precise and reliable phenotyping for WFR is extremely useful; additionally, the evaluation of large collections of plants in a short time makes the analysis more reliable, delivering quantitative measures such as nymph counts and leaf area occupied by nymphs. These highly accurate quantitative measurements of WFR are ideally suited to genomic and genetic studies searching for resistance genes, using QTLs or GWAS analysis (Kayondo et al., 2018; Nzuki et al., 2017). The automated methodology of Nymphstar eliminates errors and bias that can be caused by manual counting, making it a more reliable tool for quantifying the number and area of *A. socialis* nymphs on cassava. Previous QTL analyses for whitefly resistance relied on field testing using scales of damage and population of whiteflies as phenotypic data. However, these scales did not provide the best measure for quantitative analysis. They have allowed for the characterization of cassava germplasm into resistant and susceptible categories, which were validated by later studies (Parsa et al., 2015). In contrast, the quantitative and continuous data provided by Nymphstar offer a better resolution and can account for the variability of WFR across this segregating population.

Nymphstar offers several advantages over traditional methods of whitefly evaluation. It is quicker, less laborious, and allows for the visual control of the outcome. Moreover, it can provide additional parameters, such as the leaf area occupied by nymphs, which can be useful in determining the level of whitefly infestation.

The use of Nymphstar is not limited to working hours, as it can be used in batch mode without human supervision. This feature makes it a valuable tool for the fast and accurate screening of multiple breeding populations to select superior genotypes with decreased whitefly infestation. By reducing the population size of whiteflies in cassava cropping systems, Nymphstar can help in minimizing the potential for insect-transmitted diseases.

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4.4 | Future field screening: Opportunities and challenges

Nymphstar, an efficient tool for glasshouse-based whitefly resistance (WFR) phenotyping, holds immense promise for future field applications in agriculture. Transitioning from controlled environments to open fields presents both opportunities and challenges.

Incorporating Nymphstar into field screening workflows demands robust field deployment strategies. The tool needs to withstand unpredictable environmental conditions, including variable lighting, weather, and field-specific challenges such as pest pressure.

Furthermore, field screening encompasses a vast diversity of pest species, host plants, and environmental conditions. Adapting Nymphstar to identify and count various whitefly species while accommodating the variability in host plant characteristics is a formidable challenge. It necessitates continuous research and algorithm refinement to ensure its versatility and effectiveness across different crops and regions.

To unleash Nymphstar's full potential in field screening, addressing challenges related to data management, scalability, integration with complementary technologies, user training, and accessibility is imperative. Implementing robust data handling solutions, ensuring scalability for large-scale agriculture, and integrating Nymphstar with other sensor technologies for comprehensive crop assessment are essential steps. Effective training programs and user-friendly interfaces will empower farmers, extensionists, and researchers to harness Nymphstar's capabilities, making it a valuable asset in addressing pest-related challenges in the field. Ultimately, overcoming these challenges will pave the way for Nymphstar to revolutionize pest management and crop breeding, contributing to global food security and sustainable agriculture practices.

5 | CONCLUSIONS

Nymphstar is a high-throughput image analysis-based tool that has been proven highly efficient in obtaining quantitative measurements such as the number of third- and fourth-instar nymphs and the leaf area they occupy. It is used with glasshouse-based WFR bioassay for the fast and accurate screening of multiple breeding populations to select superior genotypes for decreasing the relative population size of whiteflies in cassava cropping systems, thus reducing the potential for insect-transmitted viruses (CMD and/or CBSD) to mutate into more virulent forms.

Overall, Nymphstar represents a significant advancement in the field of cassava breeding and offers an efficient and effective way to evaluate whitefly resistance, which has important implications for improving the productivity and sustainability of cassava production in many parts of the world.

AUTHOR CONTRIBUTIONS

Adriana Bohorquez-Chaux: Conceptualization; data curation; formal analysis; investigation; methodology; supervision; writing—original draft. **Maria Isabel Gomez-Jimenez**: Data curation; investigation; validation; writing—original draft. **Luisa Fernanda Leiva-Sandoval**: Methodology; software; validation; visualization; writing—original draft. **Luis Augusto Becerra Lopez-Lavalle**: Conceptualization; funding acquisition; methodology; project administration; resources; supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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