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Full Length Research Paper

Rapid single-tube splice variants typing of the BF gene based on dual-primer RT-PCR amplification that influence resistance/susceptibility to Marek's disease in chicken

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A new effective splice variants typing based on multiplex allele-specific dual-primer RT-PCR assay was developed in a single tube for the rapid detection of the exon 7 splice variant of the BF gene. With 2 pairs of primers, one pair was used for amplifying cDNA fragments containing exon 7 of the BF gene, the other does not contain exon 7 of the BF gene. The templates were amplified in one tube and the type of splice variants was determined by the length of products to be extended and by analysis of nucleotide sequences of these BFs. Results obtained for all samples showed 100% accuracy compared to those obtained with a semi-nested PCR (snPCR) assay of 100% accuracy, but which need two round PCR assay. The dual-primer RT-PCR assay was more rapid and easy to operate than the snPCR assay.

Key words: BF gene (chicken MHC class I gene), Marek's disease (MD), splice variants, resistance, chickens.

INTRODUCTION

The Major Histocompatibility Complex (MHC) with particular traits across all jawed vertebrates (two glycoproteins of primary sorts binding peptides) came from antigens of intracellular or extracellular to the present circulating T-cells and have an integral effect on immune systems of innate and adaptive (Kelly and Trowsdale, 2019). The chicken MHC on chromosome 16 have long been referred to as a gene region which makes remarkable contribution in genetic resistance to some epidemic diseases, consisting of two regions, the polymorphic MHC-Y region and the MHC-B region. Localized into these regions, BL (chicken MHC class II) and BF (chicken MHC class I) genes are involved in resistance against viral, bacterial and protozoal diseases not only in chicken (Dawkins and Lloyd, 2019; Kaufman, 2018; Miller and Taylor, 2016; Psifidi et al., 2016).

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Marek's disease (MD) is a malignant lymphoproliferative disease of poultry caused by the Marek's disease virus (MDV), which is ubiquitous around the world. MD is characterized by a mononuclear infiltration of one or more of the following: peripheral nerves, gonad, iris, various viscera, muscle and skin (Payne and Rennie, 1973).

BF gene consists of 8 exons and 7 introns. Encoding the second segment of the mature BF protein in the cytoplasmic part, exons 7 belongs to the sequence of splice variants. The polymorphisms at position of all exon 7 in the cDNA of the BF gene encoding the MHC class I of chicken generates two allotypes BF molecules (with or without the second segment of the mature BF protein in the cytoplasmic part). As one of them, alternative exon 7 splice variant of the BF gene has not been detected in MD-resistant haplotypes, but only in the MD-susceptible haplotypes so far (Dalgaard et al., 2005; Jin et al., 2010a, 2014), suggesting a subtle correlation between the alternative exon 7 splice variant and resistance**/** susceptibility to MD in chickens.

The objective of this research was to improve the BF haplotypes detection effectively of resistance**/** susceptibility to MD in chicken by a new rapid splice variant typing based on multiplex allele-specific dual-primer polymerase chain reaction for selective breeding against MD.

MATERIALS AND METHODS

All procedures in the present study were subject to approval by the Institutional Animal Care and Use Committee of Guangxi University (Permit No. QBS-L20130319) and carried out in accordance with the approved guidelines. All efforts were made to minimize the suffering of the animals. The movement of birds that have no homogenization of the population was not restricted before the age of 14 days. For isolation of bird primary hepatocytes, the bird at 14 days were killed with an electrolethaler before harvesting their liver samples.

Experimental cDNA fragments of BF haplotypes of resistance/susceptibility to MD sample preparation

The experimental cDNA fragments containing exon 7 and not containing exon 7 of the BF gene were, respectively from the 195-bp product (GenBank accession numbers: EU746446) of the D_{12} Xiayan homozygous chickens (resistant to MD) and the 162-bp product (GenBank accession numbers: EU746447) of the A_5 Xiayan homozygous chickens (susceptible to MD) (Jin et al., 2010a, b).

Dual-primer design of reverse transcription-polymerase chain reaction (RT-PCR) for detection of exon 7 splice variant of the BF gene

Two pairs of primers were designed by Jin for dual-primer RT-PCR amplification, one pair was used for amplifying cDNA fragments containing exon 7 of the BF gene, the other for not containing exon 7 of the BF gene: The forward primer (5′-TACAACATTGCGCCCGAC-3′) and the reverse primer (5′-GGAAGCAGAATGAGATGTGAGAGG-3′) of one pair were designed to amplify a 174 bp fragment containing exon 7 of BF gene. The forward primer (5′-TACAACATTGCGCCCGGG-3′) and the reverse primer (5′-GGAAGCAGAATGAGATGTGAGAGG-3′) of the other were designed to amplify a 141 bp fragment not containing exon 7 of BF gene.

Dual-primer RT-PCR detection for sensitivity

The sensitivity of the dual-primer RT-PCR assays was measured using serially diluted 1 µl cDNA mixture of the A_5 and D_{12} homozygous Xiayan chickens from, respectively 0.5 µl cDNA (100 µg/µl) samples containing exon 7 of the BF gene and 0.5 µl cDNA (100 µg/µl) samples not containing exon 7 of the BF gene. The dynamic range of the cDNA mixture consisted of 10-fold dilutions between 10^{-1}-10^{-6}.

Optimization for dual-primer RT-PCR annealing temperature

Dual-primer RT-PCR annealing temperature optimization was measured using 10 different levels of gradient of temperature rising from 52 to 62°C.

Final optimization for dual-primer RT-PCR

Each 50 µl reaction mixtures contained Golden Easy PCR Mix 25 µl, 0.2 mM of each primer, approximately 1 ng mixture of cDNA samples (from respectively 0.5 ng cDNA sample containing exon 7 of the BF gene and 0.5 ng cDNA sample not containing exon 7 of the BF gene) and ddH_2O to 50 µl. The amplification process consisted of a 5-min initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58.25°C for 30 s, elongation at 72°C for 30 s and a final elongation at 72°C for 5 min. The purified RT-PCR product was cloned into pMD18-T vector and transformed into DH5 alpha cells. Nucleotide sequences of the positive clones were obtained by automated sequence analysis. The analysis of nucleotide sequences alignment of these BFs with the BF of B^{21} and B^{19}, respectively containing and not containing exon 7 was carried out using DNAStar software (DNASTAR, Inc., Madison, WI, USA).

Clinical samples test

The cDNA of peripheral blood leucocytes (PBL) from the 300 avian neoplastic diseases clinic qualified as MDV1 positive clinical samples (Jin et al., 2010b, 2014) was prepared. All clinical cDNA was detected by dual-primer RT-PCR and snPCR (Jin et al., 2010a) assays, and the results were analyzed both by agarose gel electrophoresis and nucleotide sequences to confirm amplification of the predicted cDNA fragment.

RESULTS

Dual-primer RT-PCR detection for sensitivity

Dual-primer RT-PCR detection for sensitivity showed that a 174-bp product and a 141-bp product from all dilutions but 10^{-6} fold dilution of the cDNA mixtures of the Xiayan chickens homozygous D_{12} and A_5 were obtained (Figure 1). So the lowest detection threshold after the dual-primer RT-PCR assays was at 10^{-5} fold dilution of the cDNA mixtures.

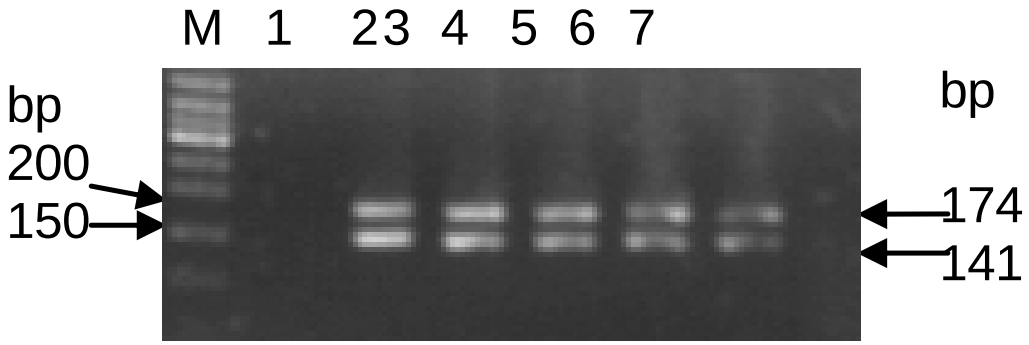

Figure 1. Dual-primer RT-PCR detection for sensitivity. M: 50 bp DNA Marker; Lane 1: negative control; 2-7: Dual-primer RT-PCR product (174 bp for BF gene containing exon 7 and 141 bp for BF gene no containing exon 7) of 10^{-1}-10^{-6} dilution of samples of cDNA mixtures.

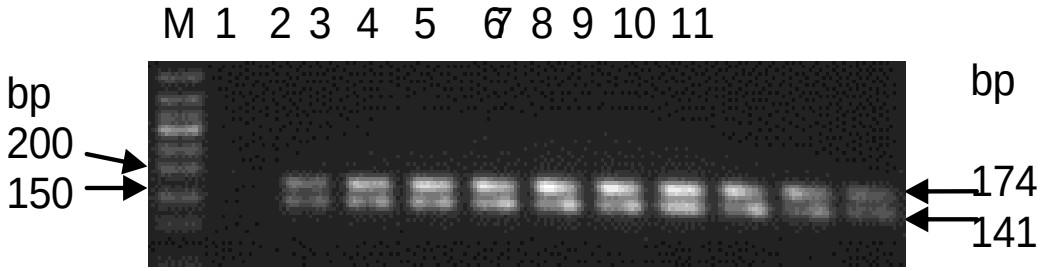

Figure 2. Optimization for dual-primer RT-PCR annealing temperature. M: 50 bp DNA Marker; Lane 1: negative control; 2-11: Dual-primer RT-PCR product (174 bp for BF gene containing exon 7 and 141 bp for BF gene no containing exon 7) of 10 different levels of gradient of temperature rising from 52 to 62°C of samples of cDNA mixtures.

Optimization for dual-primer RT-PCR annealing temperature

Dual-primer RT-PCR annealing temperature optimization measure showed that a 174-bp product and a 141-bp product from all 10 levels of gradient of temperature of the cDNA mixture of the Xiayan chickens homozygous D_{12} and A_5 were obtained, but the product that gradient of temperature is higher than 58.25°C was very faint (Figure 2). As a result, 58.25°C was the best annealing temperature.

Final optimized result for dual-primer RT-PCR assays

A 174-bp product and 141-bp product of the BF gene from samples of cDNA mixtures of the Xiayan chickens homozygous D_{12} and A_5 were obtained (Figure 3) in the final optimized result for dual-primer RT-PCR assays of BF genes. The amplified products of nucleotide sequence were confirmed by sequence alignment and analysis (data not shown).

Clinical samples test

In the sample of cDNA extracted from PBL of 300 Xiayan chickens for dual-primer RT-PCR assays, a 174-bp product and 141-bp product for dual-primer RT-PCR amplification of BF genes in 211 Xiayan chickens was obtained (Figure 4). It means that exon 7-deprived was present in those chickens. While only a 174-bp product for dual-primer RT-PCR assays of BF genes in 89 Xiayan chickens was obtained (Figure 5). It means that exon 7-deprived was not present in those chickens. The nucleotide sequence of the amplified products was confirmed by sequence alignment and analysis (data not shown).

DISCUSSION

In addition to the obvious importance to susceptibility or resistance of disease in chicken, study in the BF of chicken may be the gateway to novel insights about differential expression of the BF gene at the transcript

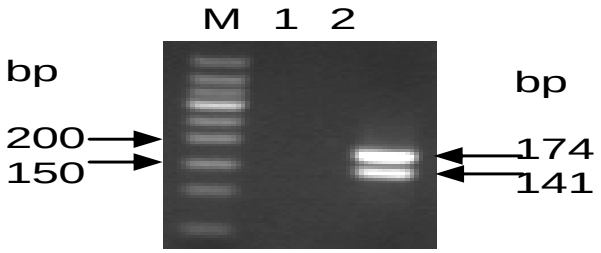

Figure 3. Final optimized results for dual-primer RT-PCR. M: 50 bp DNA Marker; Lane 1: negative control; 2: Dual-primer RT-PCR product (174 bp for BF gene containing exon 7 and 141 bp for BF gene no containing exon 7) of samples of cDNA mixtures.

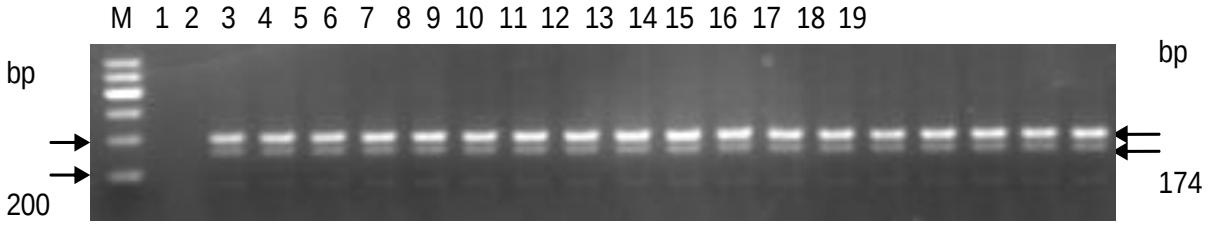

Figure 4. Some samples detection results by dual-primer RT-PCR. M: DNA Marker I; Lane 1: negative control; 2-19: Dual-primer RT-PCR product (174 bp for BF gene containing exon 7 and 141 bp for BF gene no containing exon 7) of some samples of cDNA extracted from PBL.

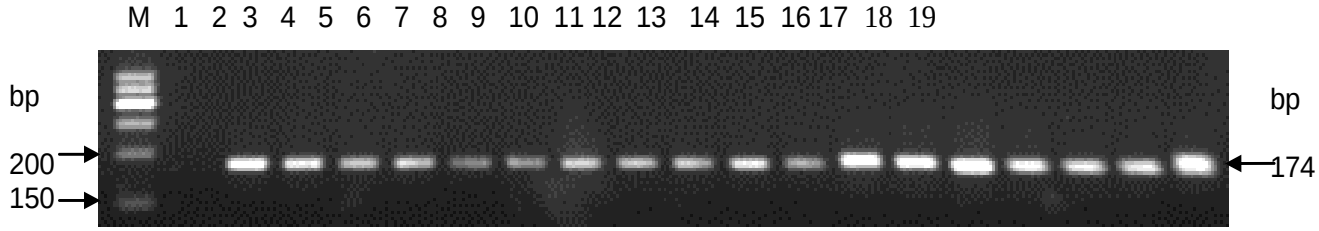

Figure 5. Some samples detection results by dual-primer RT-PCR. M: 50 bp DNA Marker; Lane 1: negative control; 2-19: Dual-primer RT-PCR product (174 bp for BF gene containing exon 7) of some samples of cDNA extracted from PBL.

level exercises a great influence on disease resistance to MD in chicken (Dalgaard et al., 2005; Jin et al., 2010a, 2014). One possible mechanism, MHC I (BF) proteins are impressionable to internalization after reaching the cell surface, then may recycle through acidic endosomes (Williams et al., 2002). The second segment of the mature BF proteins in the cytoplasmic part was of significant importance for endocytosis of HLA (Vega and Strominger, 1989), in contrast, HLA-G generating a truncated cytoplasmic tail (encoded by exon 7-deprived variant) is not internalized (Williams et al., 2002). Cyclic utilization of MHC I proteins may be the major source. Therefore, the presence of exon 7 of MHC I may be an essentially important role (Dalgaard et al., 2005).

There are already two PCR assay respectively by snPCR assay (Jin et al., 2014) and PCR assay (Dalgaard et al., 2005), which were implemented piecemeal on some chickens MHC haplotypes to detect exon 7 splice variant of the BF gene. On one hand, exon 7-deprived band was not present in B^{21} and the B^{21}-like haplotypes, the individual with which are MD resistant. On the other hand, the exon 7-deprived variant was produced in B^{19} and the B^{19}-like haplotypes, the individual with which are MD susceptible (Dalgaard et al., 2005; Jin et al., 2010a). Nevertheless, the electrophoretic bands of exon 7-deprived variant were extremely vague in B^{2}, B^{14}

and B^{15} by Dalgaard et al. (2005) as opposed to those of Jin et al. (2010a).

This is a new effective splice variants typing based on multiplex allele-specific dual-primer RT-PCR assay developed in a single tube for the rapid detection of the BF gene exon 7 splice variant. In this study, of all 300 Xiayan chickens, exon 7-deprived was present in 211 Xiayan chickens, whereas exon 7-deprived was not present in 89 Xiayan chickens by dual-primer RT-PCR assay. It is interesting to observe that we came to the same conclusion independently by snPCR assay but which need two round PCR assay (Jin et al., 2010a). Therefore, the detection of the BF gene alternative splicing of exon 7 was more effective by the dual-primer RT-PCR than by the snPCR assay. The method of the dual-primer RT-PCR assay rapid was specific and easy to operate to enhance the opportunity to pick up the MD resistant chickens.

Conclusion

The dual-primer RT-PCR assay was more rapid and easy to operate than the snPCR assay to detect the BF gene alternative splicing of exon 7.

AUTHORS CONTRIBUTION STATEMENT

Yuan-chang Jin and Yu-feng Li contributed equally to this work.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Dalgaard TS, Vitved L, Skjodt K, Thomsen B, Labouriau R, Jensen KH, Juul-Madsen HR (2005). Molecular characterization of major histocompatibility complex class I (B-F) mRNA variants from chickens differing in resistance to Marek's disease. Scandinavian Journal of Immunology 62(3):259-270.

Dawkins RLF, Lloyd SS (2019). MHC Genomics and Disease: Looking Back to Go Forward. Cells 8(9):944.

Jin YC, Huang L, Wei XX, Zhao ZY, Li Y, Wei P (2014). Relationship between alternative exon 7 splice variant of BF gene and MHC-related Marek's disease resistance in chickens. Scandinavian Journal of Immunology 80(5):323-326.

Jin YC, Wei P, Wei XX, Zhao ZY, Li Y (2010a). Rapid detection of BF haplotypes by a semi-nested polymerase chain reaction that causes to resistance/susceptibility to Marek's disease in chicken. Scandinavian Journal of Immunology 72(8):94-97.

Jin YC, Wei P, Wei XX, Zhao ZY, Li Y (2010b). Marek's disease resistant/susceptible MHC haplotypes in Xiayan chickens identified on the basis of BLB2 PCRRFLP and BLB2/BF2 sequence analyses. British Poultry Science 51(4):530-539.

Kaufman J (2018). Generalists and Specialists: A New View of How MHC Class I Molecules Fight Infectious Pathogens. Trends in Immunology 39(5):367-379.

Kelly A, Trowsdale J (2019). Genetics of antigen processing and presentation. Immunogenetics 71(3):161-170.

Miller MM, Taylor RL (2016). Brief review of the chicken Major Histocompatibility Complex: the genes, their distribution on chromosome 16, and their contributions to disease resistance. Poultry Science 95(2):375-392.

Psifidi A, Banos G, Matika O, Desta TT, Bettridge J, Hume DA, Dessie T, Christley R, Wigley P, Hanotte O, Kaiser P (2016). Genome-wide association studies of immune, disease and production traits in indigenous chicken ecotypes. Genetics Selection Evolution 48(1):74.

Payne LN, Rennie M (1973). Pathogenesis of Marek's disease in chicks with and without maternal antibody. Journal of the National Cancer Institute 51(5):1559-1573.

Vega MA, Strominger JL (1989). Constitutive endocytosis of HLA class I antigens requires a specific portion of the intracytoplasmic tail that shares structural features with other endocytosed molecules. Proceedings of the National Academy of Sciences of the United States of America 86(8):2688-2692.

Williams A, Peh CA, Elliott T (2002). The cell biology of MHC class I antigen presentation. Tissue Antigens 59(1):3-17.

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Molecular and morphological identification of fungi causing canker and dieback diseases on *Vangueria infausta* (Burch) *subsp. rotundata* (Robyns) and *Berchemia discolor* (Klotzsch) Hemsl in lower Eastern Kenya

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Drought-tolerant multipurpose fruit trees *Vangueria infausta* (Burch) subsp. *rotundata* (Robyns) and *Berchemia discolor* (Klotzsch) Hemsl are native to Kenya. These fruit tree species are suitable for dryland agroforestry and support local communities with food, medicine, fodder and other necessities. Reports by the local communities indicate that the two species suffer from diebacks and cankers. The aim of this study was to identify the fungi associated with *V. rotundata* and *B. discolor* and determine the cause of diebacks and cankers symptoms observed. Samples were collected from two sites (Tiva and Ikanga) in Kitui County and one site (Mkange) in Makueni County. Fungal isolations were carried out by incubating the samples on malt extract agar media supplemented with Streptomycin Sulphate. Morphological identification grouped the fungal isolates into 7 clusters. *Botryosphaeriaceae* and *Nectriaceae* had the highest frequency of occurrence (32.7 and 30.5%) respectively. DNA was extracted from pure fungal cultures, amplified and sequenced. Phylogenetic analysis of DNA sequences clustered the fungal isolates into seven families; *Botryosphaeriaceae, Sporocadaceae, Nectriaceae, Trichosphaeriaceae, Pleosporaceae, Diaporthaceae* and *Glomerellaceae.* Using Koch's postulates, this study showed that isolates of *Botryosphaeriaceae* within the genera *Lasiodiplodia, Alanphillipsia* and *Dothiorella* are pathogenic to *B. discolor* and other indigenous agroforestry species due to their ability to cause similar symptoms to those observed in the field. This is the first study to investigate the fungal flora linked to *V. rotundata* and *B. discolor* dieback and canker diseases.

Key words: *Vangueria rotundata*, *Berchemia discolor*, *Botryosphaeriaceae, Nectriaceae* canker, dieback, DNA and ITS primers.

INTRODUCTION

Vangueria rotundata and *Berchemia discolor* are drought tolerant multipurpose trees with potential of providing medicine, food and other commodities to drylands communities of Kenya. They are candidates for dry land agroforestry due to their ability to withstand a wide range of temperature and rainfall regimes. *Berchemia discolor* can withstand a temperature range between 14-30°C and an annual rainfall of between 200-1400 mm. On the other hand, *V. rotundata* can withstand temperatures between 12-36°C and an annual rainfall of between 700-1500 mm (Maundu 1999). The two fruit trees are of great importance during the famine and crop failure as their fruits provide a wide range of nutrients such as carbohydrates, vitamins and proteins for people residing in the arid and semi-arid areas (Feyssa et al., 2012; Eulalia et al, 2015). Cheikhyoussef et al. (2010), Maroyi (2018) and Ramavhale et al. (2018) pointed out the medicinal and nutritional value of the two species as well as their importance as food, feed and source for construction material. However, local communities have reported decreased fruit production and tree death on trees with diebacks and cankers. Moreover, Njuguna et al. (2011) had previously reported that canker and dieback were threatening the cultivation of *Grevillea robusta* in the arid and semi-arid areas.

The World Agroforestry Centre (ICRAF), in conjunction with the Kenya Forestry Research Institute (KEFRI), have initiated domestication of wild fruit trees over the years. Tree Genebanks have been established in order to promote the conservation of key indigenous tree species such as *V. rotundata* and *B. discolor* across the country (Muok et al, 2000). These Genebanks also provide healthy germplasm (Kitonga et al., 2020) for utilization in breeding programs so that high-quality tree varieties with desired traits such as drought tolerance and disease resistance can be developed for improved productivity. Moreover, domestication enhances ecosystem sustainability, improved livelihood, nutrition security and poverty reduction. (Jamnadass et al., 2019; Miller et al., 2020). However, domestication and cultivation of indigenous fruit trees face many challenges, including diseases and pests, overexploitation, low acceptance and insufficient research on their growing (Gachie et al., 2020; Omotayo and Aremu, 2020). Plant diseases play a crucial role in agriculture, horticulture and forest ecosystems and have become a worldwide concern on food security and climate change (Agrios, 2005). Diseases of plants are caused by a wide range of biotic and abiotic factors (Nazarov et al., 2020), however, disease will only manifest if the host is in an intimate relationship with a virulent pathogen and in favourable environmental conditions (Agrios, 2005). Diseases affect the productivity and vigor of the trees leading to reduction in their health, quality and quantity of tree production and causing losses that may amount to billions of US dollars (Jeger et al., 2021; Thambugala et al., 2020). However, diseases caused by biotic factors may overlap with those caused by abiotic factors (Pernezny et al., 2008) and it is therefore essential to correctly determine the actual cause based on an appropriate observation of signs and symptoms present in the field and to finally carry out laboratory isolations and diagnostics.

Major biotic factors that cause plant diseases include fungi and fungal like organisms, bacteria, viruses, nematodes and parasitic higher plants. However, fungi have been described as the most dominant causal agents of plant diseases globally (Hariharan and Prasannath, 2021). Fungal pathogens are ecologically, morphologically and genetically diverse, thus making their identification to species level quit challenging (Lücking et al., 2020; Raja et al*.,* 2021; Tekpinar and 2019) Monitoring the health of plants and diagnosing diseases of plants is crucial in controlling diseases (Nalla and Kalmer, 2020).

Fungi are usually identified both morphologically and by the use of molecular techniques. Although morphological identification is important, it can sometimes be problematic, especially for untrained mycologists and closely related genera (Raja et al*.,* 2017). The use of molecular techniques with morphological traits offers a better fungal identification (Bernreiter, 2017, Dayarathne et al., 2020). Moreover, molecular identification is a standardized method that is fast and accurate to species-level identification based on gene phylogenies (Das et al., 2015; Dulla et al., 2016). Subsequently, the ITS operon has been identified by many studies as a potential primary DNA barcode marker for most fungi due to its ease in amplification and availability of a large number of Genbank sequences (Schoch et al., 2012; Badotti et al., 2017). This study employed morphological methods to cluster fungal pathogens associated with *V. infausta subsp. rotundata* and *B. discolor* into fungal families and used molecular phylogenetics to identify species within the families.

MATERIALS AND METHODS

Study area

This study was carried out in Kitui County at the Tiva ICRAF field gene bank site (1.506° S: 38.011° E) where a selection of indigenous fruit tree species had been established in 1990 and also

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at two farmer's fields in Ikanga (2.356° S: 39.172° E) and Mkange (2.697° S: 38.817° E) within Kitui and Makueni counties respectively. The sites are located in semi-arid zones and receive a mean annual rainfall being less than 500 mm. Mean annual temperatures range between a minimum of 17°C and a maximum of 31°C. The study sites experience a bimodal type of rainfall with long rains in March to May, whereas the short rains fall between October and December. Rainfall in these areas is low, unreliable and poorly distributed. According to Jaetzold et al. (2012), these areas are mainly characterized by luvisols which are fertile soils with high cation exchange capacities and high base saturation. The major economic activities in such areas include subsistence farming, livestock rearing, and apiculture. Over the past decades, the study areas have been experiencing frequent droughts, which affect the availability of water, pasture and food for humans (Ngaina et al., 2014).

Sample collection

The general symptoms in the field in the Tiva, Ikanga and Kibwezi were characterized by dieback, canker, for both *V. rotundata* and *B. discolor* trees. Dieback symptoms were characterized by dead branches and twigs that began from the tip and progressed downwards. On the other hand, canker was characterized by cracks, dead and sunken areas on either stems or branches and when the bark was removed, the area appeared discolored (Bush, 2018). Tissue samples were collected from bark, branches, and leaves in a zigzag pattern, with a zigzag transect covering the entire farm or plantation and multiple locations placed along the transect. Samples were then collected from the front trees that were closest to the points. This was repeated for all the sites. There were a total of 71 trees that were diseased and 30 healthy trees were sampled. To conserve moisture, samples were placed in separate paper bags and enclosed in larger plastic bags until isolation was completed within 24 h. Samples that were not processed right away were maintained in a cool, dry environment or in a refrigerator at 4°C. To prevent reinfection by other pathogens, the cut piece of the tree was treated with a broad-spectrum fungicide (Bavistin) after each sampling. After each sampling, the sampling equipments were disinfected with 70% ethanol.

Tissue preparation

Leaves, branches and parts of stems showing symptoms of the disease and from healthy trees were collected and separately placed in khaki bags, sealed in larger plastic bags to retain moisture until isolation was done within 24 h. Samples were transported to KEFRI laboratories, where following standard laboratory techniques, small pieces were cut from the disease edges of the trees showing cankers on branches and stems and symptoms of dieback on shoots and branches. Samples from woody tissues and the inner bark of healthy trees were also used for fungal isolation using a modified procotol by Njuguna et al. (2011). The pieces were surface sterilized by immersing them for 1 min in 70% ethanol; they were then immersed in 33% hydrogen peroxide and rinsed three times in sterile distilled water for about 1 min for every rinse. They were then blotted dry using sterile filters paper in aseptic conditions. An antibiotic, Streptomycin sulphate (Duchefa Biochemie), was incorporated in the media to inhibit bacterial contamination. Samples from leaves, branches and bark were cut into tiny pieces and placed into plates containing MEA media in a laminar flow hood and incubated at 25ºC for fungal growth. Subculturing was done onto fresh media to obtain pure cultures. Isolated fungi were grouped based on the texture of the mycelia and the color of the colony (Jacobs and Rehner, 1998).

Morphological identification

The emergence of a young fungal colony was noted and given different numbers then isolated onto fresh MEA media. Fungal isolates were grouped based on mycelia texture and colony color. The isolates were purified through single hyphal tip isolations as described by Brown (1924). Spores in aniline blue were placed on microscope slides and examined using an Olympus SZ61 stereomicroscope to identify and group the fungi using protocols described by Jacobs and Rehner (1998) and Slippers et al. (2004). Isolation of single hyphae for DNA analysis was done according to the method described by Machua et al. (2016).

Molecular identification

DNA extraction, amplification and sequencing

Fungal isolates were sub-cultured on MEA media for 48 h at 25ºC. As detailed by Machua et al. (2016), mycelium was scraped from actively developing cultures with a sterilized surgical blade, placed into 2-ml Eppendorf tubes, freeze-dried, and ground to a powder using a Retsch Mixer MM 301. DNA was extracted using the CTAB (CetylTrimethylAmmonium Bromide) method described by Gardes and Bruns (1993). The variable internal transcribed spacer regions (ITS1, ITS2), including the complete 5.8S gene of the nuclear rDNA, were amplified using ITS1 (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) fungal primers. The ITS1 and ITS4 sequences were given as 5'-CTTGGTCATTTAGAGGAAGTAA-3' and 5'-TCCTCCGCTTATTGATATGC-3' respectively. The PCR amplicons were purified and sequenced in both forward and reverse (Inqaba biotec^{TM} SA). The sequences were modified in Bioedit 7.2 (Biological Sequence Alignment Editor) and Blast searches in GenBank [National Centre for Biotechnology Information (NCBI), of the National Institute of Health Bethesda (http://www.ncbi.nlm.nih.gov/BLAST), USA]. The sequences were then compared to sequences from closely similar species that had previously been published. Sequences with a similarity of 98-100% were chosen for further alignment using MAFFT version 7 online (https://mafft.cbrc.jp/alignment/software/). The model parameters were utilized to create a phylogenetic tree using the Maximum likelihood technique in MEGA X after the MAFFT alignment was subjected to a nucleotide substitution model test.

Phylogenetic analysis by Maximum Likelihood method

With 1000 bootstrap support, the phylogeny of the fungal isolates was inferred using the Maximum Likelihood technique and the Tamura-Nei model (Tamura and Nei., 1993). The highest log-likelihood tree (-4400.73) is shown. Next to the branches is the proportion of tree(s) in which the associated taxa clustered together. The initial tree(s) for the heuristic search were generated automatically by applying the Neighbor-Join and BioNJ algorithms on a matrix of pairwise distances calculated using the Tamura-Nei model and selecting the topology with the highest log-likelihood value. To describe evolutionary rate variations between sites (2 categories (+G, parameter = 0.3796), a discrete Gamma distribution was utilized. The branch lengths are measured in the number of substitutions per site, and the tree is depicted to scale. There were 47 nucleotide sequences in this study. In the end, there were 608 positions in the dataset. MEGA X was used to undertake evolutionary analysis (Molecular Evolutionary Genetics Analysis, Kumar et al., 2018).

Pathogenicity of the three pathogenic fungal families

Isolate pathogenicity was assessed using isolates from known

(a) (b)

Figure 1. Symptoms of canker and dieback (a) Stem canker on *B. discolor* (b) Severe shoot dieback on *V. rotundata.*

pathogenic fungal families *Botryosphaeriaceae* (3 species) and *Diaporthaceae* (1 species) inoculated on four indigenous agroforestry tree species; *B. discolor, Croton. megalocarpus*, *Tamarindus indica* and *Olea europaea.* A negative control (a sterile MEA plug) was included as a fungal treatment. *T. indica* and *C. megalocarpus are indigenous trees found in the drylands and* usually intercropped with *V. rotundata* and *B. discolor*. A highlands agroforestry tree species *O. europaea* was included in the pathogenicity test to examine the promiscuity of the test fungal pathogens. *V. rotundata* was not included in the pathogenicity test for lack of seeds or seedlings at the study time. The pathogenicity test was conducted using a method described by Njuguna et al. (2011) by inoculating healthy seedlings of approximately 30-35 cm in height and with a root collar diameter of approximately 8-10 cm. A total of 320 seedlings were used for the pathogenicity test (4 tree species × 4 fungal treatments × 20 seedlings per treatment). Inoculated and control treatments were arranged in 4 replicate blocks using a complete randomized block design containing 5 seedlings of each fungal treatment per block. The isolates were grown in MEA at 25°C for 5 days before inoculation. The stems were injured using a sterilized scalpel blade, and mycelia plugs of approximately 6 mm in diameter were placed on the wound and wrapped using Parafilm tape. Another set of seedlings were inoculated with sterile MEA plugs of 6 mm diameter as controls. All the inoculated seedlings were placed in a glasshouse and watered in the mornings and evenings. The seedlings were observed daily for 5 months and any disease symptoms were recorded. Fungal pathogens from the infected seedlings were re-isolated using the same procedure and the isolates used for molecular identification.

Statistical analysis

Data on the internal lesion sizes were transformed by square root prior to analysis. Data were analyzed using Minitab Version 10 (Minitab Inc 2010). Descriptive statistics were used to test the normality of the data set before the analysis of variance. The data were found to have a normal distribution. Significance differences in the level of virulence of the fungal isolates determined were inferred by one-way analysis of variance (ANOVA).

RESULTS

Disease symptoms in the field

The general symptoms observed in Tiva, Ikanga and Kibwezi were characterized by dieback and canker. It was noted that scattered branches of *V. rotundata* were also dying from the tip suggestive of dieback (Figure 1b). Canker was however easily identified in both *V. rotundata* and *B. discolor* trees on the stem and branches with visible discoloration on the affected areas but no resin flow (Figure 1a). In all the trees that were assessed, 54% of *V. rotundata* and 18% *B. discolor* trees had dieback and canker infections (Table 1).

Fungi associated with *B. discolor* and *V. rotundata* and their characterization

According to morphological characteristics, 7 fungal groups were identified through observation of the colony color, namely: *Botryosphaeriaceae*, *Sporocadaceae*, *Glomerellaceae, Diaporthaceae, Pleosporaceae, Trichosphaeriaceae* and *Nectriaceae* (Table 1). The most dominant fungi isolated were *Botryosphaereacea* followed by *Nectriaceae*. Since the same fungi that were isolated from dieback symptoms were also isolated from canker, there was a high probability of a connection between the two symptoms.

Molecular identification

Phylogenetic and sequence analysis of the ITS rDNA data grouped the fungal isolates into 7 families; *Botryosphaeriaceae, Diaporthaceae, Glomerellaceae, Nectriaceae, Pleosporacea, Sporocadaceae* and *Trichosphaeriaceae* (Figure 2).

Botryosphaeriaceae isolates clustered into three genera; *Diothiorella, Alanphillipsia* and *Lasiodiplodia.* Isolate MW940855 was identified as *Lasiodiplodia lignicola,* while isolate MW931778 was identified as *Alanphillipsia aloegena*. Although isolates OK036579 and OK036778 were identified with the genus *Diothiorella,* the two had strong bootstrap support for a separate species Isolate MW931851 had a very strong bootstrap suppor

Table 1. Fungal frequency Mean ± SE.

Variable	Family	Mean ± SE	Perecnt
Frequency	*Botryosphaeriaceae*	606.50 ± 6.50	32.7
	Nectriaceae	567.0 ± 17.0	30.5
	Sporocadaceae	338.0 ± 35.0	18.2
	Pleosporaceae	310.5 ± 11.5	16.7
	Glomerellaceae	19.00 ± 1.00	1.0
	Trichosphaeriaceae	10.00 ± 1.00	0.5
	Diaporthaceae	6.50 ± 1.50	0.3
Total		1857.50	100

(100) within *Diaportheceae* and was identified as *Diaporthe ganjae*. Isolate MW931878 was identified as *Colletotrichum gloeosporioides* within the *Glomerellaceae* (100 bootstrap support).

The *Nectriaceae* family formed a polyphyletic clade with *Fusarium chlamydosporum* and *Fusarium lateritium* in one group and *Fusarium proliferatum* and *Fusarium equiseti* in another group. Isolate MW931873 was identified as F. chlamydosporum (95 bootstrap support), while isolate OK036782 was identified as *F. lateritium* (99 bootstrap support). Under the *F. proliferatum* and *F. equiseti* group, isolate OK036780 was identified as *F. proliferatum* (99 bootstrap support) while isolates OK036779, OK036582, OK036583 and OK036781 were identified as *F. equiseti* (99 bootstrap support.

Pleosporaceae isolates MW931855 was identified as *Culvularia pseudoclavata* (100 bootstrap support) within the family, while MW931858 was identified as *Culvularia pseudointermedia* (92 bootstrap support). Isolates OK036580 and OK036581 were identified as *Alternaria species* (99 bootstrap support) since the ITS phylogeny could not singularly distinguish between *Alternaria alternata* and *Alternaria tenuissima* within the same clade.

Within the *Sporocadaceae*, isolates OK036684, OK036685, OK036682, OK036683, OK036593 and OK036594 were identified as belonging to the genus *Neopestalotiopsis*. However, the ITS phylogeny did not have strong bootstrap support to identify the isolates to species level within the *Neopestalotiopsis*.

The *Trichosphaeriaceae* group had two isolates OK036783 and OK036588 which were identified as *Nigrospora sphaerica* (100 bootstrap support) and *Nigrospora oryzae* (73 bootstrap support), respectively. All the identified isolates, their host species and sites of origin are presented in Table 2.

Pathogenicity test

Results showed different levels of susceptibility to the fungi. Internal lesions were defined by inner tissue discoloration and decay. The internal lesion size differed significantly ($P \leq 0.05$) among the host species and between the host species and the control species. The lesion length caused by *Lasiodiplodia lignicola* was significantly ($P \leq 0.05$) longer in all the host species. This trend was followed by *Alanphillipsia aloegena, Dothiorella* sp and *Diaporthe ganjae* was least virulent.

Croton megalocarpus and *T. indica* resulted in the longest internal lesions among the host species. *O. europaea* was the least susceptible ($P \leq 0.05$) to the test pathogens (Table 3).

DISCUSSION

This is the first report of identifying fungi causing canker and dieback on *V. infausta,* and *B. discolor* in Kenya using molecular and morphological techniques. Morphological features observed suggested that the isolates belonged to the 7 ascomycetous fungal families: *Botryosphaeriaceae, Nectriaceae, Sporocadaceae, Pleosporaceae, Glomerellaceae, Trichosphaeriaceae* and *Diaporthaceae.* However, morphological characteristics alone were insufficient for species identification of the isolates. The use of morphological features alone in fungal identification is challenging due to the limited number of phenotypic characters usually confusing within related taxa. This often leads to inaccurate identification of the fungal isolates especially below the genus level. This study incorporated the use ITS phylogeny for molecular identification since the method has been largely accepted and used as a barcode marker for identification of fungi to the species level (Kõljalg et al., 2013). The internal transcribed spacer (ITS) is found in the chromosome between the small subunit ribosomal RNA (rRNA) and large subunit rRNA genes, or in the polycistronic rRNA precursor transcript's corresponding transcribed region. Because of various advantageous qualities, such as the high degree of variation between closely related species, sequence comparison of the eukaryotic ITS regions is commonly employed in taxonomy and molecular phylogeny (Bußkamp et al., 2020). The spacer sequences are non-coding and hence have very low evolutionary pressure acting on them, thus

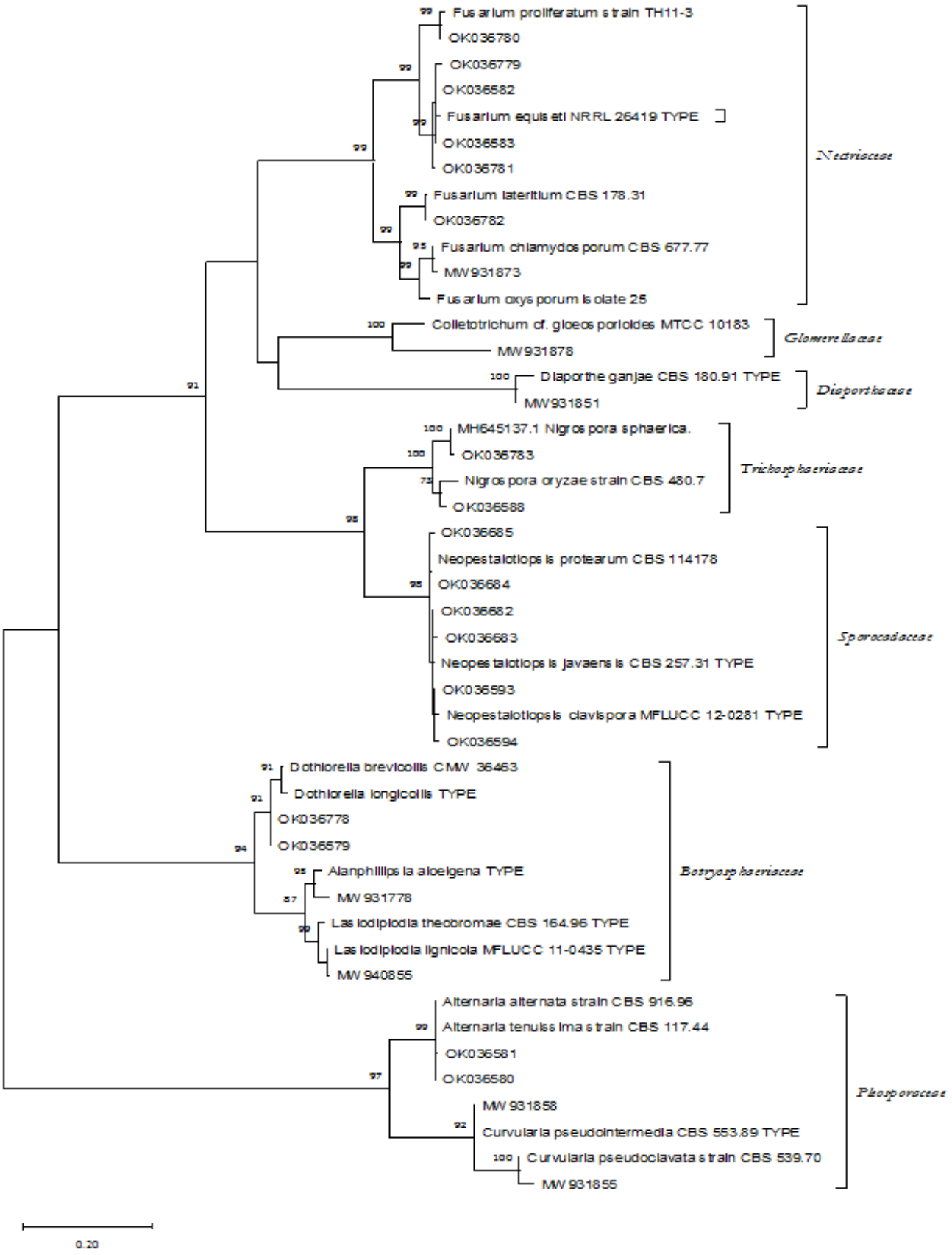

Figure 2. Molecular phylogenetic analysis by maximum likelihood method (bootstrap values less than 70 are not shown).

Table 2. Identified isolates and their host species.

Site	Host species	Isolate no.	Identification	Genbank acc. no.
Mukange	*Vangueria infausta* sub sp *rotundata*	VMT28	*Lasiodiplodia lignicola*	MW940855
Mukange	*Vangueria infausta* sub sp *rotundata*	VMT29	*Alanphillipsia aloeigena*	MW931778
Mukange	*Vangueria infausta* sub sp*. rotundata*	VML43	*Nigrospora* oryzae	OK036588
Mukange	*Vangueria infausta* sub sp*. rotundata*	VMB88	*Fusarium equiseti*	OK036583
Mukange	*Vangueria infausta* sub sp. *rotundata*	VMB11	*Neopestalotiopsis* sp	OK036594
Mukange	*Berchemia discolor*	BMB52	*Alternaria* sp	OK036580
Mukange	*Berchemia discolor*	BML53	*Curvularia pseudoclavata*	MW931855
Mukange	*Berchemia discolor*	BMT26	*Neopestalotiopsis* sp	OK036684
Mukange	*Berchemia discolor*	BMT25	*Neopestalotiopsis* sp	OK036683
Mukange	*Berchemia discolor*	BML27	*Neopestalotiopsis* sp	OK036685
Tiva	*Berchemia discolor*	BTB100	*Dothiorella* sp	OK036579
Tiva	*Berchemia discolor*	BTL82	*Diaporthe ganjae*	MW931851
Tiva	*Berchemia discolor*	BTB57	*Colletotrichum gloeosporioides*	MW931878
Tiva	*Berchemia Discolor*	BTL57	*Fusarium equiseti*	OK036582
Tiva	*Berchemia Discolor*	BTT58	*Fusarium chlamydosporum*	MW931873
Tiva	*Berchemia Discolor*	BTL33	*Nigrospora sphaerica*	OK036783
Tiva	*Vangueria infausta* sub sp. *rotundata*	BTL60	*Fusarium equiseti*	OK036779
Tiva	*Vangueria infausta sub* sp*. rotundata*	VTB66	*Fusarium lateritium*	OK036782
Tiva	*Vangueria infausta* sub sp. *rotundata*	VTT7	*Neopestalotiopsis* sp	OK036682
Ikanga	*Vangueria infausta* sub sp. *rotundata*	VIT30	*Dothiorella* sp	OK036778
Ikanga	*Vangueria infausta* sub sp. *rotundata*	VIL54	*Curvularia pseudointermedia*	MW931858
Ikanga	*Vangueria infausta* sub sp*. rotundata*	VIB70	*Fusarium equiseti*	OK036781
Ikanga	*Vangueria infausta* sub sp*. rotundata*	VIB78	*Fusarium proliferatum*	OK036780
Ikanga	*Berchemia discolor*	BIL18	*Neopestalotiopsis* sp	OK036593

Table 3. Mean internal lesion length (cm) caused by the *Botryosphariaceae* isolates.

Fungal species	Mean internal lesion length (Mean±SE)			
	C. megalocarpus	***T. indica***	***B.discolor***	***O.europaea***
Lasiodiplodia lignicola	5.39 ± 1.24^{aA}	4.773 ± 0.81^{abA}	2.260 ± 0.30^{bA}	2.01 ± 0.26^{bA}
Alanphillipsia aloegena	4.41 ± 0.71^{aAB}	3.020 ± 0.52^{abAB}	2.19 ± 0.14^{bcA}	0.95 ± 0.37^{bB}
Dothiorella sp	2.93 ± 0.47^{aA}	2.55 ± 0.38^{aBC}	1.89 ± 0.21^{abAB}	1.07 ± 0.07^{cB}
Diaporthe ganjae	1.92 ± 0.34^{aBC}	2.03 ± 0.33^{aBC}	1.82 ± 0.23^{aAB}	0.59 ± 0.11^{bB}
Control	1.37 ± 0.27^{abC}	1.040 ± 0.09^{bC}	1.11 ± 0.19^{bB}	0.83 ± 0.15bB

Means with lowercase superscripts represent significance differences among the tree species (across) while uppercase superscripts represent significance differences between the fungal species (vertically). Means with the same superscript were not significant.

helping to maintain evolutionary diversity (Baldwin et al., 1995). However, it was impossible to identify some of the fungal isolates from this study to the species level as the ITS region alone may not always provide adequate variation for fungal differentiation within a genus (Raja et al., 2017).

Botryosphaeriaceae family was most frequent on all the sites, and apparently, its isolates were the most pathogenic to the hosts tested. This family consists of saprobes, endophytes and plant pathogens (Dissanayake et al., 2016). Moreover, as endophytes, they can remain latent for an indefinitely and become pathogenic when the host suffers from physiological stress resulting in large cankers on trunks and branches of the affected trees (Moricca et al., 2010). In addition, *Botryosphaeriaceae* species have been known as the principal cause of canker and dieback in woody plants (Burgess et al., 2019).

The ability of the *Botryosphaeriaceae* isolated in this study to cause dieback and canker symptoms observed

Table 4. Description of the reference isolates used in this study.

Species	Culture no.	Genbank acc. No.	Host	Origin	Collector
Lasiodiplodia lignicola	MFLUCC 11-0435	MFLUCC 11-0435	Wood	Thailand	A.D Ariyawansa
Lasiodiplodia theobromae	CBS 164.96	NR_111174.1	Fruit along coral reef coast	Papua New Guinea	Phillips et al. (2005)
Alanphillipsia aloeigena	TYPE MATERIAL	NR_137121.1	*Aloe melanacantha*	South Africa	M.J. Wingfield
Dothiorella brevicollis	CMW 36463	NR_111703	*Acacia karroo*	South Africa	Jami F
Dothiorella longicollis	TYPE MATERIAL	NR_136999.1	*Lysiphyllum cunninghamii*	Australia	Pavlic et al. (2008)
Alternaria alternate	MH237955.1	CBS 916.96	Apple	China	Unknown
Alternaria tenuissima	CBS 117.44	MH856117.1	Unknown	Denmark	Vu, D
Curvularia pseudointermedia	CBS 188.61	MN688820.1	Decaying grass	Guadeloupe	Marin-Felix et al. (2020)
Curvularia pseudoclavata	CBS 539.70	MN688817.1	*Oryza sativa*	Denmark	S.B. Mathur
Diaporthe ganjae	CBS 180.91	NR_120259.1	*Cannabis sativa*	USA: Illinois	J.M. McPartland
Colletotrichum gloeosporioides	JF908919.1	MTCC 10183	Unknown	India	Mansoor Alam
Fusarium equiseti	CBS 307.94	NG_068575.1	*Gibberella intricans*	Germany	Vu et al. (2019)
Fusarium lateritium	CBS 178.31	MH855172.1	Unknown	unknown	Vu,D
Fusarium chlamydosporum	CBS 677.77	MH861111.1		Solomon Islands	Vu et al. (2019)
Nigrospora sphaerica	isolate COL8	MH645137.1	*Citrus reticulata*	Pakistan	Naeem, I
Nigrospora oryzae	CBS 480.7	MH860749.1	Unknown	Kazakhstan	Redet. W. Gams
Neopestalotiopsis javaensis	CBS 257.31	NR_145241.1	*Cocos nucifera*	Indonesia	R.L. Steyaert
Neopestalotiopsis clavispora	MFLUCC 12-0281	NR_111782.1	Dead plant material	China	Unknown
Neopestalotiopsis protearum	CBS 114178	LT853103.1	*Leucospermum cuneiforme*	Zimbabwe	Unknown

in the field in healthy seedlings of *B. discolor, C. megalocarpus*, *T. indica* and *O. europaea* and their re-isolation in culture media, confirmed them as the probable causal agent of symptoms that were observed in the field.

Occurrence of a pathogen with a wide range of host will pose a threat to *V. rotundata* and *B. discolor* and other agroforestry trees and crops growing in close proximity. Additionally, as Slippers and Winfield reported in 2007, the latent phase of the pathogenic fungi may sometimes be overlooked hence underestimating their ability to cause disease. The latent phase of *Botryosphaeriaceae* endophytes (Luo et al., 2019), makes them a significant threat to the two agroforestry fruit trees, farm and commercial forestry at large.

Conclusion

This is the first report of canker and dieback on *V. rotundata* and *B. discolor*. Although research on the fungi infecting these tree species is scanty, there is no doubt that these indigenous fruit trees are a host to several fungal species of economic importance. Of particular importance, it is clear that canker and dieback are widespread in the drylands of Eastern Kenya indicating that agroforestry in these regions is under serious threat if measures are not taken to mitigate the disease. Further studies should therefore be conducted on the pathogenic nature of these fungi on agricultural crops.

Morphological identification coupled with molecular phylogeny is important for accurate identification of fungal species in this study. However, Internal subscribed spacer (ITS) phylogeny alone may present some drawbacks for some fungal families and for species level identification of some isolates (Table 4). It is therefore, highly recommended that multiple gene phylogeny using genes such as elongation factor 1-α and β-tubulin be utilized for conclusive identification.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Agrios GN (2005). Plant pathology 5th Edition. Elsevier Academic Press pp. 79-103.

Badotti F, de Oliveira FS, Garcia CF, Vaz ABM, Fonseca PLC, Nahum LA, Góes-Neto, A (2017). Effectiveness of ITS and sub-regions as DNA barcode markers for the identification of Basidiomycota (Fungi). BMC Microbiology 17(1):1-12.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell

CS, Donoghue MJ (1995). The ITS Region of Nuclear Ribosomal DNA: A Valuable Source of Evidence on Angiosperm Phylogeny. Annals of the Missouri Botanical Garden 82(2):247-277.
Bernreiter A (2017). Molecular diagnostics to identify fungal plant pathogens–a review of current methods. ECUADOR ES CALIDAD-Revista Científica Ecuatoriana.
Brown W (1924). II. A method of isolating single strains of fungi by cutting out a hyphal tip. Annals of Botany 38(150):402-404.
Burgess TI, Tan YP, Garnas J, Edwards J, Scarlett KA, Shuttleworth LA, Jami F (2019). Current status of the *Botryosphaeriaceae* in Australia. Australasian Plant Pathology 48(1):35-44.
Bußkamp J, Langer GJ, Langer EJ (2020). Sphaeropsis sapinea and fungal endophyte diversity in twigs of Scots pine (Pinus sylvestris) in Germany. Mycological Progress 19(9):985-999.
Bush EA (2018). Botryosphaeria canker and dieback of trees and shrubs in the landscape.
Cheikhyoussef A, Naomab E, Potgieter S, Kahaka GK, Raidron C, MuAshekele H (2010). Phytochemical properties of a Namibian indigenous plant; Eembe (*Berchemia discolor*).
Dayarathne MC, Mridha AU, Wang Y (2020). Diagnosis of Fungal Plant Pathogens Using Conventional and Molecular Approaches. In Diagnostics of Plant Diseases. Intech Open.
Das S, Deb B (2015). DNA barcoding of fungi using ribosomal ITS marker for genetic diversity analysis: A review. International Journal of Pure and Applied Bioscience 3(3):160-167.
Dissanayake AJ, Phillips AJL, Li X H, Hyde KD (2016). Botryosphaeriaceae: Current status of genera and species. Mycosphere *7*(7):1001-1073.
Dulla EL, Kathera C, Gurijala HK, Mallakuntla TR, Srinivasan P, Prasad V, Jasti PK. (2016). Highlights of DNA Barcoding in identification of salient microorganisms like fungi. Journal de Mycologie Medicale 26(4):291-297.
Eulalia C (2015). Utilization of Wild Fruit in Mozambique – Drying of *Vangueria infausta* (African medlar). Food Technology, Engineering and Nutrition, Lund University.
Thambugala KM, Daranagama DA, Phillips AJ, Kannangara SD, Promputtha I (2020). Fungi vs. fungi in biocontrol: an overview of fungal antagonists applied against fungal plant pathogens. Frontiers in Cellular and Infection Microbiology 10:604923.
Hariharan G, Prasannath K (2021). Recent advances in molecular diagnostics of fungal plant pathogens: a mini review. Frontiers in Cellular and Infection Microbiology 10:829.
Feyssa DH, Njoka JT, Asfaw Z, Nyangito MM (2012). Nutritional Value of *Berchemia discolor*: A Potential to Food and Nutrition Security of Households. Journal of Biological Sciences 12(5):263-271.
Gachie P, Kipsat J, Cheboiwo J, Esitubi M, Mwaura J, Wairimu P, Gathogo M (2020). On-farm tree growing opportunities and constraints in Murang'a County, Kenya. East African Agricultural and Forestry Journal 84(1):53-65
Gardes M, Bruns TD (1993). ITS primers with enhanced specificity for Basidiomycetes: application to the identification of mycorrhizae and rusts. Molecular Ecology 2(2):113-118.
Jacobs KA, Rehner SA (1998). Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. Mycologia 90(4):601-610.
Jaetzold R, Hornetz B, Shisanya CA, Schmidt H (2012). Farm management handbook of Kenya Volume I-IV (Western Central Eastern Nyanza Southern Rift Valley Northern Rift Valley Coast). Nairobi: Government Printers.
Jamnadass R, Ofori DA, Dawson I.K, Tchoundjeu Z, McMullin S, Hendre PS, Theme T (2019). Enhancing agroforestry systems through tree domestication. Sustainable development through trees on farms: agroforestry in its fifth decade. Bogor, Indonesia: World Agroforestry (ICRAF) Southeast Asia Regional Program pp. 45-59.
Jeger M, Beresford R, Bock C, Brown N, Fox A, Newton A, Yuen J (2021). Global challenges facing plant pathology: multidisciplinary approaches to meet the food security and environmental challenges in the mid-twenty-first century. CABI Agriculture and Bioscience 2(1):1-18.
Kitonga K, Jamora N, Smale M, Muchugi A (2020). Use and benefits of tree germplasm from the World Agroforestry genebank for smallholder farmers in Kenya. Food Security 12:993-1003.
Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AF, Bahram M, Larsson KH (2013). Towards a unified paradigm for sequence based identification of fungi. Molecular Ecology 22(21):5271-5277.
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.
Luo Y, Lichtemberg PS, Niederholzer FJ, Lightle DM, Felts DG, Michailides TJ (2019). Understanding the process of latent infection of canker-causing pathogens in stone fruit and nut crops in California. Plant Disease 103(9):2374-2384.
Machua J, Jimu L, Njuguna J, Wingfield MJ, Mwenje E, Roux J (2016). First report of *Teratosphaeria gauchensis* causing stem canker of *Eucalyptus* in Kenya. Forest Pathology 46(2):168-170.
Maundu MP (1999). Traditional food plants of Kenya. National Museum of Kenya.
Maroyi A (2018). Nutraceutical and ethnopharmacological properties of Vangueria infausta subsp. infausta. Molecules 23(5):1089
Miller DC, Muñoz-Mora JC, Rasmussen LV, Zezza A (2020). Do trees on farms improve household wellbeing? Evidence from national panel data in Uganda. Frontiers in Forests and Global Change 3:101.
Minitab INC (2010). Minitab 16 statistical software. URL: [Computer software]. State College, PA: Minitab, Inc. (www. minitab. com).
Moricca S, Uccello A, Ragazzi A (2010). Endophytic mycocenoses in urban forests under environmental stress. Micologia Italiana 39(1):47-56.
Muok BO, Owuor B, Dawson I, Were J (2000). The potential of indigenous fruit trees: results of a survey in Kitui District, Kenya. Agroforestry Today 12(1):13-16.
Nalla K, Pothabathula SV, Kumar S (2020). Applications of Computational Methods in Plant Pathology. In Natural Remedies for Pest, Disease and Weed Control X Academic Press.
Slippers B, Crous PW, Denman S, Coutinho TA, Wingfield BD, Wingfield MJ (2004). Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. Mycologia 96(1):83-101.
Nazarov PA, Baleev DN, Ivanova MI, Sokolova LM, Karakozova MV (2020). Infectious plant diseases: Etiology, current status, problems and prospects in plant protection. Acta Naturae 12(3):46-59.
Ngaina JN, Mutua FM, Muthama NJ, Kirui JW, Sabiiti G, Mukhala E, Maingi NW, Mutai BK (2014). Drought monitoring in Kenya: A case of Tana River County.International Journal of Agricultural Science Research 3(7):126-135.
Njuguna JW, Barklund P, Ihrmark K, Stenlid J (2011). A canker and dieback disease is threatening the cultivation of Grevillea robusta on small-scale farms in Kenya. African Journal of Agricultural Research 6(3):748-756.
Lücking R, Aime MC, Robbertse B, Miller AN, Ariyawansa HA, Aoki T, Schoch CL (2020). Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding? IMA fungus 11(1):1-32.
Omotayo AO, Aremu AO (2020). Underutilized African indigenous fruit trees and food–nutrition security: Opportunities, challenges, and prospects. Food and Energy Security 9(3):e220.
Pernezny K, Elliott M, Palmateer A, Havranek N (2008). Guidelines for identification and management of plant disease problems: part II. Diagnosing plant diseases caused by fungi, bacteria and viruses. EDIS 2008(2).
Ramavhale RP (2018). Seasonal abundance and diversity of insects on *Sclerocarya birrea* and *Berchemia discolor* in Tshikundamalema, Limpopo Province, South Africa (Doctoral dissertation).
Raja HA, Miller AN, Pearce CJ, Oberlies NH (2017). Fungal identification using molecular tools: a primer for the natural products research community. Journal of Natural Products 80(3):756-770.
Raja HA, Oberlies NH, Stadler M (2021). Occasional comment: Fungal identification to species-level can be challenging. Phytochemistry 190:112855.
Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Fungal Barcoding Consortium. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences 109(16):6241-6246.
Slippers B, Crous PW, Denman S, Coutinho TA, Wingfield BD,

Wingfield MJ (2004). Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. Mycologia 96(1):83-101.
Slippers B, Winfield MJ (2007). *Botryosphaericeae* as endophytes and latent pathogens of woody plants: diversity, ecology and impact. Fungal Biology Reviews 21(2-3):90-106.
Tamura K, 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.
Tekpinar AD, Kalmer A (2019). Utility of various molecular markers in fungal identification and phylogeny. Nova Hedwigia 109(1-2):187-224.
White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal rRNA genes for phylogenetics. PCR protocols: a guide to methods and applications. Academic Press pp. 315-322.

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Isolation and characterization of multidrug resistance bacteria from hospital sewage samples, Maharashtra, India

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Multiple antibiotic resistance is a major cause of clinical infections worldwide. This study determined the pattern of multidrug bacterial resistance in hospital sewage samples from the Marathwada region of India. Forty-eight isolates of bacteria were obtained from 6 locations of Aurangabad. An antibiotic sensitivity test was carried out using the disc diffusion method. Among all the antibiotics tested, the highest level of resistance was observed in the beta lactam class (85%), followed by Tetracycline (58%), Cephalosporin (58%), quinolones (52%) and gentamycin (45%). *Escherichia coli* and *Klebsiella pneumoniae* are the most prevalent bacteria, showing antibiotic resistance to all tested antibiotics with a MAR index of 1. It is concluded that hospital sewage water could be a reservoir of antibiotic resistant bacteria, which may further contaminate drinking water bodies, potentially presenting a public health risk to the general populace.

Key words: Antibiotics, *Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas species, Staphylococcus aureus, Streptococcus pyogenes.*

INTRODUCTION

Antibiotics are compounds produced by micro-organisms and that are capable of inhibiting bacterial growth. Nowadays, the term antibiotic is broadly used for all those compounds that can be used against the bacterial infection. However, antibiotic resistance is increasing, and it is projected that by 2050, antibiotic resistant organisms will contribute to over 10 million deaths annually worldwide (De Kraker et al., 2016). This is mainly because of the slow pace of developing new antibiotics (De Kraker et al., 2011). The problem of antibiotic resistance has attracted the attention of World Health Organization (WHO) and several other stakeholders. The WHO announced in 2011 that antibiotic resistance is an urgent priority of research area (WHO, 2011) and several countries, including India (Government of India, 2017) accordingly framed their national health action plans for managing drug resistant bacteria (Smith et al., 2016).

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The problem of antibiotic resistance occurs due to the uncontrolled and excessive use of antibiotics by hospital and home care patients (Sidhu et al., 2016). This leads to the spread of resistant genes in water systems (Mukhopadhyay et al., 2012). For instance, Sidhu et al. (2016) analyzed and demonstrated the presence of *E. coli* in the drinking water from different schools in Northern India. Many multiple drug resistant bacteria like *Pseudomonas aeruginosa*, coliforms and *Enterococcus* species have been isolated from household water samples in Karnataka, India (Mukhopadhyay et al., 2012). Mulamattathil et al. (2014) documented the multiple antibiotic resistance profile in bacteria from sewage water samples (Mafikeng, South Africa).

In rural communities of developing countries like the India, untreated water from rivers, dams, and streams is directly used for drinking (Biyela et al., 2004). These water resources could possibly be contaminated with microbes from sewage water through rainfall runoff and other sources (Obi et al., 2002). Most of the areas are semi-arid with very low rainfall and a high evaporation rate, so water should be reused (Mckenzie et al., 2003). For obtaining good quality water, there is need for proper waste water treatment procedures. Biological waste water treatment processes may selectively increase the antibiotic resistance in water, which is a major health concern issue of reuse of water (Mulamattathil et al., 2000). Release of hospital sewage directly into the water ecosystem further contributes to the antibiotic resistant bacteria. Therefore, there is pressing need to focus on this aspect locally and globally. Hence, the current study has been undertaken to evaluate the presence of potential pathogenic bacteria in hospital sewage samples from Marathwada region of Maharashtra, India, as well as to determine the antibiotic resistance profiles of the isolated bacteria.

MATERIALS AND METHODS

Collection of sewage samples

One hundred milliliters of hospital sewage samples were collected from six different sites in Aurangabad, Maharashtra region, in sterile screw cap tubes and brought to the laboratory in a cooled condition. Serial dilutions [10^{-5}] of each samples were prepared, and 1 ml of each sample were spread on the nutrient agar medium. Further plates were incubated at 37°C for 24 h and different colonies were obtained. Pure bacteria were procured by streak plate method utilizing Mueller-Hinton agar (Hi-Media, India) or Nutrient Agar [Hi-Media, India]. The isolated bacteria were characterized on the basis of colony morphology study. All the pure colonies were subjected to Grams staining and distinguished bacteria in two groups, viz: Gram positive and Gram negative. These pure cultures were further subjected to biochemical tests according to Nandi and Mandal, (2016), Holt (1984) and Forbes et al. (2007). Morphologically different colonies of bacteria were maintained on Nutrient Agar or Cystine tryptone agar (Hi-Media, India) stabs, at 4°C for further studies. Of the 30 isolates obtained, eight were further used for antibiotic sensitivity testing.

Collection of samples

A total of six hospital sewage samples were collected during the study period, from different sampling sites of Marathwada region, Maharashtra, India and labelled as S1, S2, S3, S4, S5 and S6 (Figure1) in sterile plastic bottles. After dilution and inoculation of each sample on nutrient agar plates, many colonies were obtained. Three Gram positive and 5 Gram negative bacteria were isolated from each of the 6 hospital sewage samples (Table 1). Further, their sensitivity to different classes of antibiotics namely beta lactam group, cephalosporin group, tetracycline, quinolones and aminoglycosides was analyzed. It was observed that all of the 6 samples carry antibiotic resistant bacteria for different antibiotics (Tables 2 and 3).

Antibiotic sensitivity test

Isolated bacteria were further subjected to the antibiotic sensitivity tests by disc diffusion method given by Kirby-Bauer. It was performed through following steps.

Preparation of the test organisms for sensitivity test

Three colonies from each sample were taken and subcultured in sterile nutrient broth aerobically at 37°C for 24 h. Broth cultures of the isolates were centrifuged at 3000 rpm for 10 min. The sediments were diluted with sterile phosphate buffer saline (PBS) and adjusted to the 10^8 CFU/ml using McFarland matching standard (mixture of 0.6 ml of 1% $BaCl_2.H_2O$ and 99.4 ml of 1% concentrated H_2SO_4) using spectrophotometer at 540 nm.

Antibiotic sensitivity testing

100 µl of each aliquot was spread on nutrient agar or Mueller Hinton agar medium. By using sterile forceps, antibiotic sensitivity discs were applied on the surface of the medium. The set-up was incubated aerobically at 37°C for 24 h. The inhibition zone diameters were measured using meter rule after 24 h, incubated and recorded.

The results, in terms of ZDI (zone diameter of the inhibition) values of the test antibiotics, were interpreted following the guidelines of the Clinical and Laboratory Standards (CLSI) Institute (2011). As per the CLSI guidelines, bacteria were classified into three groups viz. resistant, intermediate and sensitive to a particular antibiotic.

Determination of antibiotic resistance pattern

Bacteria showing antibiotic resistance to three or more antibiotics were considered as the multiple antibiotic resistant bacteria [MAR] and MAR index value for each sample using the following formula.

MAR index = M/n

Where, M is number of antibiotics to which the isolate showed resistance and n is the number of total antibiotics used in the test (Krumperman, 1983) that was calculated. Generally, MAR index value higher than 0.2 indicates the isolate is multiple antibiotic resistant (Adefisoye and Okoh, 2017).

To know the prevalence of antibiotic resistance as per the sample collection sites, ARI [Antibacterial resistance index] was determined using the formula described by Krumperman (1983), which is mathematically expressed as

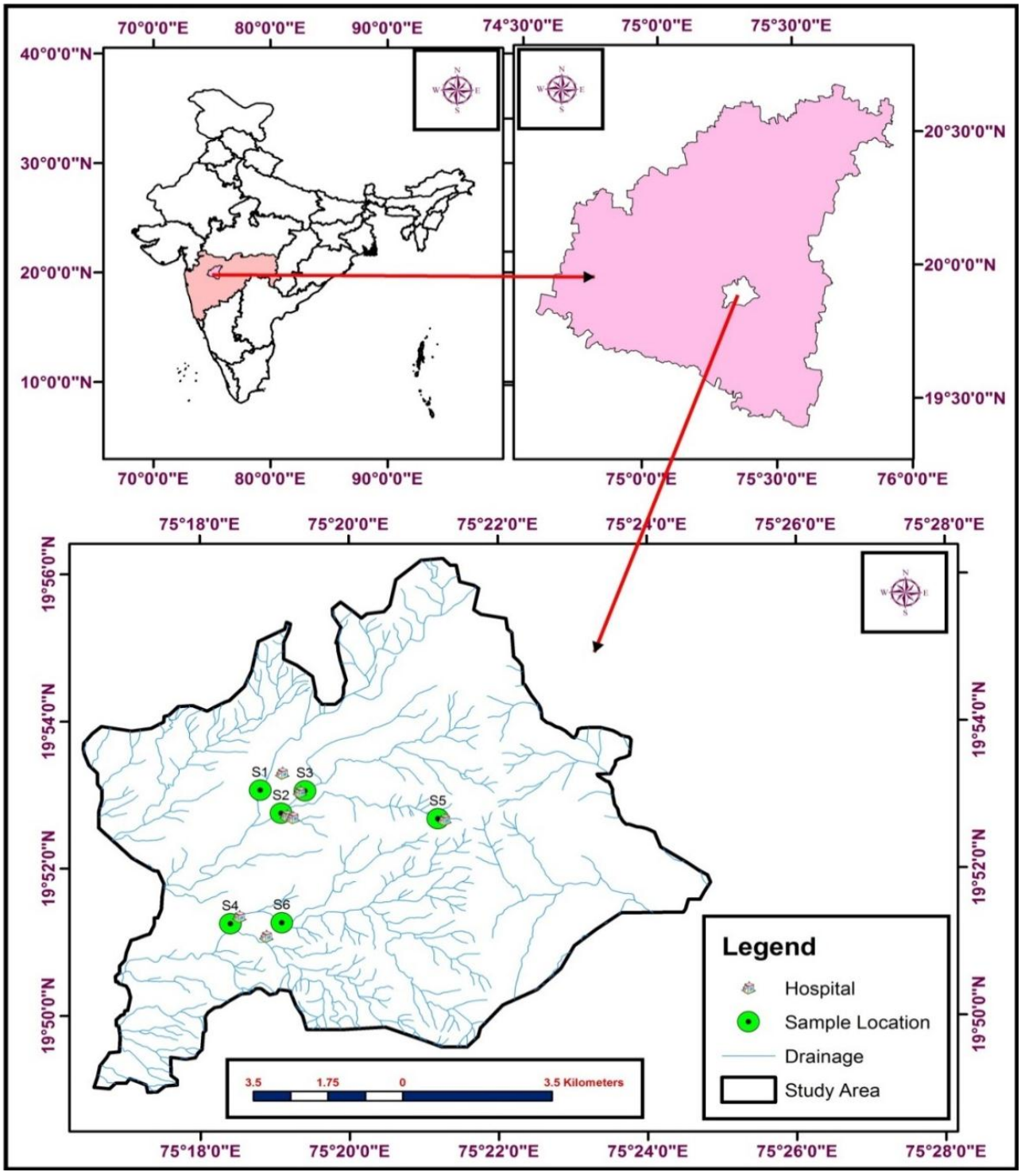


Figure 1. Hospital sewage sample location map of Aurangabad Urban Area, Maharashtra India.

ARI = y/nx

Where, y is actual number of resistance microbes in the sample, n is the number of isolates tested and x is the total antibiotics tested in sensitivity test. Generally, ARI index is directly proportional to the prevalence of antibiotic resistance as per sample collection sites.

RESULTS AND DISCUSSION

Biochemical analysis

Further, by using the biochemical tests and referring to

Table 1. Biochemical tests and identity.

Site	Sample ID	GS	Biochemical test and results								Sugar fermentation test results					Bacterial identity
			CO	CI	IN	CT	OD	MR	VP	DN	Glucose	Sucrose	Lactose	Maltose	Mannitol	
	S11	-	-	+	-	+	-	-	+	-	+	+	+	+	+	*Klebsiella pneumoniae*
	S12	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
S1	S13	-	-	+	-	+	-	-	+	-	+	+	+	+	+	*Klebsiella pneumoniae*
	S14	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S15	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S21	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S22	-	-	+	-	+	-	-	+	-	+	+	+	+	+	*Klebsiella pneumoniae*
S2	S23	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S24	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S25	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S31	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S32	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
S3	S33	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S34	-	-	+	-	+	+	-	-	-	-	-	-	-	+	*Pseudomonas species*
	S35	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S41	-	-	+	-	+	-	-	+	-	+	+	+	+	+	*Klebsiella pneumoniae*
	S42	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
S4	S43	-	-	+	-	+	-	-	+	-	+	+	+	+	+	*Klebsiella pneumoniae*
	S44	-	-	+	-	+	+	-	-	-	-	-	-	-	+	*Pseudomonas species*
	S45	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S51	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S52	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
S5	S53	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S54	-	-	+	-	+	-	-	+	-	+	+	+	+	+	*Klebsiella pneumoniae*
	S55	-	-	+	-	+	-	-	+	-	+	+	+	+	+	*Klebsiella pneumoniae*
	S61	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S62	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
S6	S63	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S64	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*
	S65	-	ND	-	+	+	-	+	-	-	+	V	+	+	+	*Escherichia coli*

GS: Gram staining, CI: Citrate, IN: Indole, CT: Catalase, OD: Oxidase; MR: Methyl red; VP: Voges–Proskauer; - Negative; +: Positive; CO: coagulase, DN: DNase, ND: No data; V= variable.

the Bergey's Manual of Systematic Bacteriology, all isolates were identified (Table 1). In biochemical tests carried out for 48 isolates, we found a total of 6 different types of bacteria out of which 3 were Gram negative and 3 were Gram positive namely, *Klebsiella pneumoniae,*

Table 2. Results of staining, biochemical tests and identity of isolates selected for further study.

Site	Sample ID	GS	Biochemical test and results								Sugar fermentation test results					Bacterial identity
			CO	CI	IN	CT	OD	MR	VP	DN	Glucose	Sucrose	Lactose	Maltose	Mannitol	
S1	S16	+	+	+	-	+	-	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
	S17	+	+	-	-	-	-	ND	+	ND	+	+	+	+	+	*Enterococcus faecalis*
	S18	+	+	-	-	-	-	ND	+	ND	+	+	+	+	+	*Enterococcus faecalis*
S2	S26	+	+	+	-	+	-	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
	S27	+	-	ND	-	-	-	+	-	+	+	+	+	+	-	*Streptococcus sp.*
	S28	+	+	+	-	+	-	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
S3	S36	+	-	ND	-	-	-	+	-	+	+	+	+	+	-	*Streptococcus pyogenes*
	S37	+	+	+	-	+	-	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
	S38	+	+	-	-	-	-	ND	+	ND	+	+	+	+	+	*Enterococcus faecalis*
S4	S46	+	+	+	-	+	-	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
	S47	+	+	+	-	+	-	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
	S48	+	+	-	-	-	-	ND	+	ND	+	+	+	+	+	*Enterococcus faecalis*
S5	S56	+	+	+	-	+	-	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
	S57	+	+	+	-	+	-	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
	S58	+	+	+	-	+	-	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
S6	S66	+	+	+	-	+	-	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
	S67	+	+	-	-	-	-	ND	+	ND	+	+	+	+	+	*Enterococcus faecalis*
	S68	+	+	-	-	-	-	ND	+	ND	+	+	+	+	+	*Enterococcus faecalis*

CI: Citrate, IN: Indole, CT: Catalase, OD: Oxidase; MR: Methyl red; VP: Voges–Proskauer; - Negative; +: Positive; CO: coagulase, DN: Dnase, ND: No data; V= variable.

Escherichia coli, Pseudomonas species, Staphylococcus aureus, Enterococcus faecalis and *Streptococcus pyogenes.* Among all bacteria obtained, Gram negative *E. coli* and Gram positive bacteria *S. aureus* were the most prevalent bacteria found at all sites.

Antibiotic resistance pattern

Based on the zone of inhibition (mm) obtained, antibiotic sensitivity of isolates as per the guidelines given by Clinical and Laboratory Standards Institute (CLSI, 2009) mentioned in Table 3 (Gram negative bacteria) and Table 4 (Gram positive bacteria), the isolates are divided into 2 types, viz: resistant and sensitive. Isolates with intermediate phenotypes obtained in AST were considered as antibiotic resistant bacteria. Among all the antibiotics tested, highest level of resistance was observed for beta lactam class [85%], then for Tetracycline [58%], Cephalosporin [58%], quinolones [52%] and gentamycin [45%] (Figure 2). *E. coli* was the most prevalent bacteria showing antibiotic resistance to all tested antibiotics.

Multiple antibiotic resistance pattern

Further MAR index values were calculated for all the isolates. MAR index values indicate the number of antibiotics to which the isolate is showing resistance. When the MAR index value is >0.2, the bacteria is considered as multiple antibiotic resistant bacteria. From the MAR index values we can predict that around 41 out of 48 isolates show multiple antibiotic resistance and only four are not MAR (Tables 5 and 6). Some of the isolates S11, S22, S34, S35, S38, S54, S55, S56 and S64 were found to be resistant to all the

Table 3. Results of antibiotic sensitivity test of Gram negative isolates.

Site	Sample ID	Zone of Inhibition (mm)				
		Beta lactams [Amp]	Tetracycline [Tet]	Cephalosporin	Quinolones [Cip]	Aminoglycosides
S1	S11	10	14	12	11	11
	S12	20	10	23	15	09
	S13	19	22	13	14	10
	S14	23	12	10	24	20
	S15	29	14	11	26	28
S2	S21	18	22	20	17	24
	S22	14	11	08	10	12
	S23	29	18	00	28	24
	S24	22	10	10	26	18
	S25	20	25	18	14	25
S3	S31	06	22	23	27	26
	S32	09	24	13	15	11
	S33	29	22	11	26	28
	S34	12	14	10	13	10
	S35	19	12	08	10	09
S4	S41	20	11	10	30	25
	S42	30	18	17	18	14
	S43	20	18	09	13	08
	S44	21	11	19	27	22
	S45	22	12	13	22	19
S5	S51	12	11	26	27	18
	S52	14	09	11	24	06
	S53	24	12	24	24	22
	S54	19	14	13	14	11
	S55	10	13	10	11	10
S6	S61	16	09	08	08	20
	S62	12	05	07	09	22
	S63	30	11	10	11	23
	S64	10	14	13	10	10
	S65	08	25	19	15	21

antibiotics tested in the present study while the sample S48 and S58 showed sensitivity to all the antibiotics used. Mainly, *K. pneumoniae* bacteria showed resistance to most of the tested antibiotics.

ARI index

ARI index indicates the prevalence of antibiotic resistant and sensitive bacteria as per the locations. In the present study, it was observed from the ARI (Table 7) that beta lactam resistant bacteria are more prevalent in nature as compared to other antibiotics at all the sample collection sites. The prevalence of antibiotic resistant bacteria is shown in Graph 1. Beta lactam antibiotic resistant bacteria are the more prevalently occurring bacteria in nature. Bacteria obtained from the site 1 appear more resistant to all the antibiotics as compared to other sites.

Conclusion

The present study on the hospital sewage water collected from six different locations of Aurangabad, Maharashtra, India has shown .*E coli* as the most prevalent antibiotic resistant bacteria. We obtained Gram negative bacteria more abundantly in hospital sewage samples. Isolates number 48 and 41 showed >0.2 MAR indices and hence, it is considered to have the potential to cause human infections. Most of the isolates showed multiple antibiotic resistance. Among tested antibiotics, beta lactam group antibiotic resistant bacteria were found to be most prevalent and comparatively less number of isolates was resistant to gentamycin. Therefore, there is need to prepare effective guidelines for judicious use of antibiotics and release of hospital sewage directly into the water bodies, in order to avoid the spread of bacterial multiple antibiotic resistances.

Table 4. Results of antibiotic sensitivity test of Gram positive isolates.

Site	Sample ID	Zone of Inhibition (mm)				
		Beta Lactams [Amp]	Cephalosporin [Cx]	Quinolones [Cip]	Aminoglycosides [Gen]	Tetracycline [Tet]
S1	S16	20	16	14	14	11
	S17	18	20	22	10	20
	S18	19	22	12	09	10
S2	S26	18	23	18	10	15
	S27	15	20	22	19	13
	S28	06	06	32	20	06
S3	S36	08	19	24	24	09
	S37	12	11	13	10	25
	S38	13	10	10	24	09
S4	S46	07	08	30	25	11
	S47	20	30	30	11	31
	S48	32	25	22	23	25
S5	S56	13	11	14	11	09
	S57	12	11	30	22	20
	S58	29	22	30	30	21
S6	S66	12	18	`13	16	22
	S67	20	19	21	23	22
	S68	06	20	11	18	20

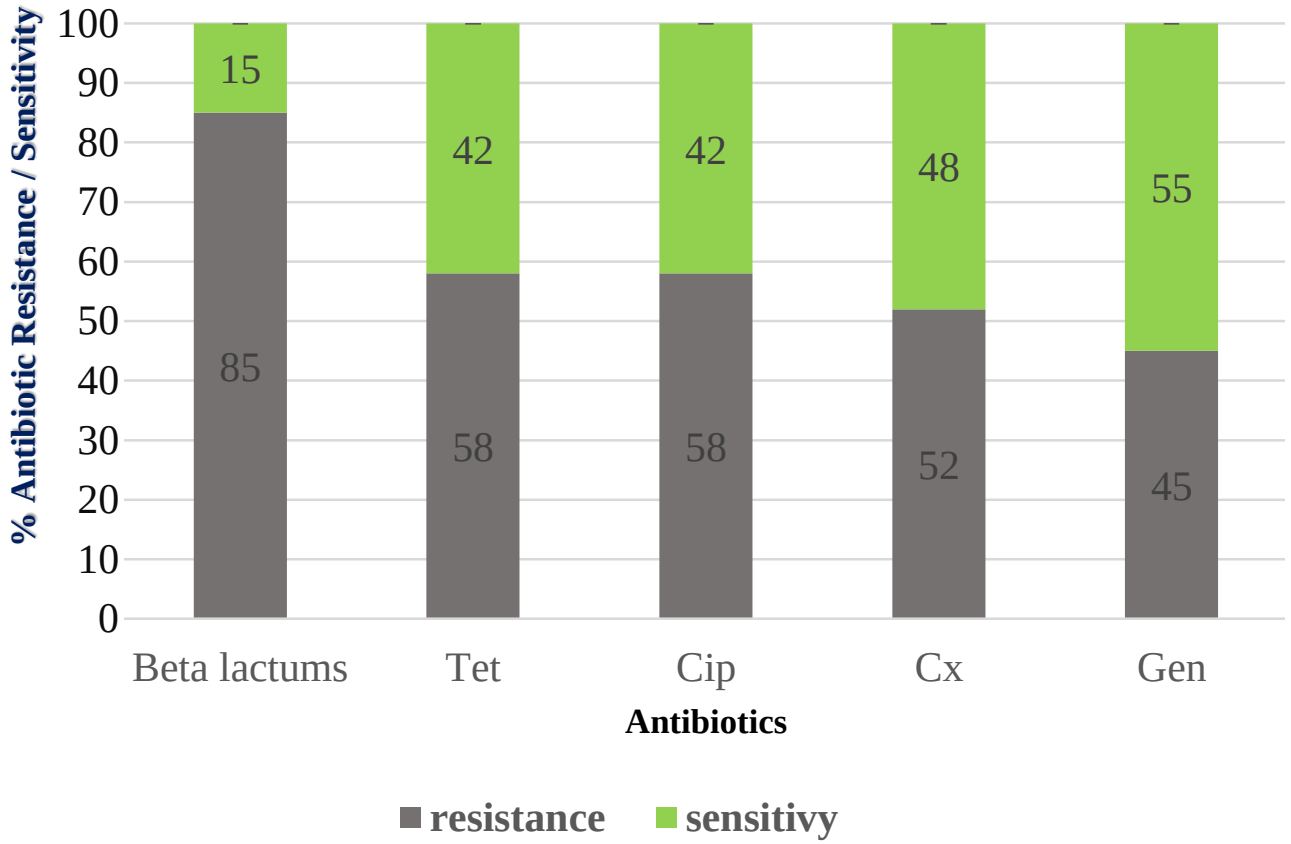


Figure 2. Prevalence of antibiotic resistance.

Table 5. MAR index values of Gram negative isolates.

Site	Sample ID	Antibiotic resistance status					MAR
		Beta lactams [Amp]	Tetracycline [Tet]	Cephalosporin [Cx]	Quinolones [Cip]	Aminoglycoside [Gen]	
S1	S11	R	R	R	R	R	1
	S12	R	R	S	R	R	0.8
	S13	R	S	R	R	R	0.8
	S14	R	R	R	S	S	0.6
	S15	S	R	R	S	S	0.4
S2	S21	R	S	S	R	S	0.4
	S22	R	R	R	R	R	1
	S23	S	S	R	S	S	0.2
	S24	R	R	R	S	R	0.8
	S25	R	S	S	R	S	0.4
S3	S31	R	S	S	S	S	0.2
	S32	R	S	R	R	R	0.8
	S33	S	S	R	S	S	0.2
	S34	R	R	R	R	R	1
	S35	R	R	R	R	R	1
S4	S41	R	R	R	S	S	0.6
	S42	S	S	S	S	R	0.2
	S43	R	S	R	R	R	0.8
	S44	R	R	S	S	S	0.4
	S45	R	R	R	S	S	0.6
S5	S51	R	R	S	S	S	0.4
	S52	R	R	R	S	R	0.8
	S53	R	R	S	S	S	0.4
	S54	R	R	R	R	R	1
	S55	R	R	R	R	R	1
S6	S61	R	R	R	R	S	0.8
	S62	R	R	R	R	S	0.8
	S63	S	R	R	R	S	0.6
	S64	R	R	R	R	R	1
	S65	R	S	S	R	S	0.4

Table 6. MAR index values of Gram positive isolates.

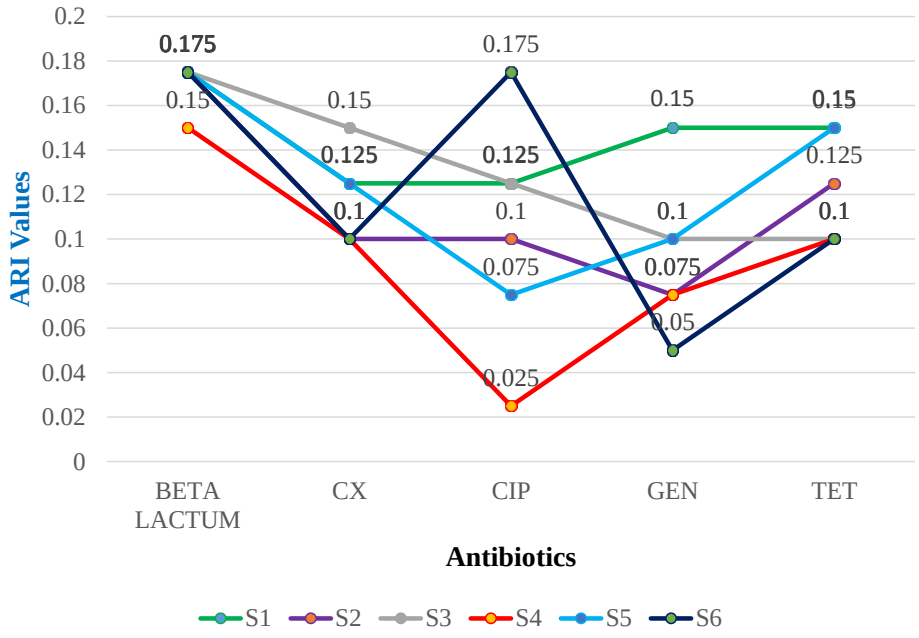
Site	Sample ID	Zone of inhibition (mm)					MAR
		Beta Lactams [Amp]	Cephalosporin [Cx]	Quinolones [Cip]	Aminoglycoside [Gen]	Tetracycline [Tet]	
S1	S16	R	I	R	I	R	0.6
	S17	R	S	S	R	S	0.4
	S18	R	S	R	R	R	0.8
S2	S26	R	S	I	R	I	0.4
	S27	R	S	S	S	R	0.4
	S28	R	R	S	S	R	0.6
S3	S36	R	S	S	S	R	0.4
	S37	R	R	R	R	S	0.8
	S38	R	R	R	S	R	1
S4	S46	R	R	S	S	R	0.6
	S47	R	S	S	R	S	0.4
	S48	S	S	S	S	S	0
S5	S56	R	R	R	R	R	1

Table 6. Contd.

	S57	R	R	S	S	S	0.4
	S58	S	S	S	S	S	0
	S66	R	S	R	I	S	0.4
S6	S67	R	S	S	S	S	0.2
	S68	R	S	R	S	S	0.4

Table 7. ARI index.

Site	ARI Values				
	Beta Lactams [Amp]	Cephalosporin [Cx]	Quinolones [Cip]	Aminoglycosides [Gen]	Tetracycline [Tet]
S1	0.175	0.125	0.125	0.15	0.15
S2	0.175	0.1	0.1	0.075	0.125
S3	0.175	0.15	0.125	0.1	0.1
S4	0.15	0.1	0.025	0.075	0.1
S5	0.175	0.125	0.075	0.1	0.15
S6	0.175	0.1	0.175	0.05	0.1



Graph 1. Prevalence of antibiotic resistance as per site of sample collection.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Adefisoye MA, Okoh AI (2017). Ecological and public health implications of the discharge of multidrug-resistant bacteria and physicochemical contaminants from treated wastewater effluents in the Eastern Cape, South Africa. Water 9(8):562.

Biyela PT, Lin J, Bezuidenhout CC (2004). The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. Water Science and Technology 50(1):45-50.

Clinical and Laboratory Standards Institute (CLSI) (2011). Performance standards for antimicrobial susceptibility testing, 21st informational supplement M100S21. CLSI, Wayne, Pa.

Clinical and Laboratory Standards Institute (CLSI) (2009). Performance Standards for Antimicrobial Susceptibility Testing M100-S19;

Nineteenth Informational Supplement. Vol. 29, Clinical and Laboratory Standards Institute, Wayne.
De Kraker ME, Stewardson AJ, Harbarth S (2016). Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050? PLoS Medicine 13(11):e1002184.
De Kraker MEA, Davey PG, Grundmann H (2011). Mortality and Hospital Stay Associated with Resistant *Staphylococcus aureus* and *Escherichia coli* Bacteremia: Estimating the Burden of Antibiotic Resistance in Europe. PLoS Medicine 8(10):e1001104.
Forbes BA, Sahm DF, Weissfeld AS (2007). Bailey and Scott's diagnostic of microbiology". 12th Edition, Mosby (Elsevier), USA.
Government of India (2017). National Action Plan on Antimicrobial Resistance (NAP-AMR) 2017-2021. [accessed on May 15, 2019] Available from: http://www.searo.who.int/india/topics/antimicrobial_resistance/nap_amr.pdf .
Holt JG (1984). Bergey's Manual of Systematic Bacteriology". Williams and Wilkins, Baltimore.
Krumperman PH (1983). Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of fecal contamination of foods. Applied and Environmental Microbiology 46(1):165-170.
Mckenzie R, Buckle J, Wegelin W, Meyer N (2003). Water demand management cookbook, rand water in collaboration with WRP, managing water for African Cities UN Habitat Programme, New York. Medical Bulletin 68:199-208.
Mukhopadhyay C, Vishwanath S, Eshwara VK, Shankaranarayana SA, Sagir A (2012). Microbial quality of well water from rural and urban households in Karnataka, India: a cross-sectional study. Journal of infection and Public Health 5(3):257-262.
Mulamattathil SG, Bezuidenhout C, Mbewe M, Ateba CN (2014). Isolation of environmental bacteria from surface and drinking water in Mafikeng, South Africa, and characterization using their antibiotic resistance profiles. Journal of Pathogens 10(4):1-11.
Mulamattathil SG, Esterhuysen H.A, Pretorius PJ (2000). Antibiotic-resistant Gram-negative bacteria in a virtually closed water reticulation system. Journal of Applied Microbiology 88(6):930-937.
Nandi S, Mandal S (2016). Bacteriological profiling of commercially available eye cosmetics and their antibiotic susceptibility pattern. Translational Biomedicine 7(3):1-8.
Obi CL, Potgieter N, Bessong PO, Matsaung G (2002). Assessment of the microbial quality of river water sources in rural Venda communities in South Africa. Water SA 28(3):287-292.
Sidhu S, Malhotra S, Devi P, Gupta A (2016). Bacteriological analysis of the drinking water from different schools in Northern India: a concern in developing countries. International Journal of Medical Resarch and Review 4(4):630-634.
Smith E, Lichten CA, Taylor J, MacLure C, Lepetit L, Harte E (2016). Evaluation of the action plan against the rising threats from antimicrobial resistance. Final Report. European Commission. 2016. [accessed on May 15, 2019]. Available from: https://www.ec.europa.eu/health/amr/sites/amr/files/amr_final-report_2016_rand.pdf .
World Health Organization (WHO) (2011). Regional office for South-East Asia. Jaipur declaration on antimicrobial resistance. 2011. [accessed on May 15, 2019]. http://www.who.int/iris/handle/10665/205397.

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Diversity of *Arbuscular mycorrhizal* fungi in the three agroecological zones of the Central African Republic

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***Arbuscular mycorrhizal* fungi (AMF) are organisms that can establish symbioses with 80% of terrestrial plants. Soils were collected in three agroecological zones (AEZ) of the Central African Republic (CAR). The aim of this research is to study the diversity of AMF in the CAR. The spore extraction and enumeration, granulometric and physicochemical analyses of each soil were performed. The results showed that AEZ3 has a large number of spores; it is therefore the densest followed by AEZ2 and AEZ1. The particle size analysis showed that AEZ1 and 2 have a sandy-clay texture and AEZ3 is sandy-silty. The average water pH of the three surveyed areas is 5.13. The organic matter content is low. The total nitrogen content of the soils varies from one area to another. The C/N ratio of the studied soils varies from 9.65 to 15.5 with an average of 13.8. The total phosphorus content is lower than the norm. The best calcium content is obtained in AEZ3 followed by AEZ1. Magnesium, sodium, the sum of exchangeable cations and cation exchange capacity (CEC) were below the critical thresholds.**

Key words: Diversity, *Arbuscular mycorrhizal* fungi (AMF), agroecological zones, Central African Republic.

INTRODUCTION

The Central African Republic (CAR), in its effort to achieve significant economic development and food self-sufficiency, is in need to develop its natural soil resources. Since agriculture is the basis of the country's economy, the study of soils is of paramount importance for obtaining basic cartographic documents that are essential to be able to plan agriculture in the framework of establishing the director plan for land use (MDR,

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2014). Agriculture in the 21st century faces several challenges in order to meet an ever-increasing demand for food. Nowadays the main challenges today are to reduce the use of unsustainable chemical fertilizers, limit water consumption, and improve the resilience of agricultural crops, thus increasing the productivity of agricultural systems. Endomycorrhizal symbiosis helps to address these different objectives (Crossay, 2018). Mycorrhizae are symbiotic associations that promote plant growth and development. Among the different types of mycorrhizal symbioses, *Arbuscular mycorrhizae* (endomycorrhizal) are the most abundant, and the most ecologically and economically important symbiosis associated with herbacsous crops (Schüβler et al., 2001).

The interest in using and preserving (AMF), with a view to use them as bioinoculants for sustainable agriculture, is becoming increasingly evident, as the proper management of these symbiotic fungi could decrease the use of chemical fertilizers that harm the environment and the health of living organisms (including our own) (Crossay, 2018). The use of AMF represents a high initial cost, however in the long run this method is economically viable and less expensive than conventional methods of fertilizing agricultural soils and even saves money for agricultural producers and restoration of degraded soils (Ngonkeu, 2013). Among symbiotic microorganisms, AMF associate with more than 80% of terrestrial plants (Smith and Read, 1997; Strullu, 1991). There are now over 150 species in this group of fungi (Selosse and Le Tacon, 1998).

Despite their importance, these symbiotic associations are untapped and almost ignored by farmers (Smith and Read, 1997; Ngonkeu et al., 2013). AMF promote soil fertility, vegetation restoration of degraded soils, protection against some soil-borne pathogens, mineral nutrition of plants, and plant protection against drought is well demonstrated (Sieverding, 1991; Strullu, 1991). AMF allow the plant to acquire mineral elements, especially, elements that are not very mobile in the soil such as phosphorus, copper and zinc (Fagbola et al., 2001; Saïdou et al., 2009, 2012; Haougui et al., 2013; Bossou et al., 2019). The last one would indeed reduce expenditures by up to 70% for phosphate fertilizers and by 30 to 40% for nitrogen, potassium and trace elements (Johnson and Menge, 1982). In CAR, no research has been conducted on the diversity of AMF to our knowledge. Thus, the aim of this research is to study the diversity of AMF in the three agroecological zones of the CAR.

MATERIALS AND METHODS

Study zones

The study was conducted in 3 zones of the 4 agroecological zones in CAR. These agroecological zones are: the forest zone, known as the forest-coffee zone, which covers the southwestern and southeastern parts of the country (AEZ1); the savannah zone, known as the food-livestock zone (AEZ2), which extends over the central-western part of the country; and the cotton-livestock zone (AEZ3), which extends from west to east, to the north of the food-livestock zone and the coffee zone (Figure 1).

Soil sampling and collection

In each surveyed agroecological zone, three localities were chosen taking into account their soil type in order to show the sample variability. At each location, two fields were surveyed and three samples were taken from each field. Soil samples were taken with an auger to a depth of 30 cm. Four soil samples were collected in each field and a composite sample of 300 g was prepared for spore extraction to determine the AMF density and spore diversity and for chemical analysis.

Microbiological analysis of soils

Spores extraction

Spores were extracted from each composite soil sample using the wet sieving method described by Gerdemann and Nicolson (1963). A quantity of 100 g of each soil sample was suspended in 500 ml of tap water. The resulting mixture was passed through a series of sieves of decreasing mesh size (250, 100, 60, and 40 µm). To the filtrate obtained by sieving, a 5 ml of two solutions with different concentrations of sucrose (40 and 60%) was added, and the mixture was centrifuged at 3000 rpm for 30 min and at a temperature of 4°C (Oehl et al., 2003). The operation was repeated three times to retain the maximum number of spores (Figure 2).

Soil sampling and collection

In each agroecological zone prospected, three localities were chosen taking into account their soil type in order to have sample variability. At the level of each locality, two fields were prospected and in each field three samples were taken. In each locality, two different fields were prospected and at the level of each field, soil samples were taken with an auger to a depth of 30 cm. The four soil samples taken from the field are put together to form a composite sample of 300 g. This composite sample of 300 g of soil thus formed per site was used for the extraction of spores for the determination of the density and spore diversity of AMFs as well as for chemical analyses.

Spores enumeration

The method used for spore enumeration was as described by Bossou et al. (2019). Spores abundance was assessed under a binocular microscope from the centrifuged sucrose filtrate and placed in a gridded Petri dish (5 cm diameter). Spores were counted according to their size, color, and mode of attachment of the hypha to the spore. The identification and description of the listed species was done using the identification key of the "International Culture Collection of Vesicular and *Arbuscular mycorrhizal* fungi" (INVAM, http//www.invam.caf.wdu.edu). Different indices of biological diversity were calculated on the basis of the number of spores obtained by type of color and differentiation.

Figure 1. The major agroecological zones of the Central African Republic (MDR, 2014).

These are the Shannon diversity index (H') (Shannon and Weaver, 1962), the Simpson diversity index (1-D) (Simpson, 1949) and the Hill diversity index (1-Hill). These indices were used to extract the maximum information and better understand the AMF communities.

Physicochemical analysis of soils

The analyses were carried out at Laboratory of Soil Analysis of Arras 273 street of Cambrai 62000 Arras INRA Dijon UMR Agroecological (France) in September 2018. The collected soil samples were air-dried under cover for 10 days, then crushed, sieved to 2 mm and kept cool at 4°C. The analytical methods were those described by Bocoum (2004). The chemical analysis of soil included organic carbon (C), total nitrogen (N), pH, EC, salinity, exchangeable bases (Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}) and CEC. The measurement of pH and EC was performed on soil suspensions with soil/water ratios of 1/2.5 and 1/10, respectively. The particle size was determined by the sieving method for coarse elements and by densimetry with a Robinson pipette for fine elements. Exchangeable bases were determined by the ammonium acetate extraction method (pH < 7), CEC by the NH^{4+} saturation method. Organic carbon was determined by the Anne method and N by the Kjeldahl method. C content was determined at the C.H.N Microanalyzer by total combustion of the analytical soil sample at 1050°C under oxygen flow. Na and K were determined photometrically with a spectrophotometer. Ca and Mg were determined by volumetry with a chelating agent, EDTA.

Statistical analysis

All data obtained were analyzed by ANOVA using SPSS 20 software for Windows. Turkey's test was used to determine any significant difference between the different varieties at the threshold of $p<0.05$. Results were expressed as means ± standard deviation. All experiments were performed in four replicates.

RESULTS

Abundance and density

The extraction of spores from the different soils allowed us to know the spore density of the soils by agroecological zone (AEZ) (Table 1). There were significant differences at the 5% level among the ZAEs in terms of spore number and spore density per gram of soil (Table 1). The comparison of means among the different AEZs showed that AEZ3 had a large number of spores

Table 1. Abundance and density of *Arbuscular mycorrhizal* spores per agroecological zone.

Agroecological zone	*Glomus*	*Gigaspora*	*Sclerocystis*	*Scutellospora*	Abundance (spores)	Density (spores/g soil
AEZ1	347[b]	3[b]	4[b]	0[b]	354[c]	3.54[c]
AEZ2	384[b]	52[a]	0[c]	3[c]	439[b]	4.39[b]
AEZ3	689[a]	4[b]	6[a]	0[b]	699[a]	6.99[a]

Values with the same superscript in a column are not significantly different (P>0.05). Tukey test.

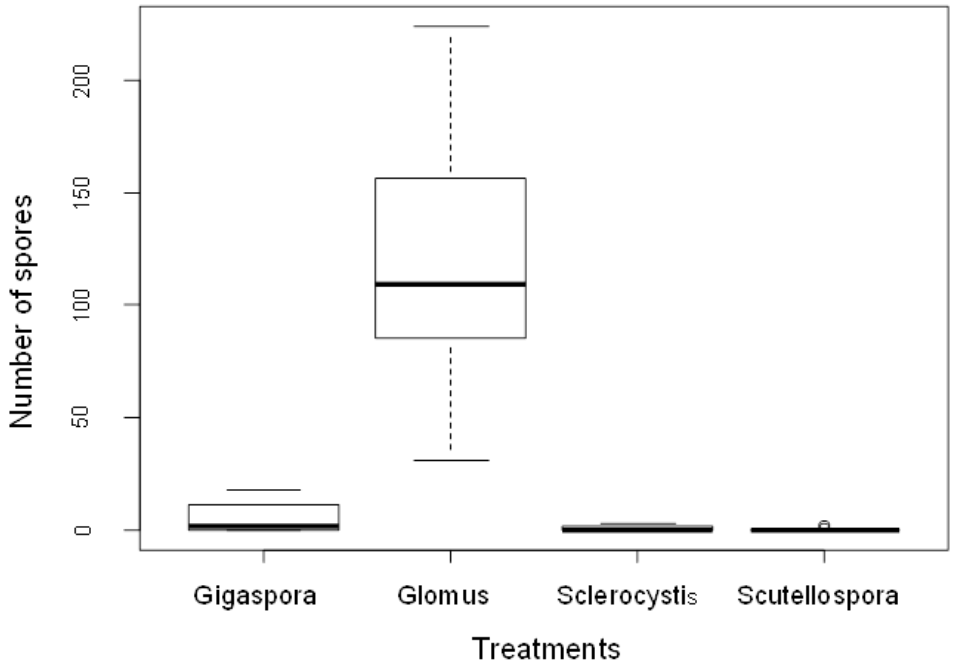

Figure 2. Average number of spores in different genus of *Arbuscular mycorrhizal* fungi.

(699 per 100 g of dry soil), it is therefore the densest followed by AEZ2 (439 per 100 g of dry soil) and AEZ1 (354 per 100 g of dry soil) (Table 1).

Morphological characteristics of the spores

All morphological characteristics of the spores, described by Giovannetti and Gianinazzi-Pearson (1994) play a very important role in the identification, description and classification of new AMF species. After analysis of these different soil samples, it was globally noticed a dominance of 4 genera of fungal species such as: *Glomus* on average 95.18% (Figure 4A), compared to those of *Gigaspora* (3.95%) (Figure 4B), *Sclerocystis* (0.67%) (Figure 4C) and those of *Scutellospora* (0.20%) (Figure 4D). Thus, the average number of spores of the genus *Glomus* was significantly higher in the three surveyed zones compared to the other genera (Figure 3).

Physico-chemical characteristics of soils

The physicochemical analysis of soils was done on three soil samples taken in each agroecological zones of CAR (AEZ1, AEZ2 and AEZ3).

Granulometry

The distribution of the different granulometric fractions makes it possible to classify the soil in a class of texture which defines certain parameters of physical behavior,

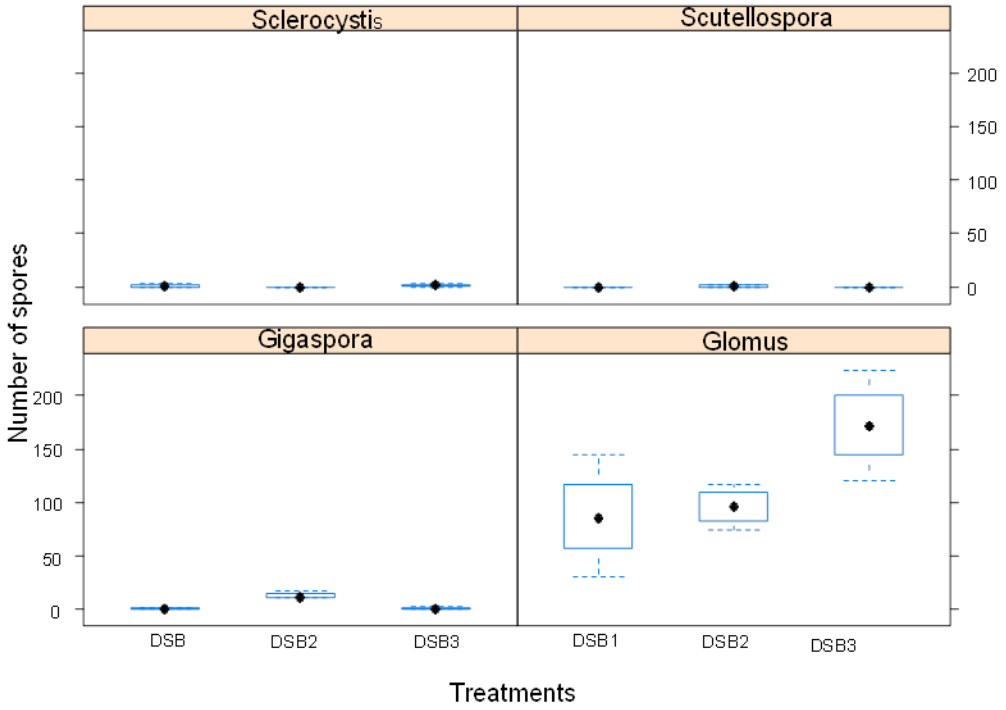

Figure 3. Number of spores of *Arbuscular mycorrhizal* fungi species in the different agroecological zones of the Central African Republic.

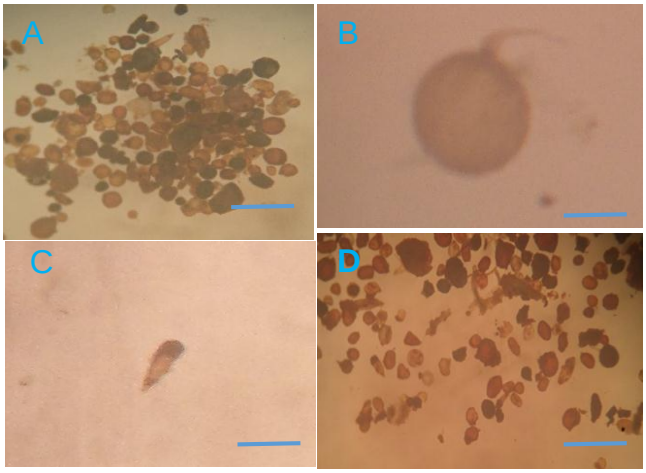

Figure 4. Diversity of AMF spore genera (A: *Glomus* spp.; B: *Gigaspora* spp.; C: *Sclerocystis* spp..; D: *Scutellospora* spp.); Line = 0.5 mm.

useful water retention, capacity to store fertilizing elements, risks of losses of these elements by leaching. The results showed, according to the textural triangle of Duchaufour (1991), that the soils have a clayey-sandy texture (AEZ1 and AEZ2) with a variable percentage of sands (95.31 and 97.20%), clays (3.25 and 8.75%) and silts (1.44 and 4.5%), respectively. The soil of AEZ3 is of the sandy-silty type with 88.50% sand, 9.50% silt and 2%

Table 2. Granulometric analysis of the soils of the 3 agroecological zones of the Central African Republic.

Parameter	Clay (%)	Silt (%)	Sand (%)
AEZ.1	3.25[b]	1.44[c]	95.31[a]
AEZ.2	8.75[a]	4.50[b]	87.20[b]
AEZ.3	2.00[c]	9.50[a]	88.50[b]

Values with the same superscript in a column are not significantly different (P>0.05). Tukey test.

Table 3. Chemical characteristics of soil in each agroecological zone of the Central African Republic.

Soil characteristics	Agroecological zones			Standards
	AEZ1	AEZ2	AEZ3	
pH (H_2O) 1/2,5	4.32	5.01	6.08	-
OM (%)	21.70	19.10	19.30	20-30
C ($g.kg^{-1}$)	12.50	11.10	11.10	12.6-25
N ($g.kg^{-1}$)	1.30	0.79	0.72	1.2-2.2
C/N	9.65	14.10	15.5	11-15
P ($g.kg^{-1}$)	0.02	0.00	0.01	0.20-0.23
K ($g.kg^{-1}$)	0.14	0.057	0.22	0.15-0.25

clay. According to the analysis of variance performed, the difference observed in general is significant between the different parameters (P<0.05) (Table 2).

pH of the surveyed soils

The lowest pH was observed in AEZ1 (4.32) indicating a very acidic soil compared to the acidic soil at AEZ2 (5.01) and slightly acid soil at AEZ3 (6.08) (Table 3).

Analysis of soil organic matter and total mineral elements

The soils of the three agroecological zones studied have a low organic matter concentration of 19.10 and 19.30%, respectively for AEZ2 and AEZ3, while AEZ1 had 21.70% of organic matter concentration in the range of 20 to 30% corresponding to the normal range of values (Table 3). The total N concentration of the soils varied from one zone to another. Of the samples analyzed, AEZ1 (1.30 $g.kg^{-1}$) had a good total N concentration, falling within the reference range of 1.20 to 2.20 $g.kg^{-1}$, followed by AEZ2 (0.79 $g.kg^{-1}$) and AEZ3 (0.72 $g.kg^{-1}$) (Table 3). The C/N ratio of the studied soil samples varied from 9.65 to 15.5. This indicates a slow mineralization potential of organic matter by soil microorganisms in the surveyed sites (Table 3). Total P concentration ranged from 0.02$g.kg^{-1}$ (AEZ1) to 0.00 $g.kg^{-1}$ (AEZ2) and 0.01 $g.kg^{-1}$ (AEZ3). Of the soil samples analyzed, none had total P levels within the critical threshold range of 0.20 to 0.23 $g.kg^{-1}$ (Table 3). The AEZ1 (0.11 $g.kg^{-1}$) and AEZ2 (0.05 $g.kg^{-1}$) zones have exchangeable K values below the critical threshold (0.15-0.25 $g.kg^{-1}$). Only sample AEZ3 (0.22 $g.kg^{-1}$) is within the normal range (Table 3).

Analysis of exchangeable cations and cation exchange capacity

The analysis of the exchangeable bases of the three prospected zones reveals that the best Ca concentration was obtained at AEZ3 (3.02 cmol+.kg^{-1}) followed by AEZ1 (1.72 $cmol^{+}.kg^{-1}$) then AEZ2 (0.49 $cmol^{+}.kg^{-1}$). All these samples fall within the range of reference values 5to 8. The Mg^{2+}, Na^{+}, sum of exchangeable cations (Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}), and cation exchange capacity (CEC) of the soil samples have values below the critical thresholds of 1.50 to 3.00 and 0.3-07 $cmol^{+}.kg^{-1}$, 7.50-150, and 10≤CEC≤ 20 $cmol^{+}.kg^{-1}$, respectively (Table 4). Mn concentration is 16.2 mg.kg for AEZ1 falls within the range of reference values 12 to 35 while the other samples AEZ2 (3.7 mg.kg) and AEZ3 (11.1 mg.kg) have

Table 4. Total exchangeable cations, sum of bases and cation exchange capacity of soils in the 3 agroecological zones of Central African Republic.

Soil characteristics	Agroecological zones			Standards
	AEZ1	AEZ2	AEZ3	
Ca ($cmol^+.kg^{-1}$)	1.72	0.49	3.02	5-8
Mg ($cmol^+.kg^{-1}$)	0.324	0.219	0.866	1.5-3.0
Na ($cmol^+.kg^{-1}$)	0.011	0.010	<0.005<0.0011	0.3-0.7
Mn ($cmol^+.kg^{-1}$)	16.2	3.7	11.1	12-35
S (Ca^{2+},Mg^{2+},K^+,Na^+) ($cmol^+.kg^{-1}$)	1.446	1.602	2.127	7.5-15
CEC ($cmol^+.kg^{-1}$)	5.46	4.36	5.2	10≤CEC≤20

values below the critical thresholds. The K concentration of AEZ1 (0.36 $cmol^+.kg^{-1}$) and AEZ3 (0.22 $cmol^+.kg^{-1}$) are within the reference range 0.15to 0.25 $cmol^+.kg^{-1}$. The content of AEZ2 (0.14 $cmol^+.kg^{-1}$) is below the standard (Table 4).

DISCUSSION

This study was conducted with the objective of characterizing the diversity of AMF in the 3 agroecological zones of the CAR. The results of this work reveal a very high density and number of spores (about 699 spores per 100 g of soil) for AEZ3 compared to the other AEZ. This density and number are significantly lower than those obtained by Bossou et al. (2019) (about 134156 per 100 g of soil) in the different agroecological zones of Ivory Coast, by Johnson et al., (2013) (about 4045 spores per 100 g of soil) under cowpea culture in the different agroecological zones of Benin, by Bivoko et al. (2014) under cassava cultivation in the Azaguié region of southeastern Ivory Coast, by Balogoun et al. (2015), under cashew plantation in central Benin as well as the one found in the classified forest of Wari-Maro in northern Benin under Isoberlinia doka by Houngnandan et al., (2009).

The first descriptions of AMF diversity were based on morphological characters of spores (color, shape. These criteria led to the classification of AMF into six genera (Morton and Benny, 1990). Morphological characteristics allowed us to identify four genera of AMF from the 3 agroecological zones of the CAR: *Glomus, Gigaspora, Sclerocystis* and *Scutellospora*. These results show a strong representation of the average number of spores of the genus *Glomus* in relation to the genera obtained. Similar results were obtained by some authors such as Bossou et al. (2019) on the diversity of AMF associated with maize (*Zea mays* L.) cultivation in Benin, Mbogne et al. (2015) and Johnson et al. (2013), Ngonkeu (2003) on AMF species identified using morphological criteria in 5 agroecological zones of Cameroon.. The results reported in the present study indicate that fungal spore diversity varies little from one agroecosystem to another with a dominance of spores of the genus Glomus extracted from soils of the different AEZ. The predominance of *Glomus* species in most ecosystems suggests a better adaptation of this genus either to the most hostile conditions such as drought, salinity and other environmental stresses or to a wide range of ecological niches (Houngnandan et al., 2009). Indeed, the genera *Glomus* would spread much more by spores which are forms of resistance of AMF to harsh conditions while the genera *Gigaspora*, *Sclerocystis* and *Scutellospora* would spread more with other types of propagules such as hyphae and extra root mycelial fragments (Brito et al., 2012).

The results of the granulometric analysis of the soils of the three agroecological zones of CAR studied showed that AEZ1 and AEZ2 have a sandy-clay texture while AEZ3 has a sandy-silt structure. These textures (sandy-clay and silty-clay) are suitable for growing several crops. These results confirm those obtained by Ballot (2006) who considers that these textures are very favorable for cassava cultivation and some authors (Buol et al., 2011a; Pypers et al., 2011) have reported that loamy soil texture or free soil is excellent and suitable for most crops. pH is a key component of soil chemistry and determines the availability of nutrients to plants and soil microorganisms (Borah et al., 2010). The water pH of the studied soils from the three areas is 5.13 and moderately acidic. This result is similar to Ballot (2006) who obtained a pH=5.5 in Damara, CAR that this pH is a favorable element for cassava cultivation and Ognalaga et al. (2017). Organic matter is one of the main drivers for improving soil fertility and yield of cassava (Akanza et al., 2011). The results of this study show low organic matter (OM) content in AEZ2 and AEZ3 in contrast AEZ1 at OM content in the range of 20 to 30% corresponding to the normative values. Some authors have reported that CO plays a physical role in the soil for cohesion, structure, porosity, water retention or storage, etc. It also plays a chemical role in the

development of the soil. It also plays a chemical role in plant nutrition through degradation actions, mineralization, etc. (Ballot, 2016; Hubert and Schaub, 2011). The N contents of AEZ2 and AEZ3 are lower than the normative value (1.2 - 2.2), which can be explained by the fact that these plots have been extensively used for agricultural activities, except for AEZ1 (N=1.3), where the plot was not used for years due to the military-political events that the country experienced. These results are in agreement with the work of Ognalaga et al. (2017) and Ballot (2016). The organic matter allows plant nutrition by releasing adsorbed mineral elements. Therefore, it prevents the leaching of mineral elements due to its low adsorption capacity of mineral colloids. Boyer (1982) showed in his previous studies that the value of the ratio between C/N characterizes the degree of mineralization of OM. According to Duchaufour (1997), the results obtained in this study in the AEZ1 and AEZ2 zones on C/N, which are lower than 15, indicate a slow or even difficult decomposition of OM mineralization. Results confirm those obtained by some authors (Diallo et al., 2015; Ballot, 2016). The values of total Phosphorus obtained from the studied soil samples are not in the reference range (0.20 - 0.23). These results can be explained by the fact that organic matter does not allow a good immobilization of phosphorus in soils. These results are in agreement with those obtained by other authors (Bertrand and Gigou, 2000; Luciens et al., 2012; Ballot, 2016).

Exchangeable bases showed that there is a deficiency of Ca^{2+} and Mg^{2+} and Na^{2+} in the different soil samples compared to the respective threshold values of 5 - 8, 1.5 - 3 and 0.30 - 0.70 $cmol^{+}.kg^{-1}$. Ballot (2016) work on integrated fertility management of cassava (*Manihot esculenta* Crantz) growing soils in the Damara savanna zone of CAR confirms these results. The values of the sum of exchangeable actions and cation exchange capacity, are low in the three soil samples analyzed, compared to the respective reference values of 7.50 to 15 $cmol^{+}.kg^{-1}$ and $10 \leq CEC \leq 20$. Such values were reported by Ballot (2016) in soils from the commune of Damara (CAR).

Conclusion

At the end of this study which focused on the diversity of AMF in the three agroecological zones of the CAR, it is apparent that the survey carried out in the three agroecological zones allowed the identification of four genera of AMF: *Glomus*; *Gigaspora*; *Sclerocystis*; *Scutellospora*. Part of the specific diversity of AMF was described with a strong representation of the genus *Glomus* in the different zones. The granulometric analyses of the soils showed that the soils of AEZ1 and AEZ2 have a clayey-sandy texture and AEZ3 has a silty-sandy structure. These textures (sandy-clay and silty-clay) are suitable for the cultivation of several crops. Physico-chemical analyses showed that the composition of C, N, organic matter and exchangeable bases also varied according to the agroecological zones surveyed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

MDR (2014). Report of the Ministry of the Rural Development of the Central African Republic 85 p.

Akanza P, N'zue B, Anguete K (2011). Influence de la fumure minérale et de la litière de volaille sur la production du manioc (Manihot esculenta Crantz) en Côte d'Ivoire. Agronomie Africaine 14(2):79-89.

Ballot CSA (2016). Gestion intégrée de la fertilité des sols de culture du manioc (Manihot esculenta CRANTZ) dans la zone de savane de damara en République Centrafricaine. Thèse de doctorat. Université de Lomé 170 p.

Balogoun I, Saïdou A, Kindohoundé NS, Ahoton EL, Amadji GL, Ahohuendo BC, Babatoundé S, Chougourou D, BabaMoussa L, Ahanchédé A (2015). Soil Fertility and Biodiversity of *Arbuscular mycorrhizal* Fungi Associated with Cashew's Anacardium occidentale L. Cultivars Characteristics in Benin West Africa. International Journal of Plant and Soil Science 51:50-63. DOI: 10.9734/IJPSS/2015/13817

Bertrand R, Gigou J (2000). Fertilité des sols tropicaux. Paris (France) Maisonneuve et Larose 397 p.

Bivoko D, Ahonzo-Niamke S, Zeze A (2014). Impact des propriétés physicochimiques des sols de culture du manioc sur l'abondance et la diversité des communautés de champignons mycorhiziens à arbuscules dans la zone agroécologique d'azaguie, Sud-Est de la Côte D'Ivoire. Agronomie Africaine 25(3):251-264

Bocoum M (2004). Méthodes d'analyses des sols. Doc de travail. Institut National de Pédologie, Dakar - Sénégal 55 p.

Borah KK, Bhuyan B, Sarma HP (2010). Lead, arsenic, fluoride, and iron contamination of drinking water in the tea garden belt of Darrang district, Assam, India. Environmental Monitoring and Assessment 169(1-4):347-35 doi: 10.1007/s10661-009-1176-2.

Bossou LDR, Houngnandan HB, Adandonon A, Zoundji C, Houngnand. P (2019). Diversité des champignons mycorhiziens arbusculaires associés à la culture du maïs (*Zea mays* L.) au Bénin. International Journal Biological and Chemical Sciences 13(2):597-609. DOI: https://dx.doi.org/10.4314/ijbcs.v13i2.2

Boyer J (1982). Les sols ferralitiques: facteur de fertilité et utilisation des sols. Init. Doc. Tech. ORSTOM, Paris 52, 384 p.

Brito B. Plaza-Diaz J, Munoz-Quezada S, Gomez-Llorente C, Gil A (2012). Probiotic mechanisms of action. Nutrition and Metabolism 1:160-174. doi: 10.1159/000342079 .

Buol SW, Southard RJ, Graham RC, McDaniel PA (2011a). Morphology and Composition of Soils. In : Soil Genesis and Classification, Sixth Edition pp. 35-87.

Crossay T (2018). Caractérisation taxonomique des champignons mycorhiziens à arbuscules natifs des sols ultramafiques de Nouvelle-Calédonie; analyse de leur synergie permettant l'adaptation desplantes à ces milieux extrêmes. Thèse de doctorat. Université de la. Nouvelle-Calédonie 293 p.

Diallo MD, Ngamb T, Tine AK, Guisse M, Ndiaye O, Mahamat Saleh M, Diallo A, Seck S, Diop A, Guisse A (2005). Caractérisation

agroécologique des sols de Mboltime dans la zone des Niayes (Sénégal), Agronomie Africaine 2(1):57-67.
Duchaufour (1991) Duchaufour P. 1991. Pédologie : sol, végétation, environnement. Troisième édition. Masson, Paris 189 p.
Duchaufour P (1997). Pédologie : sol, végétation, environnement. Abrégés de Pédologie. Masson 5ème édition 291 p.
Fagbola O, Osonubi O, Mulongoy K, Odunfa SA (2001). Effects of drought stress and *Arbuscular mycorrhiza* on the growth of Glirincidiasepium Jacq, Walp and *Leucaena leucocephala* Lam de Wit, in simulated eroded soil conditions. Mycorrhiza 11(5):215-223. DOI: http ://doi.org/10.1007/s005720100114.
Gerdemann JW, Nicholson TH (1963). Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. Transactions of British Mycological Society 46(2):235-244 https://doi.org/10.1016/S0007-1536(63)80079-0
Giovannetti M, Gianinazzi-Pearson V (1994). Biodiversity in *Arbuscular mycorrhizal* fungi. Mycological Research 98(7):705-715
Haougui A, Souniabe PS, Doumma A, Adam T (2013). Evolution des populations des champignons endomycorhiziens sur les adventices de quatre sites maraîchers de la région de Maradi au Niger. International Journal of Biological and Chemical Sciences 14(3):1065-1073. DOI: 10.4314/ijbcs.v7i2.12
Houngnandan P, Yemadje RGH, Kane A, Boeckx P, Van Cleemput O (2009). Les glomales indigènes de la forêt claire à Isoberlinia dokaCraibet Stapf à WariMaro au centre du Bénin. Tropicultura 27(2):83-87.
Hubert G, Schaub C (2011). La fertilisants des sols. L'importance de la matière organique. Chambre d'Agriculture, Bas-Rhin. Service Environnement-Innovation 46 p.
Johnson JM, Houngnandan P, Kane A, Sanon K, Neyra M (2013). Diversity patterns of indigenous *Arbuscular mycorrhizal* fungi associated with rhizosphere of cowpea Vigna unguiculata L. Walp. in Benin, West Africa. Pedobiologia 56(3):121-128. DOI: https://doi.org/10.1016/j.pedobi.2013.03. 003.
Johnson CP, Menge JA (1982). Mycorrhizae may save fertilizer dollars. American Nurseryman 156:79-86.
Luciens NK, Yannick US, Michel MM, David BM, Emery KL, Louis BL (2012). Effets des apports des doses variées de fertilisants inorganiques (NPKS et Urée) sur le rendement et la rentabilité économique de nouvelles variétés de Zea mays L. à Lubumbashi, Sud-Est de la RD Congo. Journal of Applied Biosciences 59:4286-4296.
Mbogne TJ, Temegne C, Hougnandan P, Youmbi E, Tonfack LB, NtsombohNtsefong G (2015). Biodiversity of *Arbuscular mycorrhizal* fungi of pumpkins Cucurbita spp. under the influence of fertilizers in ferralitic soils of Cameroon and Benin. Journal of Applied Biology and Biotechnology 3(5):1-10. DOI: 10.7324/JABB.2015.3501.
Morton B, Benny GL (1990). Revised classification of arbusculars mycorrhizal fungi (Zygomycètes): New order, Glomales, two new families, Acaulosporaceae and Gigasporaceae, with an amendation of Glomaceae. Mycotaxon 37:471-491.
Ngonkeu MEL (2003). Biodiversité et potential des mycorhizes à arbuscules de vertaines zones agro-écologiques du Cameroun. Thèse de Doctorat 3ème cycle, Université de Yaoundé I Cameroun 259 p.
Ngonkeu MEL, Nwaga D, Adamou S, Fokom R, Tchameni NS, Onguene NA, Nana WL, Chaintreuil C, The C, Amougou A, Moulin L, Prin Y, Lebrun M, Dreyfus B (2013). Diversité des champignons mycorhiziens arbusculaires du Cameroun. Institut de Recherche pour Le Développement 15 p.
Oehl F, Sieverding E, Ineichen K, Mäder P, Boller T, Wiemken A (2003). Impact of land use intensity on the species diversity of *Arbuscular mycorrhizal* fungi in agroecosystems of central Europe. Applied and Environmental Microbiology 69(5):2816-2824. DOI: 10.1128/AEM.69.5.2816-2824.2003.
Ognalaga M, M'Akoué DM, Daudet Medza Mve S, Ondo Ovono P (2017). Effet de la bouse de vaches, du NPK 15 15 15 et de l'urée à 46% sur la croissance et la production du manioc (*Manihot esculenta* Crantz var 0018) au Sud-Est du Gabon (Franceville). Journal of Animal and Plant Sciences 31(3):5063-5073.
Pypers P, Sanginga JM, Kasereka B, Walangululu M, Vanlauwe B (2011). Increased productivity through integrated soil fertility management in cassava–legume intercropping systems in the highlands of Sud-Kivu, DR Congo. Field Crops Research 120(1):76-85.
Saïdou A, Etèka AC, Amadji GL, Hougni DGJ, Kossou D (2012). Dynamique des champignons endomycorhiziens dans les jachères manioc sur sols ferrugineux tropicaux du Centre Bénin. Annales des Sciences Agronomiques 16(2):215-228.
Saïdou A, Kossou D, Azontondé A, Hougni DGJ (2009). Effet de la nature de la jachère sur la colonisation de la culture subséquente par les champignons endomycorhiziens: cas du système 'jachère' manioc sur sols ferrugineux tropicaux du Bénin. International Journal of Biological and Chemical Sciences 3(3):587-597. DOI: 10.4314/ijbcs.v3i3.45330
Schüßler A, Schwarzott D, Walker C (2001). A new fungal phylum, the Glomeromycota : phylogeny and evolution. Mycological Research 105(12):1413-1421. https://doi.org/10.1017/S0953756201005196
Selosse MA, Le Tacon F (1998). The land flora: a phototroph-fungus partnership? Tree 13(1):15-19
Shannon CE, Weaver W (1962). The mathematical theory of communication, university of Illinois Press, Urbana.
Sieverding E (1991). Vesicular-arbuscular mycorrhiza Management. In Tropical Agrosystems. GTZ n° 224 FRG 28
Simpson EH. (1949). Measurement of diversity. Nature 163:688.
Smith SE, Read DJ (1997). Mycorrhizal Symbiosis. 2nd Edition, Academic Press, London.
Strullu DG (1991). Les mycorhizes des arbres et des plantes cultivées. Lavoisier Paris. 3e edit 249 p.

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Identification of potential seed storage protein responsible for bruchid resistance in common bean landraces from Tanzania and Malawi

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Bean bruchids are among the most devastating insect pests of common bean that can inflict huge losses in storage. To identify potential resistance to these pests, screening was performed at Sokoine University of Agriculture. Two resistant landraces were identified, viz Kalubungula and KK25. Recombinant inbred (RI) KSy, KSw and ML populations were created from crosses between Soya × Kalubungula, Soworo × Kalubungula and Nagaga × KK25, respectively. Seed storage proteins were characterized and sequenced in RI population progenies to determine if phenotypic resistance was associated with α-amylase inhibitor – phytohemagglutinin – arcelin (APA) storage proteins. We found no association between the seed storage proteins observed in Kalubungula and its recombinant inbred lines with an APA protein. KK25 and its progenies had Arcelin-5, Leucoagglutinin, Erythroagglutinin and a hypothetical seed storage protein that conditions antibiosis effects as a resistance mechanism. The hypothetical seed storage protein observed in these lines may contribute to enhanced resistance.

Key words: α-amylase, phytohemagglutinin, arcelin, *Acanthoscelides obtectus*, *Zabrotes subfasciatus*, common beans.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is the principal grain legume grown as a major source of protein as well as an important source of income to many farmers in developing countries (Broughton et al., 2003). Common bean is mostly cultivated by small scale farmers who cannot afford the technologies that would enhance their ability to grow and securely store their crop. Consequently, they store the grains on-farm under open conditions where they incur a wide range of postharvest losses including insect pest infestations (Cardona et al., 2005). The most serious storage pests of beans are the bean seed weevils. Two species, namely *Acanthoscelides obtectus* and *Zabrotes subfasciatus* are the major species infesting beans in Tanzania. Their distribution is

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temperature dependent, with Z. *subfasciatus* being confined to warmer areas and *A. obtectus* being confined to cooler areas (Blair et al., 2010). Most of small-scale farmers use pesticidal plants and other indigenous methods for bruchid control of small quantities of seeds (Mbongo et al., 2013; Mutungi et al., 2020; Kusolwa et al., 2013). These methods are less efficient in most cases due to poor availability of pesticidal plants. One of the promising ways of controlling them is using host plant resistance.

Legume seeds contain different compounds that are essential for embryo and seed development as well as for defense against insect pests. Among these compounds are tannins, cyanogenic glucosides, non-protein amino acids and proteins such as protease, α-amylase inhibitor, lectins, chitinases, β-1, 3-glucanases, phaseolin and arcelins. Some of these compounds have nutritional value and others are antinutritional with antibiosis effects against both vertebrate and invertebrate seed consumers (Baldin et al., 2017; Sales et al., 2000). In common bean, the important seed storage proteins that have been identified and characterized are phaseolin, lectins, trypsin inhibitor and lectin-like proteins (Gepts and Bliss, 1988; Lioi et al., 2003).

Phaseolin is the major storage protein in common bean that account for 50% of total seed storage protein and provides essential amino acids to seed consumers (Bollini and Chrispeels, 1978). Lectins and lectin-like proteins are the anti-nutritional seed storage proteins that defend bean seeds against insect pests. The lectin and lectin-like proteins include phytohemagglutinin, arcelins and α-amylase inhibitors. α-amylase inhibitors are a lectin-like proteins that acts as pesticides in bean due to their ability to prevent carbohydrate digestion. These proteins also possess chitinolytic activity by hydrolysing chitinous exoskeletons as well as internal perithropic gut membranes of insect pests (Dayler et al., 2005). Arcelins are also lectin-like proteins made up of polypeptides that are closely related to phytohemagglutinins and α-amylase inhibitors but possess different intrinsic specificities for complex sugars that make it toxic to insect pests (Minney et al., 1990).

Generally, phytohemagglutinin and α-amylase inhibitor are present in wild and cultivated genotypes of common bean while arcelins are found only in wild genotypes of common bean (Sparvoli, et al., 2001). Different arcelin alleles have been introduced from wild common bean as well as tepary bean (*P. acutifolius*) into experimental lines, and some of these lines have been deployed to breeding programs in Africa (Tigist et al., 2021). Accession G40199 of tepary bean is among the wild accessions found to confer high level of resistance to bruchid infestation (Kusolwa and Myers, 2012). A previous survey by Sokoine University Agriculture found two landraces (Kalubungula from Tanzania and KK25 from Malawi) to be resistant to bruchids. The mechanisms of resistance and possible storage proteins related to resistance in these landraces are unknown. This study focused on characterizing and investigating the seed storage proteins related to bruchid resistance in KK25, Kalubungula and derived progenies from KK25 and Kalubungula crosses with susceptible parents.

MATERIALS AND METHODS

Study area

The study was conducted at Sokoine University Agriculture (SUA) and Oregon State University (OSU). Seed was multiplied at SUA whereby after crossing parents and producing F_1 seeds, F_2 seeds were advanced to F_3 generation. After harvest and drying, the grains were stored at -20ºC for two days in order to eliminate any field acquired bruchid infestations. The F_3 seeds were then taken to OSU for laboratory analysis of seed storage proteins.

Plant materials

The bean landraces used in this study included bruchid resistant, red-seeded 'Kalubungula' and 'KK25' from Tanzania and Malawi respectively collected by Bean Bruchid Resistance Project supported by McKnight Foundation at SUA. The susceptible landraces used in crosses were two farmers' preferred varieties 'Soya' and 'Soworo' from Tanzania and crossed to Kalubungula, and 'Nagaga' from Malawi and crossed to KK25. These landraces were part of the major bean collection from farmers' saved seed in major bean growing regions in Tanzania and Chitedze Agriculture Research Station (CARS) in Malawi. The Soya × Kalubungula cross was designated KSy while the Soworo × Kalubungula cross was designated KSw. From the 101 $F_{2:3}$ families obtained, 53 genotypes were from Soya × Kalubungula and 48 from Soworo × Kalubungula. Nagaga × KK25 population was generated by Kananji (2007) and consisted of 3 genotypes in the F_3 generation. These bean genotypes together with the Tanzanian lines were used in protein profiling and sequencing.

Protein extraction

The samples were prepared as described by Osborn et al. (1986) with some modification. Cotyledons of individual seeds were scraped on sandpaper to obtain a fine powder. Ten milligrams (10 mg) of the cotyledon flour of each seed were placed in the microfuge tube and suspended in 200 µl of extraction solution (0.5 M NaCl, pH 2.4), shaken vigorously and vortexed. The mixture was allowed to settle at room temperature for 30 min and centrifuged at 11200 x g for 2 min. Thereafter, 3 µl of the supernatant was mixed in a microfuge tube with 3µl of 0.5 M NaCl pH 2.4 and 6 µl of 2x protein-based sample buffer from BIORAD (65.8 mM tris HCl pH 6.8, 26.3% [w/v] glycerol, 2.1% SDS, 0.5% 2-mercarptoethanol, 0.01% Bromophenol blue). The mixtures were transferred to the Polymerase Chain Reaction (PCR) plates and heated for 5 min in a thermocycler at 94°C to denature the tertiary protein structures into primary structures. Ten microliter (10 µl) of each sample was immediately loaded onto a 15% pre-cast Tris-glycine SDS-PAGE running gel (BIORAD) and was electrophoresed at 200 V constant for 50 min in 1x Laemmli SDS-PAGE running buffer (25 mM Tris-base, 192 mM glycine, 0.1% w/v SDS, pH 8.3). The gels were placed in a sealable plastic container with 100 ml of staining

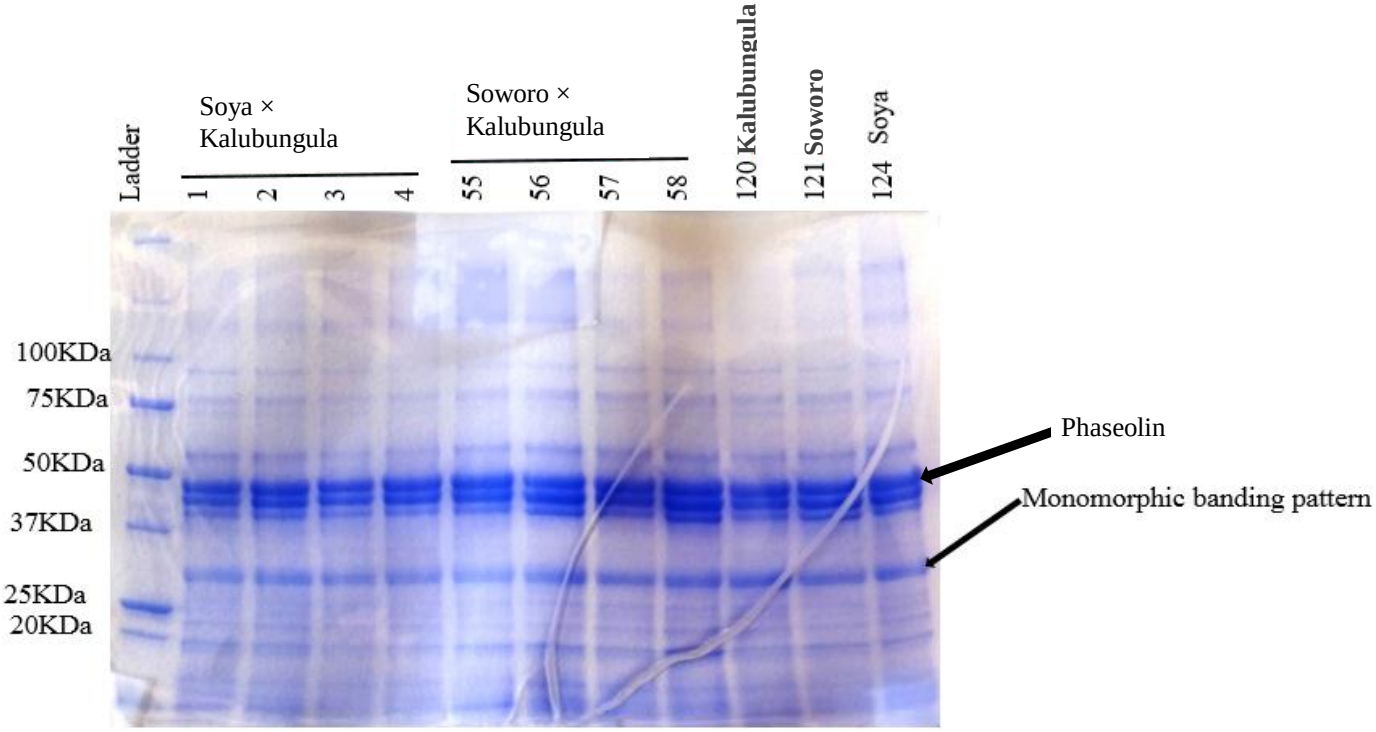

Figure 1. Bean seed storage profile separated on 15% SDS-PAGE gel with an arrow indicating a 33kDa monomorphic banding pattern between parents and progenies. Protein ladder molecular weight on left, lanes 1-6 are Kalubungula × Soya RILs, 55-58 are Kalubungula × Soworo RILs, 120 is Kalubungula, 121 is Soworo and 124 is Soya

solution (40% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue R-250®). They were agitated for 1-2 h on a platform shaker at low speed and destained (40% methanol, 10% acetic acid) overnight. The gels were then washed three times and shaken gently in deionized water for 15 min then placed between pre-wetted cellophane (BIORAD) to dry. The gels were scored with reference to a 33kDa protein subunit based on electrophoretic mobility of size standard proteins. Gels were photographed to retain permanent documentation.

Protein isolation from SDS-PAGE gels and sequencing

We isolated and sequenced protein as described by Kusolwa (2007) with little modification. Unique bands from the gels were excised with a sterile scalpel and cut into 1-mm pieces then placed in microfuge tubes. The gel plugs were washed twice, whereby 200 µl of deionized water were added, soaked for 15 min, vortexed occasionally and centrifuged for 5 min. The liquid was then removed by a pipette after each spin. The gel plugs were washed 2 times to remove Coomassie brilliant blue stain. We added 200 µl of a 50% of acetonitrile mixed with 50% of 50 mM NH_4HCO_3 solution, soaked for 30 min, occasionally vortexed and centrifuged for 5 min. The liquid was then removed by a pipette. To dehydrate the gel plugs, we added 500 µl acetonitrile and left the mixtures to stand, vortexed occasionally until they turned opaque, centrifuged for 5 min, and removed the liquid. The plugs were dried for 30 min in a vacuum centrifuge. Thereafter the plugs were rehydrated by adding 25 mM NH_4HCO_3 containing 20 ng/µl trypsin pH 8.0, chilled on ice for 45 min. Buffer was added to ensure thorough rehydration of the plugs followed by trypsin digestion for six hours in the dark at 37°C. The supernatant was extracted to new microfuge tubes, and the gel plugs were extracted 3 times whereby 50 µl of 50% acetonitrile were added; thereafter the mixtures were vortexed briefly and centrifuged for 5 min. The supernatant was combined in a new centrifuge tube. The samples were submitted to Mass Spectrophotometry Laboratory (MS-MS Lab.) for sequencing at OSU.

RESULTS

Protein profiles

The total seed storage protein from cotyledons of the Tanzanian and Malawian landraces and their progenies was profiled by one-dimension SDS-PAGE gels. There were no polymorphic bands of seed storage proteins observed in Tanzanian landraces, progenies, and susceptible checks. Instead, a monomorphic band was observed in the region of 33kDa, where arcelins and α-amylase inhibitors typically are found (Figure 1). This same band was present in Malawian RILs, but a second polymorphic band was observed at approximately 26kDa in Nagaga x KK25 progenies (Figure 2). This band was present in the KK25 landrace as well but absent from the other Malawian landrace (Nagaga) as well as all

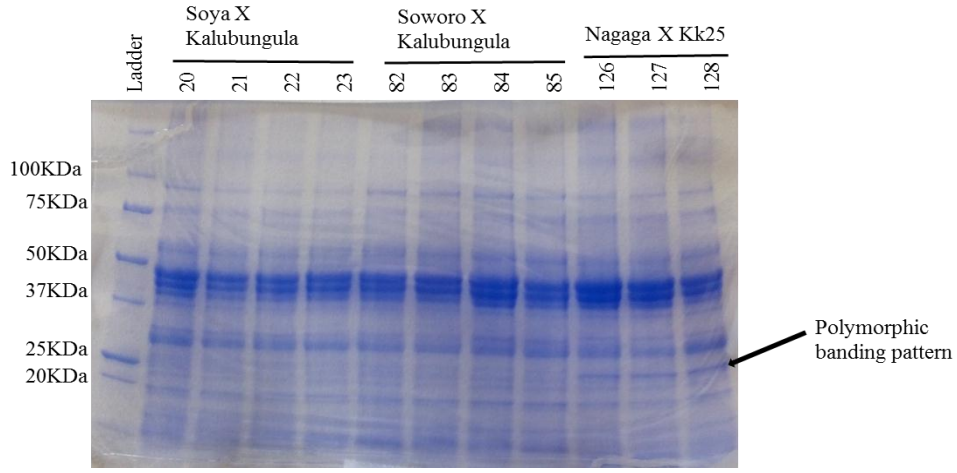

Figure 2. Bean seed storage proteins separated on 15% SDS-PAGE gel. Protein ladder molecular weight in first lane, 20-23 are Soya × Kalubungula RILs, 82-85 are Kalubungula X Soworo RILs, 126-127 are Nagaga X KK25 RILs. An arrow indicates the unique ~26kDa band observed.

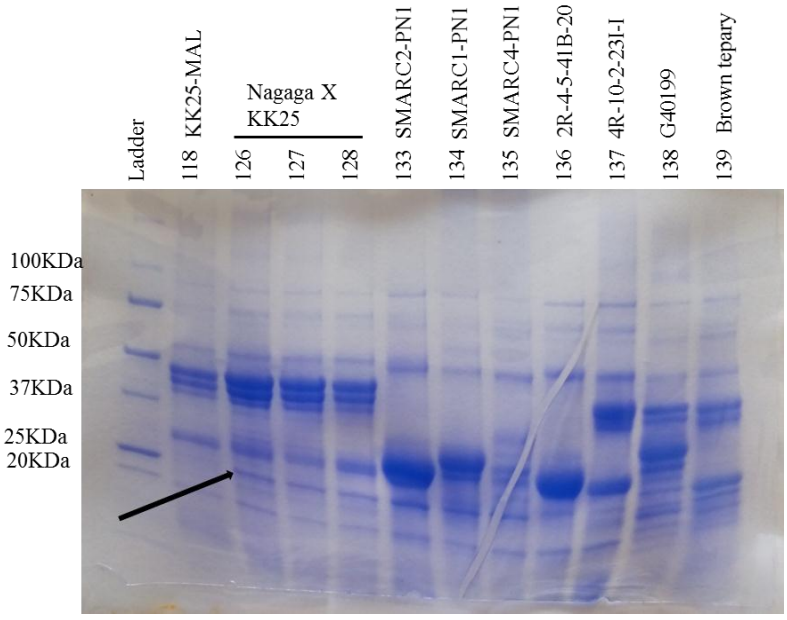

Figure 3. Bean seed storage profile separated on 15% SDS-PAGE gel. Protein ladder molecular weight on left, 118 is KK25- MAL, 126-127 are Nagaga/KK25 RILs, and 133-139 are the arcelin-2, arcelin-1 and arcelin-4, phaseolin null SMARC lines. The last four lanes (136 – 139) show seed storage protein patterns derived from tepary bean and introgressed into common bean (136 – 137) as well as two tepary parents (138 – 139). Arrow points to a band found in KK25 and Nagaga/KK25 RILs that is at a similar location to the arcelin containing SMARC lines.

Tanzanian landraces and progenies. Arcelin-containing bean lines used as bruchid resistant checks were compared for banding pattern with KK25 and progeny, and the banding pattern at 26kDa was similar for the SMARC lines but not the tepary and tepary-derived lines (Figure 3). Both Kalubungula, Nagaga and KK25 and

Table 1. Amino acid sequences produced from 26 kDa protein fragments of Nagaga X KK25 RILs with their matching proteins from NCBI database.

Protein size (kDa)	Observed	Peptide sequence	Match sequence	Matched protein	Reference
		ML3(Nagaga x KK25)			
26kDa	25.58kDa	YTDDMELDDAVHTAILTLKEGFEGQISGK	1	ARC-5	Hamelryck et al. (1996)
	26.35kDa	HSLLGASGEISDFQEILRYLDELILYDNMWDDGNSLGPK	6	PHA-e	Nagae et al. (2016)
	26.35kDa	FNPLWNALVLGGVK	3	PHA-l	Hamelryck et al., 1996
		ML10 (Nagaga x KK25)			
26kDa	26.77kDa	ATFLGEIITSLPTLGAGQSAFK	1	ARC-5	Hamelryck et al. (1996)
26kDa	26.77kDa	IYDYDVYDNLGDPDK	1	PHA-l	Chrispeels and Raikhel. (1991)
26kDa	26.77kDa	LDSQVYGDHTSQITK	-	Hypothetical *Phaseolus vulgaris* protein	This work

their progenies were of the Andean Center of Domestication based on the triplet banding pattern of phaseolin (Figures 1 and 2) located between 37-50kDa.

Amino acid sequencing

Sequencing of the excised protein bands at 26kDa from Nagaga × KK25 recombinant inbred lines revealed the presence of both arcelin and phytohemagglutinin. One of the amino acid sequences from ML10 (indicated by 127 in Figures 2 and 3) did not correspond to any previously reported proteins in the genus *Phaseolus* but appears to be an uncharacterized or hypothetical *Phaseolus vulgaris* protein. The observed amino acid sequences and their corresponding protein matches from NCBI are shown in Table 1.

BLAST search of the observed amino acid (aa) (Figure 4) sequences revealed that the sequences were 100% identical to Phytohemagglutinin sequence of the *P. vulgaris* accession 101A and 1FAT; and with above 96.0% aa identity and similarity to CAJ34351, CAD28838, CAD28674, respectively. Two sequences from ML3 (Nagaga x KK25) also matched sequence to leucoagglutinin with matching ranging from one to three matches. One sequence resembled the erythroagglutinin type of phytohemagglutinin with six sequence matches (Figures 5 and 6). Leucoagglutinin (PHA-l) (Figure 4) from Tanzania genotypes had above 96.4% similarity score with 1FAT-A, CAJ34351, CAD28838, CAD286774 PHA-l from *P. vulgaris*, and distantly similar to *P. acutifolius and P. costaricensis* by 93.1 and 92.3% respectively (Supplementary Figures 1 and 2). The erythroagglutinin (PHA-e) sequence from ML3 (Figure 5) had higher amino acid identity with Erythroagglutinating phytohemagglutinin sequences from GenBank, including 5AVA_A, XP_007152771, P05088, AHB17899, and CAD28837 with 100, 99.6, 98.9, 98.2 and 97.8%, respectively (Supplementary Figures 3 and 4). The observed similarity indicate there is little variation between the observed amino acid sequences compared to the reference protein sequence in NCBI while the sequence matching to arcelin-5 from ML3 and ML10 (Figure 6) had 'aa' identity of 100, 99.6 and 96.2% with sequences of 1IOA_a, Q42460.2, and Q41116.1, respectively, from GenBank (Supplementary Figures 5 and 6).

DISCUSSION

Protein characterization from KSy and KSw populations showed the presence of a monomorphic band at approximately 33kDa. This band that was also observed in susceptible bean genotypes checks had a relatively similar molecular size like arcelins-like proteins. However, the banding pattern was different from that typical of the APA proteins. This and the fact that both susceptible and resistant parents had the band indicated that the near-33kDa storage proteins in these bean lines was not likely related to resistance to bruchids. Kananji (2007) identified bean landraces (KK35, KK73 and KK90) that lacked arcelins but exhibited resistance. In an

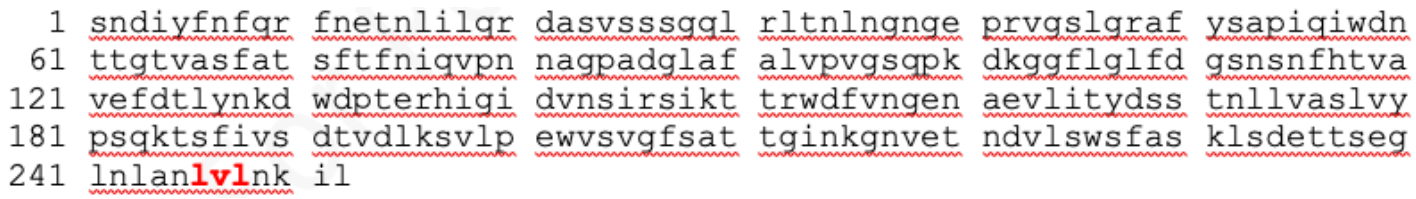

Figure 4. Amino acid sequences of the 25kDa protein band from ML3 and ML10, the Nagaga × KK25 RIL's matched to leucoagglutinin (PHA-l) of *Phaseolus vulgaris.* Matched sequences are shown in red colour and bold.

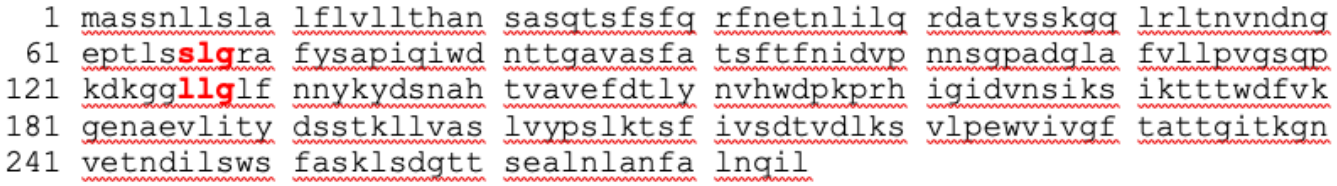

Figure 5. Amino acid sequences of the 25KDa protein band from ML3 and ML10, the Nagaga × KK25 RIL's matched to erythroagglutinin (PHA-e) of *Phaseolus vulgaris.* Matched sequences are shown in red colour and bold.

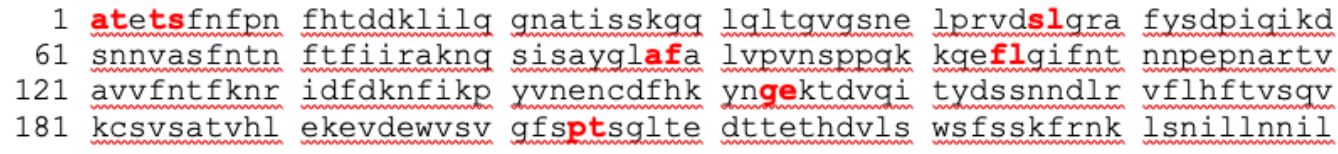

Figure 6. Amino acid sequences of the 25KDa protein band from ML3 and ML10, the Nagaga × KK25 RIL's matched to arcelin-5 of *Phaseolus vulgaris.* Matched sequences are shown in red color and bold.

experiment with those lines, increased numbers of *A. obtectus* adult bruchids emerged when the seed coats were removed, suggesting that resistance was conferred by the seed coat. He concluded that the seed coat acted as a physical and/or chemical barrier to attacks by bruchids.

Sales et al. (2000) found that the presence of vicillins and legumins in the seed coat of broad bean *Vicia faba* deterred development of the first instar larvae of cowpea weevils *Callosobruchus maculatus*. Silva et al. (2004) provided supporting evidence that vicillins or phaseolin present in the seed coat of *P. vulgaris* were detrimental to *C. maculatus* development. They found that the seed coat thickness was not important but high vicillins concentration in the seed coat was an important factor for resistance. Lattanzio et al. (2005) reported that high concentration of tannins in undamaged seeds of cowpea conferred a biochemical defense that deterred, poisoned, or starved bruchid larvae. We believe that the seed coat confers resistance in the Tanzanian Kalubungula landrace and its progenies, but further studies are needed to confirm this assertion.

Presence of seed storage proteins at 26kDa in Nagaga × KK25 and its recombinant inbred lines suggested that resistance was storage protein based. Amino acid sequencing from the trypsin digested protein fragments from the 26kDa band revealed the presence of trace amount of protein peptides corresponding to arcelin-5, like that observed by Hamelryck et al. (1996), phytohemagglutinin-l observed by Chrispeels and Raikhel (1991) and phytohemagglutinin-e of *P. vulgaris* similarly observed by Nagae et al. (2016). These protein peptides have a special property of binding glycan in a complex structure of a back-fold conformation which affects activities of glycosyltransferases enzymes and localization of carrier glycoproteins in an insect. These seed storage proteins are known to defend common bean against bruchids by interacting with the glycoprotein, interfering with carbohydrate digestion, and binding to the intestinal cells of insect. It is possible that the mechanism of

resistance in these Malawian landraces is antibiosis conferred by presence of an arcelin/ phytohemagglutinin-like protein though other factors might be involved. Kusolwa and Myers (2012) observed the presence of multiple variants of the antibiosis seed storage proteins of the complex APA locus in progenies of crosses between wild tepary bean (*Phaseolus acutifolius*) accession G40199 highly resistant to bean bruchids and a susceptible common bean cultivar (ICA Pijao). Our protein peptide sequencing demonstrated low and weak sequence matching (1-6 match) with reference proteins in the databank, which suggests that the protein observed in KK25 and progeny may be quite novel and different from previously characterized proteins of similar size. This novel storage protein may not be the only source of resistance in these line and other factors may have contributed to the observed resistance. The presence of an uncharacterized sequence in one of the KK25 progeny may be of importance to breeders and may contribute to new knowledge about seed storage proteins and bruchid resistance.

Conclusion

Intriguing is the lack of seed storage proteins conferring bruchid resistance in the Tanzanian lines. We recommend that the role of the seed coat and its potential to mitigate bruchid damage be assessed. Further investigation is needed to determine if the unique 26kDa proteins are responsible for bruchid resistance in Malawian lines or if there are other factors involved. Further studies are needed to better define the storage proteins observed in Malawian lines.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

FUNDING AND ACKNOWLEDGEMENTS

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REFERENCES

Baldin EL, Lara FM, Camargo RS, Pannuti LE (2017). Characterization of resistance to the bean weevil Acanthoscelides obtectus Say, 1831 (Coleoptera: Bruchidae) in common bean genotypes. Arthropod-Plant Interactions 11(6):861-870. DOI 10.1007/s11829-017-9540-6

Blair MW, Munoz C, Hector FB, Jose F, Juan MB, Cardona C (2010). Genetic mapping of microsatellite markers around the arcelin bruchid resistance locus in common bean. Theoretical and applied genetics, 121(2):393-402.

Broughton, WJ, Hernandez G, Blair M, Beebe S, Gepts P, Vanderleyden J (2003). Bean (*Phaseolus* spp.)-model food legumes. Plant and Soil Journal 252:55-128. https://doi: 10.1007/s00122-010-1318-5

Broughton WJ, Hernandez G, Blair M, Beebe S, Gepts P, Vanderleyden J (2003). Bean (*Phaseolus* spp.) model food legumes. Plant and Soil Journal 252:55-128. https://doi.org/10.1023/A:1024146710611

Bollini R, Chrispeels MJ (1978). Characterization and subcellular localization of vicilin and phytohemagglutinin, the two major reserve proteins of *Phaseolus vulgaris* L. Planta 142(3):291-298. https://doi.org/10.1007/BF00385080

Cardona C, Valor JF, Mejia-Jiménez A, Beebe S, Tohme J (2005). Developing germplasm with resistance to pests: *Zabrotes*, *Acanthoscelides* - bruchids. CIAT- Annual Report pp. 53-59.

Chrispeels MJ, Raikhel NV (1991). Lectins, lectin genes, and their role in plant defense. Plant Cell 3(1):1-9. https://doi.org/10.1105/tpc.3.1.1

Dayler CS, Mendes PA, Prates MV, Bloch C, Franco OL, Grossi-de-Sá, MF (2005). Identification of a novel bean α-amylase inhibitor with chitinolytic activity. FEBS Letters 579(25):5616-5620. https://doi.org/10.1016/j.febslet.2005.09.030

Gepts P, Bliss FA (1988). Dissemination pathways of common bean (*Phaseolus vulgaris*, Fabaceae) deduced from phaseolin electrophoretic variability. II. Europe and Africa. Economic Botany 42(1):86-104. https://doi.org/10.1007/BF02859038

Hamelryck TW, Dao-Thi MH, Poortmans F, Chrispeels MJ, Wyns L, Loris R (1996). The crystallographic structure of phytohemagglutinin-L. Journal of Biological Chemistry 271(34):20479-20485. https://doi.org/10.1074/jbc.271.34.20479

Kananji GAD (2007). A Study of Bruchid Resistance and Its Inheritance in Malawian Dry Bean Germplasm. PhD. Thesis, University of KwaZulu-Natal, Republic of South Africa. https://researchspace.ukzn.ac.za/handle/10413/716

Kusolwa PM, Myers JR (2012). Peptide sequences from seed storage proteins of tepary bean (*Phaseolus acutifolius*) accession G40199 demonstrate the presence of multiple variants of APA proteins. International Journal of Biochemistry and Biotechnology 1(1):012-018.

Kusolwa PM, Mwatawala MW, Mgembe ER, Mwaitulo S, Muhamba TG, Mwakalobo ABS, Mndolwa EJ (2013). Efficacy of selected botanical insecticides for reduced post-harvest losses of common beans by bruchid species in farmers' households in Tanzania. Harnessing pesticidal plant technologies for improved livelihoods. The First International Conference on Pesticidal Plants. January 21 -24 ICIPE Nairobi Kenya.

Kusolwa PM (2007). Breeding for bruchid resistance in common bean (*Phaseolus vulgaris* L.): Interspecific introgression of lectin-like seed proteins from tepary bean (*P. acutifolius* A. Gray), genetic control and bruchid characterization. Ph.D. Thesis, Oregon State University, Corvallis, Oregon, USA.

Lattanzio V, Terzano R, Cicco N, Cardinali A, Venere DD, Linsalata V (2005). Seed coat tannins and bruchid resistance in stored cowpea seeds. Journal of the Science of Food and Agriculture 85(5):839-846. https://doi.org/10.1002/jsfa.2024

Lioi L, Sparvoli F, Galasso I, Lanave C, Bollini R (2003). Lectin related resistance factors against bruchids evolved through a number of duplication events. Theoretical and Applied Genetics 107(5):814-822. https://doi.org/10.1007/s00122-003-1343-8

Mbongo DB, Mwatawala MW, Kusolwa PM (2013). Efficacy of pesticidal plants in controlling bruchids (Coleoptera: Bruchidae) in common beans (*Phaseolus vulgaris* L.). Harnessing pesticidal plant technologies for improved livelihoods. The First International Conference on Pesticidal Plants. January 21 -24 ICIPE Nairobi Kenya.

Minney BHP, Gatehouse AMR, Dobie P, Dendy J, Cardona C, Gatehouse, JA (1990). Biochemical bases of seed resistance to *Zabrotes subfasciatus* (bean weevil) in *Phaseolus vulgaris* (common

bean); a mechanism for arcelin toxicity. Journal of Insect Physiology 36:757-767. https://doi.org/10.1016/0022-1910(90)90049-L

Mutungi C, Chamwilambo M, Masanja S, Massam C, Wayda P, Tungu J, Gaspar A, Bekunda M, Abass A (2020). Quality and storability of common beans in small-holders farm stores in Northern Tanzania: A multivariate analysis of agro-location, variety, and storage method effects. Journal of Stored Products Research 89:101723. https://doi.org/10.1016/j.jspr.2020.101723

Nagae M, Kanagawa M, Morita-Matsumoto K, Hanashima S, Kizuka Y, Taniguchi N, Yamaguchi Y (2016). Atomic visualization of a flipped-back conformation of bisected glycans bound to specific lectins. Scientific Reports 6(1):1-11. https://doi.org/10.1038/srep22973

Osborn TC, Blake T, Gepts P, Bliss FA (1986). Bean arcelin. Theoretical and Applied Genetics 71(6):847-855. https://doi.org/10.1007/BF00276428

Sales MP, Gerhardt IR, Grossi-de-Sá MF, Xavier-Filho J (2000). Do legume storage proteins play a role in defending seeds against bruchids? Plant Physiology 124(2):515-522. https://doi.org/10.1104/pp.124.2.515

Sparvoli F, Lanave C, Santucci A, Bollini R, Lioi L (2001). Lectin and lectin-related proteins in lima bean (*Phaseolus lunatus* L.) seeds: biochemical and evolutionary studies. Plant Molecular Biology 45(5):587-597. https://doi.org/10.1023/A:1010647310311.

Silva LB, Sales MP, Oliveira AE, Machado OL, Fernandes KV, Xavier-Filho J (2004). The seed coat of *Phaseolus vulgaris* interferes with the development of the cowpea weevil [*Callosobruchus maculatus* (F.) (Coleoptera:Bruchidae)]. Anais da Academia Brasileira de Ciências 76(1):57-65.https://doi:10.1590/s0001-37652004000100006

Tigist SG, Raatz B, Assefa A, Melis R, Sibiya J, Keneni G, Mukankusi C, Fenta B, Ketema S, Tsegaye D (2021). Introgression of bruchid (*Zabrotes subfasciatus*) resistance into small red common bean (Phaseolus vulgaris) background and validation of the BRU_00261 (snpPV0007) resistance marker. Plant Breeding 140(6):1081-1089. DOI: 10.1111/pbr.12969.

SUPPLEMENTARY FIGURES

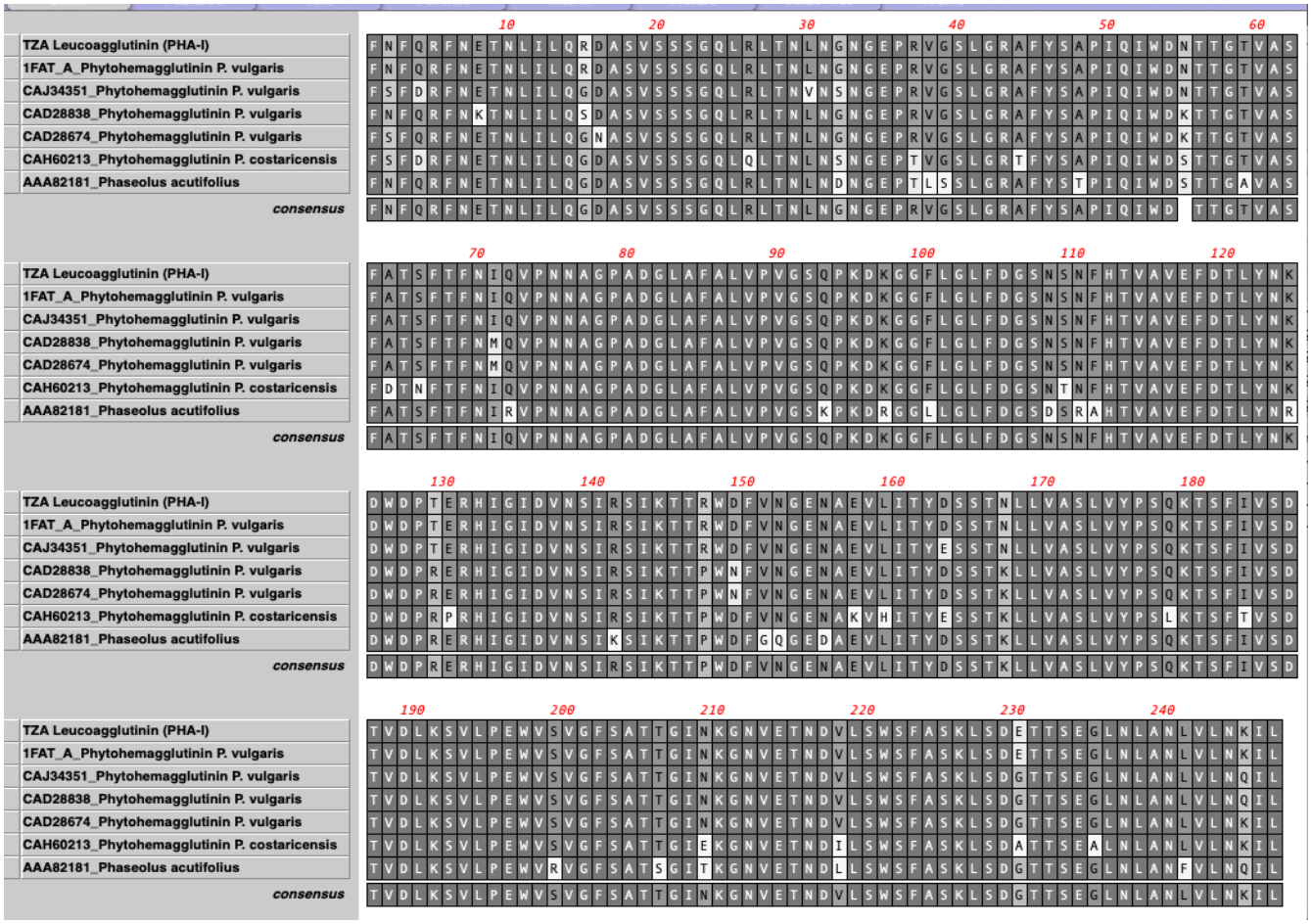

Figure 1. Amino acid alignment of TZA Phytohemagglutinin leucoagglutinin (PHA-l) with GenBank deposited Phytohemagglutinin sequences.

```
                                                                ** Identity Scores (%) **
                                                TZA Leu  1FAT_A_  CAJ3435  CAD2883  CAD2867  CAH6021  AAA8218
                                                coagglu  Phytohe  1_Phyto  8_Phyto  4_Phyto  3_Phyto  1_Phase
                                                tinin (  magglut  hemaggl  hemaggl  hemaggl  hemaggl  olus ac
                                                PHA-l)   inin P.  utinin  utinin   utinin   utinin   utifoli
                                                          vulgar  P. vulg  P. vulg  P. vulg  P. cost  us
TZA Leucoagglutinin (PHA-l)                       100.0    100.0     96.8     96.0     96.0     90.3     87.9
1FAT_A_Phytohemagglutinin P. vulgaris             100.0    100.0     96.8     96.0     96.0     90.3     87.9
CAJ34351_Phytohemagglutinin P. vulgaris            98.0     98.0    100.0     94.7     95.1     91.5     87.4
CAD28838_Phytohemagglutinin P. vulgaris            96.8     96.8     96.0    100.0     98.0     89.9     88.7
CAD28674_Phytohemagglutinin P. vulgaris            96.4     96.4     96.8     98.4    100.0     91.1     88.3
CAH60213_Phytohemagglutinin P. costaricensis       92.3     92.3     93.9     92.7     93.5    100.0     84.6
AAA82181_Phaseolus acutifolius                     93.1     93.1     93.1     93.9     93.9     91.1    100.0
                                                ** Similarity Scores (%) **

**Similarity Scores(s) are shown below the diagonal (x) with Identity Scores(I) above**
  a b c d e
a x i i i i
b s x i i i
c s s x i i
d s s s x i
e s s s s x
```

Figure 2. Similarity matrix of TZA Phytohemagglutinin leucoagglutinin (PHA-l) sequences with GenBank deposited Phytohemagglutinin sequences.

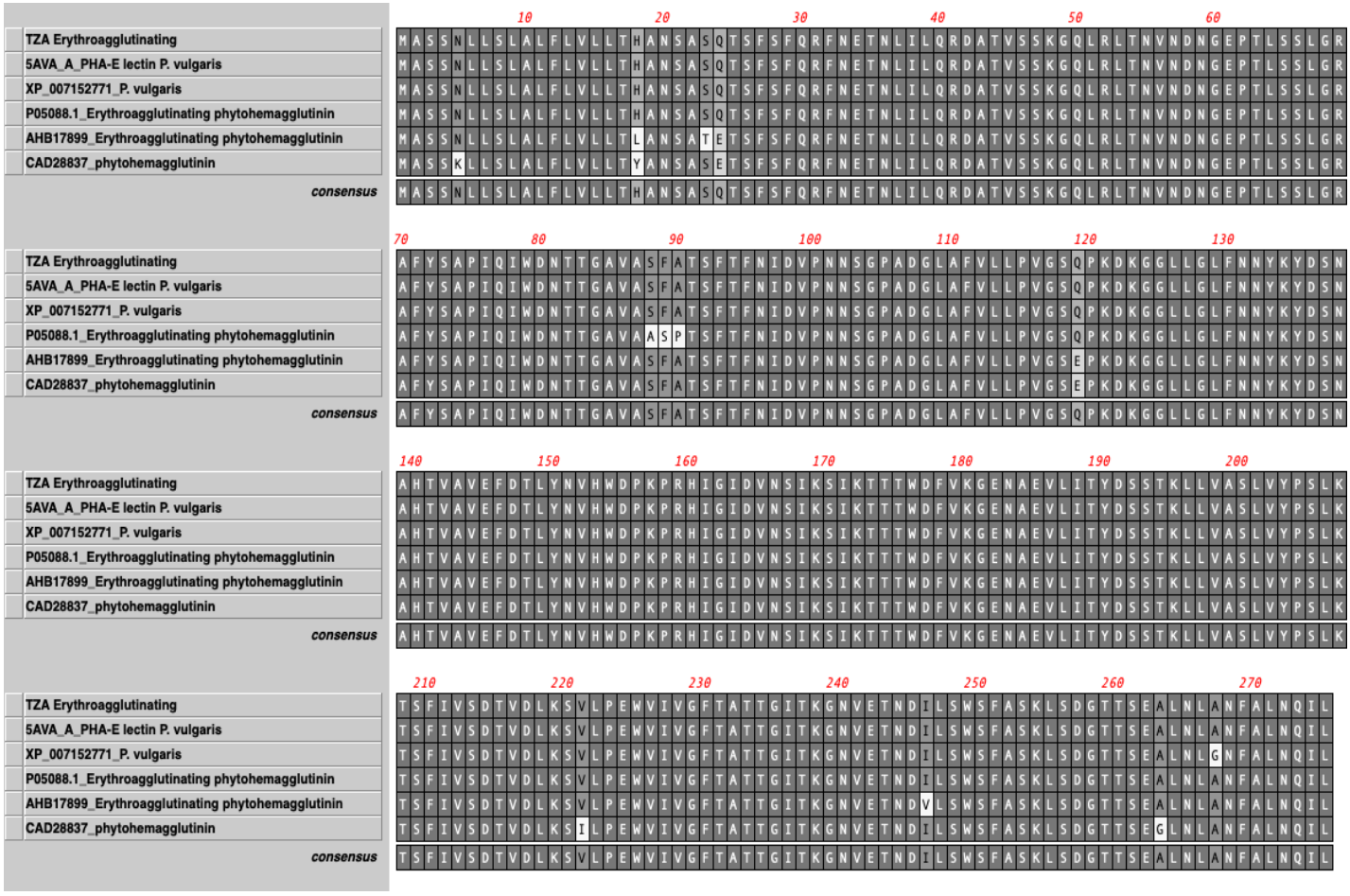

Figure 3. Amino acid alignment of TZA Phytohemagglutinin erythroagglutinin (PHA-e) with GenBank deposited erythroagglutinin sequences.

```
                                                    ** Identity Scores (%) **
                                                TZA Ery  5AVA_A_  XP_0071  P05088.  AHB1789  CAD2883
                                                throagg  PHA-E l  52771_P  1_Eryth  9_Eryth  7_phyto
                                                lutinat  ectin P  . vulga  roagglu  roagglu  hemaggl
                                                ing (PH  . vulga  ris      tinatin  tinatin  utinin
                                                A-e)     ris              g phyto  g phyto
TZA Erythroagglutinating (PHA-e)                 100.0    100.0     99.6     98.9     98.2     97.8
5AVA_A_PHA-E lectin P. vulgaris                  100.0    100.0     99.6     98.9     98.2     97.8
XP_007152771_P. vulgaris                          99.6     99.6    100.0     98.5     97.8     97.5
P05088.1_Erythroagglutinating phytohemagglutinin  99.3     99.3     98.9    100.0     97.1     96.7
AHB17899_Erythroagglutinating phytohemagglutinin  99.6     99.6     99.3     98.9    100.0     97.8
CAD28837_phytohemagglutinin                       98.9     98.9     98.5     98.2     98.9    100.0
                                               ** Similarity Scores (%) **

**Similarity Scores(s) are shown below the diagonal (x) with Identity Scores(I) above**
  a b c d e
a x i i i i
b s x i i i
c s s x i i
d s s s x i
e s s s s x
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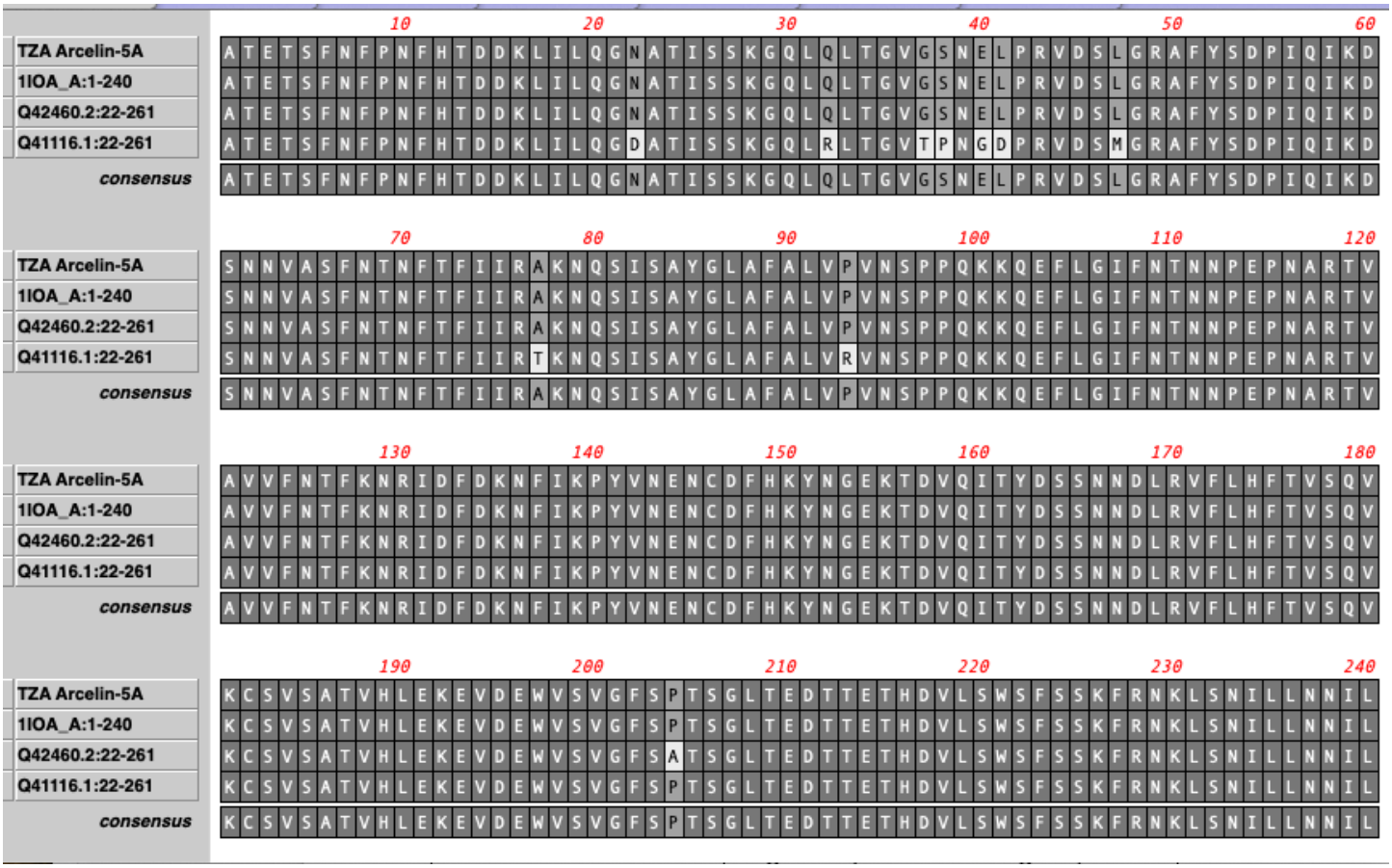
Figure 4. Similarity matrix of TZA Phytohemagglutinin erythroagglutinin (PHA-e) sequences with GenBank deposited erythroagglutinin sequences.



Supplementary Figure 5. Amino acid alignment of TZA Arcelin-5A with GenBank deposited Arcelin sequences.

```
                    ** Identity Scores (%) **
                 TZA Arc   1IOA_A:   Q42460.   Q41116.
                 elin-5A   1-240     2:22-26   1:22-26
                                     1         1
TZA Arcelin-5A     100.0     100.0     99.6      96.2
1IOA_A:1-240       100.0     100.0     99.6      96.2
Q42460.2:22-261     99.6      99.6    100.0      95.8
Q41116.1:22-261     97.5      97.5     97.1     100.0
               ** Similarity Scores (%) **

**Similarity Scores(s) are shown below the diagonal (x) with Identity Scores(I) above**
  a b c d e
a x i i i i
b s x i i i
c s s x i i
d s s s x i
e s s s s x
```

Supplementary Figure 6. Similarity matrix of TZA Arcelin-5A sequences with GenBank deposited Arcelin sequences.

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