Plant Disease • 2019 • 103:1544-1550 • https://doi.org/10.1094/PDIS-10-18-1747-RE

e-Xtra*

First Report of *Septoria* sp. Infecting *Stevia rebaudiana* in France and Screening of *Stevia rebaudiana* Genotypes for Host Resistance

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Abstract

Stevia rebaudiana, for which cultivation is on the increase worldwide, accumulates acaloric intense sweeteners called steviol glycosides (SGs) in its leaves. Yields can be affected by Septoria leaf spot (SLS) caused by *Septoria* spp. The objectives of the research were (1) to morphologically and genetically characterize five isolates of *Septoria* sp. found for the first time from outbreaks of *Septoria* in stevia fields in Southwestern France and (2) to screen *S. rebaudiana* germplasm from diverse origins through an automated inoculation method using one of the isolates. Multilocus sequence typing grouped the five isolates obtained from symptomatic plants, closely related to *Septoria lycopersici* and *Septoria apiicola*. The response to *Septoria* sp. of 10 genotypes from different origins was assessed for disease severity (DS), either by visually scoring the symptomatic portion of the whole plants or the portion of symptomatic foliar area (PLSA) determined by image analysis, and the area under the disease progress curve (AUDPC) calculated on the basis of the disease severity rating taken 12, 15, 18, and 21 days after inoculation. No genotypes with complete resistance were identified. Moderately susceptible genotypes "Gawi" and "Esplac1" exhibited only 10 to 15% of symptomatic part on whole plant and the slowest disease development. They could be distinguished from highly susceptible ones "E8", "C", and "E161718" exhibiting up to 40% of symptomatic part on whole plant. The variability of response to *Septoria* sp. that exists in *S. rebaudiana* opens up the field of breeding strategies for the development of new cultivars for sustainable and organic *S. rebaudiana* production.

Stevia rebaudiana (Bertoni), an herbaceous perennial plant from the Asteraceae family, is native to Paraguay, South America. *S. rebaudiana* accumulates steviol glycosides (SGs) in its leaves, which impart an intense and persistent sweet taste, up to 300 times sweeter than sucrose. In Europe and the United States, health agencies recommend reducing daily sugar intake in order to limit obesity and diabetes (Carocho et al. 2017). *S. rebaudiana* and its acaloric sweet molecules therefore constitute a natural alternative to synthetic sweeteners (Carrera-Lanestosa et al. 2017). Since 2011, SGs have been accepted by the European Union as a food additive (Commission Regulation (EU) 2011), and more recently in 2017, dried leaves were also accepted for consumption in Europe (EC 2017). The global stevia market reached nearly US$350 million in 2014 and is estimated to surpass US$565 million by the end of 2020 (PMR 2017). This increase is being driven by many national food-based dietary guidelines providing context-specific advice and principles on healthy diets and wellness food. In France, stevia has been produced commercially on less than 20 hectares since 2011, but has room for expansion to accommodate the increasing market. The primary foliar pathogens which reduce overall plant biomass and therefore yield include *Alternaria alternata* (Maiti et al. 2007) and *Botrytis cinerea* (Garibaldi et al. 2009), which have been described in India and Italy, respectively. However, Septoria leaf spot (SLS) caused by *Septoria* spp. is the most prominent foliar disease described worldwide but has not been previously reported in France.

SLS has been described on *S. rebaudiana* in Japan (Ishiba et al. 1982), the United States (Lovering and Reeleder 1996), Canada (Reeleder 1999), and more recently in India (Bhandari and Harsh 2006) and Paraguay (Veia Sanabria and Orrego Fuente 2013). SLS starts as small black or brown lesions surrounded by chlorotic tissue on the older leaves. The leaves become necrotic, abscise from the plant, and the disease progresses upward (Reeleder 1999).

S. rebaudiana can be cultivated for 5 to 6 years (Andolfi et al. 2006). In temperate climates, *Septoria* sp. represents a real threat to stevia crops due to overwintering capacity and spread during rain events, leading to potential direct yield losses estimated at 50% of the destroyed foliage for susceptible cultivars in Canadian experiments (Reeleder 1999). Extensive study of *Septoria* spp. on other crops provides knowledge of the spread of the disease in the field (Fones and Gurr 2015). Conidia of Septoria tritici blotch from wheat debris spread the infection vertically up the plant by splash dispersal until it reaches the heads (Ficke et al. 2018). For successful infection, the pathogen requires moisture on the leaf surface from either very wet days (>10 mm rainfall) or 3 days with at least 1 mm of rain (Fones and Gurr 2015), which is not unusual in southwestern France. Rain splash events are also important for epidemic development in tomato, as demonstrated in a field trial with *Septoria lycopersici* inoculation (Parker et al. 1997). Disease progress in the field is fast and has been assessed at between 0.3 and 1.3 m/day in 1992 and 1993 at the Iowa State University horticulture farm (Parker et al. 1997). In northern Germany, an average temperature of 13°C, leaf wetness of 92%, and precipitation of 0.4 mm per day were observed 20 days before epidemic outbreaks on wheat (Henze et al. 2007). The overwintering capacity and favorable climate conditions make *Septoria* a real threat for stevia in southwestern France.

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Funding: C. Hastoy was supported by ANRT no. 2014/0915 funding and Oviatis SA, France. The Nouvelle-Aquitaine Region supported the work through specific CIFRE funding. Work was partly supported by the Research Federation on Integrative Biology and Ecology (Bordeaux University, France). We thank Philippe Boutié and Pierre Jannot for financial and technical support (Oviatis SA and Rouages, Agen, France).

GenBank accession numbers for *Septoria* sequences: MH352355-MH352389.

*The *e*-Xtra logo stands for "electronic extra" and indicates that two supplementary tables and two supplementary figures are published online.

The authors declare no conflict of interest.

Accepted for publication 12 January 2019.

The in-season application of chemicals is effective, but options for organic production are limited (Angelini et al. 2018; Koehler and Shew 2018). Several cultural practices aiming at limiting secondary spread of inoculum are also recommended (Angelini et al. 2018). Irrigation and residue management in particular appear to be key factors in managing the disease (Ficke et al. 2018). However, the foundation of many disease management programs is the use of host resistance (Lammerts van Bueren et al. 2011). At present, the research literature on *S. rebaudiana* breeding is limited. Yadav et al. (2011) reviewed breeding programs developed mainly in Canada, the United States, Malaysia, China, Japan, and India. These breeding programs were focused on total SG content and biomass production, rather than on disease resistance (Yadav et al. 2011). Then, in an effort to develop host resistance based solutions, a new method for inoculation of foliar pathogens was developed. In July 2014, first symptoms similar to those caused by *Septoria* sp. were observed by our team in an experimental stevia field in Liposthey, France. Samples were collected from symptomatic plants in experimental research trials from 2014 to 2017. Therefore, the objectives of this research were (1) to use morphological and molecular techniques to characterize these isolates and evaluate their pathogenicity, and (2) to screen *S. rebaudiana* germplasm from diverse origins through an optimized inoculation method under controlled conditions.

Materials and Methods

Identification and characterization of fungal isolates from *S. rebaudiana*. *Fungal isolation.* Symptomatic leaf samples were collected from experimental fields in Liposthey (44°17′56.9″N 0°53′14.7″W) and in Sainte Livrade-sur-Lot (44°25′43.6″N 0°36′53.6″E) located in southwestern France. In total, five isolates, Sep-14-LIV, Sep-15-LIV, Sep-16-LIV, Sep-17-LIV, and Sep-17-LIP (Table 1) were obtained, each coming from one symptomatic leaf selected based on outbreaks observed on different genotypes from 2014 to 2017. The samples were incubated at room temperature for 48 h in a chamber with moistened paper in order to promote pathogen development. Strains were isolated by spreading exudate from one sporulating pycnidium on MacConkey medium (MacConkey 1905). Monospore isolates were obtained through three successive propagation steps from one sporulating pycnidium. Cultures were incubated in a growth chamber at 22°C with a 12-h photoperiod. These five isolates were further studied (Table 1).

Morphological characterization of Septoria *sp.* For the morphological characterization of each isolate, mature pycnidia with exuded conidia were removed and placed on a microscope slide with cotton blue (lactic acid with blue methylene) and observed under a microscope (Olympus BH2 series, OSI, France; magnification 1,000; 100×; oil microscope objective) using a micrometric scale. Color and shape of pycnidia were observed and compared with Hayward and Waterston (1966). The size of 30 conidia per isolate were scored using the NIKON DS-FI3 camera and the NIS-Elements software.

Validation of Koch's postulates. The Sep-15-LIP isolate was selected to complete Koch's postulates, as it was the first isolate for which the hosting genotype (E161718) was known (Table 1). The isolate was maintained on Mac medium at 4°C. Conidia from sporulating pycnidia were spread on 10 plates of solid Oat Agar Chloramphenicol (OAC; 50 mg/liter Chloramphenicol) medium using a sterile Pasteur pipette with curved tip. Cultures were incubated at 22°C with a 12-h photoperiod for 3 weeks. Conidia were harvested by adding sterile distilled water, breaking pycnidia with a Pasteur pipette, and agitating by hand. The total resulting suspension was filtered at 450 μm to discard the pycnidia. Conidia enumeration was performed on a Malassez cell. Inoculum concentration was adjusted to 10^5 conidia/ml, and Tween 20 was added at 0.01% for plantlet inoculation.

Five 9-week-old *S. rebaudiana* plants, genotype E161718 (Table 2), were inoculated with a conidial spray (8 ml per plant) using a hand-sprayer. Five negative control plants were sprayed with sterile water. Plants were placed in an isolated growth chamber with a sodium lamp for 21 days at 20 to 25°C, with a 16-h photoperiod and a relative humidity between 80 and 85%. Twenty-one days after inoculation, symptomatic leaves were collected and incubated in a Plexiglas moist chamber. The isolate was reisolated and morphologically characterized as previously described.

Genotypic characterization through multilocus sequence typing (MLST). *DNA extraction.* Cultures from monospore of each of the five isolates were incubated on solid OAC media for 10 days at 22°C with a 12-h photoperiod. Pycnidia and sterile mycelium were harvested and transferred to Eppendorf tubes. The materials were frozen at –80°C and lyophilized in a freeze-dryer overnight (Alpha 1-4 LDplus, Christ, Germany). Dried tissue was ground twice for 30 s by glass balls with a grinder (TissueLyser II, Qiagen, France). The resulting powder was extracted by an adapted CTAB method (Zolan and Pukkila 1986). Two milliliters of extract was used to quantify the amount of DNA by an Epoch spectrophotometer (BioTek, U.S.A.). For each of the five isolates, DNA concentration was adjusted to 10 ng/μl and 200 ng/μl for PCR.

PCR amplification and sequencing. Septoria species identification followed the protocol described in Verkley et al. (2013). Seven loci, actin (ACT), calmodulin (CAL), β-tubulin (β-TUB), internal transcribed spacer (ITS), Translation elongation factor 1-α (EF-1α), 28S nrDNA (LSU), and RNA polymerase II second largest subunit (RPB2) were amplified using primers described by Verkley et al. (2013). PCR amplification was performed in a final volume of 15 μl, containing 20 ng of extracted DNA (or 400 ng only for RPB2 amplification), 1× Buffer DyNAzyme, 0.2 mM of mixed dNTPs, 0.4 μM of each primer, and 0.5 U/μl of DyNAzyme II (Thermo Scientific). The PCR amplification steps were: 2 min at 94°C followed by 40 cycles at 96°C for 45 s, primer annealing as described in Verkley et al. (2013), elongation at 72°C for 90 s, and a final elongation step at 72°C for 10 min. For ACT and RPB2 loci, in the first 10 cycles, primer annealing started at annealing temperature + 10°C, and the temperature was reduced by 1°C per cycle, for 30 s. For the 30 remaining cycles, primer annealing was as described in Verkley et al. (2013). For each locus, two independent PCR amplifications were performed. The resulting fragments were sequenced by GENEWIZ (U.S.A.) with forward and reverse primer.

Sequence alignment and phylogenetic analysis. Sequence alignment and phylogenetic analysis followed the method developed by Verkley et al. (2013). Basic alignment of our isolate sequences was done with the complete sequences of the seven loci found in NCBI for 241 *Septoria* species (Verkley et al. 2013) using MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/index.html) for each locus. MEGA 7 (Kumar et al. 2016) was used to build the tree with concatenated sequences by the Neighbor-Joining method (Saitou and Nei 1987). The test of phylogeny was done through a bootstrap test (1,000 replicates). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004).

Screening of *S. rebaudiana* response. S. rebaudiana *genotypes.* Ten *S. rebaudiana* genotypes were selected from a collection whose

Table 1. Isolates of *Septoria* sp. isolated from *Stevia rebaudiana* experimental fields in southwestern France from 2014 to 2017

Isolate identification	Year of isolation	Experimental field location	Genotype on which isolate has been sampled
Sep-14-LIV	2014	Ste-Livrade-sur-lot, France	Unknown[z]
Sep-15-LIP	2015	Liposthey, France	E161718
Sep-16-LIV	2016	Ste-Livrade-sur-lot, France	E161718
Sep-17-LIV	2017	Ste-Livrade-sur-lot, France	Unknown[z]
Sep-17-LIP	2017	Liposthey, France	Unknown[z]

[z] Progeny from open pollinated cross.

performances in terms of biomass and SG yield have been evaluated in southwestern France conditions (Hastoy et al. 2019). These 10 genotypes have been chosen for their different origins to obtain genetic diversity (Table 2).

Plant production for controlled inoculation. For the evaluation of *S. rebaudiana* susceptibility under controlled conditions, the 10 selected genotypes were propagated by stem-cuttings from mother plants in greenhouse. Stem-cuttings were placed in commercial plug trays containing Peltracom soil (Greenyard Horticulture France SAS), without nutrient supply. Plantlets were grown in greenhouse conditions under a 15-h photoperiod for 4 weeks, with variable temperatures according to time: 6 h to 9 h: 18°C, 9 h to18 h: 22°C, 18 h to 21 h: 20°C, 21 h to 6 h: 16°C. Axillary meristems were removed during vegetative growth in order to avoid secondary ramifications. Then, the plantlets were placed in a nonregulated greenhouse and transplanted into 10 × 10 cm pots after 5 weeks of growing and then used for inoculation 1 week later.

Inoculum production. The Sep-15-LIP isolate used to establish Koch's postulates was selected for the germplasm evaluation. The inoculum was prepared as detailed for conducting Koch's postulates.

Inoculation in a spray chamber. A fixed spray chamber was used for inoculation of *S. rebaudiana* genotypes with *Septoria* isolate Sep-15-LIP (Euro-pulvé, Aspach, France) (Supplementary Fig. S1). The parameters of the spray chamber were set to 4 s of spraying with a flow rate of 1.8 liters/min and pressure at 3 bars. Five flat jet nozzles Teejet XR 8001VS (TeeJet Technologies), which produce fine droplets, were used to provide uniform distribution throughout the flat spray pattern. A maximum of 10 plants (1 plant per genotype) were arranged in staggered file in the middle of the spray chamber. Automatic spraying enabled the whole plants to be sprayed until run-off. A total of 16 plants per genotype were inoculated with prepared inoculum, and 4 negative control plants per genotype were sprayed with sterile water. The inoculated plants were air-dried and transferred to a growth chamber. The inoculation experiment was repeated three times during spring 2017. After each inoculation series, the spray chamber system and walls were washed out with sterile water and surface-sterilized using 70% ethanol. The spray nozzles were soaked in 70% ethanol overnight.

Experimental design in the growth chamber. For each experiment, the plants were placed in a unique growth chamber with a 400W sodium lamp for 21 days at 20 to 25°C, with a 16-h photoperiod and 80% relative humidity. Humidity was maintained by placing water in the bottom and sterile distilled water sprayed on the Plexiglas walls. The growth chamber was arranged into five randomized blocks. One block was dedicated to the negative control plants (four plants/genotype/experiment), sprayed with water first and immediately transferred to the growth chamber. The inoculated plants were distributed between the four remaining blocks (16 plants/genotype/experiment). The four plants of each genotype were randomized within the block. Plants were subirrigated every 5 days during the experiment.

Table 2. Genetic resources of *Stevia rebaudiana* evaluated for their response to *Septoria* leaf spot through controlled inoculation

Provider	Country of the provider	Name[z]	Year of introduction in the collection	Origin
EUSTAS gene bank	Germany	C	2016	Colombia
		Gawi	2016	Unknown
STEVIA STORE	Paraguay	Eirete	2015	Paraguay
OVIATIS collection	France	E161718	2011	Argentina
		E8	2011	Argentina
		EspLac1	2013	Spain
		FP	2011	Argentina
		S6030-1	2015	Israel
		Lac4	2013	Argentina
		Larrère	2013	Argentina

[z] These genotypes have previously been phenotyped for biomass and SG yield in Hastoy et al. (2019).

Evaluation of S. rebaudiana *response.* Disease development was assessed at 12, 15, 18, and 21 days after inoculation (DAI). Disease severity (DS) was rated for each plant based on the percentage of plant tissue showing symptoms, using a 0 to 100% scale. These four ratings were used to calculate the area under the disease progress curve (AUDPC), as described in Jeger and Viljanen-Rollinson (2001).

At 21 DAI, in order to obtain a precise and quantitative response in controlled conditions, leaves from inoculated plants were separated for color scanning at 300 dpi with an Epson Expression 1640 XL flatbed scan. The resulting scans were analyzed with ImageJ software (version 1.51). Total foliar area was detected through binary image transformation, where whole leaves were detected automatically. Selection of the symptomatic area was performed on colored images by color threshold, with hue between 0 and 47, saturation between 37 and 255, and brightness between 0 and 204. These settings allowed the necrotic and chlorotic parts of leaves to be selected according to their color (Fig. 1). Symptomatic foliar area was measured and reported in cm^2. Disease severity was calculated as the proportion of symptomatic leaf area (PSLA) in relation to the total foliar area (%).

Statistical analysis. Statistical analyses were performed using R version 3.4.3 software (Mangiafico 2015; R Core Team 2015). The outliers were deleted using a Bonferroni test on Studentized residuals of a linear model from the "car" package (Fox and Weisberg 2011). Analysis of variance was applied to a mixed linear model by using the "lme4" (Bates et al. 2015) and "car" (Fox and Weisberg 2011) packages:

$$lmer(y \sim A + (1|B) + (1|C) + (1|A:B) + (1|A:C) + (1|B:C) + (1|A:B:C))$$

where y is the quantitative response, A is the fixed effect (genotype or time after inoculation), and B and C are random effects (experiment, randomized block, and their interaction).

Estimated marginal means and standard errors were calculated from mixed models, and significant differences between genotypes were determined by Tukey's Honestly Significant Difference (HSD) test from the "emmeans" package (Lenth et al. 2018). Significant differences in symptom development according to time were

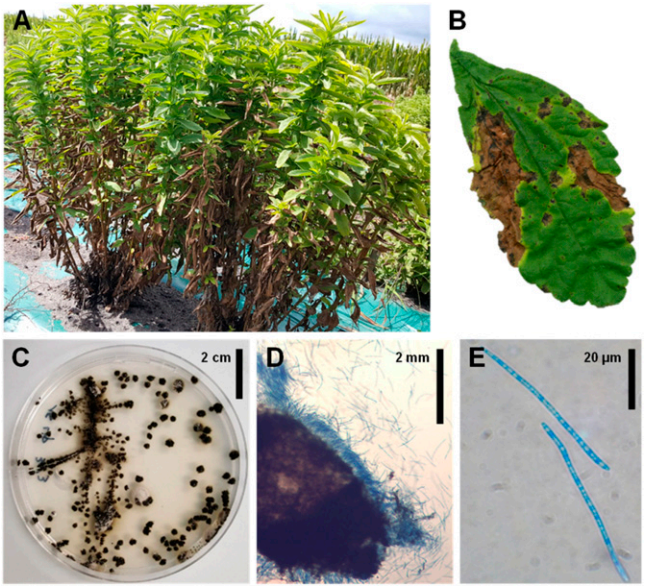

Fig. 1. Identification of Septoria leaf spot disease on *Stevia rebaudiana* in the southwest of France. (**A**) Symptoms under field conditions in Liposthey (France), (**B**) symptomatic leaves after 48 h of incubation in a humidity chamber, (**C**) colony morphology on Murashige & Skoog medium, (**D**) characteristic brown pycnidia, and (**E**) conidia of the Sep-15-LIP isolate.

analyzed through pairwise comparison of the regression slope with the "emmeans" package (Lenth et al. 2018). For data representation, "ggplot2", "cowplot", and "extrafont" packages were used (Chang 2014; Wickham 2009; Wilke 2017).

Results

Characterization of *Septoria* sp. isolated from experimental field in France on *S. rebaudiana*. In experimental fields, black leaf spots with surrounding chlorosis were observed on the lower older leaves and progressed upward (Fig. 1A and B), corresponding to symptoms previously described as SLS on *S. rebaudiana* (Bhandari and Harsh 2006; Ishiba et al. 1982; Lovering and Reeleder 1996; Reeleder 1999; Veia Sanabria and Orrego Fuente 2013). Based on morphological observations of pure culture, the isolates were determined to be from the genus *Septoria* (Fig. 1C to E). They produced black and spherical pycnidia, containing hyaline and threadlike pycnidiospora of mean length 52.7 µm (SD +/– 13.3) × mean width 1.6 µm (SD +/– 0.3) corresponding to partitioned conidia, linked to pycnidia by conidiogenous cells. These morphological characteristics correspond to the genus *Septoria* (Quaedvlieg et al. 2013). The teleomorph was not observed.

Koch's postulates were fulfilled, as it could be demonstrated that the same symptoms as those observed in the field could be obtained (Fig. 2) after inoculation with isolate conidia. No symptom was observed on control plants. Macroscopic description and ITS sequencing confirmed *Septoria* sp. (Fig. 3).

Phylogenetic classification of Septoria *isolates using multilocus sequence typing.* Multilocus sequence typing (MLST) approach provided the highest species resolution of the genus *Septoria* (Verkley et al. 2013). Based on this previous study, the five isolates (Table 1) were sequenced for seven specific loci and compared with the sequences of 241 *Septoria* isolates (Supplementary Fig. S2). Phylogenetic analysis indicated that the five from stevia fell among the clades of *Septoria* spp. described by Verkley et al. (2013), thus we confirmed that the isolates from *S. rebaudiana* belonged to the genus *Septoria* and were phylogenetically related to subclade 4A (Fig. 3). This subclade contains species from miscellaneous host plants including *Septoria lycopersici* (Solanaceae), *S. paridis* (Trilliaceae and Violaceae), and *S. apiicola* (Apiaceae). On the basis of MLST analysis, it could be concluded that these isolates are closely related to *S. lycopersici* and *S. apiicola*. Despite different field origins and years of isolation, the five French isolates appear to be phylogenetically related.

Evaluation of *S. rebaudiana* genotypic response to *Septoria* isolate under controlled conditions. The response of 10 *S. rebaudiana* genotypes to *Septoria* isolate 15-LIP was characterized by three separate automated inoculations. Control plants inoculated with sterile water remained asymptomatic during the experiments, confirming that the *Septoria* isolate was responsible for the appearance of symptoms. Small black lesions developed approximately 10 days after inoculation. As disease developed, the larger necrotic lesions became surrounded by a small chlorotic halo.

At 12 DAI, all the tested genotypes were symptomatic, indicating the absence of complete resistance among these genotypes (Fig. 4). Analysis of DS score variance indicated a strong genotype effect at the first scoring period 12 DAI ($P < 0.001$; Supplementary Table S2). At 12 DAI, "Gawi" and "EspLac1" were the least symptomatic, with less than 5% of the whole plants showing visible symptoms. They were significantly different from "E8" and "E161718", with the highest DS ranging from 5 to 10% (Fig. 4). At 21 DAI, "Gawi" and "EspLac1" remained the least symptomatic genotypes, with less than 10 to 15% of the whole plants affected, whereas "E161718", "E8", and "C" appeared to be the more susceptible, reaching a 40% DS score ($P < 0.05$; Fig. 4).

Fig. 2. Septoria leafspot symptoms on E161718 *Stevia rebaudiana* genotype after *Septoria* sp. isolation and reinoculation.

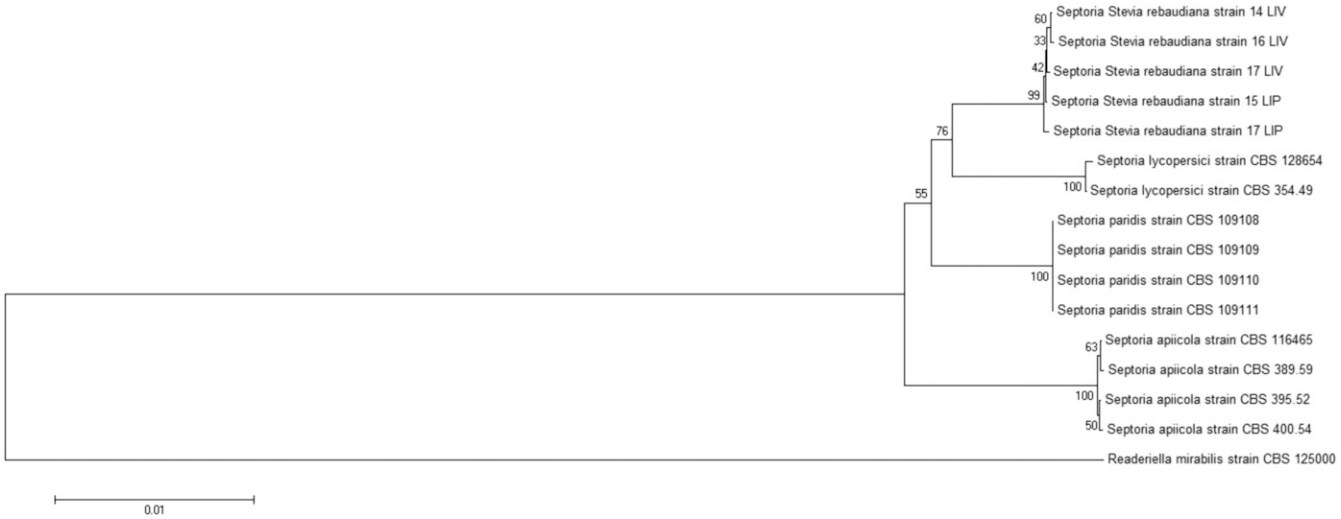

Fig. 3. Phylogenetic classification of *Septoria* strains isolated from *Stevia rebaudiana* in France using multilocus sequence typing of seven loci. Subclade 4A established by Verkley et al. (2013) is represented. The tree was obtained on concatenate sequences of 241 *Septoria* strains for 7 loci using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

As visual scoring can be subjective and experimenter-dependent, the scoring was also conducted using a more precise symptomatic foliar area proportion (PSLA) quantification by image analysis at 21 DAI (Fig. 5). Analysis of variance confirmed the genotype effect ($P < 0.001$). There was no difference in genotype ranking between the visual scoring and image quantification methods. Multiple comparisons showed only significant differences between the most susceptible genotypes "E161718" and "C" and the less susceptible ones, "Gawi" and "EspLac1" (Fig. 5). The susceptible genotypes were scored around 15% symptomatic through PSLA image analysis quantification, whereas the least susceptible developed symptoms on only 3% of the total leaf area (Fig. 5).

In order to assess disease progress per genotype, DS was scored between 12 and 21 DAI. It appeared to be linear over time, and the regression slopes were significantly different according to the genotype (Fig. 4; Table 3). "C" and "E161718" presented the significant highest slope coefficients over 3.0, which corresponded to the faster symptom development under our controlled conditions. This was confirmed by AUDPC that was calculated from DS recorded four times. "E161718", "C", and "E8" presented the highest AUDPC (Fig. 6). On the contrary, symptoms developed significantly more slowly for "Gawi" and "EspLac1" genotypes, which presented regression slopes of 1.07 and 1.46, respectively (Fig. 4; Table 3). Their limited susceptibility was confirmed by AUDPC: these two genotypes presented the smallest AUDPC (Fig. 6).

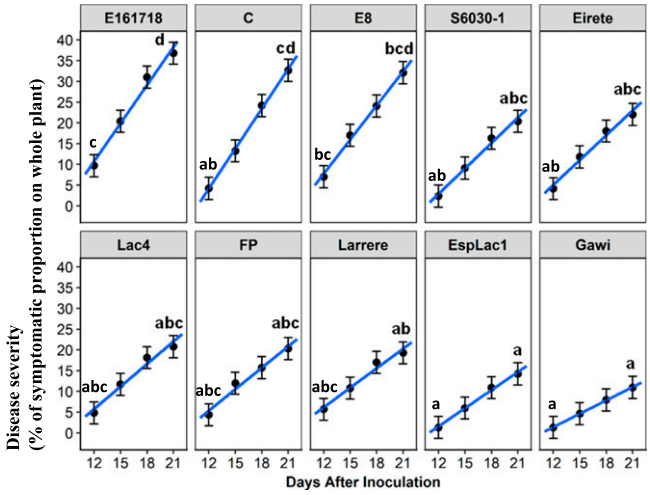

Fig. 4. Disease severity of Septoria leaf spot between 12 and 21 days after inoculation on 10 genotypes of *Stevia rebaudiana* after automated inoculation under controlled conditions with Sep-15-LIP *Septoria* isolate from southwestern France. Disease severity was scored visually on a 0 to 100% scale based on the percentage of total plant showing symptoms. Results are expressed as Estimated Marginal Means of 16 plants per genotype, repeated 3 times, with standard error, according to time after inoculation. Blue line represents linear mixed model regression. Black letters show the result of multiple comparison at 12 and 21 DAI. At each DAI, genotypes with same letter are not significantly different at $P = 0.05$ level.

Discussion

This is the first report on Septoria leaf spot (SLS) caused by *Septoria* sp. on *S. rebaudiana* in France. As no teleomorph of *Septoria* sp. is known on Asteraceae (Verkley and Starink-Willemse 2004), only anamorphs were observed. The MLST approach grouped the French *Septoria* isolates phylogenetically with the subclade 4A of the genus *Septoria* (Verkley et al. 2013), which contains species from a number of miscellaneous host plants (Solanaceae, Apiaceae, Violaceae, Trilliaceae, and here Asteraceae). Historically, host specificity was the principal criterion for species delimitation in this genus. Isolates previously identified from *S. rebaudiana* were therefore named *Septoria steviae* (Ishiba et al. 1982). However, host occurrence should not be used as the only character for species identification (Verkley et al. 2013). Many *Septoria* spp. can infect multiple plant species within the same genus, but also allied families and genera (Beach 1919; Quaedvlieg et al. 2013; Teterevnikova-Babayan 1987). More recently, multiple family associations for a single species have been confirmed by a DNA-based approach (Verkley et al. 2013). Consequently, it was expected that a certain number of previously described taxa would be synonyms of species (Quaedvlieg et al. 2013). The MLST approach identified *S. lycopersici* as the closest related species to the French *Septoria* isolates. Two hypotheses therefore remain for the dissemination of *Septoria* on *S. rebaudiana*. The first hypothesis is that the fungi were present on other hosts before the introduction of *S. rebaudiana* in western countries (Reeleder 1999). The *Septoria* species does have the ability to jump successfully to hosts in a new family, but this genetic basis is still unclear (Verkley et al. 2013). As tomato is largely produced in southwestern France, it could therefore be hypothesized that *Septoria* on *S. rebaudiana* could belong to the species complex of *Septoria lycopersici*. Nevertheless, although *S. lycopersici* is present, it has very rarely been described in France (Blancard 2017). Therefore, a second

Table 3. Linear regression parameter of disease severity score according to time after inoculation for 10 genotypes of *Stevia rebaudiana* (Fig. 4). Result of multiple comparison on slope coefficient by HSD's Tukey test are indicated by letter. Same letter shows no significant difference at $P = 0.05$ level.

Genotype	Linear regression slope
Gawi	1.07 ± 0.21 a
EspLac1	1.46 ± 0.22 ab
Larrère	1.57 ± 0.21 ab
FP	1.72 ± 0.21 ab
Lac4	1.81 ± 0.21 b
Eirete	1.99 ± 0.21 b
S6030-1	2.04 ± 0.21 bc
E8	2.74 ± 0.21 cd
E161718	3.06 ± 0.21 d
C	3.20 ± 0.22 d

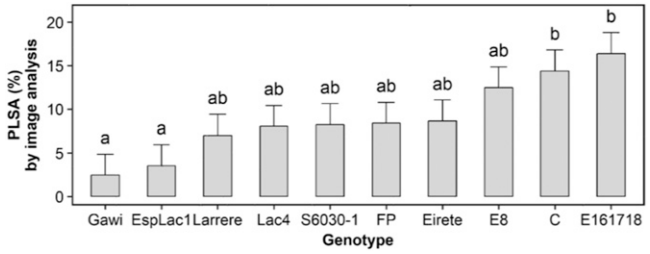

Fig. 5. Proportion of Symptomatic Foliar Area (PLSA) at 21 DAI calculated by image analysis for 10 *Stevia rebaudiana* genotypes inoculated under controlled conditions with Sep-15-LIP *Septoria* isolate from southwestern France. Results represent Estimated Marginal Means of 16 plants per genotype, repeated 3 times, with standard error. Black letters show the result of multiple comparison by Tukey's HSD. Genotypes with same letter are not significantly different at $P = 0.05$ level.

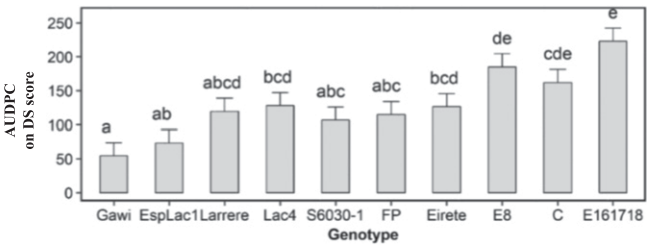

Fig. 6. AUDPC calculated from Disease severity scores at 12, 15, 18, and 21 DAI of 10 *Stevia rebaudiana* genotypes inoculated under controlled conditions with the Sep-15-LIP *Septoria* isolate from southwestern France. Results represent Estimated Marginal Means of 16 plants, repeated 3 times, with standard error. Black letters show the result of multiple comparison by Tukey's HSD. Genotypes with same letter are not significantly different at $P = 0.05$ level.

hypothesis could emerge. The infection of *S. rebaudiana* by *Septoria* has already been described in Japan (Ishiba et al. 1982). It has also been reported in North Carolina (Lovering and Reeleder 1996), Canada (Reeleder 1999), India (Bhandari and Harsh 2006), and Paraguay (Veia Sanabria and Orrego Fuente 2013), in accordance with the wide geographic distribution of genus *Septoria* (Quaedvlieg et al. 2013). It can therefore be supposed that this fungus coexists with the host in the country of origin of the plant (Paraguay) and could be present worldwide in the seed or plantlet material that is distributed (Reeleder 1999). This hypothesis is supported by the description of *Septoria* in Paraguay since 1996 (Veia Sanabria and Orrego Fuente 2013) and by seedborne *Septoria* disease on other crops, such as lettuce or wheat (Bertus 1972; Shah et al. 1995). However, there is no evidence of seedborne *Septoria* on *S. rebaudiana*. None of these studies produced molecular characterizations of the described isolates, thereby preventing any comparison between their isolates and those isolated in France. The stevia septoria sequences present in the databases ("*Septoria" steviae* CBS 120132) are those published in Quaedvlieg et al. (2013). As demonstrated by Quaedvlieg et al. (2013), these sequences were incorrectly characterized as *Septoria steviae*, as they are related to *Phoma* genus. Therefore, they could not be included in the analysis. For further study, it would be interesting to characterize *Septoria* isolated worldwide from stevia by MLST, particularly including isolates from Paraguay, which is the country of origin of *S. rebaudiana*. This knowledge would be helpful to conclude a new species in the *Septoria* genus as proposed by Ishiba et al. (1982) and its worldwide appearance.

An automated germplasm screening procedure under controlled conditions was used for the evaluation of the response of *S. rebaudiana* genotypes to *Septoria*. This automated procedure made the inoculation highly reliable and homogeneous, as we observed throughout our three independent experiments. In the literature, there is only one other study that evaluated the host resistance of 13 arbitrarily selected clonal lines of *S. rebaudiana* available from a breeding program (Reeleder 1999). A greenhouse-based assay was developed through manual spraying of combined isolates on 6-week-old cuttings. They also evaluated *S. rebaudiana* genotypes in field conditions. *S. rebaudiana* response was scored through the proportion of symptomatic leaf area, the percentage of symptomatic leaves, and the mean number of lesions per leaf. The two studies differ in terms of the length of the experiment, 21 DAI and 14 DAI, the inoculation method, automated versus manual, in our study and in Reeleder (1999), respectively, the inoculated strain and the tested germplasm. Nevertheless, they both conclude that a quantitatively variable response to *Septoria* sp. exists in *S. rebaudiana*. The quantitative gradient in the percentage of diseased leaf area is similar in the two studies, from around 3 to 50%. Nevertheless, Reeleder (1999) found two genotypes expressing complete resistance, which was not the case in our study.

Our results clearly identified moderately susceptible genotypes "Gawi" and "EspLac1", which both had lower disease severity ratings and a slower rate of disease development. On the contrary, "E161718", "E8", and "C" were determined to be highly susceptible with rapid disease progress. The other genotypes that were tested present an intermediate response, such as the widely produced "Eirete" type.

Disease response was phenotyped mainly in terms of the proportion of diseased plants, the proportion of symptomatic foliar area, and disease progress. In wheat, quantitative resistance to Septoria tritici blotch was phenotyped in terms of the degree of host damage, such as the percentage of leaf area covered with lesions, but also of variables related to pathogen reproduction, such as the density of pycnidia produced within lesions (Karisto et al. 2018). These two phenotypes are independent. They could help to provide a more detailed understanding of the response in *S. rebaudiana/Septoria* interactions.

In our study, the genotypes were screened against one of five isolates. In the future, for durable and effective quantitative resistance to be identified, screening against a diverse set of isolates will be necessary. This was demonstrated by Abrinbana (2018), where differences in aggressiveness were demonstrated between five populations of *Zymnoseptoria tritici* (causal agent of Septoria tritici blotch) from Iran on two wheat cultivars.

The combination of an automated inoculation procedure and quantitative scoring by researcher scoring or image analysis provides a very convenient tool for germplasm screening. Researcher scoring allows us to score the response at regular time slots but can be greatly experimenter dependent. Image analysis allows the scoring of accurate quantitative data through automated scripts. As it is independent from experimenter skills and gives quantitative data, it could be integrated into the screening of a larger *S. rebaudiana* germplasm collection, breeding programs and quantitative trait loci (QTL) detection. However, in our inoculation protocol, the material production and whole plant scanning remain time-consuming. It would be therefore necessary to develop a faster method in order to screen segregating populations, as done in poplar, for example, using excised leaf disks (Maxwell et al. 1997). Phenotyping could also be optimized by improving colored image analysis, as in wheat (Karisto et al. 2018). In order to demonstrate fungi development within living tissue precisely, in vivo chlorophyll fluorescence image analysis could also be used (Rousseau et al. 2013), providing a powerful nondestructive method.

The genotype ranking will also have to be confirmed in field trials under natural infection, even though promising initial results have been observed in experimental fields in France. The consequences of infection in terms of quantity and quality of SGs in the leaves also has to be investigated.

This study is the first report of *Septoria* sp. outbreak on *Stevia rebaudiana* in France. Isolates were found to be closely related to *Septoria lycopersici* and *S. apiicola*. The use of an automated inoculation and scoring process led to screen the response of 10 diverse genotypes of *S. rebaudiana*. It constitutes a promising opening for the improvement of *S. rebaudiana* resistance to SLS. The optimization of phenotyping tools and screening of large germplasm open the field to breeding strategies focusing on SLS response and the development of sustainable, organic *S. rebaudiana* production.

Acknowledgments

The authors thank Coralie Chesseron (INRA, Bordeaux, France), Justine Peirotte (Fraise Concept', Douville, France), and Mathieu Mouravy (INVENIO, Douville, France) for plant production.

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