2019-04

Detection and characterization of cashew leaf and nut blight disease in the Eastern and the Southern Zones of Tanzania

Majune, Dadili

Research Journal of Agriculture and Forestry

Research Journal of Agriculture and Forestry Sciences _____ISSN 2320 – 6063
Vol. **7(2),** 36-44, April **(2019)** *Res. J. Agriculture and Forestry Sci.*

Detection and characterization of cashew leaf and nut blight disease in the Eastern and the Southern Zones of Tanzania

Majune D.J.[1,2*], Masawe P.A.L[2], Kafiriti E.[2] and Mbega E.R.[1]
[1]School of Life sciences and Bio-engineering the Nelson Mandela African Institution of Science and Technology (NM-AIST), P. O. Box 447, Arusha-Tanzania
[2]Tanzania Agricultural Research Institute, P. O. Box 509, Mtwara – Tanzania
majuned@nm-aist.ac.tz

Available online at: www.isca.in
Received 10th December 2018, revised 15th March 2019, accepted 4th April 2019

Abstract

Cashew (Anacardium occidentale Linn.) is export crop and source of income for many households in Southern and Eastern zones of Tanzania. However, its productivity is facing a number of challenges among them are diseases and insect pests. Previously cashew powdery mildew was known to be the main disease of cashew. However, cashew Leaf and Nut Blight Disease (CLNBD) has been cited as one of the most devastating diseases in cashew industry. The disease leads into lower cashew nut yields and poor nut qualities. A study was carried out to investigate detection and characterization of CLNBD in eastern and southern zones of Tanzania. A total of ninety leaf samples infected by the disease were collected for isolation of the fungus. The fungus was isolated by direct conidial transfer method on potato dextrose agar (PDA) medium and every procedure was carried out in aseptic conditions to prevent micro-organisms contamination. DNA was extracted and amplified using Polymerise Chain Reaction (PCR). The PCR results by using primer ITS5 (forward) and ITS4 (reverse) was amplified at above 500 bp and results by using primer ITS1 (forward) and ITS4 (reverse) was also amplified at above 320 bp and bands were observed respectively. Twenty four DNA samples from three regions were sent for sequencing. The nucleotide BLAST analysis showed that ten isolates had maximum nucleotide identities of 97-99% of cashew leaf and nut blight disease and were deposited in the gene bank. The phylogenetic relationship tree was reconstructed using the nucleotide sequences. The neighbour-joining and bootstrap methods show that CLNBD have more than one fungal strain which makes to be a complex disease.

Keywords: Cashew, CLNBD, fungal, PCR, phylogenetic tree.

Introduction

Cashew (*Anacardium occidentale* Linn.) is a tropical nut crop that belongs to Anacardiaceae family, which consists about 75 genera and 700 species[1]. *Anacardium* contains eight species all of which are native to the coastal parts of north eastern Brazil[2]. Itis now cultivated in Tanzania and Mozambique[3-5]. The crop is important for income generation worldwide[6]. The apples and nutsare important products obtained from the cashew plants. The nuts are processed to produce kernels which are consumed mostly as snacks. The apples produce juice, jam, candy and alcoholic beverages like wine, gin and brandy[7]. Apart from being a source of food, employment and income; cashew nut tree provides firewood and charcoal[8]. Opeke[9] reported that main cashew producing in the world are India, Tanzania, Mozambique, Nigeria, and Guinea-Bissau countries. However, other countries like Ivory Coast, Brazil, Benin and Ghana are also among the major producers of the crop.

It is the leading export crop in Tanzania and the main cash crop for over 500,000 households in South-Eastern Tanzania[10]. It is estimated that more than 80% of the national cashew production comes from Mtwara, Lindi and Ruvuma (Tunduru District) regions[11]. The crop is grown on an estimated area of more than 400,000 hectares in mono or mixed crop production systems[11]. However, the area under cashew has increased many folds because the crop is now grown almost in all regions of Tanzania[12]. An average cashew farmer owns 1-2 hectares of cashew trees[13]. The average crop yield in farmers' ranges from 500kg/ha to 800kg/ha[5]. The production increased in the 1960s up to mid-1970s, recording as high as 145,000Mt. Thereafter, production decline up to 16,400Mt in 1973/1974. The reasons for the decline in production were cited to be powdery mildew disease, insect pests and lack of improved planting materials[14,15]. The cashew powdery mildew disease was the main reason behind decline in the cashew production[16,17]. The disease control measures developed was adopted by cashew farmers, the loss is 70 to 100% if not controlled[18-20].

In 2003, a survey carried out by Pathologists at Tanzania Agricultural Research Institute (TARI) in Tanzania revealed a new disease as "cashew leaf and nut blight" (CLNBD). The disease is caused by a fungal pathogen *Cryptosporiopsis* spp and was reported to attack cashew for the first time in Tanzania[21,22]. The symptoms appear on plant which are tender including young nuts and apples, also can cause annual crop

loss up to 48.4% if not controlled. The disease is most active during wet weather especially during off-season rains, where severe infections affect emerging young tender leaves[22]. The disease portrays angular lesions, dark tan with a dark reddish brown margin formed on leaves. Lesions subsequently enlarge and coalesce leading to large necrotic lesions and finally defoliation. If it rains during fruit setting, the disease causes rapid blackening and abscission of nuts[22-24].

Despite the importance of CLNBD in Tanzania, the disease has not been assessed using phylogenetic methods; hence the basis for conducting this study. The study was performed based on CLNBD isolates associated with cashew tree. These isolates were obtained from symptomatic leaves in eastern and southern zones in Tanzania. The morphological, pathogenic and molecular characterization on these isolates was performed. The study will aidin identifying fungal strains causing CLNBD which will enable developing management strategies of the disease.

The study objective was detection and characterization of CLNBD in southern and eastern zones of Tanzania. The ultimate goal was determination of phylogenetic relationship of existing CLNBD isolated from diseased leaf samples materials in southern and eastern zones of Tanzania.

Methodology

Nachingwea (Lindi region), Bagamoyo (Coast region) and Mtwara (Mtwara region) districts are area studied (Figure-1). These districts were selected because cashew is widely grown and CLNBD is highly prevalent. Also, these sites were selected because they had been used for establishing cashew genetic trials of elite cashew hybrids developed in 1996 and 1998when released as new cashew varieties in Tanzania.

Sample collection and culturing: A total of ninety leaf samples infected by CLNBD were collected from different fields in Nachingwea and Mtwara (southern zone) and Bagamoyo (eastern zone). In each district, thirty leaf samples were collected from farmers' fields. The samples were well labeled and transferred to Mikocheni Agriculture Research Institute (MARI) laboratory in Tanzania for fungal isolations. The fungal was isolated by direct conidial transfer method using potato dextrose agar (PDA) medium using MARI'S protocal. The medium were prepared in flask dissolved in one litre of water, were autoclaved for 15 minutes in 121^{o}C and then poured into sterilized petri dishes for solidification. Every procedure was carried out in aseptic conditions, in a separate culture room free from dust. The laminar flow was wiped with 70% alcohol, hands were cleaned and tools such as scalpels, forceps and knife were dipped in alcohol and flamed to prevent micro-organisms contamination.

Figure-1: The cashew growing areas and study of CLNBD in Tanzania.

The samples were cut into small square tissues of 1.5 cm section from the margin of lesions, so that they contain both diseased and healthy tissues. The tissue surface sterilization was done using disinfectant solution of 2% sodium hypochlorite for one minute. Using flamed forceps, leaf pieces were washed in sterilized distilled water through three rinses to remove excessive disinfectant. These pieces were placed on sterile filter paper to dry before being placed on petri dishes containing a culture medium, for incubation in a growth incubator under constant fluorescent. The leaf bits were placed in petri dishes containing sterilized PDA and incubated for four days at 25 ± 2^{o}C. The sporulated fungal on leaf bits were sub-cultured onto new sterilized PDA medium in petri dishes and incubated for five days at 25 ± 2^{o}C. After obtaining pure colonies, isolates were transferred to another petri dish containing a culture medium and cultivated for five days again until sporulation. Fungal mycelia were scooped using a needle into 1.5ml Eppendorf tubes for DNA extraction and the remaining isolation were maintained on PDA slants for references. Below are plates showing effects of CLNBD on leaves and nuts in the farm (Plate 1 A to F). Plate 1 G to L indicates the growth of fungal mycelia from leaves. 24 CLNBD isolates were obtained from districts. Samples collected from Mtwara were belonging to the Ministry of Agriculture Training Institute (MATI) Mtwarafarms, whereas Nachingwea and Bagamoyo samples were obtained from Tanzania Agricultural Research Institute (TARI) cashew genetic trials.

Plate-1: A-F showing cashew leaf and nut blight disease on leaves and nuts.


A. Disease symptoms on young cashew leaves


B. Disease symptoms on mature cashew leaves


C. Severe disease symptoms on cashew leaves


D. Symptoms of disease on young cashew nuts


E. Severe symptoms of disease on cashew nuts

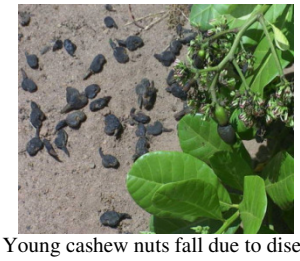
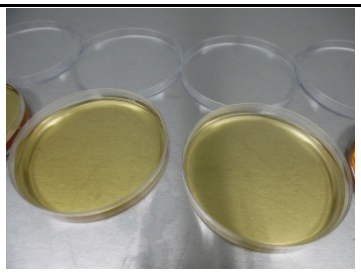

F. Young cashew nuts fall due to disease

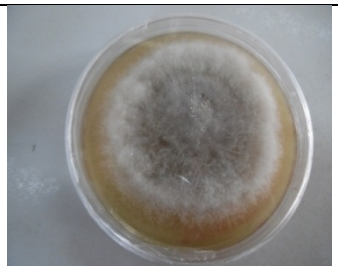
Plate 1 G-L show the growth of fungal mycelia from leaves


G. Sterilized potato dextrose agar


H. Diseased Leaf bits incubated in PDA


I. Sporulated fungal on leaves


J. Sporulated fungal on new PDA


K. Mature fungal mycelia

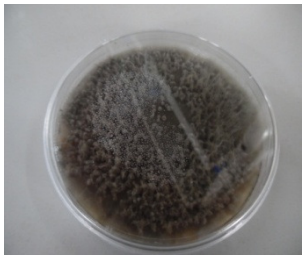

L. More matured fungal mycelia

Table-1: CLNBD isolates obtained from lesions of infected cashew leaves.

Collection code	Collection site	Collection date
Lindi1	Nachingwea	March 2018
Lindi2	Nachingwea	March 2018
Lindi3	Nachingwea	March 2018
Lindi4	Nachingwea	March 2018
Lindi5	Nachingwea	March 2018
Lindi6	Nachingwea	March 2018
Lindi7	Nachingwea	March 2018
Lindi8	Nachingwea	March 2018
Mtwara1	Naliendele	March 2018
Mtwara2	Naliendele	March 2018
Mtwara3	Naliendele	March 2018
Mtwara4	Naliendele	March 2018
Mtwara5	Naliendele	March 2018
Mtwara6	Naliendele	March 2018
Mtwara7	Naliendele	March 2018
Mtwara8	Naliendele	March 2018
Coastal1	Chambezi	March 2018
Coastal2	Chambezi	March 2018
Coastal3	Chambezi	March 2018
Coastal4	Chambezi	March 2018
Coastal5	Chambezi	March 2018
Coastal6	Chambezi	March 2018
Coastal7	Chambezi	March 2018
Coastal8	Chambezi	March 2018

DNA extraction: The total genomic fungal isolates of DNA were extracted using chloroform procedure methods. Fungal mycelia were scooped into 1.5ml Eppendorf tubes containing 400 ul of extraction buffer, 50 ul of RNase A, and 20 µl of SDS (20%). The mycelia were crushed using plastic pestles before they were vigorously vortexed. The samples were then incubated at 65^{o}C for 30 minutes. Samples were removed from heating blocks and left at recommended temperature for cooling process and were then centrifuged at maximum speed for 10 minutes. Equal amount of chloroform (phenol excluded) were added and then samples were vigorously shaken left for seven minutes on ice. The samples obtained were centrifuged at required maximum speed for two minutes and supernatant were placed into a new 1.5ml Eppendorf. Two volumes isopropanol were slowly added then mixed by inversion. 1/10 of 3 M NaOAC was added to the mixture inverted two to three times and kept at -20^{o}C for ten minutes to allow for DNA precipitation. The centrifuge took place at 1200 rpm for 15 min. Supernatant found were discarded and DNA pellets with 700 µl of 70% ethanol were washed then centrifuged two minutes at recommended maximum speed. Ethanol was discarded and the DNA pellets were dried at recommended air room temperature for 40 minutes. A DNA pellet wase-suspended with 50 µl of TE buffer. DNA obtained was stored at -20^{o}C for PCR analysis.

Polymerase Chain Reaction: The primers that target the Internal Transcribed Spacer (ITS) were used. Two types of primers were used number one primer was ITS1 (CTTGGTCATTTAGAGGAAGTAA) Forward and ITS4 (GCTGCGTTCTTCATCGATGC) reverse and number two primer was ITS5 GGAAGTAAAAGTCGTAACAAGG (forward) and ITS4 GCTGCGTTCTTCATCGATGC (reverse) for fungal[25]. To Accu Power R PCR Pre Mix (20µl tubes), 16µl of water, 1µl of ITS5 forward, 1µl of ITS4 reverse and 2µl of DNA for each. The PCR program was as follows: denaturation at 94^{o}C for two minutes and then was followed by 35 cycles at 94^{o}C for 30 seconds, 52^{o}C for 30 seconds, 72^{o}C for 1 minute and 72^{o}C for 10 minutes. Product obtained was visualized using UV light in a Trans illuminator. The PCR products obtained were well parked and labelled sent for sequencing on both strands at Mbeya Referral hospital Laboratory. 24 DNA samples from three regions were sent for sequencing, however, eight DNA samples from each region were sequenced and the results were obtained after ten days.

Results and discussion

The PCR product results by using primer ITS5 (forward) and ITS4 (reverse) was amplified at above 500 bp and bands were observed as below indicated (Figure-2). The PCR product results by using primer ITS1 (forward) and ITS4 (reverse) was amplified at above 320 bp and bands were observed as below indicated (Figure-3). The phylogenetic relationship tree was reconstructed using the nucleotide sequences obtained from three regions the neighbour-joining and bootstrap methods were used. The nucleotide BLAST analysis showed that ten isolates from three regions have maximum nucleotide identities of 97-99% of cashew leaf and nut blight disease and have been deposited in Gene bank (Table-1).

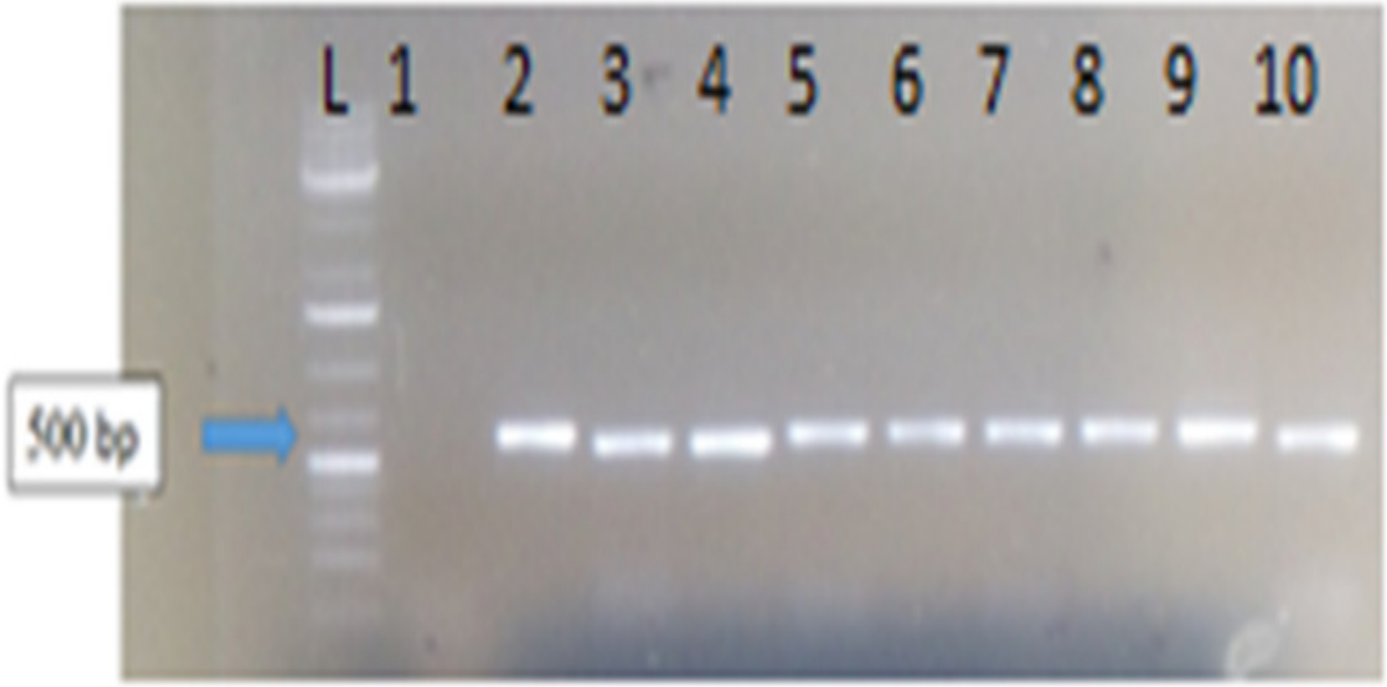


Figure-2: Amplification of a sample of pathogenicity positive isolates by PCR using ITS5 and ITS4 primers. Where L is DNA ladder, 1 is a control, 2-10 (Lindi1, Lindi2, Lindi3, Lindi4, Lindi5, Lindi6, Lindi7, Lindi8, Lindi9 and Lindi10) samples for detection of CLNBD causing fungi.

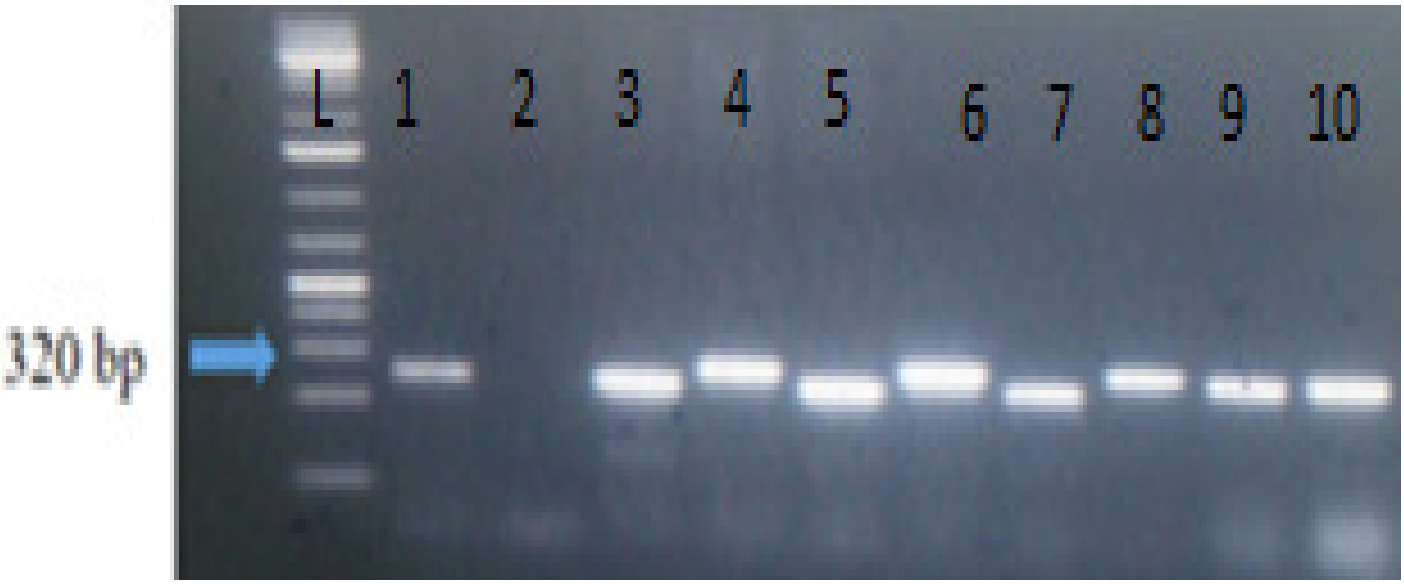


Figure-3: Amplification of a sample of pathogenicity positive isolates by PCR using ITS1 and ITS4 primers. Where L is DNA ladder, 1-10 (1 Mtwara1, 2 control, 3 Mtwara2, 4 Mtwara3, 5 Mtwara4, 6 Mtwara5, 7 Coast1, 8 Coast2, 9 Coast3 and 10 coast4) samples for detection of CLNBD causing fungi.

Evolutionary was inferred using the Maximum Likelihood method on the Tamura-Nei model[26]. The tree with the highest log likelihood (-894.71) is shown. The percentage of associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 132 positions in the final dataset. Evolutionary analyses were conducted in MEGA7[27]

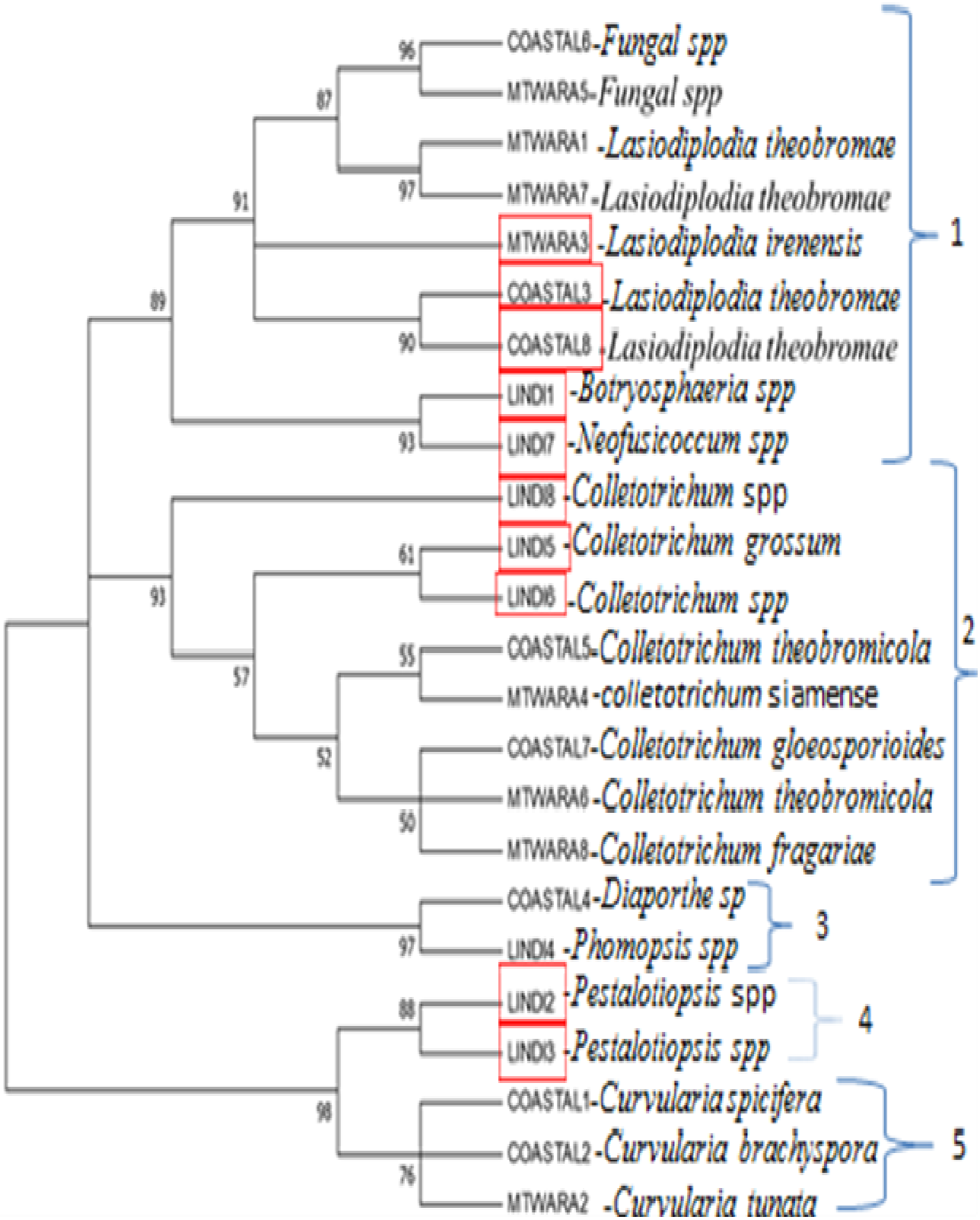


Figure-4: Phylogenetic tree reconstructed using the nucleotide sequences of CLNBD isolates. Phylogenetic tree were constructed with a neighbour-joining method in MEGA7 using bootstrap values over 50%, red blocks shows samples sequenced with 97-99% identity to known fungal species.

Table-2: showing species with maximum nucleotide identities of 97-99% of CLNBD.

Species Description	Max score	Query cover	Identy	Accession
Botryosphaeria spp	1011	98%	99%	EU330628.1
Pestalotiopsis spp	942	97%	99%	HQ607806.1
Pestalotiopsis spp	958	98%	99%	KF179297.1
Colletotrichum gloeosporiodes	998	97%	99%	JX258732.1
Colletotrichum fragariae	1000	98%	99%	KC209101.1
Neofusicoccum spp	978	97%	99%	KC706920.1
Colletotrichum spp	975	98%	99%	KJ493232.1
Lasiodiplodia iranensis	376	97%	99%	KU997384.1
Lasiodiplodia theobromae	942	99%	99%	KJ596523.1
Lasiodiplodia theobromae	41374	97%	99%	FJ904840.1

Detection and characterization of CLNBD: Using the ITS primers and sequence information, it was evident that the CLNBD in Tanzania is caused by different fungal organisms including *Botryosphaeria* spp, *Pestalotiopsis* spp, *Colletotrichumgloeosporioides*, *Colletotrichumfragariae*, *Neofusicoccum* spp, *Colletotrichum* spp, *Lasiodiplodiairanensis* and *Lasiodiplodia.*Phylogenetic clattered the isolates into five main groups. Cluster 1 = *Lasiodiplodia* spp, claster 2 = *Colletotrichum* spp, claster 3 = *Diaporthe* spp and *Phomopsis*, claster 4 = *Pestalotiopsis* spp and Claster 5 = *Curvularia* spp. Sequences for each organism have been deposited and assigned Gene bank accession numbers at NCBI as follows: MH715267, MH715268, MH715269, MH715270, MH715271, MH715272, MH715273, MH715274, MH715275, and MH715276 respectively. These results show a number of studies that indicate pathogenic nature of the identified organisms (Uaciquete, 2003; Sijaona, 2013; Zhongrun and Masawe, 2014). Though the sequence identity for some other isolates were below 95%, it remains possible that two more or three more fungal species such as *Curvularia* spp and others are associated with the CLNBD in Tanzania. Based on these results the following fungal microorganisms namely *Botryosphaeria* spp, *Pestalotiopsis* spp, *Colletotrichumgloeosporioides*, *Colletotrichumfragariae*, *Neofusicoccum* spp, *Colletotrichum* spp, *Lasiodiplodiairanensis* and *Lasiodiplodiatheobromae* are reported for the first time to be associated with CLNBD in Tanzania.

Pathogenicity testing: Cashew seedlings with one month old were inoculated with $1x10^6$ mL^{-1} conidial suspension of selected CLNBD isolates at temperature of 25-28°C and 95% RH within 24 hours. All inoculated cashew seedlings indicates leaf lesions similarly to those observed in cashew farmers field. Cashew seedlings sprayed with water did not develop any symptoms during the experimental period. The pathogen was consistently re-isolated from infected cashew leaves and grown on PDA medium then to fresh cashew seedlings, thus completing Koch's postulates. It can be concluded that all CLNBD isolates used in this study are plant pathogenic and can trigger CLNBD on leaves with similar characteristics as those from which they were obtained.

Conclusion

The findings clearly indicate that cashew materials in the studied districts appear to be susceptible to CLNBD. Further, the study has confirmed that CLNBD has more than one fungal strain, which makes the disease more complex henceforth difficult to control by conventional practices. These results suggest that farmers in the study areas and those with similar climatic conditions in Tanzania should use integrated approach in the management of the disease, including planting resistant or tolerant cashew varieties. It is recommended that the study to be undertaken in all cashew growing areas in Tanzania to validate these findings as a basis against which to develop and up-scale management strategies of CLNBD in areas where the disease threatens growth and development of the crop.

Acknowledgements

I would like to acknowledge NM-AIST for providing an opportunity to undertake a course in Applied Plant Pathology which created grounds for development of this document. Highly appreciation for Tanzania government under TARI – Naliendele for financial support on this study.

References

1. Ohler J.G. (1979). Cashew. Communication No. 71. Department of Agricultural Research, Royal Tropical Institute. Amsterdam, Holland.

2. Azam-Ali S. and Judge E. (2000). The global cashew industry-opportunities for small-scale processors. In: Proceedings of the international workshop on cashew production and processing-Cashing in on Cashew, 1-10. Marawila: Sri Lanka.http://www.academicjournals.org /JYFR. DOI: 10.5897/JYFR12.006

3. Masawe P.A.L. (1994). Aspect of breeding and selecting improved cashew genotypes (*Anacardium occidentale* L.). PhD Thesis, University of Reading, Department of Agricultural Botany.

4. Topper C. and Boma F. (1997). Evaluation of fungicides for the control of powdery on cashew. A paper submitted to registrar of pesticides TPRI, P O Box 3024, Arusha, Tanzania.

5. Masawe P.A.L. (2006). Tanzanian Cashew Cultivars Selected Clones. Cashew Research Programme, Naliendele Agricultural Research Institute, Mtwara, Tanzania. ISBN 9987-446-01-9

6. Dominic M., Makobe M., Agboton B., Shomari S. and Tiedemann A. (2014). Biology and infection mechanisms of Cryptosporiopsis spp. fungus causing blight disease on cashew (Anacardium Occidentale L.). *J. Plant Sci*, 2, 266-275. DOI: 10.11648/j.jps.20140206.12

7. Sobhana A., Matthew J. and Mini C. (2010). Utilisation of cashew apple in the food industry in India. *Proceedings of the Second International Cashew Conference*, Kampala, Uganda, 26-29 April 2010, 150-156.

8. Akinwale T.O. (2000). Cashew apple juice: Its use in fortifying the nutritional quality of some tropical fruits. *Eur. Food Res. Technol*., 211(3), 205-207.

9. Opeke L.K. (2005). Tropical Commodity Tree Crops. Spectrum Books Limited, Ibadan, Nigeria. 360, 371-373.

10. Kasuga L.J. (2013). Status of the cashew nut industry in Tanzania. In Proceedings of the Second Cashew International Conference, Kampala, Uganda, 26-29 April 2010. CAB International, Wallingford, UK. ISBN 13: 988-9987-446-05-6

11. CBT. (2015). Cashewnut Board of Tanzania procurement records 2015/16 season.

12. Anonymous (2017). Annual Cashew Research Report, for 2017/2018. *Ministry of Agriculture, Livestock and Fisheries*, Dodoma, Tanzania.

13. Topper C.P., Boma F. and Mhando H. (1997). Evaluation of fungicides for the control of powdery mildew (*Oidium anacardii* Noack) on cashew in Tanzania. B. On-farm testing of fungicide control strategies. In Topper C.P., Calig-ari P.D.S., Kullaya A.K., Shomari S.H., Kasuga L.J., Masawe P.A.L. and Mpunami A.A., *Proceedings of the International Cashew and Coconut Conference*, Dar es Salaam, Tanzania, 17-21 February 1997, 270-276.

14. Ellias F. (1980). A preliminary analysis of the decline in cashew nut production, 1974-1979: Causes, possible remedies and lessons for rural development policy. *Economic Research Bureau*, University of Dar es Salaam, 79.

15. Brown L.C., Minja E. and Hamad A.S. (1984). Cashew production in East Africa. Paper presented at CABI's First Scientific Conference on *Advancing Agricultural Production in Africa*, Arusha, Tanzania, 12-18.

16. Castellani E. and Casulli F. (1981). Osservazioni Preliminari su Oidium Anacardii Noack agente del mal bianco dell'anacardio. *Rivista di Agricoltura Subtropicale e Tropicale*, 75(2-3), 211-222.

17. Intini M. (1987). Phytopathological aspects of cashew (*Anacardiu moccidentale* L.) in Tanzania. *International Journal of Tropical Plant Diseases*, 5, 115-130.

18. Sijaona M.E.R. and Shomari S.H. (1987). The powdery mildew disease of cashew in Tanzania. TARO *Newsletter*, 2(3), 4-5.

19. Sijaona M.E.R. (1984). Investigation into the effectiveness of sulphur W.P. against *Oidium Anacardii* Noack on five-cashew tree types at Naliendele. *Rivista di Agricoltura Subtropicale e Tropicale,*78(2), 197-209.

20. Shomari S.H. (1988). A review of cashew research in Tanzania. Paper presented at the Tanzanian Agricultural Research Masterplan Conference, Arusha, 12-15 December 1988.

21. Sijaona M.E.R., Reeder R.H. and Waller J.M. (2005). Cashew leaf and nut blight – A new disease of cashew in Tanzania caused by *Cryptosporiopsis*spp. New Disease Reports, 12, 13. http:www.bspp.org.uk/ndr/jan2006/2005-75.asp

22. Sijaona M.E.R., Reeder R.H. and Waller J.M. (2006). Cashew leaf and nut blight – A new disease of cashew (*Anacardium occidentale* L.) in Tanzania caused by *Cryptosporiopsis*sp. (http://www.bsapp.org.uk/ndr/jan 2006 /2005-75.asp)

23. Menge D., Martha M., Bonaventure A., Shamte S. and Andreas T. (2013). Effect of environmental conditions on the growth of *Cryptosporiopsis* spp. causing leaf and nut blight on cashew (*Anacardium occidentale* L.). *Journal of Yeast and Fungal Research*, 4(2), 12-20.

24. Dominic M., Makobe M., Agboton B., Shomari S. and Tiedemann A. (2014). Biology and infection mechanisms of Cryptosporiopsis spp. fungus causing blight disease on cashew (Anacardium Occidentale L.). *J. Plant Sci*, 2, 266-275. DOI: 10.11648/j.jps.20140206.12

25. White T.J., Bruns T., Lee S.J.W.T. and Taylor J.L. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, 18(1), 315-322.

26. Tamura K. and Nei M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular biology and evolution*, 10(3), 512-526.

27. Kumar S., Stecher G. and Tamura K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, 33(7), 1870-1874.