# Seed-borne Fungi of the Afromontane Tree Species *Podocarpus falcatus* and *Prunus africana* in Ethiopia

Abdella Gure
*Department of Forest Mycology and Pathology*
*Uppsala*

**Doctoral thesis**
**Swedish University of Agricultural Sciences**
**Uppsala 2004**

**Acta Universitatis Agriculturae Sueciae**
Silvestria 334

ISSN 1401-6230
ISBN 91-576-6718-7

Tryck: SLU Service/Repro, Uppsala 2004

# Abstract

Gure, A. 2004. Seed-borne fungi of the afromontane tree species *Podocarpus falcatus* and *Prunus africana* in Ethiopia. Doctor's dissertation.
ISSN 1401-6230, ISBN 91-576-6718-7.

This thesis is comprised of four studies regarding seed-borne fungi of the afromontane forest trees, *Podocarpus falcatus* (Thunb. Mirb.) and *Prunus africana* (Hook. F.) Kalkman, in Ethiopia.

Based on morphology and molecular data from the rDNA (ITS) region, a diverse group of mainly Ascomycota, some Basidiomycota and a few Zygomycota were identified. Phylogenetic analysis of the ITS sequences revealed several clades differentiated according to the host. Some of these fungi were previously reported as seed-borne pathogens from other parts of the world. More fungi were isolated from *P. falcatus* than from *P. africana*.

Four *Botryosphaeria* spp., three of them from *P. falcatus* and one from *P. africana* were identified. The *Botryosphaeria* sp. from *P. africana* was a previously undescribed species and named *Diplodia rosulata* sp. nov. One of the three *Botryosphaeria* spp. from *P. falcatus* was identified as *B. parva* while the other two were previously undescribed *Botryosphaeria* spp. with *Diplodia* and *Dothiorella* anamorphs. *Botryosphaeria parva* was previously identified as a major cause of Botryosphaeria stem canker on Eucalyptus plantations in Ethiopia and elsewhere. The implications of the occurrence of *B. parva* both on native and exotic tree species were discussed.

Based on seed and seedling inoculation tests on *P. falcatus*, five categories of fungi ranging from strong pathogens to germination promoters were identified. Among the tested fungi, *Fusarium oxysporum* and *Polyporus* sp. were strongly pathogenic and caused both seed rotting and seedling damping-off, while *Diaporthe* sp. Po21 and *Diaporthe* sp. Po84 promoted seed germination.

Dual culture tests among seed-borne fungi of *P. falcatus* on 2 % malt extract agar resulted in deadlock or replacement interactions. Co-inoculation of seeds with two germination promoters and six pathogenic isolates resulted in improved germination in some of the combinations. Bioassay tests with HPLC fractions of culture filtrates of the two germination promoting *Diaporthe* spp. indicated antifungal activity against *F. oxysporum* and *Ulocladium chartarum*.

From these studies it was concluded that different groups of fungi could be associated with seeds of forest trees. There are still many undescribed fungal species that may impact forest establishment and development. Therefore, more investigations are necessary in these areas.

Key words: Seed-borne fungi, *Botryosphaeria*, Seed-borne pathogens, *Fusarium oxysporum, Diaporthe* sp., afromontane forest trees, *Podocarpus falcatus*, *Prunus africana*, interspecific interactions

*Author's address*: Abdella Gure, Department of Forest Mycology & Pathology, SLU, P.O. Box 7026, SE-750 07, Uppsala, Sweden (Wondo Genet College of Forestry, P.O. Box 128, Shashamane, Ethiopia).
E-mail: Abdella.Gure@mykopat.slu.se or wgcf@trelecom.net.et.

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## Appendix

## Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

**I.** Gure A., Wahlström, K., Barklund, P. & Stenlid, J. Fungi Associated with Seeds of Afromontane *Podocarpus falcatus* and *Prunus africana* in Ethiopia. (Submitted manuscript).

**II.** Gure, A., Slippers, B., & Stenlid, J. Seed-borne *Botryosphaeria* spp. from native *Prunus* and *Podocarpus* trees in Ethiopia, with a description of the anamorph *Diplodia rosulata* sp. nov. (Submitted manuscript).

**III.** A. Gure, K. Wahlström and J. Stenlid. Pathogenicity of seed-associated fungi to *Podocarpus falcatus in vitro* Forest Pathology. In press.

**IV.** A. Gure and J. Stenlid. *In vitro* interactions among seed-borne fungi of *Podocarpus falcatus*. (Submitted manuscript).

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# 1. Introduction

## 1.1 Afromontane forests and deforestation in Ethiopia

Montane forests are the main constituents of the natural vegetation in the Ethiopian highlands (Breitenbach, 1963). Most afromontane tree species have a wide geographical distributions and ecological amplitudes. Many also exhibit a wide range of growth forms (White, 1983). Afromontane forests can be subdivided into afromontane rain forests, undifferentiated afromontane forests, single dominant afromontane forests and dry transitional montane forests, (Friis, 1992). In Northeast Africa, dry afromontane forests are found at altitudes from 2000 – 2700 m with an average annual temperatures of 15-20 $^{\circ}$C and a mean annual rainfall of 800 – 1200 mm (Friis, 1992).

In Ethiopia, today, most of the afromontane forests have been cleared for agriculture and only a very small fraction of the original vegetation remains, usually on inaccessible steep or rocky terrain (Friis, 1992, Demel & Granström, 1995). Although not fully consistent, reports indicate drastic reduction in the extent of past forest cover in Ethiopia. According to Sayer, *et al*.(1992), about 87 % of the highlands had forest cover which was reduced to 40 % by 1950; and to just 5.6 % by 1980. The Ethiopian Forestry Action Programme (EFAP, 1993) stated that about 66 % of the country was originally covered by high forests and woodlands. More recent estimates of the existing forest cover give 4.2 % (FAO, 2001).

Rapid population growth, loss of productivity of pasture and arable lands, increased demands for productive arable land, removal of wood for fuel and construction and logging are the main causes of deforestation in Ethiopia (Tewoldeberhan, 1989). Moreover, although published accounts are not available, it is generally known that illegal felling for pit-sawing of certain valuable indigenous species such as *Podocarpus falcatus* (Thunb.) Mirb., *Prunus africana* (Hook. F.) Kalkman, *Cordia africana* Lam., and *Aningeria adolfi-friederici* (Engl.) Robyns & Gilbert to mention just a few, has been a very serious contributing factor to the decimation of the remaining afromontane forests of the country. Furthermore, deforestation has brought about the decline in Ethiopian forests in size and species richness eroding the biological diversity to such an extent that certain plants are faced with local extinction (Eshetu, 2002). Studies from the highlands of Ethiopia also indicate that deforestation and subsequent cultivation of land has led to the deterioration of the soil seed bank due to low seed viability and under-representation of the soil seed flora of woody species (Teketay, 1997, Lemenih, 2004).

*Podocarpus falcatus* and *Prunus africana* are among the afromontane forest trees species that are locally threatened by extinction due to deforestation. These species have been extensively and unwisely exploited (Negash, 1995). The annual rate of deforestation estimated to be 160,000 – 200,000 ha year $^{-1}$, is one of the

highest in tropical Africa (Reusing, 1998). Whatever the causes of such extreme rates of deforestation might be the bottom line is that concerted efforts need to be directed towards afforestation and reforestation of the degraded landscapes and conservation of the natural vegetation.

From an environmental rehabilitation stand point and due to their value to society, important native tree species should receive particular attention. However, several factors have made it difficult to use native tree species for large-scale afforestation programs. These include the lack or scarcity of information on seed biology of tropical forest trees, lack of technical skills and facilities for seed handling and treatments, incomplete knowledge about the influence of biotic factors such as pests and diseases on seeds and seedlings health and quality (Kamra, 1989). Although some efforts have been made towards improving some of the above factors, the problems remain in most of the cases. Thus, multifaceted efforts should be made in order to try to alleviate the problems by approaching them from different angles. The purpose of this thesis work, therefore, is to contribute towards that goal with studies on the fungal flora of the seeds of *P. falcatus* and *P. africana*.

## 1.2 Seed-borne fungi of forest trees

The majority of the fungi recorded from seeds of forest trees so far belong to the conidial states of Ascomycota (Anderson, 1986, Mittal, Anderson & Mathur, 1990). A number of ecological groups of fungi can be found associated with seeds. The grouping can, for example, be based on where the association begins, including those fungi commonly known as field fungi and storage fungi; or, alternatively, on the nutritional relationships with the host, encompassing pathogenic, endophytic or saprobic fungi. The term seed-borne describes the state of any micoorganism being carried with, on or in the seed, while the term seed-transmitted includes the act of infection of the seedlings from seed-borne inoculum (Agarwal & Sinclair, 1997, Sutherland, Diekmann & Berjak, 2002). The rate of seed transmission depends upon the host, pathogen, environment, vectors, and their interaction over time (Agarwal & Sinclair, 1997).

Fungal propagules gain access to the seed tissues at any time from flowering to the post-shedding phase (Sutherland et al., 2002, Dhingra *et al.*, 2003). This can happen while the fruits are still on a tree, after falling to the ground, during collection and processing, during transit or in storage (Sen-Sarma, Thakur & Sehgal, 1988, Singh, 1996, Dhingra et al., 2003). Seeds may be internally infected by fungi *ab initio* by systemic transmission via the parent plant (Mycock & Berjak, 1992, Kabeere, Hampton & Hill, 1997) or through the stigma-style continuum during flowering (Marsh & Payne, 1984). Although all types of seeds can be contaminated or colonised by fungi, recalcitrant seeds and probably all other non-orthodox seeds offer a further advantage to opportunistic invading fungi since they are shed at high moisture content. This makes them very prone to

contamination once on the ground, or in storage containers (Sutherland et al., 2002).

## 1.3 The role of seed-borne fungi in forestry

One of the strategies to counteract the alarmingly high rate of deforestation and the ensuing suite of economic and environmental consequences is to increase the rate of afforestation or reforestation. This necessitates raising a large number of seedlings, produced from a supply of high quality seeds. A constant and reliable supply of healthy and high quality seeds may be difficult to achieve for a number of reasons. Firstly, seed production by forest trees is so variable that for some species there may be no annual production of seeds, or production may be very small or of poor quality (Edwards, 1984). Secondly, like all other seeds, forest tree seeds are also exposed to different biotic and abiotic factors that can affect the normal developmental process. Among biotic factors, seeds get infected or contaminated by propagules of various microorganisms (Sutherland et al., 2002). So far the majority of the fungi recorded from seeds of forest trees belong to the conidial states of Ascomycota (Anderson, 1986, Mittal et al., 1990).

It is recognised that seed diseases are important and inflict a significant amount of damage to forests and their development, and the forest environment (Kamra, 1989, Singh & Mathur, 1993, Singh, 1996). The most commonly reported negative impacts of seed-borne fungi include reduction in storage lifespan of seeds, seed rotting, reduction in seed vigour, reduction in germination and damping-off in the nurseries (Fraedrich & Miller, 1995, Lilja, Hallaksela & Heinonen, 1995, Sateesh & Bhat, 1999, Berjak, 2000, Mamatha, Lokesh & Rai, 2000, Santos, Medeiros & Santana, 2001, Santos, Sobrosa & Costa, 2001, Dhingra *et al.*, 2002, Schroeder, Kehr & Hüttermann, 2002, Dhingra et al., 2003). Several fungal species that are generally considered to be saprophytes, do behave as pathogens under certain conditions. Such conditions include injury to the seed or seed coat, moisture and temperature conditions which favour fungal growth and increase physiological and physical vulnerability of tree cone/ fruit, seed, or seedling to infection (Singh & Mathur, 1993). Moreover, seed-borne fungi can weaken and predispose seeds and seedlings to a variety of soil-borne pathogens (Mamatha et al., 2000). Other harmful effects of seed-borne fungi include diseases of reproductive structures resulting in reduced seed production (Webb, 1983, Brown, 2000), twig dieback and stem-cankers (Smith, Kemp & Wingfield, 1994, Burgess & Wingfield, 2002).

Unhealthy seeds have the potential of introducing dangerous diseases to new areas by serving as an effective means of transport of plant pathogens over long distances. Several pathogens of forest trees have already been introduced to new areas through importation of seeds and other planting materials contaminated with or infected by seed-borne fungi (Cilliers, Swart & Wingfield, 1993, Storer, Gordon & Clark, 1998, Denman, Crous & Wingfield, 1999, Schroeder et al., 2002, Denman *et al.*, 2003). *Dothistroma septospora* (Dotoguine) Morelet, *Cryphonectria cubensis* (Burner) C. S. Hodges, and *Fusarium circinatum*

Nirenberg and O`Donnell are examples that have raised a concern for strict quarantine regulations (Burgess & Wingfield, 2002). For instance, *F. circinatum* has decimated its host (*Pinus radiata*) in its natural environment on the west coast of USA and is feared as potentially dangerous pathogen in the southern hemisphere. *Dothistroma* was introduced into pine growing regions over much of the southern hemisphere (Burgess & Wingfield, 2002). *Diplodia pinea* and *Botryosphaeria dothidea* are considered endophytes in *Pinus* and *Eucalyptus* spp. and have been introduced into various new environments worldwide (Smith *et al.*, 1996, Burgess & Wingfield, 2002). Forest pathogens associated with plant species that are extensively moved around by man for instance, *Leucaenia leucocephala* (Lam.) wit, and *Eucalyptus* spp. have greater chances to be distributed over wider geographic areas (Lenne, 1991, Smith, Wingfield & Petrini, 1996). Once established, introduced fungi can become serious threats to exotic as well as native forest species in their new environments (Burgess & Wingfield, 2002).

Apart from the harmful effects, some beneficial effects of fungi associated with seeds and fruits of forest trees have also been noted. Yamaji et al. showed that *Pencillium* spp., epiphytically associated with seeds of *Picea glehnii*, protect seedlings from damping-off. Mittal and Wang (1993) also reported a considerable increase in germination of seeds of *Pinus strobus* inoculated with seed-borne fungi such as *Alternaria alternata*, *Cladosporium cladosporoides*, *Epicoccum purpurascens* and *Mucor hiemalis* in unsterilised soil. Inoculation of surface sterilised seeds of *Podocarpus falcatus* with *Diaporthe* spp. isolated from seeds of the same host has been showed significantly increase germination (Paper II).

It appears that high frequency of seed importation from different sources could increase the probability of incursion by exotic fungi that can threaten both exotic and native forest trees (Burgess & Wingfield, 2002). The influences of fungi associated with seeds of forest trees, are and should be of great concern to gene banks, forest nurseries and, tree improvement programmes through importing germplasm since these activities rely on the health of seeds, seedlings and forest plantations. Plant quarantine officials should also know if seeds being moved domestically or internationally harbour pathogens of importance to local forests (Sutherland et al., 2002).

## 1.4 Fungal interactions

*In vitro* studies of fungal interactions on different substrates indicate that, when fungi colonize fresh substrates, they may grow and expand their domains freely for a brief period that would be followed by interactions among the fungi. Fungal communities colonizing organic substrates engage in an array of interactions (Rayner & Webber, 1984). The most common types are competitive interactions that can be further divided into primary resource capture and combat (Cuero, Smith & Lacey, 1987, Boddy, 2000). The success in primary resource capture ahead of competitors depends on the ability of the species to grow rapidly, sporulate abundantly and possess appropriate extracellular enzymes (Ramakrishna,

Lacey & Smith, 1993, Marin *et al.*, 1998). The outcome of competitive interactions could be a deadlock or replacement (Boddy, 2000). In the case of deadlock, none of the interacting fungi makes a headway gain whereas in replacement, the stronger competitor replaces the weaker one partially or completely.

Studies of interspecific interactions among field and storage fungi on cereal grains have shown that environmental factors such as temperature and moisture have marked effects on the outcomes of the interactions (Ramakrishna et al., 1993, Ramakrishna, Lacey & Smith, 1996a, 1996b, Marin et al., 1998, Lee & Magan, 1999, 2000). Ramakrishna et al. (1993) suggested that fungi in stored grains may form discrete, "mutually inhibited" colonies, similar to the discrete zones formed by wood-rotting fungi in wood blocks although on a smaller scale unless particular conditions enable one species to grow over the other. The implication for seed-borne fungi could thus be that several beneficial and/or pathogenic fungi might colonize the same seed, and in addition, antagonistic, synergistic or both interactions might occur between them (Ernst, Wendgen & Wirsel, 2003). The prevailing moisture and temperature regimes could influence the balance between the different groups and perhaps their diversity too. Therefore, depending on which group of fungi dominate the scene and the influence of the environmental variables on their interactions, seed-borne fungi could impact forest establishment either positively or negatively through their influence on seed viability, germination, seedling health and performance in the field after out planting.

# 2. Objectives of the study

The overall aim of the study was to assess the nature and impacts of fungi associated with seeds of tropical afromontane forest trees, *Podocarpus falcatus* and *Prunus africana* on seed and seedling health. An additional intention of the study was to highlight the significance of such knowledge in forest establishment and development.

## The specific objectives of the present study were:

- to isolate and identify fungi associated with seeds of *Podocarpus falcatus* and *Prunus africana*;
- to investigate the impacts of seed mycoflora of *P. falcatus* and *P. africana* on seed and seedlings;
- to describe a new *Botryosphaeria* species isolated from seeds of *P. africana*;
- to investigate the interaction among the seed mycoflora and to assess the potential of selected isolates for biocontrol.

# 3. Materials and methods

## 3.1 Isolation and identification of seed-borne fungi

Mature fruits of *P. falcatus* and *P. africana* (Fig. 1) were collected from two sites namely, Gambo ca 240 km south of Addis Ababa, and Badale ca 450 km southwest of Addis Ababa, Ethiopia (Fig. 2). The Gambo site is regarded as dry afromontane forest with altitude ca. 2100 –2700 m and mean annual rainfall of 1250 mm and mean annual temperature of 15 – 20 ºC (Demel & Granström, 1995) while the Badale site is part of the moist afromontane forest type with altitude between 1500 and 2500, mean annual rain fall 1500 – 2000 mm and mean annual temperature of 18 – 20 ºC (Friis, 1992). Seed-borne fungi were isolated from both surface sterilised and unsterilised fruits and seeds. Surface sterilisation was performed using either hydrogen peroxide ($H_2O_2$) 33 % (v/v) or sodium hypochlorite (chlorox) 13% (v/v) for 5 min followed by thorough rinsing with sterilised distilled water and blotting on sterile filter paper. After incubation of the seeds on potato dextrose agar, malt extract agar or water agar plates for 5-10 days, fungi were isolated onto malt extract agar plates. Morphological identification based on spore morphology was done either at CABI, UK or at the Department of Forest Mycology & Pathology, SLU, Sweden. Molecular identification was based on the extraction, amplification and sequencing of the nuclear internal transcribed spacer (ITS) region of rDNA from pure cultures.

**Fig. 1**. Mature female cones (fruits) of *P. falcatus* (left) and *P. africana* (right).

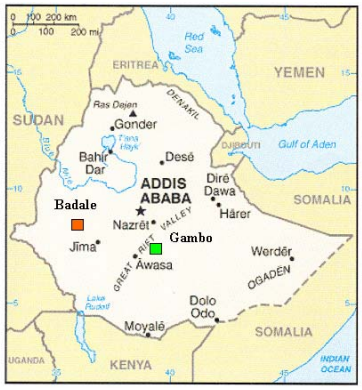


**Fig. 2**. Map of Ethiopia showing the study sites.

## Molecular identification of fungi

### *Genomic DNA extraction*

Pure cultures grown on Hagem agar (Stenlid, 1985) plates were used for DNA extraction. DNA was extracted using the CTAB and Phenol-Chloroform DNA extraction method (Gardes & Bruns, 1993) modified by omitting β-mercapto ethanol.

### *Amplification and sequencing of the ITS rDNA*

The ITS region of the rDNA was amplified by polymerase chain reaction (PCR) using universal primers ITS1 and ITS4 (White *et al.*, 1990)as described in Ihrmark (2001). The PCR products were purified using QIAquick PCR purification kit (QIAGEN GmbH HILDEN, Germany). The purified products were then sequenced on an Applied Biosystems 310 automated DNA sequencer using ABI PRISM™ BigDye™ 10X Terminator Cycle Sequencing Ready Reaction Kit v.2.0 (Perkin-Elmer, USA).

Sequence analysis was done the ABI PRISM™ Genetic Analyser. DNA sequences were assembled using the program SeqmanII from the DNASTAR software package (GATC GmbH, Konstanz, Germany). Sequences were compared with sequences in public database were performed using BLAST II algorithm http://www.ncbi.nlm.nih.gov/BLAST/.

### *Phylogenetic analysis*

Multiple alignments of the sequences were created with ClustalW algorithm in MegAlign module in the DNASTAR and were exported to PAUP version 4.0b10 (Swofford, 2002). Gaps were treated as missing data and all characters were unordered and of equal weight. Maximum parsimony trees were obtained using heuristic searches through stepwise (random) addition and tree bisection and reconstruction (TBR) as branch swapping algorithm. Maximum trees were unlimited and, branches of zero length were collapsed. For each analysis, 1000 bootstrap replicates were performed to assess statistical support for each tree.

## 3.2 Characterization of *Botryosphaeria* species from *P. falcatus* and *P. africana*

Isolates of *Botryosphaeria* spp. from seeds of *P. falcatus* and *P. africana* were characterized based on conidial morphology, cultural characteristics and phylogenetic analysis of rDNA ITS. Morphological identification of the isolates was based on conidial morphology from cultures grown on 2 % water agar. In order to induce sporulation, sterilised pine needles (autoclaved twice) were included in the plates. Furthermore, for the isolates from *P. africana*, sterilised seeds (by soaking in hydrogen peroxide (33 % v/v) over-night on a magnetic stirrer plate and followed by a thorough rinsing in sterilised water) were added to separate 2 % water agar plates for sporulation. The plates were incubated at 25 ºC in darkness for 2 wk and transferred to a chamber with alternating cycles of 12 h of near-UV light and darkness at a room temperature of around 20 ºC. Studies of cultural characteristics such as colony morphology, colour and mycelial extension rate were based on cultures grown on 2 % malt extract agar (MEA) at temperatures of 20 ºC and 25 ºC in darkness.

## 3.3 Impacts of seed-borne fungi on seeds and seedlings

Fungal isolates representing the different groups identified (Paper I) were tested for their impacts on seeds and seedlings of *P. falcatus* under laboratory conditions. For pathogenicity tests on seeds, seed coats were manually removed. The seeds were then surface sterilised with hydrogen peroxide (33 % v/v) followed by rinsing in sterilised water and thereafter inoculated by soaking them in a homogenised mycelial suspension of the test fungus. They were then placed on 1 % water agar plates (WA) and subsequently incubated at 25 ºC in darkness. Fifty seeds (ten per plate) were used for each fungus. Data on germination and any germling damage caused by the fungi were recorded. A pathogenicity test on seedlings was conducted using 4 -7 day old seedlings of *P. falcatus* grown under aseptic conditions. Seedlings were aseptically transferred to slants of WA in a capped glass test tube (one seedling per tube). Ten ─ 20 of these seedlings were inoculated per test fungus with a 5 mm mycelial plug from an actively growing culture. The test tubes were incubated in a growth chamber with daytime temperature of about 22 $^{\circ}$C and 10 $^{\circ}$C at night with alternating light and dark periods of 12 hrs. Seedlings grown under the same conditions but without inoculation of fungi served as controls. Seedlings were finally evaluated according to the ISTA rules for seedling evaluation (Bekendam & Grob, 1979).

## 3. 4 *In vitro* interactions among seed-borne fungi of *P. falcatus*

A full matrix of the pairing tests on MEA plates were performed using 26 fungal isolates that were previously tested for pathogenicity on seeds of *P. falcatus*. Five mm mycelial plugs were aseptically cut with a cork borer from actively growing margins of the mycelia and placed about 5 cm apart on 9-cm plastic Petri plates.

The plates were sealed with parafilm and incubated at 25 °C in darkness. The plates were examined at 15 and 30 days after inoculation and the observations on the outcomes of the interaction were recorded and digitally photographed.

Co-inoculation of seeds with 2 fungi that promoted germination and 6 that exhibited negative impacts on seeds and seedlings of *P. falcatus* was performed in order to evaluate if the pathogenic fungi could be suppressed by the germination promoters, thereby resulting in better germination. Each of the germination promoters was paired with each of the six pathogenic fungi on seeds by inoculating the seed first with mycelial suspension of the germination promoter followed by the respective pathogenic fungus, and *vice versa*, by dipping the seeds in mycelial suspension of the test fungi. Seeds were subsequently plated on WA plates and incubated at 25 °C in darkness. Throughout the experiment, aseptic conditions were maintained. Observations were recorded 60 days post inoculation. At the end of the experiment, five seeds were randomly selected from each treatment and plated on MEA to check whether or not the co-inoculated fungi could still grow out of the seed onto the medium.

A bioassay of antifungal activity of culture filtrates of the germination promoter *Diaporthe* sp. Po21 and Po84 was conducted using HPLC fractions inoculated with $10^6$ spores $ml^{-1}$ of *Ulocladium chartarum* Po52 and *Fusarium oxysporum* Po23. Microtiter plates with 96 wells containing 200 µl of HPLC fractions of culture filtrates were inoculated with 100 µl of spore suspension and incubated at 25 °C in darkness for 24 and 48 hrs. Antifungal activity of the fractions was measured by scanning the plates with the help of spectrophotometer coupled with examination under a stereomicroscope of spore germination and mycelial growth. Spectrophotometer readings of inoculated wells were compared with those from control wells containing only growth medium inoculated with spores of the target fungus.

# 4. Results and discussion

## 4.1 Isolation and identification of seed-borne fungi (Paper I)

Over 250 isolates of seed-associated fungi belonging to the Ascomycota, Basidiomycota and Zygomycota were obtained. The isolates were identified at least to the genus level with the help of morphological characteristics and phylogenetic analysis. Based on cultural features, the isolates were provisionally separated into 32 groups of which 19 could be identified morphologically to the genus level. All the groups were then identified on the basis of the molecular data. The morphological grouping was generally concordant with the analysis of the ITS sequence data. Furthermore, the ITS sequence analysis linked almost all the isolates to known taxa. The molecular data also resolved most of the groups to the species level and distinct phylogenetic groups were obtained; for instance, the *Diaporthe*/*Phomopsis* group and *Botryosphaeria* group were further resolved into separate groups. A combination of morphological features and molecular data to

characterise fungi has become a useful approach (Guo, Hyde & Liew, 2000, Zhou & Stanosz, 2001, Denman et al., 2003, Gezahgne *et al.*, 2004).

Among the Ascomycota, the genera identified were *Phomopsis*/*Diaporthe*, *Phoma*, *Pestalotiopsis*, *Fusarium*, *Alternaria*, *Botryosphaeria*, *Cytospora*, *Cladosporium*, *Ulocladium*, *Nectria*, *Verticillium* and *Penicillium*. *Peniophora*, *Polyporus* and *Stereum* represented the Basidiomycota, while *Mucor* was the only genus reported among the Zygomycota. Most of these genera have been recorded from seeds of many different trees in different countries as endophytes, saprobes or pathogens causing damages to seeds and seedlings (Singh & Mittal, 1989, Mittal et al., 1990, Smith et al., 1994, Lilja et al., 1995, Yuan & Mohammed, 1999, Mamatha et al., 2000, Strobel *et al.*, 2002, Dhingra *et al.*, 2003, Gezahgne et al., 2004). From the phylogenetic analysis, it appeared that some of the groups represented previously undescribed species. In the present study, one of the groups was characterised (paper II).

More fungi were isolated from seeds and fruits of *P. falcatus* than from *P. africana*, and more isolates originated from the Gambo site than from the Badale site. There seemed to be some specialised association with the host and geographic location; however, since the current data does not include samples from all the range of these species, this has not been definitely confirmed in the present study and requires more investigation.

Ecological factors also may have played some role in the disparity in the type and number of fungi from those sites. A previous study (Taylor, Hyde & Jones, 2000) indicated that differences in climate and the degree of disturbance of habitats in which the host grows could influence the diversity of microfungi.

## 4.2 Characterization of *Botryosphaeria* species from *P. falcatus* and *P. africana* (Paper II)

According to the cultural and conidial morphological data and phylogenetic studies, *Botryosphaeria* isolates considered in the present study could be separated into four distinct species. Three of these species were isolated from seeds of *P. falcatus* and one from *P. africana*. On the basis of the aforementioned criteria, three of the species were judged to be previously undescribed species while the fourth species was identified as *B. parva*.

*Botryosphaeria* sp. from *P. africana* is described as new species with the descriptions of the anamorph as *Diplodia rosulata* sp. nov. The four isolates examined for the study exhibited cultural and morphological characters distinct from the rest of the isolates in the study (Fig. 3). According to the ITS phylogenetic analysis, this fungus occupied a distinct and well-supported clade within the *Diplodia* group close of *B. corticola* and *B. tsugae* (Fig. 4). However, *D. rosulata* is different from these close relatives on accounts of morphology, phylogeny, host-range and geography. A detailed description is given in the taxonomy section in Paper II.

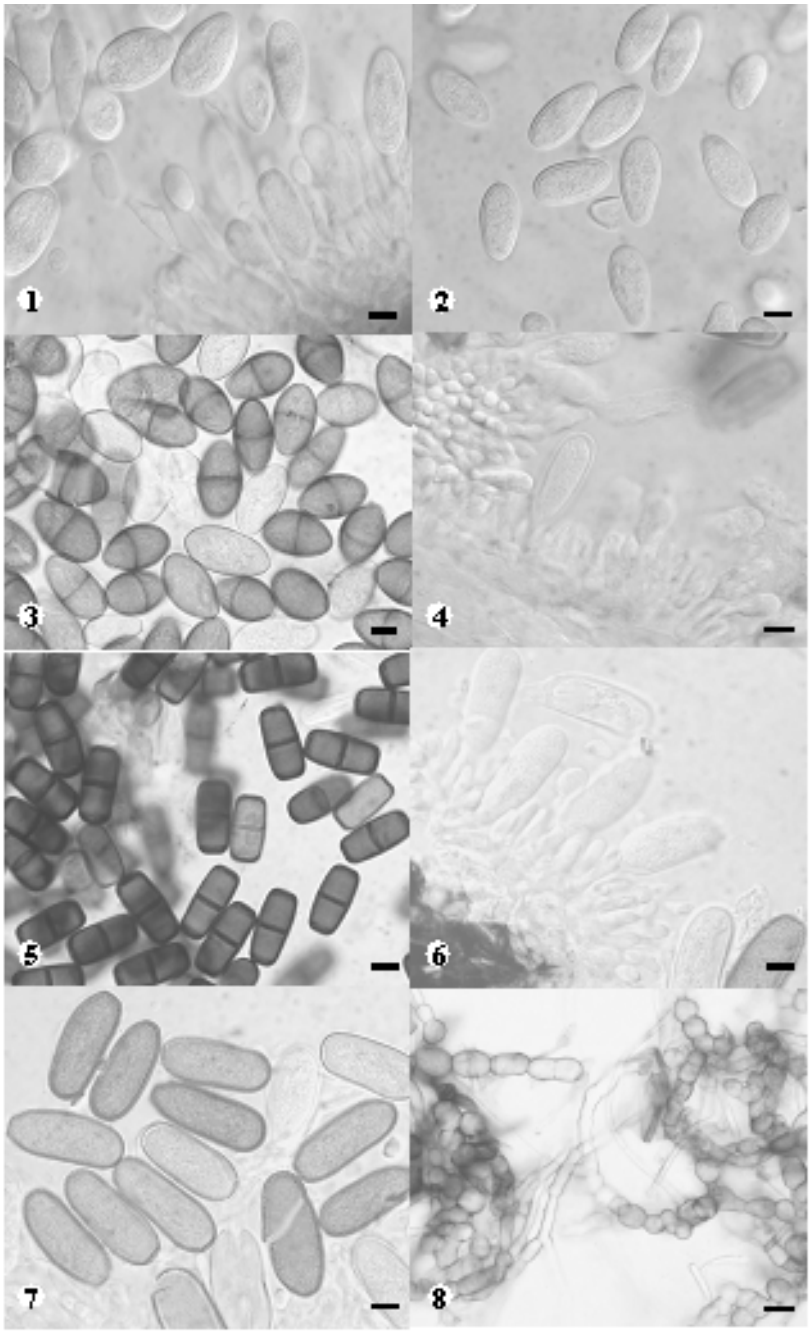


**Fig. 3**. *D. rosulata* Conidiogenous cells and aseptate conidia (1); Aseptate conidia (2); 1-septate conidia (3); *Botryosphaeria* sp. Po20 Conidiogenous cells and conidia (4); Conidia (5); *Botryosphaeria* sp. Po16 Conidiogenous cells and conidia (6); Conidia (7); Inflated hyphal cells in the culture (8). Scale bar = 10 µm.

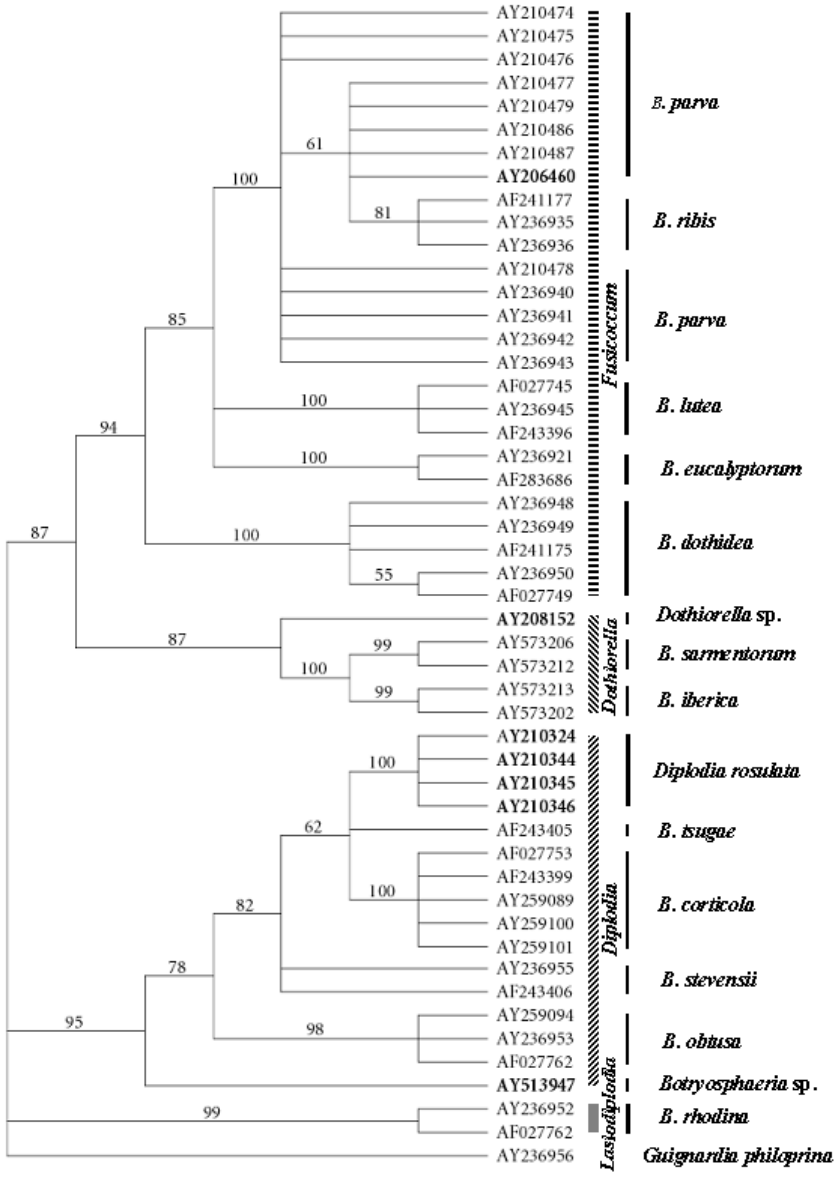


**Fig. 4.** Strict consensus of 12 most parsimonious trees obtained from ITS1, 5.8S and ITS2 rDNA sequence data. Bootstrap support values (1000 replicates) are indicated above the nodes. The tree is rooted with *Guignardia philoprina* as an outgroup.

*Botryosphaeria* sp. Po20 has anamorph features that correspond to the recently re-described genus *Dothiorella*. This genus was re-erected to accommodate anamorphs of *Botryosphaeria* spp. that produce conidia that become brown and one-septate soon after they are formed (Phillips *et al.*, 2004). Conidial morphological features of *Botryosphaeria* sp. Po20 differ from those of *Diplodia* and *Fusicoccum* in that they are aseptate or septate light- brown and become mainly septate and dark-brown before discharge.

*Botryosphaeria* sp. Po16 has conidial morphology similar to *B. obtusa*, but in the ITS phylogenetic analysis *Botryosphaeria* sp. Po16 was distinctly separated (Fig.4). Both *Botryosphaeria* sp. Po16 and *B. obtusa* have *Diplodia* anamorphs. However, *Botryosphaeria* spp. Po16 and Po20 could not be described in the current study as they were represented by a single isolate each, which is not adequate to detect any possible variation.

In the present study, *B. parva* Po66 was isolated from a native afromontane species; however, in a previous study (Gezahgne et al., 2004), it was isolated from *Eucalyptus* plantations in Ethiopia and was shown to be a major cause of Botryosphaeria stem-canker. The occurrence of this fungus on both native and exotic forest trees that are usually planted adjacent to the remaining natural forests raises the question whether or not *B. parva* is an introduced or natively evolved fungus.

There could be different scenarios and implications related to this issue.
1. If *B. parva* is an introduced fungus, it might have spread from the exotic hosts to the native tree species. In this case, if the pathogen could overcome the resistance of the native trees, it might cause disease outbreak on the native species; or the pathogen might have infected native species without causing severe damage. There could be inoculum build up on the native species that could lead to periodic disease outbreak (Burgess, Wingfield & Wingfield, 2004).

2. If *B. parva* is a native fungus that evolved in native forests as an endophyte, saprophyte or a pathogen, then it might have spread to exotic plantations. The consequence of this scenario would be that the exotic tree species could be susceptible to the pathogen leading to disease outbreak. This would require either breeding for resistance or introducing genotypes that are less susceptible to the native pathogen(s).

3. Since *B. parva* has a wide geographic distribution and host range, it is probable that this fungus existed in Ethiopia even before the introduction of *Eucalyptus* to the country. If this is the case, introduction of eucalypts to Ethiopia might have led to introductions of new genotypes of the fungus that might have increased the genetic diversity of the pathogen with the possibility of crossing with native genotypes. This could lead in the long run to the emergence of more aggressive genotypes that overcome the resistance of the tree species including the native ones (Burgess & Wingfield, 2002). The above scenarios and their implications for

both native and exotic forest tree species require further studies in order to develop good management strategies for the future.

There could be different scenarios and implications involving this issue.
1) If *B. parva* was an introduced fungus, it might have spread from the exotic hosts to the native tree species. In this case, if the pathogen could overcome the resistance of the native trees, it might have caused disease outbreak on both native and exotic species; or the pathogen might have infected native species without causing severe damage. There could be inoculum build up that could lead to periodic disease outbreak.

2) If *B. parva* is a native fungus that survived in native forests as endophyte, saprophyte or a weak pathogen, then it might have spread to exotic plantations that are usually planted adjacent to the remaining natural forests. The consequence of this scenario would be that the exotic trees species would become susceptible to the pathogen leading to disease outbreak. This would require either breeding for resistance or introducing genotypes that are less susceptible to the native pathogen(s).

## 4.3 Impacts of seed-borne fungi on seeds and seedlings (Paper III)

Seed-borne fungi of *P. falcatus* exhibited variable effects on seeds and seedlings. Based on the results from *in vitro* seed inoculation tests, the fungi could be grouped into five categories namely, I) isolates that were pathogenic only to seeds and had no obvious impacts on the germlings; II) isolates that were pathogenic only to the germlings; III) isolates that were pathogenic both to seeds and the emerging germlings; IV) isolates that were more or less harmless both to seeds and seedlings; and V) isolates that were germination promoters. Sutherland (1995) also categorised seed-borne fungi of conifers in a similar way.

The fungi in category I were *Cercospora* sp. isolate No.20, *Phoma* sp. isolate No. 16, *Guignardia* sp. isolate No.21, *Ulocladium botrytis* isolate No.15, and *U. chartarum* isolate No.22 reduced germination. Among them, *U. chartarum* caused a reduction of 50 % as compared to the control. In the category II, *Pestalotiopsis* sp. isolate No.10 severely damaged the roots of the emerging germlings followed by *Pestalotiopsis* spp. isolates No.8 and 5. They caused browning and necrosis of lateral roots of the seedlings, while colonization of the root collar region by *Phomopsis viticola* isolate No.14 caused necrotic spots on the hypocotyls. Among the isolates in category III, *Fusarium oxysporum* isolate No. 26, and *Polyporus* sp. isolates No.23 and 25 caused the most noticeable damage in both seed and seedling inoculation tests. *Fusarium oxysporum* No.26 caused total loss of seed germination and root rot in all the inoculated seedlings. This fungus has a wide host range and has been recorded from seeds of many forest tree species (Mittal et al., 1990) associated with seed rot and damping-off (Axelrood *et al.*, 1995, Fraedrich & Miller, 1995, Lilja et al., 1995, Dick & Dobbie, 2002). Certain soil borne *F. oxysporum* isolates, however, have been implied to serve as biocontrol

agents in suppressive soil (Farvel, Olivain & Alabouvettee, 2003). The *Polyporus* sp. isolates caused both pre- and post-emergence damping-off resulting in no surviving seedlings (Fig. 5). Isolates of this species were highly pathogenic under


**Fig. 5**. Left: seedlings inoculated with *F. oxysporum* isolate PoC23 (a) and seedling from the control treatment(b). Right: seedlings inoculated with *Polyporus* sp. isolate Po-95 (a) and seedling from the control treatment (b).

laboratory conditions. This is the first report of *Polyporus* sp. as seed-borne pathogen that can be transmitted from seed to seedling. Other fungi in this category also caused less pronounced disease symptoms (Paper III, Table 2 and 3). Isolates in the category IV were more or less harmless.

Fungi in category V increased seed germination; however, only *Diaporthe* spp. isolates No.1 resulted in significant increase. The mechanism involved may be through production of some compounds that stimulate seeds to germinate and/or production of antifungal metabolites (Paper III). Positive effects of fungi on seed germination were also recorded in some earlier studies (Mittal & Wang, 1993, Gramss, 1997).

The findings from this study give a good indication of the potential damage that can be incurred by seed-borne fungi and also the possibilities for the beneficial effects of certain others in forest development. However, since the current study was based on laboratory observations, the pathogenicity of these fungi needs to be tested under field condition.

## 4.4 Interactions among seed-borne fungi (Paper IV)

The outcomes of an *In vitro* pairing test on MEA revealed that seed-borne fungi from seeds of *P. falcatus* exhibited interspecific deadlock and replacement

interactions. There were variations among the fungi with respect to their strength either in deadlocking and/or replacing other competing species.

Deadlock interactions could occur either as a result of production of volatile and diffusible metabolites or gross mycelial contact between the interacting mycelia, leading to the formation of an inhibition zone or a barrage zone, respectively (Boddy, 2000). Among the fungi involved in deadlock, *Penicillium brevicompactum* Po81 was responsible for the production of diffusible metabolites (Fig. 6a), where as *Phomopsis* sp. Po49, *Pestalotiopsis* spp. Po26, Po37, *Pestalotiopsis neglecta* Po91, *Phoma glomerata* Po69, *Cladosporium oxysporum* Po51, *Botryosphaeria parva* Po66, *Diaporthe* sp. Po21, *Alternaria tenuissima* Po4 and *Pestalotiopsis guepinii* Po94 exhibited deadlock due to gross mycelial contact (Fig. 6b). These fungi resulted in the least number of cases replacing others, as well as being replaced by others.

Replacement interactions included both partial and complete replacement. *Polyporus* sp. Po78 and Po95, *Diaporthe* sp. Po84, *Nectria gliocladioides* Po90 and *Cytospora* sp. Po19, exhibited the strongest replacement cases. *Polyporus* sp. Po78 and Po95 were the strongest followed by *Diaporthe* sp. Po84 ( Paper III, Table 2). *Polyporus* sp. Po78 and Po95 completely replaced all but *P. brevicompactum* Po81, while the others were involved mostly in partial replacement. Replacement interactions are one form of combative interaction characterised by the occupation of domain belonging to another individual by secondary resource capture (Cooke & Rayner, 1984). The results of the present study on MEA plates generally correspond with those from other studies, mainly on wood inhabiting basidiomycetes (Dowson, Rayner & Boddy, 1988, Rayner & Boddy, 1988, Robinson, Dighton & Frankland, 1993, Owens, Reddy & Grethlein, 1994).

Furthermore, interspecific interactions could be markedly influenced by environmental factors such as temperature and humidity (Marin et al., 1998, Lee & Magan, 1999, 2000). Moreover, interactions among fungi on agar plates and organic substrates may not always give similar results and therefore require cautious interpretations (Holmer & Stenlid, 1993).

Co-inoculation of seed with selected pathogenic fungi and putative antagonistic *Diaporthe* spp. Po21 and Po84 resulted in higher germination as compared to the corresponding single-fungus inoculation with the pathogenic isolates. Seed inoculation with the putative antagonistic fungi gave slightly better results than the reverse method of inoculation. However, the result was statistically significant only in the case of *Diaporthe* sp. Po84 co-inoculated with *Alternaria tenuissima* Po4. This is consistent with other observations in biocontrol tests where pre-inoculation of the host with the antagonist gave better results (Capieau, 2004).

The suppressive activity of the *Diaporthe* spp. was also supported by the results of bioassay for antifungal activities of HPLC fractions of culture filtrates from the *Diaporthe* spp. Po21 and Po84 against *F. oxysporum* Po23 and *U. chartarum* Po52. Some fractions of the culture filtrates showed inhibition of spore

germination and mycelial growth of test fungi (Fig. 7). Fractions from *Diaporthe* sp. Po84 showed stronger inhibitory effects on both the test fungi than those from *Diaporthe* sp. Po21. This is consistent with results from pathogenicity tests (Paper II) as well as those from dual culture tests on MEA and seed co-inoculation tests (Paper IV).

Evidence from earlier studies with fungal metabolites suggests that the effect of the metabolites from test cultures depends on their concentration (Liu *et al.*, 2001). Thus, if concentrated, fractions from *Diaporthe* sp. Po84 may give better results than in the crude form. Other studies have also showed that some seed-associated fungi produce antifungal metabolites that have inhibitory effects on damping-off fungi such as *Pythium*, *Fusarium* and *Rhizoctonia* species (Alstrom, 2000, Yamaji *et al.*, 2001). Therefore, *Diaporthe* sp. Po84 needs to be tested further for its potential as biocontrol agent. Previous studies have also shown that seed inoculation with seed-borne endophytic fungi enhanced the biomass and vigour of seedlings of their hosts (Bose, 1947, Boursnell, 1950, Ernst et al., 2003).

# 5. Conclusions

1. The present study revealed that a highly diverse group of fungi was associated with seeds of the two host species. It is likely that several of these fungi may constitute new taxa. Information on mycoflora associated with seeds of such highly valued, but locally threatened forest tree species as *P. falcatus* and *P. africana* could be vital in the reforestation efforts especially in the handling and treatment of seeds since propagation of the species is mainly by seeds, which are in limited supply. From the variation in species and number of fungi isolated from the host species in this study, it appeared that climatic and other ecological factors in and around the sites of seed collection might influence the host-fungus association. However, the present observations and suggestions should be confirmed with more data drawn from all ranges of the hosts. This kind of information would be vital in identifying seed production areas and selecting mother trees for production of healthy and vigorous seeds.

2. To characterize one of the groups of seed-borne fungi encountered during the present study, four *Botryosphaeria* spp. were identified, three of which were previously undescribed. One of the *Botryosphaeria* sp., namely *B. parva*, has been previously reported to be a pathogen on forest trees of commercial importance in many countries, including Ethiopia. It was possible to identify many of the fungi isolated in the first part of the study with a fair degree of certainty only to the genus level. Therefore, it is likely that a number of fungal species are associated with afromontane forest tree seeds still remain undescribed. Thus, it is important to systematically study fungi that influence forest development and establishment to extract essential information on potentially dangerous pathogens that can impact the environment and the livelihood of the local people.

3. *In vitro* pathogenicity tests on seed-borne fungi of *P. falcatus* indicated that seed-borne fungal communities could be separated into five categories based on their impact on seed germination. The most notable impacts of seed-borne fungi in this study were seed rotting, pre- and post-emergence damping-off, and increased seed germination. Under lab conditions, *Fusarium oxysporium* Po23, *Polyporus* sp. Po78 and Po95 were strongly pathogenic while *Diaporthe* spp. Po21 and Po84 promoted germination. However, these results should be assessed in more natural settings. Furthermore, investigations on the incidence and severity of diseases due to seed-borne and/or other fungi in forest nurseries need to be carried out in the future.

4. The interactions observed among seed-borne fungi of *P. falcatus* probably indicate different strategies for co-inhabitation or exclusion of each other by the different groups that infect and colonize the seed tissues as manifested by deadlock and/or replacement of one of the competitors. Seed co-inoculation tests using the germination promoters and the pathogenic isolates resulted in higher germination as compared to seed inoculation with the corresponding pathogenic isolates alone. Furthermore, the antifungal activities displayed by fractions of the culture filtrates of both *Diaporthe* spp. Po21 and Po84 on spore germination and mycelial growth of *Fusarium oxysporum* Po23 and *Ulocladium chartarum* Po52 demonstrated the need for further consideration to test these isolates for their potential as biocontrol agents.

# 6. Future research perspectives

- Further studies are necessary on the diversity of fungi associated with seeds and fruits of important forest trees in order to give more complete data on fungal diversity and distribution. Due considerations should also be given to ecological factors and the degree of disturbance of the habitat that may influence diversity at different sampling areas.

- Further survey involving both exotic and native plantation forests, and the remnant natural forests should be conducted with the view of assessing the extent of cross infection, genetic diversity and the significance of the associated fungi as pathogens in the forests and the implications for forest management and planning of future plantations.

- With the globalisation of markets, the risks of spreading pathogens and pests of forest trees with importation of seeds and other planting materials from abroad could be great unless effective quarantine regulation are implemented. However, such measures need to be based on the knowledge of the organism involved and the potential risk it might pose if introduced. In Ethiopia, there is a gap in research in that respect.

- The present study showed that both pathogenic and beneficial fungi were associated with the seeds and fruits of *P. falcatus*. These results need to be tested in the nursery. Furthermore, the relevance of the pathogenic and beneficial fungi from the present and future studies in forest nurseries and plantations need to be evaluated.

- The mode of spread of the seed-borne fungi on trees in tropical afromontane forests has not been studied. However, during the current survey it has been noted that certain fruit and seed boring insects were constantly associated with the hosts. Therefore, it is important to investigate this aspect including if those insects serve as vectors for the seed-borne fungi.

- The diversity and impacts of wood rotting fungi in plantations and natural forests of the country are also important areas that have not been studied and require attention.

# 7. References


Agarwal, V. K. & Sinclair, J. B. 1997. *Principles of Seed Pathology.*2nd. CRC Press, Inc. Boca Raton, FL., 539 pp.

Alstrom, S. 2000. Root-colonizing fungi from oilseed rape and their inhibition of *Verticillium dahliae*. *Journal of Phytopathology* 148, 417-423.

Alves, A., Correia, A., Luque, J. & Phillips, A. 2004. *Botryosphaeria corticola*, sp. nov. on *Quercus* species, with notes and description of *Botryosphaeria stevensii* and its anamorph, *Diplodia mutila*. *Mycologia* 96, 598-613.

Anderson, R. L. 1986. Checklist of micro-organisms associated with tree seeds in the world, 1985. *General Technical Report, Southeastern Forest Experiment Station, USDA Forest Service* 34 p.

Arx, J. A. V. & Müller, E. 1975. A re-evaluation of the bitunicate ascomycetes with keys to families and genera. *Studies in Mycology* 9, 1-159.

Axelrood, P. E., Neumann, M., Trotter, D., Radley, R., Shrimpton, G. & Dennis, J. 1995. Seedborne *Fusarium* on Douglas-fir: pathogenicity and seed stratification method to decrease *Fusarium* contamination. *New Forests* 9, 35-51.

Berjak, P. 2000. The effects of micofloral infection on the viability and ultrastructure of wet-stored recalcitrant seeds of *Avicennia marina* (Forssk.) Vierh. *Seed Science Research* 10, 341-353.

Boddy, L. 2000. Interspecific combative interactions between wood- decaying basidiomyctes. *FEMS Microbiology Ecology* 31, 185 - 194.

Bose, S. R. 1947. Hereditary (seed-borne) symbiosis in *Casuarina equisetifolia* Forst. *Nature* 159, 512 - 514.

Boursnell, G. 1950. The symbiotic seed-borne fungus in the *Cistaceae*. *Annals of Botany* 14, 217-247.

Breitenbach, V. F. 1963. *The Indigenous Trees of Ethiopia.*Ethiopian Forestry Association. Addis Ababa, 305 pp.

Brown, B. N. 2000. Diseases and fungi of the reproductive structures of eucalypts. In: Keane, P. J., Kile, G. A. & Podger, F. D. (Ed.). *Diseases and Pathogens of Eucalypts*. CSIRO Publishing, Collingwood, Australia. pp. 103-118.

Burgess, T. & Wingfield, M. J. 2002. Quarantine is important in restricting the spread of exotic seed-borne pathogens in the southern hemisphere. *International Forestry Review* 4, 56-65.

Capieau, K. 2004. *Biological control of grey mould in Swedish forest nurseries.* PhD Thesis, Swedish University of Agricultural Sciences. Department of Forest Mycology & Pathology, Uppsala.

Cilliers, A. J., Swart, W. J. & Wingfield, M. J. 1993. A review of *Lasiodiplodia theobrome* with particular reference to its occurrence on coniferous seeds. *South African Forestry Journal* 166, 47-52.

Cooke, R. C. & Rayner, A. D. M. 1984. *The Ecology of Saprotrophic Fungi.*Longman. London, 415 pp.

Crous, P. W. & Palm, M. E. 1999. Reassessment of the anamorph genera *Botryodiplodia*, *Dothiorella* and *Fusicoccum*. *Sydowia* 51, 167-175.

Cuero, R. G., Smith, J. E. & Lacey, J. 1987. Stimulation by *Hyphopichia burtonii* and *Bacillus amyloliquefaciens* of aflatoxin production by *Aspergillus flavaus* in irradiated maize and rice grains. *Appllied environmental microbiology* 53, 1142 - 1146.

Demel, T. & Granström, A. 1995. Soil seed banks in dry afromontane forests of Ethiopia. *Journal of Vegetation Science* 6, 777-786.

Denman, S., Crous, P. W., Groenwald, J. Z., Slippers, B., Wingfield, B. D. & Wingfield, M. J. 2003. Circumscription of *Botryosphaeria* species associated with *Proteaceae* based on morphology and DNA sequence data. *Mycologia* 95, 294-307.

Denman, S., Crous, P. W. & Wingfield, M. J. 1999. A taxonomic reassessment of *Phyllachora proteae*, a leaf pathogen of *Proteaceae*. *Mycologia* 91, 510-516.

Dhingra, O. D., Lustosa, D. C., Maia, C. B. & Mesquita, J. B. 2003. Seedborne fungal pathogens of jacaranda (*Dalbergia nigra*) tree. *Seed Science and Technology* 31, 341-349.

Dhingra, O. D., Maia, C. B., Lustosa, D. C. & Mesquita, J. B. 2002. Seedborne pathogenic fungi that affect seedling quality of red angico (*Anadenanthera macrocarpa*) trees in Brazil. *Journal of Phytopathology* 150, 451-455.

Dick, M. A. & Dobbie, K. 2002. Species of *Fusarium* on *Pinus radiata* in New Zealand. In: (Ed.) *Proc. 55th New Zealand Plant Protection Society Incorporated Conf.* Rotoruga, New Zealand, August, 12 -15.

Dowson, C. G., Rayner, A. D. M. & Boddy, L. 1988. The form and outcomes of mycelial interactions involving cord-forming decomposer basidiomycetes in homogenous and heterogenous environments. *New Phytologist* 109, 423 - 432.

Edwards, D. G. W. 1984. The role seeds and seed research in combating the exploitation of the world's forest resources. *Seed Science ana Technology* 12, 757-765.

EFAP. 1993. Ethiopian Forestry Action Program Volume II. The challenge for development. Ministry of Natural Resources Development and Environmental Protection,

Ernst, M., Wendgen, K. W. & Wirsel, S. G. R. 2003. Endophytic fungal mutualists: seed-borne *Stagonospora* spp. enhance reed biomass production in axenic microcosms. *Molecular Plant-Microbe Interactions* 16, 580 - 587.

Eshetu, Y. 2002. Restoration of the native woody-species diversity using plantation species as foster trees in the degraded highlands of Ethiopia. Doctoral dissertation, University of Helsinki. Viikki Tropical Resources Institute, Department of Forest Ecology, Helsinki.

FAO. 2001. Global Forest Resource Assessment 2000. FAO Forestry paper 140. FAO,

Farvel, D., Olivain, C. & Alabouvettee, C. 2003. Research Review: *Fusarium oxysporum* and its biocontrol. *New Phytologist* 157, 493 - 502.

Fraedrich, S. W. & Miller, T. 1995. Mycoflora associated with slash-pine seeds from cones collected at seed orchards and cone-processing facilities in the south-eastern USA. *European journal of Forest Pathology* 25, 73-82.

Friis, I. 1992. Forests and Forest trees of Northeast Tropical Africa. London, 396 pp.

Gardes, M. & Bruns, T. D. 1993. ITS primers with enhanced specificity for *Basidiomycetes* - application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2, 113-118.

Gezahgne, A., Roux, J., Slippers, B. & Wingfield, M. J. 2004. Identification of the causal agent of Botryosphaeria stem canker in Ethiopian Eucalyptus plantations. *South African journal of botany* 70, 241-248.

Gezahgne, A., Roux, J. & Wingfield, M. J. 2003. Diseases of exotic *Eucalyptus* and *Pinus* species in Ethiopian plantations. *South African journal of science* 99, 29-33.

Gramss, G. 1997. Activity of oxidative enzymes in fungal mycelia from grassland and forest soils. *Journal of basic microbiology* 37, 407-423.

Guo, L. D., Hyde, K. D. & Liew, E. C. Y. 2000. Identification of endophytic fungi from *Livistonia chinensis* based on morphology and rDNA sequences. *New phytologist* 147, 617-630.

Holmer, L. & Stenlid, J. 1993. The importance of inoculum size for the competitive ability of wood decomposing fungi. *FEMS Microbiology ecology* 12, 169 -176.

Ihrmark, K. 2001. *Double-stranded RNA Elements in the Root Rot Fungus Heterobasidion annosum.* Doctoral thesis, Swedish University of Agricultural Sciences. Department of Forest Mycology & Pathology, Uppsala.

Jacobs, K. A. & Rehner, S. A. 1998. Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. *Mycologia* 90, 601-610.

Kabeere, F., Hampton, J. G. & Hill, M. J. 1997. Transmission of *Fusarium graminearum* (Schwabe) from maize seeds to seedlings. *Seed Science & Technology* 25, 245 - 252.

Kamra, S. K. 1989. Improving the forest seed situation in some African countries. In: Turnbull, J. W. (Ed.) *Tropical Tree Seed Research.* Gympie, Australia,

Lee, H. B. & Magan, N. 1999. Environmental factors influence *in vitro* interspecific interactions between *A. ochraceus* and other maize spoilage fungi, growth and ochratoxin production. *Mycopathologia* 146, 43 - 47.

Lee, H. B. & Magan, N. 2000. Impact of environment and interspecific interactions between spoilage fungi and *Aspergillus ochraceus* on growth and production of ochratoxin production in maize garin. *International journal of food microbiology* 61, 11 - 16.

Lemenih, M. 2004. Effects of Land Use Changes on Soil Quality and Native Flora Degradation and Restoration in the Highlands of Ethiopia. Doctoral Thesis, Swedish University of Agricultural Sciences. Department of Forest Soils, Uppsala.

Lenne, J. M. 1991. Diseases of *Leucaena* Species. *Tropical pest management* 37, 281-289.

Lilja, A., Hallaksela, A. M. & Heinonen, R. 1995. Fungi colonizing Scots-pine cone scales and seeds and their pathogenicity. *European journal of forest pathology* 25, 38-46.

Liu, C. H., Zou, W. X., Lu, H. & Tan, R. X. 2001. Antifungal activity of *Artemisia annua* endophyte cultures against phytopathogenic fungi. *Journal of biotechnology* 88, 277-282.

Mamatha, T., Lokesh, S. & Rai, V. R. 2000. Impact of seed mycoflora of forest tree seeds on seed quality and their management. *Seed research* 28, 59-67.

Marin, S., Sanchis, V., Ramos, A. J., Vinas, I. & Magan, N. 1998. Environmental factors, *in vitro* interactions, niche overlap between *Fusarium moniliforme*, *F. proliferatum*, and *F. graminearam*, *Aspergillus* and *Penicillium* species from maize grain. *Mycological research* 102, 831 - 837.

Marsh, S. F. & Payne, G. A. 1984. Preharvest infection of corn silks and kernels by Aspergillus flavus. *Phytopathology* 60, 1775 - 1777.

Mittal, R. K., Anderson, R. L. & Mathur, S. B. 1990. Mico-organisms associated with tree seeds, world checklist 1990. Petawawa National Forestry Institute, PI-X-96E.

Mittal, R. K. & Wang, B. S. P. 1993. Effects of some seed-borne fungi on *Picea glauca* and *Pinus strobus* seeds. *European journal of forest pathology* 23, 138-146.

Mycock, D. J. & Berjak, P. 1992. Paradoxical behavior of seed storage and field fungi: an overview. *Soth African journal of science* 88, 371-375.

Negash, L. 1995. Indigenous trees of Ethiopia: Biology, uses and Propagation techniques.SLU Reprocentralen. Umea, Sweden, 285 pp.

Owens, E. M., Reddy, C. A. & Grethlein, H. E. 1994. Outcome of interspecific interactions among brown-rot and white-rot wood decay fungi. *FEMS microbiology ecology* 14, 19 - 24.

Pennycook, S. R. & Samuels, G. J. 1985. *Botryosphaeria* and *Fusicoccum* species associated with ripe fruit rot of *Actinidia deliciosa* (Kiwifruit) in New Zealand. *Mycotaxon* 24, 445 - 458.

Phillips, A., Alves, A., Correia, A. & Luque, J. 2004. Two new species of *Botryosphaeria* with brown, one-septate ascospores and *Dothiorella* anamorphs. *Mycologia* (In Press),

Ramakrishna, N., Lacey, J. & Smith, J. E. 1993. Effects of water activity and temperature on the growth of fungi interacting on barley grain. *Mycological research* 97, 1393 - 1402.

Ramakrishna, N., Lacey, J. & Smith, J. E. 1996a. Colonization of barley grain by *Penicillium verrucosum* and ochratoxin formation in the presence of competing fungi. *Journal of food Protection* 59, 1311 - 1317.

Ramakrishna, N., Lacey, J. & Smith, J. E. 1996b. The effects of fungal competition on colonization of barley grain by *Fusarium sporotrichioides* on T-2 toxin formation. *Food additives and contaminants* 13, 939 - 948.

Rayner, A. D. M. & Boddy, L. 1988. Fungal communities in the decay of wood. *Advances in microbial ecology* 10, 115 -166.

Rayner, A. D. M. & Webber, J. 1984. Interspecific mycelial interactions - an overview. In: Jennings, D. H. & Rayner, A. D. M. (Ed.). *The Ecology and Physiology of the Fungal Mycelium*. Cambridge University Press, Cambridge. pp. 383 - 417.

Reusing, M. 1998. Monitoring Forest Resources in Ethiopia. Ministry of Agriculture,

Robinson, C. H., Dighton, J. & Frankland, J. C. 1993. Resource capture by interacting fungal colonizers of straw. *Mycological research* 97, 547 - 558.

Roux, J., Coutinho, T. A., Byabashaija, D. M. & Wingfield, M. J. 2001. Diseases of plantation Eucalyptus in Uganda. *South African journal of science* 97, 16-18.

Roux, J. & Wingfield, M. J. 1997. Survey and virulence of fungi occurring on diseased *Acacia mearnsii* in South Africa. *Forest ecology and management* 99, 327-336.

Santos, A. F. d., Medeiros, A. C. d. S. & Santana, D. L. d. Q. 2001. Seed-borne fungi associated with native tree seeds from Brazilian Atlantic forest. *Boletim de Pesquisa Florestal* 42, 51-59.

Santos, F. E. M., Sobrosa, R. d. C. & Costa, I. F. D. 2001. Pathogenic fungi detection in seed of black wattle (*Acacia mearnsii* De Wild). *Ciencia Floresta* 11, 13-20.

Sateesh, M. K. & Bhat, S. S. 1999. Detection of seed-borne *Phomopsis azadrachtae* and its transmission in *Azadrachta indica*(Neem). *Seed science and technology* 27, 753-759.

Sayer, A. J., Harcourt, S. C. & Collins, M. N. 1992. The conservation atlas of tropical forests of Africa. IUCN, Cambridge, UK, 288 p.

Schroeder, T., Kehr, R. & Hüttermann, A. 2002. First report of the seed-pathogen *Geniculodendron pyriforme*, the imperfect state of the ascomycete *Caloscypha fulgens*, on imported conifer seeds in Germany. *Forest pathology* 32, 225-230.

Sen-Sarma, P. K., Thakur, M. L. & Sehgal, H. S. 1988. Protection of forest seeds against insect pests and fungi during storage. *Journal of tropical forestry* 4, 350-356.

Singh, P. 1996. Tree seed pathogens and seed diseases: their detection and management in sustainable forestry. In: Procházková, Z. & Sutherland, J. R. (Ed.) *Proceedings of International Seed Testing Association Tree Seed Pathology Meeting*. Opocno, Czech Republic, October, 9-11.

Singh, P. & Mathur, S. B. 1993. Disease problems of forest tree seeds: diagnosis and management. In: Somé, L. M. & de Kam, M. (Ed.) *Proceedings of IUFRO Symposium on Tree Seed Problems, with special reference to Africa. Project Group P.2.04.00 - Seed Problems*. Ogadougou, Burkina Faso, November, 23 - 28.

Singh, P. & Mittal, R. K. 1989. Influence of seed-borne fungi on the nutrient composition and growth of conifer seedlings. *European journal of forest pathology* 19, 65-77.

Slippers, B., Crous, P. W., Denman, S., Coutinho, T. A., Wingfield, B. D. & Wingfield, M. J. 2004a. Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. *Mycologia* 96, 83-101.

Smith, D. R. & Stanosz, G. R. 2001. Molecular and morphological differentiation of *Botryosphaeria dothidea* (anamorph *Fusicoccum aesculi*) from some other fungi with *Fusicoccum* anamorphs. *Mycologia* 93, 505-515.
Smith, H., Kemp, G. H. J. & Wingfield, M. J. 1994. Canker and dieback of Eucalyptus in South Africa caused by *Botryosphaeria dothidea*. *Plant pathology* 43, 1031-1034.
Smith, H., Wingfield, M. J., Crous, P. W. & Coutinho, T. A. 1996. *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *South African journal of botany* 62, 86-88.
Smith, H., Wingfield, M. J. & Petrini, O. 1996. *Botryosphaeria dothidea* endophytic in *Eucalyptus grandis* and *Eucalyptus nitens* in South Africa. *Forest ecology and management* 89, 189-195.
Stenlid, J. 1985. Population structure of *Heterobasidion annosum* as determined by somatic incompatability, sexual incompatability and isozyme patterns. *Canadian journal of botany* 63, 2268-2273.
Storer, A. J., Gordon, T. R. & Clark, S. L. 1998. Association of pitch canker fungus, Fusarium subglutinans f.sp. pini, with Monterey pine seeds and seedlings in California. *Plant pathology* 47, 649-656.
Strobel, G., Ford, E., Worapong, J., Harper, J. K., Arif, A. M., Grant, D. M., Fung, P. C. W. & Chau, R. M. W. 2002. Isopestacin, an isobenzofuranone from *Pestalotiopsis microspora*, possessing antifungal and antioxidant activities. *Phytochemistry* 60, 179-183.
Sutherland, J. R. 1995. Seed-borne fungi of conifers. http://www.rngr.net/Publications/ttsm/ch6; pp 177-190.
Taylor, J. E., Hyde, K. D. & Jones, E. B. G. 2000. The biogeographical distribution of microfungi associated with three palm species from tropical and temperate habitats. *Jounal of biogeography* 27, 297-310.
Teketay, D. 1997. Seedling population and regeneration of woody species in dry afromontane forests in Ethiopia. *Forest ecology and management* 98, 149-165.
Tewoldeberhan, G. 1989. The environmental variables which led to ecological crisis in Ethiopia. *Coenoses* 4, 61 - 67.
Webb, R. S. 1983. Seed capsule abortion and twig dieback of *Eucalyptus camaldulensis* in South Florida induced by *Botryosphaeria ribis*. *Plant disease* 67, 108-109.
White, F. 1983. The Vegetation of Africa. A descriptive memoir to accompany the UNESCO/AETFA/UNSO Vegetation map of Africa. Paris, UNESCO, 356 p.
White, T. J., Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: A., I. M., Gelfand, D. H., Snisky, J. J. & White, T. J. (Ed.). *PCR protocols: a guide to methods and applications.* Academic Press, San Diego. pp. 315 - 322.
Yamaji, K., Fukushi, Y., Hashidoko, Y., Yoshida, T. & Tahara, S. 2001. *Penicillium* fungi from *Picea glehnii* seeds protect the seedlings from damping-off. *New phytologist* 152, 521-531.
Yuan, Z. Q. & Mohammed, C. 1999. Pathogenicity of fungi associated with stem cankers of eucalypts in Tasmania, Australia. *Plant disease* 83, 1063-1069.
Zhou, S. G. & Stanosz, G. R. 2001. Relationships among *Botryosphaeria* species and associated anamorphic fungi inferred from the analyses of ITS and 5.8S rDNA sequences. *Mycologia* 93, 516-527.

# Acknowledgements

First of all, I would like to express my heartfelt gratitude to my main supervisor Prof. Jan Stenlid, without whose guidance, encouragement and support, this thesis would not have been materialised. Janne will also be remembered for teaching me ice-skating. I thank my assistant supervisors Drs Pia Barklund and Kjell Wahlström for their assistance in the course of the study. I would also like to thank all of my supervisors, and their families for their hospitality during my stay in Sweden.

I am grateful to all mykopats for the social atmosphere and a wonderful working environment. You have all contributed positively to the realisation of this thesis through useful comments. Thank you so much indeed! I would be wrong if I fail to acknowledge the constant help I received from Karin Backström from the time of my arrival at the department up until now. I am grateful to Prof. Roger Finlay for his encouragement and willingness to help whenever I sought his help. I owe special thanks to Katarina Ihrmark, Anna Rosling and Maria Johansson for their assistance around the PCR lab and for their efforts in tutoring in Svenska. Kristof Capieau has been extremely helpful in several matters and particularly in the final stage of the formatting of the thesis. Anna Rosling and Jenny Arnerup have been nice officemates. Petra, Christina, Jonas, Magnus, Andy, Les, Jane, Frank, Olov, Johan, Vaidas, Audrius, Rimvis, Remis, Ăke, Guosheng, Elna, Marianne, Kalle, Michael, Johanna, Ina, Ylva, Mårten, Nicklas, Fredrik, Cajsa, Björn, Greg and Pajand I thank you all for your help in one way or another. I thank Fred Asiegbu and Nils Högberg and their families for their hospitality especially during Christmas period. I am grateful to Bernard Slippers for his unreserved assistance and stimulating collaboration on the characterisation of Botryosphaeria species.

I thank Anders Broberg and Anton Pohanka at the Department of Chemistry for their assistance with the HPLC work .

Haji Roba Amuma and his wife Addee Haloo Sheik Mohammed and their family Abdulatif (Abe), Halima, Ahmad, Sultan, Adam, Mamush, Caalaa and Sa'ida have all made me feel at home. I thank them all for being extremely nice to me. In fact, I was treated as one of their family members and made my stay in Sweden more pleasant. The presence of friends and fellow PhD students Jama Haji and Hussien Hamda was a plus to that. Thanks are also due to many others who have helped me in one way or another.

I am grateful to Wondo Genet College of Forestry and its staff for the provision of facilities during the study. I thank all the former and the present PhD students from Wondo Genet and AUA for creating a good social atmosphere and nice discussions of general interest. I am grateful to the Swedish International Development Authority for the provision of finance for my PhD study. I would also like to thank all the former and the present staff of the SLU Liaison Office in Ethiopia and the Programme Directors, for their cooperation during the study. I am grateful to the National Tree Seed Centre, Forestry Research, Ethiopia and its

staff for their assistance during the field work in Ethiopia. I am also grateful to Munessa Shashamane Forest Industry and its staff for their cooperation.

My relatives and friends in Ethiopia and abroad have always been the source of inspiration and encouragement. I am grateful to all of them. I am permanently indebted to my parents for their love, efforts and inspirations in my education. Wabe Haji's moral and financial support has greatly contributed to my education and will never be forgotten. I am obliged to mention Eyob Guye, who as a teacher, inspired and shaped my education especially during my early school age. Finally, I am indebted to my wife, Madina Jima, for her love, patience and strength in shouldering a heavy burden of responsibilities in my absence and for caring for our little ones Anakiyyaa and Qalbbii Abdella. Ayyoo tiyya baayi'ee sijaaladha!