ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Fungi associated with shoot dieback of *Podocarpus falcatus*

By

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A Thesis Presented to the School of Graduate Studies of the Addis Ababa University, in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

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July, 2006

This thesis is dedicated to

Ato Yirgu Wondimtegengne, Wizero Bekelech Asessa
and
Tigist Abera

ACKNOWLEDGEMENT

I would like to thank Drs. Alemu Gezahgne and Tesfaye Alemu for their unreserved assistance during my day-to-day activity and providing me their reading and reference materials, Professor Legesse Negash for allowing me to carry out the pathogenicity test both at AAU and at Addis Alem, Drs. Gurja Belay and Seyoum Leta for assisting with preparation of photographs, Girma Taye for helping me in statistical analysis, Zenebech, Birtukan, Hirut, Merid, Woinshet, Yohannes and the staff of FRC at EIAR for their technical support.

I would like to extend my great respect and thanks to Tigist Abera, Jemanesh Yirgu, alemitu Mengistu, Mezgebu Amha, Medhanet Bekele and Tadesse Yirgu for their encouragement and financial assistance to carry out my study. My friends Fedlu and Zenebe Teka are also thanked.

Finally I would like to thanks the Department of Biology for all rounded assistance and the School of Graduate Studies of Addis Ababa University deserve special thanks for financing this work.

Above all I thanks ***GOD*** *for giving me life and endurance in harsh environment.*

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ABSTRACT

In Ethiopia, information available on tree disease is limited and scanty. In the field survey conducted in Menagesha-Suba and Munesa-shashemene natural forests the shoot dieback was observed on *Podocarpus falcatus.* Symptoms were initially observed on leaves and two-three small circular brown spots were found at the very tip of the leading shoot. Gradually the color of the leaves changes into yellow, and to light brown as the shoots die. This symptom of dieback was observed both on seedlings, sapling and mature trees. A study was conducted to understand which fungi was associated with shoot dieback. Isolation was made from the symptomatic plant part. Culture and spore morphology was used to characterize the most common and frequently isolated fungi. Accordingly, it was understood that 3 isolates belong to *Alternaria* genus, 4 isolates that grouped into genus *Phoma,* 1 isolate to *Pestalotiopsis* and 1 isolate to genus *Fusarium* was found. Among these isolates of *Alternaria,* isolates Pf-24 and 32 seem to be close to *A.alternata* and the other one isolate Pf-53 seems to be close to *A. arborescens.* Two of the isolates in the *Phoma,* Pf-42 and Pf-22 seems to be related with *P. exigua* and the other one resemble *P.leveillei.* The other two isolates Pf-12 and 21 were unidentified. In the inoculation test all the four groups of fungi produced lesion only on wounded leaves. The importance of these fungi in relation with shoot dieback, however, needs further detailed investigation. As far as our knowledge is concerned this is the first report of shoot dieback on *P.falcatus* in the natural forest of Ethiopia. The impact of these diseases on *P.falcatus* should get due attention in the management of *P.falcatus* forest in Ethiopia.

KEY WORDS: *Podocarpus falcatus, Alternaria* species, *Phoma* species, *Pestalotiopsis species, Fusarium* species, Shoot dieback

1. INTRODUCTION

Forests are the most important components of the ecosystems and play significant role in sustaining life on earth (Campbell *et al.*, 2003). World wide they cover about 3.9 billions of hectares of land that is equivalent to one third of the total earth surface. Forests are invaluable resource for various ecological, social and economical aspects. They play significant role in regulating climate, stabilizing soil, and treating wastes. Forests also offer recreational opportunities, serve as sanctuary for wildlife, provide fuel wood, pulp and paper, medicines and lumber (Spanos, *et al.*, 1999; Eyles, *et al.*, 2003; Noël, *et al.*, 2005).

Despite these diverse values, the forest cover is declining at alarming rate. According to FAO (2005), the total world's deforestation rate was 8,885,000 ha per year in the years 1999-2000 and this had increased to about 36,586, 000 ha between 2000-2005. The deforestation condition in Africa has similar increasing pattern, which was 4,375,000 ha and increase to about 20, 201,000 ha per year in the respective periods.

In Ethiopia, the total deforestation rate between 1990-2000 was 141,000 ha per year and was 705,000 ha between 2000-2005 (FAO, 2005). A number of factors including rapid population growth led to increased demand for arable farmland, construction material, fuel wood and timber production and this have contributed to the accelerated forest destruction. Currently the major forests are depleted and only patches of natural forest are found. The area coverage and the population of most indigenous tree species are declining at alarming rate. One such tree species is *Podocarpus falcatus*. The area coverage of this tree declined to 0.9% of its original size in recent years by the continuous logging of the species for timber production (Kassa Semagn and Legesse Negash, 1996). Hence, there is an urgent need towards conserving and encouraging the use of indigenous tree species for reforestation practice. In order to facilitate the development of indigenous trees plantation the problems associated with poor seed germination, slow growth rate and poor performance in nurseries and out in the field should be improved (Legesse Negash, 1995; Abdella Gure, 2004).

In addition to this, to meet the increasing demands for wood and wood products, both natural forests and plantation forests have to be protected and managed. Threats against these wood sources by different factors such as fire, indiscriminate cutting, encroachment, pests and diseases should also need appropriate attention.

The occurrence of pathogens on woody plants is a common phenomenon on trees growing in natural forests, plantations and ornamentals tree and has resulted in serious losses to forestry programs worldwide (Manion, 1981; Wingfield, 1990). In Ethiopia, the significance of pathogens in tree health has had little attention in the past. Poor growth performance including stunted growth, death of seedlings and failure of seed germination are usually ascribed to poor site conditions, adverse climatic conditions and inadequate management practice. The role of biotic agents in causing retarded growth and death of trees both in natural and plantation forest of Ethiopia was not studied adequately. Generally, limited information is available on the prevalence of disease causing organisms both on exotic and indigenous tree species. The experience from the rest of the world highlights the importance of understanding the damage caused by pathogens. Therefore, it is essential to study the prevalence and importance of tree diseases in order to develop appropriate disease management strategies. This study was, therefore deal with shoot dieback of *Podocarpus falcatus* that was commonly observed in Menagesha and Munesa Shashemene forests.

2. OBJECTIVES

2.1. General objectives

- To identify and characterize the fungi associated with shoot dieback of *P. falcatus* in central and south-eastern parts of Ethiopia.

2.2. Specific objectives

- To characterize and identify fungi commonly found associated with shoot dieback on *P. falcatus*
- To evaluate the antagonistic potential of selected biocontrol agents against the fungal isolates obtained from shoots of *P. falcatus*

3. LITERATURE REVIEW

3.1. Biology, ecology and economic importance *Podocarpus falcatus*

Podocarpus falcatus (Thunb.) Mirb. (Synonym: *Podocarpus gracilior* Pilg.) belongs to the family Podocarpaceae that embarrasses an evergreen trees or shrubs of 7 genera and more than 90 species (Friis, 1992; Bailey, 1964). *P. falcatus* is known as podo or east African yellow wood (Legesse Negash, 2002).

Podocarpus falcatus is a dioecious tree that can attain a height of up to 35 m and occasionally occur in undifferentiated afromontane forests. It either exists as a dominant or a co-dominant forest tree along *Juniperus* species. It grows at an altitude of 1550 to 2800 m.a.s.l. and where the mean annual rainfall is between 1000-2000 mm per year. The species is distributed throughout the mountains of the tropics and temperate region in the southern hemisphere with a wide range of habitat tolerance (Legesse Negash, 2002). In Africa, it is found in Ethiopia, Rwanda, Burundi, Uganda, Kenya, Tanzania, Malawi, Mozambique and South Africa. Though the size has diminished today, *P. falcatus* was once grown in Wellega, Wollo, Gojjam, Shoa, Sidamo, Hararghe and Arsi areas (Friis, 1992; Legesse Negash, 1995).

Podo has both ecological and economical values. Ecologically, it is useful in preventing soil erosion, serving as source of food and shelter for many bird and Colobus monkey. On the other hand, the high class-softwood makes it more preferable in the construction of different house ware furniture and making match sticks (Kassa Semagn and Legesse Negash, 1996; Legesse Negash, 2002).

3.2. Plant and Pathogen association

Plants can have a mutualistic, saprophytic and parasitic association with fungal species, among which, the parasitic way of association often cause damage on several valuable tree species (Spanos, *et al.*, 1999).

Trees might be exposed to disease as a result of stress inducing factors such as drought, frost, water logging and insect damage (Kay, *et al.*, 2002). On the other hand, some pathogens can even attack the host by breaking the inherent immunity and natural protective structure of trees (Burgess and Wingfield, 2002). Forest regeneration becomes a serious task under situations where pathogens used to attack seedlings in nurseries, which have tender tissues and often difficulty in establishing themselves (Rai and Mamatha, 2005). Therefore, the prevalence of pathogen in an area affects forest composition, structure, function and dynamics of plant communities and cause subsequent disturbance of the given ecology, evolution and economy of the world (Orwig, 2002; Chakraborty, 2005).

3.2.1. The influence of native pathogens on native flora

The association between indigenous pathogens and native trees go back for centuries. Through this time native trees adopted mechanisms that help to resist the impact of pathogens and live in balance with each other. Despite this several reports indicated that native trees are exposed to the attack of native pathogen at different levels (Burgess and Wingfield, 2002). The problem is aggravated by the global climatic change and the influence of human activity. These factors bring about change in the composition, structure and diversity of the plant pathogen (Holdenrieder, *et al.*, 2004; Chakraborty, 2005). In this process a pathogen, which was considered as weak pathogen in the ecosystem may become a serious pathogen as a consequence of genetical recombination of the mild pathogen by more virulent gene. On the other hand the formations of monoculture plantation that increase genetic uniformity facilitate the incidence of disease outbreak (Burgess and Wingfield, 2002). The importance of such study is exemplified from the experience of *Armillaria* and *Endothia gyrosa* in Australia.

Armillaria is one of the genera that affect the roots of several native woody plants throughout temperate as well as most tropical regions of the world. Many of the species in the genera are serious pathogens of a wide range of native and planted conifer and hardwood trees and shrubs in forests, orchards and gardens (Coetzee, *et al.*, 2001) and some are weak pathogens. However, these weak pathogens never remain so. For instance, in southwestern Australia an indigenous pathogen namely *Armillaria luteobubalina* was associated with dead stumps and roots in forest communities only. This weak pathogen became a serious primary pathogen when the *Eucalyptus* forests in the region were subjected to heavy logging. Furthermore, it limited the reafforestation activity and regeneration of *Eucalyptus diversicolar* seedlings in areas where there was an incidence of infection (Burgess and Wingfield, 2002).

The other example was *Endothia gyrosa*. This organism was generally recognized as weak or opportunistic pathogen in natural *Eucalyptus* forests in Australia. However, it was found as primary pathogen after changing its behavior and become responsible for severe stem canker outbreak that resulted in a significant level of mortality in *E. nitens* (Eyles, *et al.*, 2003).

3.2.2. The effect of exotic pathogens on native species and native pathogen on introduced plants

The development of science and technology over the last 100 years changed the geographical barriers that restricted the distribution of world's biota for millions of years. This improvement allowed the movement of different materials from one corner of the world to the other cheaply and easily. However, some problems appeared into picture along this movement. This is mainly related to the introduction of unwanted exotic pathogen that threatens the indigenous tree species.

The impact of introduced pathogens on native trees can be exemplified from the experience of *Armillaria* that were introduce by early Dutch settlers into South Africa (Coetzee, *et al.*, 2001); *Ophiostoma ulmi* and *O. novo-ulmi*, the Dutch elm disease into North America and Europe (Brasier, 1991); *Cryphonectria parasitica*, the chestnut blight fungus, into North America and Europe and *Cronartium ribicola* (the white blister rust fungus) in North America and Canada (Palm, 1999; Burgess and Wingfield, 2002; Myers and Bazely, 2003).

Cronartium ribicola is a native fungal pathogen in Asia and Europe. The native species of pines namely Macedonian pine, Swiss pine and Blue pine are mostly resistant to the attack of this fungus in its native range (Wikipedia, 2006). After the accidental introduction of the fungus to North America with infected nursery stock grown in Europe, it became a devastating pathogen both in disturbed and undisturbed ecosystem. The pathogen infected nearly all white pines in North America. The damage was particularly heavy in western white pine (*P. monticola*), sugar pine (*P. lamertiana*), limber pine (*P. flexilis*), white bark pine (*P. albicaulis*), (Scharpf, 1993; Wikipedia, 2006). Very few species of the American pine one in 10,000 are found to be resistant due to lack of development of genetic balance by the host. Hence, the size of this commercially and ecologically valuable tree depleted to the point where one no longer considered that this tree was native to the area (Kim, *et al.*, 2003; Smith, *et al.*, 2004). Similar incidence also occurred with the introduction of *Colletotricum truncatum* with seeds of lentil from Canada and U.S.A. to Australia (Ford, *et al.*, 2004). Such kind of problems can even occur during the transfer of native species within a country or a continent. To this end there are many reports as to the attack of *Eucalyptus* species during intra continental transfer inside Australia (Burgess and Wingfield, 2002).

On the other hand the introduction of plant can be threatened by an indigenous pathogen. *Endothia gyrosa* is a native pathogen in North America. It was frequently associated with pin oak (*Quercus palustis* Muench.). This pathogen was reported to cause serious canker disease on exotic species *Liquidambar formosana*, *L. styraciflua* and *Castanea* sp. that were introduced to North America (Venter, *et al.*, 2001). These kinds of experience forced a country to develop strict quarantine system that avoids the co-transmission of pathogens along plant materials (Poole, 1989). By doing so a country minimizes the impact of pathogens in its national economy as well as in its (Liebhold, *et al.*, 1995; Dukes and Mooney, 2004).

3.2.3. Disease records in forests of Ethiopia

Observation made in nurseries and field condition indicated that biotic factors could contribute to the decline of the population and area coverage of *P. falcatus*. The damping off on seedlings and decay of seeds are good examples. Abdella Gure (2004) isolated about 183 fungal species from the seeds of *P.falcatus*. These fungi belong to three phyla namely Ascomycotina, Basidiomycotina and Zygomycotina, out of which Ascomycotina dominates. The most frequently isolated genera include *Ampelomyces/Phoma* spp, *Phomopsis/Diaporthe*, *Pestalotiopsis*, *Polyporus*, *Stereum*, *Alternaria*, *Botryospaeria*, *Guignardia* sp., *Cytospora*, *Ulocladium* and *Nectria.*

As Alemu Gezahgne *et al.* (2004) reviewed, few records of tree diseases in Ethiopia can be found associated with some important indigenous tree species. Several of these deal with *Armillaria* species. It was reported that *Armillaria* species were found on recently cleared and planted sites and where shade trees have been removed (Eshetu, *et al.*, 2000).

The prevalence of symptoms of Armillaria root rot in plantations at Wondo Genet, Munesa Shashemen, Belete/Jima, Bedele and Aman/Mizan was reported by Alemu *et al.* (2004). Armillaria root rot was found associated with *Pinus patula*, *Acacia abyssinica* Hochest, *Cordia alliodora* (Ruiz & Pav) Oken and *Cedrela odorata.* Basidiocarps were collected from the stumps of *Juniperus exelsa* trees, in a plantation at Wondo Genet. A recent population study on *Armillaria* spp. in Ethiopia reported that *A. mellea sensu stricto* is responsible for root rot on hardwood trees in the Jima and Kerita areas (Ota *et al.* 2000).

Some reports that dealt with diseases of native tree species in Ethiopia reported the occurrence of *Antrodia juniperina* (Murrill) Niemela & Ryvarden on *Juniperus exelsa/ procera* Hochest. Ex. Endl. *A. juniperina* is reported to be parasitic and saprophytic on stems of *J. exelsa* and to cause heart rot and necrosis of the butt (Niemela & Ryvarden, 1975). In another report Niemela *et al.*(1998) recorded several decay fungi in natural stands of *Hagenia abyssinica* (Bruce) J. F Gmel. in East Africa, including Ethiopia. This report only mentioned fungi involved in decaying *H. abyssinica.* They include *Hymenochaete ochromarginata* Talbot. collected from living trunks and stumps. *H. ochromarginata* is considered to be the main cause of decay of living *Hagenia* trees. The non-primary pathogens that were considered as rot fungi, *Phellinus ferruginosus* (Schrad. Fr.) Bourdot and Galizn and *Trametes socotrana* Cooke, were isolated from fallen branches and stems of *Hagenia* tree. A number of *Corticioid* fungi have also been recorded from *H. abyssinica* branches and stems. They include *Asterostroma medium*

Bres., *Cystidiodontia isabellina* (Berk. & Broome) Hjortstam and *Dichostereum kenyense* Boidin & Lanq (Niemela, Renvall & Hjortstam 1998).

3.3. Isolation techniques of disease causing agents

The process of isolation of microorganisms begins with collection of symptomatic plant materials. Commonly the process of isolation of disease causing organism commence with a through washing of plant tissues in sterile distilled water (SDW) and undergo surface sterilization in 2 % NaOCl for 1 min, 96 % ethanol for 10s (EPPO, 2005) or 0.5 % cupric chloride, 70 % alcohol each for 1 min (Przybyl, 2002). This is usually followed by rinsing the plant material in sterile distilled water and allowed to dry on sterile tissue paper.

The isolation of fungi can be done by direct, indirect or both ways. In the direct methods of isolation, the fruiting bodies or small excised plant parts from the margins of healthy and infected part will be transferred into general or selective media (Vannini and Vettraino, 2000).

The indirect means of isolation can be done at least in two ways. The first one is done by attaching symptomatic leaf discs to the cover of petri plates with the pseudothecia facing downwards in order to release its spores directly to the isolation media (Alemu Gezahgne, *et al.*, 2003). Secondly, segments from infected plant body first transfer into moist chamber to promote growth of mycelium, fruiting bodies or to sporulate out of infected tissue (Roux, *et al.*, 2004). In both isolation methods the plates that contains the isolates will be incubated and subsequent routine subculture will be done till pure colony is obtained.

3.4. Identification and Characterization

There are two common approaches in the identification and characterization of fungal species. These are classical and modern approaches. The traditional technique of identification is done based on comparison of culture characteristics such as color, production of soluble pigments, shape of colony margin, surface texture and growth rate on different media. In addition to this, it employees spore morphology that emphasize spore shape, size, color and structure of septa (Barnett and Hunter, 1972; Sutton, 1980; Nelson, *et al.*, 1983; Burgess, *et al.*, 1994; Old, *et al.*, 2000).

Molecular markers are the second alternatives in the taxonomy of fungi. It utilizes the genomic variation of organisms based on DNA fingerprinting, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) markers and DNA sequence analysis (Taylor, *et al.*, 2000; Ford, *et al.*, 2004). Especially the ITS region of the rDNA possess characteristics that are suitable for the detection of fungi at the species level since it possesses a highly stable and conserved genomic sequence within species and show high degree of polymorphism between species. Hence, this technique will help more than classical methods (Abd-elsalam, *et al.*, 2003).

3.5. Sporulation

This is one of the important steps in the process of identification, characterization and preparation of spores for pathogenicity test. Spores often are produced under conditions that are adverse to vegetative growth (Dhingra and Sinclair, 1985). Some of these conditions could be light, including ultraviolet radiation, fluctuating temperature conditions (Nelson, *et al.*, 1983), high humidity or nutrient poor medium (Vettraino, *et al.*, 2002; Kwas'na, 2003; Ray, *et al.*, 2005; van Nieker, *et al.*, 2005; Paulus, *et al.*, 2006).

The use of near ultra violet (black light) would be best if it is alternatively used on the bases of 12 hrs light and 12 hour dark inside an incubator. Regarding the sporulating media, many species sporulate better on natural substrata such as wheat straw, macerated leaves and lupine stems than agar (Sutton, 1980). In addition to this, nutritionally weak media such as Potato Carrot Agar, Oat Agar, and Water Agar are recommended for many fungi to sporulate successfully (CABI International, 2005).

3.6. Vegetative Compatibility Groups

Level of genotypic diversity can be studied using molecular markers or by determining vegetative compatibility groups (Smith, *et al.*, 2000). By vegetative compatibility the paired fungi come in contact with its counter part so as to fuse and exchange their cytoplasm or nuclear material (Proffer and Hart, 1988). This enables to determine the genetic variability of isolates within fungal population (Smith, *et al.*, 2000). Those fungi that have common alleles at one or more of their loci form heterokaryons (Puhalla and

Hummel, 1983; Proffer and Hart, 1988) are called vegetatively compatible. Under such condition, mycelium of the two pair will grow together forming confluent mycelium without barrage zone (Anagnostakis, *et al.*, 1986). On the contrary, fungi that grow to meeting point in the agar but remained separated by gap, line-gap and barrage line composed of dead cells with no covering aerial mycelium (Adams, *et al.*, 1990; Anagnostakis, *et al.*, 1986; Deng, *et al.*, 2000) are called vegetatively incompatible. This kind of test also enables to establish biological control using viruses since it dictates the transfer of dsRNA. In addition to these it provides information regarding the chance of occurrence of sexual and/or asexual reproduction (Smith, *et al.*, 2000).

3.7. Pathogenicity Tests

In order to substantiate that an organism, a group of organisms or combination of organisms and environmental factors are the causal agents for a disease one must fulfill Koch's postulates (Agrios, 2004; Partridge, 1999). The pathogenicity test begins with preparation of the spore or raw mycelium from the test organism. Different authors used different amount of spore loads during this test: $2 - 4 \times 10^{5}$ zoospores ml^{-1} (Denman, *et al.*, 2005), 0.5×10^{6} spores ml^{-1} (Ray, *et al.*, 2005), 1×10^{5} conidia ml^{-1} (Belisario, *et al.*, 1999), 5×10^{6} (Bohár and Schwarczinger, 1999), 10^{4}-10^{5} conidia ml^{-1} (Holdenridede and Kowalski, 1989) and 5×10^{4} propagules ml^{-1} (Hutton and Mayers, 1988).

The spore or mycelium could be loaded with or without wounding the plant (Denman, *et al.*, 2005). The nonwound trial helps to determine whether infection could develop without previous damage (Luque, *et al.*, 2000).

During inoculation of leaves, the adjusted spore suspension should be placed on the upper surface of leaf where cuticle was removed or on the surface where there is maximal number of stomata. However, in most cases wounding is less important in the pathogenicity tests of leaf for fungal pathogens. This is because of the direct penetration of the fungus through stomata, epidermal cells or at the junctions between epidermal cells (Dhingra and Sinclair, 1985).

According to Dhingra and Sinclair (1985), the spore can be loaded on leaves either by spraying, brushing, rubbing or dipping the leaf into a spore suspension, which is rapid and useful for inoculating large number of seedlings in small pots. Rubbing healthy leaf with infected leaf having fruiting bodies is an important method of inoculation in the pathogencity test of obligate parasites.

Covering the inoculated leaves with sterile plastic bag and paper bag will be followed in those leaves where spores do not adhere to the leaf. Hence, the covering will allow maintaining high relative humidity and optimum temperature for the pathogen to develop penetration peg. On the other hand the spores could be fixed to the target site of inoculation using a low-temperature gelling SeaPlaque agarose. This allowed the spores to cause local lesions on leaves and stems. This technique is important for its being accurate in the number of spores used, precise and easiness to handle the inoculated plants (Xu and Ko, 1998).

Finally those leaves that develop the symptoms should under go re-isolation to confirm whether they are similar to the original inoculants (Smith and Cole, 1991; Kaitera, 2003). This will finalize the Koch's postulate.

3.8. *In vitro* Biological Controls

The use of chemical controls grows concern for the hazards it causes on environment, human health and development of resistance by the pathogen (Okigbo and Osuinde, 2003). Some of these concerns could be reduced or eliminated by the use of biological control. Moreover, the use of biological control has also economical advantage by being low cost and durability than chemical controls (Okigbo and Osuinde, 2003). Leifert *et al.* (1993) suggested that the use of yeast, bacteria and fungi as biological control increased over recent years in the post-harvest fungal pathogen.

Bioagents affect the growth and development of the pathogen directly or indirectly (Roco and Perez, 2001). Some of the mode of action by the bioagent includes the release of wide range of enzymes that degrade the wall of the target organisms or exudates such as antibiotic and the competition for nutrients and space and mycoparasitism (Roco and Perez, 2001; Herman, *et al.*, 2004).

4. MATERIALS AND METHODS

4.1. Sample collection

Samples were collected in September 2005 and February 2006 from Menagesha-Suba State Forest, Munesa-Shashemene State Forest and the compound of Addis Ababa University (AAU), Science Faculty. Samples were collected from twenty symptomatic trees per site. From these trees, symptomatic leaves and twigs were collected and packed in envelope and brought to AAU mycological laboratory for further laboratory works.

4.2. Description of the study site

Menagesha-Suba State Forest, which was established during Emperor Zera Yacob (1434-1468), is located 40 km South West of Addis Ababa in Oromya Regional State. The altitude of the area ranges from 2200-3385 m.a.s.l., $8^0$57'N and $038^0$32'E (using etrex Germin global positioning system (GPS), Personal navigator). The area has a mean annual temperature of 14^0C and 1017 mm rainfall. The main rain season of the area starts in June and end in September even though there are some rains in other months too (Sebsebe Demissew, 1990; Alemu Gezahgne, *et al.*, 2003).

The total area of Menagesha forest is 7360-9248 ha, of which 2500 ha of land is covered by natural forest and 1000 ha is plantation. The remaining land is currently used for settlement, farmland and grazing (Sebsebe Demissew, 1990).

The area is dominant by two indigenous conifers namely *Juniperus procera* along the ridges and higher elevation and *P. falcatus* at the low elevation 2500 m.a.s.l. and in sheltered valleys. Other than these two species, there are a number of other trees, birds and mammals in the forest (Kassa Semagn and Legesse Negash, 1996; Sebsebe Demissew, 1990; Legesse Negash, 2002).

Munesa-Shashemene State Forest is located in Oromya Regional State. It is located about 240 km south of Addis Ababa. The area has an elevation of 2100-2700 m.a.s.l. and located $7^0$13'N and $38^0$37'E. In addition to the short rainy season that extends from March to mid

April, the main rainy season of the area extends from July to October with highest rainfall between July and February. In general the mean annual temperature and rainfall of the area ranges from 15-20 ^{0}C and 1200-1250 mm respectively (Lüttge, *et al.*, 2003; Masresha Fetene and Beck, 2004).

Podocarpus falcatus is found mixed with *Juniperus* and *Pinus* spp. that dominates the area. During sample collection, there was high infestation of aphid-related pathogens in *Cupresus lusitanica* plantation that resulted in the mass clearing of infested and dead trees from most of its plantation area.

The Science Faculty of AAU is located in the capital of Ethiopia: 2444 m.a.s.l. with a mean annual rainfall of 1196mm and located 9^0 01 N and 038^0 45 E. The maximum and minimum temperatures were 9.9^0C to 24.6^0C (Sebsebe Demissew, 1990; Fisseha Itana and Olsson, 2004). The compound had different indigenous tree such as *Podocarpus falcatus, Hagenia abyssinica* (Bruce), *Cordia africana* Lam., *Prunus africana* and others.

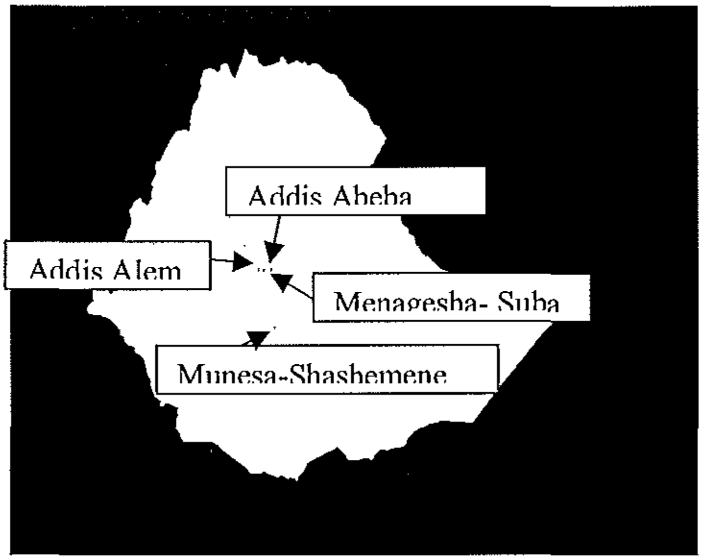

Fig. 1. Geographical location of study sites

4.3. Isolation technique

Four symptomatic leaves and twigs per tree were selected and used to isolate fungal organisms. Of these plant materials, 4 small pieces of leaves and twigs from the margin of infected and healthy regions were excised. These small portions were washed in tap water for 10 min in separate plates to minimize contaminants on the surface of samples. These samples were dipped in 70 % alcohol for 1 min to disinfect the surface and rinsed three times in sterile distilled water to remove the remaining disinfectant (Agrios, 2004).

Leaves and twigs collected from the same tree were directly transferred into a growth media containing 2 % Malt Extract Agar (MEA) (20 g L^{-1} in Oxoid, Hampshire, England) amended with 60mg L^{-1} streptomycin in order to suppress the growth of bacteria. The twigs were incubated in moist chamber to induce growth of mycelium and fruiting structures. Each growth chamber was supplied with sufficient amount of sterile water. Both of these groups were incubated in dark at 25^0C and allowed to grow for five to seven days. Each of the emerging mycelium or fruiting bodies was transferred to fresh growth media. The pure colony from the isolation were incubated and kept for further laboratory work.

4.4. Characterization and Identification

The identification of the fungal isolates from *P. falcatus* was made based on morphological characterization that emphasize on colony characteristics, conidial features and vegetative compatibility of isolates within a genus.

4.4.1. Cultural characteristics

In the cultural characteristics surface texture, topography, colony color/pigmentation, radial growth rate at different temperature, growth response on MEA and PDA and three pH levels were considered. Conidial characteristics including spore septation, shape, size, wall texture and color were examined. Based on these features, the fungi were identified to the genus level using keys developed by Barnett and Hunter (1972), Sutton (1980), Nelson, *et al.* (1983), Hanlin (1998), Burgess, *et al.* (1994) and Old, *et al.* (2000).

The growth response of the isolates obtained from symptomatic trees of *Podocarpus* was evaluated at different temperature levels including 15, 22 and 29 ^{0}C; growth response on

PDA and MEA and growth response on Potato Dextrose Broth (PDB) medium with pHs 4.5, 5.5, 6 and pH 6.5 was evaluated.

To this effect each of the isolated fungi were grown on MEA and PDA. The isolates were incubated on each growth media for 7 days at 25^0C. Mycelial disks with 5 mm diameter of each fungus were taken from the edges of the colony and the discs were inverted and placed in the center of 90 mm diameter petri plates containing MEA and PDA. The plates were sealed with parafilm and incubated in darkness in three incubators adjusted with the three temperatures levels. This was replicated three times. Colony diameter of each fungus per plate was measured in centimeter at right angle in two days interval. The average growth of the three replicates at the 8th day was finally calculated after subtracting the diameter of the plug.

Potato dextrose broth (PDB) medium (400 g L^{-1} potato, 20 g L^{-1} dextrose) was prepared to evaluate the mycelial growth at the three-pH levels. These range were adjusted using 1N HCl and 1N NaOH. The media were sterilized at 121^0 C for 15 min and 0.2 g L^{-1} streptomycin was added into the medium. Three discs with 5mm diameter were taken from the margin of 7 days old culture grown on PDA and inoculated into flask containing 50 ml PDB with three replications. The pH of the Potato dextrose broth was measured (pH 6) and was used as controls. Each pH this treatments were incubated in room temperature (25 ^{0}C). All these treatments were put into rotary shaker operating at 120 revolutions per min. After a week, the mycelium was collected using Whatman 1 filter paper and the dried in an oven having a temperature of 60-65 ^{0}C for 12 hrs. The dry weight of the isolates was measured with Wagtech WAS 220/C/2.

4.4.2. Conidial characterization

Each fungus was grown in 2% MEA and incubated at 25^0C for 4 days. Blocks of the respective cultures were transferred into sterilized water agar containing 20 g agar on 1 liter of water for sporulation. Sporulation was induced by subjecting the plates to near UV light (black light) of 366 nm in an alternating cycle of 12 hrs UV and 12 hrs continuous darkness (Sutton, 1980; CABI International, 2005). The radiation was made using UV-LW

366nm Konrad Benda D-6908 Wiesloch. The spores features were examined under microscope with 400x magnification (Olympus, Germany). The measurements of the spores were made by the Digital Solutions for Imaging and Microscopy, Soft image System.Corp., USA (http://www.soft-imaging.net.).

The process of sporulation was further analyzed in an additional three media namely Corn Meal Agar (17g L^{-1}), Podo leaf Juice Agar and Water agar that have needle. The Juice agar was prepared from the young leaves of *Podocarpus falcatus*. Leaves were thoroughly washed in tap water for 10 min and boiled in distilled water for 30 min. The liquid filtrate was collected and 20 g L^{-1} of agar were added to solidify. The last medium differs from media containing only water agar by the presence of autoclaved needles of *Casuarina equistifolia*. Three needles were laid on the surface of water agar during inoculation of fungi. Level of sporulation in this medium was considered only on the surface of the needle. The comparison of the four sporulating media was made in 3 replications by incubating at 25^0C without the induction of black light.

4.4.3. Vegetative Compatibility Groups

Cultures were grown on MEA for 5 days at 22^0C. Plugs (5 mm diameter) from colony margins of the cultures were paired on MEA in all possible combination with themselves and with all others to determine vegetative compatibility (Adams, *et al.*, 1990). Four isolates separated by 5.5 cm were placed in 14 cm petri plates. These plates were incubated at 22^0C in dark for 10 days. Based on the absence or presence of barrage zone or line between isolates, the vegetative compatibility was rated as compatible or incompatible, respectively (Adams, *et al.*, 1990, Smith, *et al.*, 2000).

4.5. Pathogenicity test

In order to check the role of the fungi found associated with shoot dieback of *P. falcatus* in causing the dieback a pathogenicity test was conducted. This test was conducted both in the laboratory and out in the field.

4.5.1. Preparation of inoculum of the test pathogens

Each fungus was first grown on 2% MEA for 4 days and transferred to 20 g L^{-1}water agar medium and allowed to sporulate under near UV light. Spore suspension of each fungus was prepared by washing the cultures with sterile distilled water. The concentration of these spores was adjusted to 1 x 10^6 spores ml^{-1}using a haemocytometer.

4.5.2. Inoculation and pathogenicity study

4.5.2.1. Inoculation on detached leaves in Growth chamber

Non-symptomatic and undamaged young leaves were collected from *P. falcatus*. These leaves were thoroughly washed in running tap water for 10 min to remove dust particles. Surface sterilization of leaves was made by immersing the leaves in 70 % ethanol for 2 min. It was then rinsed 3 times in sterile distilled water and blotted on sterile tissue paper. The wound was made on the surface of the leaf at two points using a sterile needle. Spore suspension was loaded on the point of wound. The control group was treated with sterile distilled water. All the experimental plates were regularly supplied with enough amount of sterile distilled water on the surface of filter paper to regulate the humidity inside the plates. Plates were incubated at 25^0C for a month. The treatments were replicated four times (Jackson, *et al.*, 2004).

4.5.2.2. Inoculation on seedlings in the nursery

In nursery inoculation was carried out on 9-month-old *P. falcatus* seedlings in the nursery of Science Faculty at AAU on 9th of May 2006. Four non-symptomatic leaves per tree were selected for the inoculation study. The wounded and unwounded treatment was carried out on the same plant. The inoculation was made on five seedlings and additional five seedlings were served as controls. Before inoculation each the leaves were properly washed using distilled water. The leaves were wounded by sterilized needles at two points. The spore suspension was placed on the surfaces of both wounded and unwounded leaves. The controls were inoculated with sterile distilled water. Each treated seedlings were covered with sterile plastic and paper bag overnight in order to provide favorable environment for the formation penetration pegs (Pethybridge, *et al*., 2004). Finally, each treated seedling was marked and lesion development was evaluated after 7 and 15 days. All symptomatic leaves were collected after month for re-isolation.

4.5.2.3. Inoculation on saplings

The field inoculation was conducted on April 2006 at the Center for Indigenous Trees Propagation and Biodiversity Development Tulu Korma at Addis Alem (2170 m.a.s.l, 09^0 01' N and 038^0 21' E), 42 km west of Addis Ababa.

This experiment was conducted on 2-years-old saplings having an average root collar diameter of 7.37 cm and 1.07 m height. The study was conducted on seedlings planted at 2 m spacing. Ten trees were inoculated with fungal isolates and ten trees were inoculated with sterile distilled water as control. The inoculation was made randomly after selection on branches randomly selected between the tip and half a meter above the ground to avoid contact of the branch with the ground. Five leaves each for wounded and unwounded treatment were selected and inoculated with spore suspension. The remaining technique of inoculation was made according to the procedure for inoculation on nursery.

4.6. Re-isolation

Leaves that developed lesions were selected and undergo re-isolation according to the methods described in section 4.3 except additional disinfections were made using 5% $NaOCl_3$. Cultural and spore characteristics of the re-isolated fungus were compared with the original source of inoculum.

4.7. *In vitro* Antagonistic Activity

4.7.1. Antagonism of bacteria against fungal leaf pathogens

Two bacteria, *Bacillus* spp and *Pseudomonas fluorescence* were used. The *Bacillus* sp. was isolated from soil by serial dilution method whereas *P. fluorescence* was collected from Applied Microbiology laboratory. Dual culture of a bacterium and fungus were used in all combination. A 5 mm mycelial disc of each fungus was put at 20 mm distance from the bacterium. Each bacterium was streaked on a 20 mm line perpendicular to the fungus.

King's B medium (20 g protease peptone, 2.5 g K_2 $HP0_4$, 15 ml glycerol, 6g $MgSO_4.7H_2O$, 20 g agar and 1L water) was used to cultivate *P. fluorescence* (Dhingra and Sinclair, 1985). After the fungal isolates were checked for their growth on this medium, each fungus were crossed with *P. fluorescence.* The antagonism reaction between each fungus and *Bacillus* sp was observed on 2 % MEA.

4.7.2. Antagonism of fungus against fungal leaf pathogens

Trichoderma harzianum, which is supplied by Dr. Tesfaye Alemu, was used as biological control agent in dual culture with the pathogens. A 5 mm mycelial disc from the periphery of 7 days culture of the bioagent were placed 30 mm away from fungal isolates in 2% MEA. The isolated fungi were inoculated 48 hrs prior to the placement of the bioagents to establish and balance the growth of isolated fungus with *T. harzianum.*

Three replicates and additional plates having only the test fungal isolates were used as control for each category of section 4.7.1 and 4.7.2. All these plates were incubated at 22^{O}C. Growth inhibition zones were examined using stereomicroscope every 2 days and the actual measurements were taken after 7 and 9 days of inoculations.

Radial growth reduction (% of inhibition) was calculated according to Montealegre, *et al.* (2003) in relation to growth of the control as follows:

% Inhibition = {1 – [fungal growth/ control growth]} x 100

4.8. Statistical Analysis

The statistical analysis of growth characteristics of isolates at different temperature and media and the comparison of isolates based on different pH were conducted using the GLM procedures of SAS statistical analysis software (SAS institute Inc., Cary, NC) version 8. Differences between treatments were determined by using least squared means comparisons with ($p<0.001$).

5. RESULTS

5.1. Symptoms of shoot dieback

Disease symptom was observed on the tip of leaves of the leader shoot. Initially very small brown spots developed at the tip, along the mid veins and margin of the leaf. Gradually as the lesions developed, the color of the leaves changed to yellow and end with reddish brown. This consequently led to the death of leading shoots. The infection then spread down to other leaves and twigs. These disease symptoms were found on seedlings, saplings and mature trees.

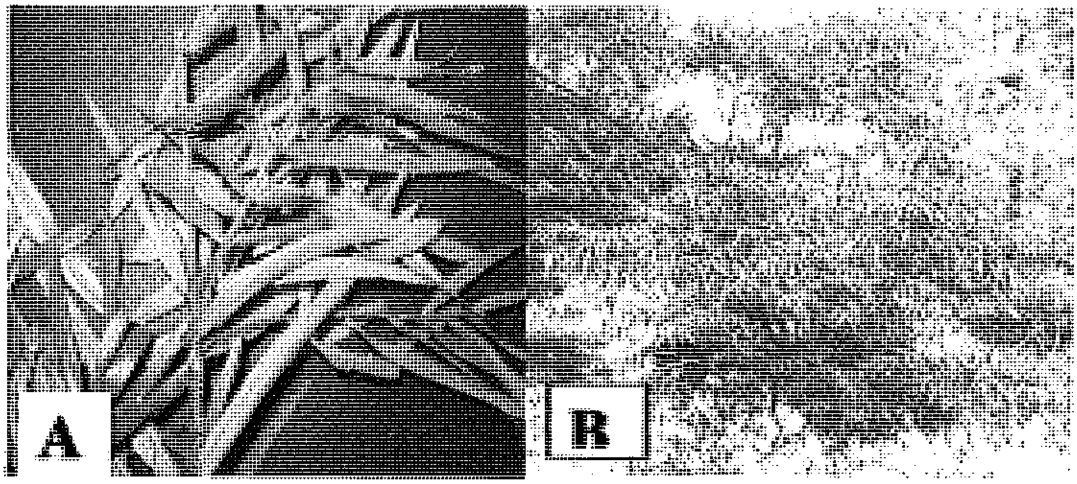

Fig. 2. Symptomatic leaves (A) and branches (B) of *P. falcatus* at Menagesha-Suba

5.2. Sample collection

The samples were collected from Menagesha-Suba and Munesa-Shashemene State Forests between 2349-2604 and 2130-2307 m.a.s.l., respectively. It was observed that infection occurred both on seedlings, sapling and matured trees of *Podocarpus falcatus*. The symptom of dieback was evident in these study sites.

5.3 Isolation

A number of fungal isolates were isolated from leaves and twigs of *P. falcatus* trees from the three study sites. Among these, nine of the most common and frequently isolated fungi were selected for further work (Table 1).

Table 1. Fungal isolates from leaves and twigs of *P. falcatus*

ISOLATE CODE	PART OF PLANT
Pf-24	Leaf
Pf-32	Twigs
Pf-53	Twigs
Pf-42	Twigs
Pf-12	Twigs and Leaf
Pf-1	Leaf
Pf-21	Leaf
Pf-22	Leaf
Pf-9	Twigs

5.4 Characterization and Identification of the fungal isolates

5.4.1. Cultural characteristics

5.4.1.1. Colony morphology

The nine most frequently isolated fungal isolates were first categorized into four groups based on the pigmentation of their colony on MEA. Accordingly isolates Pf-12, 24, 32 and 53 were assigned to group I; Pf-21 and 22 into group II; Pf- 1and 42 into group III and Pf-9 into group IV.

Isolates in-group I had gray to black color in the top with dark pigmentation at the reverse side. Among group I isolates, Pf-53 was relatively darker than the other three isolates. The mycelia of each isolate were fluffy and raised in the top. The colony had lobbed and undulating margins (Fig.3).

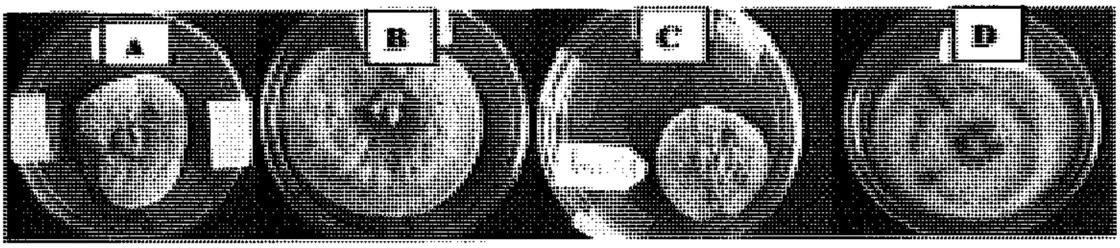

Fig.3. Colony morphology: A) Pf-24, B) Pf-32, C) Pf-53 and D) Pf-12

Isolates Pf-21 and 22 had brown pigmentation both in the top and reverse side of the colony. These two isolates were categorized into group II. They had difference at their younger and older age of the culture. Pf-21 had rifts that out radiate to the periphery whereas Pf-22 had no such structure. At the later age of the culture, Pf-22 form concentric circles while Pf-21 fail to do so (fig. 4).

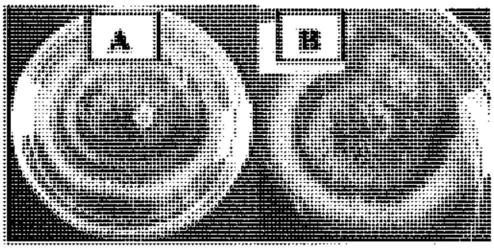

Fig.4. Colony morphology: A) Pf-21 and B) Pf-22

Isolates in-group III had white aerial mycelium in the top. Pf-42 had white, fluffy and cottony mycelium that form circular colony. Pf-1 had fluffy and raised snow-white mycelium. These two isolates differ from one another in the texture and color of aerial mycelium. Pf-42 was smooth and white at the younger age and changed to pink, as it got old. On the other hand Pf –1 had rough textured mycelium that became yellow when the colony gets old. In addition to this the colony of Pf-42 forms pink circle in the center earlier than the peripheral mycelia, which had none in Pf-10. The reverse pigmentation of the two isolates did have pigmentation on the growth medium.

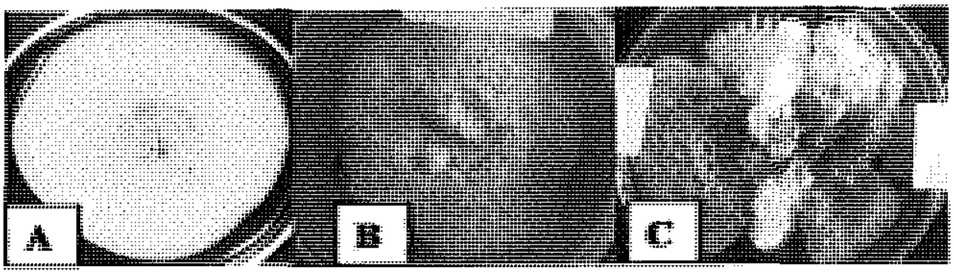

Fig. 5. Colony morphology: A) Pf-42, B) Pf-1 and C) Pf-9

The only isolate found in-group IV was Pf-9. It has an extensive and cottony mycelium. The aerial mycelium had yellow to brown pigmentation. This pigmentation gradually turned to pink, as it grows old. On the reverse side, the pigmentation was red on MEA, and there was diffusion of pigmentation ahead of the growing mycelium.

5.4.1.1 Growth characteristics of isolates at different temperature and media

All the isolate showed good growth performance on both MEA and PDA. However, some isolates showed difference in their performance at specific combination of temperature and media (Table 2). Isolates Pf-24, 32, 53, 42 and 1 showed no preference to any of the two growth media whereas isolate Pf-12, 21, 22 and 9 grew better on MEA. Isolate Pf-24, 32 and 53 preferred 29^0C for better mycelial growth whereas, Pf-12, 21, 22, 42, 1 and 9 had better colony growth at 22^0C (Table 2).

Table 2. Colony diameters of fungi at three temperatures and two media in 8 days

Isolate code	**Temperature (^{0}C)**					
	15		**22**		**29**	
	Media (cm)					
	MEA	**PDA**	**MEA**	**PDA**	**MEA**	**PDA**
Pf-24	2.38	2.13	4.62	4.28	5.2	7.15
Pf-32	2.7	3.78	6.6	6.63	7.07	6.57
Pf-53	2.73	2.77	4.22	5.00	5.72	5.00
Pf-42	4.43	4.45	7.71	8.25	6.35	7.23
Pf-12	4.48	4.15	8.33	6.40	2.67	0.68
Pf-1	3.6	3.78	7.38	7.25	2.28	1.87
Pf-21	4.11	3.28	8.45	7.03	3.75	1.74
Pf-22	4.02	3.63	8.42	5.37	3.62	2.08
Pf-9	4.7	1.83	6.85	1.67	2.83	1.88

5.3.1.3. The effect of pH on mycelial growth of the isolates

For most isolates the largest biomass production was recorded at pH 6. But isolate Pf-1 preferred pH 6.5. Three isolates namely Pf-24, 32 and 53, showed similar pattern of growth at all pH range. The minimum growth rate of these isolates was measured at pH 6.5. There was some variation in growth rate of isolates Pf-1, 9, 12, 21, 22 and 42. The lowest records of dry weight for the six isolates were observed at pH 5.5 for Pf-21, 6.5 for Pf-22, 4.5 for Pf-42, 6 for Pf-1 and 4.5 for Pf-9.

Table 3. Impact of pH on mycelial biomass production

Isolate code	Final pH				Mean mycelial dry weight (g)			
	Control (pH 6)	4.5	5.5	6.5	Control (pH 6)	4.5	5.5	6.5
Pf-24	6.87	6.87	6.24	5.98	0.870	0.721	0.615	0.55
Pf-32	6.78	6.24	6.47	6.54	0.827	0.714	0.636	0.435
Pf-53	6.23	6.17	6.51	6.76	0.971	0.77	0.704	0.304
Pf-42	6.42	5.53	6.12	6.75	2.001	0.575	0.651	0.657
Pf-12	6.41	6.70	6.87	7.06	0.805	0.553	0.430	0.398
Pf-1	5.13	5.07	5.41	5.65	1.310	1.626	1.535	1.680
Pf-21	6.34	6.50	6.68	6.73	0.680	0.646	0.539	0.551
Pf-22	6.24	6.47	6.63	6.72	0.753	0.619	0.665	0.576
Pf-9	5.74	5.50	6.38	5.86	0.660	0.356	0.517	0.450

5.4.2 Conidial Characterization

Analysis of the morphological characteristics of the spores indicated that the above nine isolates can be grouped into four with some rearrangements of the earlier categorization. Based on the new groupings, group I constitute of Pf –24, 32 and 53; group II-Pf-12, 21, 22 and 42; group III- Pf-1 and group IV- Pf-9.

The spores in group-I was broader near the base and gradually tapered to an elongated beak, providing a club-like appearance (fig.7. A-C). The conidia had brown to dark pigmentation and were rough. The conidia have both transverse and longitudinal septa walls. The septa occasionally produce a zigzag appearance. These conidia were born in chains with brown or dark pigmentation. However, the dimension of these conidia was different among the three isolates (Table 4).

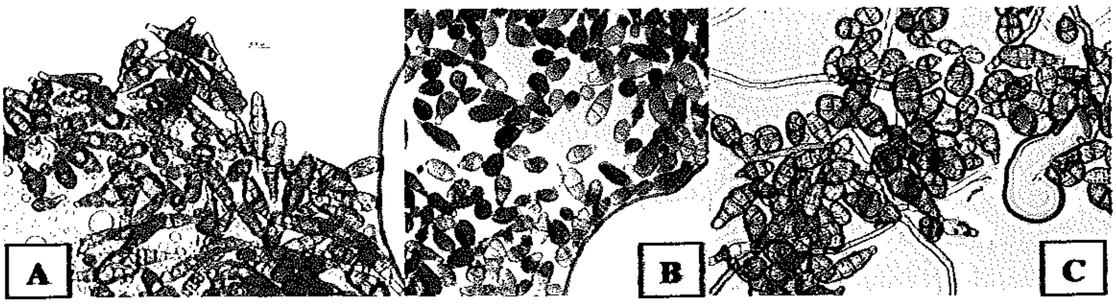

Fig. 6. Spore morphology: A) Pf-24, B) Pf-32 and C) Pf-53

The spores of isolates in group-II were embedded inside a globular structured pycnidium. In general the isolates of this group have rounded to pyriform, brown to black pycnidia. The pycnidia were located superficially or partly immersed in water agar with conspicuous ostia. The pycnidia of Pf-21, 22, and Pf-42 were located both in the surface and inside the media. However, the pycnidia of Pf-12 were limited to the surface of water agar. Moreover, Pf-12 had unusually two ostia whereas the other isolates have single ostia. The conidia that released through ostia had ellipsoid to cylindrical shape in Pf-21, cylindrical in Pf-22, ellipsoid in Pf-12 and Pf-42. All these pycnidiospores were hyaline in color and smooth in texture (Fig.8). Except Pf-21 that had two-celled, the remaining three isolates produced one-celled conidia. In addition to these features, the isolates in this group had many-celled apical chlamydospores with regular septation.

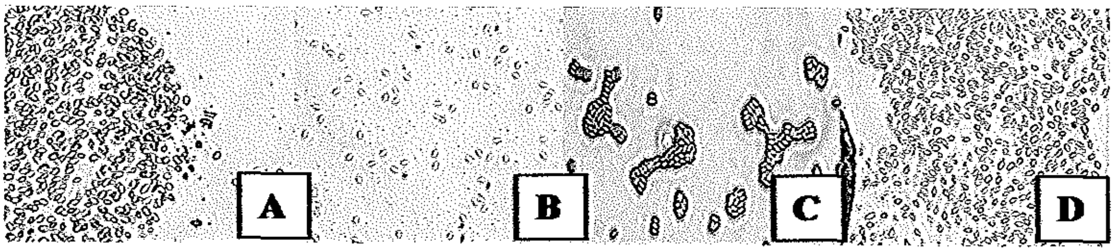

Fig.7. Spore morphology A) Pf-12, B) Pf-21, C) Pf-22 and D) Pf-42

The spores of isolate Pf-1 were different in shape from any of the isolates. Hence it stands in a separate group labeled as group-III. The spores of this isolate were imbedded inside acervulus. The acervulus had an appearance of black ball under 40X stereomicroscope. It can be easily seen by naked eye as small dots on surface of MEA. The conidia while inside the acervulus were not easily visible under any of the magnification. The conidia become visible only when the acervulus bursts. These conidia are five-celled. The five cells of Pf-1 are divided into three darkly pigmented center cells and two clear or hyaline pointed end cells. The spores had three setae or appendages in the upper terminal and a short pedicle or appendage at the other terminal. The conidia had a spindle or clavate shape (Fig. 8A). These characteristics were the basis for its identification.

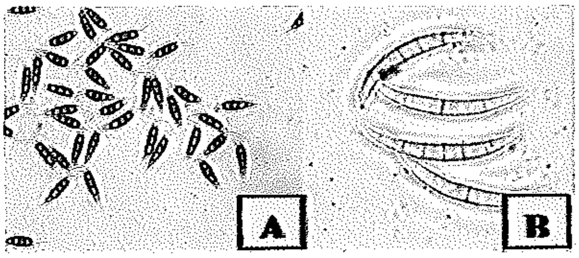

Fig. 8. Spore morphology A) Pf-1 and B) Pf-9

The isolate in-group IV had slender shaped macroconidium. This macroconidium have three distinct septa and hyaline pigmentation. It had tapering apical and foot shaped elongated basal cells (Fig.8B).

Table 4. Conidial dimensions of the isolated fungi

Isolate	Conidial length (μm)				Conidial width (μm)			
code	Mean	Range	SD	SE	Mean	Range	SD	SE
Pf-24	119.8	68.7-191.4	33.4	6.7	44.9	32.1-58.5	6.4	1.3
Pf-32	102.8	78.3-156.1	21.2	4.2	38.4	28.9-49.5	4.8	1.0
Pf-53	111.9	79.0-145.8	22.1	4.4	51.5	36.6-71.3	8.8	1.8
Pf-42	30.4	26.3-37.8	3.3	0.7	17.4	14.8-20.6	1.8	0.4
Pf-12	26.8	23.1-30.2	2.2	0.4	17.4	13.5-19.3	1.7	0.3
Pf-1	100.1	73.2-113.7	8.5	1.7	26.4	17.3-32.4	3.8	0.8
Pf-21	23.7	18.6-30.2	2.5	0.5	14.8	10.3-19.3	2.1	0.4
Pf-22	20.3	17.3-23.8	1.8	0.4	10.1	7.1-16.1	2.0	0.4
Pf-9*	-	-	-	-	-	-	-	-

Spore sizes in water agar and 400X   * The dimension of this isolate were not measured

The mean conidial dimension of the isolates within group I ranges from 102.8-119.8μm x 38.4-51.5μm, in-group II 20.3-30.4μm x10.1μm x 17.4 μm and for group III 100.1μm x26.4μm.

5.5. Vegetative Compatibility Groups

Among the three isolates of group I, Pf-24 and 32 did not form barrage, and this indicates that they are compatible. Whereas, isolates Pf-53 developed black barrage line with both Pf-24 and 32 indicating that it was not compatible with these two isolates.

All the isolates in group II (Pf-12, 21, 22 and 42) developed barrage zone along the common border. Hence, it indicates that the isolates in this group are vegetatively incompatible suggesting that these are not identical.

Table 5. Summary of classification for the isolates

Groups	Characterization of isolates		Genus
	Pigmentation	Spore morphology	
I	Pf-12, 24, 32 and 53	Pf-24, 32 and 53	*Alternaria.*
II	Pf-21 and 22	Pf-12, 21, 22 and 42	*Phoma*
III	Pf-1 and 42	Pf-1	*Pestalotiopsis*
IV	Pf-9	Pf-9	*Fusarium*

Based on colony pigmentation and spore morphology isolate Pf-24, 32 and 53 were categorized into genus *Alternaria* (Appendix 1), Pf-12, 21, 22 and 42 into genus *Phoma* (Appendix 2), Pf-1 into genus *Pestalotiopsis* (Appendix 3) and isolate Pf-9 into the genus *Fusarium* (Appendix 4).

5.6 Sporulation Performance

In addition to the media used for sporulation, isolate coded as Pf-24, 32, 53, 21, 22 and 9 produced conidia on MEA within five days. Isolate Pf-1 produced spore on MEA as it gets old. Isolates Pf-12 and 42 did not produce conidia even after extended incubation period. Moreover, the spores produced on MEA were not many as compared to the media used for sporulation in this experiment.

Fungi assocated with shoot dieback of *Podocarpus falcatus*

All the isolated fungi sporulated in both corn meal agar and water agar media within 5 days except for Pf-1. This isolate did not produce spore under the absence of UV light. However, there was difference in time of sporulation, size and number of spores produced on the two media. Among the isolates Pf-24, 32 and 53 sporulated faster than the rest of isolates.

Spore formation within 1cm radius area showed variation between the two media. Accordingly, Pf-42 formed equal sized pycnidia within the given period of time on both water agar and corn meal agar. There was also large number of pycnidia, more than 200, in both media even though there were a little more on water agar than corn meal agar. On the other hand Pf-12 showed difference in size and number of pycnidium between the two media where there was better performance on corn meal agar than water agar. In corn meal agar, there were more than 600 pycnidia per 1 cm radius around the disc area. In general Pf-21 had a relatively smaller number (less than 200) pycnidia in both media as compared to Pf-22. In both cases, large sized pycnidia were formed on corn meal agar. More number of pycnidia was counted on water agar than corn meal agar. The pycnidia of Pf-22 were fewer (less than 200) and smaller dimension on water agar. The number and size of pycnidia formed on water agar were relatively less than the number pycnida in corn meal agar.

In general there were large amount of pycnidium production both in size and number on corn meal agar than water agar. While a medium numbers of spores were counted inside 1-cm radius of the corn meal.

All the isolated fungi except Pf-42 were sporulated on the surface of the needle lied on water agar. Isolate Pf-24, 32 and 53 produced conidia and Pf-12, Pf-1, 21 and 22 produced pycnidia. These pycnidia were grown on the surface of the needle within 6 days. However, isolate Pf 1 producted large number of acervulus after 6th day of inoculation. In Pf-9, no pycnidia or conidia formation was observed on the needle even when examined under 40X stereomicroscope.

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All isolates sporulated on juice agar. The number of pycnidia and conidia were nearly equal to that on water agar. However, the color of the media made speculation difficult.

5.7. Pathogenicity test

Development of lesion was observed on both wounded and unwounded treatment on growth chamber experiment. Whereas, at the field and nursery condition lesion development was common on wounded leaves (Table 6).

5.7.1 Inoculation on detached leaves in Growth chamber

Lesion development was observed both around the wounded area and on spots where spores were inoculated on the unwounded leaf.

Table 6. Percentage of positive results with inoculation of fungal isolates

Isolate	Growth chamber		Nursery		Saplings	
code	Wounded	Unwounded	Wounded	Unwounded	Wounded	Unwounded
Control	0	0	0	0	0	0
Pf-24	50.00	75.00	33.33	-	67.86	-
Pf-32	75.00	50.00	80.00	-	69.81	-
Pf-53	100.00	100.00	50.00	-	75.00	-
Pf-42	75.00	50.00	22.22	-	67.44	-
Pf-12	100.00	100.00	44.44	-	44.12	-
Pf-1	100.00	100.00	57.14	-	-	-
Pf-21	66.67	33.33	0	-	96.15	-
Pf-22	33.33	75.00	-	-	94.44	-
Pf-9	100.00	100.00	50.00	-	53.33	-

5.7.2. Inoculation on seedlings in the nursery and saplings

The results of inoculation study in the nurseries and out in the field showed no lesions in the unwounded leaves. On wounded leaves lesion development was observed after two weeks of inoculation.

Re-isolation from lesions on growth chamber, nursery and saplings were identical with the morphological characteristics of the original isolates. Hence, it is highly probable that these isolates might have been associated with shoot dieback of *P. falcatus*. The result in table 7 demonstrates that inoculation on seedlings and saplings failed to produce lesion on unwounded leaves. Whereas, lesion development was observed on wounded leaves and this may indicate that these fungi could be a weak pathogens that attack plants under stress condition.

5.8. *In vitro* antagonistic activity

In vitro antagonistic activity of the three biological agents was evaluated against the nine isolates included in the study. The result of this study indicated the prevalence of differences in inhibiting the rate of growth of the isolates. Of these bio-agents, *T. harzianum*, has rapid growth that covers the mycelium of the combined isolates. Hence, there was no development of inhibition zone. In addition to this the activity of mycoparasitism was also not evident in its interaction with the isolates. Therefore, these results indicate that the interaction between this bioagent and the isolates was competition for space and nutrition rather than forming inhibitory zones and mycoparasitism.

The interaction of the isolates with Bacillus sp. and *P. fluorescence* was different from *T. harzianum*. Some of the isolates had inhibitory zone and the others did not. Isolates Pf-1, Pf-24 and Pf-53 developed a clear inhibition zone in the interaction with the given *Bacillus* species, whereas isolate Pf-9, Pf-21, Pf-22 and Pf-32 did not exhibit any inhibition zone. More over there was change in color of the reverse side in all isolates except Pf-1, Pf-9 isolates along the frontline of interaction.

During the interaction of the fungus with the bacillus species, isolates such as Pf-21 and Pf-22 even missed the characteristic edge of their colony. In general, there was unequal growth rate of mycelium in all isolates. That is the active growing hyphae of the isolates had limited growth in the direction of interactions. *Pseudomonas fluorescence* did not have any apparent inhibition effect against in almost all isolates. However, the bacterium was in position to grow faster than the fungus so that most fungi were near to complete surrendered by the bacterium within four days of inoculation (Table.7).

Table 7. Percent of growth reduction by the different bioagents at 22^0C on MEA

ISOLATE CODE	*P. florescence*		*Bacillus* sp.	
	Day –7	Day-9	Day-7	Day-9
Pf-24	37.20	43.65	12.50	22.80
Pf-32	28.73	31.01	25.44	27.41
Pf-53	25.96	32.18	47.37	52.66
Pf-42	44.25	44.93	9.40	21.26
Pf-12	54.72	61.02	63.02	60.55
Pf-1	42.83	45.65	21.46	20.40
Pf-21	41.27	42.71	33.03	42.67
Pf-22	36.82	36.94	18.62	36.07
Pf-9	10.96	20.38	20.95	24.68

Data are the mean of three replications

6. DISCUSSION

Generally, there is no adequate information on disease of most indigenous tree species including *Podocarpus falcatus*. In the field survey made in Menagesha and Munessa Shahshemene forests, prevalence of a wide spread death of leader shoots of *Podocarpus falcatus* was observed. The same problem was also found on *Podocarpus* trees in the compound of Science Faculty, at Addis Ababa University. Several groups of fungi were successfully isolated from the symptomatic plant parts mainly from leaves and the tender twigs. The main symptoms observed on leaves were characterized by formation of small brown spots on the surface of the leaves. This study provides information as to which fungal species are involved in causing the disease symptoms.

Based on the visual appearance of the fungal colony on the growth media, nine different types of fungal isolates were frequently and commonly isolated from most samples collected from the three sites. Further analysis of colony morphology and spore characteristics of the isolate showed that these fungal isolates were categorized under four distinct groups. Three of the isolates are grouped into the genus *Alternaria* Nees. The spore shape, pigmentation and septation pattern of these isolates were similar to the description of *Alternaria* described by Pryor (2003) and Barnett (1962).

The fungi in the genera *Alternaria* exhibited the diagnostic characteristics of the genus: abundant, thick, grayish to greenish aerial mycelium, long catenated, sometimes branched chains of conidia (Mirkova and Konstantinova, 2003). The spore dimension and shape of the fungal isolates in this group showed variability. According to Mirkova and Konstantinova (2003), this is the common characteristic of the genus. The spores of the isolates in this group had both longitudinal and transverse septa (Hanlin, 2001). The number of the septa was within the range given by Pryor (2003) for its longitudinal septa. The transverse septum of the isolates from *P. falcatus* was 1-5 compared to 3-6 septa described by Mirkova and Konstantinova (2003).

The other difference encountered in the group designated in genus *Alternaria* was the dimensions of the conidia. The isolates in this group had spore dimension in a range of 102.8-119.8 x 38.4-51.5 μm while the spore dimension recorded for *Alternaria* genus was between 25–35 × 5–10 μm (Mirkova and Konstantinova, 2003). The possible cause of difference for these isolates could be the difference in the scale of the measurement used, the media used, incubation time and temperature, humidity, species difference or the morphological plasticity of *Alternaria* species (Mirkova and Konstantinova, 2003). This means, in addition to the intrinsic factors, environmental conditions also affect the morphological characteristics of the genus (Thomma, 2003).

Comparison of spores of the three isolates from *P. falcatus* with the illustration of spores in the picture gallery of *Alternaria* online
http://ag.arizona.edu/PLP/alternaria/online/picturelibrary.htm indicated that these isolates closely resemble to *A. alternata* and *A. arborescence*. Abdella Gure (2004) has recorded *A. alternata* and *A. arborescence from seeds of Podocarpus falcatus,* however, this needs further investigation.

Alternaria has a wide distribution worldwide. The species in this genus exist as pathogen, saprophyte and endophyte (Thomma, 2003). Several *Alternaria* species are reported to be saprophytes. Some other species acquired pathogenic capacities on plants and cause a range of economical damage such as stem canker, leaf blight or leaf spot on vegetables, fruit trees and ornamentals (Thomma, 2003; Przybyl, 2002).

Alternaria brassicae (Berk.) Sacc. that caused yield losses on Indian mustard (Meena, *et al*., 2004); *A. alternata* and *A. panax* on Calathea, Brassica and their relatives, *A. saponariae* as leaf spot disease on Saponaria plant (Mirkova and Konstantinova, 2003), Abdella (2004) reported the occurrence of *A.alternata*, *A.arborescens*, *A .tenuissima* from seeds of *P.falcatus*., out of which *Alternaria tenuissima* caused necrosis on the seedling of *P.falcatus* Abdella (2004).

Similarly the inoculation test conducted on detached leaves, seedlings and saplings of *P.falcatus* that the three isolates in the genera *Alternaria* produced lesions on inoculated leaves. The development of lesion alone could not prove that these fungi are responsible for shoot dieback on Podo. The role of these fungi in relation to shoot dieback needs to be investigated further.

The second group comprised four fungal isolates namely Pf-12, Pf-21, Pf-22 and Pf-42. Based on the mycelial and spore characteristics of the isolates are closely related to the characteristics of genus *Phoma* Desm. described by Sutton (1980) and Barnett (1962). The colony morphology and spore characteristic of isolate Pf-42 was closely related to the description of *Phoma exigua* Desm. (Sutton, 1980). The result from vegetative compatibility groupings indicated that these four isolates were incompatible. This indicates that there is a high chance that these isolates might not be closely related to each other. Isolate Pf-22 also resemble to the description of *P. leveillei* Boerema and Bollen. (Sutton, 1980). However, there were differences in the dimension of the conidia of these two species (5.5-10 x 2.5-3.5μ and 2.5-3.5 x 1.5μ, respectively) to the isolates collected from *P.falcatus*. Categorizing isolates Pf-12 and 21 into species level was difficult due to limitation of information.

Phoma species are known as a pathogen, saprophyte and secondary invader of diseased tissue (Roustaee, 2000). Some of the important opportunistic plant pathogens are *P. exigua*, *P. macrostoma*, *P. multirostrata* and *P .sorghina* (common in the tropics and subtropics) (Boerema, *et al.*, 2004), *P. ligulicola* as the cause of severe spring dieback of foliage of commercial Pyrethrum crops (*Tanacetum cinerariifolium* (Trev.) Schultz Bip.) in Kenya, Tanganyika and Papua New Guinea (Pethybridge and Hay, 2001), *Leptosphaeria maculans* (anamorph=*Phoma lingam*) caused leaf and stem lesions of crops of canola (Sosnowski, *et al.*, 2004), *P. sorghina* as the aetiology of leaf spot on maize in Brazil (Amaral, *et al.*, 2004). The four isolates that belong to this genus developed lesion on leaves of *P. falcatus*. This result however, is not sufficient to suggest that these groups of fungi are responsible for shoot dieback on *P. falcatus* trees.

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The identification of Pf-1 was based on the characteristic of five-celled spores (Old, *et al.*, 2000) and the description of Barnett (1962) and Sutton (1980). This isolate belongs to the genus *Pestalotiopsis* Stey. The grouping of this isolate into specific species was found difficult for limitation of information.

The genus *Pestalotiopsis* has parasitic and saprophytic records. The saprophytes are recorded on seedlings and exposed sapwood of larger trees after wounding. For instance, *P. microspora*, has been recorded as a widespread saprophyte on bark and decaying plant material, and found as an endophyte in many plant species (Metz, *et al.*, 2000).

The pathogenic species of *Pestalotiopsis* attacks all parts of the leaf from base to tip. Some of these records include *P. acaciae* on *Acacia crassicarpa*, *P. neglecta* on *A. crassicarpa*, *Pestalotiopsis sp.* on *A. mangium* and *A. crassicarpa* (Old, *et al.*, 2000). In order to establish infection, some species of *Pestalotiopsis* require wound for infection are wound pathogens (Elliott, 2006). Pf-1 was also found to produce lesion on inoculated leaves of *P. falcatus*. Its involvement in causing the dieback is not yet confirmed.

The shape and characteristics of macroconidia of isolate Pf-9 agrees with the description made by Burgess *et al.* (1994) and Nelson *et al.* (1983) for *Fusarium* Link.spp. The complexity of the genus due to its genetic make-up (Nelson, *et al.*, 1983) made it difficult to suggest the species it resembles to. This genus has a worldwide distribution. It is composed of pathogenic and saprophytic species on higher plants and on decaying materials respectively (Barnett and Hunter, 1972). The pathogenic species have been recorded from a large number of higher plants at all stage of the tree development. The effect was evident in several of native species in the United States (Enebak and Stanosz, 2003).

In conifers *Fusarium* spp. are mainly known as nursery pathogens, causing pre-emergence and post-emergence damping off, root rot of older seedlings and stunting. *F. subglutinans* f.sp.*pini* threat *Pinus radiata* D. Don in plantation (Dick and Dobbie, 2002), *F. proliferatum* (T. Matsushima) Nirenberg and *F. oxysporum* Schlechtend.: Fr.f.sp.*asparagi*

S.I. Cohen caused *Fusarium* crown and root rot of asparagus (Reid, *et al.*, 2002). From the inoculation test it was observed that isolate Pf-9 produced necrotic lesion on *Podocarpus* leaves. The importance of this fungus as a cause of shoot dieback remains unresolved.
Most of the fungi obtained in this study are known to be both pathogenic and saprophytic on different trees and annual crops (Thomma, 2003; Roustaee, 2000; Metz, et al., 2000; Enebak and Stanosz, 2003). The inoculation test conducted on leaves of Podo revealed that these fungi have a capacity of producing lesions mainly on wounded leaves. This implies that these fungi have important association with the disease symptom of shoot dieback. Therefore, the relationship of these fungi in relation to shoot dieback of Podo should be studied in detail.

In relation to growth performance at different temperature, media and pH, isolates within the same genus had common preference of these growth factors. For instance the optimum temperature for the three *Alternaria* isolates was 29^{0}C and was 22^{0}C for *Phoma*, *Fusarium* and *Pestalotiopsis*. Similarly, the three isolates in the genus *Alternaria* had no special preference between the two media used. Unlike *Alternaria* isolates, the four isolates that were grouped into *Phoma* prefer MEA than PDA. Hence, the preference of temperature and media within a group showed more similarity than out groups.

Trichoderma spp. is free-living fungi. It has the potential of producing potent volatile and non-volatile antibiotic substances and also computes for key exudates of plants (Roco and Perez, 2001; Herman, *et al.*, 2004). Member of this genus have been extensively studied to their ability for the action of biocontrol (Monaco, *et al.*, 2004).

The mode of action of *T. harzianum* against the nine isolates agrees with the results obtained by Herman *et al.* (2004) and Kexiang *et al.* (2002) that indicated the growth of the active hyphae of *T. harzianum* quickly colonized the fungal isolates.

In vitro evaluation off antagonistic effect exhibited by *Bacillus* bioagent has the potential to inhibit the growth of the isolates. Such kind of inhibitory property is the result of secretion of different compounds. Okigbo (2005) reported that *Bacillus* spp such as *B.*

subtilis are well known for their secretion of iturin A, surfactin, Bacillopepins and Bacillomycin. Among which iturin A has antifungal properties that used to inhibit spore germination and germ tube swellings against many fungi species. The antagonistic effect of *Pseudomonas fluorescence* against the fungal isolates was not efficiently seen forming an inhibitory zone.

7. CONCLUSIONS AND RECOMMENDATIONS

As far as our knowledge is concerned this is the first report of shoot dieback on the leaves and twigs of *Podocarpus falcatus* in Ethiopia.

In this study the frequent and most commonly encountered genera were identified and characterized. This has its own input about fungal species most commonly and frequently associated the indigenous *P. falcatus* trees. This study also emphasized the need for generating basic data and information disease management in natural forest.

In order to have a good picture on the importance of the fungal species in these natural forests it requires further study especially on identifying the primary causative agent of the disease, its severity and incidence by analyzing various samples collected at different seasons of the year.

The occurrence of similar kinds of fungi on seeds and leaves of *P. falcatus* is interesting. The cause and effect of these associations needs further study.

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9.Appendix

Appendix 1. The synoptic key of genus *Alternaria* and its similarity with the isolates

	Specific characters	**Pf- 24**	**Pf-32**	**Pf-53**
Mycelium	Aerial pigmentation*	Gray	Black	Black
	Reverse pigmentation	Black	Black	Black
	Branching	Branched	Branched	Branched
	Hyphae	Septated	Septated	Septated
	Growth rate	Relatively fast	Relatively fast	Relatively fast
Conidia	Terminology	Phragmosporae Septated	Phragmosporae Septated	Phragmosporae Septated
	Shape	obpyriform,	obpyriform	obpyriform
	Color	Pale brown	Pale brown	Pale brown
	Beak	Short and conical	Short and conical	Short and conical
	Chain formation	Yes Form chain	Yes	Yes
	Septation	Both transverse and longitudinal	Both transverse and longitudinal	Both transverse and longitudinal
	Texture	-	-	-

* there was gradual change of pigmentation as the culture get old

Source: Mycology online. (2005). Alternaria sp. The university of Adelaide. http://www.adelaide .edu.au/legals/copyright.htm.

Appendix 2. The synoptic key of genus *Phoma* and its similarity with the isolates

Synoptic key	Specific characters	Pf-12	Pf-21	Pf-22	Pf-42
Mycelial character		Superficial	Superficial	Superficial	Superficial
Conidomatal character	Position in relation to substrate	Undetermined	Undetermined	Undetermined	Undetermined
	Tissue type	-	-	-	-
	Dehiscence	Ostiolate, Papillate	Ostiolate, Papillate	Ostiolate, Papillate	Ostiolate, Papillate
	Shape	Globose	Globose	Globose	Globose
	Wall-thickness	Thin	Thin	Thin	Thin
	Pigmentation	Gray-dark	Brown	Brown	White
	Setal characters	-	-	-	-
	Branching	Branched	Branched	Branched	Branched
Conidiogenous cell characters	Insertion	-	-	-	-
	Shape	-	-	-	-
	Pigmentation	-	-	-	-
	Other characters	-	-	-	-
Conidiophore characters	Shape	-	-	-	-
Conidial characters	Septation	Aseptate	Medianly 1septate	Aseptate	Aseptate
	Shape	Oval-ellipsoid	Oval-ellipsoid	Cylindrical	Oval-ellipsoid
	Guttulation	Eguttulate	Eguttulate	Eguttulate	Eguttulate
	Wall-thickness	Thin	Thin	Thin	Thin
	Ornamentation	Smooth	Smooth	Smooth	Smooth
	Pigmentation	Hyaline *	Hyaline*	Hyaline*	Hyaline*
	Appendages	-	-	-	-
Paraphyses characters	-	-	-	-	-
Sclerotia	-	-	-	-	-

Source: Sutton, 1980.

Appendix 3. The synoptic key of genus *Pestalotiopsis* and its similarity with Pf-1

General characteristics	**Specific characters**	**Pestalotiopsis**
Conidial characters	Position in relation to substrate	Not determined
	Dehiscence	Ostiolate, non-papilate
	Pigmentation	dark (Barnett and hunter, 1972)
	Type of stroma	Acervular (dark Barnett and Hunter, 1972)
	Locular confugration	Not determined
Conidiogenous cell characters	Insertion	-
	Shape	-
	Pigmentation	-
	Ornamentation	-
	Annellide morphology	-
Conidiophore characters	Shape	-
	Pigmentation	-
	Ornamentation	-
Conidial characters	Pigmentation	Brown
	Septation	4 euseptate
	Shape	Fusiform, straight or slightly curved
	Ornamentation	Verrucose
	Guttulation	-
	Appendages	-
	Wall thickness	-
Mycelium	-	Immersed, branched, septated, hyaline to pale brown
	Dehiscence	Irregular
	Conidiophores	Hyaline, branched and septated at the base and above, cylindrical or lageniform, formed from the upper cells of the pseudoparaenchyma
	Basal cell	Hyaline, truncate, with an endogenous, cellular, simple or rarely branched appendage
	Apical cell	Conic, hyaline, with 2 or more apical , simple or branched , spathulate or espathulate appendage
	Median cells	Brown,

Source: Sutton, 1980

Appendix 4. The synoptic key of genus *Fusarium* and its similarity with Pf-9

Keys	**Specific characters**	**Pf-9**
Cultural characteristics	Rate of growth	Very slow
	Aerial mycelium	Present
	Color of aerial mycelium	Tan
	Color of colony from below*	Dark red (carmine red)
	Color of spore masses	Orange to yellow to tan
Macroconidia from sporodochia	Size	Medium long, generally 3-7 septate
	Shape	Without marked dorsi-ventral curvature with the sides relatively straight and parallel for most of spore length
	Shape of basal and apical cell	Barely notched (not notched or blunt) basal cell and papillate apical cell
Microconida from aerial mycelium	Present/absent	??
	In chains/ false heads	??
	Shape	??
Conidiophores	-	-
Chlamydospores	Present/ absent	-
	Arrangement	-

Source: Nelson, *et al.* (1983); Burgess, *et al.*, 1994
* The reverse side had such color was due to pigment production

N.B. The color of the reverse side of Pf-9 diffuse into the agar often in advance of colony