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Survey Report
Neofusicoccum parvum Causing *Eucalyptus* Canker and Die-back Diseases in Ethiopia

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Abstract

Background and Objectives: *Botryosphaeriaceae* is comprised of fungal species that have a wide geographic distribution and extensive host range, including *Eucalyptus* spp. A large number of species have been reported from *Eucalyptus* spp. *Neofusicoccum* sp. are among the most aggressive members of *Botryosphaeriaceae*. The main objective of this research was to expand knowledge on *Eucalyptus* disease in Ethiopia by providing reliable information help for effective management of the diseases in the country. **Materials and Methods:** Survey of plantation forest diseases was made in Ethiopia during 2016 and 2017 growing seasons. Diseased samples with clear canker and dieback symptoms were collected from *E. globulus* and *E. grandis* plants surface sterilized and cultured on malt extract agar for identification. Four leaves/branch, 4 branches and one increment core from one tree/species were sampled for identification. Isolates were characterized based on the molecular sequence data, colony morphology and anamorph structures. **Results:** Fungal cultures developed abundant, aerial mycelium that became dark grey after 2-3 days. Black, globular pycnidia were formed on the surface of branch and main stem cultured using moist chamber method after 2 weeks. Conidia were aseptate, becoming septate or darker with age and fusiform. **Conclusion:** The result of cultural, conidial morphology and molecular sequence analysis data of the fungus revealed the diseases symptoms observed on the *Eucalyptus* tree species were due to *Neofusicoccum parvum*. This study confirms that *Neofusicoccum* sp. are currently among fungal pathogens seriously affecting the quality and quantity of *Eucalyptus* tree plantation in Ethiopia.

Key words: *Eucalyptus* disease, fungus, *Botryosphaeriaceae*, survey, identification, morphology, DNA

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The *Botryosphaeriaceae* is comprised of fungal species that have a wide geographic distribution and extensive host range, including *Eucalyptus* spp. (*Myrtaceae*)[1]. These fungi are latent and opportunistic pathogens that occur as endophytes in symptomless plant tissues and they can cause rapid disease development when plants are exposed to unsuitable environmental conditions such as drought, freezing, hot or cold winds, hail wounds or damage caused by insects or other pathogens[2].

Species of the *Botryosphaeriaceae* are pathogens of many plantation trees, including species of *Acacia*, *Eucalyptus* and Pinus[3]. Species of the *Botryosphaeriaceae* cause a wide variety of symptoms on all parts of *Eucalyptus* trees and on trees of all ages, but are mostly associated with cankers and dieback followed by extensive production of kino, a dark-red tree sap and in severe cases mortality of trees[4,5].

The *Botryosphaeriaceae* that cause diseases on *Eucalyptus* trees spp. in different parts of the world includes, *Botryosphaeria mamane*, *Neofusicoccum andium*, *Neofusicoccum parvum*, *N. pseudofusicoccum*, *N. stromaticum*, *Lasiodiplodia theobromae*, *L. crassispora* and *Lasiodiplodia pseudotheobromae*[6]. *Neofusicoccum* was introduced by Crous *et al.*[7] to accommodate species morphologically similar to, but phylogenetically distinct from *Botryosphaeria*. As in other parts of the world, pest and pathogens are rapidly emerging as one of the greatest threats to *Eucalyptus* plantation in Ethiopia.

Alemu *et al.*[4] reported that most well-known disease of *Eucalypt*s in Ethiopia is caused by *Mycosphaerella* and *Botryosphaeriaceae* species. Earlier reports of these fungi represent species such as *Botryosphaeria parvum* and *Botryosphaeria dothidea* but DNA sequence comparisons have now shown that many of the early identification were possibly incorrect. There is, however, little is known about current status of diseases on this host and this is a key motivation to expand knowledge on *Eucalyptus* disease in Ethiopia.

MATERIALS AND METHODS

Survey of plantation forest diseases was made in Oromia and Amhara regional states of Ethiopia during 2016 and 2017 growing seasons. A canker and die-back disease was observed on *Eucalyptus globulus* and *Eucalyptus grandis* in Arsi Negelle and Illubabor zones of Oromia and Tarmaber and keyit of Amhara, regional states.

Diseased samples were collected from *E. globulus* and *E. grandis* plants using the procedures of Mohali *et al.*[3], Iturritxa *et al.*[8] and Pillay *et al.*[6] during the 2016 and 2017 growing seasons. Four leaves per branch, 4 branches and one increment core from one tree/species were sampled for identification. The plant tissues were placed in paper bags and transferred to the laboratory for further processing. Plant tissue was surface disinfected in 70% ethanol for 30 sec, after which it was rinsed in sterile water for 1 min. Pieces of tissue were cut from the specimens and placed on 2% malt extract agar (MEA). The plates were incubated at 25°C for approximately 10 days. Samples from the stem and branches were also cut and placed in moist chamber for fruiting body formation. Pycnidia and conidia produced were mounted on microscope slides, examined under the microscope and pictures were taken. Fruiting bodies were aseptically transferred to MEA for pure culture isolation. Growth of endophytic fungi from the plant tissue was checked daily to isolate slow growing fungi before they were overgrown by those that are faster growing. Plant tissues that did not show any initial fungal growth were monitored for a month. Isolates were characterized based on colony morphology and anamorph structures. Single-conidial and ascospores isolate of *Botryosphaeriaceae* were made and used for DNA extraction and sequencing at Centre for Agriculture and Biosciences International (CABI), UK.

DNA isolation, amplification and identification: Molecular assays are carried out on each sample using nucleic acid as a template at CABI. A proprietary formulation [microLYSIS®-PLUS (MLP), Microzone, UK] is subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA). Following DNA extraction, polymerase chain reaction (PCR) is employed to amplify copies of the rDNA *in vitro*. The quality of the PCR product is assessed by undertaking gel electrophoresis. Sequencing reactions are undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilises fluorescent labelling of the chain terminator ddNTPs, to permit sequencing. AB 3130 Genetic Analyzer and sequencing undertaken. Following sequencing, identifications are undertaken by comparing the sequence obtained with those available from the European Molecular Biology Laboratory (EMBL) database via the European Bioinformatics Institute (EBI).

The pathogenicity of isolates obtained from *Eucalyptus* trees was tested following the methodology of the method described by Pérez *et al.*[9] using the mycelial-plug technique

on the corresponding seedlings of the trees. The region of site to be inoculated was surface-sterilized with 70% ethyl alcohol and wounded on the stems of 4-month-old plants of a *Eucalyptus grandis* and *Eucalyptus globulus* clone, approximately 10 cm above the soil between 2 nodes using a 5 mm-diameter cork borer. Mycelia plugs from pure cultures grown for a week on 2% MEA were taken using the same cork-borer size and placed into the wound with the mycelial surface and sealed. Plugs of sterile MEA were inoculated into stems of 3 trees as controls. Inoculated trees were maintained in the greenhouse and followed for symptom development.

RESULTS AND DISCUSSION

Symptoms observed on the diseased *Eucalyptus* plants were similar to those reported by Iturritxa *et al.*[8], die-back of shoots and branches, lesion and canker formation on the stems and brown and red exudates of Kino on stems and branches (Fig. 1a-b). Pure isolates were established from cultures. These isolates were initially selected based on culture characteristics typical of the *Botryosphaeriaceae*, namely fast-growing fluffy white aerial mycelium becoming grey to black with grey to indigo-grey or black pigment visible from the reverse side of petri dishes. Fungal isolates developed abundant, aerial mycelium that became dark grey after 2-3 days (Fig. 1d). Black, globular pycnidia (fruiting bodies) were formed on the surface of branch and main stem cultured using moist chamber method after 2 weeks (Fig. 1c). Conidia were aseptate, becoming septate or darker with age, thin walled, granular surface golden colure and fusiform (Fig. 1e, f). Microscopic and macroscopic features of culture and conidia morphologies indicate the fungal isolates to be the genus *Neofusicoccum* which coincide with the results of Pillay *et al.*[6] and Lopes *et al.*[10]. Pure cultures of the genus were sent to CABI for molecular identification to the species level. The result of cultural, conidial morphology and sequence analysis the fungus showed that the symptoms observed on the *Eucalyptus* tree species were due to *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and A.J.L. Phillips. The result of pathogenicity tests indicates all the fungal isolates showed diseases symptoms in the entire inoculated clone forming lesions. Re isolation and identification of the fungus confirm *Neofusicoccum parvum* was the cause for the diseased symptoms observed in *Eucalyptus* tree plants.

This study indicates fungal a species *Neofusicoccum parvum* was the cause for *Eucalyptus* tree diseases symptoms observed in plantation sites of Ethiopia. Research findings of Phillips *et al.*[11] and Lopes *et al.*[10] described the *N. parvum* species as widely distributed and reported from 90 hosts in 29 countries and commonly isolated from *Eucalyptus* tree plant hosts. The morphological study of the fungal culture indicates Asci clavate, 8-spored, bitunicate, pycnidia erumpent through the bark, globose, with a short, conical papilla, dark brown to black, smooth, thick-walled while Conidia were observed ellipsoidal with apex round and base flat, unicellular, hyaline, old conidia becoming 1-2-septate hyaline, or light brown with the middle cell darker than the terminal cells, this morphological description agrees with the description of Crous *et al.*[7].

Isolation of DNA and investigation of the ITS rDNA sequence obtained from the pure culture showed top matches at 99-100% identity to multiple sequences of *Neofusicoccum parvum*. The best matches included the sequence of *Neofusicoccum parvum* (JQ772034) which is similar to the results of the study of Abdollahzadeh *et al.*[12].

The finding of cultural, conidial morphology and sequence analysis of the fungus indicates that fungal species of *N. parvum* were the cause for die-back of shoots and branches, lesion and canker, diseases in *E. globulus* and *E. grandis* plants which agree with the findings of Vu *et al.*[13]. *Neofusicoccum parvum* is probably the species within the genus with the widest geographic distribution, host range and proven ability to cause disease[11,14]. Several studies show the fungal species has been found associated with many forest species such as conifers, Syzygium and *Eucalyptus* plants[6,8,15,16]. To the best of our knowledge, this is the first report of *N. parvum* causing *Eucalyptus* canker and dieback diseases in Ethiopia. Slippers *et al.*[17] reported that family of *Botryosphaeriaceae* including the genus *Neofusicoccum* were endophytes, opportunistic and have emerged as important pathogens of *Eucalyptus* in area where these trees are native, as well as where they have been introduced into new environments. Several research findings reveal the increasing findings of new species and importance of the *Botryosphaeriaceae* in the light of global climate change[2,17]. Mohali *et al.*[3] also reported *Neofusicoccuum* species such as *Neofusicoccum parvum*, *N. australis*, *N. eucalyptorum* and *N. eucalypticola* are the dominant species associated with canker and die-back diseases of *Eucalyptus*. This study shows findings of fungal pathogens that cause canker and diebacks diseases in limited plantation sites of the country; more plantation sites will needs to be assessed help to have more comprehensive information on the status of diseases, casual pathogens and study of appropriate management options at the national level.

Fig. 1(a-f): *Eucalyptus* plants from survey sites, showing (a) Advanced stem canker, (b) Exudation of kino on stem with severe canker, (c) Fruiting bodies of *Neofusicoccum parvum* growing on the branching stems, (d) Cultures of the isolates on the MEA and (e, f) Conidial appearance on the MEA

CONCLUSION

The presence of fungal species on wide diversity of hosts confirms that *Neofusicoccum* species are opportunistic fungi that can potentially colonize and cause diseases in most plants at stressed conditions. This study confirms that *Neofusicoccum* species are currently among fungal pathogens seriously affecting the quality and quantity of *Eucalyptus* tree plantation in Ethiopia. Accurate identification of the *Neofusicoccum* to species level needs advanced DNA sequencing technology in addition to conventional morphological approach.

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SIGNIFICANCE STATEMENT

This study discovered the fungal pathogen, *Neofusicoccum parvum* causing serious damage to *Eucalyptus* trees plants using advanced DNA technology for the first time. The finding from this study provides reliable preliminary and baseline information for further research and management studies of the diseases in the country.

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