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Morphological and Phylogenetic Characterization of Whip Smut on Commercial Sugarcane Cultivars and Assessing the Resistance to *Sporisorium scitamineum*

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ABSTRACT

Whip smut, which is caused by *Sporisorium scitamineum*, is an important disease in areas where sugarcane is cultivated in Iran, particularly in the Khuzestan province. The pathogen significantly reduces sugarcane yield, and the use of resistant cultivars is the most cost-effective strategy for managing the disease. The present study characterized the *S. scitamineum* strains collected from five commercial sugarcane cultivars (CP69-1062, CP57-614, CP48-103, SP70-1143, and NCo310) based on their morphological and phylogenetic features. The sporidial cultures of the strains appeared in two growth forms: cottony colony and yeast-like. All strains were found to be identical based on the DNA sequences of ITS, COX3, GAPDH, and EF1α regions, and revealed that all strains were identical (100%) to the reference strain *of S. scitamineum*. The disease incidence of the cultivars varied from 5 to 43% during two consecutive years. Statistical analysis of the growth rates of the strains indicated significant differences. Combined analysis of variance (ANOVA) suggested that the effects of year, strain, cultivar, and the interaction effect of strain × cultivar were significant at a 1% probability level. Our results suggest that IRK310 was the most virulent among all cultivars, with different pathogenicity percentages, while the strain IRK70 had the lowest level of virulence among all strains. Among the tested cultivars, SP70-1143 and CP57-614 showed high resistance to smut. In this research, teliospore populations of whip smut were identified, and disease reactions of the cultivars were assayed. Screening and selecting smut-resistant cultivars can help reduce disease damage in cultivated areas and can serve as a basis for further research on plant disease management.

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Introduction

Sugarcane (*Saccharum officinarum* L.), which belongs to the Poaceae family, is cultivated for sugar production (Gravois *et al*., 2014). Sugarcane is the most important sugar crop, accounting for over 80% of the world's sugar supply (Dotaniya *et al*., 2016). In Iran, sugarcane contributes to more than 50% of sugar production (Mansouri and Hassanzadeh, 2021). Fungal agents that cause leaf spots and blights, root and crown rots, smuts, and wilting are the most prevalent and extensively spread diseases in regions where this plant grows (Tiwari *et al*., 2017). Sugarcane whip smut, caused by *Sporisorium scitamineum* (Sydow) M. Piepenbr.,

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M. Stoll and Oberw. (Syn: *Ustilago scitamiea* H. & P. Sydow), is an airborne fungal disease threatening the sugarcane fields. This disease first occurred in Natal, South Africa in 1877 and then spread to other regions under cultivation all over the world (Croft & Braithwaite, 2006). Whip smut was reported for the first time in Iran in 1971 in the sugarcane fields of Hafttapeh, the major sugarcane-growing area in the Khuzestan Province of Iran (Ershad & Bani-Abbasi, 1971).

Smut-infected sugarcane plants become shorter and tend to generate a high number of tillers (50 to 100) per plant, each with a smut sorus (Huang, 2004). The disease can cause significant yield loss in susceptible cultivars (Comstock *et al*., 2000). Whip smut is still the most destructive disease in many sugarcane-growing areas of the world, especially in Iran (Sundar *et al*., 2012). A combination of strategies, including the use of resistant cultivars, fungicidal dip of planting setts (Pearse, 1989), hot water treatment of planting setts (Harlapur *et al*., 1992), manipulation of environmental factors (Hoy *et al*., 1993), crop rotation (Abdou *et al*., 1990), delayed irrigation, late planting (Viswanathan & Rao, 2011), and proper land preparation (Suzuki *et al*., 1994), can be effective in decreasing the incidence of sugarcane smut worldwide. Among these, host plant resistance is the preferred strategy to control smut infection (Croft & Braithwaite, 2006). Pathogenic races of sugarcane smut have been studied in several countries, including Hawaii, Taiwan, Brazil, and Pakistan (Bhuiyan *et al*., 2015). Identifying new races or strains of whip smut in each area is an important step toward screening resistant cultivars (Rajput *et al*., 2021). Due to the annual increase in the prevalence of sugarcane smut disease, the development of resistant sugarcane cultivars has become a top priority (Rajput *et al*., 2021). For a successful management strategy using resistant cultivars, it is important to identify the pathogen variants present in each climate zone (Rajput *et al*., 2021).

In recent years, whip smut has become widespread in the sugarcane fields of Khuzestan province, Iran. This study aims to: 1) identify five teliospore populations of whip smut collected from five different sugarcane cultivars, 2) perform multi-locus phylogenetic analyses (MLSA) in combination with morphology, and 3) assay the disease reaction to the strains of *S. scitamineum* in field conditions.

Materials and Methods

Strains and viability assessment

Teliospores of *S. scitamineum* were gathered from whips of the infected commercial cultivars, including CP69-1062, CP57-614, CP48-103, SP70-1143, and NCo310, that were planted in the fields of Iranian Sugarcane Research and Training Institute (ISCRTI, 30°58'10.7"N 48°33'05.1"E). Smut teliospores collected from each plant were dried separately in a sterile warm chamber for two weeks and then stored at 4°C in a refrigerator (Abera, 1991). To cultivate and purify *S. scitamineum* isolates, a dilute teliospore suspension of each sample was first prepared in 0.1% Tween-20 solution supplemented with streptomycin (30 mgL^{-1}) and washed thrice with sterile distilled water. Then teliospores were plated on slides bearing a thin layer of potato dextrose agar medium supplemented with streptomycin 30 mgL^{-1} using a sterile loop. The slides were placed in sterile Petri dishes and incubated at 28-30 °C in the dark. After 12 hours of incubation, the germination percentage was calculated by dividing the number of germinated teliospores by the total number of teliospores observed on the slides and multiplying by 100. A germinated teliospore of each isolate was transferred to the new PDA medium as a single-spore isolate of sporidial culture.

Growth studies

The rate of mycelial growth on the PDA medium was determined for each isolate. Mycelial disks (5 mm in diameter) from the actively growing edge of the 7-day colony of each isolate were prepared using a cork borer and cultured in the center of 80-mm PDA medium amended with streptomycin sulfate. The plates were placed in a randomized complete block design (RCBD) where each isolate was repeated four times and incubated at 28 °C (Izadi and Moosawi-Jorf, 2007). The measurement of the colony's diameter was conducted daily for at least seven days. Data on the cross diameters of each isolate were analyzed using the SAS computer software package (SAS, 2002).

Fragment amplification

The biomass of each isolate grown on the surface of the PDA medium was collected using a sterile glass slide and powdered in liquid nitrogen. Genomic DNA was extracted from about 100 mg of ground mycelia using an organic method described by Raeder and Broda (1985), with some amendments (Ahmadpour *et al*., 2017), and then stored at -20 °C. The quality of the extracted DNA was checked using a loading on the 1.0% agarose gel in electrophoresis. The four loci, including cytochrome C oxidase III (*COX3*), the internal transcribed spacer 1 and 2 with the 5.8S nuclear ribosomal DNA (ITS), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and elongation factor 1α (*EF1α*), were selected to amplify. The primer pairs of *ITS1* (5’-TCCGTAGGTGAACCTGCGG) and *ITS4* (5’-TCCTCCGCTTATTGATATGC) for ITS region (Toju *et al*. 2012), *COX3F* (5’-WGTTACACCKAGYCCWTGGC) and *COX3R* (5’-TAGGAATAGCCAAACWACATC) for *COX3* gene (McTaggart 2010), *GAPDHF* (5’-CGGTCGTATCGGMCGTATC) and *GAPDHR* (5’-GTARCCCCACTCGTTGTCGTA) for *GAPDH* gene (McTaggart 2010), and *EF1αF* (5’-GCCCTMTGGAAGTTCGAGACYCCCA) and *EF1αR* (5'-GAYACCGACAGCRACGGTCTG) for *EF1α* gene (McTaggart 2010) was used. Each polymerase chain reaction (PCR) was performed in a 25 µl volume, which included 12.5 µl of Ampliqon *Taq* DNA Polymerase Master Mix Red (2X, Ampliqon), 9.5 µl of Mili Q water, 1 µl of DNA template (around 100 ng), and 1 µl of each primer (10 µM). Amplicons were produced in an automated thermal cycler (MJ MiniTM Gradient Thermal Cycler) with an initial melting step at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primers annealing at 60 °C (*COX3*), 58 °C (ITS), 65 °C (*GAPDH*), or 62 °C (*EF1α*) for 40 s, and primer extension at 72 °C for 60 s, and a final extension at 72 °C for 10 min. Then, five µl of PCR product obtained was run on 1% agarose gel.

Phylogenetic analyses

The PCR reactions were sequenced using both forward and reverse primers by Cardiogenetic Research Center (Tehran, Iran). Obtained sequences were assembled using BioEdit v. 7.0.9.0 (Hall, 2008) and DNA Baser Sequence Assembler v4 (2013, Heracle BioSoft, https://www.dnabaser.com/index.html), respectively. Consensus sequences were deposited in GenBank. The phylogenetic analysis was performed using reference sequences from the known species of *Sporisorium* (Table 1).

Table 1. Taxa under survey and their sequence accession numbers employed in the phylogenetic analyses.

Species	Strains no.	Source	Origin	GenBank accession numbers			
				ITS	*EF1α*	*GAPDH*	*COX3*
Sporisorium scitamineum	IRK57	*Saccharum officinarum*	Iran	ON843917	ON866513	ON866508	ON866503
S. scitamineum	IRK69	*S. officinarum*	Iran	ON843918	ON866514	ON866509	ON866504
S. scitamineum	IRK48	*S. officinarum*	Iran	ON843919	ON866515	ON866510	ON866505
S. scitamineum	IRK30	*S. officinarum*	Iran	ON843915	ON866511	ON866506	ON866501
S. scitamineum	IRK70	*S. officinarum*	Iran	ON843916	ON866512	ON866507	ON866502

Ustilago hordei was used as outgroup taxa. The ITS, *GAPDH, EF1α*, and *COX3* sequence alignments were performed with Clustal W in BioEdit v. 7.0.9.0 (Hall, 2008) and combined in a multilocus dataset. Maximum-likelihood analysis was performed with raxmlGUI 2.0 beta (Edler *et al*., 2021) using a general time reversible model with gamma-distributed and invariant sites (GTR + G +I) and 1000 bootstrap replicates (MLBS). The maximum parsimony (MP) algorithm was performed in MEGA7 (Tamura *et al*., 2013) with 1000 bootstrapping replication (MPBS). Bayesian inference partitioned analysis (BI) was performed with MrBayes v.3.2.6 (Ronquist *et al*., 2012) using the best fit-model of evolution for each locus proposed by jModelTest 2 (Darriba *et al*., 2012), including GTR + G model for ITS region, GTR + I for COX3, and GTR+ G+ I for both the *GAPDH* and *EF1α.* The Markov Chain Monte Carlo (MCMC) analysis was performed with four Markov chains, two simultaneous runs of 5 M generations, sampling every 1000 generations, the standard deviation below 0.01, and removing

the first 25 % of trees for calculating posterior probabilities values (BPP).

List of sugarcane cultivars and inoculation

The cultivars CP69-1062 (CP62-374× CP56-63; Canal Point, Florida), CP57-614 (CL47-143× CP53-17; Canal Point, Florida), CP48-103 (CP29-320× Co29; Canal Point, Florida), SP70-1143 (IAC 48-65× PC; Brazilian Sugarcane Breeding Center), and NCo310 (Co421× Co312; Natal, South Africa), which had been shown to have different field responses to smut, were selected in this study. These five studied sugarcane cultivars are widely planted in Khuzestan Province, where most of Iran's sugarcane is grown. The cultivars were received from the cultivar museum of the Iranian Sugarcane Research and Training Institute. In this study, one hundred and five budded sets of each cultivar were subjected to hot water treatment at 52 °C in a water bath for 30 min (Fauconnier, 1993), followed by cooling and drying in the sterile air stream. Inoculation was performed by the dip method, as described by Xu and Chen (2000). A concentration of approximately 1.5×10^6 teliospores per milliliter was prepared and used for the dip inoculation. Stalks of sugarcane were stripped to reveal the bud, and then cut into one-bud sets using gardening scissors. The sugarcane sets of each cultivar were put in mesh bags and immersed in a teliospore suspension for 40 min, followed by drying in a sterile condition. A collection of non-inoculated setts from each cultivar was used as a control.

Experiment design

The experiment was conducted for two consecutive years in March and April 2020 and 2021. The sugarcane sets of each cultivar are carefully planted in three kg pots with a mixture of peat, soil, and perlite (1: 1: 1), three sets per pot. The pots were kept at 28-30 °C in a glasshouse for one month. After that, the pots were moved to the field. Plants were watered every day or every two days, depending on the weather conditions. Plants were fertilized every two months. The temperature and relative humidity of the field were recorded. The pots were kept under these conditions for data collection every week for 9 months. A combined analysis of variance of factorial design for the disease incidence observed on the five cultivars inoculated with the five *Sporisorium scitamineum* isolates, based on a randomized complete block arrangement with seven replicates across two years, was implemented. Due to the non-normal nature of collected data (disease incidence), the log transformation method was applied to meet the normality assumption. Data were analyzed using the R ver. 4.2.2 package.

Disease evaluation

The disease was evaluated after 4-8 weeks of the inoculation, as soon as the first smut sours were seen; the results were reviewed twice a week. The plants in each pot were inspected for the formation of smut sori, and shoots with the whip-like structure were trimmed at the footing and removed at each data recording. Data collection was ended when no new smut sori were obeyed for 10 months. Eventually, disease incidence (DI %) was calculated as employed in Bhuiyan *et al*., (2015): DI= (I/T)× 100, where I= number of plants infected, and T= total number of plants counted.

Results

Morphological characterization

Sori formed by colonization of the inflorescences, whip-like, covered by a thin membrane, silver to silver-grey, with very different lengths and rupturing irregularly with age. Spores semi-agglutinated to powdery masses, initially aggregated or mass powdery, separating later, globose to subglobose, reddish-brown to dark brown, punctate, 5.80-10.60 (av. 7.50, n = 50) µm in diameter (Fig. 1). The mean colony growth of *S. scitamineum* strains after seven days of incubation at 28 °C showed a significant difference ($P \leq 0.01$) in radial growth among strains (Table 2). The strains were classified as medium-growing (IRK70) and slow-growing (IRK57, IRK69, IRK48, and IRK310) fungi. None of the strains grew rapidly. The evaluation of colony appearance and color strain suggested that the sporidial cultures of *S. scitamineum* appeared in two growth forms including cottony colony with aerial mycelia for the strains IRK57, IRK69, IRK48, and IRK310,

and yeast-like for the strain IRK70 (Table 2; Fig. 1).

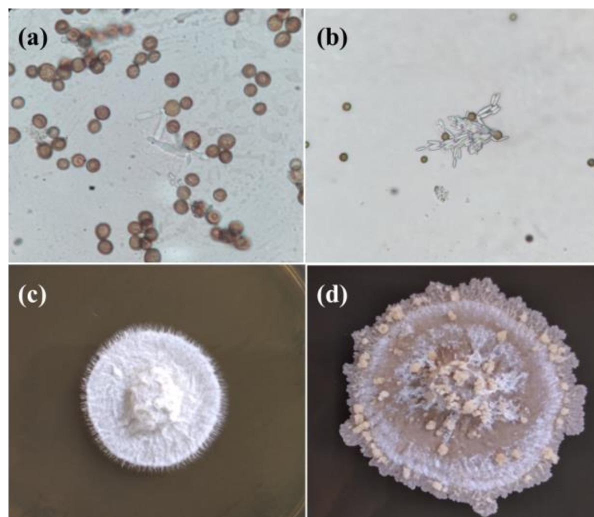

Fig. 1. Microscopic characteristics and different growth of *S. scitamineum* isolates on PDA culture medium: a) Spores (teliospores); b) Teliospore germination; c) White mycelial growth; d) Yeast-like colony.

Material examined: IRAN, Khuzestan Province, *Saccharum officinarum*, 12-31 May- 2018, A. Fazliarab, strains: IRK48 (from cultivar CP48-103), IRK57 (from cultivar CP57-614), IRK69 (from cultivar CP69-1062), IRK310 (from cultivar NCo310), and IRK70 (from cultivar SP70-1143).

Table 2. Mean colony diameter of *S. scitamineum* on PDA after seven days of incubation at 28 °C.

Strains name	Mean colony diameter (cm)*	Colony shape
IRK310	1.80 (c)	cottony colony with aerial mycelia
IRK48	1.94 (bc)	cottony colony with aerial mycelia
IRK57	1.76 (c)	cottony colony with aerial mycelia
IRK69	2.30 (b)	cottony colony with aerial mycelia
IRK70	3.74 (a)	yeast-like colony

*Means with the same letter are not significantly different.

Phylogenetic analysis

The BLASTn search, based on the sequenced regions, revealed that our strains were identical (100%) to the reference strain of *S. scitamineum*. In the phylogenetic tree based on combined ITS, *COX3*, *GAPDH*, and *EF1α* sequences, our strains clustered with the reference strains of *S. scitamineum* (CBS 131463 and Br532) and formed a well-supported monophyletic clade (MLBS 100%, MPBS 81%, BPP 1.00) that showed in figure 2. The ITS, *COX3*, *GAPDH*, and *EF1α* sequences of the strains under study were deposited in GenBank (Table 1).

Resistance response to *S. scitamineum* strains

Analysis of variance showed that the effect of year, strain, cultivar, and interaction effect of strain × cultivar was significant (Table 3). The disease incidence of sugarcane smut in the five cultivars was significant in the following order using the Duncan test: SP70-1143< CP57-614< CP69-1062< CP48-103< NCo310 (Fig. 3). Accordingly, the lowest and highest infection percentage were observed for the SP70-1143 (≅ 5%) and NCo310 (≅ 43%) cultivars, respectively (Fig. 3).

Table 3. Combined factorial analysis of disease incidence on five cultivars inoculated with five *S. scitamineum* strains over two years.

Source	DF	Type III SS	Mean Square
Year	1	0.31778489	0.317**
Replication (Year)	12	0.14970186	0.0125 ns
Cultivar	4	0.53930023	0.135**
Strain	5	0.77666066	0.155**
Cultivar*Strain	20	0.53454249	0.026**
Year*Cultivar	4	0.08697487	0.022 ns
Year*Strain	5	0.11330405	0.023 ns
Year*Cultivar*Strain	20	0.14043520	0.007 ns

**Significant at the 0.01 level; ns: non-significant; † Data underwent square root transformation.

Pathogenicity level of *S. scitamineum*

The reaction of the cultivar CP57-614 to other strains was not significantly different and mentioned strains could not be virulent to this cultivar. For the cultivar CP69-1062, all the strains except the strain IRK70 were pathogenic. The strains of IRK57 and IRK48 had a similar reaction to the cultivar CP48-103. The same thing is happening for the IRK69 and IRK310 strains. These four strains on this cultivar were able to be pathogenic showing different degrees of pathogenicity. However, the IRK70 was not significantly different from the negative control.

Cultivar NCo310 showed a high degree of the disease to strain IRK310 and was sensitive to this strain, interestingly, this strain could be placed in a separate group and could be known as the virulence strain. After that, strains of IRK57 and IRK69 revealed different percentages of the disease. Nevertheless, IRK70 and IRK48 strains were hypovirulent and had no significant

difference compared to the control. Finally, only IRK310 was virulent on cultivar SP70-1143.

In general, IRK310 was more virulent on all cultivars with different pathogenicity percentages. In contrast, strain IRK70 had the lowest level of virulence among all the strains; therefore, this strain could be pathogenic only on the most sensitive cultivars namely NCo310 and CP48-103 cultivars (Fig. 3 and Table 4).

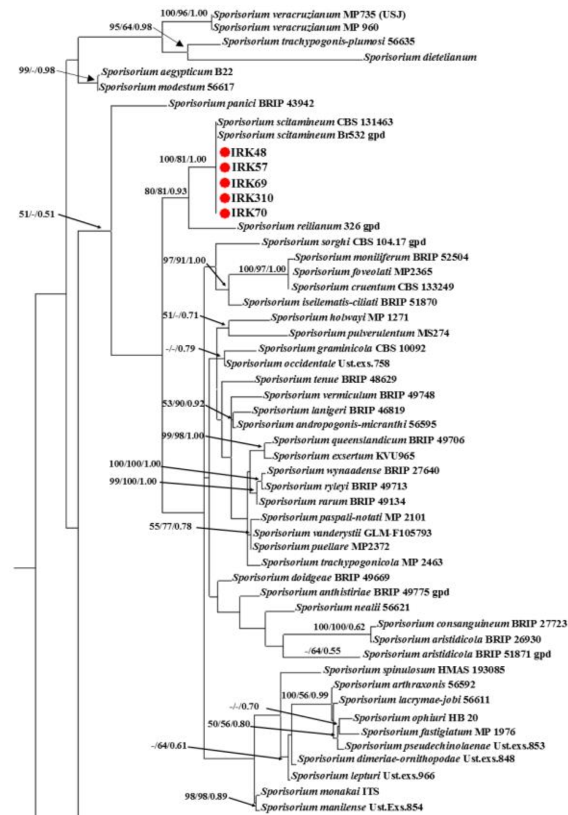

Fig. 2. ML analysis based on a concatenated alignment of ITS, GPDH, Cox3 and EF1α sequences of *S. scitamineum* strains: analysis indicated most previously known species and the tree was rooted with *Ustilago hordei*. Bootstrap support values for maximum likelihood (MLBS) and maximum parsimony (MPBS) ≥ 50% and Bayesian posterior probabilities (BPP) ≥ 0.95 are shown as MP/ML/BI above or below the nodes.

Discussion

Whip smut is considered the most prevalent and severe threat in sugarcane fields worldwide. In this study, we investigated the morphological, pathogenicity, and molecular characteristics of *S. scitamineum* strains collected from five important and commercially grown cultivars of sugarcane in Khuzestan province, Iran. The sori and teliospores of the newly collected strains in this study were similar in their morphological characteristics to the type and reference strains of *S. scitamineum* (Ainsworth, 1965), with an average size of 7.50 µm (ranging from 5.80 to 10.60 µm). The size of teliospores in this research was similar to the size of teliospores obtained from Ainsworth (1965). Two growth forms of sporidial cultures were observed in this study, including mycelial and yeast-like colonies. These observations are consistent with the results of Zekarias *et al*. (2013) and Jacques-Edouard *et al*. (2020) studies, in which the strains isolated from sugarcane in Kenya and Côte d'Ivoire had both mycelial and yeast-like colonies. In the above-mentioned studies and other studies (Fereol, 1984; Abera, 1991; Izadi and Moosawi-Jorf, 2007; Ricaud *et al*., 2012), mycelial-form growth was more common than yeast-like.

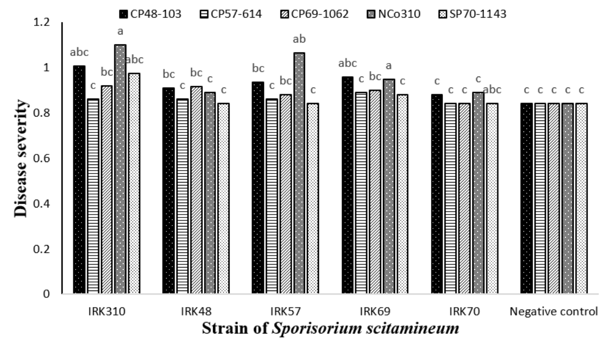

Fig. 3. The disease severity resulting from the interaction of *S. scitamineum* strains and five sugarcane commercial cultivars (*Means followed by same letters are not statistically different).

In this study, the molecular identification of *S. scitamineum* strains was performed by examining housekeeping genes such as ITS, COX3, GAPDH, and EF1α. In the present work, none of the five strains differed from each other in these genomic regions. All the strains under survey formed a monophyletic group with the authentic strains of *S. scitamineum* (CBS 131463 and Br532). The phylogenetic analyses based on concatenated DNA sequences of these four genes were able to separate the studied strains of *S. scitamineum* from the rest of the species in the genus Sporisorium, which is consistent with the results of McTaggart (2010).

Table 4. Comparing disease incidence on five cultivars inoculated with five *Sporisorium scitamineum* strains over two years.

Cultivar/strain	IRK57[†]	IRK69	IRK48	IRK310	IRK70	Negative control
CP57-614	0.86 (c)	0.88 (c)	0.86 (c)	0.86 (c)	0.84 (c)	0.84 (c)
CP69-1062	0.88 (c)	0.89 (bc)	0.92 (bc)	0.92 (bc)	0.84 (c)	0.84 (c)
CP48-103	0.93 (bc)	0.95 (abc)	0.91 (bc)	1.01 (abc)	0.88 (c)	0.84 (c)
NCo310	1.06 (ab)	0.95 (abc)	0.89 (c)	1.10 (a)	0.89 (c)	0.84 (c)
SP70-1143	0.84 (c)	0.88 (c)	0.84 (c)	0.97 (abc)	0.84 (c)	0.84 (c)

[†]Data underwent square root transformation; Values within a column followed by letters are significantly different at the 0.01 level using the Duncan test; Values within a column followed by the same letter are not significantly different using the Duncan test.

Data analysis of the disease incidence caused by each strain indicated a significant difference in the pathogenicity of each isolate on different cultivars, which is in accordance with the results reported by Syed *et al*. (2015), and Deng *et al*. (2018). In this study, the resistance response of five cultivars of sugarcane was investigated after they were inoculated with different strains of *S. scitamineum*. Since a large body of literature has reported that the use of resistant cultivars could be the most cost-effective strategy for disease management (Scortecci *et al*., 2012; Sundar *et al*., 2012), the main goal of this study was to identify more resistant cultivars against whip smut. A significant difference in the disease incidence of the five sugarcane cultivars inoculated by *S. scitamineum* was observed, indicating their different levels of potential smut resistance. This result is consistent with previous findings in different studies (Wada *et al*., 1999; Thokoane and Rutherford, 2001; Tafesse *et al*., 2009; Zekarias *et al*., 2013).

In this study, the cultivar SP70-1143 was identified as resistant to the prevalent strains of S. *scitamineum* in the areas under investigation. This cultivar was also previously reported to be the most resistant among the 16 experimental cultivars of sugarcane in the Ivory Coast (KOUAMÉ *et al*., 2012). Accordingly, the SP70-1143 cultivar has been selected for sugarcane breeding programs (Mario *et al*., 1995; Pereira, 2001; Matsuoka *et al*., 2009). The NCo310 cultivar was shown to be the most sensitive among the five tested cultivars of sugarcane, which is consistent with the results of previous studies in India, Japan, and Sudan (Singh *et al*., 2005a; Hassin, 2016; Sakaigaichi *et al*., 2019).

This study aimed to evaluate the resistance of commercially grown sugarcane cultivars to the common strains of *Sporisorium scitamineum* and estimate the disease risk under current pedoclimatic conditions. The obtained results will contribute to improving field crop management for further studies.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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