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Molecular epidemiology of sisal bole rot disease suggests a potential phytosanitary crisis in Brazilian production areas

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Sisal bole rot disease is the major phytosanitary problem of *Agave* plantations in Brazil. The disease is caused by a cryptic species of *Aspergillus*: *A. welwitschiae*. To date, the only way to diagnose the disease was to observe external symptoms, visible only when the plant is already compromised, or through the isolation and sequencing of the pathogen, which requires cutting the entire plant for bole tissue sampling. We developed a new primer set based on a unique gene region of *A. welwitschiae*, which can detect the phytopathogenic strains through PCR directly from sisal leaves. Using the new marker to study the main sisal-producing areas in Brazil, we discovered a troublesome situation. The main producing areas of this crop had a pathogen incidence of 78%–88%. The dispersion index indicates a regular spatial pattern for disease distribution, suggesting that the use of contaminated suckers to establish new fields may be the main disease-spreading mechanism. Altogether, the high incidence of the pathogen, the unavailability of clean plants, the unpredictability of disease progression, and the low investment capacity of farmers reveal the vulnerability of this sector to a potential phytosanitary crisis. By correlating the disease symptomatology with soil nutritional traits, we suggest that higher potassium availability might decrease visual symptoms, while phosphorus may have the opposite effect. Also, we observe a potential cultivar effect, suggesting that common sisal may be more susceptible than hybrid cultivars (especially H400). This new molecular tool is a significant advance for understanding the disease, enabling the implementation of a monitoring program and studies that may lead to pathogen control strategies and changes in the Brazilian production model.

KEYWORDS

Aspergillus, *Agave*, PCR diagnosis, plant-pathogen interaction, natural fibers

1 Introduction

Sisal is the popular name for a range of hard natural fibers derived from *Agave* leaves, usually extracted from *Agave sisalana* (common sisal), *A. fourcroydes*, or hybrid cultivars, like the H11648 [(*A. amaniensis* x *A. angustifolia*) x *A. amaniensis*] (Raya et al., 2021). In the past, sisal was a very relevant source of hard fibers, which were gradually replaced by the synthetic fibers, such as polypropylene (Davis and Long, 2015). However, given the widespread comprehension of climate change and the need for biorenewables, *Agave* might experience a resurgence to its former status (Raya et al., 2022), reclaiming global significance not only as a source of hard fibers, but also through profoundly diversified utilization strategies of these resilient plants (Morán et al., 2008; de Paula et al., 2012; Santos et al., 2015; Pérez-Zavala et al., 2020).

Since the 1960s, Brazil is the world's leading producer of sisal fiber, despite undergoing a significant decline in production. While in 1990 the production was 300,200 Mg annually (Silva and Beltrão, 1999), today Brazil produces around 98,000 Mg per year. This currently constitutes around 36% of the global annual sisal fiber production (FAO, 2023), with the State of Bahia alone accounting for approximately 94% of this output (IBGE, 2021). Sisal cultivation is socioeconomically relevant by providing, directly or indirectly, the main economic driver for a population of over 850,000 people in the Brazilian semiarid, where it is often the only agricultural product available (Broeren et al., 2017). Although other sisal fiber producing countries, such as China and Tanzania, cultivate the H11648 (Monja-Mio et al., 2019), Brazilian fiber production is based on the large-scale cultivation of undefined *A. sisalana* plants, with lower fiber yield (Azzini et al., 1989; Silva and Beltrão, 1999; Suinaga et al., 2007). One of the main reasons for this scenario is the production model based on a laborious fiber extraction with the utilization of equipment that demands manual operation (the “Paraibana” machine—see Silva and Beltrão, 1999). Therefore, the higher the leaf fiber content, the greater the worker's effort to accomplish their job. In other countries, such as Tanzania or China, fiber extraction is accomplished by stationary machines, with the leaves being transported to small industries where they are processed (Broeren et al., 2017). In this case, high fiber content becomes an advantage and not a restriction. Nevertheless, some more productive *Agave* cultivars like H11648 or H400 leaves (“*híbrido 400 folhas*” in Portuguese, named simply H400 from this point forward) can eventually be found in sisal fields in Brazil (Silva and Beltrão, 1999; Alvarenga Jr, 2012) but at a much lower proportion. The origin of H400, also known as the “white hybrid,” is not clear, but seems to be closely related to the H11648 (Souza et al., 2018).

For the last 2 decades, sisal fiber production in Brazil has significantly declined due to the bole rot disease, which represents the primary phytosanitary issue in the country's sisal fields (Abreu, 2010; Soares et al., 2020). This disease is caused by *Aspergillus welwitschiae*, a saprotrophic fungus that switches to a necrotrophic lifestyle when infecting the wounded sisal bole, destroying the parenchymal tissue (Duarte et al., 2018). The primary symptoms of this disease involve the decay of both the bole and leaf base, characterized by the appearance of internal tissue exhibiting hues that span from red to a spectrum of light and dark brown discoloration. Subsequently, secondary indications become apparent through

the yellowing and wilting of leaves, eventually leading to the demise of the entire plant (Duarte et al., 2018).

Drawing from extensive field observations and the documented spread of the bole rot after an intense drought period spanning 2011–2012 (both extensively detailed in prior publications by our research group), we believe that the establishment of the necrotrophic phase of the disease hinges on physiological stress. This stress can result from nutritional deficiencies, the pressures induced by drought, or even mechanical damage arising from plant manipulation during leaf removal for fiber extraction or sucker elimination. Notably, the influence of an insect, such as *Bemisia tabaci* (Quintanilha-Peixoto et al., 2021), or yet by an infestation of pests, such as *Dysmicoccus spp.* (Wang et al., 2022), could also contribute to this injury-induced phase.

Our research group has recently discussed some molecular characteristics and mechanisms possibly involved in the pathogenicity of *A. welwitschiae* in sisal (Quintanilha-Peixoto et al., 2019; Quintanilha-Peixoto et al., 2022b), but there are still open questions on the disease cycle and susceptibility of fiber-producing agaves. The identification of the etiological agent, *A. welwitschiae*, was described only in 2018, along with a comprehensive overview of the global prevalence of sisal bole rot disease and its associated pathogen (Duarte et al., 2018). This is due to the fact that within the Nigri section of the *Aspergillus* genus, *A. welwitschiae* is considered a cryptic species of *A. niger*. This cryptic nature implies that distinguishing between these two species is not feasible using conventional microbiological methods like assessing colony appearance and spore morphology (Varga et al., 2011), or even relying on the primary fungal DNA barcode, nrITS (Susca et al., 2016). Consequently, the current standard for identification and comprehensive examination of the phylogeny and taxonomy of these fungi relies on the meticulous sequencing of the calmodulin (CaM) gene—a secondary marker gene for some fungal clades. This identification method, applied by Duarte et al. (2018), entails cultivating the fungus in a pure culture, extracting its DNA, amplifying the CaM gene via PCR, and subsequently subjecting it to sequencing using the Sanger technique. Efforts have been undertaken to design specific primers targeting the CaM gene for *A. welwitschiae* (Gherbawy et al., 2015; Palumbo and O'Keeffe, 2015), particularly for discerning strains associated with food contamination. Nevertheless, as elucidated further, these primers might exhibit amplification across other species within the Nigri section.

In this study, our aim was to develop a more reliable and user-friendly molecular marker for accurate *A. welwitschiae* identification. To achieve this, we diverged from the conventional approach of seeking minor polymorphisms within traditional genes, opting instead to identify exclusive regions within the genomes of *A. welwitschiae*, which have recently been released. Once this novel marker was validated, we employed the test on samples obtained from Brazil's main sisal-producing regions, conducting an epidemiological investigation. The new test offers an unprecedented level of insight into bole rot disease in Brazil, shedding light on potential correlations between disease symptomatology and nutritional status, and even revealing potential cultivar-specific effects. This new tool presents novel opportunities for disease management, not only within Brazil but also in sisal-producing regions worldwide.

2 Materials and methods

2.1 *A. welwitschiae* unique regions detection, primer design, and validation

For the development of a set of primers targeting the detection of *A. welwitschiae*, we performed an analysis of orthologous genes using the gene-coding sequences (CDS) of 40 *Aspergillus* strains and two other fungal species, *Neurospora crassa* and *Penicillium rubens*. In this analysis, three strains of *A. welwitschiae* were used, being CBS159.1 isolated from *Welwitschia mirabilis* and CCMB 663 and CCMB 674 isolated from sisal plants. We used the same set of species (Supplementary Table S1) as seen in Quintanilha-Peixoto et al. (2022b) to find unique genes in *A. welwitschiae* and design primers that could be used to diagnose the bole rot disease. We used OrthoFinder v. 2.5.2 (Emms and Kelly, 2018) to perform the orthologous analysis, using the parameter “-d” for DNA input. From the results, we selected families that contained only *A. welwitschiae* genes.

In order to design the primer sequences, we used Primer-BLAST (available at <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) under default settings and “*A. welwitschiae*” as the organism option. Considering the housekeeping function of the gene selected as a marker in *A. welwitschiae*, we complemented OrthoFinder ortholog designation with a search for paralogs in *A. fumigatus* Af293, *A. nidulans* FGSC A4, and *A. niger* N402, which were also compared against *A. welwitschiae* CCMB 674 and *A. welwitschiae* CCMB 663 in the primer set validation.

Selected sets of primers were tested and then validated with ECRA HIFI DNA Polymerase (ECRA Biotec), following manufacturers procedures. After an initial *in vitro* screening, one primer set (Aw_8 F: CACCTGATCCTGGAGGGAGA, Aw_8 R: AGCCACCTGGAGAAGCAATC) was selected and optimized by adding DMSO to the reaction mix and testing different annealing temperatures to increase specificity. Primers previously described in the literature as capable of segregating *A. welwitschiae* and *A. niger* were also used as external controls. One pair of primers described by Palumbo & O’Keeffe (2015) (Aw F: GGGATTTCGACAGCATTTCAGAAATT and Aw R: GATAAAACCATTGTTGTCGCGGTC A) and the other by Gherbaway et al. (2015) (awaspec: ATTTTCGACAGCATTTCAGAAATTA and cmd6: CCGATAGAGGTCATAACGTGG) were selected.

2.2 Plant material and testing

A total of 179 samples of sisal plants from the main producing areas in Bahia were tested. Samples were collected in August 2019 from three regions known as “identity territories” (Figure 1) demarcated by environmental, economic, and cultural criteria (Secretaria do Planejamento do Estado da Bahia, 2016). The first territory, *Piemonte da Diamantina*, is in the North of Bahia at an mean altitude of 740 m, has an average annual maximum air temperature of 29.8°C and a minimum of 16.2°C (Fick and Hijmans, 2017), and an average annual rainfall of 492 mm (INMET, 2023). Sampling sites of *Piemonte da Diamantina* included the municipalities of Jacobina, Várzea

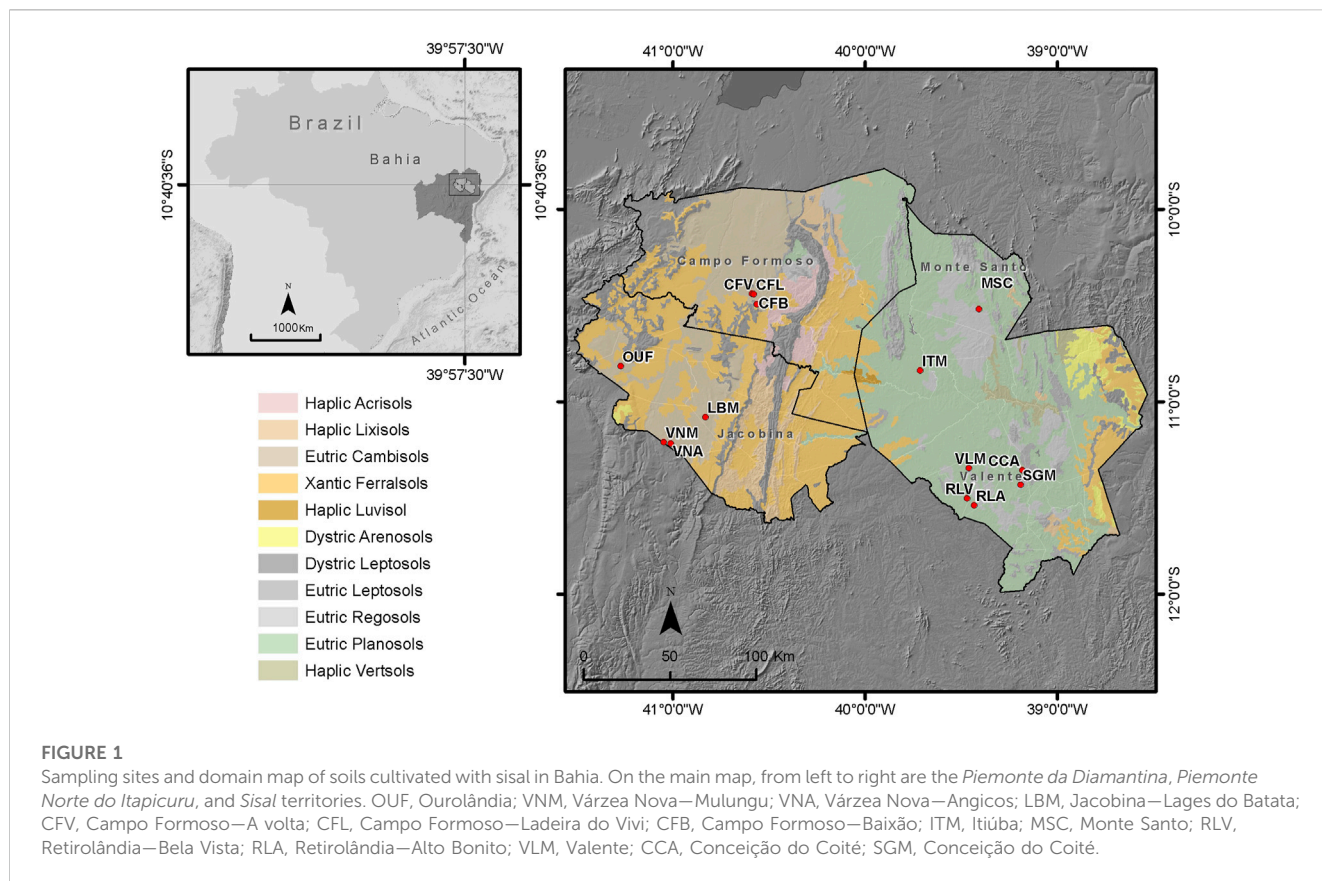
Nova, and Ouroândia, which jointly account for 11,457 Mg of sisal fiber produced per year (IBGE, 2021). The second territory, *Piemonte Norte do Itapicuru*, is farther north at an average altitude of 680 m, with a maximum annual air temperature of up to 30.7°C and a minimum of 16.3°C (Fick and Hijmans, 2017), and average annual rainfall of 580 mm (INMET, 2023). The sampling sites in this territory include only different areas of the municipality of Campo Formoso, the largest sisal-producing municipality in Brazil, with 22,546 Mg of fiber produced per year (IBGE, 2021). The third territory, *Sisal*, which is farther east than the others, has lower mean altitude (600 m) and an annual maximum air temperature of 30.3°C and a minimum of 17.4°C (Fick and Hijmans, 2017) with an average annual rainfall of 511 mm (INMET, 2023). Samples were collected from the municipalities of Conceição do Coité, Valente, Retirolândia, Itiúba, and Monte Santo, which are responsible for the annual production of 20,397 Mg of fibers (IBGE, 2021). For each territory, 45, 43, and 91 samples were tested from *Piemonte da Diamantina*, *Piemonte Norte do Itapicuru*, and *Sisal* territories respectively. In the *Sisal* territory, more samples were collected as production areas with hybrid cultivars were found during the survey, specifically in Itiúba. The three sisal cultivars sampled are represented in Figure 2.

Leaf samples were collected and maintained in styrofoam coolers with ice, until arrival at the laboratory, where they were frozen in liquid nitrogen. Total DNA was extracted from leaves using the Sahu et al. (2012) protocol. *A. welwitschiae* detection was performed as the conditions described in the previous section, but using *Agave’s* 18S (Martinez-Hernandez et al., 2010) (Atq-rRNA18SF CTGCCGTCCCGTCCCTGCCGTCCCGTCCCTTCTG C and Atq-rRNA18SR CCTGGTGGTGCCCTTCCGTCAA) as an endogenous control and DNA extracted from *A. welwitschiae* strain CCMB 674 was selected as an exogenous control. The results were evaluated according to the presence (positive) or absence (negative) of PCR product at the correct molecular weight.

2.3 Soil analyses

The sisal-producing areas, inserted in the semi-arid region of Bahia, display a great environmental diversity, especially in relation to climate, geology, geomorphology, and local vegetation, exhibiting a high diversity of soil classes. Soils cultivated with sisal range from undeveloped, shallow, fertile, and stony to well-weathered, deep, chemically poor, and clayey soils. According to the updated soil map of Bahia (IBGE, 2018) and the soil reclassification according to FAO/WRB (2015), predominate in the territories of *Piemonte da Diamantina* and *Piemonte Norte do Itapicuru* the Xantic Ferralsols (Dystric) and the Eutric Cambisols, with less occurrence of Dystric Leptosols, which is considered of low potential for sisal cultivation.

Soil samples were collected from Eutric Planosols (ITM and RLV), Eutric Cambisols (CFB, CFV, CFL, VNA, VNM, LBM, and OUF) and Eutric Regosols (VLM and CCA). For each site, composite samples were collected, from 0–20 cm, within 1 ha (Raij et al., 2022) and sent to LAGRO—Laboratório Agronômico S/C Ltda (Indaiatuba, SP) for determination of



soil granulometry and fertility (macro and micronutrients), according to the methodology described by Raji et al. (2001). Considering that there are no reference values available for classifying soil nutrients in terms of availability as low, medium

and high, the guidelines for Cerrado soils were used as the standard for macronutrients (Lopes and Guimarães, 1989). For micronutrients, the references described for perennial species in the State of São Paulo were used (Raji et al., 2022).

2.4 Data integration and analysis

Epidemiological parameters were calculated according to Vale et al. (2004). The incidence of the pathogen (PI) was calculated through:

$$PI = \frac{i_p}{i_s} \quad (1)$$

where i_p is the total number of positive individuals for *A. welwitschiae* and i_s is the total number of sampled individuals.

The prevalence (P) was defined by:

$$P = \frac{A_n}{A_t} \quad (2)$$

where A_n is the number of areas containing at least one plant diagnosed with the disease and A_t is total number of sampled areas.

The dispersion index (DI) for each territory was calculated from the pattern of contamination agglomeration in the collection sites, as given by Eq. 3:

$$DI = \frac{\sigma_t^2}{\bar{x}} \quad (3)$$

where σ_t^2 is the variance and \bar{x} is the mean of pathogen incidence per territory.

In order to understand the potential effect of soil characteristics on the incidence of *A. welwitschiae* and persistence of bole rot symptoms, we applied multivariate linear regression to the dataset. Because of the small sample of fields ($n = 11$), the dependent variables set of the regression model had to be reduced, so that the system would not be overdetermined. Therefore, we selected the top nine soil characteristics based on the highest Pearson correlation with pathogen incidence and symptom (Supplementary Figure S1) and possible direct implications on plant health. All selected characteristics had correlation coefficients (r -values) higher than 0.4. With the selected soil characteristics (P, K, Ca, Mg, CEC, V%, Na, Mn, Cu), we used the multivariate model for both (i) pathogen incidence per field and (ii) the proportion of symptomatic individuals in that field:

$$y = \alpha + \sum_i \beta_i x_i \quad (4)$$

Where α is the intercept, β_i is the effect coefficient of the dependent variable x_i , and y is the predictor equation of proportion of symptomatic or infected individuals. In order to avoid biases deriving from the different cultivars and over/sub represented areas, we discarded the data from fields containing the hybrid cultivars. All analyses were done using the R software R v4.1.3 (R Core Team, 2022) and PAST software version 4.3 (Hammer et al., 2020).

3 Results

3.1 Detection of unique regions for the diagnosis of the bole rot disease in *Agave sisalana*

Initial tests with primers described in the literature by Palumbo and O'Keefe (2015) and Gherbawy et al. (2015) did not excel at differentiating our strains of *A. welwitschiae* (CCMB 674 and CCMB

663) from *A. fumigatus* Af293, *A. nidulans* FGSC A4, and *A. niger* N402 (Figure 3). Meanwhile, our orthologous analysis using nucleotide sequences initially described 51 orthologous clusters exclusive to our 3 *A. welwitschiae* strains. 35 orthologous clusters, initially annotated as "hypothetical protein," were tested on PrimerBLAST for obtaining common PCR products. Among these, genes in four clusters did not share enough similarity for primer design, and 13 produced off-target hits in the *A. welwitschiae* genome. The top 10 longest PCR products from the remaining clusters were selected for *in vitro* testing. Among the preselected primers, only one primer set, Aw_08, was able to differentiate *A. welwitschiae* from *A. niger* (Supplementary Figure S2), producing a polymorphic PCR product with higher molecular weight. For this primer set, we performed further reaction optimization tests to improve specificity, and subsequently tested against *A. niger*, *A. fumigatus*, and *A. nidulans* (Figure 3). By increasing the annealing temperature to 64°C, Aw_8 was able to identify only the *A. welwitschiae* phytopathogenic strains (Supplementary Figure S3) (Quintanilha-Peixoto et al., 2022a), and with a sensitivity up to 0.75 ng μL^{-1} of DNA per sample (Supplementary Figure S3).

The full annotation of the proposed marker gene can be found in Supplementary Table S2. To further investigate our primer set specificity we searched for PC5R paralogs in the other genomes used for validation (*A. fumigatus* Af293, *A. nidulans* FGSC A4, and *A. niger* N402). This paralog analysis revealed other PC5R sequences sharing similarity with the *A. welwitschiae* sequences clustered by OrthoFinder. However, such sequences were highly divergent (Figure 4C), which explains our primer specificity. The closest sequence, XM_677456.1 from *A. nidulans* FGSC A4, shares a 69% identity region, which covers only 52% of the *A. welwitschiae* sequence.

3.2 Molecular epidemiology

The developed molecular marker successfully identified the presence of *A. welwitschiae* in sisal leaves. Out of all the collected samples, those showing evident symptoms of bole rot disease consistently tested positive for *A. welwitschiae*, whereas negative results were obtained from visually healthy plants. Through molecular analysis, we observed a pathogen prevalence of 100% in sisal production areas. *A. welwitschiae* incidence within the sampling sites varied from 25% to 100% (Table 1). In contrast, symptoms occurrence ranged from 0% to 56%. The territories of *Piemonte Norte do Itapicuru* and *Sisal* are traditionally known for the more intense cultivation of sisal, in particular the municipalities of Campo Formoso (largest producer) and Valente (largest fiber industrial units). In these territories, approximately 17% of the sampled plants showed bole rot symptoms. Among all municipalities, Retirolândia (*Sisal* territory) had the highest occurrence of sisal bole rot symptoms. The territory that presented the higher incidence was *Sisal*, with 88%. *Piemonte da Diamantina* had the lowest incidence, with 78%, and it was the territory with the highest asymptomatic rate. The lowest incidence was found in Ourorândia (*Piemonte da Diamantina* territory), the municipality farthest from the territory with the highest incidence of the disease (*Sisal*). For all territories, we encountered a dispersion index lower than 1, indicating that this disease has a regular dispersion pattern.

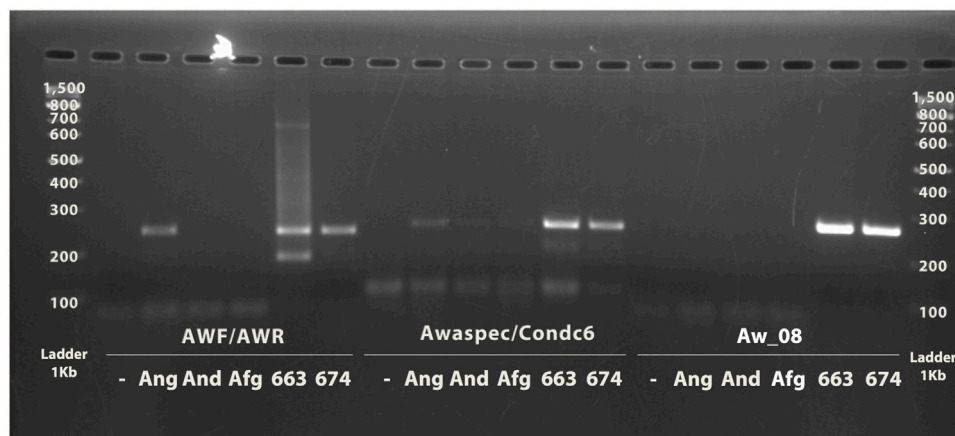


FIGURE 3

In vitro analysis of candidate primers for molecular diagnosis of sisal bole rot disease. -: negative control, Ang: *Aspergillus niger* N402, And: *Aspergillus nidulans* FGSC A4, Afg: *Aspergillus fumigatus* Af293, 663: *Aspergillus welwitschiae* strain CCMB 663, 674: *Aspergillus welwitschiae* strain CCMB 674, AWF/AWR: primers set described by Palumbo and O’Keeffe, (2015), Awaspec/Condc6: primers set described by Gherbaway et al. (2015), Aw_08: candidate primers set, and Ladder 1kb: 1Kb KASVI marker.

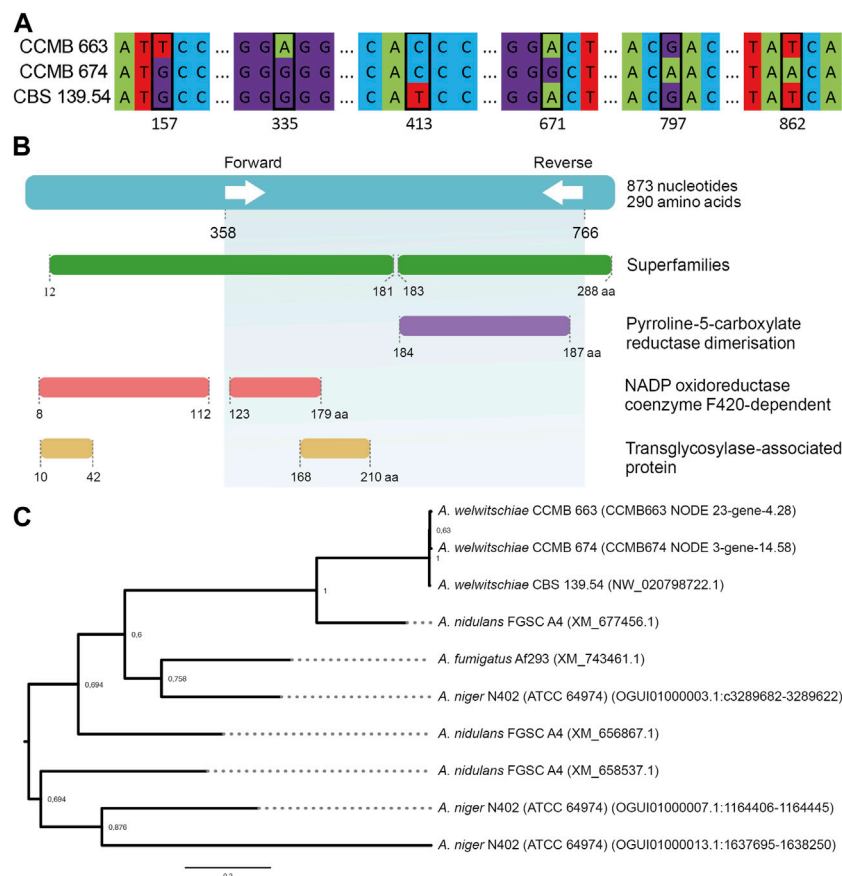


FIGURE 4

(A) SNPs in the PC5R genes of the 3 *A. welwitschiae* isolates (B) Representation of the flanking regions of the selected primer set (first layer and green shadow), protein superfamilies, and protein domains in the selected gene, according to hmmscan (C) PC5R paralogs phylogeny with bootstrap support.

TABLE 1 Epidemiology of sisal bole root disease in the main sisal-producing territories of Bahia.

Territory/Municipalities	Incidence of <i>A. welwitschiae</i> (%)	Asymptomatic plants (%)	Dispersion index
Piemonte da Diamantina	78	89	0.11045
Ourolândia	25	100	
Jacobina—Lages do Batata	71	70	
Varzea Nova—Angicos	82	93	
Varzea Nova—Mulungu	100	100	
Piemonte Norte do Itapicuru	84	81	0.00352
Campo Formoso—Baixão	86	72	
Campo Formoso—A Volta	90	78	
Campo Formoso—Ladeira do Vivi	75	100	
Sisal	88	81	0.01100
Itiúba	81	94	
Monte Santo	100	100	
Valente	100	83	
Conceição do Coité	100	79	
Retirolândia—Alto Bonito	75	50	
Retirolândia—Bela Vista	90	44	

The incidence of *A. welwitschiae* and the prevalence of asymptomatic plants within each territory (Piemonte da Diamantina, Piemonte Norte do Itapicuru, and Sisal) are calculated as averages for the municipalities encompassed by them.

TABLE 2 Incidence of *A. welwitschiae* in sisal cultivars.

Cultivars	Incidence (%)	Symptomatic (%)	Asymptomatic (%)	Number of individuals
<i>Agave sisalana</i>	84	16	84	128
H11648	76	10	90	29
H400	95	0	100	22

In addition, the incidence of *A. welwitschiae* and occurrence of symptoms were compared amongst the sisal cultivars (*A. sisalana*, H11648, and H400) (Table 2). Among them, the common sisal (*A. sisalana*) showed the highest proportion of symptomatic individuals, while no symptoms were observed for H400. Furthermore, *A. welwitschiae* was detected in 76% of the H11648 plants analyzed, but only 10% of this population showed symptoms. Nonetheless, it is important to point out that the presence of hybrid cultivars in commercial plantations is uncommon, and 82% of the hybrid cultivars encountered occurred in the same municipality (Itiúba).

3.3 Physical and chemical properties of soil samples

The fertility analysis revealed acid to weak alkaline soils (pH 4.9–7.3), with average organic matter, and rich in Ca and Mg (Table 3). Also, no Al was found, and the potential acidity ($H^+ + Al^{3+}$) was low, which is consistent with the pH. Base saturation (V%) was classified as high to very

high (>90), with Ca and Mg levels as major contributors. Accordingly, the cation exchange capacity (CEC) was average to high, indicating soils with overall good natural fertility. Soil P availability varied from 6 to 54 mg dm^{-3} among the sites, which is considered low for most perennial crops (Raij et al., 2022). Only Retirolândia and Conceição do Coité exhibited P levels above the standard. For K, we found high variations, from 0.6 to 6.9 mmolc dm^{-3} and CFV, ITM, VLM, and RLV displayed the lowest values. Sulphur ranges were low to medium, ranging from 2.3 to 8.0 mg dm^{-3} .

For micronutrients, soil Mn and B levels were mostly high, while Cu and Zn were low (Table 4). As for Fe, the samples ranged from 4 to 49 mg dm^{-3} . Specifically for the *Sisal* territory, only high Fe levels were found. It is important to emphasize that pH directly influences the availability of several nutrients, and soils with higher alkalinity have less availability of micronutrients, such as Fe, Zn, Cu, and Mn, and more availability of Ca, Mg, K, P, S, and B. Soil Na varied from 0.2 to 2.4 mmolc dm^{-3} among the sites. Granulometric analysis of soil samples indicated a predominant texture of sandy to medium, with sandy clay loam textural class being more frequent (Supplementary Table S3).

TABLE 3 Soil fertility analysis.

Sampling sites	pH _{CaCl2}	O.M _{oxi}	P _{resin}	K _{resin}	Ca _{resin}	Mg _{resin}	Al ³⁺ _{KCl}	H ⁺ + Al ³⁺	CEC	V	S _{F, Ca}
		g/dm ³	mg/dm ³	mmolc/dm ³					%	mg/dm ³	
Ourolândia	6.1	19	18	3	35	13	0	14	65.2	79	4.0
Jacobina—Lages do Batata	7.2	29	10	2.5	87	15	0	9	113.8	92	3.0
Varzia Nova—Angicos	7.1	23	9	6	106	13	0	11	136.2	92	8.0
Varzia Nova—Mulungu	6.3	22	9	6.9	108	24	0	16	155.3	90	4.0
Campo Formoso—Baixão	6.9	18.2	28.6	3.62	49.4	15.4	0	11	79.86	83.8	5.4
Campo Formoso—A Volta	7.3	33	7	0.7	129	9	0	9	148.1	94	6.0
Campo Formoso—Ladeira do Vivi	7.2	25	6	2.2	130	13	0	10	155.7	94	6.0
Itiúba	5.6	12.3	7.7	1.13	40	19.7	0	16	77.7	79.3	3.0
Valente	4.9	17	17	0.6	103	54	0	34	193	82	6.0
Conceição do Coité	6.7	32	41	4	109	100	0	16	230.7	93	3.0
Retirolândia	7.1	19	54	1.3	220	81	0	12	316.7	96	2.3

Data presented in **bold** are values considered above the reference standards, and in **red** are values considered below.

TABLE 4 Micronutrients and sodium determination.

Sampling sites	Na _{Mehlich}	Fe _{DTPA}	Mn _{DTPA}	Cu _{DTPA}	Zn _{DTPA}	B _{Hot water}
	mmolc/dm ³	mg/dm ³				
Ourolândia	0.2	15	46.0	0.7	0.6	1.1
Jacobina—Lages do Batata	0.3	4	21.0	0.6	0.8	2.2
Varzia Nova—Angicos	0.2	8	15.6	0.7	0.8	1.9
Varzia Nova—Mulungu	0.4	7	20.6	1.0	0.7	1.6
Campo Formoso—Baixão	0.4	6	44.9	1.4	1.0	1.6
Campo Formoso—A Volta	0.4	6	4.0	0.4	0.8	1.4
Campo Formoso—Ladeira do Vivi	0.5	4	17.0	0.8	1.1	2.3
Valente	1.4	42	32.0	2.6	0.4	1.1
Conceição do Coité	1.7	17	22.6	2.5	0.9	1.8
Retirolândia	2.4	12	12.0	1.2	0.7	1.5
Itiúba	0.9	49	7.4	0.8	0.4	0.8

Data presented in **bold** are values considered above the reference standards, and in **red** are values considered below.

The PCA grouped the soil samples according to textural class, separating the sandier soils on the surface: Eutric Planosols (ITM), Eutric Regosols (VLM) and some Eutric Cambisols (CFB and VNA), from those Cambisols with medium to clayey texture (Figure 5), showing that the soil granulometry parameters (clay, silt, and sand) were the main factor that contributed to sample segregation followed by CEC and Ca. Despite this grouping, the greater dispersion of soils demonstrates that there is diversity in soil composition within territories and even within the same municipality, as can be seen in the segregation between Varzea Nova samples. The studied Cambisols, associated with the Xantic Ferralsols (Eutrics), with great occurrence in the municipalities of Campo Formoso and Jacobina, are considered soils

of medium to high potential for the cultivation of sisal since they are from little to very deep, medium to high fertility, medium to clayey texture and then have reasonable water holding capacity. There are also occurrences of Dystric Leptosols in these territories, which predominate in the Sisal territory, along with Eutric Planosols, Eutric Regosols, and Abruptic or Haplic Solonetz. These less weathered soils are considered of low potential for the cultivation of sisal, as they present medium to low fertility, reduced effective depth, sandy texture, low water retention capacity, and/or high salinity, factors that, associated with the semi-arid climate of the region, lead to greater stress for cultivated plants.

Multivariate linear regression analysis revealed significant (p -value ≤ 0.1) interactions between symptom occurrence and soil

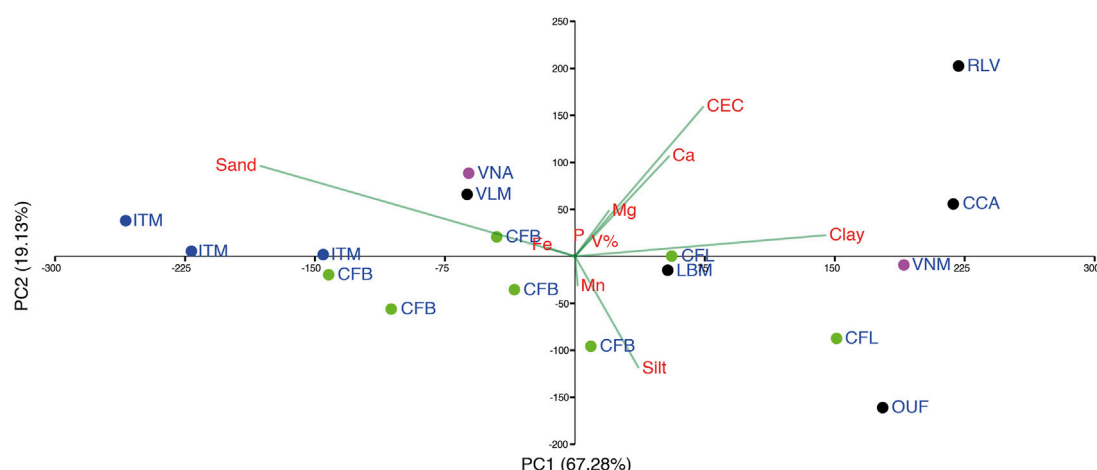


FIGURE 5

Principal component analysis of soil samples. In red are the soil traits that most contribute to the separation of the samples, and the green lines of these represent the factor contribution. OUF, Ouro-lândia; VNM, Várzea Nova—Mulungu; VNA, Várzea Nova—Angicos; LBM, Jacobina—Lages do Batata; CFB, Campo Formoso—A volta; CFL, Campo Formoso—Ladeira do Vivi; CFB, Campo Formoso—Baixão; ITM, Itiúba; MSC, Monte Santo; RLV, Retirolândia—Bela Vista; RLA, Retirolândia—Alto Bonito; VLM, Valente; CCA, Conceição do Coité; SGM, Conceição do Coité.

TABLE 5 Multivariate linear regression analysis for the presence of bole rot symptoms against soil characteristics.

	Estimate	Std. Error	t value	Pr (> t)
Intercept	-8.707943	1.297001	-6.714	0.0941*
P	0.030838	0.003970	7.768	0.0815*
K	-0.091716	0.011966	-7.664	0.0826*
Ca	-0.080246	0.011729	-6.842	0.0924*
Mg	-0.082511	0.011543	-7.148	0.0885*
CEC	0.068762	0.010146	6.777	0.0933*
V%	0.103527	0.015078	6.866	0.0921*
Na	0.227607	0.137325	1.657	0.3456
Mn	-0.003730	0.002522	-1.479	0.3784
Cu	-0.319310	0.081089	-3.938	0.1583

*Significance at $p < 0.1$.

characteristics (Table 5). The adjusted model presented multiple R^2 of 0.9936 and adjusted R^2 of 0.9363. The generated matrix (Supplementary Figure S4) displayed the expected correlations between the nutrient availability and pH (Hartemink and Barrow, 2023), e.g., P, K, Ca, and Mg, demonstrating that the soil data are consistent. The soil traits with significance were P, K, Ca, Mg, CEC, and V%. Among them, K, Ca, and Mg presented negative correlations with symptom occurrence. The analysis summary for interaction with pathogen incidence is depicted in Supplementary Table S4.

4 Discussion

Primer development for the detection and differentiation of *Aspergillus* species has proven to be a challenge. Even though a

couple of studies have tackled this issue (Gherbawy et al., 2015; Palumbo and O'Keeffe, 2015), both studies developed primer sets based on the Calmodulin gene and validated them with local samples. Calmodulin is a housekeeping gene present in all eukaryotes, and its application for phylogenetic studies of the *Aspergillus* genus is a consensus because it can be easily amplified and a large dataset is available in public databases (Samson et al., 2014; Tam et al., 2014; Alshehri and Palanisamy, 2020). Nonetheless, the proper identification of several *Aspergillus* species of section Nigri is often based on single-nucleotide polymorphisms (SNPs) of the CaM gene. Relying on such small nuances might decrease PCR specificity, leading to false positive results. When testing those literature primers, we observed PCR amplification for *A. nidulans*, *A. fumigatus*, and *A. niger* (Figure 3), which can be misleading, since these species can behave as saprotrophs (Tekaiia and Latgé, 2005) and/or as endophytes (Vijayanandan and Balakrishnan, 2018; Xu et al., 2018; Aziz et al., 2021) and, therefore, can easily be found in complex samples. In this study, we prospected potential targets and designed new primers from orthologous clusters unique for the 3 *A. welwitschiae* strains within their available genomes. Among the developed primer sets, Aw_08 presented the best outcome, proving to be specific, highly sensitive, and effective in complex samples. This new marker gene codes for a D1-Pyrroline-5-Carboxylate Reductase (P5CR) (Figure 4B), which participates in the proline metabolism (Porcel et al., 2004; Yadav et al., 2020) and has also been associated with pathogenicity in *Cryphonectria parasitica* (Yao et al., 2013). The PC5R sequences are nearly identical in all three examined strains of *A. welwitschiae*, differing by only five nucleotides (Figure 4A). These variations do not exert a significant impact on the amino acid sequence or structure. The phylogenetic analysis of Aw-P5CR indicates that the closest sequences present in *A. fumigatus* Af293, *A. nidulans* FGSC A4, and *A. niger* N402 genomes are highly divergent paralogs (Figure 4C), which might contribute to the specificity of this target in comparison with other *Aspergillus* species.

The new primer set (Aw_8) was able to amplify the Aw-P5CR gene from complex samples. With high sensitivity, the test not only

detected *A. welwitschiae* in leaves of symptomatic plants but also in samples of apparently healthy plants. This is a significant advancement for research with sisal bole rot disease, regarding epidemiological and control studies, since, until date, the pathogen's proper identification depended on a combination of microbiology techniques with Sanger sequencing (Duarte et al., 2018). The traditional identification methods are also destructive, requiring internal stem samples that implicate cutting and destroying the whole plant. Using leaf samples is an alternative that allows periodic assessments to detect the pathogen at the early stages of plant infection and colonization, before bole rot symptoms become visible. Indeed, our epidemiological study with the Aw_08 primer set detected *A. welwitschiae* in 81%–89% of *A. sisalana* asymptomatic plants, as well as in the asymptomatic hybrids H11648 and H400.

The last published research quantifying sisal bole rot disease in Bahia described a 100% prevalence, while the disease incidence varied from 5% to 40% (Coutinho et al., 2006; Abreu, 2010). Despite the gap of over 10 years, our numbers are consistent with those previous results. In our dataset, there is a prevalence of 100%, while symptom occurrence ranges from 0% to 56% (Table 1). Furthermore, the use of molecular epidemiology techniques brought a higher resolution to this analysis. In our data, 81%–89% of sampled sisal plants did not present any visual symptoms but tested positive for the presence of *A. welwitschiae*. Moreover, this pathogen was also detected in the hybrids H11648 and H400, although only 10% of H11648 plants were symptomatic and all plants of H400 were asymptomatic. According to the dispersion index, the incidence of the disease is regular, indicating, for example, that the spread of the disease may be related to the propagation of contaminated suckers (Duarte et al., 2018; Soares et al., 2020). Appropriately, the location with the lowest disease incidence is the farthest from the territory with the highest incidence. No significant correlation was found between pathogen incidence and soil nutritional analysis, possibly due to the high infection rate, which suggests that the incidence is probably more influenced by the common use of contaminated plant suckers by sisal farmers as planting material.

With the sisal market crisis triggered by the development of synthetic fibers in the late 1960s, investments in research with this crop were either reduced or canceled. In Brazil, the main example of this process was the *Agave* breeding program. From the 1950s–80s, the Agronomic Institute (IAC) was the country's leader in the development of new *Agave* cultivars (Medina, 1954; Medina, 1956; Medina, 1959; Ciaramello et al., 1975; Azzini and Ciaramello, 1977; Salgado et al., 1979; Raya et al., 2023). However, with the lack of investment and the retirement of the leading researchers, there was no continuity of this program (Raya et al., 2021; Raya et al., 2023). Currently, there is no sisal varietal control in Brazil and producers are not concerned with this topic. Nonetheless, with the advancement of bole rot disease, this scenario begins to change. For instance, in Itiúba the high incidence of disease and the susceptibility of the common sisal made many producers lose their entire fields, which pushed those farmers to invest in planting hybrid cultivars, which, according to their popular knowledge, would be more resistant to the disease. Accordingly, we observed a difference in symptomatology between common sisal and hybrid cultivars, especially H400 which did not present any

symptoms (Table 2). Still, further experiments need to be performed to confirm this possible resistance or tolerance. Hybrid cultivars are not widespread in Brazil's sisal farms, mainly because farmers claim that fiber extraction is hampered by leaf rigidity and the foul smell of the bagasse, which could be easily overcome with the adoption of stationary machines.

There is an important parallel between the tequila industry in Mexico and the Brazilian sisal situation that should be pointed out. In the late 1990s, the combination of two pathogens (*Fusarium oxysporum* and *Erwinia* spp.) together with a significant weather event wiped out 1/5 of all *Agave tequilana* planted in Jalisco (Jiménez-Hidalgo et al., 2004; Valenzuela-Zapata and Nabhan, 2004; Vega-Ramos et al., 2013; María de Jesús et al., 2017). In both cases, there was the indiscriminate use of only one clonal line and the presence of opportunistic necrotrophic pathogens. In the Brazilian scenario, this situation could be potentially worse. As the *A. sisalana* cultivated worldwide originated from plants taken to Florida by Henry Perrine in 1836 (Medina, 1959), *A. sisalana* may have higher genetic drift than other cultivated agaves. According to our data, 84% of the *A. sisalana* plants are contaminated with *A. welwitschiae*, and 16% present visible symptoms of bole rot disease (Table 2). The process by which bole rot disease changes from an asymptomatic to a symptomatic phase is complex and yet to be described. However, disease progression might be triggered by nutritional deficiency and/or other abiotic stresses such as water deficit, or even changes in the microbiome (Medina, 1954; Duarte et al., 2018; Quintanilha-Peixoto et al., 2021).

Based on our analysis, it appears that plant nutrition could play a significant role in this pathosystem. Increased K in soil might inhibit bole rot disease symptoms manifestation. Overall, K supplementation has shown to improve plant resistance to pathogens, decreasing the incidence of fungal diseases by 70% (Perrenoud, 1977), and even disease severity (Williams and Smith, 2001), possibly by modulating either basal susceptibility or constitutive defense mechanisms, such as cell wall architecture or secondary metabolites production (Amtmann et al., 2008; Wang et al., 2013). In contrast, we found that higher P could increase symptoms occurrence. Generally, P tends to improve plant health; however, it can also increase pest infestation or disease severity in several models, possibly by improving pathogen nutrition (Perrenoud, 1990; Develash and Sughra, 1997; Walters and Bingham, 2007).

We also noticed that CEC and V% appear to contribute to an increase in symptom occurrence, whereas Ca and Mg seem to have a mitigating effect. Both Ca and Mg are essential minerals for plants, and their balance has direct effects on pathogen-host interactions. For instance, *Fusarium* wilt, a necrotrophic pathogen, presents less severe infections when adequate Mg is available (Huber and Jones, 2013) and Ca is not only crucial for early signaling events for plant defense responses but its supplementation has been shown to reduce *Phytophthora* infections (Sugimoto et al., 2010; Zhang et al., 2014). In our data, soil Ca and Mg were found in concentrations considered to be high for most crops (Raj et al., 2022). CEC and V% measurements are both dependent on Ca, Mg, K, and Al levels. Since no Al was detected, and K varied among soil samples, Ca and Mg were the main contributing factors for CEC and V% correlation to symptom occurrence, which is confirmed by the correlation matrix (Supplementary Figure S4). Ca and Mg might contribute

to overall soil salinity, which can decrease biotic stress responses, increasing plant susceptibility to pathogens (Bai et al., 2018). Although Ca-deficiency was previously described as a possible aggravating factor for bole rot disease (Medina, 1954; Duarte et al., 2018), higher soil Ca levels may be detrimental. Besides possible implications on the host health, Ca levels could be influencing the pathogen's physiology and modulating its virulence. For instance, the recent study of the *A. welwitschiae* genome revealed that Na⁺/Ca²⁺ exchanger genes were under positive selection on phytopathogenic strains (Quintanilha-Peixoto et al., 2022b). Also, *Agave* accumulates calcium oxalate as a herbivory defense (Salinas et al., 2001; Corbin et al., 2015), but fungal necrotrophic pathogens can use calcium oxalate crystals to improve pathogenicity (Uloth et al., 2015). Nutrient uptake is a complex process, and its relationships can be quite specific to each pathosystem. This challenge becomes even more pronounced when studying semiarid crops that naturally employ different strategies to cope with abiotic stresses, such as salinity and drought. The multivariate regression analysis provides intriguing insights into the nutrient-health relationship. However, the limited data hampers confidence in obtaining more meaningful results. In order to comprehend the interplay between disease progression and mineral nutrition, a comprehensive investigation of the nutritional requirements for sisal cultivars is still necessary and would greatly benefit from a thorough study.

The hybrid cultivars are more productive than the common sisal, producing 30% more leaves per year and heavier leaves (Silva and Beltrão, 1999). Productivity is a multifactor trait being the outcome of several processes like photosynthesis, nutrient absorption, or stress tolerance. Differences between these cultivars' metabolism are beginning to be described and would be crucial to understand their agronomical performance. For instance, the H11648 biomass was described as more recalcitrant than *A. sisalana* (Raya et al., 2021), which can enhance resistance to necrotrophic pathogens (Miedes et al., 2014) or abiotic stresses (Moura et al., 2010). Also, cultivar interactions with sisal microbiome might be unique. Even though taxonomically similar microorganisms in *A. sisalana* and H11648 were found, transcriptomic analysis of these fungi revealed that the same groups might be using different metabolisms in each cultivar (Marone et al., 2022). The combination of these specific mechanisms may contribute to robust hybrids that simply make them healthier and, therefore, more able to deal with pathogenic microorganisms, such as *A. welwitschiae*.

The sisal market crises have changed the profile of sisal plantations in Brazil. Originally, sisal farms were large-scale operations (Medina, 1954; Silva and Beltrão, 1999). Now, the fiber is mainly produced by smallholders with little to no agricultural management (Medina, 1954; Silva and Beltrão, 1999; Broeren et al., 2017). Although Brazil is still the world's largest producer, this agro-extractivism system directly reflects in the country's fiber productivity (FAO, 2023), being five times lower than the first in the ranking (China). The lack of disease control measures and low level of technology used in sisal field management, particularly the low quality of sisal plants used for planting new fields, have contributed to the spread of bole rot disease (Soares et al., 2020) but also limits farmers investment capacity. In this scenario, a potential phytosanitary crisis arises. The current propagation system

of this crop is through rhizomes or suckers that may already be contaminated (Kritzinger et al., 1997; Silva and Beltrão, 1999; Applequist, 2003; Li et al., 2017). Another aggravating factor is that there are no nurseries or biofactories (large-scale tissue culture laboratories) producing sisal plants with high phytosanitary quality. Even with access to healthy plants, currently economic fragility of sisal producers is a barrier that blocks the plantations from being renewed with proper field management technologies.

In order to prevent this possible crisis, it is urgent for the sector to mobilize. Through public policies, it would be possible to restore farmers' investment capacity and encourage the production of healthy plants through tissue culture techniques, given that this technology is already mastered for *Agave* (Das, 1992; Bautista-Montes et al., 2022) and is routinely used in other crops (Gerald et al., 2007; Gerald et al., 2011). It is paramount for farmers to be able to count on a broader variety of species/genotypes and affordable clean plants. If the country wishes to maintain the long-term feasibility of sisal production or even correspond to *Agave* as biorenewables feedstock prospects (Raya et al., 2022), the *Agave* breeding program must be re-established. In fact, the sisal production system in Brazil needs to be rethought, as the current one depends on decentralized and semi-automatic fiber extraction process, which makes it difficult to introduce and disseminate more robust productive varieties with greater tolerance to the disease. Nonetheless, field removal of plants with bole rot symptoms and the use of healthy plants for sisal plantations are still the main disease control strategies recommended and farmers need to be encouraged (and able) to follow it. Finally, the set of primers developed in our study is an important tool to implement a disease monitoring program using adequate and more precise techniques, such as detection of the pathogen by PCR, before symptoms manifestation. This approach can significantly enhance early detection and management strategies.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#), further inquiries can be directed to the corresponding author.

Author contributions

FR: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing- Original draft preparation, Writing- Review and editing, Visualization. GQ-P: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing- Original draft preparation, Writing- Review and editing, Visualization. AdO: Conceptualization, Methodology, Formal analysis, Investigation. MM: Methodology, Software, Investigation, Data curation, Writing- Original draft preparation, Writing- Review and editing. WdR: Methodology, Validation, Formal analysis, Investigation. JM: Methodology, Software, Formal analysis, Writing-Original Draft, Writing-Review and Editing. RA: Formal analysis, Writing-Original Draft, Writing-Review and Editing. CF: Writing- Original draft preparation, Writing- Review and editing, Visualization. OC: Resources, Writing- Original draft preparation,

Writing- Review and editing, Visualization. RR: Writing- Original draft preparation, Writing- Review and editing, Supervision. AS: Conceptualization, Writing- Original draft preparation, Writing- Review and editing, Resources, Project administration, Funding acquisition. AG-N: Conceptualization, Writing- Original draft preparation, Writing- Review and editing, Resources, Project administration, Funding acquisition. GP: Conceptualization, Writing- Original draft preparation, Writing- Review and editing, Resources, Project administration, Funding acquisition. All authors contributed to the article and approved the submitted version.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fceng.2023.1174689/full#supplementary-material>

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