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Research Paper

Distribution, diversity and identification of hot spot of *Fusarium* spp. associated with onion (*Allium cepa* L.) in Burkina Faso

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Abstract. In Burkina Faso, onion (*Allium cepa* L.) crops are increasingly confronted with numerous biotic threats and diseases caused by fungi are among the main factors constraining onion production. Observation of various *Fusarium*-like symptoms including seedling melt and basal rot, on onion, suggests the involvement of several species and underlines the pressing need for additional information on their diversity, frequency and distribution. Three hundred thirty infected onion samples were collected from north to south in the central region of Burkina Faso and two hundred and thirty-one isolates of *Fusarium* species were recovered. The highest frequency of 86.66% was recorded in the Yatenga province and the lowest in the Zoundwéogo with 53.33%. From North to South, a drop in the frequency of *Fusarium* infection has been observed. Forty isolates were selected for molecular characterization based on the amplification of the translation elongation factor 1α (TEF-1α) gene. Among them, 30 isolates contained an amplified region with approximately 420 bp and showed 86.21% to 100% similarity to TEF-1α with NCBI reference strains. Twenty isolates were identified as *Fusarium oxysporum* (3 isolates represented specialized form *Fusarium oxysporum* f.sp. *cepae*), *Fusarium acutatum*, *Fusarium falciforme*, *Fusarium solani*, *Fusarium proliferatum*. Sequences with similarity rates <98% were not identified and were named *Fusarium* sp. *Fusarium solani* and *Fusarium falciforme* were the most distributed species and corresponded to 30% of all identified isolates. Sanguié province shows the largest diversity of species.

Keywords: Burkina Faso, *Fusarium* spp., molecular characterization, onion (*Allium cepa* L.).

INTRODUCTION

Vegetable production is one of the main activities for farmers in Burkina Faso during the dry season and the supply chain involves many actors, including smallholder farmers and sellers of vegetable products. The generated income made vegetable production became a strategic activity for poverty and food insecurity reduction, in rural and peri-urban areas (DPSAA, 2011). The common onion (*Allium cepa* L.) is one of the most important vegetables grown in Burkina Faso since 2007 (Tarpaga, 2012) especially in Sudano-Sahelian and Sudano areas of Burkina Faso, with a national average yield of 19 t/ha (DGESS, 2019). However, onion production is increasingly confronted with numerous biotic constraints including fungal diseases. These diseases caused considerable economic loss to onion production in recent years. They include basal rot, wilt and seedling melt caused by fungal

species belonging to the *Fusarium genus* (Ozer *et al.*, 2004; Shwartz and Mohan, 2008). Basal rot has been associated with different species of *Fusarium*, including *F. oxysporum, F. proliferatum, F. solani, F. acuminatum, F. redolens, F. verticillioides, F. equiseti, F. culmorum, F. falciforme, F. brachygibbosum, F. acutatum, F. fujikuroi, F. anthophilium* and *F. thapsinum* (Haapalainen *et al.*, 2016; Kintega *et al.*, 2020b; Kalman *et al.*, 2020; Le *et al*., 2021). Indeed *Fusarium oxysporum* f. sp. *cepae* and *Fusarium acuminatum* have been reported to be the cause of basal rot, slight discoloration of the basal plate, death of older leaves and whole plant (Cramer, 2000; Delgado-Ortiz *et al*., 2016). The species *Fusarium proliferatum* caused bulb rot of stored onions and salmon-pink discoloration of bulbs (Galván *et al*., 2008). Moreover, *Fusarium falciforme* produced yellowish and chlorotic leaves from the tip which expand, progressively to all the leaves, softening the bulb's consistency, necrosis in the basal part of the bulbs, root system reduction (Tirado-Ramírez *et al.*, 2018b). Thus, the genetic, morphology and pathogenicity characterization of *Fusarium* species in Burkina Faso will be useful for the efficient management of onion production. Hence, it is critical to associate the morphological and molecular characterization to better understand the distribution of the Fusarium species since their morphology change depending on the nutrient substrate making the morphological identification problematic (Champion, 1997; O'Donnell *et al.*, 1997; Lin *et al*., 2014; Kee *et al*., 2020; Kalman *et al.*, 2020). The molecular method based on PCR and sequencing of the small subunit ribosomal RNA gene, universal internal-transcribed spacer (ITS) is mainly used for the identification of many pathogenic species as *Fusarium* (Montri *et al*., 2009; Afanador-Kafuri *et al*., 2014). This method based on ITS underestimates the phylogenic diversity because the loci are highly conserved (Balajee *et al.*, 2009). To overcome these limitations, the translation elongation factor 1-alpha (TEF1) gene was targeted. This gene is a monocopy locus in the *Fusarium* genus that encodes an essential part of the protein machinery (Debourgogne, 2013). The complex etiology of onion diseases and the recent observation of diverse and unusual fungus-like symptoms on fields point to the urgent need for additional information on the diversity and distribution of *Fusarium* species and the etiology of the associated disease based on a large-scale survey across agroclimatic regions. So this study aimed to inventory *Fusarium* species associated with onion diseases in Burkina Faso.

MATERIALS AND METHODS

Sample collection

Surveys were conducted from August 2019 to February 2020 in the Sahelian zone (annual rainfall between 400 and 600 mm) and the sub-humid Sudano-Sahelian zone (annual rainfall between 600 and 900 mm) of Burkina Faso. During the surveys, most collection sites were located around water reservoirs. The name of the locality, farmer, variety and geographical coordinates (latitude, longitude) were recorded. A total of 330 onion samples with *Fusarium*. like symptoms were collected from nursery to ripening stage in plots. Forty-five samples were collected from 3 localities of Yatenga province in the Sahelian zone, and 285 samples from 19 localities from seven provinces in the Sudano-Sahelian zone (Figure 1). Three fields were surveyed per locality. Five symptomatic onion plants were collected from each field. All samples were placed in minigrip bags and stored in an icebox to retain humidity till further laboratory analyses.

Fungus isolation

Fungus Isolation was performed as described by Davet and Rouxel (1997) with slight modifications. Root tray, bulb and leaf sections have been taken from samples, separately. To eliminate saprophytes, each organ section was surface sterilized with 5% sodium hypochlorite for three minutes. washed with sterile distilled water, then three to five sections per organ and per sample were placed in Petri dishes containing Potato Dextrose Agar (PDA) medium amended with spectinomycin (one disk per dish) at room temperature (28 ± 2°C) for a week. Pure cultures of isolated fungi were obtained by repeated transfer to a fresh PDA medium. Macroscopic characteristics of culture (colony staining, mycelium appearance) were observed. A table Primo Star - Zeiss microscope was also used to observe the spore shapes. Manuals of Champion (1997) and Leslie and Summerell (2006) were used for identification. The frequency of *Fusarium* spp. on symptomatic onion plants was recorded as follow:

$$\text{Frequency (\%)} = \frac{\text{Number of } \textit{Fusarium} \text{ infected samples}}{\text{Total number of collected samples in the site}} \times 100$$

Molecular characterization of *Fusarium* spp

Dna extraction

Single-spore cultures for molecular characterization were performed as described by Rapilly (1968) with slight modifications. Four disks of seven-day-old culture on PDA were inoculated in 100 ml of Potato Dextrose Broth (PDB) medium for one week as described by Kintega *et al.* (2020b), and the fungal mycelia were harvested by filtering the broth by using a vacuum pump. Finally, the mycelia were collected in Eppendorf tubes and DNA was extracted

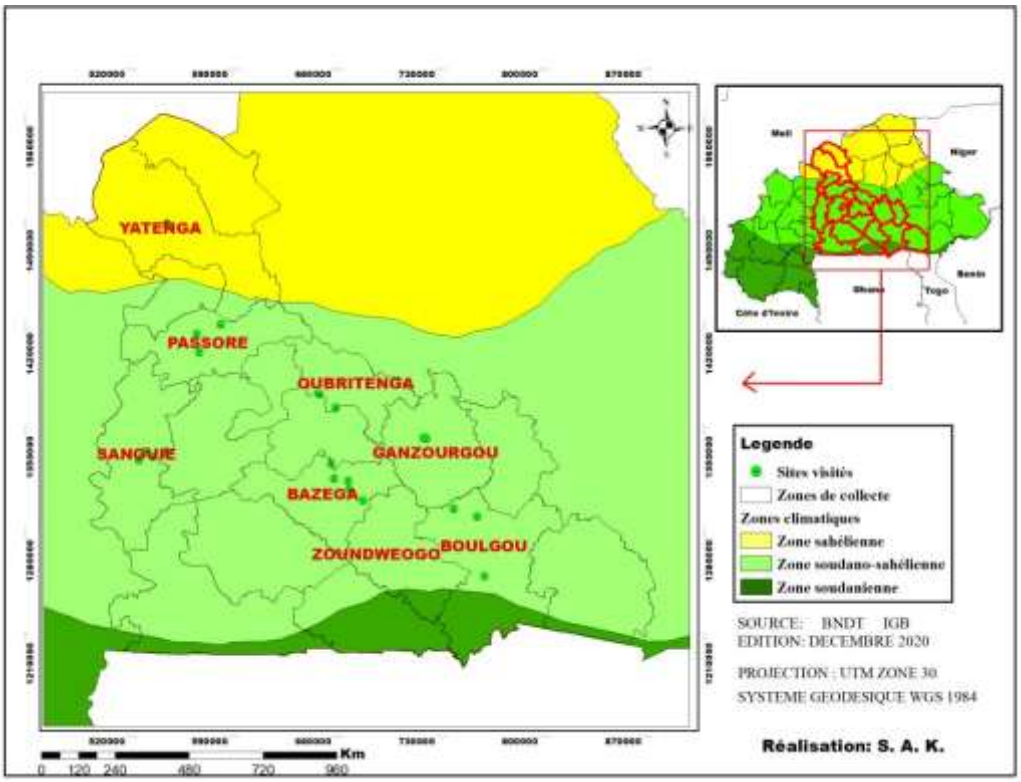

Figure 1. Map showing the different sites where onion plant samples were collected.

using Cetyl Trimethyl Ammonium Bromide (CTAB) method (O'Donnell *et al*., 1997) as recommended by Geiser *et al*. (2004). One hundred micrograms (100 mg) of mycelium for each isolates was grinded in 600 µL of CTAB extraction buffer (CTAB, 2%; NaCl, 1.4 M; β-mercapto-ethanol, 0.2%; EDTA, 20 mM; Tris-HCl, pH 8, 100 mM). Tubes were placed in a water bath at 60°C for 30 min. After cooling at room temperature, 450 µl of phenol and 450 µl of chloroform isoamyl alcohol were added to each tube and then mixed by inversion of tubes until emulsion appear. The mixture was then centrifuged at 13000 ×g for five minutes at 25°C. Five hundred microliters of supernatant were added to 400 µl of chloroform isoamyl alcohol in new tubes and then, centrifuged at 13000 ×g for two minutes at 25°C. The new supernatants were transferred to other tubes and 350 µl of isopropanol was added to precipitate. After further centrifugation of the mixture, the pellet obtained was twice rinsed with 500 µL of 75% alcohol and centrifuged for five minutes at 13,000 ×g at 25°C, before being dried at room temperature. The resulting DNA was stored at -20°C in 50 µl of distilled water.

Polymerase chain reaction and sequencing

TEF-1α elongation factor gene was amplified using the primers TEF-Fu3r (TAGTAGCGGGGAGTCTCGAA) and TEF-Fu3f (GGTATCGACAAGCGAACCAT) synthesized by Eurogentec, according to Arif *et al*. (2012). The amplification reaction was performed in a 24 µl reaction volume containing 2 µl of the stock DNA, 5 µl of buffer [FIREPol ® DNA polymerase, 5X reaction buffer B, MgCl2 (12.5mM), dNTPs (1mM), blue dye, yellow dye], 0.5 µl each of both forward and reverse primers (10 µM), and complete to the final volume by sterile distilled water. The amplification reaction was performed using a thermocycler (BIOER-LifeTouch). The PCR program was as follows: pre-denaturation at 94°C for 5 min followed by 35 consecutive cycles, denaturation at 94°C for 30 s, primer specific hybridization at 58°C for 30 s and elongation at 72°C for 45 min and post-elongation at 72°C for 10 min. Electrophoresis was performed on a 1% (W/V) agarose gel which was prepared with 1 X TBE buffer (Tris Borate EDTA at pH 8). Amplification products were plated in the presence of a molecular weight marker with a size from 300 to 10000 bp. Electrophoresis was performed at 100 V for 1 h in 0.5 X TBE buffer. Gel visualization was performed by staining in an ethidium bromide (BET) bath for 15 min followed by distaining in a water bath for 10 minutes. Once ready, the gel was photographed under UV light from a UVDI-01-254 trans-illuminator with a 10-megapixel camera.

The resulting PCR amplicons were sequenced by the Sanger sequencing method using primer-walking when

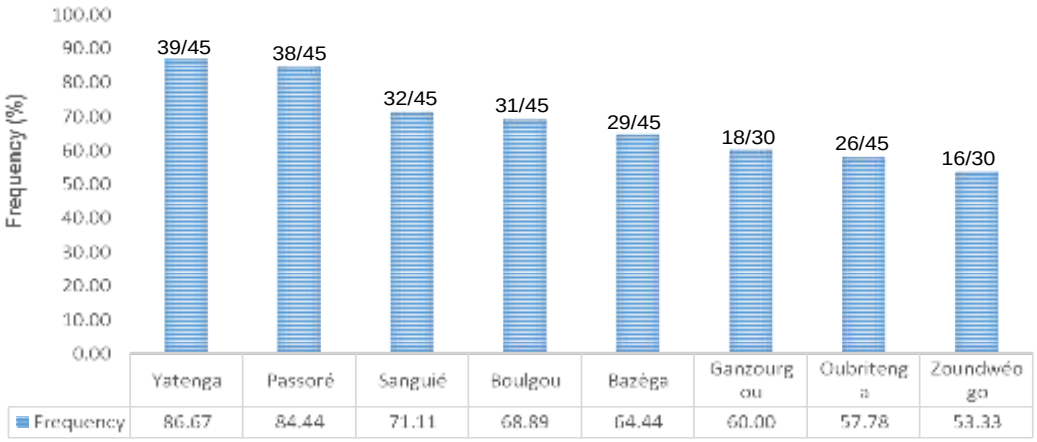

Figure 2. Frequency of *Fusarium* spp. in eight provinces of Burkina Faso.

needed (Macrogen, Europe).

Phylogenetic analysis

Resulted sequences were first assembled using Geneious R8 software (Biomatters Ltd, New Zealand). Representative TEF1-α sequences of *Fusarium* species from the NCBI nucleotide sequence database were close to our consensus sequences obtained after processing and cleaning. Sequences were aligned by MUSCLE (Edgar, 2004) with MEGA 7.0.21 (Kumar *et al.*, 2016). The phylogenetic tree was constructed using FigTree v1.4.4 using the Maximum Likelihood (ML) algorithm. Pairwise identity comparisons of nucleotide sequences were performed using SDT v1.2 with pairwise gap deletion.

RESULTS

Morphological characterization of *Fusarium* spp. with high frequency in surveyed provinces

Morphological characterization, based on microscopic observation of fungus, shows that 231 samples were found to be contaminated with *Fusarium* spp., with a rate of 70%. Two hundred and sixty-seven *Fusarium* spp. isolates were obtained from these samples including co-infection cases. Infection frequencies of samples ranged from 53 to 87% obtained from Zoudwéogo and Yatenga provinces, respectively (Figure 2). Interestingly, fusarium infection prevalence decreased according to north to south gradian.

Identification and molecular characterisation of a complex of least five *Fusarium* species

Based on the identification keys using a light microscope, 40 isolates (five isolates per province) out of the 267 fungi isolates belonging to *Fusarium* genus were selected for molecular characterization. Using the PCR protocol for TEF-1α elongation factor gene amplification, agarose gel electrophoresis showed bands for 31 isolates with an amplified region size that was approximated to 420 bp, corresponding to the amplicon.

After amplicons sequencing and sequences analysis, 30 DNA sequences were obtained from 30 out of the 31 amplicons. Sequences analysis showed percentages of identity ranging from 86.21% to 100% were found (Figure 3).

They revealed that four DNA sequences (BF-Fus12-2021-SKH, BF-Fus19-2021-SKH, BF-Fus23-2021-SKH and BF-Fus26-2021-SKH) were isolates of *Fusarium oxysporum* with similarities ranging from 98 to 99%. Three sequences (BF-Fus10-2021-SKH, BF-Fus14-2021-SKH, and BF-Fus21-2021-SKH) shared 98 to 99% similarity with *Fusarium acutatum*. Six sequences (BF-Fus7-2021-SKH, BF-Fus9-2021-SKH, BF-Fus22-2021-SKH, BF-Fus29-2021-SKH, BF-Fus30-2021-SKH and BF-Fus31-2021-SKH) were members of *Fusarium falciforme* species with similarities comprising between 98 to 100 %. Six sequences (BF-Fus1-2021-SKH, BF-Fus3-2021-SKH, BF-Fus4-2021-SKH, BF-Fus5-2021-SKH, BF-Fus17-2021-SKH and BF-Fus25-2021-SKH) were closed to *Fusarium solani* species with 98 to 100% of similarity. A sequence (BF-Fus11-2021-SKH) was assigned to *Fusarium proliferatum* with 99.70% of similarity. The results of phylogenetic analysis grouped these 20 sequences in five clades corresponding to *Fusarium proliferatum, Fusarium solani, Fusarium oxysporum, Fusarium acutatum* and *Fusarium falciforme* (Figure 3).

The other ten DNA sequences (BF-Fus2-2021-SKH, BF-Fus6-2021-SKH, BF-Fus8-2021-SKH, BF-Fus13-2021-SKH, BF-Fus15-2021-SKH, BF-Fus16-2021- SKH, BF-Fus18-2021-SKH, BF-Fus20-2021-SKH, BF-Fus27-2021-SKH and BF-Fus28-2021-SKH) showed similarity rates

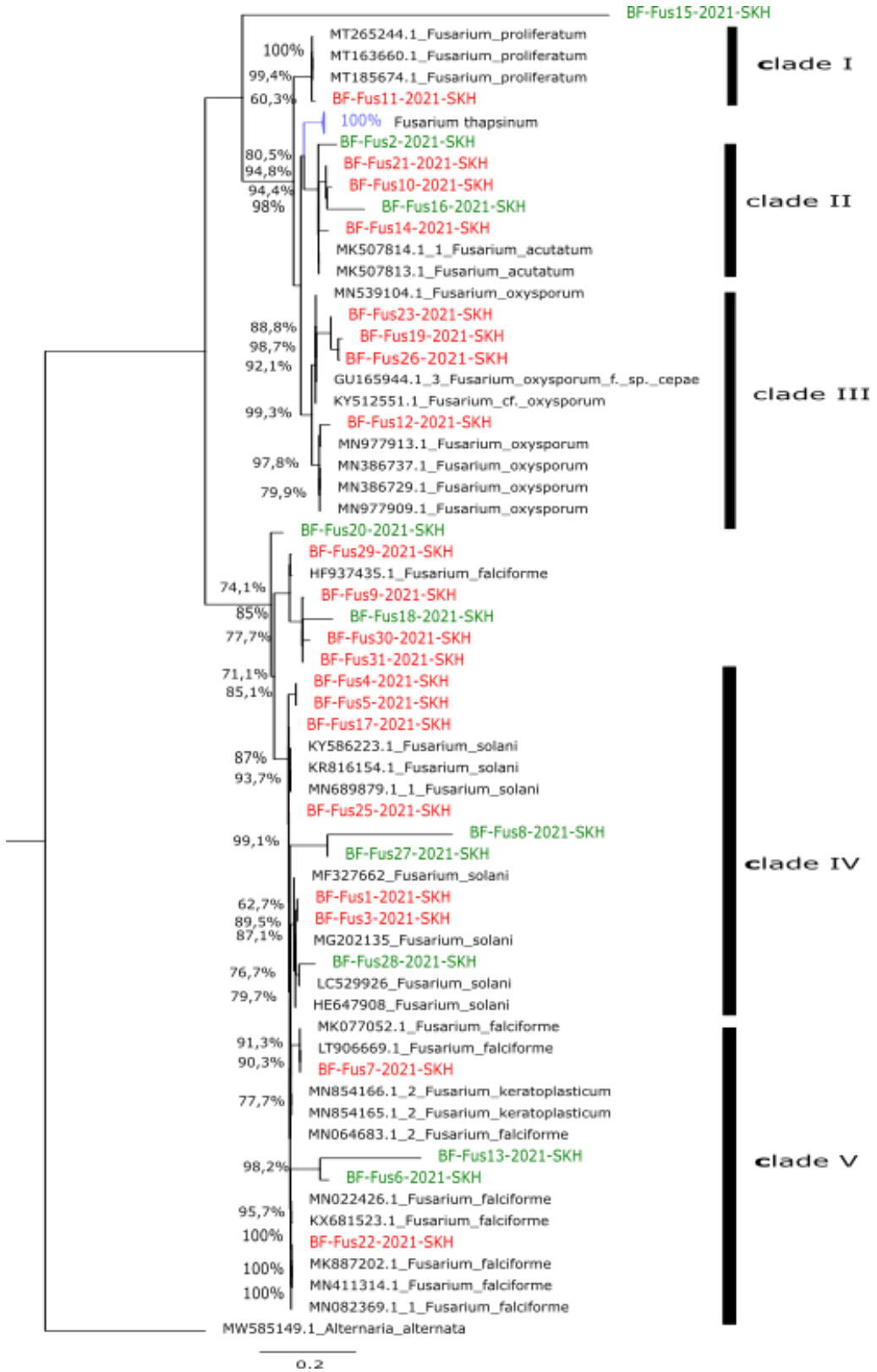

Figure 3. Phylogenetic tree generated from the sequence set by the maximum likelihood (ML) method using FastTree. The sequences in red represent the identified sequences and in green the unidentified ones. The "Out group" sequence is

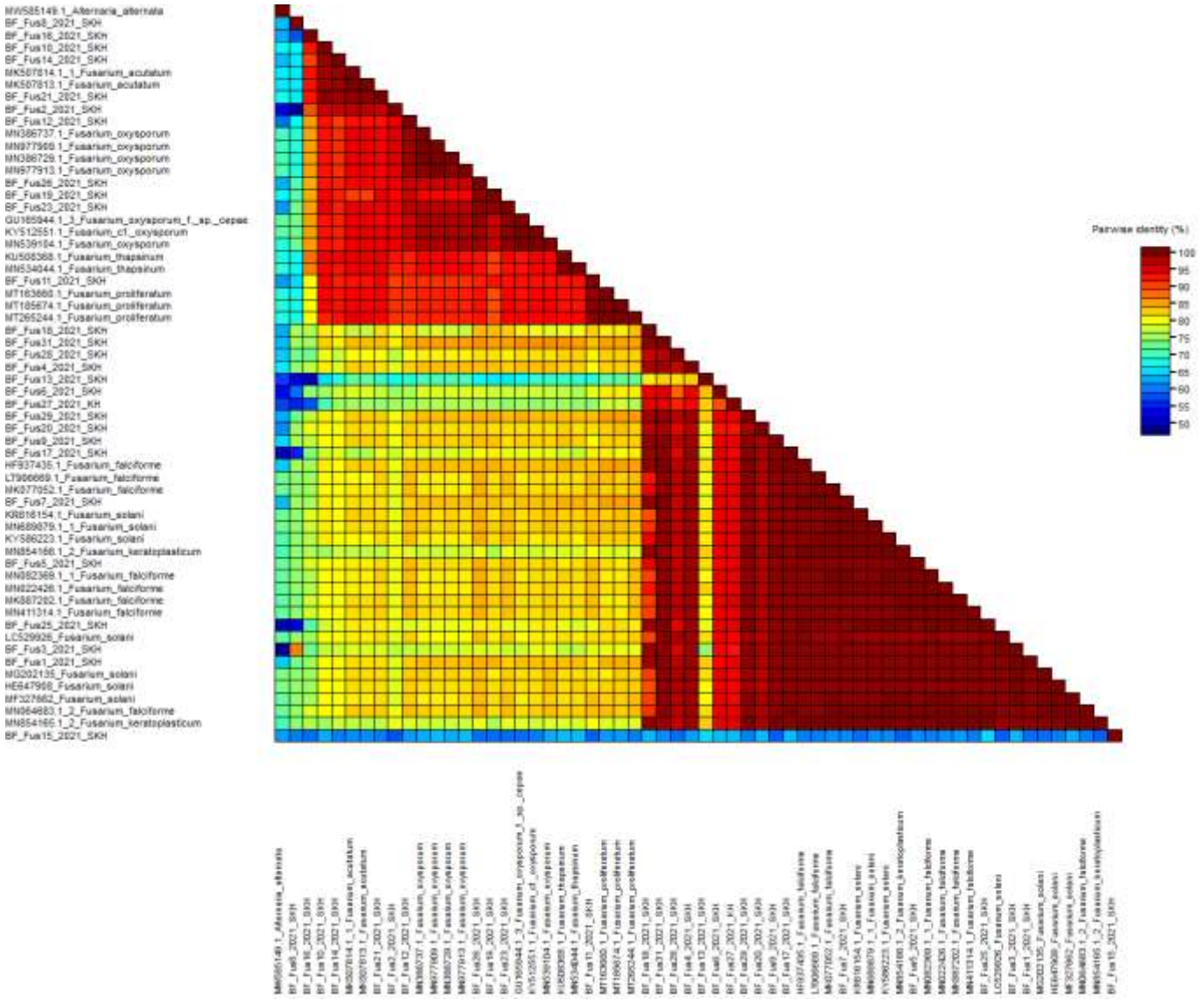

Figure 4. Colour-coded matrix of pairwise nucleotide identity deduced from alignments of partial DNA sequences of the *Fusarium* spp. elongation factor gene TEF-1 alpha with selected reference sequences from NCBI. Rates are calculated without taking gaps into account.

ranging from 86.21 to 97.73% with *Fusarium* species sequences. Thus, these sequences belonged *Fusarium* genus. Phylogenetic analysis showed the distribution of these sequences between the five previous clades excepted BF-Fus15-2021-SKH.

For the identification of fungal species no threshold based on the similarity rate of the TEF1-alpha region has been set (Geiser *et al.*, 2004). Variation around 1.5% can be considered as the same species. In most cases, an identical match with 99 to 100% coverage can be interpreted as a definitive species identification (O'Donnell *et al.*, 2015; Zarrin *et al.*, 2016). Thus, in the present study, a sequence with a percentage similarity between 98 and 100% to a GenBank species was considered to belong to that species if it falls within the same clade with a robust Bootstrap. Similarity analysis rates between sequences performed with SDT show base-pair identities without taking gaps into account (Figure 4). It showed similarity rates approximately equal to those obtained by the phylogenetic analysis. This comparison showed that the isolates belonged to five *Fusarium* species: *Fusarium oxysporum* Schltdl. (4 isolates) of which 3 represent the specialized form *Fusarium oxysporum* f.sp. *cepae* (Mart.) Sacc., *Fusarium acutatum* Nirenberg & O'Donnell (3 isolates), *Fusarium falciforme* (6 isolates), *Fusarium solani* (Mart.) Sacc. (6 isolates), *Fusarium proliferatum*

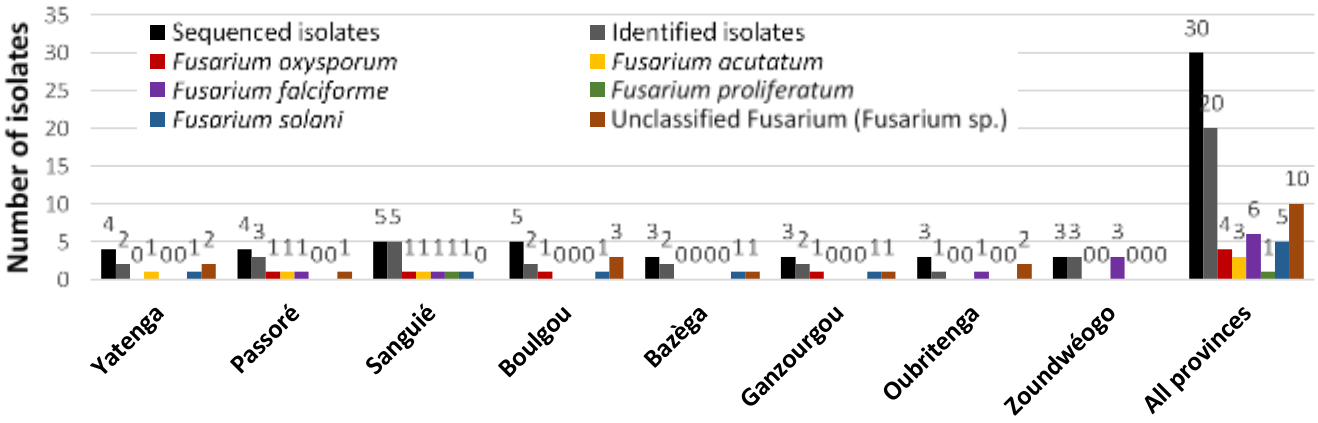

Figure 5. Distribution of *Fusarium* species in provinces from Burkina Faso.

(Matsush.) Nirenberg (1 isolate). Sequences with similarity rates < 98% were identified to the generic level, as *Fusarium* sp.

Diversity and distribution of *Fusarium* species in provinces

Considering all provinces, sequencing data showed that *Fusarium solani* and *Fusarium falciforme* were the most present species with five and six isolates out of the 20 identified isolates, respectively (Figure 5). These species were also the most distributed. *Fusarium solani* was detected in five (Yatenga, Sanguié, Boulgou, Bazèga, Ganzourgou) out of the height provinces; while, *Fusarium falciforme* was present in four provinces (Passoré, Sanguié, Oubritenga, Zoundwéogo). *Fusarium oxysporum*, *Fusarium acutatum* and *Fusrium proliferatum* represent 20% (4/20), 15% (3/20) and 5% (1/20) of the identified species and were detected in four, three and only one province(s), respectively.

Interestingly, Sanguié province was identified as a great hot spot with the detection of all of the five species of *Fusarium.* However, none unclassified *Fusarium* isolate was found there; while 10 unclassified *Fusarium* isolates (*Fusarium* sp.) were found with different proportions in the other provinces excepted Zoundwéogo. Onion, in this latest province, was only infected by *Fusarium falciforme*.

DISCUSSION

Onion basal rot caused by fungal species belonging to the *Fusarium genus* induced considerable economic damage to onion production in recent years. They include basal rot, wilt and seedling melt. This work aimed to study the distribution and the diversity of *Fusarium* species associated with onion diseases in Burkina Faso. First fungal isolations confirmed the presence of *Fusarium* fungi in Burkina Faso with an infection frequency of 70% of symptomatic collected samples. This highlights the great frequency of pathogens associated with onion basal rot in Burkina Faso. These results are in agreement with those of other studies (Dabiré, 2017; Kintega *et al.* 2020b) conducted in Burkina Faso. High frequency of *Fusarium* spp. was recorded in Yatenga followed by Passoré and Sanguié. This could be explained by the fact that these provinces are among the major producers' areas in Burkina Faso. Indeed, large areas have been set aside for onion production for several years in these localities. In addition, poor cultivation practices favor the installation of pathogens. Very few producers practice rotation or good cultural practices from nursery to transplanting. Most *Fusarium* spp. are telluric and can remain in the soil for several years in large quantities (Jiménez-Fernadez *et al*., 2010). In addition, climatic conditions such as temperature and relative humidity contribute to the installation of these fungus pathogens. Samples did not show a rate of 100% of *Fusarium* spp. Infections. This could be due to the infection of the onion by other fungal pathogens such as *Sclerotium cepivorum* and *Phoma terretris* (Schwartz and Mohan, 2008; González *et al.*, 2010).

Morphological identification made it possible to recognize the isolates of *Fusarirum* genus but our results confirmed the difficulties in identifying *Fusarium* species because of their morphological similarity. Hence the need to integrate the molecular characterization is required for an accurate classification (Geiser *et al*., 2004; Zarrin *et al.*, 2016). Molecular characterization in this study shows that onion basal rot is associated with a complex of five species (*F. oxysporum*, *F. solani*, *F. acutatum*, *F. proliferatum* and *F. falciforme*) and unclassified isolates belonging to *Fusarium* genus. This result highlights the existence of a large diversity of *Fusarium* species in Burkina Faso. *F. oxysporum* (Dabiré, 2017; Kintega *et al.*, 2020b), *F. solani* (Dabiré,2017; Kintega *et al*., 2020b) and *F. proliferatum*

(Kintega *et al*., 2020b) have already been reported in Burkina Faso in addition to *F. fujikuroi* and *F. thapsinum* (Kintega *et al*., 2020b) on onion. This study updates the number of *Fusarium* species infecting onion in Burkina Faso to seven with two species known as *F. acutatum*, and *F. falciforme* and shows the efficiency of the PCR diagnosis based on the TEF gene. In addition, the unclassified *Fusarium* isolates we obtain could suggest the existence of other *Fusarium* species on onions in Burkina Faso. This highlights the lack of knowledge of the exact diversity of *Fusarium* fungi and the need to continue with epidemiological surveillance of these pathogens.

Results in this study showed that *F. solani* and *F. falciforme* were the largest distributed species. In contrast, an earlier study concluded that *F. oxysporum* and *F. proliferatum* were strongly distributed (Kintega *et al.*, 2020b). However, Dabiré (2017) showed that *F. solani* was the most encountered species at all the sites he visited. Taken together, it exists an evolution within the groups of *Fusarium* species and this underlines the need for regular epidemiological monitoring. The large distribution of *F. solani* and *F. falciforme* in prospected provinces can be related to the use of already contaminated seed and other host plants that harbor *Fusarium* spp. The seedling melting and other symptoms associated with *Fusarium* would therefore be the work of several species. This synergy of action has been reported by other studies (Champion, 1997; Conn *et al.*, 2012; Dabiré, 2017; O'Donnell *et al.*, 2015, Kee *et al.*, 2020; Dongzhen *et al.*, 2020). This study confirmed the presence of *F. solani*, *F. oxysporum* and *F. proliferatum* on onion in Burkina Faso. These results are in agreement with previous findings (Dabiré, 2017; Kientéga *et al*., 2020b) who had already reported the presence of these species using EF1 and EF2 primers. On the other hand, *F. acutatum* and *F. falciforme* were reported for the first time through this study. These species are probably being introduced into Burkina Faso from the multiple seed varieties that exist in the country with diverse origins. Kalman *et al*. in 2020 showed that *F. acutatum* can prevent the germination of onion seeds. According to Tirado-Ramirez *et al.* (2021), *F. falciforme* is a new agent of onion bulb rot and is more virulent on the bulb than *F. oxysporum*.

The province of Sanguié showed the greatest diversity of *Fusarium* species and can be considered as the hot spot of *Fusarium* fungus. The intensification of onion production in the farms of this province could be one of the causes in addition to the favorable conditions for the development of the pathogen.

CONCLUSION

Using molecular analysis based on TEF-1α was possible to confirm that several *Fusarium* species were associated with fusariosis of onion in Burkina Faso. During this study, five species were identified: *F. solani*, *F. oxysporum*, *F. falciforme*, *F. acutatum* and *F. proliferatum*. Ten isolates belonging in *Fusarium* genus were not assigned to species. Frequency ranged from 53.33 to 86.66%. Yatenga, Passoré and Sanguié provinces recorded the highest prevalence with 86.66, 84.44 and 71.11%, respectively. Sanguié province was the hot spot with a strong diversity of *Fusarium* species. It was followed by Passoré. From North to South, the frequency of *Fusarium* infection decreased. *Fusarium solani* and *Fusarium falciforme* were the most encountered in the provinces visited. In order to establish a complete etiology of *Fusarium* wilt, it would be necessary to check their pathogenicity on onion seeds, seedlings and bulbs to access the definitive species assignment of an unclassified isolate of fusarium obtained from this study.

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