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Molecular Characterization of Fungi Associated with Pod Rot of the African Locust Beans (*Parkia biglobosa*)

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ABSTRACT

Parkia biglobosa is the African locust bean, native to West Africa and majorly used in the production of "Iru", a local soup sweetener in various African delicacies. Iru is used, as an alternative to monosodium glutamate (MSG), which has detrimental long-term effects in the body. However, pod rot disease of the *P. biglobosa* pods leads to considerable losses in "Iru" production. This study was therefore carried out to identify the causal pathogen associated with *Parkia biglobosa* pod rot disease using sanger sequencing. Twelve diseased pods of *P. biglobosa* were collected and processed to isolate the fungal pathogen based on their morphological characteristics. Genomic DNA was extracted from the isolate, amplified with primers ITS5/ITS4 and Sanger sequencing of the internal transcribed spacer (ITS) region of the pathogen was used to identify the fungal pathogen with the molecular analysis software Molecular Evolutionary Genetics Analysis (MEGA). Pathogenicity test was carried out to confirm the potential of the isolated pathogen to cause the implied disease. Using the GenBank database, the pathogen was identified as *Pseudofusicoccum violaceum* with 562 bp and 99-100 % sequence identity. Koch's postulates confirmed the pathogenicity of the isolate on *P. biglobosa* pods. This study constitutes the first report of *Pseudofusicoccum violaceum* as the causal pathogen of *P. biglobosa* pod rot in Nigeria. The findings from this study provide information on the ecological significance of *Pseudofusicoccum violaceum* as a pathogen of P. biglobosa and possible control strategies for the consequent disease.

Keywords: African trees, Sustainable use, Pathogens, Endophytes, MSG, *Pseudofusicoccum*.

INTRODUCTION

Parkia biglobosa, also known as the African locust bean, is a perennial, deciduous tree of the family Fabaceae (Janick, 2008). It is primarily grown for its pods containing sweet pulp and valuable seeds. Locust beans is native to West

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Africa and have been used to produce the fermented beans "Iru" since the 14th century (Diawara et al., 2000). Locust beans are a cheap alternative to monosodium glutamate (MSG) in Africa (Airaodion, 2019) where it is used in African delicacies (Arinola et al., 2019; Gernmah et al., 2007). This fermented bean "Iru" in African gastronomy is a traditional condiment of high significance and an essential ingredient in local cuisine across the African continent. It plays a crucial role in flavour enhancement and is a valuable source of protein and vitamins, thus contributing to the nutritional intake of its consumers (Olasupo et al., 2019). *Parkia biglobosa* is also used to treat several ailments such as diabetes, hypertension, chronic piles (Koura et al., 2011; Saleh et al., 2021) and coccidiosis in poultry with wound-healing properties (Adetutu et al., 2011). However, the amount of locust beans lost to diseases has not been documented. The products of African locust bean are important in local trades across West Africa where the dried or fermented seeds are transported far from the production sites, often across country borders (Ajayeoba, 2002).

Proper and accurate pathogen identification is an important step in effective disease management strategies (Mondal & Shanmugam, 2013). Knowing the specific causal pathogen of a disease aids in early detection, minimizes the use of broad-spectrum pesticides, thereby reducing the adverse environmental impact and preventing economic losses (Gill et al., 2014; Karlsson Green et al., 2020; Tudi et al., 2021). Diseases of economic trees in Africa constitute a major challenge, impacting rural livelihoods, economic development, and biodiversity across the continent (Graziosi et al., 2020). It has been reported that trees are crucial for Africa's future (Graziosi et al., 2020), however, this is being defeated as pests and diseases have the potential to seriously compromise the food and nutritional security of millions of people in Africa (Coraf, 2020).

Previous studies on post-harvest deterioration of locust beans products revealed several species of bacteria and fungi (Omafuvbe et al., 2000). These species can occur on the growing tree as well as harvested produce leading to damage ranging from rancidity, odour and flavour changes and germ layer destruction. Popoola & Akueshi (1985) identified the bacterial and fungal flora of deteriorated *Parkia* and maggot infested samples of fermented locust bean seeds as *Aspergillus niger, Aspergillus flavus, Penicillium, Rhizopus and Candida* sp. Setamou *et al*. (2000) recorded several generations of *M. nigrivenella* on pods of *P. biglobosa.* Infection of *Parkia biglobosa* leads to rot of the pod, which results in considerable losses, and the extent of damage caused by the pathogen may depend on the nature of the disease. High disease density will drastically reduce the yield of this tree and its economic value. However, for effective management, the first step remains the

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identification of the causal pathogen of the disease. This study therefore aimed to investigate the pathogen associated with *Parkia biglobosa* pod rot using molecular techniques and to confirm its pathogenicity.

MATERIALS AND METHODS

Sample Collection

Parkia biglobosa pods showing rot symptoms were collected in the University of Ilorin campus from January to February 2019 and then processed for fungal isolation at the Mycology laboratory, department of Plant Biology, University of Ilorin.

Fungal Isolation and Characterisation

Isolation of the fungi causing pod rot on *P. biglobosa* pods was carried out on Potato Dextrose Agar (PDA) medium. The infected pod samples collected were washed with distilled water in order to remove any dirt (Lateef et al., 2019). The infected portions of the samples were then surface sterilized with 70 % ethanol, excised with a flamed scissors and allowed to dry before plated on PDA. Inoculation was done in 5 petri dish replicates. Cultures were incubated at room temperature. Colony growth after 48-72 hrs was observed and transferred to new PDA media to obtain pure cultures. Isolates obtained from pure cultures were characterised and grouped based on their cultural and morphological features.

Pathogenicity Test

Pathogenicity of the isolate was done to confirm the observed symptoms on the diseased pods (Zhang et al., 2012). Healthy pods of *Parkia biglobosa* were collected, washed and disinfected in 70 % ethanol. The disinfected pods were then rinsed in sterile distilled water and dried before inoculation. The pods were each pierced with sterilized needle in three places. Afterwards, a small fragment of mycelia of the fungal isolates was dropped on the wounded portion using a sterile inoculating needle, sealed in Petri dishes containing sterile tissue paper with a sponge sprayed with sterilized distilled water to maintain at least 95 % relative humidity and incubated for 7 days at room temperature. Control pods were inoculated with sterile distilled water and disease symptoms were evaluated after 7 days.

DNA Extraction, Polymerase Chain Reaction and Sequencing

DNA was extracted from the isolated fungi. The extraction was done using the DNA mini-prep extraction kit (Zymo Research, USA) protocol. 50 µl DNA Elution buffer was used to get the ultra-pure DNA which was used for the PCR.

PCR and sanger sequencing of the isolate were carried out at the African genomics company, Inqabba Biotech (South Africa) with ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) as the forward and reverse primers respectively

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(White et al., 1990). The PCR cocktail consisted of 12.5 µl of Taq DNA polymerase 2X PCR master mix, 1 µl (10 µM) of the primers each, 1 µl of template DNA and then made up to 25 µl by adding 9.5 µl of double-sterilised distilled water (ddH_2O). ddH_2O was used for the control PCR. The PCR programme followed as; thus, 2 mins at 94 ˚C, followed by 35 cycles at 94 ˚C for 1 min, 55 ˚C for 1 min, 72 ˚C for 1 min and final extension for 10 mins at 72˚C. Sequencing of the PCR products were done in an ABI PRISM sequencer using the same primers as for the PCR.

Bioinformatics and Phylogenetic Analysis

Raw sequences from the sequencing machine were analysed using several bioinformatics softwares. Firstly, the raw sequences were confirmed to tally with the morphological features of the fungal isolate. SeqTrace 9.0 (Stucky, 2012)was used to view the forward and reverse raw sequence data and generate the consensus sequence. The consensus sequence was then used for similarity search using the BLAST-n tool on the GenBank website (http://www.blast.ncbi.nlm.nih.gov).

Similar sequences were then downloaded and aligned using Aliview 1.17-beta1 (Larsson, 2014). The aligned sequences were further used for phylogenetic tree analysis using MEGA X (Kumar et al., 2018). The maximum likelihood method with Tamura-Nei model was used for the phylogenetic tree construction. This analysis involved 13 nucleotide sequences including the outgroup. There was a total of 653 positions in the final dataset. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The sequence of the isolate obtained in this study was submitted to GenBank to obtain the accession number.

RESULTS

Isolation and Morphological Characterisation

In this study, the causal agent of *Parkia biglobosa* pod rot was isolated and the pathogen was identified based on their morphological characteristics. All the isolates (five) obtained were observed to have the same cultural and morphological features. The morphological characteristics of the isolated fungi was initially white but later turned grey-dark with whitish mycelia at the top. The reverse of the culture was brown to dark (Figure 1).

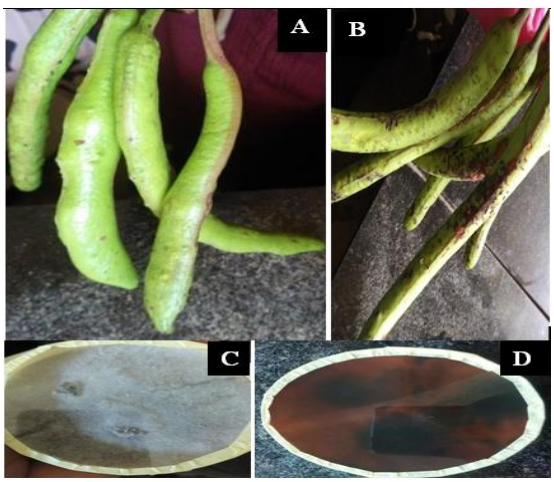

Figure 1: (A) Healthy *Parkia biglobosa*. (B) *Parkia biglobosa* with pod rot disease.

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(C & D) Fungi isolated from *Parkia biglobosa* pod rot on PDA (Front view and back view).

Pathogenicity

The pathogenicity test of the fungal isolate showed symptoms after 6-7 days similar to those observed in *P. biglobosa* pods, which developed on all the wounded and inoculated pods with the pathogen but not on the control (wounded and non-inoculated pods). Small lesions developed on the inoculated pods, turning brown-black, and continued to expand in a circular shape, thus causing large lesions. The same fungus was re-isolated from the lesions.

DNA Sequencing and Molecular Phylogenetics

The length of the internal transcribed spacer (ITS) sequence of the isolate was 562 bp (accession number: OL414950). The sequence had 99-100 % identity with several species of *Pseudofusicoccum violaceum* and *Fusicoccum* in the GenBank repository (Table 1). Further phylogenetic analysis of the isolate with various species of *Pseudofusicoccum* supported its identity as *Pseudofusicoccum violaceum* with high bootstrap values (Figure 2). Thirteen species were used for the maximum likelihood tree which resulted in two major clades: one for *Pseudofusicoccum* and the other for the *Fusicoccum* with an outgroup. The *Pseudofusicoccum* clade then later separated into sub-clades as *P. violaceum, P. kimberleyense, P. stromaticum and P. adansoniae*. The isolate from this study clustered in the *P. violaceum* clade and thus clearly identified as *P. violaceum* isolate.

Table 1: GenBank sequences used for the phylogenetic analysis including their accession numbers

S/N	Species	Isolate	GenBank accession number (ITS)
1	*Pseudofusicoccum violaceum*	MAN22312	KU997461.1
2	*Pseudofusicoccum violaceum*	MAN22138	KU997555.1
3	***Pseudofusicoccum violaceum***	**UIL 94**	**OL414950 (From this study)**
4	*Pseudofusicoccum ardesiacum*	CBS 122062	KF766221.1
5	*Pseudofusicoccum kimberleyense*	CBS 122058	KF766222.1
6	*Pseudofusicoccum kimberleyense*	CBS 122058	MH863170.1
7	*Pseudofusicoccum stromaticum*	UAOLM18LMM21	MK166049.1
8	*Pseudofusicoccum adansoniae*	W26147	KF766220.1
9	*Pseudofusicoccum adansoniae*	MMI00064	JQ585586.1
10	*Pseudofusicoccum adansoniae*	C1536	KT968485.14
11	*Fusicoccum dimidiatum*	CBS 204.33	AY819728.1
12	*Fusicoccum macroclavatum*	WAC12444	DQ093196.1
13	*Phaeosphaeria ammophilae**	CBS 114595	KF766146.1 (Outgroup)

* Outgroup, ITS = Internal Transcribed Spacer.

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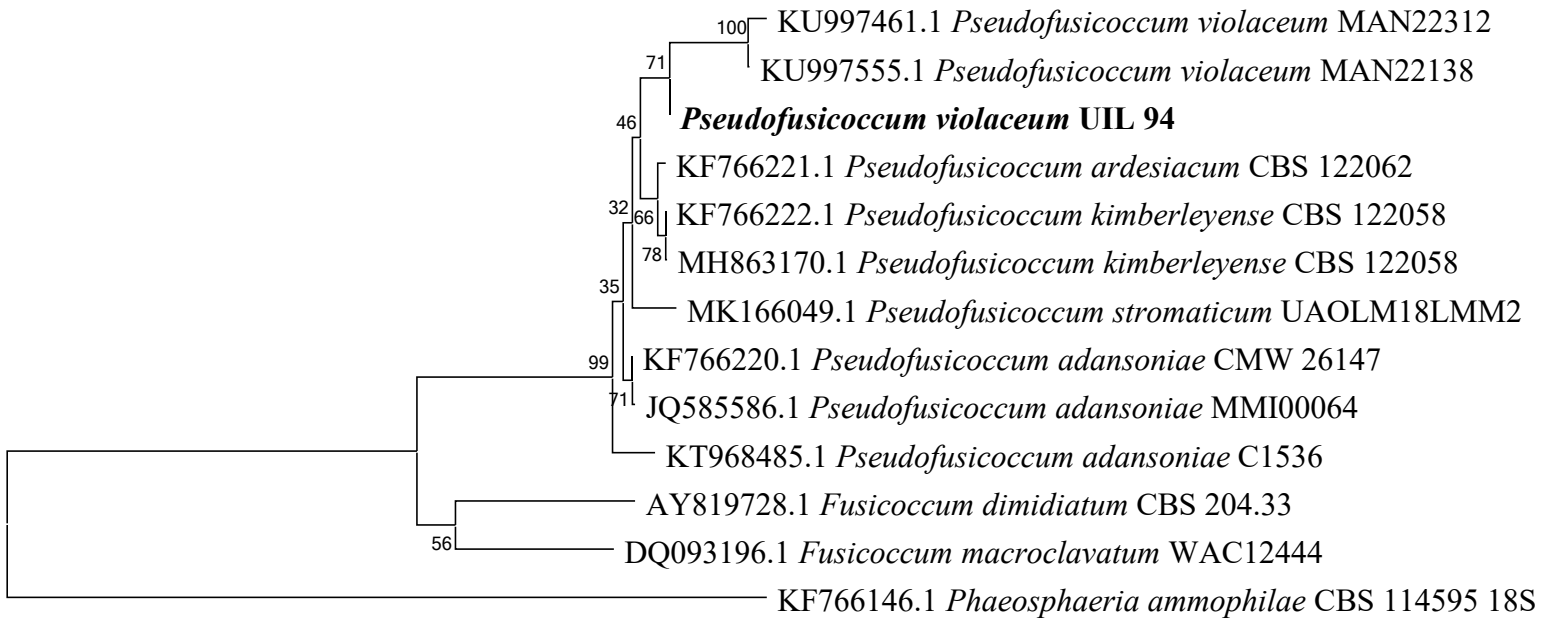

Figure 2: The phylogenetic tree showing the molecular identification of the fungal pathogen causing pod rot of *Parkia biglobosa* isolated from this study. The isolate in this study in bold. Maximum likelihood bootstrap values are shown on the tree branches.

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DISCUSSION

Fruit tree diseases in Africa are a major challenge and threat to local sustainability, livelihood, and economic development. The isolation of *Pseudofusicoccum violaceum* causing pod rot of *Parkia biglobosa* represents a key step in the sustainable use of *P. biglobosa* seeds as a local alternative to MSG. *Pseudofusicoccum violaceum* isolated from *P. biglobosa* pod rot was characterized based on their morphological features and molecular phylogenetics. This study, therefore, represents the first record of *P. fusicoccum* occurring in Nigeria. *Pseudofusicoccum* currently has 9 species and is a member of the botryosphaeraceous fungi resembling Botryosphaeria although with mucilagenous sheath surrounding their conidia (Pavlic et al., 2008). It has been reported that Pseudofusicoccum is also a pathogen on *Acacia, Pinus* and *Eucalyptus* plants (Mohali *et al.,* 2006).

Pseudofusicoccum has been associated with the dieback of mango and Malay apple (Silveria et al., 2017) as well as dieback of cassava, guava and mango in Brazil (Coutinho et al., 2018). Additionally, Sessa et al. (2021) reported *Pseudofusicoccum* as a pathogen responsible for the shoot cancer of peach in Uruguay.

In this study, the pathogenicity test confirms the Koch's postulate as *Pseudofusicoccum* was re-isolated from the inoculated *P. biglobosa* pods. This is in line with the latest recommendation of disease pathogenicity (Bhunjun et al., 2021) and it is important to state that to improve accuracy and inter-studies comparison, pathogens should be reported as associated with the disease symptoms (Bhunjun et al., 2021; Falkow, 2004; Segre, 2013) especially when introducing a new taxon.

Interestingly, Mishra et al. (2018) reported *P. andansoniae* as an endophytic fungus residing in the photosynthetic root of *Tinospora cordifolia.* This is implying *Pseudofusicoccum* spp. occurrence as pathogens in one plant and then as endophytes in other plants and points to the possibility of the organism's pathogenicity being influenced by some external factors, which may include the environment. It has also been reported that between endophytism and pathogenicity is just a thin line (Salvatore et al., 2020) which might be triggered by different factors. In the case of *Pseudofusicoccum violaceum*, studies have indicated its high frequency of being found acting as an endophyte as opposed to its role as a primary pathogen (Sharma et al., 2013; Mehl et al., 2011).

Therefore, the role of *Pseudofusicoccum violaceum* as an endophyte then suggests its possible capacity to produce secondary metabolites as observed with several other endophytes. However, this study points to the likelihood of *P. violaceum* as a latent pathogen as opposed to being an endophyte (Slippers & Wingfield, 2007).

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This study thus revealed the occurrence of *P. violaceum* on *Parkia biglobosa* and its disease-causing ability on its pods.

CONCLUSION

The current study used molecular techniques to successfully characterise the pathogen associated with *Parkia biglobosa* pod rot. The nucleotide sequence of the pathogen was compared with sequences available in the GenBank database and the fungus was correctly identified as *Pseudofusicoccum violaceum*. This represents the first in-depth analysis of *Pseudofusicoccum violaceum* causing disease on *Parkia biglobosa* and the first report of this fungus occurring in Nigeria. The result of this study will also aid in proffering ways to enhance the prevention/control of diseases associated with African fruit trees and *P. biglobosa* pod in particular. Further studies on the endophytic lifestyle of *P. violaceum* and the conditions that may prompt its switch from an endophytic role to its pathogenic activity are needed.

REFERENCES

Adetutu, A., Morgan, W. A., & Corcoran, O. (2011). Ethnopharmacological survey and in vitro evaluation of wound-healing plants used in South-western Nigeria. *Journal of Ethnopharmacology*, 137(1), 50–56. https://doi.org/10.1016/j.jep.2011.03.073

Airaodion, A. I. (2019). Toxicological Effect of Monosodium Glutamate in Seasonings on Human Health. *Global Journal of Nutrition & Food Science,* 1(5): 2019. *https://doi.org/10.33552/GJNFS.2019.01.000522.*

Arinola, S. O., Oje, O. J., & Omowaye-Taiwo, O. A. (2019). *Evaluation of Physicochemical Properties and Phytochemical Composition of African Locust Bean (Parkia biglobosa) Pulp. Applied Tropical Agriculture,* 24(1), 64-69.

Bhunjun, C. S., Phillips, A. J. L., Jayawardena, R. S., Promputtha, I., & Hyde, K. D. (2021). Importance of Molecular Data to Identify Fungal Plant Pathogens and Guidelines for Pathogenicity Testing Based on Koch's Postulates. *Pathogens*, 10(9), Article 9. https://doi.org/10.3390/pathogens10091096

Coraf. (2020). *Fighting Against Plant Pests & Diseases in West and Central Africa – CORAF* [Http://www.coraf.org/2020/10/14/fighting-against-plant-pests-diseases-in-west-and-central-africa/]. http://www.coraf.org/2020/10/14/fighting-against-plant-pests-diseases-in-west-and-central-africa/

Diawara, B., Sawadogo, L., Amoa-Awua, W. F., & Jakobsen, M. (2000). Capability building for research and development in quality

DOI: 10.56892/bima.v8i3B.1000

assurance and fermentation technology for African fermented foods. HACCP system for traditional African fermented foods: Soumbala. Taastrup: WAITRO, 60 p.

Falkow, S. (2004). Molecular Koch's postulates applied to bacterial pathogenicity—A personal recollection 15 years later. *Nature Reviews Microbiology*, 2(1), 67–72. https://doi.org/10.1038/nrmicro799

Gernmah, D. I., Atolagbe, M. O., & Echegwo, C. C. (2007). Nutritional composition of the African locust bean (*Parkia biglobosa*) fruit pulp. *Nigerian Food Journal*, *25*(1), 190-196. https://doi.org/10.4314/nifoj.v25i1.33669

Gill, H. K., Garg, H., Gill, H. K., & Garg, H. (2014). Pesticides: Environmental Impacts and Management Strategies. In *Pesticides—Toxic Aspects*. Intech Open. https://doi.org/10.5772/57399

Graziosi, I., Tembo, M., Kuate, J., & Muchugi, A. (2020). Pests and diseases of trees in Africa: A growing continental emergency. *PLANTS, PEOPLE, PLANET*, *2*(1), 14–28. https://doi.org/10.1002/ppp3.31

Karlsson Green, K., Stenberg, J. A., & Lankinen, Å. (2020). Making sense of Integrated Pest Management (IPM) in the light of evolution. *Evolutionary Applications*, 13(8), 1791–1805. https://doi.org/10.1111/eva.13067

Koura, K., Ganglo, J. C., Assogbadjo, A. E., & Agbangla, C. (2011). Ethnic differences in use values and use patterns of Parkia biglobosa in Northern Benin. *Journal of Ethnobiology and Ethnomedicine*, *7*(1), 42. https://doi.org/10.1186/1746-4269-7-42

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Larsson, A. (2014). AliView: A fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics (Oxford, England)*, *30*. https://doi.org/10.1093/bioinformatics/btu531

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*.

DOI: 10.56892/bima.v8i3B.1000

Mondal, K. K., & Shanmugam, V. (2013). Advancements in the diagnosis of bacterial plant pathogens: An overview. *Biotechnology and Molecular Biology Reviews*, *8*(1), 1–11. https://doi.org/10.5897/BMBR12.007

Olasupo, N. A., Okorie, P. C., Olasupo, N. A., & Okorie, P. C. (2019). African Fermented Food Condiments: Microbiology Impacts on Their Nutritional Values. In *Frontiers and New Trends in the Science of Fermented Food and Beverages*. IntechOpen. https://doi.org/10.5772/intechopen.83466

Omafuvbe, B. O., Shonukan, O. O., & Abiose, S. H. (2000). Microbiological and biochemical changes in the traditional fermentation of soybean for 'soy-daddawa'—Nigerian food condiment. *Food Microbiology*, *17*(5), 469–474. https://doi.org/10.1006/fmic.1999.0332

Popoola, T. O. S., & Akueshi, C. O. (1985). Microorganisms associated with the fermentation of soyabean for the production of soyabean daddawa a condiment. *Nigerian Food Journal*, *3*, 194–196.

Saleh, M. S. M., Jalil, J., Zainalabidin, S., Asmadi, A. Y., Mustafa, N. H., & Kamisah, Y. (2021). Genus Parkia: Phytochemical, Medicinal Uses, and Pharmacological Properties. *International Journal of Molecular Sciences*, *22*(2). https://doi.org/10.3390/ijms22020618

Salvatore, M. M., Andolfi, A., & Nicoletti, R. (2020). The Thin Line between Pathogenicity and Endophytism: The Case of Lasiodiplodia theobromae. *Agriculture*, *10*(10), Article 10. https://doi.org/10.3390/agriculture10100488

Segre, J. A. (2013). What does it take to satisfy Koch's postulates two centuries later? Microbial genomics and Propionibacteria acnes. *The Journal of Investigative Dermatology*, *133*(9), 2141. https://doi.org/10.1038/jid.2013.260

Slippers, B., & Wingfield, M. J. (2007). Botryosphaeriaceae as endophytes and latent pathogens of woody plants: Diversity, ecology and impact. *Fungal Biology Reviews*, *21*(2–3), 90–106. https://doi.org/10.1016/j.fbr.2007.06.002

Stucky, B. J. (2012). SeqTrace: A graphical tool for rapidly processing DNA sequencing chromatograms. *Journal of Biomolecular Techniques : JBT*, *23*(3), 90–93. PubMed.

DOI: 10.56892/bima.v8i3B.1000

https://doi.org/10.7171/jbt.12-2303-004

Tudi, M., Daniel Ruan, H., Wang, L., Lyu, J., Sadler, R., Connell, D., Chu, C., & Phung, D. T. (2021). Agriculture Development, Pesticide Application and Its Impact on the Environment. *International Journal of Environmental Research and Public Health*, *18*(3), 1112. https://doi.org/10.3390/ijerph18031112

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and Direct Sequencing of fungal Ribosomal RNA genes for Phylogenetics. In *PCR Protocols: A Guide to methods and Applications.* (pp. 315–322). Academic Press.

Zhang, L. X., Li, S. S., Tan, G. J., Shen, J. T., & He, T. (2012). First Report of Nigrospora oryzae Causing Leaf Spot of Cotton in China. *Plant Disease*, *96*(9), 1379. https://doi.org/10.1094/PDIS-04-12-0349-PDN