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Research

Screening of *Stevia rebaudiana* Susceptibility to *Septoria steviae* Through Leaf Disk Inoculation

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Abstract

Stevia rebaudiana Bertoni is a perennial cultivated plant that produces steviol glycosides, which are natural acaloric sweet compounds in its leaves. Many pathogens can infect this species. Among these pathogens, *Septoria steviae*, the causal agent of Septoria leaf spot, is the most widely described on *S. rebaudiana*. The objective of this study is to validate a leaf disk screening method for *S. rebaudiana* response to *S. steviae* infection on 10 *S. rebaudiana* genotypes with contrasting responses to the fungus. Three independent experiments were carried out in which 40 leaf discs per genotype were infected with an inoculum at a concentration of 10^5 conidia/ml or with water. The response of the 10 genotypes was scored through image analysis. Image analysis was conducted at 9, 12, 15, 18 and 21 days after inoculation (DAI), allowing the percentage of leaf symptomatic area (PLSA) and the area under the disease progress curve (AUDPC) to be quantified.

The evaluation of symptom speed development was realized through a linear regression between PLSA and time for each tested genotype. Genotypes exhibited the lowest susceptibility at 21 DAI, with Cult102_SPA and Cult76_GER displaying 3% of the surface symptomatic. Genotypes with the highest susceptibility were Cult33_FRA and Cult75_GER, with 24% severity. These results correlate with visual scoring performed at 21 DAI and with previous inoculation protocols on *S. rebaudiana* cuttings by maintaining the ranking of the different genotypes regardless of the test. The development of a leaf-disk inoculation method offers new avenues for breeding strategies to develop tolerant or resistant *S. rebaudiana* cultivars.

Keywords: leaf-disk inoculation, *Septoria steviae*, *Stevia rebaudiana*

Stevia rebaudiana Bertoni (2n = 22) (Bertoni 1905) is a perennial plant from Paraguay and is traditionally used as a natural source of sweetener by Guarani natives (Lewis et al. 1992; Soejarto 2001). This species produces steviol glycosides (SGs), which are natural

acaloric sweet compounds that are 250 to 300 times sweeter than sucrose (Ceunen and Geuns 2013a). These components are mostly accumulated in leaves (Ceunen and Geuns 2013b), which are the harvested part of the plant (Angelini et al. 2018).

The increasing demand for SGs leads to an increase of *S. rebaudiana* production area all over the world (Future Market Insights 2025). China is the main *S. rebaudiana* leaf supplier, with a production up to 80% of global production (Sun 2016). Other countries in the world can be counted as significant *S. rebaudiana* producers, such as India, Indonesia, Brazil, Argentina, the United States, Paraguay, and Canada (Gantait et al. 2018). In Europe, recent studies proved that perennial *S. rebaudiana* production was viable in an agronomic and economic way (Andolfi et al. 2006; Angelini et al. 2018; Hastoy et al. 2019a; Libik-Konieczny et al. 2018; Munz et al. 2018).

Pathogens have a huge ability to infect new hosts through host-range expansion (Corredor-Moreno and Saunders 2020). A rise in production acreage has corresponded with increased pathogen reports in *S. rebaudiana*. Indeed, various *S. rebaudiana* pathogens are present in every cultivation area, leading to yield losses. This includes viruses such as *Tomato spotted wild virus* in the United States and Greece (Chatzivassiliou et al. 2007; Koehler et al. 2016) and fungi within the phylum Ascomycota such as *Alternaria alternata* in India (Maiti et al. 2007), *Botrytis cinerea* in Italy (Garibaldi et al. 2009) and belonging to Basidiomycota such as *Athelia rolfsii* (*Sclerotium rolfsii*) in the United States, Greece, or

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India (Chatzivassiliou et al. 2016; Kamalakannan et al. 2007; Koehler and Shew 2014) and in France (Le Bihan et al. 2020b). In addition to these pathogens, the fungal pathogen *Septoria steviae*, the causal agent of Septoria leaf spot, is the most widely described foliar disease of *S. rebaudiana* across the world. Symptoms of Septoria leaf spot include foliar chlorosis and necrosis (Reeleder 1999). First described in Japan (Ishiba et al. 1982), the disease was then described in all new production areas, such as in the United States (Koehler et al. 2019; Lovering and Reeleder 1996), in Canada (Reeleder 1999), in India (Bhandari and Harsh 2006), in Paraguay (Veia Sanabria and Orrego Fuente 2013), and recently in France (Hastoy et al. 2019b). Nowadays, the defoliation from this disease is a major threat to yield potential in *S. rebaudiana*, and approaches to control the disease development are needed (Koehler and Shew 2019).

Septoria fungi are classified in the order Mycosphaerellales (Koehler et al. 2019); they are characterized by pycnidial conidiomata and filiform multiseptate conidia (Sutton 1980; Verkley et al. 2013). The development and spread of this fungus were investigated on crops such as wheat, with the fungal agent being *Mycosphaerella tritici* and *S. tritici*; parsley, with the fungal agent being *S. petroselini*; and tomato, with the fungal agent being *S. lycopersici* (Dhangar and Choudhury 2022; Kurt and Tok 2006; Sanderson 1972). First, conidia form hyphae on the adaxial leaves before entering the plant through the stomata. The fungus then spreads within the host plant via the intercellular spaces (Kema et al. 1996). Cohen and Eyal (1993) demonstrate on wheat that infection remains asymptomatic during 5 to 9 days after inoculation (DAI). Around 10 to 12 DAI, the fungus starts forming sporulated pycnidia visible on the leaf surface (Cohen and Eyal 1993).

Pycnidia will develop from the residues, producing conidia that will infect leaves close to the soil through water by splash dispersal. The problem of maintaining the inoculum between two production periods is that contamination from one year to the next is a possibility when producing this perennial species. During the *S. rebaudiana* growth, fungus develop upward in the canopy (Hastoy et al. 2019b; Koehler and Shew 2018). Infection will be boosted with favorable environmental conditions, such as an leaf humidity (Chungu et al. 2001), due to rainfall or aspersion irrigation for example, and a temperature between 20 and 23°C (Fones and Gurr 2015).

Cultivation of *S. rebaudiana* is developing in southwestern France, where humid and warm environmental conditions are particularly well-suited to the development of Septoria leaf spot of *S. rebaudiana* (Hastoy et al. 2019b). Additionally, *S. rebaudiana* is a perennial plant cultivated over 5 to 6 years in southern European conditions (Andolfi et al. 2006; Le Bihan et al. 2020a), which means that the *S. rebaudiana* plants remain in the same field. Moreover, the *S. rebaudiana* fields are mostly irrigated by water dispersion (Angelini et al. 2018), leading to good maintenance of *S. steviae* inoculum during winter and an efficient and quick fungus propagation.

To control *S. steviae*, different methods can be considered. The application of fungicide with active ingredients, such as azoxystrobin, chlorothalonil, or difenoconazole, were evaluated as effective in the United States to maintain disease severity under 5% of damage (Koehler and Shew 2018; Sanabria-Velazquez et al. 2023). Furthermore, European legislation is limiting the use of phytosanitary products with the regulation (EC) 1107/2009, where products must be evaluated by the EFSA agency at the European level and ANSES at the French level before being allowed on a specific crop. On *S. rebaudiana*, no phytosanitary product is allowed so far. Moreover, for organic crop management in Europe, the use of chemicals is prohibited (Regulation [EC] 834/2007). Other solutions, such as the use of copper or biocontrol agents, are available, but their effi-

cacy is only preventive and limited (Chiou and Hsu 2019). Among prophylactic methods, irrigation technics (drop-to-drop), residue management, and long crop rotation are mainly used, and genetic control remains one of the most important means of control.

Genetic improvement for disease resistance relies on the availability of resistance alleles among the genetic resources and the introgression of resistance alleles to selected genotypes through a breeding program. However, the precise characterization of the resistance response requires the implementation of a precise, simple, and inexpensive phenotyping protocol.

So far, *S. rebaudiana* breeding programs mainly focused on yield component traits, such as leaf production and SG content in leaves, as well as the SG diversity produced by *S. rebaudiana* plants (Yadav et al. 2011). However, tolerance/resistance to pathogens has not been studied much. Few *S. rebaudiana* varieties are described as tolerant to foliar diseases in patents (Alvarez Britos 2012, 2013) without any details given on the type of resistance and the scoring method used. Evaluation of the response of *S. rebaudiana* genotypes to *S. steviae* infection in the field was performed by visual scores (Koehler et al. 2019; Reeleder 1999). However, in a screening program, the outcome is subject to the randomness of a natural infection that cannot be controlled and may depend on the experimenter's skill with regard to visual score. To include the response to *S. steviae* in a French *S. rebaudiana* breeding program, an optimized phenotyping method was developed in France (Hastoy et al. 2019b). This method is based on an inoculation method of 3-week-old isolate suspension on 6-week-old *S. rebaudiana* cuttings through a spray chamber. However, this method is time consuming and demands the production of a large quantity of *S. rebaudiana* cuttings, which is possible only at a certain time in the year. This limits the number of plants or progeny that can be evaluated in a given season.

Leaf disks inoculation assay is a reliable screening procedure already developed on germplasm and progenies screening on many crops and their associated foliar pathogens such as *Alternaria solani* on *Solanum tuberosum* (Bussey and Stevenson 1991), *Sclerotinia trifoliorum* on *Medicago sativa* (Pratt 1996), *Melampsora larici-populina* on *Populus* sp. (Dowkiw et al. 2003), *Plasmopara viticola* on *Vitis vinifera* (Deglène-Benbrahim et al. 2010), and more recently *Phytophthora colocasiae* on *Colocasia esculenta* (Nath et al. 2016), *Podosphaera* sp. on *Phlox paniculata* (Farinas et al. 2019) and *Alternaria alternata* on *Butomus umbellatus* (Harms et al. 2020). This method, which can be applied to a large number of plants and a large number of replicates while remaining compact, as compared with a field estimate, was largely adopted on many pathosystems and showed its efficiency, explaining the choice to adapt the protocol to *S. rebaudiana*. The aim of this study is to validate a leaf disk screening method for *S. rebaudiana* response to *S. steviae* infection, on 10 genotypes with contrasting responses to the fungus.

Materials and Methods

Plant and fungal material

Ten *S. rebaudiana* genotypes that were already evaluated for their susceptibility to *S. steviae* through cutting inoculation (Hastoy et al. 2019b) were chosen to perform this test (Table 1). The Sep-15-LIP isolate, characterized in Hastoy et al. (2019b), was cultivated on oat agar chloramphenicol medium (50 mg/liter of chloramphenicol) in a growing chamber (22°C and 12-h photoperiod). This strain was used to validate Koch's postulates and to characterize the response of 10 genotypes in an ex situ test on pot plants (Hastoy et al. 2019b). *S. rebaudiana* genotypes were grown under controlled conditions in INRAE greenhouses. Mother plants were grown in 5-liter pots with

Peltracom media (Greenyard Horticulture France SAS). MULTICOTE nutrient supply 18-6-12 (Haifa) was administrated twice a year. Greenhouse conditions were as follows: a 15-h photoperiod, with temperatures of 18°C for 3 h and 22°C for 9 h during the day and 20°C for 3 h and 16°C for 9 h at night. Three months before inoculation, apical dominance was removed from each plant by cutting the growing tip to favor secondary stems and to increase the number of young leaves.

Preparation of leaf disks for in vitro inoculation

The protocol is adapted from the one developed for vine testing by Debieu et al. (1995). On the day of the inoculation, young leaves, from the apex until the fourth node below the apex, were collected on mother plants. All following steps were performed under a sterile flow hood. Leaves were sterilized by immersion in a calcium hypochlorite (Ca[ClO]2) solution that was concentrated at 40 g liter-1 for 10 min and were rinsed three times in sterile distilled water. Sterile leaves were dried on sterile blotting paper. Leaf disks with a 15-mm diameter were cut from *S. rebaudiana* leaves (main leaf vein in the middle of leaf disk) using a sharp disk cutter. Leaf disks were immediately placed on the abaxial side in 9-cm-diameter Petri dishes containing 15 ml of agar medium that were concentrated at 8 g liter-1 of agar and 0.03 mg liter-1 of difenoconazole (a fungicide). The difenoconazole was added to the medium

to prevent the fungus from developing in the medium. The measure of a precise 15-ml volume of medium guaranteed a similar agar thickness in every Petri dish.

Inoculum production

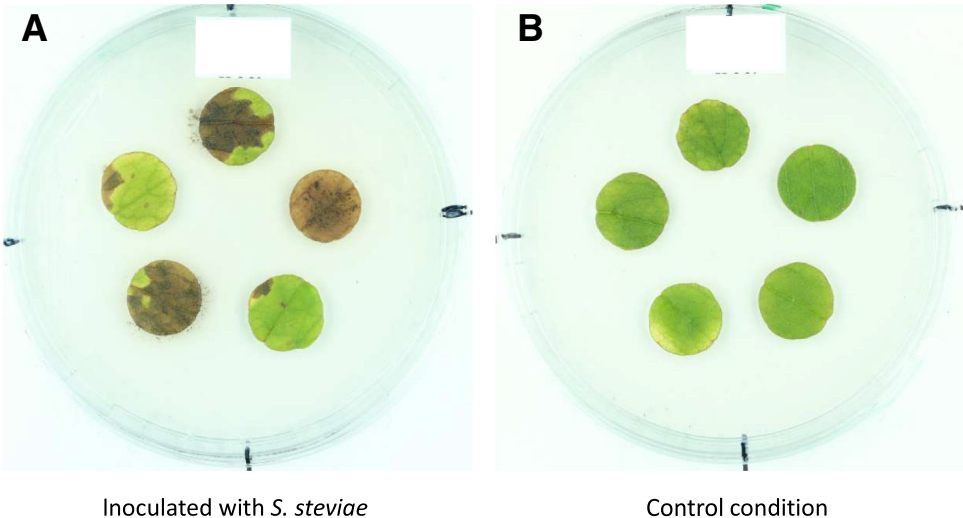
For each inoculation, four to five 3-week-old fungal cultures were used for conidia harvest. Under sterile conditions, sterile distilled water with 0.01% Tween 20 was poured into plates. A Pasteur pipette was used to break pycnidia and suspend conidia into sterile water. The solution was filtered through a 450-µm filter. A conidia count was performed on a Malassez cell to adjust the inoculum concentration at 105 conidia ml-1.

Leaf-disk test

Five leaf disks were placed per Petri dish following a specific pattern to facilitate symptoms scoring and measurement (Fig. 1). The leaf disks were inoculated on the adaxial side by depositing three drops of 10 µl of the *S. steviae* suspension, which were concentrated at 105 conidia ml-1. After inoculation, inoculated Petri dishes were placed in a growth chamber (22°C, 12-h photoperiod). Negative control Petri dishes were prepared with three drops of 10 µl of sterile water, with Tween 20 added at 0.01%. The contact duration between inoculum drops or water drops and leaves was 48 h, and inoculum or water drops were removed after this time.

TABLE 1 Genetic resources of <i>Stevia rebaudiana</i> tested in leaf-disk test, including their names, origin before selection, providers, and country				
Name according to Cosson et al. (2019)	Origin before selection	Providers	Country	Name according to Hastoy et al. (2019b)
Cult75_GER	Colombia	EUSTAS gene bank	Germany	C
Cult76_GER	Unknown	EUSTAS gene bank	Germany	Gawi
Cult33_FRA	Argentina	Oviatis	France	E161718
Cult34_FRA	Argentina	Oviatis	France	E8
Cult102_SPA	Spain	Oviatis	France	EspLac1
Cult35_FRA	Argentina	Oviatis	France	FP
Cult12_CAN	Israel	Oviatis	France	S6030-1
Cult36_FRA	Argentina	Oviatis	France	Lac4
Cult37_FRA	Argentina	Oviatis	France	Larrère
Cult51_FRA	Paraguay	<i>S. rebaudiana</i> store	Paraguay	Eirete

FIGURE 1
Symptoms observed on **A**, genotype CULT51_FRA 21 days after inoculation (DAI) with *Septoria steviae* on excised leaf discs and **B**, the control condition.



Three independent inoculation experiments were performed. Each inoculation experiment was carried out on leaves from the 10 *S. rebaudiana* genotypes (Table 1): five Petri dishes of five leaf disks ($n = 25$)/genotype/experiment were inoculated with a *Septoria* suspension, and three Petri dishes of five leaf disks ($n = 15$)/genotype/experiment were water-inoculated and considered as a negative control, corresponding to 40 leaves per genotype per inoculation experiment. Control and inoculated Petri dishes were randomly placed in the growth chamber.

Disease severity assessment

Visible spectrum image analysis (VIS). A first picture of each Petri dish was taken at 2 DAI. Pictures were then taken every 3 days from 9 DAI until 21 DAI (9, 12, 15, 18, and 21 DAI). Petri dishes were placed into a dark room under white light on a white background, with 55 cm under the camera. Pictures were taken using a Canon EOS 760D camera and a Canon lens EF-S 60 mm f/2.8 Macro USM with speed = 100, open = 9.0, and ISO = 200. Pictures were analyzed using ImageJ software (version 1.8.0). Firstly, a binary transformation of pictures was performed to obtain the total leaf area. Then, a threshold based on color was performed on pictures to select only chlorotic and necrotic leaf areas (hue: 0 to 47; saturation: 37 to 255; brightness: 0 to 204) on inoculated and control leaves. To take account of the effect of leaf handling, the chlorotic and necrotic surfaces of the controls were averaged by genotype and experiment ($n = 15$). Those measures were used to calculate the percentage of leaf symptomatic area (PLSA) as follows:

$$PLSA_{ij} = [(Symptomatic\ inoculated\ leaf\ surface_{ij} - Average\ of\ symptomatic\ control\ leaf\ surface_{ij}) / Total\ leaf\ surface_{ij}] \times 100$$

with leaf *i* of genotype *j*/inoculation experiment.

The area under the disease progress curve (AUDPC) was calculated using the formula presented by Das et al. (1992) on PLSA (%) values.

Visual estimation of disease severity. A score of symptoms through visual evaluations using a symptoms scale with six levels, from 0 to 5 (Table 2) based on a partial standard area diagram (SAD) (Bock et al. 2020), was performed on each Petri dish at 21 DAI.

Comparison of results obtained from leaf disks experiments and those obtained from whole plants

To validate the leaf disk inoculation method as a screening of *S. rebaudiana* response to *S. steviae*, the results of this study were compared with those obtained on whole potted plants (Hastoy et al. 2019b). Given the difference between the experiments, with one carried out on excised leaves in Petri dishes and the other on potted plants, the comparison was made on the end point of each experiment, 21 DAI, on the PLSA variable. The correlation between the two inoculation tests on *S. rebaudiana*, the leaf cuttings test (Hastoy et al. 2019b) and leaf disks test (this study), was controlled with a Spearman correlation test. The correlation test was implemented on the estimated marginal means (emmeans) of the PLSA data obtained from the two tests. The emmeans were calculated from the analysis of variances applied to the mixed models, as presented above and in Hastoy et al. (2019b). Based on the PLSA data, a reaction norm was constructed to illustrate the ranking of the genotypes in the two studies.

Statistical analysis

Statistical analyses were performed on R version 3.6.3 software (R Core Team 2018). Analysis of variance type II was applied to the

mixed model using the “lme4” (Bates et al. 2014) and “car” (Fox and Weisberg 2011) packages:

$$y \sim A \times B + \frac{1}{C}$$

TABLE 2 Visual rating scale for symptoms related to the development of <i>Septoria steviae</i> on <i>Stevia rebaudiana</i> leaf discs ^z			
Symptom score	Description	SAD	Illustration
0	No symptoms	0%	
1	One lesion	5%	
2	One necrotic area with its chlorotic halo	10%	
	Several necrotic areas	10%	
3	One necrotic area on 1/3 of leaf surface	30%	
	Necrotic areas are connecting	30%	
4	Necrotic area is developed on 50 to 75% of leaf surface	50 to 75%	
5	Necrotic area is developed on more than 75% of leaf area	Over 75%	

^z The symptom score is assigned on the basis of the description and a standard area diagram (SAD). An illustration is provided for each score.

where y is the measured variable, A is the fixed effect genotype, B is the fixed effect time of scoring, and C is the random effect inoculation. Random effects of inoculation allow the consideration of the variability resulting from the experiment effect.

For the AUDPC variable, the time of scoring effect was not used into the model.

Emmeans and standard errors were calculated from models using the package “emmeans” (Lenth 2018). A multiple comparison was performed to obtain significant differences between genotypes with a Tukey’s honestly significant difference (HSD) test in the “emmeans” package. The evaluation of symptom speed development was realized through a linear regression between PLSA and time for each tested genotype. The “emmeans” package was used to perform a pairwise comparison of the genotype regression coefficients.

Graphics were represented using the “ggplot2” package in R.

Results

Genotypic response to leaf disk assay

The response of the 10 *S. rebaudiana* genotypes to an inoculation of *S. steviae* isolate 15-LIP was characterized through three independent experiments. For each inoculation, control Petri dishes

were inoculated with sterile water and did not develop symptoms during the 21 days of the experiment (Fig. 1). Some control leaf disks showed a slight necrosis line on the edge of the disk, at the location of the excision. This slight necrotic area was taken into account in the calculation of symptoms percentage. The first symptoms, necrotic lesions, were observed 8 to 10 DAI according to the genotype. Then, the necrotic area extended on the leaf surface and was surrounded by a chlorotic area.

Kinetics of *S. steviae* development on *S. rebaudiana* genetic resources

An image analysis was performed from 9 until 21 DAI for every Petri dish, allowing for the calculation of the PLSA at each scoring time and for the following of the development of the symptoms for each tested genotype (Fig. 2). At 9 DAI, most of the genotypes present a low level of symptoms, with a PLSA under 5%. However, a range of responses is already observed, starting with the least symptomatic genotypes, Cult102_SPA and Cult76_GER, at 1% of symptoms, until the most symptomatic genotypes, Cult33_FRA and Cult75_GER at PLSA 5% (Fig. 2). For each genotype, an increase of the symptomatic foliar area is measured until 21 DAI. The variability measured at 21 DAI is ranging from 3% for Cult102_SPA to 24% for Cult33_FRA, confirming the same extreme phenotypes

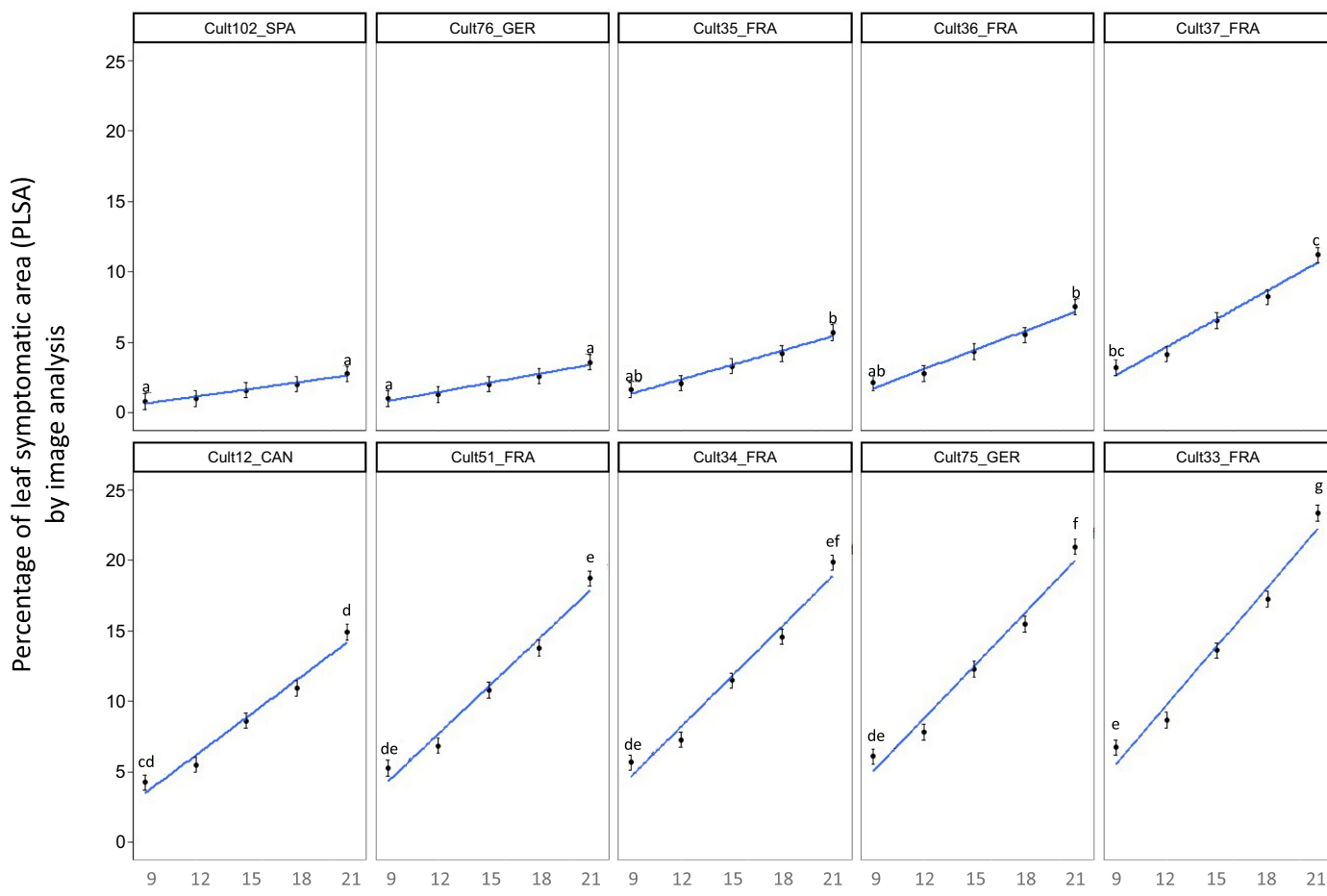


FIGURE 2

Symptoms evolution of *Septoria* leaf spot on 10 *Stevia rebaudiana* genotypes inoculated in a controlled-condition leaf-disk assay. The percentage of leaf symptomatic area (PLSA) was measured through image analysis on pictures of the inoculated leaf-disk Petri dishes from 9 days after inoculation (DAI) until 21 DAI. Results are estimated as the estimated marginal means of five Petri dishes per genotype ($n = 25$), which are repeated three times, with standard error, according to the number of DAI. Blue lines represent linear regression. Letters show the results of multiple comparison at 9 and 21 DAI. At each date, genotypes with the same letter are not significantly different at the $P = 0.05$ level.

observed at 9 DAI (Fig. 2). The variance analysis performed on this trait confirmed the strong and significant genotype effect (Table 3), as well as a significant DAI effect. Multiple comparisons performed on 21 DAI scorings confirm the difference between the least susceptible genotypes, Cult102_SPA and Cult76_GER, and the most susceptible ones, which are Cult33_FRA, Cult75_GER, and Cult34_FRA (Fig. 2).

The symptoms evolution from 9 to 21 DAI varied a lot among the tested genotypes. The evolution of fungus development is presented in Figure 2 and indicates a linear increase over the time of scoring. The regression slopes were calculated for every genotype and appear significantly different according to the genotypes (Table 4). Cult33_FRA and Cult75_GER presented the highest slope coefficients at 1.39 and 1.24, while Cult102_SPA and Cult76_GER presented the lowest slope coefficients at 0.16 and 0.21 (Table 4).

PLSA scorings from 9, 12, 15, 18, and 21 DAI were used to calculate the AUDPC. This variable is an interesting indicator because it synthesizes the information of the level of symptoms as well as the timing of symptoms development. As presented in Figure 3, the AUDPC varies from 19.2 for Cult102_SPA to 163.6 for Cult33_FRA. This variance analysis confirms the results on PLSA (Table 3), showing a similar susceptibility classification among tested genotypes (Fig. 3).

Classification of genotypes by visual scoring and image analysis

A visual scoring of symptoms was also performed at 21 DAI (Fig. 4) using a visual scale (Table 2). The visual end-point notation (21 DAI) by the experimenter makes it possible to clas-

sify the genotypes (Fig. 4). It shows that the extreme susceptible (Cult33_FRA, Cult75_GER, and Cult34_FRA) and the least susceptible (Cult102_SPA and Cult35_FRA) genotypes are the same as those determined by image analysis. Only the classification of the five intermediate genotypes is slightly modified.

Comparison between classification through leaf-disk test and cutting inoculation

The genotype classifications obtained with the leaf-disk inoculation methods were compared with the results obtained by Hastoy et al. (2019b), who performed a genotype screening directly on *S. rebaudiana* cuttings. The results of both tests were compared based on the PLSA scoring at 21 DAI to determine if those inoculation tests provide comparable information. The correlation coefficient between the two variables, leaf disks PLSA and leaf cuttings PLSA, is 0.842 (*P* value of 0.0045). The genotype ranking was compared through a reaction norm (Fig. 5). The results indicate that the level of symptoms can vary slightly from one test to another. However, the genotypes are still classified in the same groups. The first group gathers the least susceptible genotypes: Cult102_SPA and Cult76_GER. The second group is characterized by intermediately susceptible genotypes: Cult35_FRA, Cult36_FRA, Cult37_FRA, and Cult12_CAN. Finally, the third group gathers the most susceptible genotypes, which are Cult33_FRA, Cult75_FRA, Cult34_FRA, and Cult51_FRA. The genotype classification is similar in both inoculation tests.

Discussion

The genetic improvement of *S. rebaudiana* until now has been essentially based on the quantity and quality of SG production. The development of this crop in many parts of the world with very different pedoclimatic conditions makes the problem of interaction with pathogens a major issue. Once the disease and the causative pathogen have been characterized, a simple, inexpensive, and reproducible test should be available to assess the response of genetic resources, parents, and offspring to these pathogens. For many crops, the first tests to describe the response to fungal infection are realized through field trials or cutting trials. For example, the study of wheat cultivars response to *Septoria nodorum* was first realized in fields (Cooke and Jones 1971; Scott and Benedikz 1977). Recently, a study by Sanabria-Velazquez et al. (2023) validated the use of standard area diagrams in the field for rating the response of *S. rebaudiana* to *S. steviae*. While those protocols permit a very good description of a crop's response to fungal infection in a cultivation context, they can be complicated to carry out. Field trials are especially difficult to interpret due to the lack of control of inoculum source and propagation.

For perennial plants, planting of progenies in the field is time-consuming and expensive (McClure et al. 2014). For *S. rebaudiana*, as in many perennials, another constraint is its allogamy, which imposes realizing cuttings to obtain clones when repeats are needed. All these constraints are limiting factors for the development of progenies field trials, and inoculating *S. steviae* isolate on field-planted progenies is not an option. Detached leaf inoculation methods in controlled conditions are a necessity to investigate the variability of the response to leaf pathogens (Griffiths et al. 1985; Mebrate and Cooke 2001). It is within this framework that we have adapted a grapevine leaf disk inoculation test to study the response of *S. rebaudiana* genetic resources to *S. steviae*.

On *S. rebaudiana*, the first inoculation protocol developed to score the response of genetic resources to *S. steviae* infection was developed on cuttings through a spray chamber inoculation by

TABLE 3 Type II analysis of variance (ANOVA) on mixed linear models over percentage of leaf symptomatic area (PLSA) and area under the disease progress curve (AUDPC) on genetic resources and days after inoculation (DAI)				
Factors	PLSA		AUDPC	
	Probability	Significance	Probability	Significance
Genotypes	2.2e-16	<i>P</i> < 0.001	2.2e-16	<i>P</i> < 0.001
DAI	2.2e-16	<i>P</i> < 0.001	–	–
Genotypes × DAI	2.2e-16	<i>P</i> < 0.001	–	–

TABLE 4 Linear regression coefficient of percentage of leaf symptomatic area (PLSA) according to days after inoculation for the 10 genotypes of <i>Stevia rebaudiana</i> ^z	
Genotype	Regression slope coefficient
Cult102_SPA	0.16 ± 0.09 a
Cult76_GER	0.21 ± 0.09 a
Cult35_FRA	0.34 ± 0.09 ab
Cult36_FRA	0.45 ± 0.1 bc
Cult37_FRA	0.67 ± 0.09 c
Cult12_CAN	0.89 ± 0.09 d
Cult51_FRA	1.13 ± 0.2 e
Cult34_FRA	1.19 ± 0.09 ef
Cult75_GER	1.25 ± 0.09 ef
Cult33_FRA	1.39 ± 0.09 f

^z Results of multiple comparison on slope coefficient by Tukey's honestly significant difference (HSD) test are indicated by letter. The same letter shows no significant difference at the *P* = 0.05 level.

Hastoy et al. (2019b). This first test was developed on 10 genetic resources, allowing a first phenotypic evaluation of the variability of the response to *S. steviae*. The validation of the *S. rebaudiana* leaf-disk test was performed as the symptoms scoring method through image analysis, and it is precise and shows the same genotypes classification at 21 DAI as the one obtained with the cuttings inoculation test performed by Hastoy et al. (2019b). Moreover, the symptoms evolution according to DAI was analyzed through a linear regression, which was identical to the study on *S. rebaudiana* cuttings. Even if the regression slopes calculated in this study are smaller than the coefficients calculated in the study by Hastoy et al. (2019b), they indicate a slower symptom increase for the leaf-disk inoculation. Finally, the genotype classification obtained through the visual scale-based scorings is similar for the extreme phenotypes but differs for the intermediate ones. This difference is probably linked to the construction of the visual scoring scale for leaf-disk inoculation, which is composed of six grades from 0 to 5, which limits the precision in the description of variability.

This leaf-disk inoculation method presents numerous advantages compared with the growth chamber-sprayed cuttings inoculation

method or the field inoculation with natural or artificial infection. First of all, a small quantity of fungal material is needed. The inoculum volume for the leaf-disk inoculation is only a few milliliters, which corresponds to an important reduction compared with the previous inoculation method on cuttings, using 3 liters of inoculum per inoculation. Second, a diminution of the quantity of plant material used is observed as well. Indeed, for the leaf-disk experiment, the number of leaves needed is equal to the number of leaf disks used, corresponding to 40 leaves per genotype per inoculation experiment. The plant material is available any time, as mother plants are grown in greenhouses. For progenies, the small quantity of plant material needed allows one to work on leaves directly sampled from the field experiment. This is a main advantage compared with the cutting inoculation method, for which 6-week-old cuttings are used. The production of cuttings requires space, a higher number of mother plants, and is only possible from March until July, as the reduction in daylight hours induces flowering. Another advantage of the leaf-disk inoculation method compared with the cutting method is the reduction of space used in the growing chamber, allowing for testing a higher number of genotypes at the same time. However,

FIGURE 3

The area under the disease progress curve (AUDPC) was calculated from percentage of leaf symptomatic area (PLSA) measured at 9, 12, 15, 18, and 21 days after inoculation (DAI) of 10 *Stevia rebaudiana* genotypes inoculated with *Septoria steviae* in a controlled-condition leaf-disk assay. Results are estimated as the estimated marginal means of five Petri dishes per genotype ($n = 25$), which are repeated three times, with standard error. Letters show the result of multiple comparison by Tukey's honestly significant difference (HSD) test, and genotypes with the same letter are not significantly different at the $P = 0.05$ level.

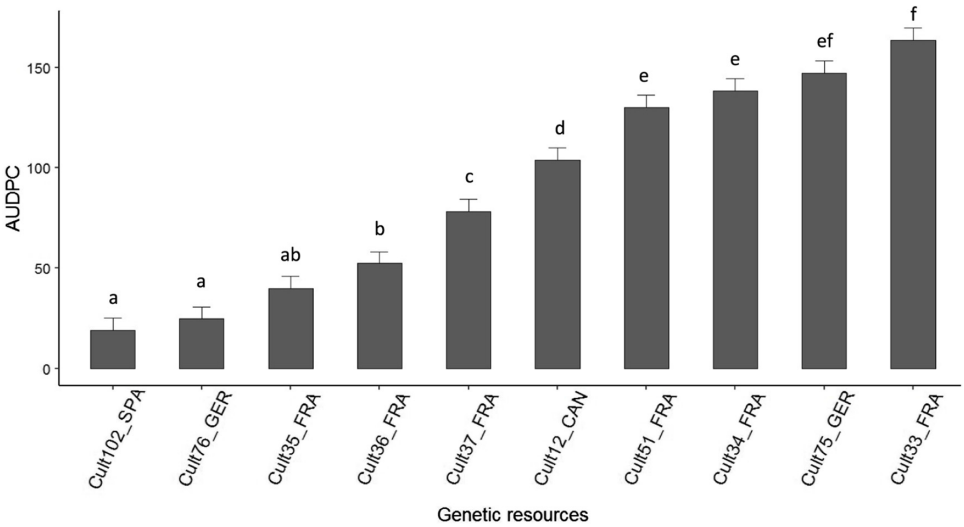
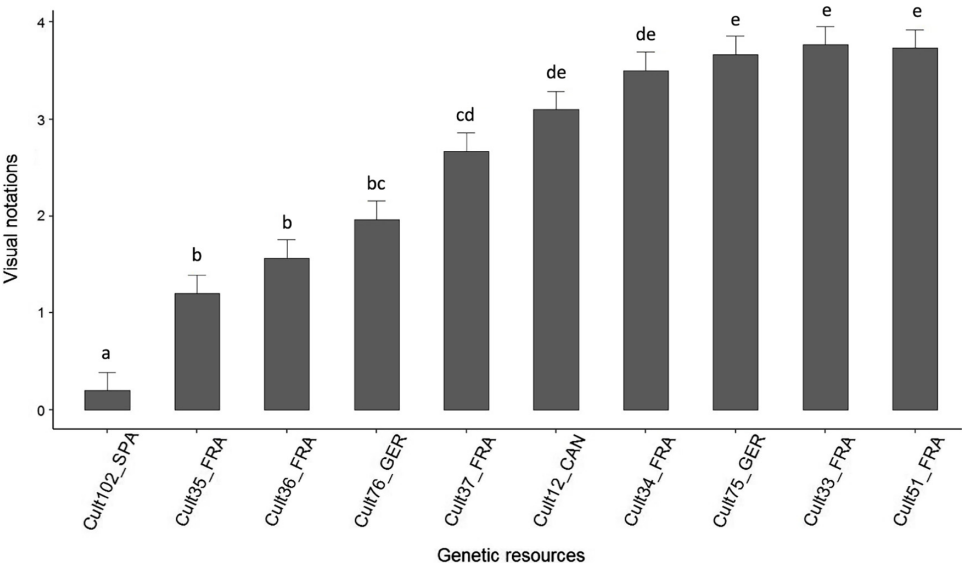


FIGURE 4

Visual scorings were performed according to a six-level scale on 10 *Stevia rebaudiana* genotypes inoculated with *Septoria steviae* in a controlled-condition leaf-disk assay. Results are estimated as the estimated marginal means of five Petri dishes per genotype ($n = 25$), which are repeated three times, with standard error. Letters show the result of multiple comparison by Tukey's honestly significant difference (HSD) test, and genotypes with the same letter are not significantly different at the $P = 0.05$ level.



this method is certainly more expensive than visual assessment in the field and requires laboratory equipment. It is certainly less suitable for assessing the severity of the disease under field conditions (Sanabria-Velazquez et al. 2023) but is very well suited to screening progenies in the case of genetic improvement programs.

Two scoring methods were performed in the leaf-disk protocol: a visual scoring method based on a six-level scale and an image analysis method based on pictures. The visual scoring method is reliable for performing symptom phenotyping and evaluates the global answer of the tested individuals. This method has the merit of being quicker and is easier to execute. This explains why the phenotyping method is widely employed in the literature, with various degrees of precision. Visual scorings were performed to evaluate the *Brassica oleracea* susceptibility to *Peronospora parasitica* on three responses: resistant, hypersensitive response, or moderately susceptible (Agnola et al. 2003). Some more precise visual scorings were performed to estimate the percentages of *Vitis vinifera* disk-leaf area infected by downy mildew *Plasmopara viticola* (Musetti et al. 2006). Identically to the present study, the inoculation of *Podosphaera clandestina* on *Prunus avium* L. was evaluated through visual scorings on the percentage of symptoms for the leaf-disk inoculation and a five-level scale for the field screening (Olmstead et al. 2000). A leaf-disk inoculation of powdery mildew on *Cucumis melo* L. progenies was evaluated by visual scorings using a six-level scale. This phenotyping protocol associated with linkage map construction permits the detection of two quantitative trait loci (QTLs) associated with the susceptibility to powdery mildew (Fukino et al. 2008). Agnola et al. (2003) used a leaf-disk assay to screen, through visual scorings, a broccoli F2 population for the susceptibility to six *Peronospora parasitica* isolates and identified a dominant gene controlling resistance to the fungus isolates.

Nevertheless, although it is more complicated to implement and is more time-consuming, image analysis offers many advantages. This method offers similar results independent from the experi-

menter. Indeed, Olmstead et al. (2000) adapted the scoring of the inoculation of *Podosphaera clandestina* on *Prunus avium* L.; they performed an image analysis on scanning the leaf disk to calculate the infected foliar area and obtained the same results to visual scoring. Image analysis provides a fine quantitative variable, allowing the implementation of QTL mapping detection strategies. It also allows a more precise monitoring of the fungus development and thus allows the production of hypotheses on the type of interaction highlighted according to the genotype tested. Moreover, a precise evaluation of the kinetics development of the fungus is also possible. Leandro et al. (2001) studied the germination and sporulation of *Colletotrichum acutatum* on strawberry. They performed a count of the number of germinated and ungerminated conidia on the inoculated leaves observed by microscope. The image analysis method, though, requests more material, such as a darkroom equipped with neon tubes and a camera, and more time. In the future, the scoring method can be selected according to the needed precision, the available time, and the material.

The development of this simplified inoculation method opens the field to a number of opportunities for *S. rebaudiana* phenotyping and breeding. In the present test, only one *S. steviae* isolate was inoculated. However, a genotype's susceptibility can vary a lot according to the isolate inoculated; for example, in wheat, the cultivar 'Longbow' is susceptible to the IPO323 isolate but resistant to the IPO88004 isolate (Orton et al. 2017). Moreover, in the field condition studies on wheat, it showed that the inoculum was actually a mix of several isolates of the fungus (McDonald and Martinez 1990; Stukenbrock et al. 2012). In the present study, the isolate Sep-15-LIP was inoculated, but other isolates could be tested, alone or in an inoculum mix, to mimic field conditions (Hastoy et al. 2019b). This test would answer short- and long-term questions for *S. rebaudiana* production: do the less susceptible genotypes have the same response to other isolates? Does interaction among isolates reduce or increase symptoms on production genotypes?

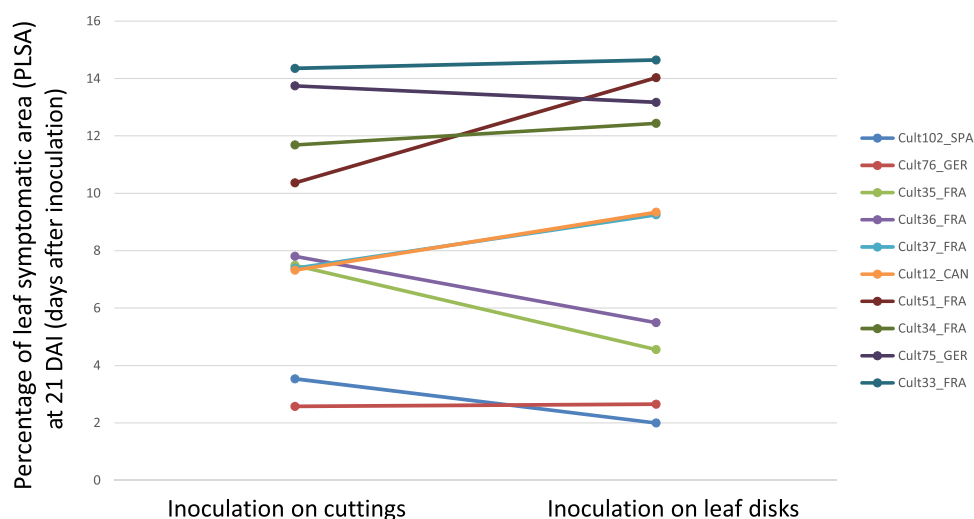


FIGURE 5

Reaction norm of scorings of percentage of leaf symptomatic area (PLSA) at 21 days after inoculation (DAI) for the 10 *Stevia rebaudiana* genotypes inoculated through two inoculation methods. The results presented for the leaf-disk inoculation were obtained in this paper, and the results presented for the cutting inoculation were obtained in the Hastoy et al. (2019b) analyses. Each genotype mean is represented for both inoculation methods. The correlation between the two inoculation tests on *S. rebaudiana*, leaf cuttings test (Hastoy et al. 2019b) and leaf disks test (this study), was controlled with a Spearman correlation test implemented on the estimated marginal means (emmeans) of the PLSA data obtained from the two tests. The emmeans were calculated from the analysis of variances applied to the mixed models, as presented in Hastoy et al. (2019b).

Moreover, an opportunity accessible with the development of this method is also the test of new phytosanitary products or methods to avoid or limit the fungus development. Batta (2003) researched an inoculation of *Alternaria cucumerina* on *Cucumis sativus* L. leaf disks with the application of four different fungicides, allowing for the selection of the most efficient to control the fungus development. This first result was validated through a field experiment, indicating that a leaf-disk assay was an effective preliminary test. Musetti et al. (2006) used a leaf-disk assay to investigate the inhibition potential of endophytic fungus of grapevine, *A. alternata*, on the downy mildew *Plasmopara viticola*. Their results indicate that *A. alternata* is effective in inhibiting *P. viticola* sporulation; the authors concluded that this endophytic fungus has good potential to be used as a biocontrol agent. A similar experiment was conducted on a leaf-disk assay and field trial by Carisse et al. (2000) to control *Venturia inaequalis* of *Malus domestica* Borkh, showing that fungal antagonists reduced the *V. inaequalis* ascospore production. In organic agriculture, this development of disease control methods can help to maintain a low level of fungal development. This method could also be extended to screen the response to other leaf fungi already described on *S. rebaudiana*.

Finally, in an *S. rebaudiana* breeding program for this trait, a first visual scoring could be performed on progenies to obtain an elite set of progenies. The small number of plants could be evaluated a second time by image analysis. This will give a more precise phenotyping and open the field to research on the fine phenotyping of the interaction to find its cellular and molecular determinants.

The comparison with the *S. rebaudiana* cuttings inoculation method confirms that the leaf-disk inoculation method is reliable and efficient for the screening test of *S. rebaudiana* response to *S. steviae* infection. Visual scorings appeared to be a quick reliable scoring method when adapted to test a high number of plants, such as progenies. However, the image analysis scorings are more precise and give the opportunity to investigate new research paths, such as the study of the pathogen kinetics. Finally, the development and validation of this new inoculation protocol opens up new opportunities for the *S. rebaudiana* research community.

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