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ORIGINAL ARTICLE

Multiplex qPCR Assay for Detection and Relative Quantification of *Diaporthe aspalathi*, *D. caulivora*, *D. longicolla* and *D. miriciae* in Soybean Tissues

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Received: 23 December 2024 | **Revised:** 6 March 2025 | **Accepted:** 7 March 2025

Funding: This work was funded by Agencia Nacional de Investigación e Innovación (ANII), grant FCE_3_2022_1_172688, Uruguay, and Programa de Desarrollo de las Ciencias Básicas (PEDECIBA) Uruguay.

Keywords: *Diaporthe* | molecular diagnosis | qPCR | soybean stem canker

ABSTRACT

Soybean (*Glycine max*) is one of the most economically important crops in Uruguay and is affected by soybean stem canker (SSC) disease. The main pathogens involved are *Diaporthe aspalathi*, *D. caulivora*, *D. longicolla*, and *D. miriciae*, causing similar symptoms, including brown-to-reddish necrotic lesions on stems and wrinkled, cracked seed coats. The presence of these pathogens in asymptomatic plants, along with their similar morphology, constitutes a challenge for conventional disease diagnosis. Currently, no resistant cultivars are available for all *Diaporthe* species, and effective control methods are lacking, making prevention the primary strategy. Therefore, early detection and accurate identification of the specific *Diaporthe* species involved are essential for minimising losses. This study aimed to detect and quantify the three most prevalent *Diaporthe* species in Uruguay, *D. caulivora*, *D. longicolla*, and *D. miriciae*, and identify *D. aspalathi* in stem canker lesions using multiplex quantitative PCR (qPCR). Species-specific TaqMan primer-probe sets targeting the translation elongation factor 1-alpha gene (*tEF1a*) were designed for each *Diaporthe* spp. The specificity and sensitivity of the primer-probe sets were tested using genomic DNA. The assay was validated using stem tissues and seed samples artificially inoculated with one or more *Diaporthe* species. Additionally, the multiplex qPCR assay was evaluated in field-collected soybean stem and seed samples in 2024, and seed lots from different origins. Our results demonstrate that this multiplex qPCR assay effectively detects and quantifies *Diaporthe* species individually and simultaneously, providing a valuable diagnostic tool for seed certification, pathogen surveillance, and the development of effective strategies for SSC management.

1 | Introduction

Fungal *Diaporthe* species cause widespread and destructive soybean diseases, including seed decay caused by *D. longicolla*, pod and stem blight caused by *D. sojae*, and stem canker caused by *D. aspalathi*, *D. caulivora*, and *D. longicolla*, among others (Hosseini et al. 2020; Udayanga et al. 2015). Soybean stem canker (SSC) disease symptoms caused by these *Diaporthe* spp. are similar and consist of necrotic lesions on the stems, wilting, and plant death due to stem girdling (Mena et al. 2024b). Infected soybean seeds are smaller, more elongated, and lighter than healthy seeds, and exhibit reduced germination rates (Hosseini et al. 2020; Sinclair 1993). Some seeds have wrinkled and cracked seed coats, while others are symptomless (Sinclair 1992). A pathogen survey of symptomatic soybean plants conducted over several years revealed

that *D. caulivora*, *D. longicolla*, and *D. miriciae* are the most prevalent species causing SSC in Uruguay (Mena et al. 2020). Recently, two isolates of *D. masirevicii* were detected in stem canker lesions (Mena et al. 2024a). While resistance loci have been identified for *D. aspalathi* (locus *Rdm 1–6*) and *D. caulivora* (locus *Rdc1*) (Pioli et al. 2003; Peruzzo et al. 2019), only soybean cultivars with *Rdm 1–6* are currently available for commercial use. The presence of these resistant genotypes in Uruguayan farms likely contributes to the reduced prevalence of *D. aspalathi* isolates and the absence of this species in the survey (Mena et al. 2020). Thus, sanitation practices and prevention are the available management strategies in the absence of other resistant cultivars and effective chemical control measures (Mena et al. 2024b; Roth et al. 2020). Furthermore, the similar morphological and disease characteristics of *Diaporthe* pathogens constitute a challenge for conventional disease diagnosis. Therefore, the accessibility of a fast, accurate, and sensitive molecular method to effectively identify and quantify *Diaporthe* spp. associated with SSC is required.

The internal transcribed spacer (ITS) region of the ribosomal DNA and genes encoding translation elongation factor 1-α gene (*tEF1a*), actin, β-tubulin, histone H3, and calmodulin are commonly used in the identification and phylogenetic study of *Diaporthe* spp. (Hosseini et al. 2020; Mena et al. 2020; Udayanga et al. 2014). Conventional PCR and loop-mediated isothermal amplification are simple and reproducible techniques that have been previously used to detect *Diaporthe* in infected soybean tissues (Mena et al. 2020; Vechiato et al. 2006; Zhang et al. 1999). Real-time quantitative PCR (qPCR) incorporates quantitative measurements and offers additional advantages, including high sensitivity, which enables the detection of pathogens in the early stages of infection, even in asymptomatic plants (Schaad and Frederick 2002). qPCR assays developed for two *Diaporthe* pathogens responsible for Phomopsis stem canker in sunflowers have proven to be reliable diagnostic tools and have facilitated the screening of sunflower germplasm for disease resistance (Elverson et al. 2020). More recently, a multiplex qPCR assay was developed to distinguish between *D. longicolla*, *D. caulivora*, *D. eres*, and *D. novem* in soybean seeds (Hosseini et al. 2021). The use of TaqMan probes with different fluorescent dyes enabled the detection of several *Diaporthe* DNAs in a single reaction and discrimination between different species in the same multiplex qPCR assay (Hosseini et al. 2021), which reduces reagent costs and time. Notably, up to three different *Diaporthe* species were identified in a single seed sample (Hosseini et al. 2021). This method has been previously used for the detection of oomycete, fungal, bacterial, and viral plant pathogens (Hosseini et al. 2021; Schaad and Frederick 2002; Schena et al. 2006).

In the present study, we obtained additional *Diaporthe* isolates from soybean stems with SSC lesions, demonstrating that *D. caulivora*, *D. longicolla*, and *D. miriciae* are the most prevalent *Diaporthe* species causing SSC in Uruguay. Then, we designed and validated a new multiplex TaqMan qPCR assay for the detection and quantification of these three *Diaporthe* spp. This assay was tested on seed and stem samples from soybean plants cultivated by Uruguayan farmers, as well as on seed lots from various origins. In addition, we included a TaqMan probe for *D. aspalathi* in the qPCR assay to detect its presence in Uruguay.

2 | Materials and Methods

2.1 | *Diaporthe* Isolates Obtained From SSC Lesions

In order to study the prevalence of *Diaporthe* spp. associated with SSC symptoms in Uruguay, *Diaporthe* spp. were recovered from soybean stem tissues with SSC in farms during 2022–2023 and 2024 (Table S1). Briefly, symptomatic soybean stems were collected and disinfected by submerging them in 70% ethanol for 30 s. Tissue fragments of 5 mm were cut out from the upper edge of diseased stem cankers (10–15 mm from the lesion) and placed onto Petri dishes containing potato dextrose agar (PDA; Difco). PDA plates were incubated at 25°C for 3–5 days, and isolates were purified by the single hyphal tip method. All the fungal species were grown on PDA plates at 25°C and were maintained in 30% glycerol at −80°C until use.

2.2 | DNA Extraction From Mycelium

For DNA extraction, approximately 100 mg of mycelium was recovered from 8-day-old cultures grown on PDA. Samples were ground in liquid nitrogen, and total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. DNA concentrations were determined by measuring the absorption at 260/280 nm in a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

2.3 | Identification of *Diaporthe* Species

A partial sequence of the *tEF1a* gene and, in some cases, the ITS region of nuclear rDNA, was amplified for each isolate using the fungal primers EF1-728F and EF1-986R (Carbone and Kohn 1999) or ITS4 and ITS5 (White et al. 1990), respectively. The PCR conditions used are as described by Mena et al. (2020). The sizes of the amplified DNA products were determined by electrophoresis in a 1.5% agarose gel in Tris/acetate/EDTA (TAE) at 120 V for 30 min, stained with ethidium bromide and visualised by UV light. PCR products were purified, sequenced in both directions and BLAST searches performed by Macrogen (Korea).

2.4 | Inoculation and Symptom Evaluation of Soybean Plants Grown Under Controlled Conditions

Soybean susceptible plants and seeds of cv. Williams (PI 548631) were used for inoculation under controlled conditions. Plants were grown in a growth room under a 16 h light/8 h dark regime at 24°C. For symptom evaluation and qPCR assays, three plants at the V3 stage were inoculated with *D. aspalathi* (Dpm2), *D. caulivora* (D57), *D. longicolla* (D43.1) and *D. miriciae* (D67). For symptom evaluation, one mycelial plug of each individual isolate taken from 7-day-old

PDA cultures was placed in a 5-mm wound made on the stems (Mena et al. 2020). Inoculations with sterile PDA plugs served as negative controls. Lesion length was determined at 5, 7, and 11 days post-inoculation (dpi) according to Mena et al. (2020). The area under the disease progress curve (AUDPC) was calculated over time (5–11 dpi; Mena et al. 2020). In addition, 10 soybean seeds were artificially inoculated with each *Diaporthe* spp. after superficial disinfection in 70% ethanol for 30 s. Briefly, an individual mycelium plug of *D. aspalathi* (Dpm2), *D. caulivora* (D57), *D. longicolla* (D43.1) or *D. miriciae* (D67) was placed in the middle of a PDA plate and 10 seeds were randomly scattered around the plug. One PDA plate with a sterile PDA plug and 10 seeds was used as a control. All the PDA plates were incubated in the growth room, and the seeds were collected after 7 days for symptom evaluation, when the mycelia reached the edge of the PDA plate.

2.5 | Plant Material for Multiplex qPCR Assays

2.5.1 | Soybean Plants Inoculated Under Controlled Conditions

Three soybean plants were inoculated with *D. aspalathi* (Dpm2), *D. caulivora* (D57), *D. longicolla* (D43.1) and *D. miriciae* (D67) alone or in combinations. For multiple inoculations, two, three, or four *Diaporthe* spp. were grown together on the same PDA Petri dish, and a mycelial plug was taken from the intersection of the different mycelia and placed on a 5-mm wound on the stems under controlled conditions. In total, 16 treatments were performed (Table 1). Soybean stem samples were taken from *Diaporthe*-inoculated plants at 14 dpi, and stem samples without pathogen were used as controls. A pool of three stems of three individual plants constituted a sample.

2.5.2 | Plant Tissues With SSC Symptoms Collected From the Field and Seed Lot Samples

Stem and seed samples were collected from several local fields in the Canelones, Colonia, Flores, San José, Soriano, and Río Negro departments of Uruguay during March and April of 2024. Soybean plants with symptoms of SSC, often with pycnidia structures on the stem, were selected. In addition, two asymptomatic plants were included. Plant materials were disinfected with sodium hypochlorite (1%) and liquid soap and washed with sterile distilled water. Surface-disinfected soybean seeds were squeezed to remove their seed coats. Seed lot samples were obtained from various seed companies in October and November of 2024. In total, 200 seeds of each lot were disinfected and embedded in Milli-Q sterile water for 6 h. All material samples were stored at −20°C until use.

2.6 | DNA Extraction From Plant Samples

For plant DNA extraction, 150 mg of tissue was used, taken from a pool of three stems (3 cm) of plants grown under controlled conditions or one stem or five seeds from plants grown under field conditions. For each seed lot, 200 seeds were homogenised, and 200 mg of tissue was used. All samples were ground in liquid nitrogen, and total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. DNA concentrations were determined by measuring the absorbance at 260/280 nm in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

2.7 | Primer and Probe Design for TaqMan Assay

New primer sets and probes were designed based on the *tEF1a* DNA sequences of *D. aspalathi* (Dpm2), *D. caulivora* (D57) and *D. miriciae* (D67) that showed sufficient single-nucleotide polymorphism (SNPs; Da-tEF1a, Dc-tEF1a and Dmi-tEF1a). Primer set Dl-tEF1a for *D. longicolla* was obtained from Hosseini et al. (2021) with some modifications (Figure S1 and Table 2). Primer and probe (TaqMan) design and specificity was carried out using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). In 'Primer Pair Specificity Checking Parameters' we entered Refseq representative genomes as organisms *Diaporthe*, *Fungi* and *Glycine max*. In 'Advanced parameters', 'Primer size' was selected as 18–24 and 'GC content' as 40%–60%. Melting temperatures and potential secondary structures of primers and probes were evaluated with OligoAnalyzer (https://www.idtdna.com/pages/tools/oligoanalyzer). In order to predict potential false positive results in multiplexing, all possible combinations of oligonucleotide sequences (primer, probe) were evaluated in in silico PCR tests in Primer-BLAST for compatibility between primers and similarities to sequences of other *Diaporthe/Phomopsis* spp. or phylogenetically close fungi from

TABLE 1 | Treatments performed by artificial inoculations under controlled conditions with one isolate or combinations of two, three or all four *Diaporthe* spp., and control treatment without fungi.

No. of isolates				
0	**1**	**2**	**3**	**4**
Control without fungi	*D. aspalathi* (Dpm2)	Dpm2–D57	Dpm2–D57–D43.1	Dpm2–D57–D43.1–D67
	D. caulivora (D57)	Dpm2–D43.1	Dpm2–D57–D67	
	D. longicolla (D43.1)	Dpm2–D67	Dpm2–D43.1–D67	
	D. miriciae (D67)	D57–D43.1	D57–D43.1–D67	
		D57–D67		
		D43.1–D67		

TABLE 2 | TaqMan primer-probe combinations for detection of *D. aspalathi* (Da), *D. caulivora* (Dc), *D. longicolla* (Dl) and *D. miriciae* (Dm) in soybean samples.

Primer and probe set	Primer	Sequence (5′–3′)	Efficiency (%)	References
Da-tEF1a	Da-F	GGAGTGCTTATCTCCGCCT	93	This study
	Da-R	CGTTCTCAGAAAGAGTACCGTCG		
	Da-P	Cy5-ATCACACATGCTGTCCCCCCTGC-BHQ2		
Dc-tEF1a	Dc-F	ACCACCATTTCCGCCACCA	93	This study
	Dc-R	TAGAGAAGAGGTCAGCATCATGC		
	Dc-P	FAM-GCCGTCGCCCGCCCACATC-Dabcyl		
Dl-tEF1a	Dl-F	TGTCGCACCTTTACCACTG	98	Hosseini et al. (2021)
	Dl-R	GAACGATCCAAAAAGCTCTC		
	Dl-P	HEX-GCATCACTTTCATTCCCACTTTCTGC-BHQ1		
Dm-tEF1a	Dmi-F	GCCGCTTATCTCACACATCAAACC	90	This study
	Dmi-R	GGAACCCTTGCCCAGCTC		
	Dmi-P	ROX-CATCTGCCACCCTTCACC-BHQ2		
Soybean (*Glycine max*)	Gm-F	GCCTCTGGATACCTGCTCAAG	95	Hosseini et al. (2021)
	Gm-R	ACCTCCTCCTCAAACTCCTCTG		

other genera such as *Mazzantia*, *Ophiodiaporthe*, *Pustulomyces*, *Phaeocytostroma* and *Stenocarpella* (Gao et al. 2017). Internal control primers for soybean DNA detection were included. All oligonucleotide primers and probes were synthesised by Macrogen (Seoul, Korea) and are shown in Table 2. Primer specificity was tested in qPCR. The efficiency of all primers was between 90% and 98%.

2.8 | Specificity of Multiplex qPCR Assay

In initial tests, genomic DNA of *D. aspalathi* (Dpm2), *D. caulivora* (D57), *D. longicolla* (D43.1) and *D. miriciae* (D67) was used to optimise the qPCR conditions, using QuantiNova SYBR green PCR kit (Qiagen) and a QuantStudio 3 thermocycler (New Applied Biosystem). Each reaction consisted of 1× SYBR Green mix, 0.7 μM primer mix, and 50 ng genomic DNA in a final volume of 20 μL. The thermocycler was programmed to run for 3 min at 95°C, followed by 40 cycles of 15 s at 95°C and 45 s at 60°C. Further denaturation at 95°C for 15 s, holding at 60°C for 1 min, and heating from 60°C to 95°C for 15 s were carried out for melting curve analysis.

TaqMan qPCRs were performed in a MIC 5-plex qPCR system (Bio Molecular Systems) using 2× SensiFAST Probe No-ROX mix (Meridian Bioscience) according to the manufacturer's protocol. For plant samples, reaction mixtures in singleplex qPCR assays contained 10 μL 2× SensiFAST Probe No-ROX mix, 1.6 μL of each forward and reverse primer, 0.4 μL probe, and 50 ng template DNA. Reaction mixtures for duplex and quadruplex qPCR assays contained 10 μL 2× SensiFAST Probe No-ROX mix, a reduced amount of 0.8 μL of each forward and reverse primer, 0.2 μL of each of the two probes, and 50 ng template DNA. In addition, reaction mixtures for quantifying soybean DNA consisted of 10 μL 2× SensiFAST SYBR No-ROX mix, 1.6 μL of each forward and reverse primer for soybean and 50 ng template DNA. The qPCR protocol consisted of initial denaturation 95°C for 3 min, 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 45 s. Reactions were run in technical duplicates. No-template negative controls were included for all assays.

2.9 | Standard Curves of TaqMan qPCR Assay

To determine the amplification efficiency of each primer-probe set, we used genomic DNA of the *Diaporthe* isolates in 10-fold serial dilutions from 10 to 0.001 ng/μL. The regression equations and R^2 coefficient of determination for quantification cycle (C_q) versus the concentration of amplified genomic DNA (gDNA) were calculated. The amplification efficiency (%) was calculated by the following equation: $[10^{(-1/\text{slope})} - 1] \times 100$ based on the logarithm of gDNA concentration.

3 | Results

3.1 | *Diaporthe* Species Causing SSC in Uruguay

During a *Diaporthe* survey of symptomatic soybean plants conducted in 2022–2023 and 2024, we identified 15 isolates of *D. caulivora*, 60 isolates of *D. longicolla*, four isolates of *D. miriciae*, and three isolates of *D. masirevicii* (Table S1). When these isolates were combined with those from our previous survey (Mena

et al. 2020), the total number of *Diaporthe* isolates increased to 161. Among them, *D. caulivora*, *D. longicolla*, and *D. miriciae* were the most prevalent species in Uruguayan soybean fields, with 48, 89, and 16 isolates, respectively (Table S1).

3.2 | Disease Symptoms Following *Diaporthe* Inoculation Under Controlled Conditions

Soybean stems were inoculated with *D. aspalathi*, *D. caulivora*, *D. longicolla*, and *D. miriciae*, and symptom development was observed at 11 dpi. Disease symptoms appeared on the stems of all soybean plants as 1–2 mm spots that gradually expanded into elongated brown lesions. The presence of pycnidia on soybean stems was observed in plants inoculated with *D. longicolla* and *D. miriciae* (Figure S2A). Soybean seeds were inoculated with *D. aspalathi*, *D. caulivora*, *D. longicolla*, and *D. miriciae*, and symptom development was observed at 7 dpi. All inoculated seeds displayed a marked colour change compared to the controls, and mycelium grew on the seeds. Infected seeds exhibited a range of visible symptoms, from minimal to severe, with colours varying between cream, brown, and black. Severely infected seeds were shrivelled, cracked, and often partially covered by a whitish mould growth. The most severe symptoms were observed in seeds inoculated with *D. longicolla* and *D. caulivora* (Figure S2B). Moreover, plants inoculated with these two species exhibited significantly greater lesion length and a higher area under the disease progress curve (AUDPC) (Figure S2C,D).

3.3 | Specificity and Standard Curves for Quantification Using the TaqMan qPCR Assay

The specificity of each primer pair was evaluated through qPCR using genomic DNA from the four *Diaporthe* species (Figure 1A). In these simplex qPCR assays, a single melting temperature (Tm) peak was observed, indicating amplification specificity of the primer-probe sets. When two species of *Diaporthe* were tested together, the TaqMan primer-probe sets retained similar specificity and showed discrimination between the particular pathogens present in the duplex assay (Figure 1B). Finally, when DNA from three or four *Diaporthe* spp. was used in a single qPCR, the detection of signals corresponding to all reporting dyes demonstrated the assay's ability to simultaneously

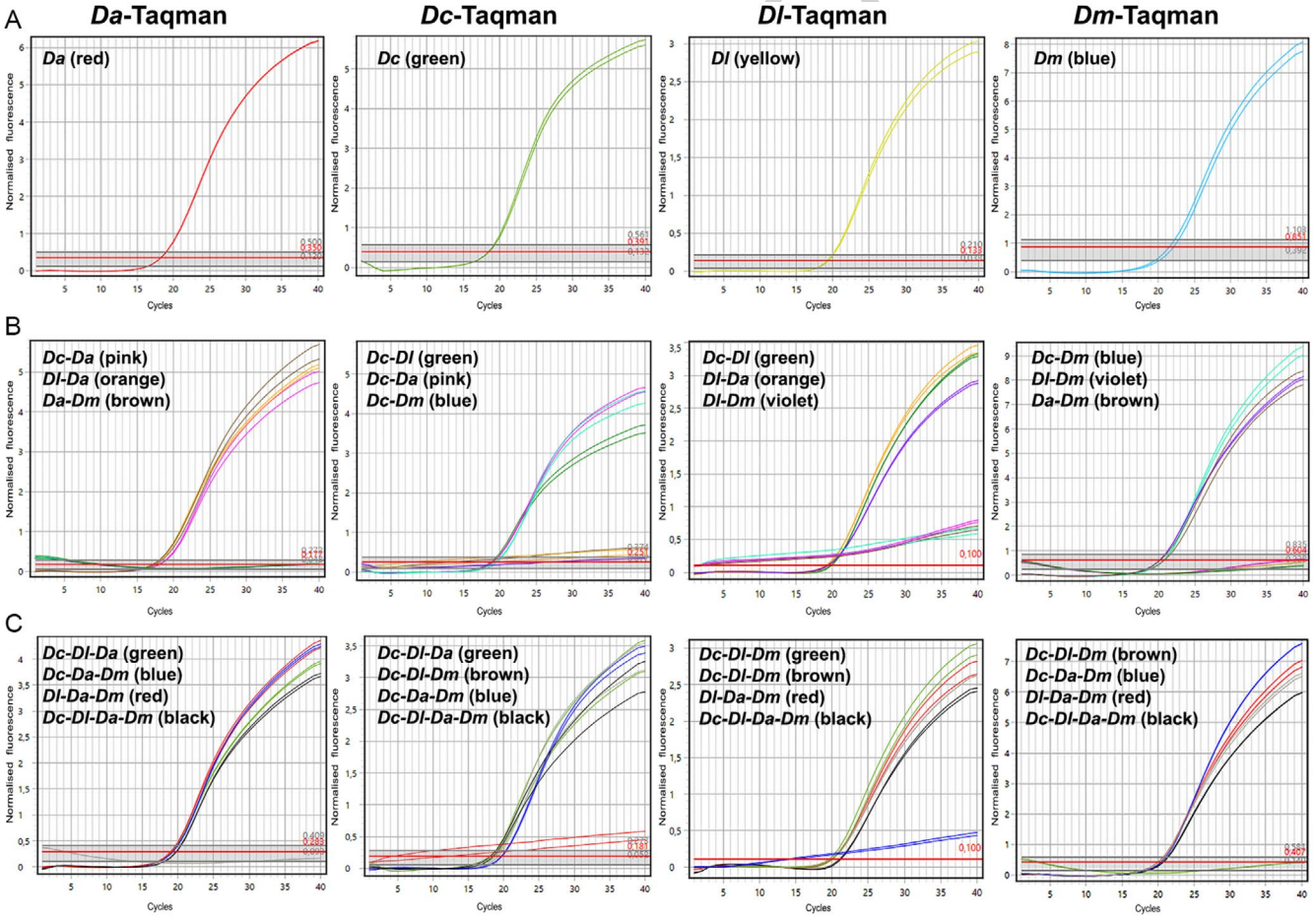

FIGURE 1 | Specificity of quantitative PCR (qPCR) assay using genomic DNA from each *Diaporthe* species: *D. aspalathi* (Da), *D. caulivora* (Dc), *D. longicolla* (Dl) and *D. miriciae* (Dm); each column represents the species-specific TaqMan primer-probe set. (A) qPCR of pure genomic DNA of *Diaporthe* species. (B) Six combinations of two *Diaporthe* species in duplex qPCR: *Dc-Dl* (green), *Dc-Da* (pink), *Dc-Dm* (blue), *Dl-Da* (orange), *Dl-Dm* (violet) and *Da-Dm* (brown). (C) Four combinations of three *Diaporthe* species or combination of all four in quadruplex qPCR: *Dc-Dl-Da* (green), *Dc-Dl-Dm* (brown), *Dc-Da-Dm* (blue), *Dl-Da-Dm* (red) and *Dc-Dl-Da-Dm* (black). In all cases, black text indicates the samples detected by the probe. Curves with a very slow increase in fluorescence are negative controls.

identify *D. aspalathi*, *D. caulivora*, *D. longicolla*, and *D. miriciae* (Figure 1C).

After testing the specificity of all primer-probe sets, we evaluated the sensitivity of the qPCR assay. The amplification of a 10-fold DNA dilution series (10 to 0.001 ng DNA/μL) showed a linear relationship between genomic DNA concentration (gDNA, ng/μL) and quantification cycle (C_q) for all *Diaporthe* primers (Figure 2). The standard curves obtained in each case were used to calculate the amount of *Diaporthe* DNA in nanograms per reaction. The quantification limit of detection (LOD) was estimated at < 0.001 ng/μL in all pathogens, corresponding to C_q values between 31 and 35.

3.4 | Validation of Multiplex qPCR in Plant Samples

To validate the quadruplex qPCR assay in plant tissues, 16 treatments were performed, including inoculations with the different *Diaporthe* spp., *D. aspalathi* (Da), *D. caulivora* (Dc), *D. longicolla* (Dl), *D. miriciae* (Dm), their combinations, and controls without fungi (Table 1). *Diaporthe* spp. were successfully detected in all tested samples with visible symptoms, and no amplification was produced for control stem samples with the primer-probe sets (Table S2). This assay proved the ability of the multiplex qPCR to detect *D. aspalathi*, *D. caulivora*, *D. longicolla*, and *D. miriciae* simultaneously (Figure S3). The internal control for soybean DNA was amplified in all samples except in negative controls.

3.5 | Multiplex qPCR Assay Evaluation in Field-Collected Soybean Plants With SSC Symptoms

A total of 52 stems exhibiting SSC symptoms with different disease levels, two asymptomatic plants (Figure S4), and 34 seed samples from these field-grown plants were analysed (Table S3 and Figure 3A). *D. caulivora* was detected in the asymptomatic plants (St6 and St54). Moreover, *Diaporthe* spp. were successfully identified in all tested samples exhibiting visible symptoms. *D. longicolla* was the predominant species detected in both stem and seed samples. In the stems, *D. longicolla* was found alone in 29 samples, followed by *D. caulivora* in 11 samples, and *D. aspalathi* was detected alone in one sample. This *D. aspalathi* isolate was recovered from a canker lesion and confirmed by *tEF1a* sequencing. *D. miriciae* was only detected with other *Diaporthe* species. Co-infections were observed in five stems with *Dl-Dc*, six with *Dl-Dm*, one with *Dl-Dc-Dm*, and one with *Dl-Da-Dm* (Figure 3B). In seeds, only *D. longicolla* and *D. caulivora* were detected; six samples showed co-infections, 19 samples were infected only with *D. longicolla*, and four with *D. caulivora* (Figure 3B). Five seed samples showed no presence of any *Diaporthe* species included in this study. These results demonstrate that co-infection of *Diaporthe* species associated with SSC can be effectively detected and distinguished using this multiplex qPCR assay. Testing of stem and seed samples from the symptomatic plants revealed that in 19 of the 20 samples with *D. longicolla* in the stems, the species was also present in the seeds. Similarly, eight of the 12 *D. caulivora*-infected plants had *D. caulivora* present in both stems and seeds. Moreover, relative quantification of *Diaporthe* species DNA in soybean tissues with respect to total DNA using standard curves revealed different amounts of pathogen DNA in the different samples, ranging from 0.005 to 3.48 in stems and from 0.001 to 2.76 in seeds (Table S4 and Figure 3C,D).

In addition, we evaluated 47 seed lots obtained from different seed companies. For each lot, we extracted the DNA from 200 seeds and quantified the relative amount of *Diaporthe* species in the seed tissues. Some seed lots contained seeds infected with *Diaporthe* spp., while others appeared to be pathogen-free. *D. longicolla* was the predominant species detected in 13/47 seed lots, while *D. miriciae* was identified in two lots. Relative quantification of *Diaporthe* DNA with respect to total DNA using standard curves ranged from 0.002 to 2.78 in the seed lots (Table S4 and Figure 3E).

4 | Discussion

In South America, Argentina and Brazil are the leading soybean producers, with additional cultivation occurring in Uruguay, Paraguay and Bolivia. Several pathogens, including *Diaporthe* spp., affect soybean production worldwide (Vidić et al. 2013). In a previous study aimed at identifying the most prevalent *Diaporthe* spp. causing SSC in Uruguay, we identified 78 *Diaporthe* isolates, including 33 isolates of *D. caulivora*, 29 isolates of *D. longicolla* and 12 isolates of *D. miriciae* from soybean stems with symptoms (Mena et al. 2020). In the current study, we identified 83 additional *Diaporthe* isolates that were obtained by isolating the fungi in PDA medium from SSC lesions, bringing the total number to 161 isolates. Among these, *D. longicolla* was

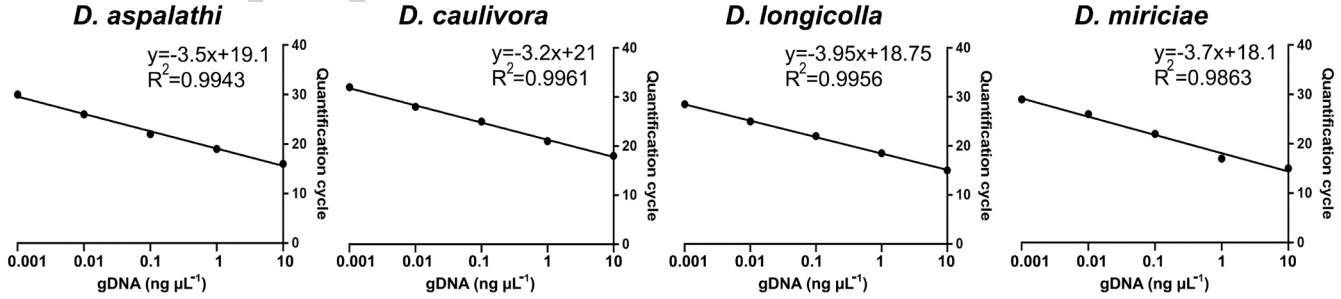

FIGURE 2 | Standard curves for quantification of *Diaporthe* species using the quadruplex quantitative PCR assay. Dots represent the mean of three technical replicates of each dilution. The regression equations and R^2 coefficient of determination for quantification cycles value versus the concentration of amplified genomic DNA is shown on the chart.

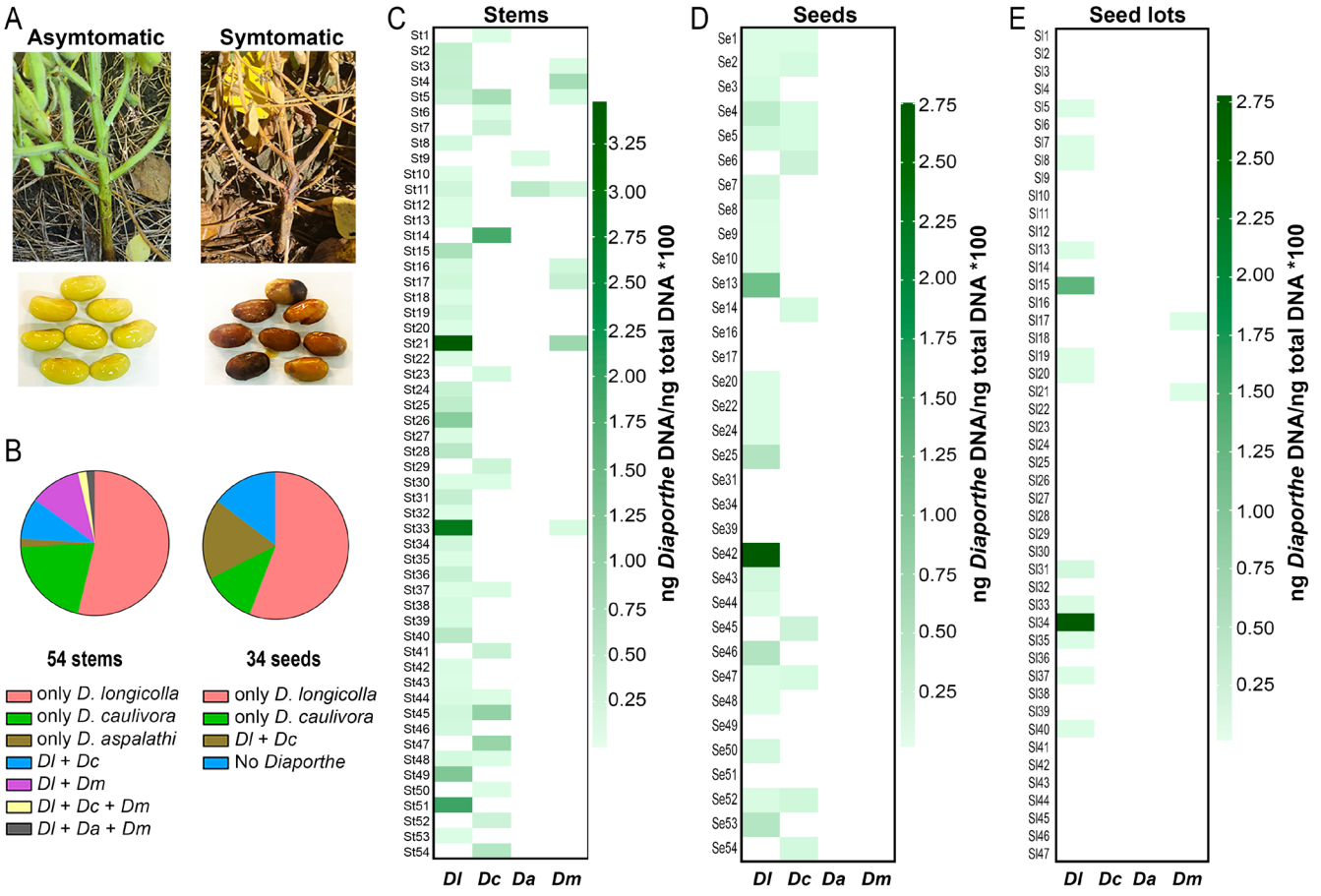

FIGURE 3 | (A) Asymptomatic and symptomatic soybean field-grown plants (stem and seeds). (B) Frequency of amplification of *Diaporthe aspalathi* (Da), *D. caulivora* (Dc), *D. longicolla* (Dl) and *D. miriciae* (Dm) DNA from naturally infected soybean plants. (C) Amplification and relative quantification (ng *Diaporthe* DNA/ng total DNA × 100) of Da, Dc, Dl and Dm in stem samples and (D) in seed samples. Se, Seed; St, Stem; identical numbers refer to the same plant. (E) Amplification and relative quantification in seed lots (Sl) obtained from different seed companies.

the most prevalent, accounting for 55.3% of the isolates, followed by *D. caulivora* at 29.8% and *D. miriciae* at 9.9%. A limitation of this approach is that not all species responsible for SSC are recovered from a single lesion. To address this limitation and facilitate broader sampling for epidemiological studies, we developed a multiplex qPCR assay capable of detecting the most prevalent *Diaporthe* pathogens causing SSC in Uruguay. TaqMan qPCR assays have been successfully employed for detecting *Diaporthe* pathogens responsible for diseases in other plants, including *D. citri*, which causes citrus melanose (Pu et al. 2024), *D. vaccinia* responsible for twig blight disease in blueberry and cranberry (Dharmaraj et al. 2022), and *D. helianthi* and *D. gulyae* causing Phomopsis stem canker in sunflower (Elverson et al. 2020).

In soybean inoculations performed under controlled conditions, our results demonstrated that the TaqMan primer-probe sets, based on the *tEF1a* gene, were efficient and specific for detecting *D. longicolla*, *D. caulivora*, *D. miriciae*, and *D. aspalathi*, both individually and simultaneously in artificially inoculated soybean stems, seeds, and pure cultures. A similar study with primer-probe sets designed in the *tEF1a* has been previously performed to detect *D. longicolla*, *D. caulivora*, *D. eres*, and *D. novem* in artificially inoculated soybean plants and two seed lots (Hosseini et al. 2021). The standard curves generated from genomic DNA of pure cultures of *D. aspalathi*, *D. caulivora*, *D. longicolla*, and *D. miriciae* enabled the quantification of these species in soybean samples. The LOD estimated was 1.0 pg, compared with 0.2 pg and 0.5 pg obtained by Hosseini et al. (2021) and Fujiwara et al. (2021), respectively, in *Diaporthe* detection by qPCR multiplex.

In symptomatic stems from field-grown plants, the multiplex qPCR assay revealed that *D. longicolla* was the predominant species, followed by *D. caulivora* and *D. miriciae*. Co-infections were observed in 24% of both stem and seed samples from field-grown plants. While co-infections involving two or three species were detected in various combinations within stem canker lesions, infected seeds only harboured *D. longicolla* and/or *D. caulivora*. These findings are supported by the fact that *D. longicolla* is the primary causal agent of Phomopsis seed decay, and that *D. caulivora* is capable of producing soybean seed decay (Dos Santos et al. 2024; Sun et al. 2012). Furthermore, the detection of double or triple co-infection in the same sample would probably have been missed using other diagnostic methods, highlighting the effectiveness of the multiplex qPCR assay. For the first time in several years of sampling, we detected *D. aspalathi* in two stems exhibiting SSC symptoms, indicating its presence, albeit in very low proportions, in Uruguayan fields. *D. aspalathi* was found in two soybean cultivars, At 6.0 (seeds from Argentina) and SCJ21112 (seeds from China), cultivated in

the Department of Colonia. Infected soybean seeds are potential carriers of pathogens for long-distance dissemination. Our findings that *D. longicolla* and *D. caulivora* can be present in both symptomless seeds and symptomatic tissues emphasises the importance of testing both types of tissue in future field studies.

The testing of both stem and seed samples from symptomatic plants revealed a strong correlation between the presence of *D. longicolla* and *D. caulivora* in both tissues. Specifically, 95% of *D. longicolla*-infected stems and 67% of *D. caulivora*-infected stems also contained the pathogen in the seed tissues. However, to the best of our knowledge, the transmission of *Diaporthe* spp. from stems to seeds has not yet been demonstrated and requires further investigation. Additionally, testing seed lots from various local companies revealed the presence of *Diaporthe* spp. in 32% of the samples, including 13 lots with *D. longicolla* and two lots with *D. miriciae*. This finding underscores the potential impact of pathogen spread on soybean production. Consequently, our multiplex qPCR assay offers a reliable method for detecting *Diaporthe* DNA in seed sanitation diagnostic tests—a critical tool that has not yet been established in Uruguay.

In conclusion, our multiplex qPCR assay provides a rapid and practical method to detect and quantify four important and common species of *Diaporthe*, causing soybean stem canker, directly in diseased plant tissues. The qPCR assay is specific for each of the fungal pathogens and can distinguish them in co-infected soybean plants. The application of our assay offers the potential to improve laboratory diagnosis of *Diaporthe* spp. in soybean.

Acknowledgements

The authors thank Ricardo Larraya and Jhon Larzabal for technical assistance, and the Engineering faculty of ORT University and Uruguayan Seed Companies for the seed materials (ADP Agronegocios del Plata, Sofoval-Agro, Lage y Cia SA, Greising y Elizarzú, INIA La Estanzuela and Erro). This work was funded by Agencia Nacional de Investigación e Innovación (ANII), grant FCE_3_2022_1_172688, Uruguay, and Programa de Desarrollo de las Ciencias Básicas (PEDECIBA) Uruguay.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data available on request from the authors.

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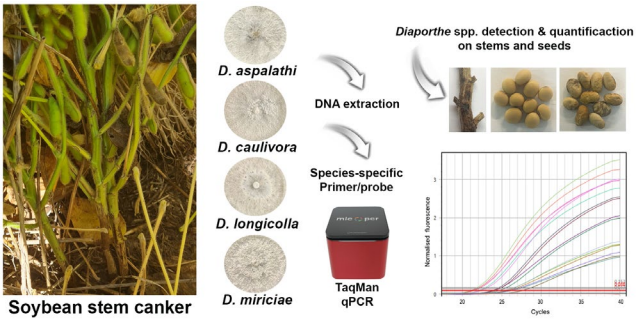

A rapid multiplex qPCR assay for detection and relative quantification of four *Diaporthe* species that cause soybean stem canker in stems and seeds tissues was developed for seed certification, pathogen surveillance and management.