MICROBIAL LOAD AND MOLECULAR CHARACTERIZATION OF FUNGI ASSOCIATED WITH SWEET PEPPER (*CAPSICUM ANNUUM* L.) DURING STORAGE

TAOFEEQ GARUBA[1*], BABATUNDE YUSUF LAWAL[2],
AZEEZ ADEBOLA LATEEF[1], GANIYU SHITTU OLAHAN[1]

Peppers are perishable and characterized with a short shelf life. Poor storage methods contribute immensely to their deterioration, leading to a great postharvest loss. This study is therefore aimed at isolation and characterization of fungi causing spoilage in sweet pepper during storage. Some healthy samples of *Capsicum annuum* fruits were collected from Lasoju farm located in Asa LGA of Kwara State, Nigeria in sterile polyethylene bags and transported to the laboratory. The fruits were kept in the refrigerator, at ambient condition and in an evaporative cooling system. The fruits were observed for spoilage at the end of the second and third week after storage. The spoiled samples were sterilized, cultured on Potato Dextrose Agar, incubated at 25°C for 3–28 days. Thereafter, the different fungal colonies resulted were sub-cultured to obtain pure cultures of each of them. It was revealed that polythene bag as storage material for *Capsicum annuum* promoted increase in microbial load, while the pure fungal isolates were identified morphologically and molecularly as *Aspergillus fumigatus, A. flavus* and *Rhizopus oryzae*. Therefore, the fresh fruits need to be properly handled and stored in order to eliminate or minimize fungal contamination.

Keywords: *Aspergillus* spp., *Capsicum annuum*, incubation, *Rhizopus oryzea*, storage conditions.

INTRODUCTION

Sweet pepper (*Capsicum annuum*) belongs to Solanaceae family and includes more than 30 species of flowering pepper plants. The fact that sweet pepper is frequently contaminated with fungi such as *Botrytis cinerea, Penicillium, Aspergillus, Alternaria, Mucor, Rhizopus* and *Alternaria alternate*, causing pre- and post-harvest diseases has put at risk, the income of small and large-scale pepper producers worldwide (Caires *et al*., 2014; Mandeel, 2005; Iqbal *et al*., 2011). These fungal species produce many spores and are present almost everywhere the sweet

[1] Department of Plant Biology, University of Ilorin, *corresponding author's e-mail: garuba.t@unilorin.edu.ng
[2] School of Science, Federal College of Education (Special), Oyo

pepper is grown; they take advantage of any damage or bruising on the fruit wall and attack the fruits at all stages from harvest to consumption (Campbell, 1985). *Capsicum* spp. are also susceptible to contamination with mycotoxins, which are toxic chemical products, formed as secondary metabolites by filamentous fungi. Several studies have described high frequency of mycotoxigenic fungal strains in *Capsicum* fruits (Dia *et al*., 2017). Furthermore, it has been reported that climatic conditions in tropical or sub-tropical regions, such as high temperature, humidity, and rainfall, contribute to the high fungal burden and mycotoxin contamination in pepper samples (Singh *et al*., 2017).

In order to minimize microbial contamination and to improve the food safety standards, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) encourage government agencies to conduct microbiological risk assessment of foods and to provide data collection and documentation for improving crop safety regulations (FAO, 1997). This study was therefore aimed at determining the microbial load and characterizing the fungi associated with sweet pepper during storage.

MATERIAL AND METHODS

Collection of *Capsicum annuum* fruits: Pepper (*Capsicum annuum*) fruits were obtained from Lasoju farm in Asa Local Government Area of Kwara State, Nigeria. Based on visual maturity determination, green mature pepper fruits with about 25% red skin were harvested manually with maximum care by experienced personnel.

Preparation of samples: The harvested fruits were washed with tap water containing 2% sodium hypochlorite solution to remove field heat, soil particles and reduce their microbial population. After surface drying with cheese cloth, the fruits were sorted into uniform sizes in populations of 100 per set. There were 30 sets of 100 prepared sweet pepper fruits.

Storage methods and packaging materials: Each set of hundred sweet pepper fruits was packaged in different packaging materials and stored in a refrigerator, at ambient temperature (AT) and inside an evaporative cooling system (ECS) in three replicates. The evaporative cooling system (ECS) was prepared following the methods described by the Nigerian Stored Products Research Institute, Ilorin. Hence, a total of 1,000 fruits were kept under each of the three storage conditions.

Experimental design: A factorial combination of four packaging materials: Polyethylene bag (PB), Jute sack (JB), Banana leaf (BL) and control (only plastic plate); and three storage environments refrigerator, Evaporative Cooling System (ECS) and ambient temperature) were used. Twelve treatments with three replications and

one control in each condition were used in the study. The treatments were arranged in a Randomized Complete Block Design (RCBD) with three replicates.

FPB = Fridge + Polyethylene bag
FBL = Fridge + banana leaf
EPB = ECS + Polyethylene bag
EBL = ECS + banana leaf
APB = Ambient + Polyethylene bag
ABL = Ambient + banana leaf
FJB = Fridge + Jute sac bag
FCL = Fridge + control
EJB = ECS + jute sac bag
ECL = ECS + control
AJB = Ambient + jute sac bag
ACL= Ambient + control

Data collection: A sample per treatment was taken to Chemical Engineering Laboratory of the University of Ilorin for both Physicochemical and Phytochemical analyses every seven days. Five (5) samples from the different conditions; refrigerator, evaporative cooling system (ECS) and Ambient Temperature, making a total of 60 samples.

Temperature and relative humidity of the storage conditions; Temperature and relative humidity of the storage conditions (Evaporative cooling system, Refrigerator and for the ambient conditions) were measured during the storage period using thermo-hygrometer.

Estimation of microbial load of a sample: Potato Dextrose Agar (PDA) and Nutrient Agar (NA) culture media were prepared according to the manufacturers' instructions. Four folds serial dilution of each of the sample was prepared. Half of a milliliter (0.5 ml) of each dilution was inoculated on the PDA and NA Plates. The nutrient agar plates were incubated at of 37^{o}C for 24 hrs, while the PDA plates were incubated at room temperature for 48 hrs for bacteria and fungi growth, respectively. The colonies of microbial growth were counted and recorded using colony a counter, while the number of colony forming unit was calculated using the formula:

Cfu = Number of colonies × $1/10^{-4}$× 1/0.5

Cfu = colony forming unit, $1/10^{-4}$ = dilution factor, 0.5ml = inoculated sample volume

Isolation of fungi from stored *Capsicum annuum*

The isolation was carried out following the method of Garuba *et al.* (2018). The samples were surface-sterilized with 70% ethanol and rinsed in two changes of sterile distilled water. A sterile scalpel was used to cut 3 mm×3 mm sections of tissue from the infectious portions of samples and these portions were aseptically placed on previously prepared Potato Dextrose Agar (PDA) in Petri dishes. Three replicates were made and they were incubated for five days at room temperature of 25 ± 3°C. After five days, a portion of each colony was picked with a sterilized needle and put into fresh disposable Petri dishes with PDA under aseptic conditions to get the pure culture.

Morphological and molecular identification of fungal isolates

For morphological identification, the wet mounts of the isolates in lacto-phenol cotton blue were examined using a binocular compound Olympus microscope model (CH) and their mycelial structure, nature of spores and colonial morphology were observed. Identifications were made with reference to Barnett and Hunter (2010) and Campell and Stewart (1980).

Molecular identification was carried out using the procedures as described by Lateef *et al.* (2019). Fungal mycelia of 100 g were taken into sterile mortal and 1 ml of DNA Extraction Buffer (DEB) containing proteinase K (0.05 mg/ml) was added and macerated with sterile pestle. The extract was then transferred into 1.5 ml Eppendorf tube. To this Eppendorf tube, 50 µl of 20% Sodium Dodecyl Sulphate (SDS) was added and incubated in a water bath at 65^{o}C for 30 minutes and was allowed to cool to room temperature. 100 µl of 7.5 M Potassium Acetate was added and mixed briefly. It was centrifuged at 13,000 rpm for 10 minutes and the supernatant was transferred into new fresh autoclaved tubes. To the supernatant add 2/3 volumes of cold Isopropanol / Isopropyl alcohol, invert the tubes 3–5 times gently and incubate the tubes at -20^{o}C for 1 hour. It was centrifuged at 13,000 rpm for 10 minutes and the supernatant was discarded. 500 µl of 70% ethanol was added and centrifuged for 5 minutes at 13,000 rpm. The supernatant was discarded carefully with the DNA pellet intact. Remaining traces of ethanol was removed and the DNA pellets was dried at 37^{o}C for 10–15 minutes. DNA pellets was re-suspended in 50 µl of Tris-EDTA (TE) buffer. Aliquot DNA was stored at -20^{o}C for further lab analysis.

PCR reaction cocktail consisted of 10 µl of 5x GoTaq colourless reaction; 3 µl of 25 Mm MgCl2; 1 µl of 10 mM of dNTPs mix; 1 µl of 10 pmol each forward primer (ITS 1)- 5’ TCC GTA GGT GAA CCT GCG G 3’and reverse primer (ITS 4)- 5’ TCC TCC GCT TAT TGA TAT GC 3’; 0.24 µl of 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water and 8 µl working DNA template. Amplification was carried out using PCR system thermal cycler (Applied Biosystem Inc., USA) with PCR profile of an initial denaturation, 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 minute 30 seconds; and a final extension at 72°C for 10 mins.

Sanger sequencing of the PCR products were carried out in an ABI PRISM sequencer at a private sequencing company (InqabbaBiotecPvt) in Pretoria, South Africa using the same forward and reverse primers.

Data analysis: Experimental values were expressed as means $\pm$ SEM. Comparison of means values between the environmental conditions, treatments and their interaction were done by one way-analysis of variance (one way ANOVA) and significant means were separated using Duncan Multiple Range Test (DMRT)

at P ≤ 0.01.d, SeqTrace version 0.9.0 (Stucky, 2012) was used to obtain a consensus DNA sequence and AliView version 1.17-beta1 software (Larsson, 2014) was used to do the alignment. Phylogenetic analyses were done using Molecular Evolutionary Genetics Analysis (MEGA version 6) software.

RESULTS AND DISCUSSION

Temperature and relative humidity: Temperature and relative humidity of the three storage conditions during the 20 days of storage are presented in Figures 1 and 2 respectively. The temperature in the refrigerator was lowest and relatively constant throughout the storage period. The average ambient temperature was higher than that of the evaporative cooling system. Highest percentage relative humidity was recorded in the ambient condition. Hardenburg *et al*. (1986) reported that peppers are very sensitive to chilling injury which limits their storage temperature to about 10°C. Workneh and Woldetsadik (2004) also reported that the evaporative cooling chamber maintained the range of temperature from 17–26°C, and relative humidity between 43–98%. These values agreed with those obtained for ECS in this study.

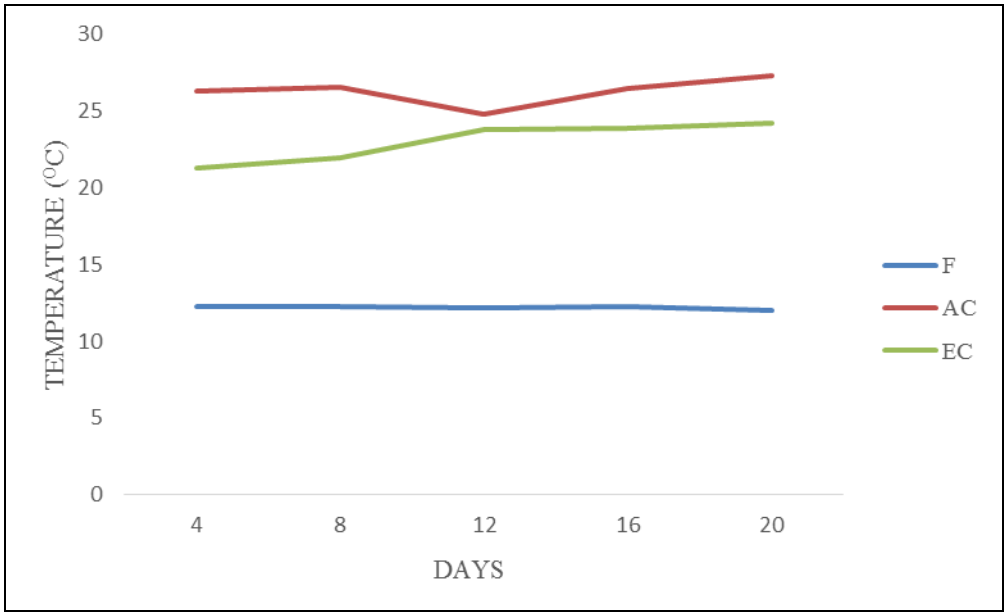

Fig. 1. The Temperature in the storage structure during storage of *Capsicum annuum*.

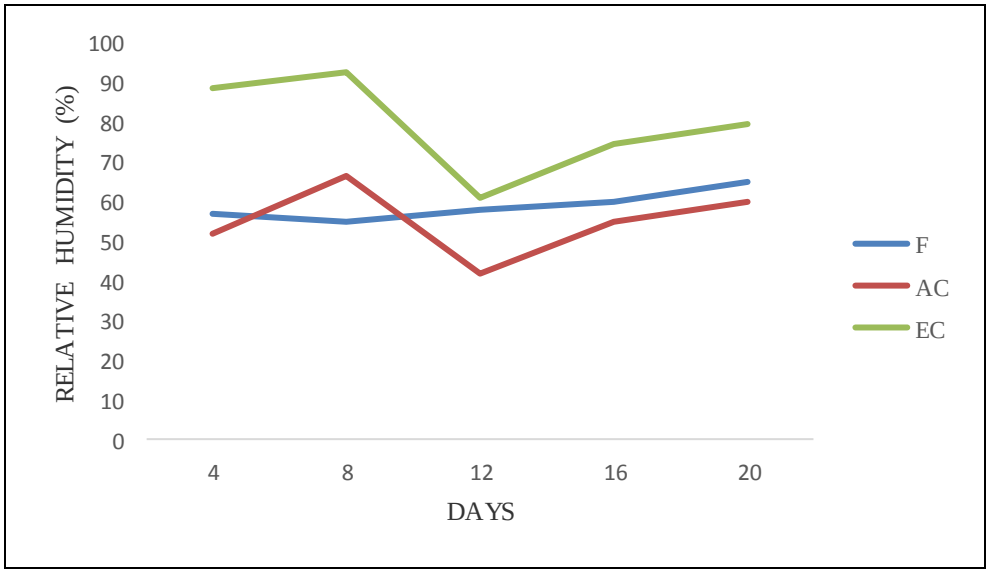

Fig. 2. Relative humidity in the storage structures during storage of *Capsicum annuum*.

Microbial populations on *Capsicum annuum* fruits: The results for microbial load are presented in Table 1. Among all treated samples on Day 7 of this study, the fruits subjected to APB had the highest bacterial load. In terms of storage structure, the bacterial population in the fruits stored in the refrigerator was comparatively lower. Fungal load of the fruits subjected to APB and EPB were too numerous to count on Day 14 of this study. This indicated that polythene bags as packaging material for *Capsicum annuum* promoted proliferation of bacteria and fungi.

High microbial population in fruits is an indication that such fruits can be mycotoxigenic especially if they contain fungal species belonging to *Aspergillus*, *Fusarium*, and *Penicillium* (Costa *et al*., 2019). Moreover, it has been previously reported that several *Aspergillus* and *Fusarium* species are responsible for the post-harvest deterioration of fresh *Capsicum* peppers, some edible fruits and vegetables collected from different markets and farm lands in Nigeria, in a study conducted by Udoh *et al*. (2015).

Identification of fungal isolates from the spoilt *Capsicum annuum* fruits

Three fungal species were isolated from all the spoilt fruits of *Capsicum annuum* in this study. Morphological characteristics of the isolates are summarized in Table 2, while their macroscopic and microscopic descriptions are given in Figures 3–5. Based on these, the fungal isolates were tentatively identified as *Aspergillus fumigatus, A. flavus* and *Rhizopus. oryzea.*

Phylogenetic analyses of ITS region of DNA sequences of the fungal isolates corroborated the identities based on their morphological characteristics. The phylogenetic trees are shown in Figures 6–8.

According to Udoh *et al.* (2015**)**, species of *Aspergillus* produce mycotoxigenic substances that cause disease in humans, animals and plants. *A. flavus* cause aspergillosis and produce aflatoxin, a carcinogenic mycotoxin. Ademoh *et al.* (2015) isolated *Aspergillus* sp. from tomato fruits belonging to the same Family with pepper. Several studies have also reported that *Aspergillus* and *Rhizopus* are associated with post-harvest rot of vegetables and fruits such as tomatoes, watermelon, pawpaw, orange, lettuce and red pepper (Udoh *et al*., 2015; Mailafia *et al*., 2017). Moss (2002) reiterated that 60 % of fruit spoilage was due to the activities of *A. niger* and *R. stolonifer*, and account for about 10% of mould deterioration of vegetables. *Rhizopus oryzae* is one of the most economically important members of Mucoromycotina (Gryganskyi *et al*., 2010). Although post-harvest infection by *Rhizopus* sp. is more common than pre-harvest infection in the absence of injury or fruit cracking, *Rhizopus* strains have been found, in some instances, to be associated with preharvest infection in several fruits (Kwon *et al.*, 2000; Zhang *et al*., 2013).

CONCLUSION

This study revealed that polythene bag, as a packaging material, promotes proliferation of microbes and consequently increases the microbial load on fruits of *Capsicum annuum*. *Aspergillus fumigatus, A. flavus* and *Rhizopus oryzae* were isolated from the spoilt pepper fruits in this study. It is however recommended that *Capsicum annuum* should be handled with care during harvesting, transportation, storage and marketing to avoid any form of mechanical injury that may pave way for fungal invasion. This will increase the fruit quality and reduce postharvest loss minimally.

Table 1

Populations of microorganisms on *Capsicum annuum* stored for 14 days (Cfu/ml)

Packaging material	**Bacteria (Cfu/ml)**		**Fungi (Cfu/ml)**	
	7	**14**	**7**	**14**
FPB	$1.12x10^6$	TNC	$2.13x10^5$	$9.73x10^5$
FBL	$1.75x10^6$	TNC	$1.13x10^5$	$1.20x10^5$
FJB	$1.23x10^6$	TNC	$4.67x10^4$	$7.0x10^4$
FCL	$1.18x10^6$	TNC	$2.0x10^4$	NG
APB	$3.03x10^6$	TNC	$1.69x10^6$	TNC
ABL	$1.17x10^6$	TNC	$2.67x10^4$	$1.24x10^6$
AJB	$2.72x10^6$	TNC	$2.20x10^5$	$2.07x10^5$
ACL	$7.0x10^5$	TNC	$6.0x10^4$	$4.0x10^4$
EPB	$2.57x10^6$	TNC	$1.96x10^6$	TNC

Table 1 (continued)

Packaging material	Bacteria (Cfu/ml)		Fungi (Cfu/ml)	
	7	14	7	14
EBL	$1.41x10^6$	TNC	$3.53x10^5$	$5.90x10^5$
EJB	$1.24x10^6$	TNC	$2.33x10^5$	$1.36x10^6$
ECL	$3.56x10^6$	TNC	$2.0x10^5$	$4.4x10^5$

Keys: FPB (Fridge + Polyethylene bag), FJB (Fridge + Jute sac bag), FBL (Fridge + banana leaf), FCL (Fridge + control), EPB (Evaporative cooler + Polyethylene bag), EJB (Evaporative cooler + jute sac bag),EBL (Evaporative cooler + banana leaf), ECL (Evaporative cooler + control), APB (Ambient + Polyethylene bag), AJB (Ambient + jute sac bag), ABL (Ambient + banana leaf), ACL (Ambient + control), TNC (Too numerous to count), NG (no growth)

Table 2

Fungal isolates from spoilt pepper

Fungi isolate	Description	Identification
Isolate F1	The colonies formed by fungus were colourless when young but turned green with maturation due to production of conidia. Colonies were round and granular when observed. They produce numerous spores. The colour of the stipes is grey around the apex. They have a smooth surface and possess small columnous globuse. The surface of the conidia is rough.	*Aspergillus fumigatus*
Isolate F2	The colonies were round and granular when observed. Microscopic examination of the fungus revealed thick-walled colourless, septate, rough and higly unbranched conidiophores, which arose from thickened foot cells. The hyphae were septate and unbranched. The conidia were greenish, oval shaped with rough surfaces. They occurred in chains of two or three.	*Aspergillus flavus*
Isolate F3	Colonies of this fungus were dusty whitish in appearance. They grew rapidly covering the whole surface of culture plate within 48 hours on PDA. Mycellia were interwoven and extensively ramifying. Microscopic examination revealed well-developed hyphae which branched freely and were caenocytic. Brown coloured, smooth walled, non-septate and erect but branched sporangiophores developed from the hyphae. The sporangia were globose, small and contained spores, which were singular or oval in shape, and black when mature. They have well developed collumellae which are globose or oval in shape. The wall is usually smooth and the colour is pale brown.	*Rhizopus oryzae*

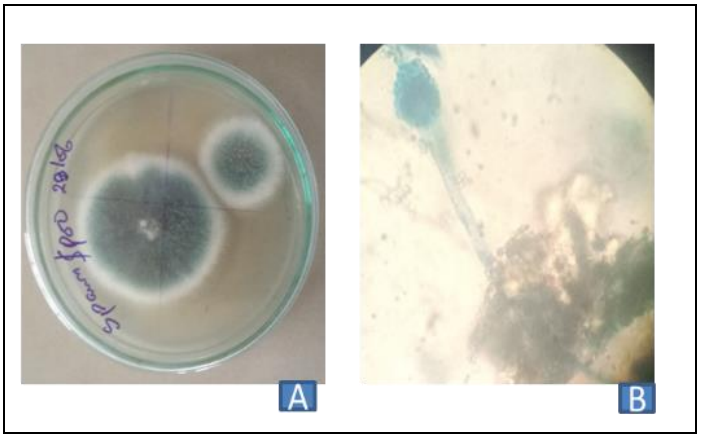

Fig. 3. *Aspergillus fumigatus* A. pure culture B. microscopic view.

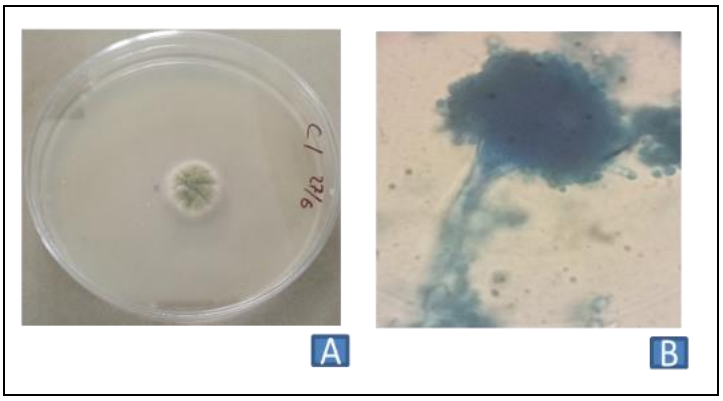

Fig. 4. *Aspergillus flavus* A. pure culture B. microscopic view.

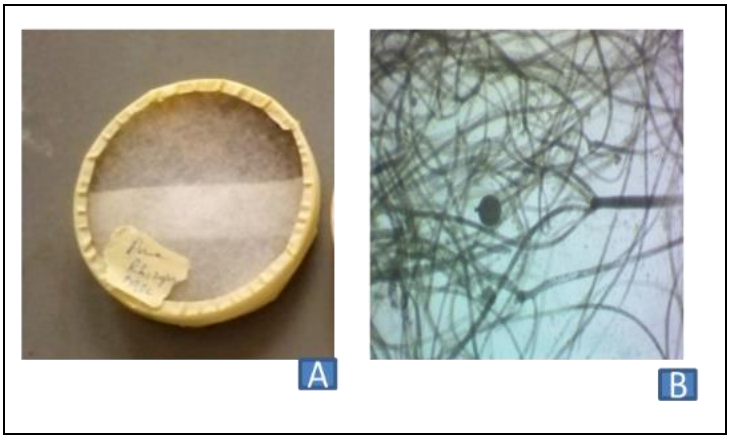

Fig. 5. *Rhizopus oryzae* A. pure culture B. microscopic view.

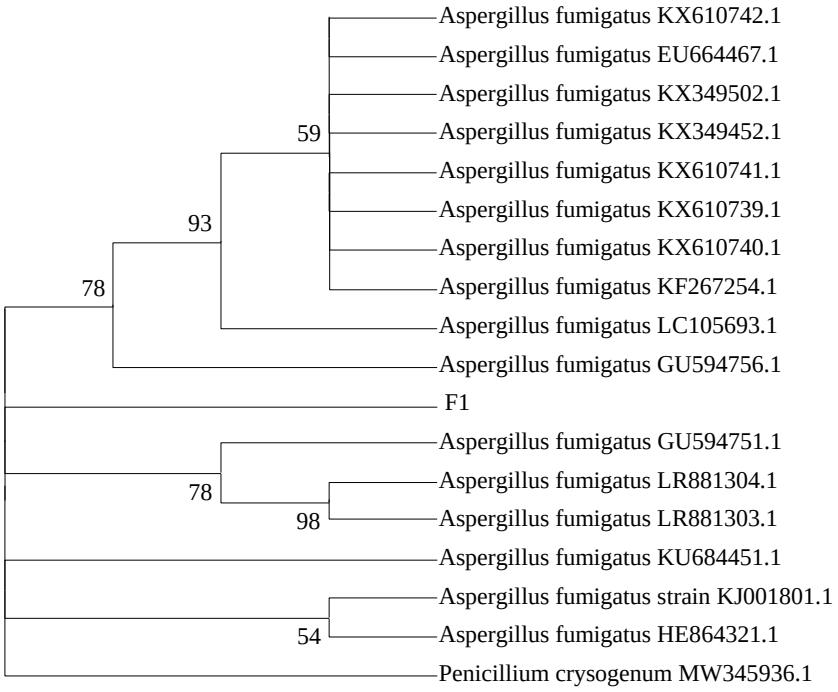

Fig. 6. Phylogenetic analysis of ITS gene sequences of fungal pathogens isolate F1.

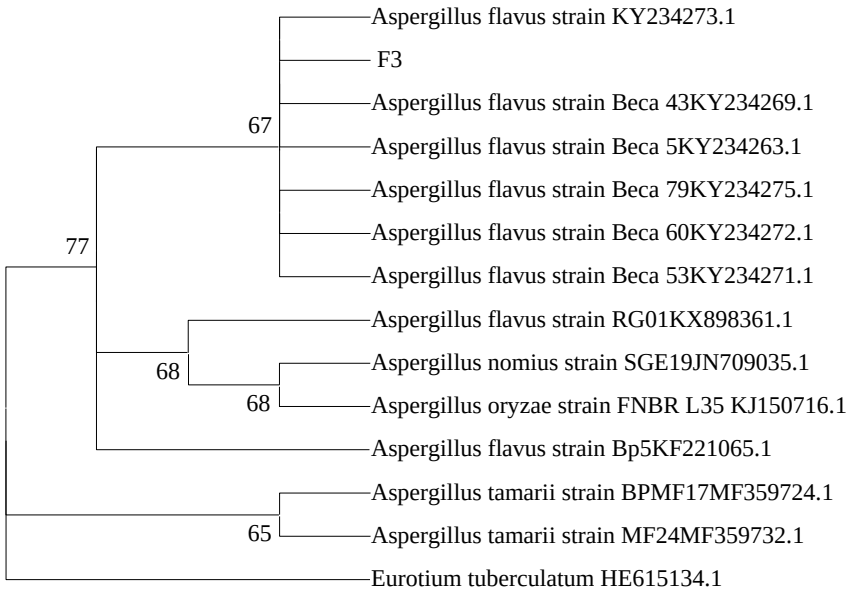

Fig. 7. Phylogenetic analysis of ITS gene sequences of fungal isolate F3.

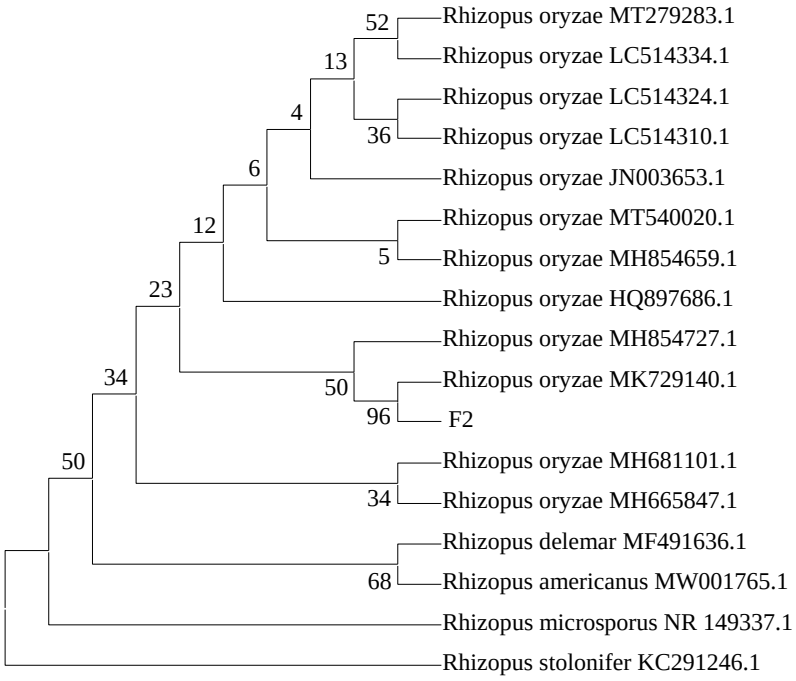

Fig. 8. Phylogenetic analysis of ITS gene sequences of fungal isolate F2.

REFERENCES

1. Ademoh O.F., Okeke C.U., Ezeabara C.A., Iiodibiam C.V., Uka C.J. 2015. Macromorphological observations in Capsicum varieties cultivated in Awka, Anambara State South Eastern, Nigeria. *American Journal of Life Science Researches*, 3(1):30–34.
2. Barnett H.L., Hunter B.B. 2010. Illustrated Genera of Imperfect Fungi (4th Edition). The American Phytopathological Society, St. Paul, Minnesota.
3. Caires N.P., Pinho D.B., Souza J.S.C., Silva M.A., Lisboa D.O. Pereira O.L., Furtado G.Q. 2014. First report of Anthracnose on pepper fruit caused by *Colletotrichum scovillei* in Brazil. *Plant Dis*, 98: 1437.
4. Campbell M.C., Stewart J.L. 1980. The Medical Mycology Handbook. A Wiley Medical Publication. John Wiley and Sons. New York.
5. Campbell, R. (1985). *Plant Microbial*. ELBS Edition.London publishers, London. pp. 38: 42.
6. Costa J., Rodríguez R., Garcia-Cela E., Medina A., Magan N., Lima N., Battilani P., Santos C. 2019. Overview of fungi and mycotoxin contamination in Capsicum pepper and in its derivatives. *Toxins*, 11: 27.
7. Dia G.B., Gomes V.M., Moraes T.M.S. 2017. Characterization of *Capsicum* species using anatomical and molecular data. *Genetics and Molecular Research*, 12(4): 6488–6501.
8. Faber J.M. 1991. Microbiological aspects of modified atmosphere packaging technology – A review. *J. Food. Prot*., 54: 58–70.
9. Food and Agricultural Organization of The United Nations (F.A.O.) (1997). Risk management and food safety. Report of a Joint FAO/WHO Consultation Rome, Italy, 27 to 31 January 1997. Available online at https://www.fao.org/3/w4982e/w4982e.pdf.

10. Garuba T., Azeez J.A., Bello M.A., Lateef A.A. 2018. Isolation and physiological studies of fungi associated with post-harvest diseases of selected solanaceous fruits in Ilorin Markets, Nigeria. *Cameroon Journal of Biological Sciences*, 26: 01–06.
11. Gryganskyi A.P., Lee S.C., Litvintseva A.P., Smith M.E.I. 2010. Structure, function, and phylogeny of the mating locus in the *Rhizopus oryzae* complex. *PloS One*, 5: 1–12.
12. Hardenburg R.E., Watada A.E., Wang C.Y. 1986. The commercial storage offruits, vegetables, and florist and nursery stocks. *U.S. Dept. Agri. Handbook 66*.
13. Iqbal Q. Amjad M., Asi M.R., Ariño A. 2011. Assessment of hot peppers for aflatoxin and mold proliferation during storage. *J. Food Prot*., 74: 830–835.
14. Lateef A.A., Garuba T., Saad G., Olesin M., Eperetun G. G., Tiamiyu B.B. 2019. Isolation and molecular identification of dominant fungal endophytes from green leaves of physic nut (*Jatropha curcas*) from Unilorin Plantation, Ilorin, Nigeria. *Sri Lankan Journal of Biology*, 4(1); 1–13.
15. Mailafia S., Okoh G.R., Olaboda H.O. K., Osanupin R. 2017. Isolation and identification of fungi associated with spoilt fruits vended in Gagwalada Market, Abuja, Nigeria. *Veterinary World*, 10(4): 393–397.
16. Mandeel Q.A. 2005. Fungal contamination of some imported spices. *Mycopathologia*, 159: 291–298.
17. Messiaen C.M. 1995. *Tropical Vegetable Garden: Principles for Improvement and Increased Production with Application to the Main Vegetables Types*. Macmillan Education Limited, London. Pp. 34–50.
18. Moss M.O. 2002. Mycotoxin review: *Aspergillus and Penicillium*. *Mycologist*, 16: 116–119.
19. Singh P., Cotty P.J. 2017. Aflatoxin contamination of dried red chilies: Contrasts between the United States and Nigeria, two markets differing in regulation enforcement. *Food Control*, 80: 374–379.
20. Udoh I.P., Eleazar C.I., Ogeneh B. O., Ohanu M.E. 2015. Studies on fungi responsible for the spoilage/deterioration of some edible fruits and vegetables. *Advances In Microbiology*, 5: 285–290.
21. Workneh T.S., Woldetsadik K. 2004. Forced ventilation evaporative cooling: a case study on banana, papaya, orange, mandarin and lemon. *Tropical Agirculture*, 81(1): 1–6.