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TECHNICAL NOTE

An objective high-throughput screening method for thrips damage quantitation using Ilastik and ImageJ

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INTRODUCTION

Quantifying insect damage is essential for identifying resistance mechanisms in plants. For chewing insects such as caterpillars and beetles, feeding damage is commonly determined by measuring the amount of removed leaf area, using freely available software such as ImageJ Fiji (https://fiji.sc) (Schindelin et al., 2012; Neves et al., 2014; Nguyen et al., 2016) or commercial programs such as Winfolia (http://regent.qc.ca/assets/winfolia_about.html) (Joshi & Tielbörger, 2012). However, cell-sucking insects such as thrips, do not remove sections of the leaf lamina, but rather cause localized discoloration of the leaf surface. It is very challenging to quantify this type of damage in an objective manner using these software programs.

Thrips are widespread sucking-piercing insects that are responsible for severe yield reduction in several vegetable crops such as cucumber, strawberry, melon, and pepper. Crops infested with thrips show stunted growth, deformation of the plant, and scarring of the fruits, resulting in reduced yield and marketing quality (Welter et al., 1990; Tommasini & Maini, 1995; Shipp et al., 1998). In addition, thrips are important vectors of plant viruses, especially tospoviruses (Whitfield et al., 2005; Rotenberg et al., 2015). In screening programs for host-plant resistance to thrips, the total damaged leaf area is used as a criterion to determine resistance levels. Damage is often characterized by silvery spots that show a high contrast with the intact leaf area, though thrips feeding can also include darker areas ranging from dark green to brown. These more gradual discolorations of the leaf are too subtle to precisely quantify with ImageJ or Winfolia alone. As a result, thrips damage is commonly scored by individuals that rate the samples. Samples are classified into categories signifying the amount of damage (Mirnezhad et al., 2010; Maharijaya et al., 2011, 2012), or damage is estimated to the nearest 1 mm^2 (Leiss et al., 2009; Mirnezhad et al., 2010; Maharijaya et al., 2011, 2012). These subjective measurements make comparison between studies/screening programs difficult. Moreover, they are time consuming and thus costly for breeding companies.

Here we present an objective, automated protocol for the screening of thrips damage on leaves. We developed a high-throughput standardized screening method to measure leaf surface damage caused by thrips using two types of freeware, ImageJ (Schindelin et al., 2012) and Ilastik (Sommer et al., 2011). Ilastik has a wide range of applications ranging from cell biology (Fabrowski et al., 2013), where it is used to compute the amount of surface flattening of epithelial cells, to biomechanics (Bongiorno et al., 2014), where it is used to identify boundaries of human mesenchymal stem cells. It is an easy-to-use, self-learning image processing program that allows segmentation and classification of two-dimensional surfaces based on labels provided by the user (Sommer et al., 2011). ImageJ is often used to quantify the amount of removed leaf area by chewing herbivores and the total leaf surface of intact leaves (Meyer &

Hull-Sanders, 2008; Morrison & Lindell, 2012). However, it is rarely used to quantify feeding damage caused by thrips, as the program is limited in quantifying more gradual color differences, for which Ilastik provides a more suitable alternative.

Analyzing thrips damage in a high-throughput manner requires a fast and reliable screening protocol. We chose to screen damage on leaf discs, as this is a widely applied experimental approach in pest resistance screening, for example, with mites (Adesanya et al., 2018), thrips (van Rijn et al., 1995), aphids (Sattar et al., 2016), whiteflies (Thakur et al., 2014), and even fungi (Fukino et al., 2013). The downside of working with leaf discs is the relatively high amount of damage that is inflicted to the leaf prior to the assay. This might induce resistance to the herbivore that is tested. However, several studies demonstrated that there was no difference in resistance scores between detached/attached leaves and leaf disc assays (Chaerle et al., 2007; Maharijaya et al., 2011; Eshraghi et al., 2014). Moreover, leaf discs are easily kept fresh for several days on a drop of water-agar, which also allows for standardization of the leaf side that is exposed to thrips feeding. A set-up with leaf discs is space-efficient because the screening assays can be conducted in refrigerator-size climate cabinets. In addition, the screening can be physically separated from plant production, and therefore the risk of thrips contamination in the greenhouse can be minimized.

We developed the leaf disc screening method using *Capsicum* spp. as the host plant. *Capsicum*, commonly known as hot or sweet pepper, suffers greatly from damage caused by various thrips species, especially in the seedling stage (Fery & Schalk, 1991). In *Capsicum*, thrips feeding causes deformation of the leaves, short internodes, chlorosis (Fery & Schalk, 1991), reduced photosynthesis, and yield losses (Shipp et al., 1998). The genus *Capsicum* contains a broad range of accessions with a wide range of resistance levels, providing a good model to develop an optimal thrips resistance screening system.

We illustrate the successful application of our screening method by addressing two research questions. Based on observations in the field (Daniel et al., 1983; Culliney, 1990; Feller et al., 2002; Tree & Walter, 2009), it is widely accepted that thrips feeding occurs mostly on the abaxial leaf side. However, we are not aware of a direct quantitative comparison of feeding damage between the two leaf sides. In this study, we therefore test our method by comparing thrips feeding damage on the abaxial and adiaxial leaf sides. Furthermore, we investigate whether thrips resistance changes over the course of plant ontogeny. Thrips resistance is mostly studied in young, vegetative plants. It is unclear whether resistance levels in early, vegetative stages can be extrapolated to the mature,

reproductive stage, which also suffers severely from thrips damage. Resistance to thrips may change due to ontogenetic changes that result in alterations in the plant's metabolism and the allocation of defense metabolites toward younger leaves and flowers (van Dam et al., 1996, 2001). We applied our novel high-throughput screening method to assess whether and how the plant's ontogenetic stage affects the level of thrips resistance.

Materials and methods

Plant material

We used five *Capsicum* species, *Capsicum annuum* L., *Capsicum chinense* Jacq., *Capsicum baccatum* L., *Capsicum pubescens* Ruiz & Pavon, and *Capsicum eximium* Hunz (Solanaceae) (Table 1). Seeds of four accessions were used in experiment 1 to compare thrips damage on the abaxial and adiaxial leaf sides. In addition, nine commercially available accessions plus accession Shanshu-2001 were used in experiment 2, assessing the ontogenetic effects on thrips damage (Table 1).

Experiment 1: Comparing thrips damage on the abaxial and adaxial leaf sides

Seeds of accessions A04750316, A14750547, 944750228, and DS were germinated on potting soil (Potting soil 4; Horticoop, Bleiswijk, The Netherlands) in trays (20 × 10 × 5.5 cm) in a climate cabinet (Snijders Labs, Tilburg, The Netherlands) at L16(30 °C):D8(20 °C) photo-thermoperiod. When the first two true leaves had developed, the seedlings were transplanted to pots (11 × 11 × 12 cm) containing the same potting soil. The pots were placed on tables in a greenhouse, inside an insect-free net cage (Rovero 0.30-mm gauze, 7.5 × 3 × 2.75 m) at L16:D8 photoperiod and minimum temperatures set to 20 (day) and 17 °C (night). Natural light was supplemented when below 200 Watt m^{-2} with Greenpower lights (400 V/1000 W; Phillips, Amsterdam, The Netherlands). Leaves for thrips assays were sampled 4 weeks after transplanting.

Experiment 2: Ontogenetic effects on thrips damage

Seeds were germinated in plastic cups (7 cm diameter) on sterile glass beads (1 mm diameter) and placed in the same cabinet used in experiment 1. When the cotyledons had fully developed, seedlings were transferred to single pots as in experiment 1 and placed on tables in the greenhouse, inside the insect-free net cage. Leaves for thrips assays were harvested 3 weeks after transplantation to test resistance in the vegetative plant stage. For the flowering stage, leaves were collected after fully opened flowers had emerged on all plants. For the fruit ripening stage, leaves were collected

Table 1 Overview of *Capsicum* accessions used in experiments 1 (comparing thrips damage on abaxial and adaxial leaf sides) and 2 (ontogenetic effects on thrips damage)

Experiment	Species	Accession	Code	Source[1]
1	*C. baccatum*	A14750547	547	SC
	C. chinense	A04750316	316	SC
	C. eximium	944750228	228	SC
	C. pubescens × *C.* spec.	DS	DS	SC
2	*C. annuum*	Golden California Wonder	GCW	PZ
		Serrano	SR	PZ
		Thai Hot Culinary	THC	PZ
		Yolo Wonder	YW	PZ
	C. baccatum	Aij Crystal	AC	PZ
	C. chinense	Fatalii Red	FR	PZ
		Habanero Red	HR	PZ
	C. pubescens	Rocoto Red	RR	PZ
	C. annuum	ShanShu-2001	SS	VRI

[1]SC, Solanaceae Collection Radboud University, Nijmegen, The Netherlands (http://www.ru.nl/bgard/solanaceae-collection/databases/solanaceae); PZ, peperzaden.nl; VRI, Vegetable Research Institute of Shanxi, Academy of Agricultural Sciences, China.

when fruit ripening reached the breaker stage. Thrips colony rearing conditions and testing conditions were kept constant over time.

Insect culture

To establish a stock colony, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) was obtained from Wageningen University, The Netherlands. Cultures were kept in glass jars (11 cm high, 7.5 cm diameter) with lids (8.3 cm diameter) with fine polyester gauze (45 μm mesh, 6 cm diameter) for aeration. Each glass jar contained five fresh green beans (*Phaseolus vulgaris* L.) and a 1.5-ml Eppendorf tube with a small amount of organic pollen grains (De Traay Imkerij, Lelystad, The Netherlands) to increase oviposition rates (Kirk, 1985; Anjum et al., 2012). Three layers of filtration paper were placed on the bottom of the jars to absorb excess moist and to prevent the beans from fouling. Thrips were transferred to clean jars weekly; beans that were still looking fresh were also transferred. The jars with thrips were kept in a climate cabinet (Economic Delux 432 L with TL lightning; Snijders Labs, Tilburg, The Netherlands) at 25 °C and L16:D8 photoperiod. All experiments were performed with synchronized L1/L2 larvae that were starved for 24 h.

No-choice leaf disc assay

Leave samples, between the fourth leaf node from the bottom of the plant and below the fourth leaf node from the top of the plant (avoiding the oldest and youngest leaves), were collected in the greenhouse of the Radboud University by cutting them off at the petiole with a sharp razor. Leaves were placed in a Ziploc-like bag (18 × 25 cm,

50 μm polyethylene 'foliezak met druksluiting'; Vink Lisse, Lisse, The Netherlands) containing 2 ml of water, and transported to the laboratory. Using a cork borer, leaf discs (1.5 cm diameter) were punched from the leaves, avoiding the mid-vein. One leaf disc per Petri dish was placed on a drop of 1.5% slightly liquid agar (Sigma Aldrich, St. Louis, MO, USA) in the center of the Petri dish. For experiment 1, leaf discs were placed either with the abaxial or adaxial side up (n = 5 Petri dishes per leaf side, per accession), whereas for experiment 2, leaf discs were placed only with the abaxial side up (n = 3 Petri dishes per accession, per ontogenetic stage). Five thrips larvae were placed on each leaf disc using a small painting brush. The Petri dish was sealed with Parafilm M and placed in the same climate cabinet as used for insect rearing. After 48 h, the thrips were removed and digital images of the leaf discs were acquired at 1 200 dpi using an Epson Expression 11000XL scanner and Epson Scan Utility v.3.4.9.9 software. Leaf discs were placed on the scanner using a grid to ensure equal distribution of the leaf discs, which is important for processing of the acquired image. Before scanning, the leaf discs were covered with black paper to obtain a dark background providing sufficient contrast and to prevent overexposure. Scan files were stored as TIFF files until further image processing.

Image analysis

Image processing and quantitation of feeding damage was performed with ImageJ Fiji (v.2.0.0 with Java 1.6.0_24) and Ilastik (v.1.1.3) (Sommer et al., 2011). Scanned images were automatically 'cut' in scans of individual leaf discs using ImageJ Fiji (Figure 1A). Before further
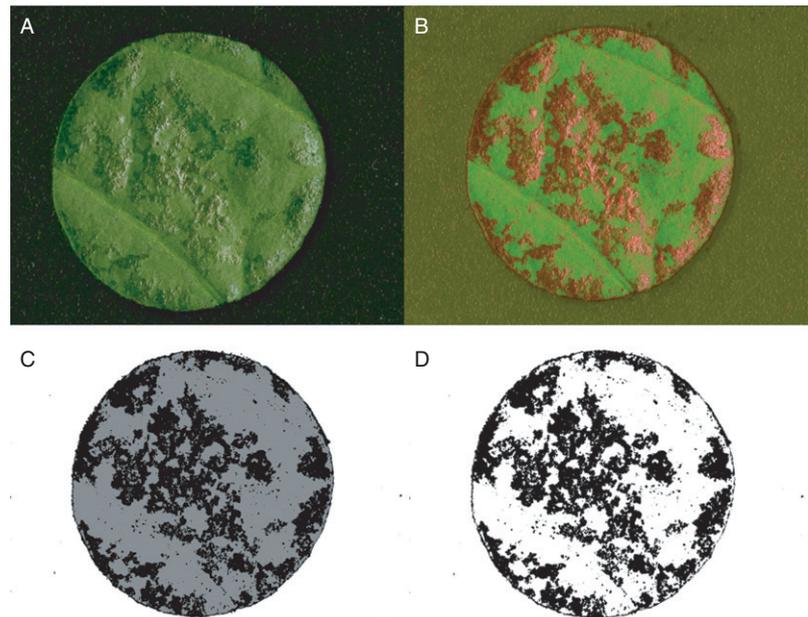
Figure 1 Example of image analyses using Illastik and ImageJ Fiji, illustrating the progression from (A) the scanned image and (B) the learning phase in Ilastik, (C) to a simple segmentation image produced in Ilastik and eventually to (D) an image that is used for calculating the thrips damage in ImageJ Fiji.

processing in Ilastik, the program was trained to recognize damage based on color/intensity, edge (based on brightness and color gradient), and texture at the level of 1 pixel. Three labels were assigned to the various color spectra that were identified: one for thrips damage, one for undamaged leaf area, and one for background. The program was trained to recognize the three segments (thrips damage, leaf disc, and background) using four leaf discs per accession with sufficient damage until the program could precisely identify the segments in the scanned images. Whether training had been sufficient was checked by making use of the 'life update' function that allows the user to switch between the scan image and an overlay image that shows the three segments based on the learning process up until that point (Figure 1B). After proper training, images were converted to JPEG files that are simple segmentations of the original image in black (thrips damage), grey (leaf disc), and white (background) (Figure 1C). In ImageJ Fiji, thrips damage was extracted resulting in a TIFF image which shows thrips damage in black (Figure 1D). Total thrips damage area was easily determined using the 'analyze particles' command that counts and measures objects in threshold images after setting the correct scale (number of pixels : length in mm) in ImageJ Fiji. A step-by-step protocol can be found at Bio-protocol (http://www.bio-pro tocol.org/; Visschers et al., 2018).

Statistical analysis

All data were analyzed using SPSS v.21.0. Prior to analysis, the distribution of the feeding damage values was normalized by performing log transformation. Transformed data were analyzed by one-way ANOVA and, if significant differences were detected, means were subjected to post-hoc Tukey's test.

Results and discussion

An effective screening method

Our protocol based on the freewares Ilastik and ImageJ resulted in effective screening for identification of thrips damage. On average, it takes ca. 4 h to process 50 images (containing a single leaf disc) of 3.08 Mb each (from scan image to a data graph in Microsoft Excel on a PC with an Intel core i7-4910MQ CPU at 2.9 GHz, and 16 GB RAM).

Training is important, as the program depends on sufficient training to accurately recognize the various components in the image. Training can take up to 30 min, with a training image processing speed of ca. 0.9 MB s^{-1} (Intel core i7-4910MQ CPU at 2.9 GHz, 16 GB RAM). The number of leaf discs necessary for training depends on the amount of thrips damage that has been inflicted. As a reference, approximate 10 cm (with the pencil tool set to 3 pixels) of thrips damage area marking is necessary for accurate learning. Once Ilastik is properly trained for one accession of a model plant, image analysis can proceed in batch mode, allowing fast analysis of leaf disc images with the same settings.

Comparing thrips damage on the abaxial and adaxial leaf sides

To validate our standardized digital damage processing method we first analyzed whether there was a difference in thrips feeding between the abaxial and adaxial leaf sides. In

general, we observed more thrips damage on the abaxial than on the adaxial side of the leaf discs in all four accessions ($F_{1,31}$ = 62.091, P<0.001) (Figure 2). This difference between leaf sides was not equally strong for each accession (interaction effect: $F_{3,31}$ = 7.081, P<0.001). Although slightly more feeding on the abaxial leaf side was observed in accession DS, the amount of thrips damage on both leaf sides did not differ significantly (t = 2.124, d.f. = 8, P = 0.066).

Our results indicate that standardizing the exposed leaf side when screening for host plant resistance is important for ranking the accessions for resistance. For example, based on the amount of thrips damage on the adaxial sides, accession 944750228 is the most resistant. However, based on feeding damage on the abaxial side the same accession would be the second most susceptible (Figure 2).

Overall, our results are in concordance with observations in the field, where it was found that thrips damage occurs more on the abaxial leaf side (Daniel et al., 1983; Culliney, 1990; Feller et al., 2002; Tree & Walter, 2009). Morphological resistance traits such as trichomes might be key players, although the relationship with thrips resistance was found to be equivocal (Yadwad et al., 2008; Maharijaya et al., 2013). In addition to morphology,
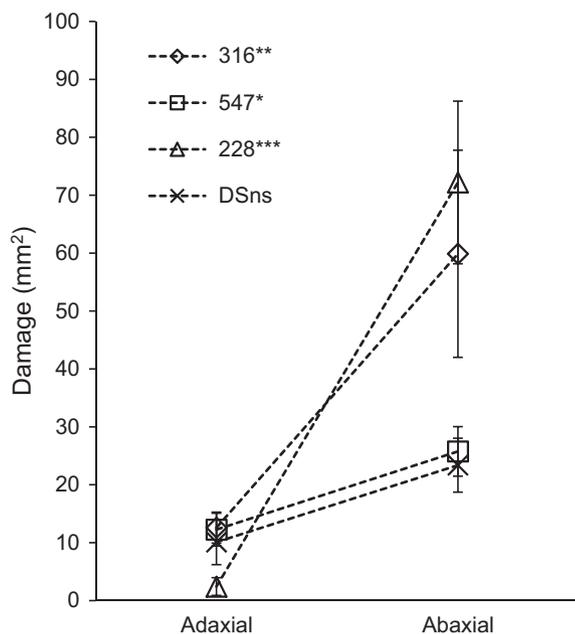
biochemical differences between leaf sides might play an important role. For example, glucosinolate concentrations on the abaxial leaf surface of *Arabidopsis thaliana* (L.) Heynh. were found to be up to 30× higher than on the adaxial leaf side which was linked to higher oviposition rates of the specialist *Pieris rapae* (L.) on the abaxial leaf side (Shroff et al., 2015). Whether similar differences in defense distribution play a role in *Capsicum* remains unknown. More in-depth research into the chemical and morphological differences between leaf sides may improve our understanding of thrips resistance mechanisms.

Ontogenetic effects on thrips damage

We further tested whether thrips resistance changes with ontogeny, by quantifying thrips damage in 10 commercial *Capsicum* accessions in the vegetative, flowering, and fruit ripening stage. Overall, we observed an effect of developmental stage and accession ($F_{9,60}$ = 20.162, P<0.001; Table 2) on thrips damage. Not all accessions displayed a similar pattern (interaction effect: $F_{17,60}$ = 6.097, P<0.001; Table 2); Yolo Wonder, Aij Crystal, Serrano, and Cayenne Long Slim were damaged by thrips more in the vegetative stage than in later stages (Table 2). Discs taken from Fatalii Red plants in the fruit ripening stage showed significantly less thrips damage compared to those taken from the same plants in the flowering stage. Shanshu showed an opposite response, with higher feeding damage observed in the fruit ripening stage (Table 2).

Consequently, ranking resistance is dependent on the ontogenetic stage and resistance ranks differ between accessions. For example, Rocoto Red when tested in the vegetative stage, would be rated as moderately susceptible (rank 6, Figure 3). However, in the reproductive stages it would be rated as resistant (rank 2). In contrast, Yolo Wonder is resistant in the vegetative stage (rank 2), but becomes more susceptible in the reproductive stages (rank 7). In addition, we observed consistency in resistance/susceptibility levels over the whole plant's ontogenetic development in Habanero Red and Aij Crystal. Especially accessions with stable constitutive thrips resistance over the whole plant's ontogenetic development may provide interesting leads for breeding programs.

Overall, our results indicate that thrips resistance is dependent on the plant's developmental stage and its genetic background. Studies on other plant species have also indicated the importance of ontogenetic stage when screening for resistance. For example, in rice, the water weevil, *Lissorhoptrus oryzophilus* Kuschel, preferred the pre-tillering stage (the stage before plants start to produce tillers for vegetative propagation) (Stout et al., 2013). However, resistance against *F. occidentalis* in cucumber (*Cucumis sativus* L.) was not dependent on

Figure 2 Mean (± SE; n = 5) feeding damage (mm^2) by *Frankliniella occidentalis* on the abaxial and adaxial leaf side of leaf discs (1.5 cm diameter) of four *Capsicum* accessions. Overall, feeding damage was the highest on the abaxial side (P<0.000 (***), P<0.01 (**), P<0.05 (*) except for accession DS (ns)). Full names of the accessions and corresponding codes are in Table 1.

Table 2 Mean (± SE; n = 3) feeding damage (mm²) by *Frankliniella occidentalis* on leaf discs (1.5 cm diameter) of 10 commercial *Capsicum* accessions in three life stages

Species	Accession	Vegetative stage	Reproductive stage	
			Flowering	Fruit ripening
C. annuum	Cayenne Long Slim	46.9 ± 11.9a	2.8 ± 0.4b	5.5 ± 1.7b
	Golden California Wonder	4.4 ± 2.2	1.3 ± 0.3	6.6 ± 3.2
	Serrano	29.3 ± 3.8a	7.3 ± 2.2b	8.1 ± 1.0b
	ShanShu	4.5 ± 1.7a	7.6 ± 2.2a,b	16.4 ± 3.5b
	Thai Hot Culinary	96.8 ± 2.5	84.4 ± 6.3	na
	Yolo Wonder	21.2 ± 8.8a	2.8 ± 0.2b	2.1 ± 1.0b
C. baccatum	Aij Crystal	17.7 ± 2.1a	7.5 ± 0.7b	6.6 ± 2.1b
C. chinense	Fatalii Red	5.8 ± 0.4a	9.0 ± 0.1b	2.7 ± 0.4c
	Habanero Red	2.0 ± 0.3	3.7 ± 1.2	2.7 ± 0.5
C. pubescens	Rocoto Red	11.2 ± 5.6	13.2 ± 1.0	12.2 ± 0.4

Means within a row followed by different letters differed significantly (Tukey's test: P<0.02).
na, not assessed.
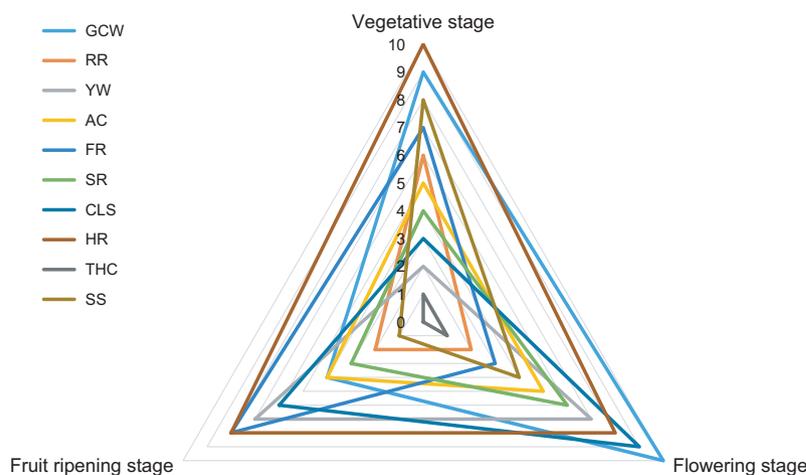
Figure 3 Three-dimensional resistance ranking of 10 commercial *Capsicum* accessions based on thrips damage using no-choice assays of leaf discs from the same plants in the vegetative, flowering, and fruit ripening stages. Rank 1 = high damage levels (susceptible); 10 = low damage levels (resistant). Full names of the accessions and corresponding codes are in Table 1.

plant age (de Kogel et al., 1997). Although the plants tested in the latter study were of different ages, it was not specified whether these plants were also in different ontogenetic stages. Our finding that resistance levels in leaves are not consistent over the plant's ontogenetic development indicates that breeding for resistance in crops should not rely on screening for resistance in one ontogenetic stage only, as it might lead to selection of accessions with unstable constitutive thrips resistance. In addition to identifying constitutive resistance in leaves, it is also important to assess thrips resistance on whole plants, especially when they are flowering. Flower thrips, such as *F. occidentalis*, can use the pistil, calyx, petals, and filaments for oviposition (Childers & Achor,

1991; Cockfield et al., 2007), and pollen as a high-quality food source (Kirk, 1984). Screening whole plants, however, requires a great investment in time, greenhouse space, and labor. Our screening method may help to reduce the number of accessions to screen as whole flowering plants, and thus contribute to a more efficient selection of *Capsicum* accessions highly resistant over the entire ontogenetic development.

Conclusion

We demonstrated the successful application of our screening method in thrips damage quantitation and the importance of standardizing resistance screening methods. The

handling of leaf discs and the use of a scanner makes it a straightforward and easy method that can be applied by different experimenters without compromising subjectivity. Especially, while screening for resistance in large-scale experiments our method provides an accurate tool, as it has been observed that researchers scoring plant resistance often change their ranking estimates when they gain experience in due time (Sushil Kumar, East-West Seed, pers. comm.). The application of our objective screening method will provide solid objective data which will allow for better comparison among test series and studies on thrips resistance. In addition, the method can be easily adapted to other plant-insect research systems such as leaf miners or any other leaf surface damaging insect. Ultimately, our method thus provides an efficient tool for screening for resistance to insects in applied research and commercial breeding.

Acknowledgements

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