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PATHOGENIC CHARACTERIZATION OF THREE FUSARIUM SPECIES ASSOCIATED WITH ONION (*ALLIUM CEPA* L.) IN BURKINA FASO

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ABSTRACT

Basal rot is a widespread onion disease caused by fungi belonging to *Fusarium* genus. The disease is known to incur onion yield loss worldwide. This study aims to identify and determine the pathogenicity of *Fusarium* species associated with basal rot of onions in Burkina Faso. Thirteen *Fusarium* isolates from seven regions of Burkina Faso were identified based on morphological observations and molecular diagnosis and their pathogenicity was assessed in laboratory and Greenhouse. Results showed that, the 13 isolates belonged to *Fusarium falciforme*, *Fusarium acutatum* and *Fusarium oxysporum* species. *F. falciforme*, *F. acutatum* and *F. oxysporum* were pathogenic on onion seeds, seedlings and bulbs. *F. falciforme* caused lower seedling damping-off rate compared to that caused by *F. acutatum* and *F. oxysporum*. All these species of *Fusarium* genus induced onion bulbs rots. The results of this study confirmed the presence of these species in Burkina Faso and that they are pathogenic on onion. Therefore, it would be useful to suggest an implement sustainable management approach of these pathogens.

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INTRODUCTION

The common onion (*Allium cepa* L.) is one of the most important vegetable crops in Burkina Faso (Tarpaga, 2012). With a national average yield of 19 t/ha, onion is more produced in Sudan–Sahel and Sudan zones of Burkina Faso. However, onion production is increasingly confronted with numerous biotic constraints, which induce significant damages and economic losses. Fungus infection is meanly responsible for onion losses. The most observed damages caused by fungi include onion basal rot, wilt and seedling melt (Özer *et al.*, 2004; Mohan and Schwartz, 1999). These diseases are induced by several fungus species which belong to *Fusarium* genus (Haapalainen *et al.*, 2016; Kintega *et al.*, 2020a; Kalman *et al.*, 2020; Le *et al.*, 2021; Sogoba *et al.*, 2021). Among the *Fusarium* species, *Fusarium oxysporum* f. sp. *cepae* is known to be highly virulent on onion and have been reported to be responsible for basal rot, basal plate slight discoloration, older leaves and the whole plant death (Delgado-Ortiz *et al.*, 2016). In addition, another species of this fungal genus, *Fusarium falciforme,* is involved in yellowing and chlorosis of leaves of onion, softening of the bulb, necrosis of the basal part of the bulbs and reduction of the root system (Tirado-Ramírez *et al.*, 2018). Previous studies conducted in Burkina Faso have reported the existence of a large diversity of

Fusarium species infecting onion. These are *F. oxysporum, F. solani, F. proliferatum, F. fujikuroi, F. thapsinum, F. acutatum* and *F. falciforme* (Dabiré, 2017; Kintega *et al.*, 2020a; Sogoba *et al.*, 2021). Among them, *F. oxysporum*, *F. falciforme* and *F. acutatum* appear to be the most virulent species causing seedling damping-off and inhibitory effect on the growth of onion seeds. Thus, a comparative study of the pathogenicity of these three *Fusarium* species is necessary to establish their epidemiology.

The aim of this study is therefore to determine and compare the pathogenicity of *F. falciforme, F. oxysporum* and *F. acutatum* isolated from onion in Burkina Faso.

MATERIALS AND METHODS

Sample Collection and Isolation of Fungus Isolates

Surveys were conducted from 2020 to 2021 in seven regions (Boulgou, Ganzourgou, Oubritenga, Passoré, Sanguié, Yatenga and Zounwéogo) in Burkina Faso. A total of 300 onion samples were randomly collected at all stages of plant development, from nursery to maturation. Basal plate and bulb were used for isolation as described by Sogoba *et al.* (2021). Organ fragments were surface sterilized briefly and washed using 3% sodium hypochlorite and distilled water, respectively. The organ fragments were then used to obtain pure cultures of fungus isolates base on a repeating transfer technique using Potato Dextrose Agar (PDA) medium with an antibiotic commercially named spectinomycin (Sogoba *et al.*, 2021). The obtained pure cultures of fungus isolates were used for the morphological and molecular identifications as describe below.

Molecular Identification of *Fusarium* spp. and Phylogenetic Analysis

Total DNA was extracted of fungal mycelia of each collected isolate using the Cetyl Trimethyl Ammonium Bromide (CTAB) extraction procedure as described by Sogoba *et al.* (2021). Resulting DNA was stored at -20°C in 50 µl of distilled water.

For gene amplification by Polymerase Chain Reaction, 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). Amplification reaction was performed as previously described by Sogoba *et al.* (2021). The resulting PCR amplicons were sent to Macrogen Europe for sequencing by the Sanger sequencing method using primer-walking when needed (Macrogen, Europe). Obtained sequences were analyzed and phylogenetic tree were built as described by Sogoba *et al.* (2021).

Morphological Characterization of *Fusarium* spp.

Microscopic characteristics of fungal isolates were examined under an optical microscope (Primo Star– Zeiss, Germany). Manuals of Champion (1997) and Leslie and Summerell (2008) were used for identification. Macroscopic characteristics were determined on PDA medium. A five replicates Fisher block design was used. Representative isolates of each species identified based on molecular method were chosen based on node strength. Identified *Fusarium* strains were incubated at room temperature in the laboratory (28 °C to 30 °C). After eight to fourteen days, the macroscopic and microscopic characteristics of the culture (length and diameter of the macro and microconidia, diameter of the mycelium, growth rate of the mycelium and the number of septa) were observed and the measurements were made at 10 x 100 magnification.

Experimental Design for Pathogenicity Assays

A completely randomized Fisher block design with five replicates was used for all the experiments. Four treatments were applied. The control consisted in using distilled water as inoculum. The three other treatments were the inoculation of *F. oxysporum* (Treatment 1), *F. falciforme* (Treatment 2) and *F. acutatum* (Treatment 3) identified based on molecular and morphological methods described above. Three experiments were taken out. They involve bulbs, seeds and seedlings of an onion accession named "*violet de galmi*" from INRAN (Niger). This accession was chosen because of its susceptibility to *Fusarium* and its wide cultivation in Burkina Faso.

Inoculum Preparation

Identified *Fusarium* strains were cultured in Petri dishes containing PDA medium and incubated at 28 °C for seven to ten days with a 12 hour photoperiod. Five milliliters of sterile distilled water was poured into each Petri dish. Mycelium and conidia were carefully scraped off with a sterile slide. Each strain suspension was adjusted to $1x10^6$ spores per milliliter with distilled water. For the determination of the concentration of the different spore suspensions, Neubauer hematimeter was used. This concentration of spores was used for the pathogenicity test on seeds, seedlings and bulbs.

Fusarium Species Effects on Seed Germination Assay

Onion seeds were washed in distilled water and shaken for 10 min in 1% sodium hypochlorite. The seeds were

then rinsed with distilled water before being shaken again for 15 min. Each group of onion seeds containing 25 seeds was transferred into a tube containing 10 ml of spore solution and soaked during 30 minutes before the whole set was poured into a sterile Whatman paper lined in Petri dish. Seeds were spread out with sterile forceps. Distilled water was added every day to maintain moisture and allow germination. After nine days of incubation at 28 °C, germination rate and coleoptile length were recorded. The stunting rate was assessed according to the following formula (Kaboré, 2014; Kalman *et al.*, 2020).

$$Stuntingrate = \frac{ACLC - ACLT}{ACLC} X100$$

ACLC = average coleoptile length of the control

ACLT = average coleoptile length of the treatment

***Fusarium* Species Effects on Seedling Development Assay**

Pots were previously filled with sterile sand mixed with sterilized compost (bokashi) and placed in a greenhouse. They were lightly watered with tap water and then manually tamped. Seeds were disinfected and inoculated with the different *Fusarium* species described above, before sowing. Control plants were soaked in distilled water. One hundred seeds were inoculated for each strain, sown equally in five pots, and grown at 25-30°C with a relative humidity of at least 50% and about 12-14 hours of daylight, following the technique of Bayraktar and Dolar (2011).

For severity scoring, the emergence rate was assessed at 14 days after sowing, and the mortality percentage was calculated for each strain at 21 and 45 days after sowing. Strains with mortality rates above 50% were considered highly virulent (Bayraktar and Dolar, 2011).

***Fusarium* Species Effects on Bulbs Assay**

Fifteen bulbs with a diameter between 3 to 3.5 cm and grown in an incubator set at 28°C were used per treatment for this test. For each *Fusarium* species, spores suspension was prepared. After removing the outer scales, the bulbs were sterilized in 70% ethanol solution for 30 seconds. Perforations approximately 5 mm deep were made in the basal plate with a 4 mm diameter sterile punch. A total of five small holes were made in each bulb. Ten microliters of each inoculum were introduced into the holes and sealed with tape. The same quantity of water was used for the controls. Rot length was observed for each treatment after two weeks of incubation and was noted by measurement (Kalman *et al.*, 2020; Kintega *et al.*, 2020b).

Statistic and Analysis

For pathogenicity analysis, germination rate was expressed as percentages and coleoptile length as centimeters. To assess the effectiveness of the treatments on coleoptile lengths, emergence rates and melting, rot lengths, descriptive statistics (means and standard deviation) were calculated. Variance analysis in comparison with the controls was performed by the least significant difference (LSD) test. Different means were compared with the control using the Tukey test at a 5% threshold with R software version 4.1.1.

RESULTS

Fungal Isolation

Surveys conducted in the seven regions of Burkina Faso confirmed the presence of fungus infecting onion. Morphological characterization, based on microscopic observation of fungus, shows that 202 out of the 300 samples were found to be contaminated with *Fusarium* spp., and 239 isolates were obtained. Based on the identification keys 13 isolates out of the 239 fungi isolates were selected for molecular identification.

Molecular Identification of *Fusarium* spp.

Polymerase chain reaction using the primers TEF-Fu3f and TEF-Fu3r was performed on 13 isolates. For each of them, an amplification signal was observed. Partial sequences obtained from sequencing of PCR products were analyzed. Based on similarity research using GenBank database, four (BF-Fus12-2021-SKH, BF-Fus19-2021-SKH, BF-Fus23-2021-SKH and BF-Fus26-2021-SKH), three (BF-Fus10-2021-SKH, BF-Fus14-2021-SKH, and BF-Fus21-2021-SKH) and six (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) consensus sequences from the three groups of isolates obtained above shared 98.23-99.48%, 98.18-99.23% and 98.28-100% of identity with the species *F. oxysporum*, *F. acutatum* and *F. falciforme*, respectively. The results of phylogenetic analysis grouped these sequences in three distinct clades corresponding to those of *F. oxysporum*, *F. acutatum* and *F. falciforme* with high Bootstrap values (Figure 1).

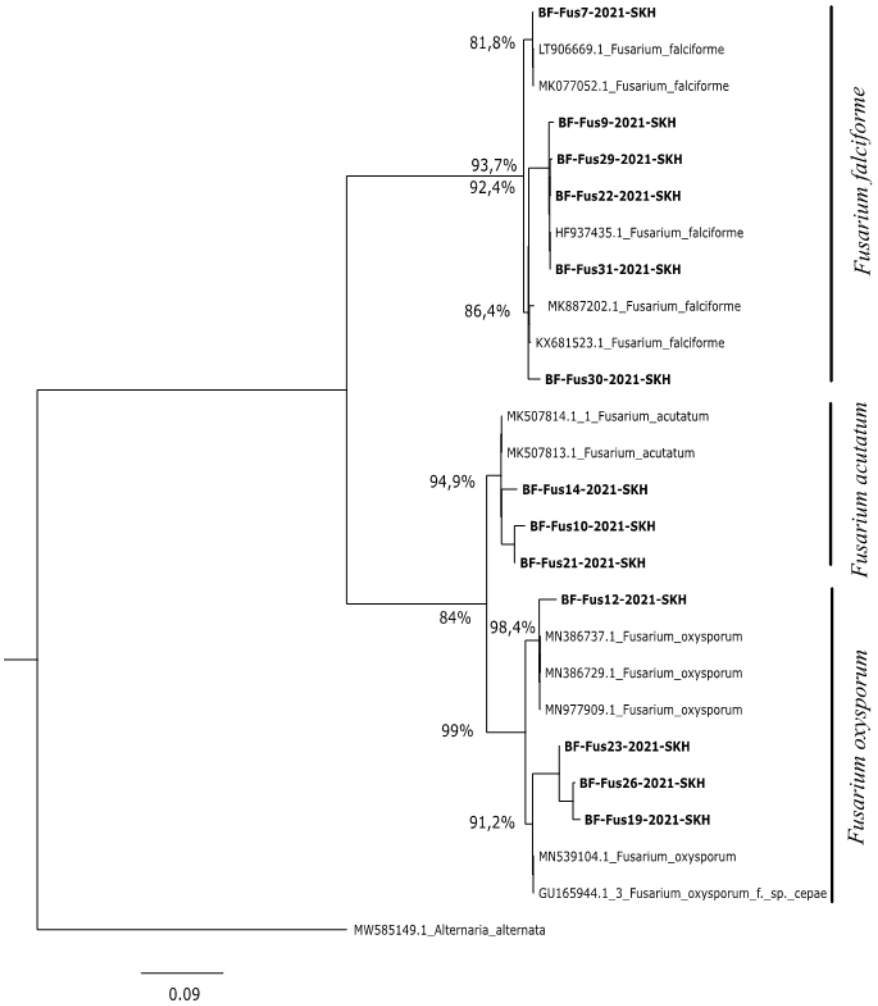

Figure 1. Maximum-likelihood phylogenetic tree inferred from alignments including representatives sequences of *Fusarium* spp. Sequences in bold represent *Fusarium spp.* identified in this study. *Alternaria alternata* sequence has been used as "Out group". Bootstrap values from these analyses are reported at tree nodes.

Morphological Characteristics of the Isolates

Cultures of fungi showed age-dependent colors changes from the top and bottom (colony maturation) of the culture medium. Typical colors of *Fusarium* species were observed (purple, white, orange, gray, brown) (Figure 2 A, B, D, E, G and H). The most common spores were microconidia without septum, with shapes ranging from oval, ellipsoid to kidney-shaped. Macroconidia were fusiform and had a number of septa comprising between two and four (Figure 2 C, F and I).

The four isolates of *F. oxysporum* exhibited a cottony colony with abundant aerial mycelium that varied in colour from white to purple towards the center of the Petri dish depending on culture age (Figure 2A). Macroconidia were slightly straight to fusiform with tapered ends. Microconidia were produced as false heads borne by monophialids. Chlamydospores were abundant, spherical to oval (Figure 2C).

The three isolates of *F. acutatum* cultures showed a colony stuck to the culture medium with sparse and less abundant mycelium than the isolates of the two other species, the colour was orange to white and lite purple according to age. It showed a pattern of concentric rings on the upper surface (Figure 2D). Macroconidia were slightly straight and fusiform with rounded tips. Microconidia were abundant and produced as false heads borne on long monophialides. Chlamydospores were spherical to oval (Figure 2F).

The six isolates of *F. falciforme* presented a cottony colony with abundant aerial mycelium which was white and pale yellow on the underside. It showed a pattern of concentric rings on the upper surface

(Figure 2G). Macroconidia were slightly straight and fusiform with rounded tips. Microconidia were abundant and produced as false heads borne on long monophialides. Chlamydospores were spherical to oval (Figure 2I).

Macroscopic and microscopic characteristics were compared amongst the three species. Mycelial growth and mycelial diameter were slightly higher for isolates belonged to species *F. acutatum* and *F. falciforme* than those of *F. oxysporum*, but no significant differences were recorded to microconidia length, width and length of macroconidia and number of septa (Table 1).

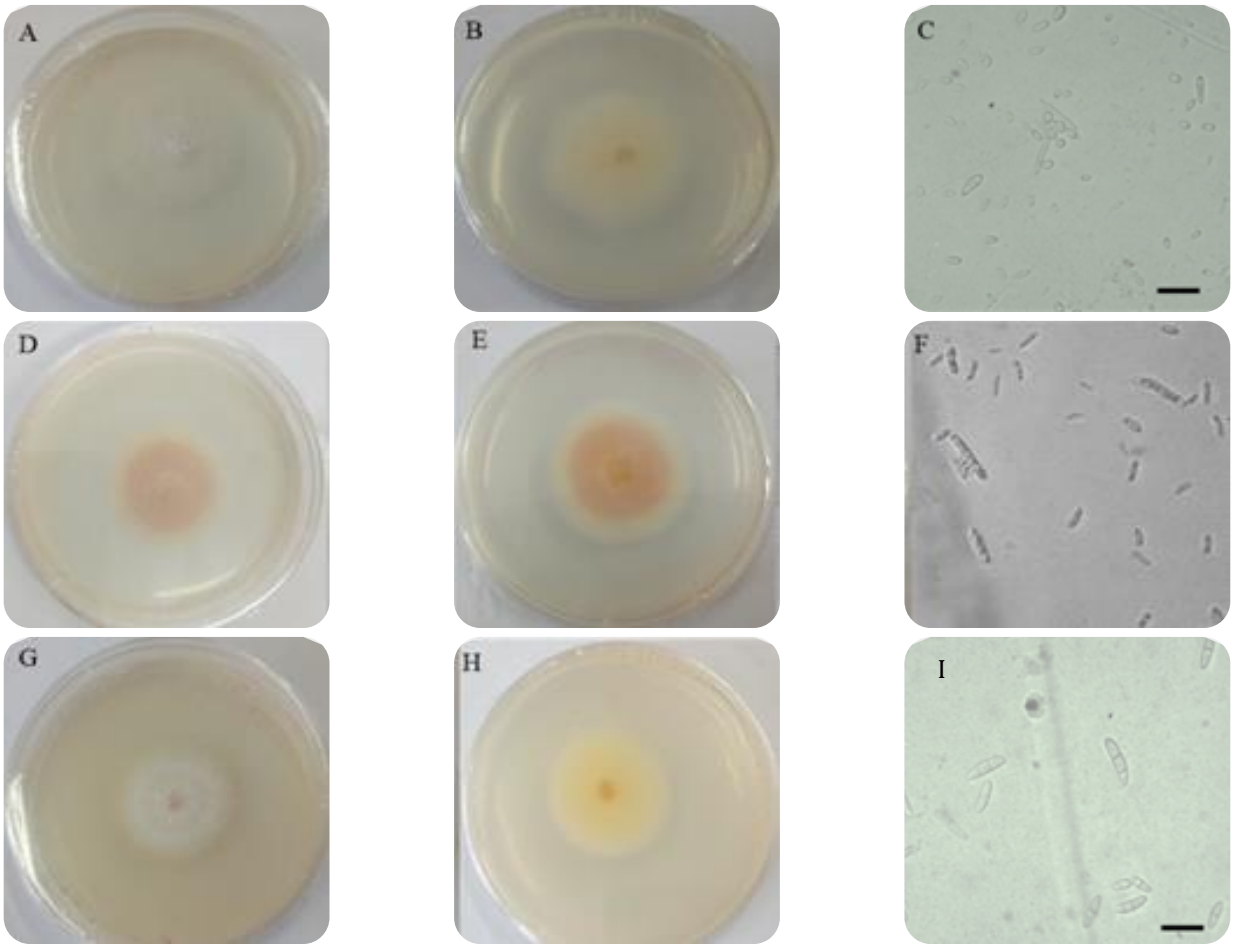

Figure 2. Macroscopic and microscopicy characteristics of *Fusarium oxysporum* (A, B) *Fusarium acutatum* (C, D) *and Fusarium falciforme* (E, F) observed after 9 days of culture on PDA medium. Macroscopic aspect of the colonies from the top (A, D, G) and bottom (B, E, H) of the Petri dish and microscopic observation, G-10 µm (C, F, I).

Table 1. Morphological characters of *Fusarium oxysporum, Fusarium acutatum* and *Fusarium falciforme* isolates obtained from root tray and bulb of diseased plants of onion.

Species Parameters	*F. oxysporum*	*F. acutatum*	*F. falciforme*	
Mycelial growth (mm)	7.96 ± 0.06 c	8.16 ± 0.08 b	8.29 ± 0.04 a	p≤ 0.001
Mycelial diameter (µm)	3.50 ± 0.10 c	4.26 ± 0.06 a	3.90 ± 0.20 b	p≤ 0.001
Microconidia width (µm)	3.50 ± 0.40 b	2.80 ± 0.04 b	4.90 ± 1.00 a	p≤ 0.017
Microconidia length (µm)	10.40 ± 0.06 a	15.58 ± 0.24 a	12.70 ± 1.00 a	p≤ 0.239
Macroconidia width (µm)	3.80 ± 0.20 a	3.14 ± 0.14 a	5.00 ± 0.50 a	p≤ 0.459
Macroconidial length (µm)	19.80 ± 1.40 a	23.72 ± 3.00 a	20.68 ± 5.00 a	p≤ 0.507
Number of septa	2.11 ± 1.00 a	2.00 ± 1.00 a	2.00 ± 1.00 a	p≤ 0.988

Means followed by the same letter are equivalent statistically at 0.05% according to Tukey test.

Effects of *Fusarium* Inoculation on Onion Seed Germination

The effect of *F. oxysporum, F. acutatum* and *F. falciforme* isolates was assessed through onion seeds inoculation assay nine days after sowing. The results showed that the germination of seeds of onion were negatively impacted by the three species of *Fusarium* (Figure 3). Emergence rates ranged from 52 to 65.6% for seeds treated with the *Fusarium* strains. In contrast, 92.80% were obtained for the control with significant difference ($p \leq 0.001$). No significant difference was recorded within the treatments with fungal strains (Table 2). Furthermore, measurements of the length of the coleoptile showed that all of the three *Fusarium* species induced a delayed growth of coleoptiles. Seed inoculation with *Fusarium* species inhibited the growth of the coleoptile in comparison with the control ($p \leq 0.001$), but no significant difference was observed between these *Fusarium* species (Table 2).

Table 2. Effect of the *Fusarium* species on coleoptile emergence and coleoptile length of onion seeds at nine days after sowing.

Treatments	Coleoptile emergence (%)	Coleoptile length (cm)
Control	92.80 ± 3.50 b	4.15 ± 0.72 b
F. oxysporum	52.00 ± 15.23 a	1.85 ± 0.59 a
F. falciforme	65.60 ± 9.21 a	2.14 ± 0.17 a
F. acutatum	61.60 ± 10.43 a	1.43 ± 0.22 a
	p≤ 0.001	p≤ 0.001

Means followed by the same letter are equivalent statistically at 0.05% according to Tukey test.

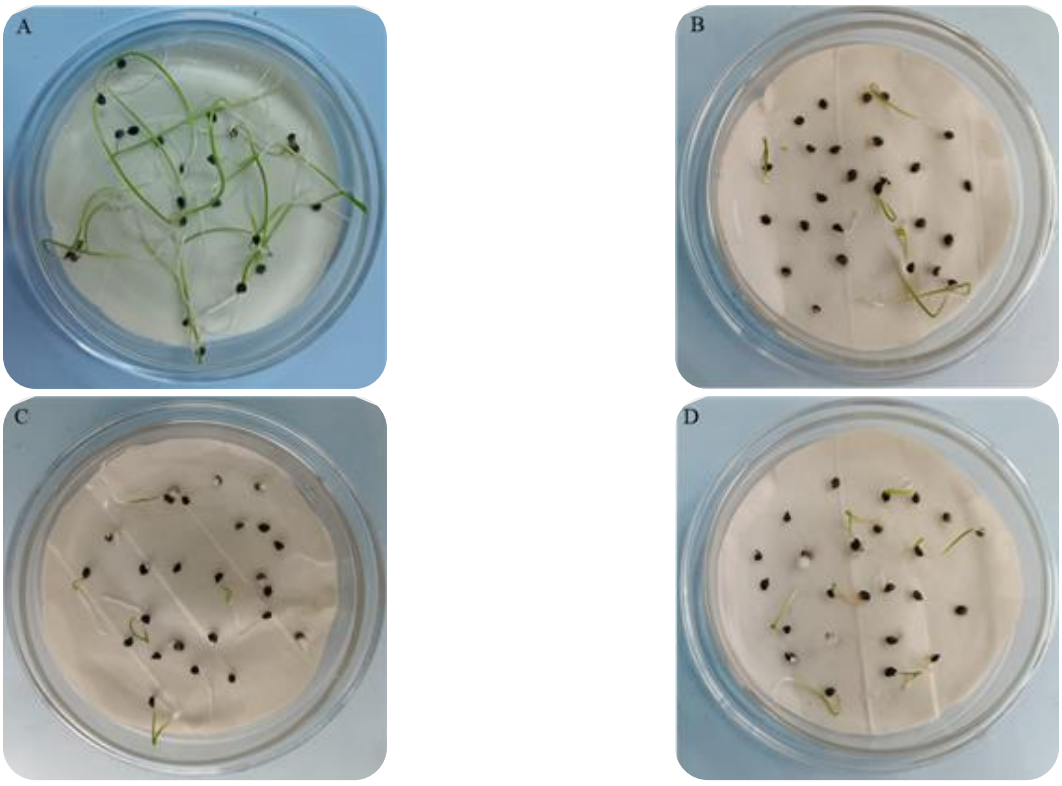

Figure 3. Effect of the three *Fusarium* species on germination of onion seeds. A = Control; B = *F. oxysporum*; C = *F. acutatum*; D = *F. falciforme.*

Aggressiveness of *Fusarium* Species towards Onion Seedlings

In the greenhouse, the percentage of emergence was recorded 14 days after planting, while damping-off rates were assessed 21 and 45 days after sowing (DAS). The results showed emergence rates ranged from 25 to 37% for the treatments associated with *Fusarium* species as against 86% recorded for the control. All

treatments associated with *Fusarium* species showed significant differences compared to the control ($p \leq 0.001$) (Table 3). Evaluation of damping-off at 21 and 45 DAS showed that *F. acutatum* induced more damping-off than *F. oxysporum* and *F. falciforme* with 84% and 91%, respectively (Table 3). *Fusarium oxysporum* was responsible for more damping-off of seeds than *F. falciforme* with 80% and 84% at 21 and 45 DAS, respectively. The lower percentage of damping-off was recorded for *F. falciforme* with 26% and 42% (Table 3). Comparative analysis showed that the treatments with *F. acutatum* and *F. oxysporum* showed significant differences compared to *F. falciforme* with $p \leq 0.009$ and $p \leq 0.019$ at 21 and 45 DAS, respectively. No significant difference was noted between *F. falciforme* and the control ($p \leq 0.938$). Moreover, the symptoms observed on onion plants were similar to those observed in the field. No symptoms were observed on treatment which received distilled water as control. The same pathogens inoculated were re-isolated from the root plates of diseased plants to fulfill Koch's postulate.

Table 3. Different parameters recorded in the greenhouse on onion seedlings inoculated with *Fusarium oxysporum, Fusarium falciforme* and *Fusarium acutatum* at 14, 21, 45 days after sowing.

Treatments	Emergence 14 DAS (%)	Damping-off 21 DAS (%)	Damping-off 45 DAS (%)
Control	86 ± 2.24 b	11.22 ± 08.40 a	14.76 ± 10.15 a
F. oxysporum	25 ± 0.00 a	80± 28.80 b	84 ± 21.91 b
F. falciforme	37 ± 2.74 a	26.78 ± 13.12 a	42.85 ± 23.72 a
F. acutatum	30 ± 9.35 a	84.50 ± 17.18 b	91 ± 12.45 b
	p≤ 0.001	p≤ 0.001	p≤ 0.001

Means followed by the same letter are equivalent statistically at 0.05% according to Tukey test DAS: days after sowing

Aggressiveness of *Fusarium* Species towards Onion Bulbs

Biological traits related to the aggressiveness of three *Fusarium* species were evaluated on onion bulbs. All *Fusarium* species were highly aggressive with different levels of rot. When inoculated with *F. falciforme* strains, onion bulbs showed rotting of the scaly leaves, the fleshy leaves and the buds at 14 days post inoculation. Within the same period, *F. oxysporum* induced rotting of scaly leaves and the fleshy leaves, while the bud remained healthy. Finally, *F. acutatum* induced partial rotting of fleshy leaves, which were touched during the inoculation process. In general, the rots were more serious with *F. falciforme* and *F. oxysporum* than *F. acutatum* (Figure 4).

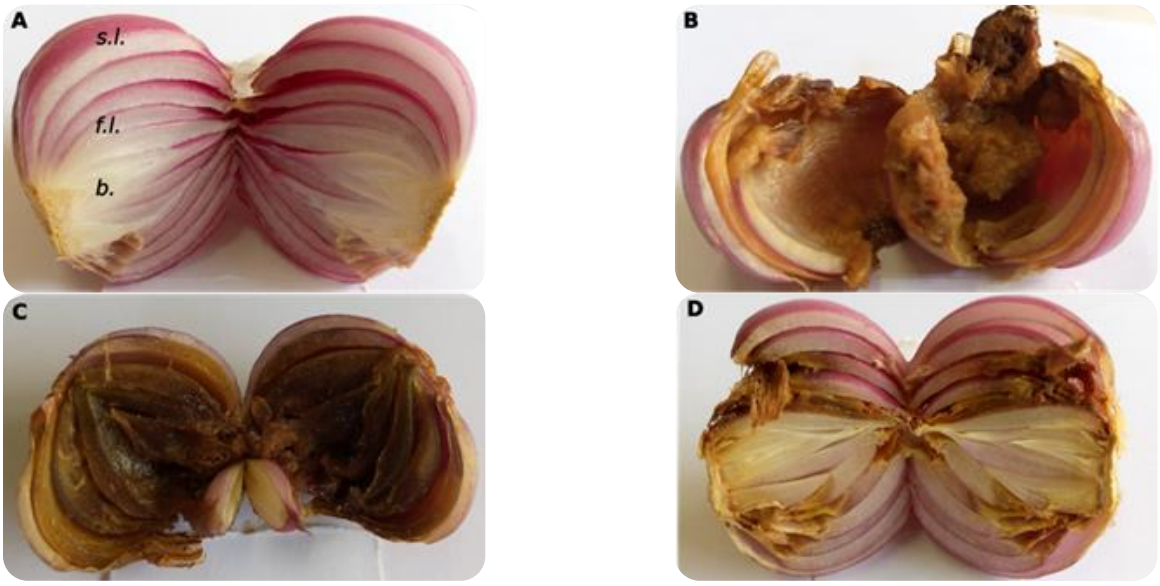

Figure 4. Bulb rot caused by *Fusarium* species on the variety violet de galmi observed after 14 days of incubation. A = Control (distilled water); B = *F. falciforme*; C = *F. oxysporum;* D = *F. acutatum*; *s.l.* = scaly leaves; *f.l.* = fleshy leaves; *b* = bud

DISCUSSION

Using molecular identification, it was easy to identify the different species of *Fusarium*. A phylogenetic tree analysis based on TEF-1α sequences was used to separate the recovered strains into distinct clades containing reference strains of different species. This analysis showed that our 13 isolates belonging to three different species (*F. falciforme, F. acutatum* and *F. oxysporum*). The morphological and microscopic analyses done on the three species confirmed that the mycelial growth rate, the mycelial diameter, the macroconidial width and the number of septa could not discriminate these three species. These results are partly in accordance with those of Kintega *et al.* (2020a) who could not discriminate *F. oxysporum, F. proliferatum* and *F. solani* on the basis of mycelial growth. As for microconidial length, *F. acutatum* was statistically different from other species. Many other authors have also shown this variability within *Fusarium* species (Lin *et al.*, 2014; Kee *et al.*, 2020; Kalman *et al.*, 2020; Kintega *et al.*, 2020a; Díaz-Nájera *et al.*, 2021). Like that of O'Donnell and Cigelnik (1997), our results highlighted that morphological analysis alone is not sufficient to identify *Fusarium* species, clearly.

The pathogenicity study results showed that all the identified *Fusarium* species had an inhibitory effect on onion seed emergence. This has been observed in laboratory and greenhouse. Thus, the pathogenicity of the species identified in this study was confirmed, as already mentioned by some authors (Haapalainen *et al.*, 2016; Nasr Esfahani, 2018; Kintega *et al.*, 2020b; Tirado-Ramirez *et al.*, 2021). All the strains used, in this study also, showed an inhibitory effect on coleoptile growth. These strains, therefore induced a stunted growth rate. Inoculations with *F. oxysporum* and *F. acutatum* caused severe seedling damping-off at 21 DAS and 45 DAS, but there was no difference between *F. falciforme* treatment and the control. Defense-related genes in seedlings were differently expressed depending on the aggressiveness of *Fusarium* isolates and the outcome of *Fusarium* basal rot is driven by the phenology of onion (Le *et al.*, 2022). These results match with those of other authors who have reported that there are *Fusarium* species responsible for seedling damping-off (O'Donnell *et al.*, 2015; Kee *et al.*, 2020; Dongzhen *et al.*, 2020). Le *et al.* (2022) reported that fumonisin B1 generate by *Fusarium* species appeared to be a virulence factor specific for the seedling stage.

Bulb assay indicated that *F. oxysporum*, *F. falciforme* and *F. acutatum* were really aggressive. This confirmed the pathogenicity of these species on onion and the results are in agreement with that of Tirado-Ramirez *et al.* (2021) who reported in their work that *F. falciforme* is a new agent of onion bulb rot and would be more aggressive on the bulb than *F. oxysporum*. Kalman *et al.* (2020) also presented in their work that *F. acutatum* could cause onion bulb rot. Pathogenicity test results revealed new pathogenic strains of *Fusarium* genus associated with onion in Burkina Faso. These strains could have a negative impact on onion production, so epidemiological monitoring is required to follow species evolution.

CONCLUSION

Molecular analysis based on translation elongation factor 1α gene (TEF-1α) allowed the positive identification of *Fusarium falciforme, F usarium oxysporum* and *F usarium acutatum* strains and confirmed their presence in several regions of Burkina Faso. Pathogenicity study revealed that the three species are pathogenic and caused emergence failure and had an inhibitory effect on coleoptile growth and induced rotting of onion bulbs. Following this pathogenicity study and depending on the virulence or aggressiveness of each species, it would be useful to suggest and implement sustainable management control methods of these pathogens.

CONFLICT OF INTEREST

The authors have not declared any conflict of interest.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally to the research and compiling the data as well as editing the manuscript.

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