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Research Article
Eucalyptus Globulus (*E. globulus*) Leaf Spot and Stem Canker Diseases Due to *Phoma* spp. In North and North West Ethiopia

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Abstract

Background and Objective: *Eucalyptus globulus* species are exotic plantations in Ethiopia covering one-third of the total plantation area of the country mainly for construction and resources. Inspite of this importance the success of growth and development of *E. globulus* plantations is currently influenced by fungal pathogens causing leaf spot and stem canker diseases. The main objective of this study was to investigate the diversity and distribution of fungal species associated with plantations causing leaf spot and stem canker diseases, so that management options to be studied for effective control and prevention of the diseases. **Methodology:** Twelve plantation sites were purposivly sampled and surveyed in Amhara and Tigray regions of Ethiopia from May-June, 2016 for disease symptoms. Samples with clear diseases symptoms were collected cut to desired size, surface sterilized and cultured on the potato dextrose agar media for morphological study. Twenty pure cultures of fungi colonies were isolated and morphologically characterized to genus and species level for identification. The collected data were summarized, ranked and expressed using simple descriptive statistics of SPSS version 16.0 and SAS Version 9.0 procedures. **Results:** Based on the results, among the isolates 60% (12 out of 20) isolates were with long chains or solitary, unicellular or multicellular, alternarioid chlamydospores while the rest of isolates were with milky conidia, two oil droplets inside, oozing in characteristic shape from dark pyinida and without chlamydospores. The morphological characteristics of the isolates were found to be genus Phoma and the species were consistent with Phoma glomerata and Phoma lingam, respectively. The mean colony diameters of the isolates were range from 7-8 cm and 7.3-8 cm for Phoma lingam and Phoma glomerata, respectively. There is no significance difference in mean colony diameter among the isolates. **Conclusion:** The result of this study demonstrated that Phoma glomerata and Phoma lingam were the cause for leaf spot and stem canker diseases of *E. globulus* tree plantations in high lands of Ethiopia, which is important in the study of management options for control and prevention of the diseases in the country.

Key words: Stem canker, leaf spot, *Phoma lingam*, *Phoma glomerata*, *E. globulus*

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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

Eucalypts of the Family Myrtaceae (subfamily Myrtoideae) is among the dominant genuses of flowering plants in the world, consisting about 800 species[1]. Planting of *Eucalypts* in Ethiopia has historically dating back to the late 1800s[2,3]. *Eucalyptus globulus* species are among exotic plantation in Ethiopia covering about one-third of the total plantation area of the country mainly for the benefit of construction, fuel, poles and an important resource for subsistence farmers[4]. Introduction of eucalypts plantations to the country was mainly due to deforestation of native forests for farm lands and construction materials[5]. It has been shown by Mengist[6] that introduction and distribution of fast growing exotic tree species such as Eucalyptus *globules* in the country was to minimize the problem of wood biomass crisis and construction materials. As already pointed out by Alemu[7], Eucalyptus plantations has become an alternative income source and employment opportunity in the cash crop devoid high land areas of Ethiopia. Among Eucalyptus species, *Eucalyptus globules* were found the most effective in terms of survival and growth[8].

According to Bekelle[9] and other researchers out of the total area of small areas of plantation, about 35% are dominated by *E. globulus* for variety of products including, leaves and small branches for fuel, poles and posts for house building and sources of income in Amhara region of Ethiopia.

Inspite of this importance the success of *E. globulus* plantations is currently influenced by several factors in Ethiopia. Among the factors that challenge growth and development of these tree species are fungal pathogens mainly from *Ascomycota*. Approximately about 700 species of fungi are associated with *Eucalyptus* leaves and stems[10]. *Cylindrocladium* spp. *Mycosphaerella* spp., *Phaeoseptoria eucalypti* spp., *Pestalotiopsis* spp. and *Pseudocercospora eucalyptorum* spps are among important fungi that cause diseases to *Eucalyptus* spp.[4,11].

Fungi of the genus *Phoma* are at present cosmopolitan in respect of geography consisting of a large number of species in varied ecological niches. From among 3000 taxa described so far, 110 are pathogenic species often infecting plant that are important from the economic point of view[10]. *Phoma* sp. are frequently found in association with symptoms of blight, leaf spots; fruit rot and stem canker throughout the world[12]. The disease is most prevalent in temporary periods of cool and wet weather associated with light and frequent rains[13].

Most leaf spot diseases and stem canker develop as small, scattered, circular to oval dead areas in the leaves; usually tan, dark brown, yellow, gray, purple, or black. Some spots are raised, shiny and coal black, others may drop out leaving ragged holes; some are marked with light and dark concentric zones (Fig. 1). Numerous spots develop yellow, purple, red or reddish brown to black margins; and later, in damp weather, increase in size and number can merge into large, angular to irregular dead areas. Dark areas and speck-sized, fungus-fruiting bodies (known as pycnidia, acervuli and perithecia) commonly form the dead tissues of many older spots (Fig. 2). Heavily infected leaves may turn yellow to brown, wither and drop early, weakening the tree. Occasionally, some leaf spotting fungi deform or kill flowers, buds, fruits, twigs, or even small branches (Fig. 1). In Ethiopia, research on tree diseases in plantation and natural forests is at infant stage, stress from climate change and other factors brought loss of plantation trees due to diseases and this is increasing from time to time in the country. To overcome this problem, research on type, prevalence and management of plantation diseases is very important. The objective of this research was to investigate the diversity and distribution of fungal species associated with plantation of *E. globulus* causing leaf spot and stem canker diseases in North and North West Ethiopia. The result of this study will help at large in the study of effective diseases management options for effective control and prevention of *E. globules* diseases. This study also aims to contribute base

Fig. 1: *E. globulus* plantations in the field showing stems canker and leaves spot symptoms

line findings and information that initiate researchers for further tree disease research in the country.

MATERIALS AND METHODS

Study areas, sampling and sampling techniques

Study areas: Amhara Region is located between 8°45'N and 13°45'N latitude and 35°46'E and 40°25'E longitude in North West Ethiopia with annual mean minimum and maximum temperatures between 15 and 21°C and the average annual rain fall of 1194 in mm[12]. Tigray forms the northernmost reaches of Ethiopia and is located between 36 and 40° east longitude, north-south extent spans 12 and a half degrees to 15° north[14]. The average annual rainfall between 450-980 in mm and the annual minimum and maximum mean temperature of the region is between 9.86 and 24.9°C[15].

Sampling and sampling techniques: *Eucalyptus* trees in commercial stands, farm lands and woodlots were surveyed in the selected high land areas of Amhara and Tigray regions in moist season from May-June, 2016 for presence of disease symptoms. The survey and sampling procedure were according to the procedures of Banito *et al*.[16] with modification. Twelve plantation sites were purposively surveyed based on severity of the problems in the areas, from each plantation sites 3-4 plants were randomly selected by walking in an "X" fashion for collection of samples. At least four to five leaves, twigs and stems were collected from each plant within the plantations for identification.

Identification and morphological characterization of fungal pathogens: Leaves, twigs and segments of stems showing disease symptoms were collected and separately placed in brown paper bags, which were sealed in larger plastic bags to retain moisture, until isolations were done. Sample culturing, identification and morphological study were done according to the procedures of Aveskamp *et al*.[13], Boerema *et al*.[17] and De Castro Silva[18]. After washing the tissues thoroughly with sterile water, the causal fungi were isolated from plant tissues exhibiting clear symptoms. The infected tissues along with adjacent small unaffected tissue was cut into small pieces (2-5 mm^2) and by using flame-sterilized forceps, they were transferred to sterile Petri dishes containing tap water, then to 90% ethanol solution and then to distilled water for surface sterilization of plant tissues for 30-60 sec. Three sterilized pieces were aseptically transferred to Petri dishes of 9 cm diameter containing Potato Dextrose Agar (PDA) in triplicate and incubated at room temperature (25-30°C) for 5-7 days and examined daily for the growth of the organism. The cultures were incubated during the first week in a thermostat without access of light at the temperature of 22°C and for the next 13 h in UV light and 11 h in darkness. Samples that could not be processed immediately were kept in cool dry conditions or in a refrigerator at ~4°C.

Morphological studies of cultures isolate on PDA were conducted following the methods described by Boerema *et al*.[17]. Colony diameters were measured after 7 days converted to mean colony diameter and colony morphologies were determined after 7-14 days of incubation. Colony colours on the surface and reverse of inoculated Petri dishes were assessed according to the colour charts of Rayner[19]. Micromorphological descriptions for 20 *Phoma* spp., culture of relevant features were carried out from mature conidiomata and conidia slides mounted in water[13,20]. Slides were prepared to make detailed observations of the morphological features, size, shape, colors of conidiomata, pyinida, conidia and chlamydospores and patterns of fungal growth *in vitro* using a compound microscope. The keys considering the current principles of the taxonomy of fungi from genus *Phoma* were used for identification[21]. Fungal cultures were identified at genus and species level on basis of macroscopic characteristics like colony morphology, color, texture, shape and appearance and microscopic characteristics like conidia shape, hyphae color, septation, concentric zone, pigmentation, fruiting bodies or any other visible structures[22-25].

Pathogenesity tests using detached leaf technique: Mycelia disks from each *Phoma* isolate were used to inoculate leaves old field-grown *E. globules*. New leaves free from leaf spot symptom, were collected, washed and surface sterilized with 70% ethanol, 0.5% sodium hypochlorite and rinsed with sterile distilled water, prior to inoculation and sprayed with the spore solution of evaluated fungal isolates. Controls were sprayed with sterile water free of fungal spores. After inoculation, leaves were placed in humid plastic bags and kept at room temperature. Symptoms were recorded 5 days after inoculation and re-isolation, was made from all resulting lesions according to Badillo-Vargas *et al*.[26] and Torres-Calzada *et al*.[27].

Data analysis: The collected data were summarized, ranked and expressed using simple descriptive statistics such as percentages and graphs. Survey data of morpho-cultural characters, relative prevalence of each pathogenic fungal species with respect to localization and others were analyzed using SAS procedure Version9.0 at probability level, $p = 0.05$[28].

RESULTS

Symptoms: On site observation of leaf spot and stem canker symptoms on *E. globulus* plantation was made in May and June, 2016. Brown to black spots, round to irregular-shaped, occurs on leaves and stems. Leaf spots were circular or irregular in shape separated or aggregated and often located at the margins with brown, pale brown to grayer coloration while stem canker is associated with elongated, grayish, hell brown to dark brown border between discolored tissues lesions (Fig. 1).

Morphological characterization of fungal isolates: A total of 20 isolate of fungi colonies consisting of *Phoma* species were identified and further characterized (Fig. 2).

The mycelium develops slowly, forming round to wedge-shaped colonies on PDA medium. After 7 days, pink to grey, green to dark green and white to grey colour mycelia sectorially or in concentric zones and round, dark brown to black Pycnidia was observed (Fig. 3). The mean colony diameter was found to be range from 7-8 cm and 7.3-8 cm for *Phoma lingam* and *Phoma glomerata*, respectively after fourteen days. The mean standard errors of the isolates show that there is no significance difference in mean colony diameter among the isolates on the PDA (Table 1).

Colony textures were recorded as either appressed with sparse aerial mycelium, flocculose with raised and slightly dense aerial mycelium, or floccose with raised and dense aerial mycelium (Fig. 3). Colony colors were observed as white, gray and pink. Colony shape was either uniform with smooth edges, irregular with rough edges or banded with sectors consisting of thin expansive mycelium. Morphological variations were observed among the isolates with specific morphological or cultural traits (Fig. 3).

Conidia were single-celled, hyaline, short cylindrical, with oil drops on both tips while multicellular chlamydospores of alternarioid shapes were also observed (Fig. 4).

Pathogenesity tests conducted under laboratory conditions showed that all *Phoma* isolates evaluated were pathogenic to *E. globulus* leaves brought from nursery. Gray, brown to black spots on leaves was observed seven to twelve days after inoculation. Control plants did not show symptom development after four weeks of periodical observations.

DISCUSSION

The survey depicted that leaf spot and stem canker diseases were found the most prevalent on *E. globulus* plantations in the highland fields of Ethiopia. The findings of this study revealed that the diseases were due to fungal

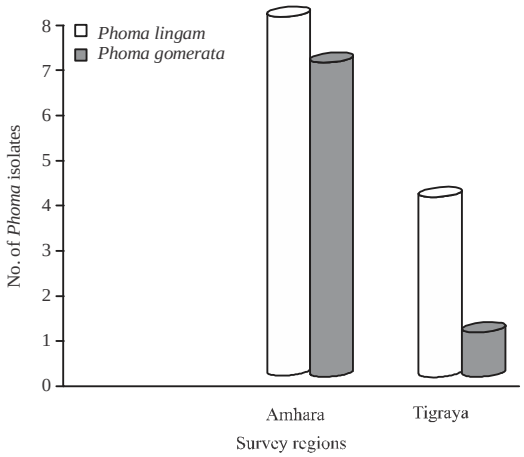

Fig. 2: Number and types of *Phoma* species isolated from Amhara and Tigraye regions

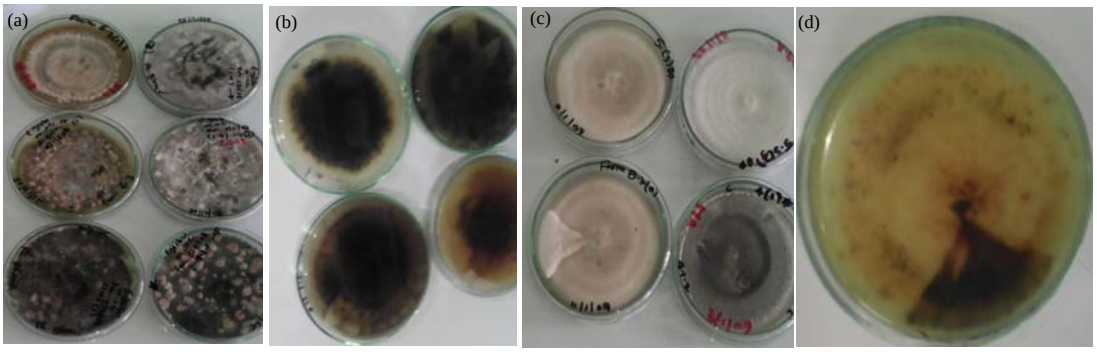

Fig. 3(a-c): Morphological variabilities among *Phoma* spp., isolate from stems and leaves of *Eucalyptus globulus*: (a, b) *Phoma glomerata* front and reverse side and (c, d) *Phoma lingam* after 7 days of incubation

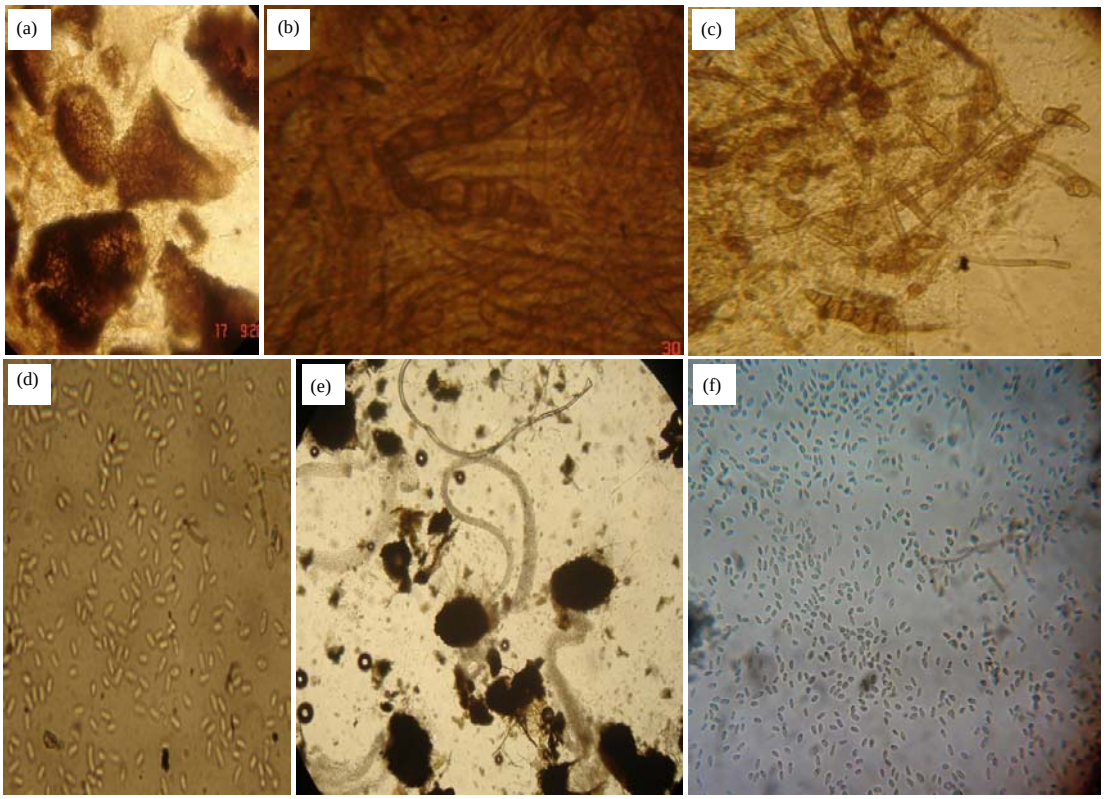

Fig. 4(a-f): *Phoma* spp., isolates microscopic structure, (a) *Phoma glomerata* Pycnidia (b, c) *Phoma glomerat*a alternarioid chlamydospores (d), *Phoma glomerata* free conidia (e, f), *Phoma lingam* Pycnidia with characteristic shape of oozing spores and free spores

pathogens belonging to genera of *Phoma*. The morphological characteristics of pure fungal culture were found to be varies in texture, color and margin. Among the isolates, majority were apprised in texture while the rest were floccules and floccus (Fig. 2). In terms of colony color, isolates were range from white to grey, light pink to white and dark green to black on PDA but, colony margins of most isolates were observed regular, this result is consistent with the findings of Aghapour *et al.*[21] showing variation among *Phoma* spp., colonies within and among groups, depending on culture medium, substrate and environmental conditions. Based on observed morphology, circular, white to pink and, dark green to grey color with long chains or solitary, unicellular or multicellular alternarioid chlamydospores. The morphological characteristics were consistent with morphological descriptions of Zhao and Kang[10], showing the *Phoma* species to be *Phoma glomerata*. The relative high prevalence of the diseases in the area is attributed to the production of conidia with a relatively fast growth rate during suitable weather condition enabling the fungi to invade large number of hosts within short period of time, which is in line with the study of Banito *et al.*[16]. The rest of pure culture isolates with milky white to gray color mycelia, associated with free conidia oozing in characteristic shape from dark pyinida, oval shaped, unicellular and each with two oil like droplets inside, without chlamydospores were identified as *Phoma lingam,* which agrees with research findings of Aveskamp *et al.*[13] and Boerema *et al.*[17]. According to the findings from morphological study *Phoma glomerata* and *Phoma lingam* are found to be the cause for leaf spot and stem canker of *E. globulus* plantations which is again in line with the study of others such as Aghapour *et al.*[21], Chen *et al.*[29] and Hammoudi *et al.*[30]. The results of this study also confirm, the cosmopolitan nature of *Phoma* spp., showing majority of plants being infected worldwide as stated by Aveskamp *et al.*[13] and Boerema *et al.*[17]. This nature of Phoma *spps* is also stated by Aveskamp *et al.*[13] as *P. eupyrena*, *P. exigua*, *P. glomerata*, *P. herbarum* and *P. macrostoma* are the most frequent occurring *Phoma species* worldwide, irrespective of the climatic conditions. This study is an evidence for *Phoma* spp., to be a cause for stem and leaf disease in *E. globulus* plantations which is also indicated in the study of Sivasithamparam *et al.*[31] and Yuan

Table 1: Morpho-cultural and microscopic characteristics of fungi isolated in the study areas

Region and zone	Locality	No. of isolates	Altitude *(masl)	Morpho-cultural characteristics on *PDA	Microscopic (conidia) characteristics	Mean colony diameter of the isolates after 14 days (cm) and SE, respectively		*Phoma* species
						cm	SE	
South Gondar, Amhara	Lomi Dur	1	2705	Rapid growth, cotton-like mycelium, colorless when young and becomes deep gray or black	Unicellular and ovoid conidia	7	0.00	*Phoma lingam*
South Wolo, Amhara	Jeme	2	2359	White cotton-like mycelium which turns to gray with time forming acervuli	Abundant unicellular, fusiform conidia	7.8	0.03	*Phoma lingam*
South Gondar, Amhara	Gelaye	2	3095	White mycelium which turns to gray on old cultures	Abundant hyaline, unicellular, oozing conidia	8	0.00	*Phoma lingam*
South Gondar, Amhara	Atrik	2	2975	White cotton-like mycelium-forming black acervuli	Hyaline, oval, unicellular conidia with oily droplets inside	7	0.00	*Phoma lingam*
Tigray	Adi Sesino	1	2635	Rapid growth, sparse mycelium colorless to gray conidia	Pyramidal branched conidiophores.	8	0.00	*Phoma lingam*
South East Tigray	May Keyah	2	2312	Abundant milky white cotton-like mycelium turns to gray on old cultures	Conidia are hyaline, oval and unicellular	7.5	0.08	*Phoma lingam*
South Gondar, Amhara	Kosso Mado	1	3206	Raised cream White cotton-like mycelium in concentric circle	Conidia, hyaline, oval, unicellular with droplets inside	8	0.09	*Phoma lingam*
South Tigray	Korem Zuria	1	2579	Raised White cotton-like mycelium in concentric circle	Single-celled, short cylindrical conidia, with oil drops inside	7.5	0.08	*Phoma lingam*
South Wollo, Amhara	Sulula	2	2290	Slow growth; whitish when young and turning progressively red to grey with age	Brown Chlamydospores in long chains	7.9	0.008	*Phoma glomerata*
North Showa, Amhara	Tarma ber	2	2215	Localized growth; whitish colonies when young and turning progressively red, then to grey with age	Chlamydospores are brown, long chains or solitary, unicellular or multicellular	7.8	0.031	*Phoma glomerata*
Tigray	Keyit	1	2880	Localized growth; colonies with twisted outlines, whitish when young light pink at the center	Chlamydospores brown, unicellular or multicellular and alternarioid	7.3	0.08	*Phoma glomerata*
North Showa, Amhara	Elu	3	2891	Slow growth, sparse mycelium initially light pinkbut rapidly turns green with profused rough green surface	Chlamydospores brown, long chains alternarioid appearance.	8	0.03	*Phoma glomerata*

*PDA-Potato Dextrose Agar, *masl-meter above sea level

and Mohammed[32]. Studies in Zhang *et al.*[33] also supported this finding showing *Phoma* fungi that cause leaf spots grows along the leaf petioles to the stems and result in stem canker during favorable environmental conditions. Researchers such as Bettucci *et al.*[34] and others also stated that *Phoma* spp., are among Endophytic fungi colonizing leaves, stem and twigs of *E. globulus* and even their seedlings, depending on the stress from environmental variables they can possibly be aggressive pathogens that cause severe losses to these plants. The research of Aveskamp *et al.*[35] also indicates, *Phoma* spp., can cause maximum yield loss usually during nursery stages and cause significant yield loss of the tree plantations when there is prolonged environmental stress. The Pathogenecity study of the *Phoma* isolates described showed that they are pathogenic to sprayed *E. globulus* leaves and produced identical symptoms to those observed in the field, confirming the *Phoma* isolates causal agents for leaf spot disease of *E. globulus* in Ethiopia, which is also in line with findings of Zimowska[36]. Beside the absence of molecular analysis and limitation of the study to specific parts of the country due to time and financial related constraints, the findings of this research from morphological study show that *Phoma* species could be the cause for leaf spot and stem canker diseases of *E. globulus* in the high land areas of Ethiopia. The findings of this study will be expected to contribute much in the study and application of effective management options with in depth research including other high land areas of the country for effective control and prevention of the diseases.

CONCLUSION AND FUTURE RECOMMENDATION

In conclusion, *E. globulus* tree plantations are important sources of economy in North and North West Ethiopia. *E. globulus* production is currently with great challenges in the regions because of pathogen related problems. On the basis of cultural and morphological characteristic of fungal isolates *Phoma* spp., were the cause for severe leaf spot and stem canker diseases on *E. globulus* tree plantations in Amhara and Tigray regions of Ethiopia. The pathogens were identified and characterized to genus and species level through morphological characteristic and found to be *Phoma glomerata* and *Phoma lingam*. The result of pathogenesity analysis revealed that *Phoma* fungi isolates were pathogenic to *E. globulus* plants forming similar symptoms with samples collected from the research sites.

Further long range research is needed for enough data of incidence, prevalence and severity of *Phoma* fungi on *E. globulus* plantations. Morphological characteristic method assisted with modern approaches including molecular tools, would provide a better understanding and reliable information of *Phoma* isolates to species level. Awareness about the spread of this disease to nursery site workers, farmers and plantation owners help the diseases to be controlled and protected at source level. Additional survey of the diseases at nursery sites is recommended for comparison of diseases parameters at plantation and nursery site. Nurseries have to be assessed for diseases symptoms and there has to be appropriate treatments for diseased *E. globulus* seedlings before distribution to society's plantation sites.

SIGNIFICANCE STATEMENTS

The researcher identified the fungal pathogens that cause the *E. globulus* diseases which provide information at large in the study of management options for future control and prevention of the diseases.

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