Armillaria root rot of tea in Kenya

Characterization of the pathogen and approaches to disease management

CENTRALE LANDBOUWCATALOGUS
0000 0889 9003

Promotor: Prof. dr. M. J. Jeger
Hoogleraar in de Ecologische Fytopathologie

Co-promotor: Dr. ir. A. J. Termorshuizen
Universitair Docent leerstoelgroep Biologische Bedrijfssystemen

Samenstelling promotiecommissie:
Prof. dr. ir. P. Struik (Wageningen Universiteit)
Dr. C. Prior (Royal Horticultural Society, Wisley, UK)
Dr. Th. W. Kuyper (Wageningen Universiteit)
Dr. ir. J. M. Raaijmakers (Wageningen Universiteit)

Armillaria root rot of tea in Kenya

Characterization of the pathogen and approaches to disease management

Washington Otieno

Proefschrift

ter verkrijging van de graad van doctor

op gezag van de rector magnificus

van Wageningen Universiteit,

Prof. dr. ir. L. Speelman

in het openbaar te verdedigen op maandag 11 februari 2002

des namiddags te vier uur in de Aula

1639573

NN08201,3144

Propositions

1. General reference to *Armillaria* as a pathogen of 'woody plants' is incorrect. This Thesis.
2. DNA sequencing techniques provide opportunity for adoption of molecular characteristics in the 'nomenclature' of *Armillaria* species. This Thesis.
3. In biological control of soil borne pathogens, extrapolation of results from pot based experiments can give erroneous conclusions.
4. The term 'incite' should not be used in aetiological literature as it implies an indirect role of the pathogen in disease.
5. Chemical pesticides and genetically modified organisms should equally be causes of concern in agriculture.
6. Over-diversification in agriculture is the result and the cause of food insecurity in small-holder farming communities.
7. Positive thinking can only be founded on criticism of good will.
8. Greatness only exists where simplicity, goodness and truth are present (Rosemary Edmond, 1962).

Washington Otieno

Armillaria root rot of tea in Kenya – characterization of the pathogen and approaches to disease management.
Wageningen, 11 February 2002

Preface

I began this PhD study in February 1997 under a 'sandwich programme' which involved Wageningen University and The Tea Research Foundation of Kenya. The goal of the study was to improve the understanding of Armillaria root rot of tea in Kenya. As I complete this phase of the study, I would like to acknowledge Wageningen University for offering me fellowships during my stay in Wageningen and the Tea Research Foundation of Kenya for funding the research and granting me study leave that enabled me to do the first and the last parts of the study. Some of the laboratory experiments were conducted at The Royal Horticultural Society, Wisley, U.K. I am very grateful to Ana-Perez Sierra and Dr. Chris Prior of (RHS) for their contribution to the success of these experiments. I also acknowledge all persons who at all or various stages took part in this work. I am particularly grateful to my promotor, Professor Mike Jeger, for guiding the progress of experimental work and for thoroughly and consistently guiding the writing of the thesis. I am also very grateful to my co-promotor, Dr. Aad Termorshuizen, for his guidance and in particular for providing keen critiques and suggestions in data analysis and interpretation. Despite very busy schedules he was always available to attend to my numerous questions.

Laboratory and field experiments were conducted at Tea Research Foundation of Kenya. I am very grateful to Dr. Francis Wachira for his contribution to the success of experiments on molecular characterization of *Armillaria* isolates. I also thank the staff of the Plant Protection and Botany departments of the Tea Research Foundation of Kenya for the roles they played in these experiments. Their personal involvement and interest in the work determined the success of these experiments. I thank Dr. Caleb O. Othieno for his useful suggestions and comments on some of the field experiments. Special thanks also go to Dr. Peter-Jan Keizer for his contribution to the taxonomic section of Chapter 2.

I had a cordial relationship with the staff at Biological Farming Systems of Wageningen University and Tea Research Foundation of Kenya. Dr. Wim Blok and members of our discussion group at Biological Farming Systems are acknowledged for their useful suggestions on some of the chapters.

Finally I thank my relatives for their understanding and encouragement. To my wife Melony and children Dora, Anne and Paul, I would praise their perseverance during the years 2000 and 2001 when I spent most of my time away from home striving to complete this work. I dedicate this thesis to them with honour.

Washington Otieno Wageningen 11 February 2002

Contents

Chapter 1	General introduction	1
Chapter 2	*Armillaria* in Africa	11
Chapter 3	Characterization of *Armillaria* isolates from tea (*Camellia sinensis*) in Kenya	27
Chapter 4	Screening of *Trichoderma* isolates for inhibition to *Armillaria* in infested wood	51
Chapter 5	Effect of *Trichoderma harzianum* and soil amendment with coffee pulp on survival of *Armillaria*	61
Chapter 6:	Efficacy of soil solarization, *Trichoderma harzianum*, and coffee pulp amendment against *Armillaria*	75
Chapter 7	General discussion	87
References		95
Summary		109
Samenvatting		113
List of publications		117
Curriculum vitae		119

Chapter 1

General introduction

Armillaria root rot of tea in Kenya is the subject of this thesis. This chapter gives background information to the subject and an outline of the thesis. The first section describes tea production in general and more specifically in Kenya. The aetiology and management of the disease are introduced in the second section. The third section outlines characteristics of the genus *Armillaria* used in species identification and introduces the problem of attempting to apply these in Africa. The possibilities for managing the disease in commercial plantations are introduced in the fourth section. The final section outlines the objectives and approach to the present study.

The tea plant

Tea (*Camellia sinensis* (L.) O. Kuntze) is a perennial evergreen beverage crop belonging to the family Theaceae. *Camellia sinensis* originated in the lower montane forest of mainland Asia from south western China (Sichuan) to north western India (Assam). The primary centre of origin is presumed to be near the source of the Irrawadi River in northern Burma. Early human interest in stimulating properties of tea may have been instrumental in its wide dispersal in Asia. The tea grown in China and Japan is *C. sinensis* var. *sinensis* ('China tea') which has smaller leaves and greater cold tolerance but grows less vigorously than *C. sinensis* var. *assamica* (Mast.) Kitamura ('Assam tea') discovered in the forests of north-eastern India in 1823.

The tea plant is a multi-stemmed, up to 3 m tall shrub (var. *sinensis*), or a 10-15-m tall tree with one main stem (var. *assamica*). Under cultivation the plants are pruned to 1-1.5 m and trained as low profusely branching and spreading bushes. Written records dating from 5th Century AD confirm widespread cultivation of tea and its general use as a refreshing beverage in central Chinese provinces. In Japan, tea cultivation started in the 9th century with the seed introduced from China. Assam tea and hybrids between the two

varieties became the basis of the tea industries in south, south-east, and west-Asia as well as for those established in Africa and South America in the 1900s. For more than 300 years all the tea drunk in the western world came from China but this monopoly on the international tea market came to an end with the development of tea plantations in India (1840), Sri Lanka (1870) and Indonesia (1880). By 1925 very little of the 300,000 tonnes of tea imported into Europe came from China. Currently Sri Lanka and the African countries export > 90% of their tea, Indonesia, 60%, China 27% (black + green), and India 20%. Of the 1.1 million tonnes of tea that goes to the world market, the leading importers, United Kingdom, Russian Federation, Pakistan, U.S.A and Egypt take up 155, 150, 110, 85, and 70 thousand tonnes respectively.

Tea cultivation

Tea is grown in an expansive region of the world between latitudes ranging from the equator to 33°S in Natal, South Africa and 49°N in Georgia (Huang, 1989) and spanning a range of altitudes from sea level in Bangladesh to over 2600 m in Kenya. The climates in these regions range from Mediterranean to tropical (Carr and Stephens, 1992). General climatic requirements for tea production have been described in detail (Willson and Clifford, 1992; Vossen and Wessel, 2000). Mean annual rainfall required for good growth of tea varies from 1500 mm (Uganda) to 3500 mm (Java). On average, economic tea production requires ≥ 1700 mm annual minimum rainfall, which should not fall below 50 mm per month for any prolonged period. Optimum temperature for shoot growth ranges from 18 - 30°C. Base temperature (Tb) below which shoot growth stops is 12.5°C on average but can vary according to genotype from 8 - 15°C. However, night frost temperatures that occur in important growing areas at higher latitudes or high elevations do not kill tea. In the absence of water stress, the thermal time (= the product of the number of days and effective temperature (T-Tb) for shoot regeneration cycle (SRC)) averages 475 day °C. This parameter is useful in predicting seasonal and geographical effects on the length of SRC and the consequent yield pattern. It is, however, not applicable at temperatures > 30°C because the accompanying high vapour pressure deficits (> 23 mbar) depress shoot growth. Tea grows on a wide range of soil types developed from diverse parent rock material under high rainfall conditions. Suitable soils

should be free draining, have a depth not less than 2 m, pH between 4.5 and 5.6, a texture of sandy loam to clay and good water holding capacity.

Tea was introduced into Kenya in the early 1900s and first planted at Limuru near Nairobi. Its successful establishment led to the spread of cultivation to other parts of the country. The tea-growing region of Kenya straddles the equator with economic production taking place at altitudes ranging from 1600 - 2600 m in the highlands west of the Great Rift Valley and Mount Kenya region. The annual rainfall in this region is > 1400 mm and well distributed, the temperatures range from 21 - 28°C, and the soil is highly leached and acid (pH ~ 5.0) nitisols. Due to the tropical climate tea is harvested every 11 - 17 days throughout the year.

Economic value of tea

Tea is grown commercially to produce a beverage made from tender shoots consisting of two expanded leaves and one terminal bud. The three main categories of tea are black, green, and oolong. All of these come from the same plant species (*C. sinensis*), the difference being only the method of processing. As at 1995-98 total annual world production of tea was 2.6 million tonnes of which 78% was black tea. About 1.1 million tonnes went to the international market of which 93% comprised of black tea. Of the total world production averaging 2.6 million tonnes from a total area of 2.5 million ha in 30 countries (22% green tea), S. Asia (India, Sri Lanka, and Bangladesh) produces about 40%, E. Asia (China, Japan, Taiwan), 27%; Africa (10 countries) 14%; W. Asia (Turkey, Iran, Georgia, Azerbeijan) 10%; S.E. Asia, 7%; and S. America (Argentina, Brazil) 2%. Table 1.1 gives a summary of world tea production for the 5 leading producing countries and their percentage share in the world market. The leading tea importing countries are the United Kingdom, Ireland, USA, and Egypt with annual per capita consumption of 2.5, 3.1, 0.3, and 1.1 respectively.

In Kenya, which is the largest producer in Africa, tea is an important foreign exchange earner and contributes 17 – 20% of total export revenue. The tea industry in Kenya consists of two main sectors: the large estates owned by multinational companies and the small holders (individual small-scale farmers). These own approximately 30 and 70% respectively of the total land planted with the crop. Small-scale farmers grow over

Table 1.1: The five leading tea producing countries and their contribution to tea trade worldwide

			Global share (%)	
Country	Planted area (ha)	Tea production (tonnes yr^{-1})	Quantity	Trade
India	425,000	755,000	29.8	17.0
China	1,100,000	580,000	22.6	17.6
Sri Lanka	187,309	250,000	10.2	21.3
Kenya	120,000	240,000	8.1	21.0
Indonesia	91,400	140,000	4.4	5.4

80% of tea. At production and processing stages, tea provides employment to over 1 million people. In the small holder sector, over 300,000 people (small holders and their families) depend on tea growing as a direct means of livelihood.

Diseases of the tea plant

A number of diseases have been recorded in various tea-growing countries but only a few are of economic importance. Blister blight (*Exobasidium vexans*), which attacks young leaves and shoots, is the most important foliar disease of tea and is of major importance in all tea growing areas of Asia, but has not been reported in Africa or South America (Arulpragasam, 1992). Anthracnose (*Colletotrichum theae-sinensis*) and net blister blight (*Exobasidium reticulum*) are also of importance mainly in Japan and Taiwan. Grey (*Pestalotia theae*) and brown (*Colletotrichum camelliae*) blights are caused by weak parasites attacking mainly mature leaves near senescence but can be a problem in mechanically harvested tea. There are several stem cankers of which *Macrophoma theicola* and *Phomopsis theae* are most common but these can be controlled by careful pruning, protecting pruning cuts with fungicidal paints, and removing infected branches. In Kenya wood rot (*Hypoxylon serpens*) and stem canker (*Phomopsis theae*) are the only important

stem diseases. Correct pruning practices and fungicidal protection of pruning cuts can manage both diseases. A number of important root pathogens (*Phellinus* (= *Fomes*) *noxius*, *Ganoderma pseudoferrum* and *Armillaria* sp.) are difficult to control in tea. In Kenya, *Armillaria* root rot persists in several farms particularly in the small holder sector, killing tea bushes and rendering the subsequent inoculum foci useless for production of many other crops.

Armillaria root rot

Armillaria root rot affects many plant species in the highland areas of Kenya. The only full descriptions of the disease on tea in Kenya were made in 1960 (Gibson, 1960a, Gibson and Goodchild, 1960; Goodchild, 1960). The disease hinders successful establishment of tea when planted shortly after deforestation. Maintenance of plant populations at the original density is also not possible in some old plantations if eradication of infected plants is not regularly and properly carried out. The principal indicators of infection in a tea plantation are gaps resulting from death of plants. Locations of these infection foci are often associated with stump and root remnants of trees and shrubs from the previous forest vegetation. Infection of tea is common shortly after forest replacement with the crop and decreases over many years (Goodchild, 1960). Where no efforts are made to check the spread of *Armillaria*, the disease persists for decades causing considerable plant mortality. Sometimes infections appear in a tea plantation after several years of disease-free production (Goodchild, 1960) showing that inoculum in plant materials at great soil depths can remain viable for many years. Viable mycelium of the pathogen can be recovered from such materials several years after tree felling (Goodchild, 1960). Incidence of *Armillaria* is less common in the large tea estates than in the small holder farms. This can be attributed to efficient and thorough removal of inoculum reservoirs from the soil by the heavy machinery used in the large estate establishments compared to the hand implements used by the small holders (Onsando *et al.*, 1997). The incidence is also higher in new plantations than in older plantations but this can be attributed to more frequent establishment of root contacts with inoculum sources and may have little to do with higher susceptibility of younger plants. There are

few records of Armillaria root rot on tea under 12 months old. Cases of infection may, however, be frequent but go unrecognised as disease casualties because they occur at times when planting failure due to other factors such as poor land preparation, improper planting and insect damage are common.

The species of *Armillaria* that cause root rot of tea in Kenya are certainly indigenous to natural forests. Little has been done to determine diversity of *Armillaria* in Kenya and until recently, the disease was arbitrarily attributed to *Armillaria mellea* (Vahl : Fr.) Kumm. *sensu lato*. Identity of the pathogen is thus still obscure. The two species, *A. heimii* Pegler and *A. mellea* 'ssp. *africana*' reported so far (Mohammed *et al.*, 1993; Mwangi *et al.*, 1993) are currently assumed to be largely responsible for the disease. However, there are indications that other species of *Armillaria* exist in Kenya suggesting that additional taxonomic groups may also be involved in the disease. Due to the general absence of basidiomata on infected tea, presence of other species that have not been described hitherto has been difficult to ascertain.

Delimitation of African *Armillaria* spp.

Delimitation of *Armillaria* species employs morphological characteristics of basidiomata. Where basidiomata are present, it is also possible to obtain haploid cultures from basidiospores. These can be used in mating tests to identify 'biological species'. Ordinarily cultures of basidiospores are haploid while those of basidiomata flesh are diploid. Haploid and diploid cultures of *Armillaria* contrast in their morphology, the haploid having a fluffy and the diploid a crustose appearance. In mating tests, crossings are made between a haploid colony of a known species (tester strain) and the unknown haploid or diploid isolate. Macroscopic change in colony morphology of the tester colony from fluffy to crustose indicates the change from haploidy to diploidy and therefore genetic similarity of the tested isolates with the tester (Korhonen, 1978; Anderson, 1986; Siepmann, 1987). The mating tests are used extensively to identify temperate *Armillaria* and to designate them as 'biological species' *sensu* Hintikka (1973). Compatibility in mating tests has led to the definition of a number of morphologically recognisable species in North America, Europe, and Australia. However, mating tests are applicable only to

heterothallic *Armillaria* because homothallic isolates lack a stable haploid condition. Most African *Armillaria* isolates appear to be homothallic. Between continents compatibility tests for isolates are routinely inconsistent (Mwangi *et al.*, 1989). Mating tests are therefore useless as a criterion for identification of African *Armillaria* isolates.

An incompatibility system based on antagonistic responses (Rizzo and Harrington, 1993) between mycelia of colonies paired on a common substrate is also used to distinguish isolates of *Armillaria*, even diploids, into somatic incompatibility groups. The incompatibility reaction can indicate inter- or intra-specific groups (Korhonen, 1978). Interspecific incompatibility is characterised by the failure of hyphae to anastomose and the production of a pigmented line of demarcation between colonies. This has been used in a few studies to distinguish among African *Armillaria* isolates (Abomo-Ndongo *et al.*, 1997; Guillaumin *et al.*, 1993). Due to the plastic nature of morphological characteristics, the absence of basidiomata and homothallism, biochemical methods particularly those based on isozyme electrophoresis (Agustian *et al.*, 1994; Mwangi *et al.*, 1989; Mwenje and Ride, 1996; 1997) and DNA molecular markers (Mohammed, 1994; Coetzee *et al.*, 1997; Chillali *et al.*, 1997) have gained popularity in attempts to characterise or identify *Armillaria* isolates from Africa.

Management of Armillaria root rot

Methods that effectively control Armillaria root rot are currently not available to tea growers. Eradicating *Armillaria* from the soil by removing plant materials that may serve as reservoirs of the inoculum as well as infected plants is recommended (Anon., 1986) and widely practised in Kenya. However, complete elimination of the pathogen from the soil by this method is difficult, particularly for inoculum occurring deeply in the soil or where field infestation is extensive and removal has to be carried out manually. The disease therefore persists in small holder farms causing considerable mortality to tea and other economic plants including fruit trees and tuber crops (Otieno, pers. observations).

In some plantations, the disease only becomes a problem when trees inter-planted with or growing in close proximity to tea are felled (Otieno, pers. observation). Factors

associated with this phenomenon have not been investigated. It is known that roots of some trees may have non-harmful associations with *Armillaria* as lesions or epiphytic rhizomorphs (Gibson, 1960b). It has been hypothesised that if such trees are first ring-barked then left to dry up slowly over a period of 18–24 months before they are felled, their roots would be depleted of starch reserves thereby exposing *Armillaria* to adverse effects of microbial competition which reduce the longevity of inoculum (Leach, 1939). This method is routinely employed in Kenya to kill trees that grow adjacent to tea plantations or on land to be planted with crops prone to the disease. The finding of active *Armillaria* mycelium in roots of indigenous hard wood trees in Zimbabwe several years after they were killed by ring-barking (Masuka, 1993) makes it questionable whether the practice has any value in controlling the disease.

In the past the use of soil disinfestation with chemicals such as methyl bromide and carbon disulphide has been a common method of controlling soil-borne pathogens. In modern agriculture these are unpopular due to hazards they pose to the environment. No record exists of any attempts to control Armillaria root rot in Kenya by use of chemicals. Even if their use was acceptable, *Armillaria* possesses mechanisms of protection that would limit the ability of chemical fumigants to attain maximum efficacy (Raziq, 2000). Development of safe and effective methods of controlling the disease is therefore desirable. In several studies it has been demonstrated that *Trichoderma* spp. can antagonise *Armillaria*. However, their use is barely documented as a practical method of disease control. Munnecke *et al.* (1981) observed that capacity of *T. viride* to infect and kill *A. mellea* was enhanced when the antagonist was applied to the soil consequent to exposure of the pathogen to sub-lethal levels of chemical fumigation, heat, or desiccation. The research described in this thesis investigated whether increasing population density of antagonistic *Trichoderma* and exposure of *Armillaria* to the high soil temperatures achieved by solarization would affect survival of the pathogen in naturally infected plant materials.

About this thesis

Despite the prevalence of Armillaria root rot over a large geographical area in highland regions of Kenya and its common incidence on various hosts, the disease has been subject of only a few studies. Identity of the causal agent and applicability of methods that have been tried elsewhere for management of the disease have remained largely uninvestigated in Kenya and other parts of Africa. In this thesis an attempt is made to characterise *Armillaria* pathogenic on tea in Kenya and to investigate the possibility of managing the disease.

Characterisation of tea pathogenic Armillaria

A survey was made on the incidence of Armillaria root rot in each of the two (the east and the west of the Great Rift Valley) major tea-growing regions of Kenya. Isolates of the fungus were collected from infected tea and a few other plants. The cultural morphological characteristics of these isolates were described and somatic incompatibility tests carried out. In addition the isolates were characterised using DNA-based molecular markers. In the few instances where basidiomata were found, their morphological characteristics were recorded.

Disease management

The search for ways of managing *Armillaria* root disease on tea by biological and cultural methods was the focus of the field experiments. The *Trichoderma* isolates used were obtained in an earlier study on antagonism to the pathogen on synthetic media (Onsando and Waudo, 1994). In this study, the *Trichoderma* isolates were tested for inhibition of the pathogen in plant materials. The isolate showing the greatest antagonism was subsequently evaluated in field experiments for effect on survival of the pathogen in naturally infected tea stumps. Two approaches were adopted in testing the hypothesis that low population density of antagonistic *Trichoderma* spp. limits their efficacy for the control of *Armillaria* (Garrett, 1958) and that resistance of the pathogen to the hyperparasite can be disrupted by partial soil disinfestation (Munnecke *et al.*, 1981). In

Guillaumin, 1994; Ota *et al.*, 2000); only some isolates of *A. heimii* have been reported as heterothallic (Abomo-Ndongo *et al.*, 1997), making mating tests inapplicable. Diagnosis of the disease is also hampered by the scarcity of rhizomorphs. Thus, although Armillaria root rot has been reported from various African countries, its diagnosis is often based only on the presence of white mycelial fans under affected bark tissue. For these reasons the occurrence of *Armillaria* in Africa and other tropical countries may have been underreported. Nevertheless, reports do suggest that Armillaria root rot can cause significant damage to several plant species in the continent but few up-to-date appraisals on the disease have been made. This review aims to reassess the existing knowledge on *Armillaria* in Africa.

Species diversity

Nomenclature of *Armillaria* species is based on the morphology of the basidiomata. Isolates that can only be identified by compatibility experiments are referred to as "biological species". Due to the homothallic nature of most African isolates, biological species cannot be designated. The following species have been reported from Africa:

Armillaria camerunensis (Hennings) Volk and Burdsall (1995)

Basionym: *Armillaria mellea* (Vahl : Fr.) P. Kumm. var. *camerunensis* Henn. In Hennings (1895).

Synonym: *Armillariella camerunensis* (Henn.) Singer (1986).

Original description

A. mellea Vahl in Fl. Dan. t. 103 var. *camerunensis* P. Henn.; pileo carnuloso, convexo-explanato, ½-1 cm diametro, rufo-brunneo, dense granulato- vel verrucoso-squamoso, squamis parvis atris subconicis vel depressis, margine primo involuto dein explanato, substriato; stipite farcto, 1-2 cm longo, 2-3 mm crasso, subsquamoso, substriato, laete brunneo, parte superiore annulo amplo, membranaceo-flocculoso, albo, patente; lamellis sinuoso-adnatis, vix decurrentibus, subconfertis, pallidis; sporis subglobolis, levibus, hyalin-8 μ, basidiis clavatis.

Kamerun, bei Bomana, c. 670 m, an faulenden Baumstämmen rasig (P. Dusén n. 1[a]-19 Juli 1892).

Eine sehr zierliche und kleine Form, die äusserlich der typischen Art sehr ähnlich, aber durch die nicht herablaufenden Lamellen etwas verschieden ist. Bei N'dian wurden von Herrn Dusén unter n. 39[a] lange Rhizomorphenstränge an faulenden Stammen gesammelt, die von derartigen Mycelien des Pilzes nicht verschieden sind und wahrscheinlich zu demselben gehören.

Translation

A. mellea Vahl. in Fl. Dan. t. 103 var. *camerunensis* P. Henn.; cap thin-fleshy, convex-expanded, ½-1 cm diameter, red-brown, densely granular- or verrucose-scaly, with small, black, slightly conical or depressed scales, with at first involute, later expanded, weakly striate margin; stem stuffed, 1 – 2 cm long, 2 – 3 mm broad, slightly squamulose [and] striate, light brown, within the upper part a large, membranaceous-flocculose, white ring, spreading; lamellae emarginate-adnexed, hardly decurrent, slightly crowded, pale; spores subglobose, smooth, hyaline, 7-8 μm, basidia clavate.

Cameroon, near Bomana (approx. 670 m), against decomposing trunks.

A very graceful and small form which is very similar to the typical species, but differs from it by its non-decurrent lamellae. Near N'dian long rhizomorphs (39a) were collected from decomposing trunks by mr. Dusén, which are not different from such mycelia of the fungus and which probably belong to it.

Discussion

A. camerunensis differs from other African *Armillaria* species by its tiny dimensions (cap 5-10 mm diam. as opposed to *A. heimii* measuring 15-25 mm diam.), dark colour of the cap, membranaceous annulus and subglobose spores. In contrast, *A. heimii* has a cream-coloured cap, fugacious annulus and ellipsoid spores. The only other *Armillaria* species for which subglobose spores have been reported is *A. fuscipes*, but this species has much larger dimensions (with cap diam. up to 60 mm) and a more bright (orange yellow) coloured cap. *A. camerunensis* has been reported only by Hennings (1895) from Cameroon and by Beeli (1927) from Congo. Watling (1992) suspected that another specimen of *Armillaria* collected from Cameroon belongs to this species. The species could have been overlooked because of its minute dimensions. More definitive

(1992) argued that *A. heimii* and *A. fuscipes* referred to the same species. Watling (1992) arrived at this conclusion after extensive microscopic investigation of the type specimens. In our opinion, however, he overvalued the microscopic characters at the expense of macroscopic characters. For most *Armillaria* species, microscopic features are not reliable for identification. It is therefore not surprising that the type collections did not differ microscopically. When inspecting the original species descriptions, macroscopically *A. heimii* has quite small dimensions, a fugacious annulus and a stipe which is white at the top turning to ochre towards the base while *A. fuscipes* has larger dimensions, a thick annulus and a stipe which is dark brown. Confusion between the two species may have arisen by the interpretation of *A. heimii* by Heim (1963), who distinguished a parasitic form of this species. In our opinion the identity of this parasitic form is doubtful because of its larger dimensions, dark-coloured cap and dark brown stipe.

Reports on *A. heimii* are difficult to interpret since its identity depends on the author's interpretation in relation to *A. fuscipes.* The species has been reported over a wide range of altitudes and hosts in Africa. It could be the most common African *Armillaria* species. Homothallic and heterothallic isolates have been reported (Abomo-Ndongo *et al.*, 1997; Agustian *et al.*, 1994; Guillaumin *et al.*, 1993).

***Armillaria mellea* (Vahl : Fr.) P. Kumm. (1871)**

Basionym: *Agaricus melleus* Vahl (1787)

Synonyms: see Termorshuizen and Arnolds (1987), Volk and Burdsall (1995) and Watling *et al.* (1982).

Original description

Agaricus melleus, pileo convexo lutescente, fusco irrorato, lamellis pallidis, stipitibus aggregatis annulatis farctis Fl: Dan. tab. 1013.

Habitat in pratis ad radicis ulmorum Bergo Norway, in Danica in truncis fagetis β in arenosis Medio Augusto et Septembris.

Translation

A honey-coloured agaric, cap convex, becoming yellowish, brown speckled, lamellae pale, stems [growing] in groups, ringed, stuffed.

Habitat in meadows on roots of elm-trees, Bergen, Norway, in Denmark on trunks in sandy beech forests, in the middle of August and September.

Discussion

A. mellea sensu stricto has been reported only very rarely from Africa and extensive descriptions of the basidiomata have not been published so far. The description of Pegler (1977) most likely does not refer to an *A. mellea s.s.* because he reported the presence of clamp connections, which are absent in European *A. mellea s.s.* Mohammed *et al.* (1993) reported that the morphology of artificially produced basidiomata from a Kenyan isolate resemble that of European *A. mellea s.s.* but they did not mention the morphology of any naturally produced basidiomata. Cultures of African *A. mellea* showed partial compatibility with testers of *A. mellea s.s.* from Europe and North America (Mwangi *et al.*, 1989) but the authors did not present their results in detail and admitted that results from haploid/diploid crossings are sometimes difficult to interpret. Several reports mention that African *A. mellea* is different from European *A. mellea* with respect to pectic isozyme profiles (Mwenje and Ride, 1997), DNA-analysis using RFLP and RAPD (Mohammed *et al.*, 1993; Mohammed and Guillaumin, 1994; Chillali *et al.*, 1997), and somatic compatibility tests and RAPD-analysis (Ota *et al.*, 2000). However, none of these authors presented any descriptions of basidiomata or else reported how the isolates they named as *A. mellea* were identified. Thus, we refrain from a definitive conclusion until more extensive descriptions of basidiomata have been published. When comparing morphology of European *A. mellea s.s.* (Termorshuizen and Arnolds, 1987) with that of *A. fuscipes* (Petch, 1909), the former differs by its somewhat larger dimensions and by having yellow-greenish tinges in the centre of the cap, absence of a nearly white margin of the cap and presence a membranaceous annulus. These differences are rather subtle and it may be that both names refer to the same species.

***Armillaria pelliculata* Beeli (1927)**

Armillaria pelliculata Beeli (1927) was found only once. The species was rejected from *Armillaria* because of the vicid veil and infundibiliform pileus (Watling, 1992).

Provisional key for *Armillaria* species in Africa

1a Annulus fugacious, hardly discernable on mature basidiomata.................... *A. heimii*

1b Annulus persistent, clearly visible on mature basidiomata.................................2

2a Average cap diam. < 25 mm, dark brown........................... *Armillaria camerunensis*

2b Average cap diam. > 30 mm, not so dark, brown yellowish brown, brownish yellow, greenish yellow or yellow...3

3a Annulus flocculose, relatively narrow; cap brown to yellow brown in the centre, turning strikingly pale towards the margin.. *A. fuscipes*

3b Annulus membranaceous, relatively wide; cap brownish yellow in the centre, or greenish yellow, not turning pale towards the margin... .... *A. mellea*

Economic importance and distribution

Significant and extensive damage by *Armillaria* has been reported in Africa (Tables 2.1 and 2.2) suggesting that *Armillaria* root rot may be an obstacle to production of several crops in many parts of the continent. In tea the disease has also been reported from other countries including India and Indonesia (Willson and Clifford, 1992), Malawi (Leach, 1939), Tanzania, and Uganda (Gibson, 1960a). Non-woody plants are equally affected by the disease and in Zimbabwe and the Congo damage is common on cassava (Makambila, 1980; Mwenje *et al.*, 1998). In Kenya, affected non-woody plants include banana, yam, and tomarillo (Otieno, pers. observations). The situation may be the same in other tropical regions. In all the African countries from which Armillaria root rot has been reported (Figure 2.1) the pathogen generally occurs in high rainfall areas and is not found in dry regions (Mohammed *et al.*, 1993). It is confined to high (> 1500 m) elevations in the eastern and western highlands of Kenya; low to high (600 - 1800 m) elevations in eastern highlands bordering Mozambique, the north eastern part of Marondera, and Harare areas in Zimbabwe; and low (< 500 m) elevations in the southern regions of the Congo (Mwangi *et al.*, 1993). In Tanzania, 900 m was reported to be the lowest altitude for occurrence of *Armillaria* (Gibson, 1960b). Within each of these countries local distribution of *Armillaria* is discontinuous in space with *A. heimii* occurring in areas with cool temperatures in Kenya and Zimbabwe but in warmer humid locations in the Congo (Mwangi *et al.* 1993).

Table 2.1: Reports of *Armillaria* in Africa

Country	Hosts	References*
Ethiopia	unidentified indigenous trees	21
Kenya	*Citrus* spp.	17
	Camellia sinensis	6, 14, 20
	Eucalyptus spp.	12, 20
	Pinus spp.	4, 5
	Coffea arabica, *Musa acuminata*, *Discorea* spp., *Persea americana*, tomarillo, *Saccharum* spp., *Cupressus* spp. *Prunus* spp. and unidentified trees	6, 11, 20
Mauritius	*Pinus* spp.	10
Malawi	*Pinus* spp.	10
Cameroon	unknown rotting wood	22
Ivory Coast	*Gmelina arborea,*	7
Gabon	*Theobroma cacao*, *Mannihot* sp., *Havea brasiliensis.*	7
Tunisia	Olives	21
Tanzania	*Camellia sinensis* and unidentified indigenous tree species	15
	Pinus sp.	21
Uganda	*Camellia sinensis* and indigenous tree species	15
S. Africa	*Pinus* spp.	10
	Litchi sinensis	2
Re Union	*Pelargonum asperum*	2, 10
Madagascar	*Coffea arabica*	3
The Congo	*Mannihot esculenta*	1, 8
	Havea braziliensis	7
Sao Tome	*Theobroma cacao*	16
Gabon	*Havea braziliensis*	7
Zimbabwe	*Pinus* spp., indigenous tree species (*Acacia xanthophloea*, *Brachystegia utilis*, *B. spaciformis*, *Bridelia micrantha*, *Ficus* sp., *Harungana madagascarensis*, *Julbernardia globiflora*, *Parinari curatelifolia*, *Tectonia grandis*, *Vernonia* sp.)	2, 9, 12, 13, 18, 19
Zambia	*Tectonia grandis*	2, 15

* [1]Abomo-Ndongo *et al.* (1997), [2]Coetzee *et al.* (1997), [3]Dadant (1963), [4]Gibson (1960b), [5]Gibson and Goodchild (1960), [6]Goochild (1960), [7]Guyot (1997), [8]Makambila (1980), [9]Masuka, (1993), [10]Ivory (1987), [11]Mwangi *et al.* (1993), [12]Mwangi (pers. comm., 1998), [13]Mwenje and Ride (1997), [14]Onsando *et al.* (1997), [15]Pegler (1977), [16] Rishbeth (1980), [17]Seif and Whittle (1984), [18]Swift (1968), [19]Swift (1972), [20]Otieno (pers. Observation), [21]Ota *et al.* (2000), [22]Watling (1992).

Table 2.2. Estimates of mortality due to *Armillaria* in some hosts in Africa

Country	Host	Mortality (%)	Source
Gabon	*Havea braziliensis*	up to 0.38	Guyot (1997)
The Congo	*Havea braziliensis*	>1	Pichel (1956)
	Mannihot esculenta	up to 100	Mwangi *et al.* (1993)
Kenya	*Camellia sinensis*	up to 50	Onsando *et al.* (1997)
	Cupressus lusitanica	up to 12.6	Mwangi *et al.* (1993)
Zimbabwe	*Pinus oocarpa*	up to 2.5	Masuka (1993)
	Plantation forests	0-25	Masuka (1993)
	Pinus elliottii	25-50	Ivory (1987)
E- and S-Africa	*Pinus elliottii*	25-50	Ivory (1987)

Distribution of the species in the Congo also appears to vary with soil type, its presence being associated only with clay but not sandy soils (Mwangi *et al.*, 1993).

Ecology

Basidiomata of *Armillaria* are rarely encountered in Africa. Although conditions affecting natural fructification by the fungus have not been investigated in this continent, high temperatures and probably limited rainfall may hamper fructification. The few basidiomata that have been found were used to name the species discussed above. Other hitherto unknown *Armillaria* species could perhaps be present at locations where basidiomata are absent. When found, basidiomata occur during the cool and moist periods of the year after the rainy seasons (Mohammed *et al.*, 1993) and are produced typically in clusters at the base of infected stumps and dead trees (Mwangi *et al.*, 1993; Mohammed *et al.*, 1993). Armillaria root rot is generally prevalent at elevations > 1500 m in Kenya, but basidiomata occur at elevations > 2000 m (Mwangi *et al.*, 1993; Otieno, pers. observation). On the other hand, basidiomata of *A. camerunensis* have been found at an elevation of 670 m in Cameroon (Hennings, 1895). As stated, basidiomata are uncommon in Africa and this may have contributed to a selective advantage of homothallism which is common in African *Armillaria.* When basidiomata are rare, the likelihood of two haploid mycelia meeting to form a diploid mycelium would be very low. Among the temperate *Armillaria* species, only *A. ectypa* is homothallic (Kile *et al.*, 1993) but unlike all the other species, it grows on peat and does not infect plants.

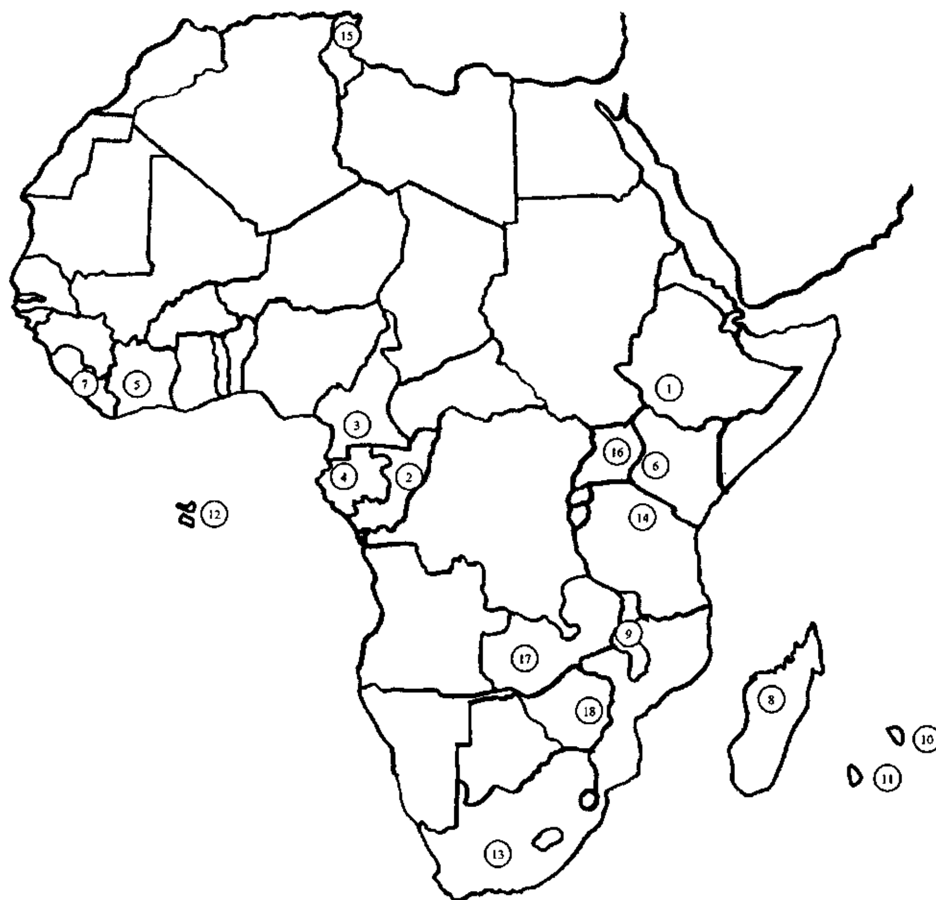
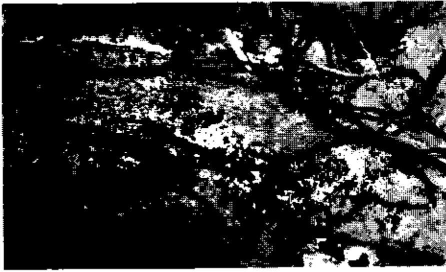


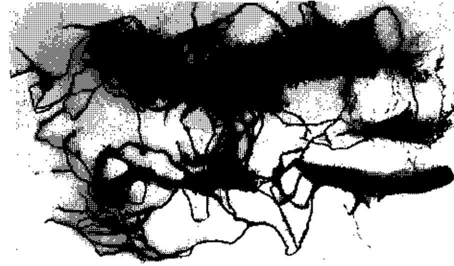
Fig. 2.1: African countries from which Armillaria root disease has been reported ([1]Ethiopia, [2]Congo [3]Cameroon, [4]Gabon, [5]Ivory Coast, [6]Kenya, [7]Liberia, [8]Madagascar, [9]Malawi, [10]Mauritius, [11]Re-Union, [12]Sao Tome, [13]South Africa, [14]Tanzania, [15]Tunisia, [16]Uganda, [17]Zambia, [18]Zimbabwe).

Rhizomorphs, a typical feature of the temperate *Armillaria* species which provide the principal means of below-ground dispersal, are rare in Africa. Their absence at low elevations in tropical Africa (Swift, 1964; Swift, 1968) has been explained by the prevailing soil temperatures which are often too high for rhizomorph growth (Rishbeth, 1978). The temperature range for rhizomorph growth varies for different species

(Rishbeth, 1978), but in general they are not formed above 28°C. Pearce and Malajczuk (1990) ascribed the paucity of rhizomorphs of *A. luteobubalina* in *Eucalyptus* forests in SW-Australia to unfavourably high temperatures and low soil moisture contents. Cultures of *A. luteobubalina* failed to form rhizomorphs at 30°C but they did so abundantly after the temperature was lowered to 20°C, suggesting an enzyme inactivation process within this range (Pearce and Malajczuk, 1990). *Armillaria* isolates from tropical lowlands do produce rhizomorphs *in vitro* at 25°C (Swift, 1968). Onsando *et al.* (1997) reported the presence of rhizomorphs in tea plantations at high elevations on residual roots of trees that were present before the tea was planted. This could be attributed to the prevailing moderate temperatures at deeper soil layers. The rhizomorphs associated with Armillaria root rot, if found on infected plants, are usually the cortical type (Figure 2.2a). Only rarely are subterranean forms (Figure 2.2b) encountered.

In the absence of basidiomata and with the scarcity of rhizomorphs, dispersal of *Armillaria* takes place mainly by root-to-root contact. Similarly when rhizomorphs, thought to be crucial for survival of *Armillaria* in the temperate climate zones (Kile *et al.*, 1991) are absent, persistence of the pathogen depends primarily on the ability to survive in infected plant tissue. It has been shown that many indigenous trees in natural forests harbour superficial lesions of *Armillaria* which apparently do not harm the trees (Gibson, 1960b). These lesions have been reported on *Parinari curatelifolia*, *Brachystegia utilis*, *B. spaciformis*, *Bridelia micrantha*, *Ficus* sp., *Harungana madagascarensis*, *Julbernardia globiflora*, as well as *Vernonia* spp. in Zimbabwe (Masuka, 1993), bamboo (*Arudinaria alpina*) and several unnamed plant species in Kenya (Gibson 1960b). They have also been reported in cultivated tea (Goodchild, 1960) as recently confirmed (Otieno, pers. observation; Figure 2.3). For *Armillaria* a range of predisposing factors including nutrient deficiency (Singh, 1983; Entry *et al.*, 1986), reduced light conditions (Redfern, 1978; Entry *et al.*, 1986, 1991), defoliation (Wargo, 1977), air pollution (Grzywacz and Wazny, 1973), changes of the nutrient status of the host caused by pruning (Popoola and Fox, 1996), and attack by insects (Huddak and Wells, 1974) have been implicated. Epiphytic lesions may enable *Armillaria* to quickly colonize weakened hosts that are unable to respond with a proper defence reaction. The ability of *Armillaria* to persist for years in a quiescent state on a healthy host can be regarded as an important survival strategy. Banik





a b

Fig 2.2: Kinds of rhizomorphs that may be found associated with roots of tea. (a) cortical rhizomorphs (more frequently encountered associated with tea roots at high elevations) and (b) subterranean rhizomorphs found in the soil (extremely rare in Kenya).

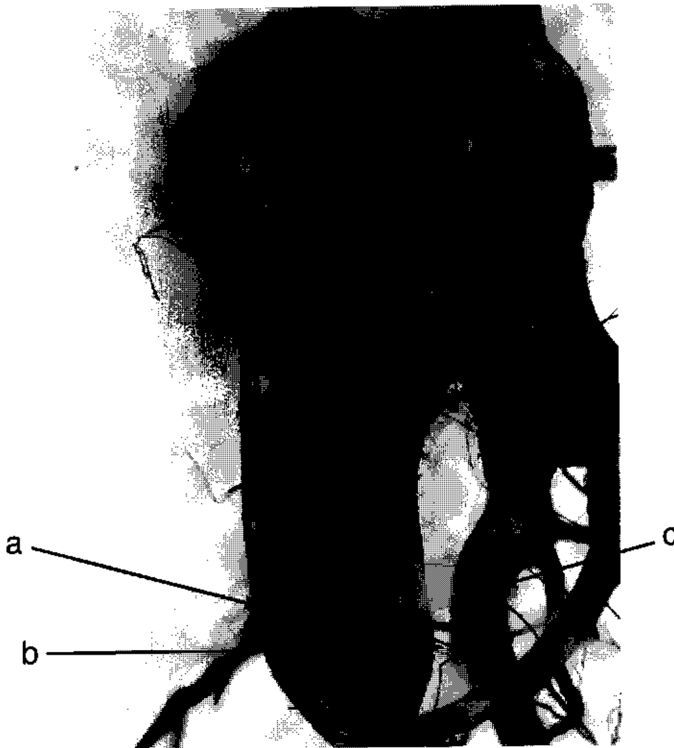
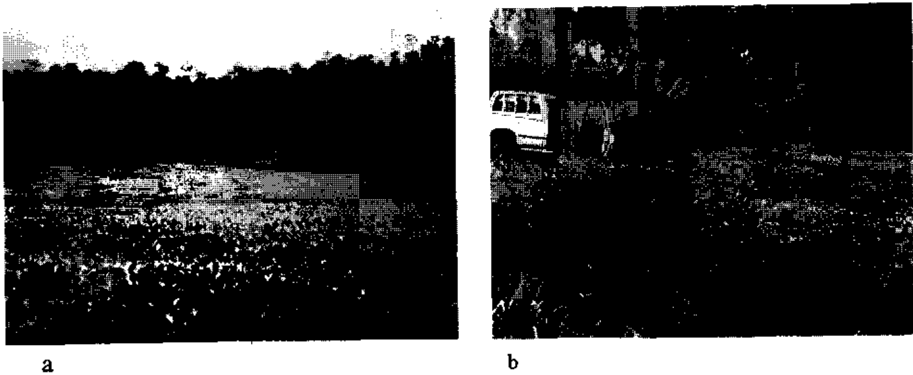


Fig. 2.3: Lesions (a and b, visible as small black spots) and epiphytic rhizomorphs (c) found on roots of tea. Some of the plants with these features do not show above-ground symptoms of the disease.

et al. (1995) were able to isolate the primary pathogen *A. ostoyae* from superficial root lesions of healthy *Populus tremuloides* trees, while no isolations were made of *A. lutea* which is a weak pathogen. This indicates that indeed primary pathogenic *Armillaria* rather than weak- or non-pathogenic species are involved in the root lesions.

The impact of *Armillaria* on undisturbed African forests is not known but as shown for commercial plantations of tea severe cases of the disease in Africa are often situated close to natural forests or have another tree species growing in close proximity (Figures 2.4 and 2.5). Studies in natural forests elsewhere indicate a limited impact (Hood and Sandberg, 1987; Kile *et al.*, 1991; Shearer *et al.*, 1997) of *Armillaria* but its presence



a b

Fig 2.4: (a) A tea plantation and an adjacent natural forest and (b) a progressing *Armillaria* infection centre associated with roots of a fuel wood species (*Grevillea robusta*) growing in close proximity to a tea plantation.

may already have resulted in a changed tree population structure in favour of species that can resist infection to some extent. Although no experiments have been carried out to elucidate pathogenic variation in African *Armillaria*, Mohammed *et al.* (1993) and Mwangi *et al.* (1993) reported that *A. heimii* and *A. mellea* 'ssp. *africana*' may cause considerable damage on many plants. Clearly more work needs to be done on the pathogenicity of African *Armillaria* species. Generally deforestation followed by planting

of exotic species such as *Pinus* spp. or tea leads to *Armillaria* problems, both at low elevations (where basidiomata and rhizomorphs are absent) and high elevations in Africa.

Fig 2.5: *Armillaria* infection centre in a tea plantation showing a dead stump (a) of an unknown tree species killed by ring-barking prior to felling. Surrounding the stump is a patch where several tea plants have died of Armillaria root rot.

The first stages of 'awakening' of the lesions and infection of the planted trees needs more investigation. Possibly the sudden reduction in the flow of photosynthates to the roots after tree felling might force the fungus to assume a more active search for nutrients. At the same time when the stump of a felled tree is dying the lesion may expand considerably before the substrate is fully invaded by saprotrophic decomposers. Lesion expansion would increase the likelihood of infection of roots of plants growing adjacent to remnants of lateral roots of the felled tree.

Conclusions

There are three reasons why *Armillaria* is poorly characterized in Africa: basidiomata are extremely rare; cultures of African *Armillaria* are usually homothallic, rendering mating tests inapplicable and hampering taxonomic research; and rhizomorphs are also extremely rare especially at low elevations. Scarcity of basidiomata and rhizomorphs hinders diagnosis of Armillaria root rot and thus the disease may have been

regularly underreported. Rarity of basidiomata means that infections by basidiospores are not likely to take place while scarcity of rhizomorphs limits the below-ground spread of the pathogen. Whereas rhizomorphs are the primary means of dispersal of *Armillaria* in temperate regions, in the tropics the fungus spreads primarily by root-to-root contacts. The danger of fragments of rhizomorphs broken during land preparation prior to planting or when infected plant parts are being removed increasing the inoculum density in the soil (Morrison, 1982; Redfern, 1973) is therefore remote in most parts of Africa. Hence the removal of infected plant tissues from the soil is a more important strategy in managing Armillaria root rot in Africa than in the temperate regions.

Chapter 3

Characterization of *Armillaria* isolates from tea (*Camellia sinensis*) in Kenya

Abstract

Armillaria is a primary root rot pathogen of tea (*Camellia sinensis*) in Kenya. The main species of the fungus described in this country are *A. mellea* and *A. heimii*. In 1997 a survey covering the 14 tea growing districts of the country was carried out and 47 isolates (41 from infected tea plants) were collected. Cultural morphology, rhizomorph characteristics, somatic incompatibility and features of basidiomata were used together with genetic analysis based on randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), and restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (IGS) and the intergenic spacer (ITS) regions, and the sequence of the IGS region were used to characterize the isolates. Based on the findings of this research it can be concluded that two *Armillaria* groups were present. The first group was morphologically similar to *A. heimii* but this was contradicted by the molecular data, suggesting that *A. heimii* could be a complex of several species. The second group was different and could be a new *Armillaria* species.

Introduction

Armillaria is a primary root rot of tea (*Camellia sinensis*) occurring in the entire geographical range where the crop is grown in Kenya causing losses as high as 50% in small holder farms (Onsando *et al.*, 1997). Identification of *Armillaria* to species level in Africa is limited by the fact that basidiomata and rhizomorphs are rarely found in the tropical regions (Gibson 1960b, Swift 1968), the fungus being frequently detected only as mycelium beneath the bark of infected plants. Occasionally basidiomata of *Armillaria* have been found in parts of East and Central Africa for the isolates that were identified as African forms of *A. mellea* (Vahl : Fr.) Kumm. and *A. heimii* Pegler (syn. *Clitocybe elegans* Heim) (Pegler, 1977). This was supported by Mohammed and Guillaumin (1994) who found isolates that corresponded to

these two species during a survey in different African countries. They also reported two additional groups of isolates from high altitude regions in Kenya, which were clearly distinct from the two species. The African *A. mellea*, unlike the species in Europe and North America, is homothallic (Mohammed *et al.*, 1993; Abomo-Ndongo *et al.*, 1997). Homothallism has also been reported in *A. mellea* isolates from Japan (Cha *et al.*, 1994; Ota *et al.*, 1998). Ota *et al.* (2000) confirmed by somatic incompatibility tests and RAPD analysis that *A. mellea* isolates from Africa were identical to *A. mellea* isolates from Japan. On the other hand *A. heimii* has been described as a variable species having both homothallic and unifactorial heterothallic forms (Abomo-Ndongo *et al.*, 1997) associated with various hosts in different regions of tropical Africa. Unifactorial heterothallism seems to be unique to this species.

The homothallic nature of some of these *Armillaria* spp. together with the rare occurrence of their basidiomata has restricted the use of mating tests (Korhonen, 1978) and basidiomata morphology for identification of unknown African species. In most basidiomycetes somatic incompatibility is also used for the study of intra-specific variability while in tetrapolar species (for instance in *Armillaria* of the temperate regions), the distinction of 'biological species' is based on sexual compatibility/incompatibility. Somatic incompatibility has been used in studies to distinguish some isolates of African *Armillaria* (Abomo-Ndongo and Guillaumin, 1997) but most attempts to characterize these have tended to employ methods that do not depend on the presence of basidiomata or haploid forms: including techniques based on the use of isozyme electrophoresis (Agustian *et al.*, 1994; Mwangi *et al.*, 1989; Mwenje and Ride, 1996, 1997) and molecular markers (Mohammed *et al.*, 1993; Chillalli *et al.*, 1997; Ota *et al.*, 2000). Techniques such as DNA restriction fragment polymorphisms (Anderson *et al.*, 1987; Smith and Anderson, 1989), nuclear DNA-DNA homology (Jahnke *et al.*, 1987), and DNA sequence analysis (Anderson and Stasovski, 1992) have been used in various taxonomic studies on *Armillaria*. Mohammed *et al.* (1993) used RAPD markers to distinguish African isolates of diverse origins. Analysis of the ribosomal DNA spacers have also been used to compare *Armillaria* isolates from various geographical areas in tropical Africa (Chillali *et al.*, 1997). Coetzee *et al.* (1997, 2000) used RFLP and nucleotide sequence data of the intergenic spacer region of ribosomal DNA operon to distinguish Southern Africa isolates of *Armillaria* showing that both nuclear and organelle DNA-based molecular markers provide a method that can aid systematics of *Armillaria* in Africa.

The present study used methods based on morphological characteristics, somatic incompatibility, and DNA profiles generated by PCR with RAPD, ISSR and RFLPs of the IGS and ITS to characterize isolates of *Armillaria* collected from tea plantations in several districts of Kenya.

Materials and methods

Origin of the isolates

Isolates were collected in the main tea growing districts in Kenya (Figure 3.1) during a survey carried out for the presence of *Armillaria* spp. between October and December 1997. The isolates (Table 3.1) were obtained from infected plants by plating mycelial fans removed from beneath the bark of roots on 3% MEA (3% Oxoid malt extract, 2 % Lab M agar No.1, in distilled water) containing 30 ppm of rose bengal (Sigma, USA) and 120 ppm of streptomycin (Sigma, USA). The isolates were stored on slants of the same medium and maintained in the dark at room temperature (21 - 23°C). The cultures were transferred to fresh slants every three months. Isolations from basidiomata found in nature were made by plating both basidiospores and pieces of mycelia removed from the pileus on 3% MEA. These provided monospore and standard somatic cultures respectively. Additional isolates K5, K8, K10 K12, K14 and ST1 from Africa were donated by J.J. Guillaumin (UMR INRA, France) for comparative purposes.

Morphological characteristics of basidiomata

Gross morphological features of basidiomata were described and dimensions of pileus, stipe, basidia, and basidiospores were recorded. Observations for clamp connections were also made.

Cultural morphology

Monospore and somatic cultures were observed on 3% MEA over a period of 4 weeks for colony characteristics. Morphological characteristics of all isolates were studied on 2% MEA and 3% MEA containing 0.06% peptone (Oxoid) (MEA + P). Petri dishes containing the two media were inoculated with mycelial plugs removed from margins of colonies growing on 3 % MEA. These were incubated in the dark at room temperature (21 - 23°C). Colony growth and morphological characteristics were observed over a period of 4 weeks

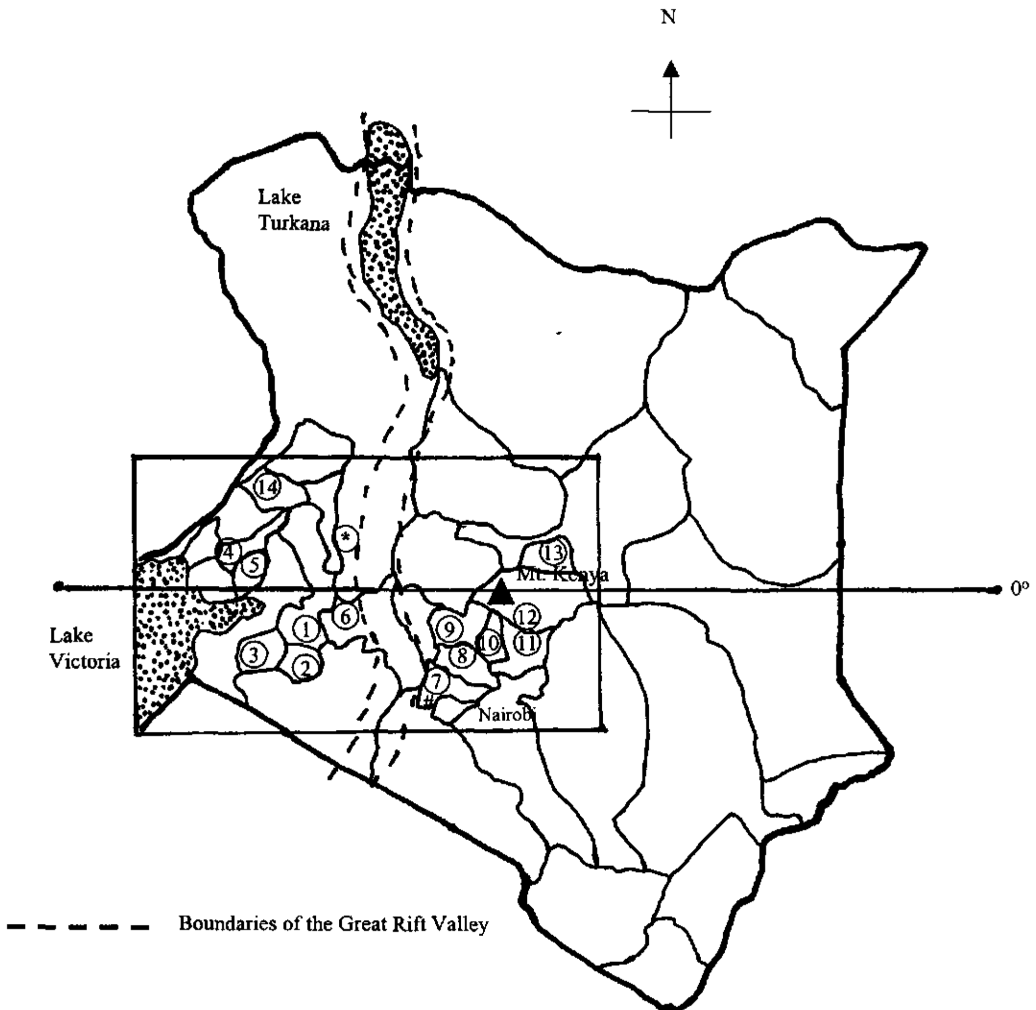


Fig. 3.1: Tea growing districts of Kenya from which *Armillaria* isolates were obtained ([1]Kericho, [2]Bomet, [3]Kisii/Nyamira,[4]Nandi, [5]Kakamega/Vihiga, [6]Nakuru, [7]Kiambu, [8]Muranga, [9]Nyeri, [10]Kirinyaga, [11]Embu, [12]Meru Central, [13]Meru North, [14]Trans. Nzoia). The isolates were obtained from all the districts except Kisii/Nyamira. Additional isolates from earlier collections originated from *Kaptagat (Keiyo district), #Muguga and Kinale (Kiambu district).

Table 3.1 Isolates of Kenyan *Armillaria* collected by Washington Otieno

District	Division	Isolate	Host	Altitude (m)	SIG[a]	MG[b]	RAPD/ISSR/RFLP[c]
Kericho	Buret	$1BU_1$	*Camellia sinensis*	1800-2000	I	I	I
	Ainamoi	$1AI_1$	*Dombeya* sp.	>2000	I	I	I
	Ainamoi	$1AI_{2-3}$ (11)[d]	*Camellia sinensis*	>2000	II	II	II
	Belgut	$1BE_1$	*Camellia sinensis*	1800-2000	II	II	II
Bomet	Konoin	2K (2)	*Camellia sinensis*	>2000	I	I	I
Nandi	E. Nandi	$4EN_1$	*Camellia sinensis*	1800-2000	I	I	I
Vihiga	Hamisi	$5H_1$	*Camellia sinensis*	<1800	I	I	I
Nakuru	Molo	$6M_1$	*Camellia sinensis*	>2400	II	II	II
Kiambu	Githunguri	7GI (6)	*Camellia sinensis*	1800-2000	I	I	I
	Gatundu	$7GU_3$	*Camellia sinensis*	1800-2000	I	I	I
	Lari	$7L_1$	*Camellia sinensis*	1800-2000	I	I	I
Muranga	Kangema	$8KA_1$	*Camellia sinensis*	1800-2000	I	I	I
Nyeri	Tetu	$9T_1$	*Camellia sinensis*	1800-2000	I	I	I
	Tetu	$9T_2$	*Camellia sinensis*	1800-2000	II	II	II
Kirinyaga	Gichugu	10G (4)	*Camellia sinensis*	1800-2000	I	I	I
	Gichugu	$10G_3$	*Eucalyptus* sp.	1800-2000	I	I	I
	Gichugu	$10G_5$	*Coffea arabica*	1800-2000	I	I	I
	Ndia	$10N_1$	*Camellia sinensis*	1800-2000	I	I	I
Embu	Manyatta	11MA (2)	*Camellia sinensis*	1800-2000	I	I	I
Meru	S. Imenti	$12SI_1$	*Dioscorea* sp.	1800-2000	I	I	I
	S. Imenti	12SI (3)	*Camellia sinensis*	1800-2000	I	I	I
	S. Imenti	$12SI_3$	*Coffea arabica*	1800-2000	I	I	I
	S. Imenti	$12SI_5$	*Musa acuminata*	1800-2000	I	I	I
Nyambene	Tigania	$13T_1$	*Camellia sinensis*	1800-2000	I	I	I
	Tigania	$13T_2$	*Camellia sinensis*	1800-2000	II	II	II
Trans Nzoia	Cheranganyi	$14C_1$	*Camellia sinensis*	1800-2000	I	I	I

[a] Somatic incompatibility group

[b] Cultural morphology group

[c] Groups discriminated using RAPD,ISSR and RFLPs of the ITS and IGS regions

[d] Number of isolates collected in the same plantation but from different plants

Table 3.2. Additional isolates for comparison purposes

Isolates	Host	Country	Altitude (m)	Collector	Identity
ST1	*Theobroma cacao*	São Tomé	1000-1500	Unknown	*A. mellea*
K5	*Cupressus funebris*	Kenya	>2000	I.A.S. Gibson	*A. mellea*
K8	*Grevillea robusta*	Kenya	Unknown	M. Ivory	*A. mellea*
K10	*Pinus patula*	Kenya	>2000	Unknown	*Armillaria* sp.
K12	*Pinus patula*	Kenya	>2000	I.A.S. Gibson	*Armillaria* sp.
K14	*Pinus radiata*	Kenya	>2000	I.A.S. Gibson	*Armillaria* sp.

and distinctive colony features used to place the isolates into cultural morphology groups.

Rhizomorphs produced by woody inocula

Stems of cassava (*Manihot esculenta*) with diameter 2.0-2.5 cm were cut into 6-cm long segments. Ten of these were placed in 1-l kilner jars containing 300 ml tap water and autoclaved for 15 minutes at 121°C then left to cool. The stems were inoculated aseptically by placing on their upper transverse surfaces a 4-mm diameter disc of agar cultures removed from one-wk-old colonies using a cork borer. The isolates used for the inoculation were $1AI_{2b}$, $1AI_{2f}$, $1BE_1$, $1BU_1$, $5H_1$, $6M_1$, $7GU_3$, $7L_1$, 9T1, $9T_2$, $13T_1$, and $14C_1$ (Table 3.1). Inoculated stems were incubated in the dark at room temperature (21 - 23°C) until fungal growth was visible at the distal ends (approx. 12 weeks). Stems colonized by *Armillaria* were used as inoculum sources. For each isolate, one of these was buried in 800 cm^3 vermiculite contained in a 1-l plastic pot placed under a bench in the greenhouse where the temperature was maintained at about 18°C. The inocula were kept moist by adding 200 ml tap water to the surface of the vermiculite in each pot once every week. Three replicate pots were used for each isolate. After 12 weeks the inocula were removed, vermiculite gently washed off then observations made for presence of rhizomorphs and their growth patterns.

Somatic incompatibility

Culture discs consisting of undifferentiated mycelia without crust or rhizomorphs were cut from the margins of two-wk-old colonies using a 4 mm diameter cork borer. The discs were placed 5-10 mm apart on the surface of 3% MEA. Self-pairings were done in the control. The cultures were incubated in the dark at room temperature (21 - 23°C) and observed over a period

Table 3.3. List of isolates used to retrieve IGS sequences from GenBank

Name	Code	Alternative code	Host	Origin	Collector	GenBank
A. mellea	B608	CMW3966, AF	Unknown	South Korea	Sung, J-M	AF163611
A. mellea	B731	CMW3967, 1003"	*Chamaecyparis* sp.	Japan	Shaw, CG.	AF163610
A. mellea	B1217	CMW4619, 216	Unknown	USA	Raabe, B.	AF163609
A. mellea	B927	CMW3964	*Quercus*	USA	Bruns, T.D.	AF163608
A. mellea	B929	CMW3962	*Mayten boria*	USA	Bruns, T.D.	AF163607
A. mellea	B1205	CMW4613, 86009/1	Unknown	Iran	Saber, M.	AF163606
A. mellea	B1212	CMW4615, 94056/1	Unknown	Hungary	Szanto, M.	AF163605
A. mellea	B186	CMW4603, BQ5F	Unknown	England	Gregory, S.	AF163604
A. mellea	B1240	CMW4624, AM1	Unknown	England	Sierra, A.P.	AF163603
A. mellea	B176	CMW3179, M1	*Rosa* sp.	England	Rishbeth, J.B.	AF163602
A. mellea	B1247	CMW4628, P-5580	*Chamaecyparis*	England	Sierra, A.P.	AF163601
A. mellea	B525	CMW3957, KD1	Unknown	France	Guillaumin, J.J.	AF163600
A. mellea	B527	CMW4607, PM8	Unknown	France	Guillaumin, J.J.	AF163599
A. mellea	B1245	CMW4627, D-5	*Prunus* sp	France	Unknown	AF163598
A. mellea	B623	CMW4609, PJZ-87-1A	Unknown	USA	Zambino, P.J.	AF163617
A. mellea	B282	CMW4605, TCH-2-1	*Betula populifolia*	USA	Harrington, T.C	AF163616
A. mellea	B497	CMW3956, NABS VI, 97-1	*Acer rubrum*	USA	Anderson, J.B.	AF163615
A. mellea	B495	CMW3155, NABS VI, 49-5	*Acer saccharum*	USA	Anderson. J.B.	AF163614
A. mellea	B917	CMW4611, A-2	Unknown	South Korea	Sung, J.M.	AF163613
A. mellea	B916	CMW4610, A-5	Unknown	South Korea	Sung, J.M.	AF163612
A. mellea ssp. *nipponica*	HUA93110		*Fraxinus lanuginosa*	Japan		D89922
A. singula	HUA9101		*Fraxinus mandshurica*	Japan		D89926
A. sinapina	HUA9124		*Ulmus japonica*	Japan		D89925
A. ostoyae	HUA9242		*Gastria elata*	Japan		D89924
A. jezoensis	HUA9116		*Ulmus japonica*	Japan		D89921
A. gallica	HUA9125		*Prunus ssiori*	Japan		D89920
A. cepistipes	F306		*Galeola septentrionalis*	Japan		D89919
A. gemina	160-8		Unknown	USA		AF243054
A. gemina	35-5		Unknown	USA		AF243053
A. borealis	331-1		*Betula* sp.	Finland		AF243056
A. ostoyae	27-1		*Picea glauca*	USA		AF243050
A. ostoyae	300-2		*Pinus strobus*	Canada		AF243048
A. calvescens	21-2		*Acer saccharum*	USA		AF243070
A. calvescens	11-9		*Acer saccharum*	USA		AF243071
A. lutea	90-4		*Fraxinus* sp.	USA		AF243066
A. cepistipes	304-1		*Alnus glutinosa*	Finland		AF243069
A. sp. NABS	206-1		Conifer	Canada		AF243061
A. cepistipes	311-1		Lawn	Finland		AF243068
A. nabsnona	207-4		Hardwood	Canada		AF24306

of 2 to 3 weeks for compatibility reactions. Somatic incompatibility was also studied among some of the isolates using the method described by Hopkin *et al.* (1989). The isolates were paired on 2% MEA (2% Merck malt extract, 1.5% agar, in distilled water). The agar was covered with 8.5 cm diameter disks of sterile cellophane (cellophane grade 350 from UCB Films), as described by Abomo-Ndongo and Guillaumin (1997). Small discs (3 mm diameter) from the edge of the colonies were placed 5 mm apart on the surface of the sterile cellophane. Two replicates of each pairing were prepared. The plates were incubated in the dark at 20°C. After 21 days the plates were opened and a 2 cm^2 square was cut around the paired isolates and immersed in a freshly prepared solution of 0.05% of L-Dopa (Sigma, UK) in a pH 7.0 potassium phosphate buffer (Sambrook *et al.*, 1989). They were incubated at 37°C for 1 h and then examined under the stereomicroscope for the presence of a black line between the thalli.

Extraction of DNA

Two methods were used for the DNA extraction.

Method A: This method was used to extract DNA for RAPD and ISSR. Approximately 8 ml of CTAB buffer (1M Tris, pH 7.5; 0.5M EDTA; pH 8.0; 5 M NaCl, 1.5 g polyvinylpyrolidone (PVP), 30 g hexatrimethylammonium bromide (CTAB) and 0.10% dithiothreitol (DTT)) were added to the freeze-dried mycelium from a single petri-dish that had been ground to a fine powder. The suspension contained in a 15-ml centrifuge tube was thoroughly mixed and incubated at 60°C for 30 min. Six ml of CIA (chloroform/isoamyl alcohol, 24:1) was then added and the suspension mixed by thorough shaking. The mixture was then centrifuged (2500 r.p.m. for 5 min). The aqueous layer was transferred to a fresh 15 ml centrifuge tube and the nucleic acids precipitated by adding two volumes of chilled isopropanol and mixed by gentle inversion. The mixture was centrifuged (2500 r.p.m. for 5 min) to pellet the precipitated nucleic acids. The pelleted nucleic acids were rinsed thrice in 1 ml 70% chilled ethanol and the pellet air dried for 1 h at room temperature before re-suspending in 1.0 ml sterile distilled water.

Method B: This method was used to extract DNA for PCR-RFLPs. The isolates were grown in liquid media (1% malt extract, 0.5% yeast extract and 1% glucose). The use of 100 ppm oxytetracyclin (Sigma, UK) and 200 ppm streptomycin (Sigma, UK) was needed to avoid bacterial contamination in some cases. The flasks were incubated in the dark at 20°C for three weeks. The flasks were not shaken during this time. The mycelium was harvested, rinsed with distilled water, frozen in liquid nitrogen and stored at -80°C. The total DNA of the isolates was

extracted from frozen mycelium using the Dneasy™ Plant Mini Kit (Qiagen, Germany). The mycelium was first mechanically disrupted by grinding to a fine powder, under liquid nitrogen, using a mortar and pestle. Then the kit protocol was followed.

Purification of DNA

DNA obtained by method A was purified. The ribonucleic acids were denatured by adding 20 μl of RNase solution (20 ng ml^{-1}) to 500 μl of the nucleic acid suspension and incubated at 55°C overnight. The DNA was further purified by adding 500 μl of CIA and shaking for 15 min before centrifugation (12000 r.p.m. for 15 min). The aqueous layer was transferred into a clean micro-centrifuge tube and precipitated by adding 700 μl isopropyl alcohol and mixing gently. This was finally centrifuged at 5000 r.p.m. for 1 min and the resulting pellet re-suspended in 200 μl sterile distilled water that was incubated overnight at 55°C and stored at 4°C.

Polymerase chain reaction (PCR) amplification

Two different methods were used for the amplification. For DNA obtained with method A, each DNA sample (0.5 μl) was added to an amplification reaction solution consisting of 5.9 μl sterile distilled water, 1.0 μl reaction buffer IV (x10), 1.0 μl $MgCl_2$, 0.5 μl dNTPS, 1.0 μl primer, and 0.1 μl Taq polymerase (Applied Biotechnologies, U.K) in a 10 μl volume PCR tube. PCRs were performed for eleven RAPD primers: OPB-07, OPC-02, OPD-05, OPD-20, OPI-13, OPU-05, OPV-17, OPW-02, OPW-06, OPG-06, OPM-04 (Operon Technologies Inc. USA) with the conditions at 94°C for 5 min (hot start) then 93°C for 1 min, 42°C for 1.5 min, and 72°C for 1 min for 40 cycles and a final extension phase of 5 min at 72°C. Six ISSR primers: dinucleotide repeats (GA)8T, (CT)8T, (GT)8CG, (AT)8T, (AG)8C and trinucleotide repeats (ACC)6 (International Livestock Research Institute, Nairobi, Kenya) were also screened with the conditions at hotstart then 94°C for 30 sec, 52°C for 45 sec, and 72°C for 2 min in 40 cycles. The amplification was performed with a Techno: Model FGENO2TD (UK) thermocycler.

For DNA obtained with method B, the intergenic spacer (IGS) of the ribosomal DNA between the 26S and 5S gene of the isolates was amplified with two different set of primers. The first set included LR12R, 5' CTG AAC GCC TCT AAG TCA GAA 3' (Veldman *et al.*, 1981) and O-1, 5' AGT CCT ATG GCC GTG GAT 3' (Duchesne and Anderson (1990) recommended

by Anderson and Stasovski (1992). The second set of primers included: P-1, 5' TTG CAG ACG ACT TGA ATG G 3' and 5S-2B, 5' CAC CGC ATC CCG TCT GAT CTG CG 3' recommended by Coetzee *et al.* (1997). The internal transcribed spacer (ITS) was amplified with the primers ITS_1 and ITS_4 as described by White *et al.* (1990). Ready-To-Go PCR beads (Amersham Pharmacia Biotech) were used for the PCR amplification. Individual reactions were brought to a final volume of 25 µl. Each reaction contained 1.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, stabilizers including BSA, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.1 µM of each primer and purified water (Sigma Chemical Co.). The PCR amplification program to amplify the IGS region consisted of 1 cycle of 95°C for 95s, followed by 35 cycles of 60°C for 60s, 72°C for 120s and 95°C for 60 s and a final extension at 72°C for 10 min. The PCR amplification program to amplify the ITS was as described by Chillali *et al.* (1997). The amplifications were performed on a Progene (Techne, UK) thermocycler.

Electrophoresis and DNA restriction

Following method A, electrophoresis was performed using 1.5% agarose (ABgene, UK) gel in Tris-boric acid-EDTA (TBE). A tracking dye was added (10:3) and 10 µl of the mixture was electrophoresed at 150V for 3 h. After electrophoresis, the gel was stained for 1 hour in ethidium bromide (Sigma, UK) (10 µl l^{-1} of water) then observed under UV and photographed using a black/white Kodak film. Following method B, the IGS and ITS amplified product was purified with a QIAquick™ Purification Kit (Qiagen, Germany) and digested with the restriction enzyme *Alu*I and *Hinf* I (Harrington and Wingfield 1995). The restriction enzyme analysis was performed by adding 5 µl of the enzymes (Promega, UK) to the purified PCR product and incubating at 37°C for at least one hour. The digested fragments were separated in 3% agarose (Sigma, UK) gel containing 7.5 µl of ethidium bromide (Sigma, UK).

Data analysis

The data were scored and entered in a computer as binary matrices where 0 coded for absence and 1 for presence of a band. Estimates of similarity are based on the total number of shared fragments (Nei and Li, 1979). The principal component and the average linkage cluster analyses were performed using GENSTAT 5 Version 4.1. The unweighted pair group method with arithmetic mean (UPGMA) was used to construct the genetic relationships.

Phylogenetic analyses

Sequencing of the IGS region between the 26S and 5S of some of the isolates was done using the primers LR12R, O-1, P-1 and 5S-2B by MWG BIOTECH AG Ebersberg (Germany). Additional sequences from the GenBank databases (Table 3.3) available through the National Center for Biotechnology Information (NCBI, Bethesda, MD) were obtained using the search facility Blast. The sequences were edited and aligned with the software for Macintosh, EditSeq and MegAlign of Lasergene (DNASTAR 2000) programs. The alignments were initially done with the CLUSTAL option in MegAlign and were manually adjusted. Insertions and deletions were coded using MacClade (Maddison and Maddison 1992). Phylogenetic analyses were performed using PAUP version 4.0b (Swofford, 1998). Heuristic searches using maximum parsimony with TBR branch swapping, MULPARS, and steepest descent options were performed in PAUP. Clade support was evaluated using Jackknife analysis (in PAUP) with 30% deletion and fast stepwise addition was calculated with 10000 replicates. Groups shown in 50% or more of the trees were retained.

Results

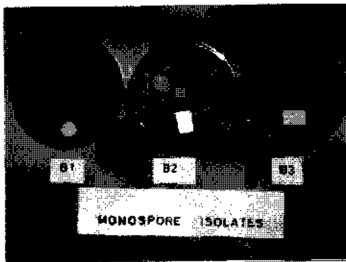
Basidiomata and rhizomorphs in nature

Basidiomata were infrequent but were found in one tea plantation (location: 0°22′S;35°21′E; altitude > 2180 m) during the rainy season. They occurred typically in clusters of 5-21, fused at the point of attachment to the base of infected plants (Figure 3.2). The pileus was 10-15 mm in diameter, convex, applanate to umbonate, with a non-striate margin, cream but dark-brown at the disk. The stipe was creamy white in colour, 45-50 x 3-6 mm and a whitish, fugacious annulus was attached to the upper quarter of the stipe. Lamellae were white cream. The spores were 5-7 x 4-6 µm, sub-globose to ovoid and basidia were elongate clavate, 28.5-36 x 6-7.5 µm, bearing four sterigmata. Very few, if any, rhizomorphs were found from the base of the stipe downwards and these were firmly in contact with the root cortex running along the surface of the bark. Rhizomorphs firmly in contact with the root were found for isolates $1AI_1$, $1AI_{3d}$, $1BE_1$, $1BU_1$, $2K_1$, $2K_2$, $4EN_1$, $5H_1$, $7GI_1$, $7GI_3$, $7GI_4$, $7GI_5$, $7GI_6$, $7GU_3$, $7L_1$, $8KA_1$, $9T_1$, $10G_1$, $10G_2$, $10G_3$, $10G_5$, $10G_6$, $10N_1$, $11MA_2$, $11MA_3$, $12SI_1$, $12SI_2$, $12SI_3$, $12SI_5$, $12SI_{4a}$, $12SI_{4b}$, $13T_1$, and $14C_1$. An extensive subterranean network of rhizomorphs was found for isolates $6M_1$ and $9T_2$.

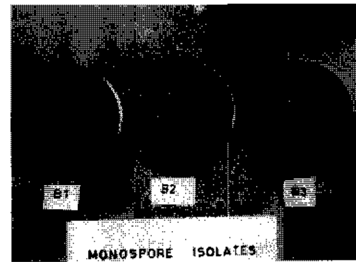
Fig 3.2: Basidiomata of *Armillaria* at the base of infected tea plant (observed at Timbilil tea estate, Kericho)

Cultural morphology

Colonies that arose from basidiospores were characteristically fluffy with raised mycelium when young but became rhizomorphic, flat, and crustose as they aged (Figure 3.3).



a



b

Fig 3.3: Monospore cultures arising from basidiospores of basidiomata in Figure 3.2 on 2% MEA (a) after 7 days and (b) after 14 days.

In contrast, colonies which arose from the pileus were consistently rhizomorphic, flat, and crustose. The appearance of colonies of individual isolates was more or less similar in the two media. On the basis of colony morphology, the isolates could be placed in two groups. Group I consisted of 31 isolates with flat, crustose, rhizomorphogenic colonies. The entire colony often turned into a network of rhizomorphs with only a small mycelial centre. White/grey mycelium was observed at the colony centres and on rhizomorph surfaces. The rhizomorphs were compact or open in appearance (due to high or low frequency of branching), both submerged and aerial, cylindrical or flat. Group II consisted of 16 isolates which had raised typically mycelial colonies with thin submerged or no rhizomorphs. Colonies of isolates in Group I were similar in appearance to those arising from isolations by plating pieces of basidiomata flesh. The majority of isolates had this type of colony. However, individual isolates showed minor differences cultural morphology.

Rhizomorph characteristics

Characteristics of rhizomorphs in culture varied only slightly with the medium, production being more abundant and branching more frequent in 3% MEA + P. The growth pattern of rhizomorphs produced in vermiculite distinguished three forms between which conspicuous differences were evident mainly in the extent of rhizomorph growth and branching patterns. The majority of isolates with abundant production of large and extensive rhizomorphs were placed in Group I. Rhizomorphs were either unbranched or branched with a palmate and/or dichotomous pattern of growth. Isolates that produced thin submerged rhizomorphs visible mainly from underneath the petri-dishes and non rhizomorphic isolates were placed in Group II.

Somatic incompatibility

Two types of reactions were observed between colonies of paired isolates: the merging of mycelia at points of contact or failure of mycelia to merge. Isolates whose colonies failed to merge and which produced a pigmented line at their interfaces were recorded as incompatible. The presence of a dark line of demarcation at the interface of the colonies was noted as the principal indicator of dissimilarity of incompatible isolates and was used to place them in distinct groups. Group I consisted of 31 isolates: $1AI_1$, $1BU_1$*, $2K_1$, $2K_2$, $4EN_1$, $5H_1$, $7GU_3$*, $7GI_1$, $7GI_3$, $7GI_4$, $7GI_5$, $7GI_6$, $7L_1$*, $8KA_1$, $9T_1$, $10G_1$, $10G_2$, $10G_3$, $10G_5$, $10G_6$, $10N_1$, $11MA_2$, $11MA_3$, $12SI_1$, $12SI_2$, $12SI_3$, $12SL_{4a}$, $12SI_{4b}$, $12SI_5$, and $13T_1$, $14C_1$*. Group II consisted of 13 isolates: $1AI_{2a}$, $1AI_{2b}$, $1AI_{2d}$, $1AI_{2i}$ $1AI_{2e}$, $1AI_{2f}$, $1AI_{2g}$, $1AI_{2j}$, $1AI_{3c}$, $1AI_{3d}$, $1BE_1$*, $6M_1$*, and

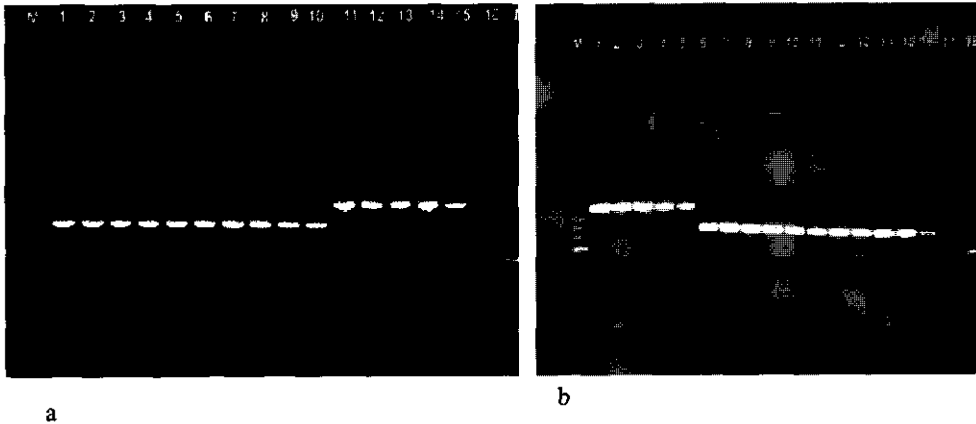
$13T_2$*, Group III consisted of isolate $9T_2$*. Isolates marked with (*) showed unclear somatic incompatibility reactions and were tested further using the method described by Hopkin *et al.* (1989). Reaction between incompatible strains showed the distinct black line, which was visible to the naked eye and easy to interpret. This was considered as an indication of different genetic constitution. All isolates were paired against themselves giving a compatible reaction. From the results obtained 6 different groups were found: Group I: $1BU_1$, $7GI_2$, $7L_1$, $11MA_2$, $14C_1$; Group II: $1AI_{2a}$, $13T_2$; Group III: $9T_2$; Group IV: $6M_1$; Group V: $1BE_1$; and Group VI: $1AI_{2e}$.

DNA amplification and polymorphism

Following method A, the total cellular DNAs of *Armillaria* were used as templates and therefore the genomic origin of the amplified RAPD and ISSR fragments in the isolates cannot be specified. Successful DNA amplification was, however, obtained with 10 RAPD and 3 ISSR primers which gave respectively a total of 181 and 39 fragments with an average of 20 and 13 fragments per primer. The amplified fragments ranged in sizes from <564 to 1977 bp for RAPD and <564 to 1685 for ISSR primers. Two RAPD and three ISSR primers failed to amplify fragments from the fungal DNA. Of the fragments 94.5% and 89.7% respectively were polymorphic for the RAPD and the ISSR primers but only 127 and 29 of the polymorphic fragments respectively were considered for further analysis to derive similarity values and construct genetic relationships.

Following method B, the amplification of the IGS region of the ribosomal DNA with the first set of primers LR12R and 0-1 only occurred with isolates $1AI_{2a}$, $1AI_{2b}$, $1AI_{2d}$, $1AI_{2e}$, $1AI_{2f}$, $1AI_{2i}$, $1AI_{2j}$, $1BE_1$, $6M_1$, $9T_2$, $13T_2$, K5, K8, K10 and K12 and gave as a result a single fragment of approximately 900 bp. Amplification with the primers P-1 and 5S-2B from the isolates $1AI_{2f}$, $1BE_1$, $6M_1$, $9T_2$, K5, K10 and K12 was very poor. The isolates $1BU_1$, $4EN_1$, $5H_1$, $7GI_2$, $7GI_3$, $7GI_4$, $7GI_5$, $7GI_6$, $7L_1$, $9T_1$, $10G_3$, $10G_6$, $11MA_2$, $11MA_3$, $12SI_1$, $12SI_{4a}$, $12SI_{4b}$ and $14C_1$ were amplified with the primers P-1 and 5S-2B and gave one single fragment over 1000 bp. Poor amplification from the isolate $11MA_2$ was obtained with the primers LR12R and 0-1. Amplification of the ITS with the primers ITS_1 and ITS_4 gave a single fragment of about 900 bp for the isolates $1AI_{2a}$, $1AI_{2e}$, $1BE_1$, $6M_1$, $9T_2$, $13T_2$, K5, K8, K10 and K12 and a single fragment of over 600 bp for the isolates $1AI_1$, $1BU_1$, $4EN_1$, $5H_1$, $7GI_2$, $7L_1$, $10G_3$, $11MA_2$, $12SI_{4a}$ and $14C_1$. The amplified fragments of the IGS and ITS regions of some of the isolates are shown in Figure 3.4 and the patterns obtained after the digestion of IGS and ITS amplification products with restriction endonucleases *Alu*I and *Hinf*I in Figure 3.5. A 100 bp DNA ladder was used as

size marker. Fragments below 100bp were only included when clearly visualised. For the IGS, the isolates $1AI_1$, $1BU_1$, $4EN_1$, $5H_1$, $7GI_2$, $7GI_3$, $7L_1$, $9T_1$, $10G_3$, $10G_6$, $7GI_4$, $7GI_5$, $7GI_6$, $11MA_3$, $11MA_2$, $12SI_1$, $12SL_{4a}$, $12SL_{4b}$, and $14C_1$ showed restriction fragments of approximately 365, 245 and 135 bp respectively. The isolates $1AI_{2a}$, $1AI_{2b}$, $1AI_{2d}$, $1AI_{2e}$, $1AI_{2f}$, $1AI_{2h}$, $1AI_{2i}$, $1AI_{2j}$, $1BE_1$, $6M_1$, $9T_2$, $13T_2$, K10 and K12 showed restriction fragments of approximately 310, 210, and 135 bp respectively and the isolates K5 and K8 showed restriction fragments approximately 310, 180, 135 bp respectively. For the ITS, the isolates $1AI_{2a}$, $1AI_{2d}$, $1AI_{2e}$, $1BE_1$, $6M_1$, $9T_2$, $13T_2$, K5, K8, K10 and K12 showed a restriction pattern of approximately 510, 225 and 95 bp, respectively, the isolates $1AI_1$, $1BU_1$, $4EN_1$, $5H_1$, $7GI_2$, $7L_1$, $9T_1$, $10G_3$, $12SL_{4a}$, $11MA_2$, and $14C_1$, showed restriction patterns of approximately 480, 160 bp respectively while isolates K5 and K8 showed restriction patterns of approximately 320, 235, 170,150 bp respectively.



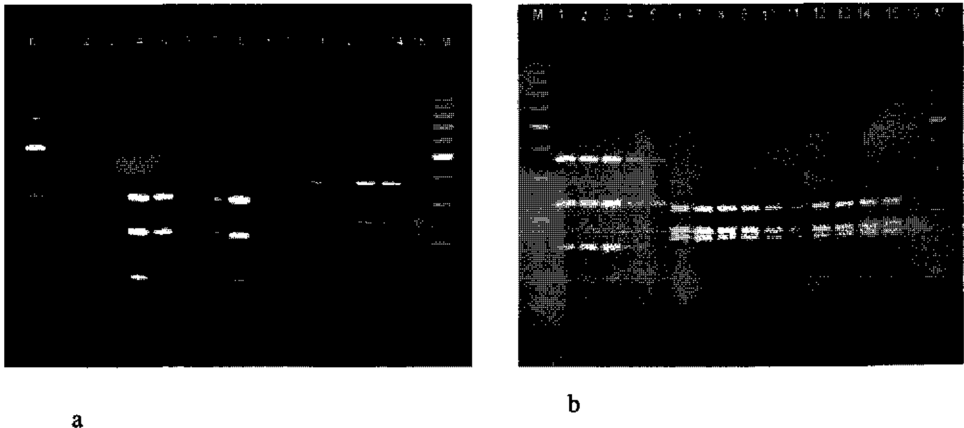
a b

Fig 3.4: Agarose gel (3%) showing (a) the amplified PCR product for (a) the IGS region of isolates ST1, K5, K8, $1AI_{2e}$, $1BE_1$, $6M_1$, $9T_2$, $13T_2$, K10, K12, $1BU_1$, $7L_1$, $7GI_2$, $11MA_2$, $14C_1$ and negative control respectively and (b) the ITS regions of the isolates $1AI_{2e}$, $1BE_1$, $6M_1$, $9T_2$, $13T_2$, $1BU_1$, $4EN_1$, $5H_1$, $7L_1$, $7GI_2$, $9T_1$, $10G_5$, $11MA_2$, $12SI_2$, $14C_1$, $1AI_1$ and negative control respectively. A 100 bp ladder was used as a marker in the first and the last line.

Genetic relationship among the isolates

The matrix of similarity coefficient values based on shared fragments shows that similarities among the isolates ranged from 95% (between isolates $10G_5$ and $10G_6$) to 29% (between isolates $1BU_1$ and $10G_3$). Dendrogram based on average linkage cluster analysis

(Figure 3.6) resolved the 47 isolates into two major clusters. The larger of these showed differences that indicate greater variability among isolates than in those within the smaller cluster. This variability is also evident in the principal components plot (Figure 3.7) where three sub-groups could be discerned within the larger cluster with isolates $12SI_3$, $12SI_{4b}$, and $10G_3$ forming the most distant sub-group. The fourteen isolates in the smaller cluster (Group II) had no major sub-clusters. The principal component plot placed all of these (isolates 1AI2a, $1AI_{2b}$, $1AI_{2d}$, $1AI_{2e}$, $1AI_{2f}$, $1AI_{2g}$, $1AI_{2i}$, $1AI_{2j}$, $1AI_{3c}$, $1AI_{3d}$, $1BE_1$, $6M_1$, $9T_2$, and $13T_2$) together.



a b

Fig 3.5: Agarose gel (3%) showing the *Alu* I restriction pattern for the IGS of isolates ST1, K5, K8, $1AI_{2e}$, $1BE_1$, $6M_1$, $9T_2$, $13T_2$, K10, K12, $1BU_1$, $7L_1$, $7GI_2$, $11MA_2$, $14C_1$ and (b) the *Hinf* I restriction pattern of the ITS region for the isolates $1AI_{2e}$, $1BE_1$, $6M_1$, $9T_2$, $13T_2$, $1BU_1$, $4EN_1$, $5H_1$, $7L_1$, $7GI_2$, $9T_1$, $10G_5$, $11MA_2$, $12SI_2$, $14C_1$, $1AI_1$ respectively. A 100 bp ladder was used as a marker in the first and the last line.

In Group I, fifteen isolates ($1AI_1$, $1BU_1$, $2K_2$, $7GI_3$, $7GI_6$, $7GU_3$, $9T_1$, $10G_4$, $10G_5$, $10G_6$, $10N_1$, $13T_1$, $12SI_5$, $14C_1$,) were also placed together in a sub-group separate from ten others (isolates $2K_1$, $4EN_1$, $5H_1$, $7GI_4$, $7GI_5$, $8KA_1$, $11MA_2$, $11MA_3$, $12SI_2$, $12SI_{4a}$,) in another sub-group and from another three (isolates $10G_3$, $12SI_3$, $12SI_{4b}$) in a third sub-group.

Phylogeny

The sequence of the IGS of the isolates in Group II were identical. The sequence obtained was compared with sequence published in GenBank using the Blast search facility and seven

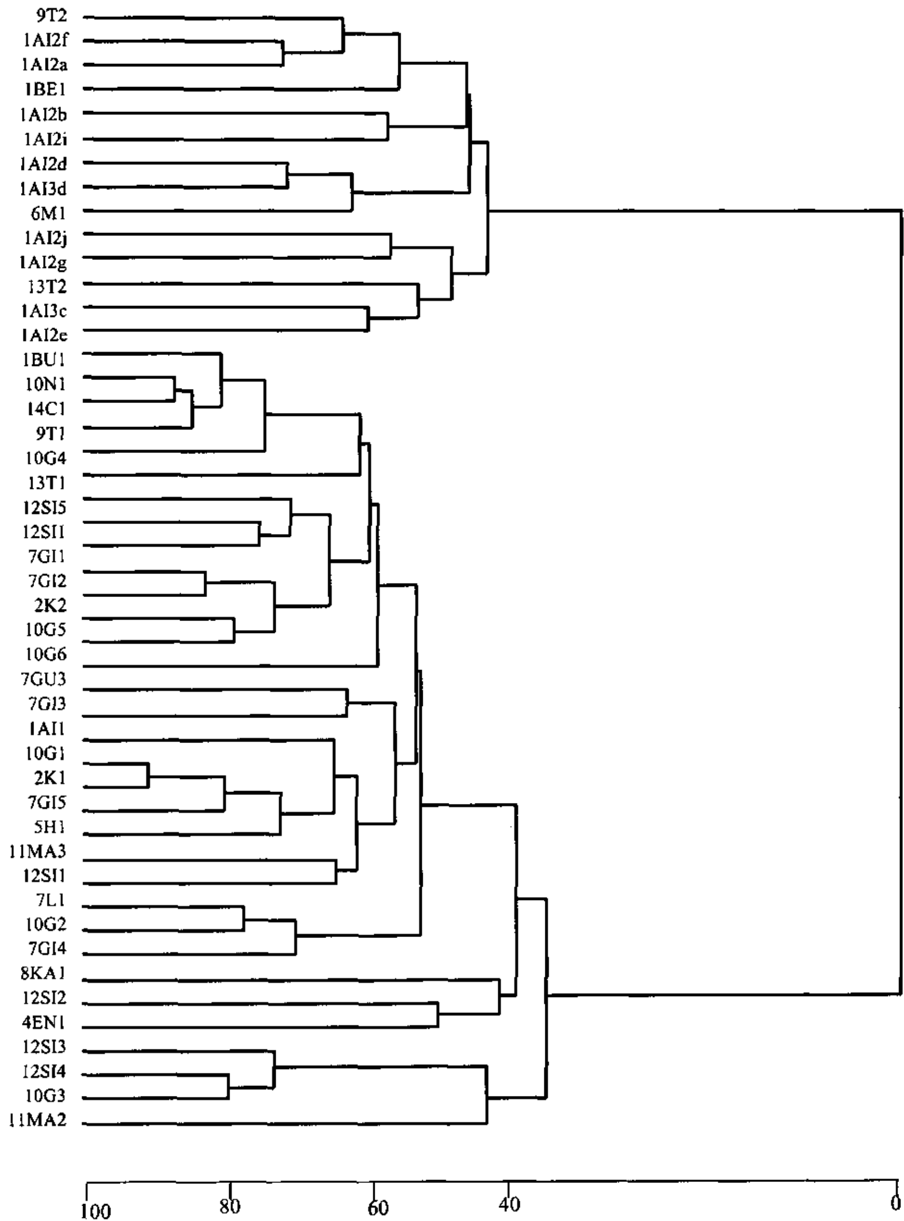


Fig. 3.6: Dendrogram of *Armillaria* isolates based on average linkage clusters analysis (Nei and Li, 1979) of shared fragment coefficients from DNA analysis using RAPD and ISSR markers. The baseline is a scale as percentage average genetic similarity between the isolates.

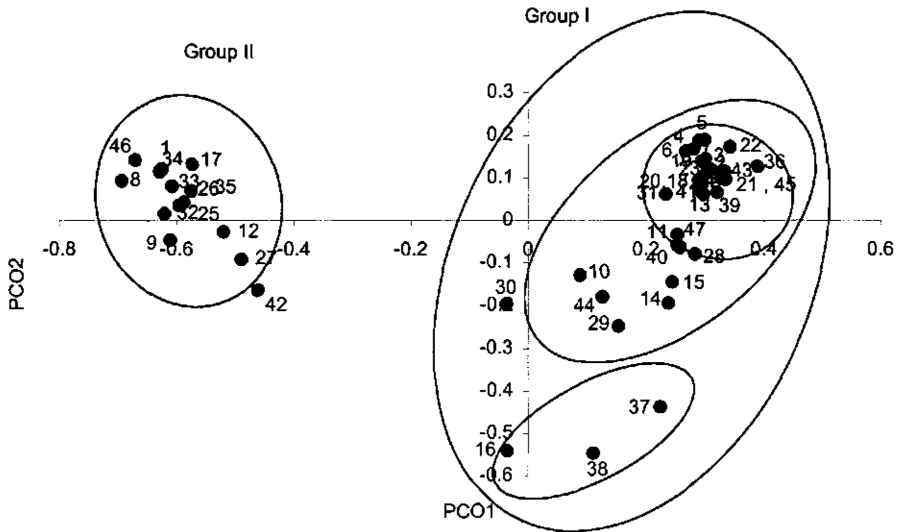


Fig. 3.7: Different clusters of 47 isolates of *Armillaria* based on similarity data from average linkage cluster analyses. The first two principal components accounted for 49% of the total variation revealed. (The isolates are indicated in the figure according to the following numbers in superscript: $^{1}9T_{2}$, $^{2}BU_{1}$, $^{3}10N_{1}$, $^{4}9T_{1}$, $^{5}14C_{1}$, $^{6}10G_{4}$, $^{7}1AI_{1}$, $^{8}1AI_{2f}$, $^{9}1AI_{2j}$, $^{10}8KA_{1}$, $^{11}5H_{1}$, $^{12}13T_{2}$, $^{13}10G_{1}$, $^{14}11MA_{3}$, $^{15}12SI_{4a}$, $^{16}12SI_{3}$, $^{17}1BE_{1}$, $^{18}13T_{1}$, $^{19}7GI_{2}$, $^{20}2K_{2}$, $^{21}10G_{5}$, $^{22}10G_{6}$, $^{23}7GU_{3}$, $^{24}7GI_{3}$, $^{25}1AI_{3d}$, $^{26}6M_{1}$, $^{27}1AI_{3c}$, $^{28}2K_{1}$, $^{29}11MA_{2}$, $^{30}12SI_{2}$, $^{31}7GI_{6}$, $^{32}1AI_{2g}$, $^{33}1AI_{2b}$, $^{34}1AI_{2d}$, $^{35}1AI_{2i}$, $12SI_{5}$, $^{37}12SI_{4b}$, $^{38}10G_{3}$, $^{39}12SI_{1}$, $^{40}7GI_{5}$, $^{41}7L_{1}$, $^{42}1AI_{2e}$, $^{43}7GI_{1}$, $^{44}4EN_{1}$, $^{45}10G_{2}$, $^{46}1AI_{2a}$, $^{47}7GI_{4}$).

Armillaria sequences, *A. gemina* (AF243053/AF243054) and *A. ostoyae* (AF243048/AF243049/AF243050/AF243051/AF243052) matched more closely to the sequence. Other *Armillaria* species selected for comparison were *A. borealis* (AF243056), *A. calvescens* (AF243070/AF243071), *A. cepistipes* (AF243068/AF243069), *A. lutea* (AF243066), *A. nabasnona* (A243060), and *A.* sp., NABS X (AF243061). Because there was the possibility of this group being a form of *A. mellea*, the sequences of the IGS of 21 strains of

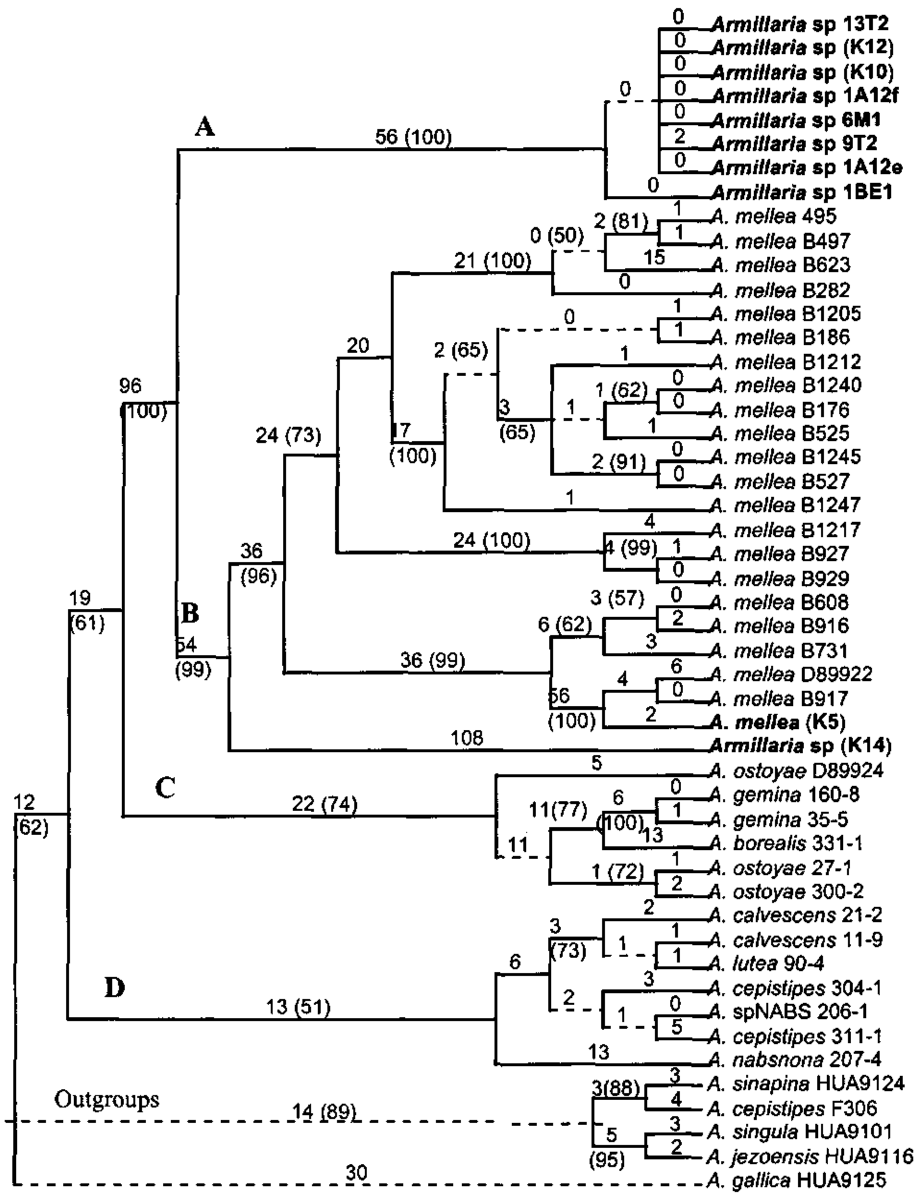


Fig. 3.8. One of the 121 equally parsimonious trees recovered using sequences of the IGS based on *A. sinapina* (D89925), *A. cepistipes* (D89919), *A. singula* (D89926) and *A. jezoensis* (D89921) as outgroups. Dashed lines indicate branches that collapsed in the strict consensus tree. Plain figures indicate branch lengths and figures in brackets show the Jackknife support value. Length = 837, CI = 0.774, RI = 0.941.

A. mellea published in GeneBank were included (Table 3.3). As outgroups *A. sinapina* (D89925), *A. singula* (D80026), *A. jezoensis* (D89921), *A. cepistipes* (D80019) and *A. gallica* (D89920) were selected. A heuristic search with all the strains yielded 121 equally parsimonious trees. A strict consensus was calculated. The alignment generated 788 characters, 336 of which were parsimony-informative (43%). The tree generated (Figure 3.8) showed four major clades (A-D). Clade A comprises the *Armillaria* isolates from Group II together with the isolates K10 and K12. This had 100% Jackknife support. Long branches (56 steps) suggested some distance in the relationship of this clade with the remaining clusters. Clade B comprises all the *A. mellea* strains, including an isolate from Kenya that had been named *A. mellea* (K5). This isolate was grouped with *A. mellea* from Japan and South Korea. The isolate K14, which was different from the rest of the isolates from Kenya, was also part of the *A. mellea* clade. The separation of clade A and B had 100 % Jackknife value. Clade C comprises the strains of *A. ostoyae*, *A. gemina and A. borealis* and had strong support (74% Jackknife value). Clade D grouped together the strains of *A. calvescens*, *A. lutea*, *A. cepistipes* and *A.* sp. NABS X and had a Jackknife support of 51%.

Discussion

Cultural morphology of the Kenyan *Armillaria* isolates separated them into two groups. Group I had crustose rhizomorphic colonies while Group II had mycelial colonies with sparse or no rhizomorphs. Basidiomata were found only in one tea plantation located at a high altitude (2180 m) in Kericho, consistent with the observations (Mohammed *et al.*, 1993; Mwangi *et al.*, 1993) that natural fructification by the fungus occurs rarely in Africa and is limited to cooler areas. The cultural morphology of colonies that arose from the basidiomata corresponded with Group I. The description of the basidiomata conformed to that of *A. heimii* (Heim, 1963). The 10-15 mm diameter pileus which was convex, applanate to umbonate, cream but dark brown at the disk; the fugacious, whitish veil attached to the upper quarter of the stipe; and the subglobose to ovoid, 5-7 x 4-6 μm spores conformed to *A. heimii*. Also the crustose rhizomorphic rather than mycelial cultural morphology of colonies of Group I resembles *A. heimii* according to Mwangi (pers. comm. 2000). Monospore isolates of Group I had light brown fluffy appearance in culture when young but turned crustose with age, indicating homothallism. This has been reported before for diverse African isolates of *A. mellea* and *A. heimii* (Mohammed *et al.*, 1993; Abomo-Ndongo *et al.*, 1997). No basidiomata

were found for Group II. The cultures of these arising from plating of mycelial fans on infected plants had white mycelial colonies that became dark brown as they aged.

Rhizomorph production has been reported to be limited in Africa (Gibson, 1960b) but Group I invariably showed presence of rhizomorphs firmly in contact with the root surface particularly at high elevations. Two isolates from Group II ($6M_1$ and $9T_2$) showed an extensive network of naturally produced subterranean rhizomorphs.

Somatic incompatibility is one of the methods that has been used for the identification of genotypes and the incompatible reaction is characterized by the presence of a black line along the demarcation zone between paired colonies. The melanized cellular contents of the hyphae constitute the black line but the mechanism which causes the hyphae to become melanized is unknown (Mallet *et al.*, 1986). The black line is usually absent in pairings between two genotypes of the same species (Guillaumin *et al.*, 1991). The tests showed compatibility for all isolates within Group I but Group II consisted of five subgroups. Of these, four consisted of one or two isolates. Although results of the initial tests did not always show clear black lines in all the incompatible combinations, the method of Hopkin *et al.* (1988), which has been devised to increase the intensity of the black line formation between incompatible colonies, clearly showed to the naked eye the incompatibility reaction between isolates within Group II. Although this phenomenon has not been reported earlier for African *Armillaria*, Abomo-Ndongo *et al.* (1997) reported a dubious reaction for isolates K10 and K12. A similar phenomenon has been observed for *Ganoderma* in oil palms where most isolates, even when taken from the same plant, were somatically incompatible with one another (Miller *et al.*, 1999). Care should therefore be taken in interpreting somatic compatibility tests aimed at delimiting species in *Armillaria*.

The molecular data based on RAPD, ISSR, and RFLPs of the ITS and IGS regions separated the isolates into two distinct groups which corresponded with the morphological groups. These techniques showed higher variability and diversity within Group I than within Group II. This may suggest that Group I is a more ancestral form of *Armillaria* in Kenya. Existence of sub-clusters in this group may be interpreted as indicating the presence of genotypic variants that could be taxonomic sub-groups among this collection of isolates. There were no sub-clusters in Group II, indicating low diversity. The isolates from Group I were widely distributed in Kenya but more prevalent in the eastern highlands. Group I isolates were found on tea and a few other other plant species between 1800-2000 m altitude except for isolates $1AI_1$, $2K_2$ and $2K_1$ that were found above 2000 m. Except for isolates $9T_2$

and $13T_2$, all isolates in Group II originated in the western highlands suggesting that this group could be more predominant in this region of Kenya. The apparent greater variability in Group I might also be due to genetic recombination by meiosis in the occasional basidiomata.

For the amplification of the IGS region, different sets of primers were used for Group I and Group II, hence the different fragment sizes, but this was consistent with all the isolates within each group. The IGS region of Group I could not be amplified with the primers LR12R and O-1 as was expected from experience with temperate *Armillaria* species (Anderson and Stasovski, 1992; Harrington and Wingfield, 1995). The primer O-1 is complementary to the position 1-18 of the 5S rRNAs of a number of basidiomycetes and is partially conserved in some *Armillaria* species (Duchesne and Anderson, 1990). It is suggested that the 5S gene is inverted in some *Armillaria* spp. and this could be the reason why this group was not amplified with the primer O-1 (Coetzee *et al.*, 2000). Group I was amplified using the primers P-1 and 5S-2B. Group II was amplified with the primers LR12R and O-1. The ITS was amplified with the primers ITS1/ITS4 and the isolates were separated into the same two groups and different band sizes were obtained for each group. In isolates K8, K5, KT10 and K12, which were obtained for comparative purposes, the IGS region was amplified with the primers LR12R and O-1 and the restriction pattern obtained for K10 and K12 with the enzyme AluI was the same as Group II and different from K8 and K5. The amplification of the ITS region of K10 and K12 showed the same fragment size as Group II.

Even though the morphology data point to our Group I being *A. heimii*, the molecular data may suggest differently. RFLPs showed the group had a similar restriction pattern as the *Armillaria* sp. described by Coetzee *et al.* (2000) and, according to these authors, it has a closer affiliation to *A. fuscipes* than to *A. heimii*. There is confusion in the identity and nomenclature of *A. heimii* and *A. fuscipes* as different species (Kile and Watling, 1989; Coetzee *et al.*, 2000). However, the original descriptions differ strongly, with *A. heimii* having smaller dimensions for the pileus, a whitish stipe at the top which changes to ochre at the base and a fugacious annulus (Heim, 1963) and *A. fuscipes* having larger dimensions, a dark brown stipe and a persistent annulus (Petch, 1909). Unfortunately, we failed to isolate DNA from the type material of *A. fuscipes* deposited at Royal Botanical Garden at Kew (UK). Three attempts were made using different methods but without success.

No conclusions about the identity of the Group II isolates can be drawn since we did not find basidiomata but the colony morphological and molecular data indicated that it could be a new African species different from Group I and also from *A. mellea* reported from

Kenya. Isolates K10 and K12 have been used in previous studies of African *Armillaria* spp. and have been suggested to be a yet unnamed species (Chillali *et al.*, 1997). Morphological and molecular data showed that these isolates are similar to our Group II. Presence of basidiomata is necessary for describing this group. Artificial induction of fructification was attempted in the course of this study but without much success. Only two isolates produced basidiomata *in vitro* but these were too aberrant to be useful in species description. The DNA sequence of the IGS region of this group of isolates is different from that of all the *Armillaria* spp. submitted to GenBank (Figure 3.8). There is strong morphological and molecular evidence that Group II could be a new *Armillaria* species. Since there was the possibility of this group being a form of *A. mellea,* the sequences of the IGS of 21 strains of *A. mellea* published in GeneBank (Table 3.3) were included in the phylogenetic analysis. The phylogenetic tree (Figure 3.8) showed four major clades (A-D) of which clade A comprises the *Armillaria* isolates from Group II together with the isolates K10 and K12 while clade B comprises all the *A. mellea* strains, including *A. mellea* from Kenya (K5) which was grouped with *A. mellea* from Japan and South Korea together with isolate K14, which was different from the rest of Kenyan isolates. Clade C comprises the strains of *A. ostoyae, A. gemina* and *A. borealis* while clade D grouped together the strains of *A. calvescens, A. lutea, A. cepistipes* and *A.* sp. NABS X. It can be concluded from this study that at least two different *Armillaria* groups cause damage in tea were present during this survey. Isolates in Group I were widely distributed and were found in all locations where tea was grown in Kenya. One of these may represent *A. heimii.* Since the groups are not characterized by location, host, or altitude, no further ecologically functional sub-groupings could be made based in our findings. Research is in progress to identify the two *Armillaria* groups.

Chapter 4

Screening of *Trichoderma* isolates for inhibition to *Armillaria* in infested wood

Abstract

Five isolates of *Trichoderma* (four *T. harzianum*, and one each of *T. koningii* and *T. longibrachiatum*) were screened for the ability to colonize plant materials and antagonise *Armillaria*. All isolates were equally able to colonize autoclaved tea stem sections. Tests for antagonism consisted of holding *Trichoderma*-colonized stem sections end-to-end with stem sections colonized by *Armillaria* and incubating the banded stem sections in soil. The tests were carried out in three experiments of which two were done in sterile soil and one in non-sterile soil. Inhibition of the ability of *Armillaria* to invade *Trichoderma*-colonized stem sections and the reduction of inoculum viability following invasion of pathogen-colonized stem sections by *Trichoderma* isolates were used as indicators of antagonism. *Trichoderma koningii* and *T. longibrachiatum* were unable to suppress *Armillaria* but all four *T. harzianum* isolates significantly reduced the incidence of the pathogen in both the *Armillaria*-inoculated and *Trichoderma*-inoculated stem sections, only isolate T4 showing complete elimination of the pathogen in sterile soil. This *Trichoderma* isolate also antagonised two different *Armillaria* isolates in non-sterile soil but its efficacy was lower than in the sterile soil. It was judged sufficiently antagonistic to warrant field testing for the effect on survival of *Armillaria* in roots of naturally infected tea.

Introduction

The variety of enzymes and fungitoxic metabolites produced by species of *Trichoderma* make them successful as competitors in the soil ecosystem. They can parasitise other fungi, are able to compete aggressively for nutrients, and produce antibiotics (Klein and Eveleigh, 1998). These attributes facilitate their ability to colonize

particular habitats in which they may hamper development of various fungal species including some plant pathogens. Strains of some *Trichoderma* spp. are able to colonize wood, using mainly the non-structural carbohydrates. As a result they modify the substrates and limit the attack by secondary invaders and wood rotting fungi (Hulme and Stranks, 1970). Antagonism of *Trichoderma* spp. to other fungi takes place mainly by antibiosis and parasitism. Hyperparasitic strains coil around hyphae of the host fungi and degrade their cell walls enzymatically. Scanning and transmission electron microscopy of hyphal interactions between *T. harzianum* and *Rhizoctonia solani* demonstrated that coiling of hyphae of the hyperparasite around the hyphae of the plant pathogen was accompanied by cell penetration, cell wall alteration, retraction of the plasma membrane, and aggregation of the cytoplasm (Benhamou and Chet, 1993). Dumas and Boyonoski (1992) observed a similar phenomenon between *T. harzianum* and *Armillaria gallica* (= *A. lutea*). In addition to the production of diverse antibiotics, these phenomena enable certain strains of *T. harzianum* to limit growth and activity of some plant pathogenic fungi, making them useful as biocontrol agents. The use of *Trichoderma* spp. has been proposed for commercial application in biocontrol of some soil-borne pathogens (Hjeljord and Tronsmo, 1998).

Antagonism of various *Trichoderma* spp. to *Armillaria*, the causal agent of root rot in a broad spectrum of shrub and tree species, has been demonstrated mostly in culture (Fox *et al.*, 1994; Li and Hood, 1992; Munnecke *et al.*, 1973, 1981; Reaves *et al.*, 1990; Onsando and Waudo, 1994) and for only a few cases in plant materials (Rishbeth, 1976; Nelson *et al.*, 1989, 1995). *Armillaria* is commonly found in the soil as mycelia or rhizomorphs associated with dead roots and stumps. To be effective in the control of Armillaria root rot, *Trichoderma* must therefore be capable of colonising and suppressing *Armillaria* inoculum that is present in infected plant tissues. Colonization of plant materials is a pre-requisite for bringing their hyphae in close proximity to hyphae of *Armillaria*, providing the opportunity for parasitization of the pathogen. This is particularly the case for *Armillaria* in tropical regions where high temperatures restrict growth of rhizomorphs (Rishbeth, 1978). The present study compared six *Trichoderma* isolates, reported earlier to be antagonistic to a tea pathogenic *Armillaria* sp. *in vitro* (Onsando and Waudo, 1994), for capability to colonize woody substrates and antagonise

Armillaria. The objective was to determine the potential of these isolates for use in controlling Armillaria root disease.

Materials and methods

Trichoderma and Armillaria isolates

Isolates of *Trichoderma* listed in Table 4.1 were obtained from tea plantation soils amended with coffee pulp and identified at the International Mycological Institute (IMI), Kew, UK (Onsando and Waudo, 1992). *Armillaria* isolates $6M_1$ and $13T_1$ (chapter 3 table 3.1) were isolated from infected tea plants and characterised in chapter 3 of this thesis.

Table 4.1: The *Trichoderma* isolates screened for antagonism to *Armillaria* in plant materials

Isolate	IMI No.	Species
3*	339495	*T. longibrachiatum*
4*	339496	*T. harzianum*
5	339497	*T. harzianum*
9*	342183	*T. koningii*
10	342184	*T. harzianum*
11	342185	*T. harzianum*

*Isolates of *Trichoderma* that appeared most antagonistic to an *Armillaria* sp. *in vitro* according to Onsando and Waudo (1994).

Experiments

Wood colonization by Trichoderma isolates

Stems of tea (diam. ≅ 3.0 cm) were cut into 6-cm-long sections, placed in 1-l kilner jars containing 300 ml tap water and autoclaved for 15 min at 121°C. After cooling to room temperature, these were inoculated with *Trichoderma* isolates as follows: A 5-mm-

diam. agar disc removed from a 4-d-old culture on 3% MEA was placed on the upper transverse surface of each stem section and the inoculated sections incubated under room conditions (21 - 23°C) for 6 wk. In Exp. 1a isolates T3, T4, and T9 were compared for efficiency in colonising the stem sections. Isolate T4 was then compared with isolates T5, T10, and T11 in Exp. 1b. The extent of colonization was determined as follows. A sample of inoculated stem sections was taken every week and surface-sterilised by immersing in 70% ethanol for 30 s and flaming. Woody chips were removed at 1, 2, 3, 4, and 5 cm from the inoculated end and plated on Martin's (1950) Rose Bengal Medium (glucose, 10 g; Lab M agar No 1, 17 g; peptone, 0.5 g; yeast extract, 0.5 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $Mg_2SO_4.7H_2O$, 0.5 g; rose bengal, 0.05 mg; streptomycin sulphate, 40 mg; distilled water, 1000 ml). The plates were incubated under room conditions (21 - 23°C) and checked regularly over a period of 1 wk for outgrowth of *Trichoderma*. The number of plated chips with fungal outgrowth was recorded. Each experiment was carried out twice.

Competitive colonization of tea stem sections by Trichoderma and Armillaria

A slightly modified method described by Nelson *et al.* (1989) was used to test the antagonism of *Trichoderma* isolates to *Armillaria*. Autoclaved tea stem sections were inoculated with *Armillaria* or *Trichoderma* isolates using the procedure described under Exp. 1 above. Stem sections inoculated with *Armillaria* were incubated in the dark under room conditions for 6-8 wk while those inoculated with *Trichoderma* were incubated as described under Exp. 1 for 4 wk.

A sample of the 0 – 30-cm layer of a local soil (nitisol) was taken and moisture content adjusted to 38 - 40%. The soil was divided into 250 cm^3 quantities, placed in 0.5-l plastic troughs and autoclaved at 121°C for 1 h. *Armillaria*-colonized stem sections were placed end-to-end with *Trichoderma*-colonized sections, held together with masking tape and placed in the soil. The troughs were covered with their lids and incubated under room conditions (21 - 23°C) for 12 wk. Sheets of cotton wool were used to wrap the troughs and were moistened weekly with sterile water to minimise moisture loss during incubation. In each experiment, the control treatment consisted of banding *Armillaria*-colonized stem sections with autoclaved uncolonized sections. Stem sections colonized

by isolates T3, T4, and T9 (Exp. 2a) or isolates T4, T5, T10, and T11 (Exp. 2b) were banded with those colonized by *Armillaria* isolate $13T_1$ and incubated in autoclaved soil. In Exp. 3, stem sections colonized by *T. harzianum* isolate T4 were banded with those colonized by *Armillaria* isolate $6M_1$ or $13T_1$ and incubated in non-sterile soil. The experiments were set up in a randomised complete block design with four replications (Exp. 2) or three replications (Exp. 3). Each experiment was carried out twice.

At the end of the period of incubation, the banded stem sections were removed from the soil and detached from each other. The sections were washed in sterile distilled water and surface sterilised by immersing in 70% ethanol for 30 s and flaming. Ten woody chips were taken from each section and bulked together for each trough. The chips were plated in lots of 6 (Exp. 2) or 10 (Exp. 3) per petri dish on Martin's Rose Bengal medium and *Armillaria* semi-selective medium (malt extract, 20 g; glucose, 20 g; Lab M agar No 1, 20 g; peptone, 6.0 g; streptomycin sulphate, 0.1 mg; rose bengal, 0.03 mg; PCNB, 0.01 mg; benomyl, 0.01 mg; distilled water, 1000 ml). Each plating was done in triplicate. The proportion of plated woody chips from which *Trichoderma* and *Armillaria* grew was recorded. The data were arcsine transformed (Snedecor and Cochran, 1989) prior to analysis using MSTAT (Version 2.10, R. D. Freal, Michigan State University).

Results

Colonization the stem sections by Trichoderma isolates

The data on colonization of the autoclaved tea stem sections by *Trichoderma* isolates is shown in Table 4.2. All the isolates colonized the stem sections and within 4 wk were recovered from all the distances at 100% isolation frequency.

Competitive colonization of stem sections by Trichoderma and Armillaria isolates

The data on recovery of *Armillaria* from the pathogen or the *Trichoderma*-colonized sections after 12-wk incubation in the soil are shown in Table 4.3. Results from the two repeats of each experiment were similar in all cases. Although *Trichoderma* could always be isolated from both stem sections in all experiments (data not shown),

Table 4.2: Average number of isolations of *Trichoderma* from tea stem sections as a function of incubation time and distance from inoculation site. At week 4 outgrowth of all *Trichoderma* isolates was 100% at all distances along the inoculated stem sections assayed (data not shown)

		Exp. 1a			Exp. 1b			
		Isolates of *Trichoderma*			Isolates of *T. harzianum*			
Incubation (wk)	Distance (cm)	T3	T4	T9	T4	T5	T10	T11
1	1	8.3[1]	7.2	6.7	7.8	7.6	7.7	7.8
	2	7.6	5.8	7.2	6.5	6.3	6.2	6.3
	3	7.2	5.1	5.7	5.8	5.6	5.7	5.8
	4	6.7	4.8	5.2	5.3	5.3	5.2	5.3
	5	6.3	4.1	4.7	4.5	4.3	4.4	4.5
2	1	8.7	9.3	9.2	9.5	9.6	9.7	9.8
	2	8.2	7.7	8.7	8.0	8.3	8.1	8.0
	3	7.6	7.3	8.2	7.5	7.6	7.7	7.5
	4	7.2	6.8	7.7	7.2	7.3	7.2	7.3
	5	6.8	6.2	7.2	6.5	6.3	6.6	6.6
3	1	9.8	9.7	10.0	9.9	10.0	9.9	9.9
	2	9.3	9.3	9.7	9.6	9.5	9.7	9.8
	3	8.8	7.9	9.2	8.4	8.0	8.2	8.3
	4	8.2	7.8	8.7	7.5	7.6	8.0	7.7
	5	7.8	7.4	8.2	7.2	7.1	7.2	7.3

[1]The number of plated woody chips were 10.

Table 4.3: Average number of chips of tea stem sections which were inoculated at one end with *Armillaria* and at the other end with *Trichoderma* showing *Armillaria* outgrowth when plated onto medium after 12 wk of incubation in the soil

	Stem section			
Treatments	*Armillaria*-infested	*Trichoderma*-infested	*Armillaria*-infested	*Trichoderma*-infested
Trichoderma isolates [1]	Exp. 1a		Exp. 1b	
T3	5.5a[2,3]	5.3a	5.0a	4.3a
T4	0.0b	0.0b	0.0b	0.0b
T9	5.5a	5.3a	5.5a	6.0a
Control	6.0a	6.0a	6.0a	6.0a
T. harzianum isolates [1]	Exp. 2a		Exp. 2b	
T4	0.0a	0.0a	0.0a	0.0a
T5	2.2b	2.2b	2.0b	2.2b
T10	2.0b	1.7b	1.5b	1.7b
T11	2.9b	2.8b	2.2b	2.2b
Control	6.0c	6.0c	6.0c	6.0c
Armillaria isolates [4]	Exp. 3a		Exp. 3b	
6M1	2.3a	2.3a	2.5a	2.4a
Control of $6M_1$	3.3b	3.2b	3.6b	3.6b
13T1	4.4A	4.3A	4.4A	4.4A
Control of $13T_1$	6.1B	5.5B	6.3B	6.2B

[1] Tested against *Armillaria* isolate $13T_1$.

[2] The number of plated woody chips was 6 in Exp. 2 and 10 in Exp. 3.

[3] In each column, means followed with different letters are significantly ($P < 0.01$) different according to Tukey's multiple comparison procedure.

[4] Tested with *Trichoderma harzianum* isolate T4.

differences in their effects on *Armillaria* were highly significant. In both Exp. 2 and 3, incidence of *Armillaria* in the initially pathogen-colonized or *Trichoderma*-colonized stem sections was more or less similar for each of the *Trichoderma* isolates. While isolates T3 and T9 had insignificant effect, isolates T4, T5, T10, and T11 strongly inhibited the pathogen and reduced its incidence in the stem sections under sterile conditions by 100, 63-67, 67-75 and 52-63% respectively. In the control treatments *Armillaria* was always isolated at 100% frequency. However, in non-sterile soil (Exp. 3), the isolation frequency from the control ranged from 32–36% and 55–63% for isolates $6M_1$ and $13T_1$ respectively. The effect of *T. harzianum* reduced ($P < 0.01$) the isolation frequencies by 30% and 22-30% respectively. Contrary to Exp. 2, the pathogen was isolated from the stem sections initially colonized by isolate T4.

Discussion

Most studies have used mycelial interactions in dual culture to screen for the biocontrol potential of *Trichoderma* spp. Onsando and Waudo (1994) used this method together with the effect of extracellularly produced metabolites to identify inhibitory capabilities of the *Trichoderma* isolates (Table 4.1) against two isolates of *Armillaria* sp. pathogenic on tea. Comparative use of a standard malt extract medium and a minimal essential medium designed to mimic the C:N ratio in wood to screen *Trichoderma* isolates for the ability to kill *Serpula lacrymans* showed that these *in vitro* methods do not simulate the natural state and may not lead to reproducible antagonism in the field (Score and Palfreyman, 1994). This indicates the need to test in woody substrates the potential of *Trichoderma* spp. for the control of wood rotting fungi. If the ultimate aim is to eradicate a pathogen from infected woody debris, as is the case for *Armillaria*, the biocontrol agent should also be able to colonize wood. All *Trichoderma* isolates used in the present study readily colonized autoclaved tea stem sections (Table 4.2) indicating a good capability to invade woody substrates. Nelson *et al.* (1989) showed that competitive colonization of autoclaved stem sections by *A. luteobubalina* and *Trichoderma* spp. is a viable criterion for evaluating biocontrol potential of the mycoparasitic fungus. This procedure is particularly suitable for *Armillaria* given that primary inoculum sources of

this pathogen are principally infected wood in the soil except in the temperate regions where rhizomorphs are also common. Thus the *Trichoderma* species antagonistic to *Armillaria* would be useful in disease control if they can colonize infected plant materials.

Only a few studies have used interactions between the pathogen and the antagonist in wood to screen *Trichoderma* isolates against wood damaging basidiomycetes (Schoeman *et al.*, 1994; Tucker *et al.*, 1997; Smouse *et al.*, 1999). The *Trichoderma* isolates colonized the stem sections regardless of the presence of the pathogen while, on the other hand, growth of *Armillaria* into *Trichoderma*-colonized sections was deterred to varying degrees by different isolates (Table 4.3). Although *Trichoderma* was always isolated from both kinds of stem sections, *Armillaria* was not found in the sections colonized by isolate T4 and did not colonize stem sections with this isolate during incubation in sterile soil (Exp. 2a and 2b). Veigh *et al.* (1994) screened naturally occurring antagonists against wood decay basidiomycetes both *in vitro* and on plane wood and found that *T. harzianum* was the most successful antagonist. The *T. koningii* (T3) and *T. longibrachiatum* (T9) isolates were not efficacious in contrast to the *T. harzianum* isolates unlike what had been observed *in vitro* (Onsando and Waudo, 1994); although parasitism of these three *Trichoderma* species to a range of soil-borne fungal pathogens has been demonstrated (Harris, 1999; Sreenivasaprasad and Manibhushanrao, 1993; Ahmed *et al.*, 2001).

Despite *T. harzianum* (isolate T4) invading the *Armillaria*-colonized stem sections in non-sterile soil, the pathogen was still recovered from the sections. Apparently its inhibition could not take place in the non-sterile soil as efficiently as in the sterile soil. It is likely that competing micro-organisms influenced the antagonism of this isolate to *Armillaria*. It might be envisaged that at points of contact of the banded stem sections, antagonistic mycelial interactions between isolate T4 and *Armillaria* completely prevented the pathogen from colonising stem sections harbouring this isolate whilst following its invasion of the *Armillaria*-colonized stem sections, the inoculum was rendered non-viable more rapidly under sterile than under non-sterile conditions. No attempts were made to determine the mechanism of the observed effect on *Armillaria*. Antagonism of *T. harzianum* to various fungi including *Rhizoctonia solani* (Benhamou

and Chet, 1993) and *Armillaria gallica* (Dumas and Boyonoski, 1992) has been attributed to the lysis of cell walls of the host fungi, a phenomenon characteristic of mycoparasitism (Siestma and Wessels, 1979). It is possible that cell wall degrading enzymes produced by *T. harzianum* could be subject to inactivation before exerting an effect on the target host in non-sterile environments, thus causing the difference observed between the effect on *Armillaria* under sterile and non-sterile conditions. Nevertheless the antagonism of isolate T4 was reproducible and this isolate was selected for further disease control experiments (chapters 5 and 6 of this thesis).

Chapter 5

Effect of *Trichoderma harzianum* and soil amendment with coffee pulp on survival of *Armillaria*

Abstract

Isolate T4 of *Trichoderma harzianum* applied to the soil as a wheat bran culture and coffee pulp as an organic soil amendment were investigated under greenhouse and field conditions for their effect on viability of *Armillaria* in woody inoculum sources. Infesting the soil surrounding the inoculum sources with *T. harzianum* significantly ($P < 0.001$) increased the incidence of the antagonist in these sources and reduced viability of *Armillaria*. Coffee pulp amendment did not affect the incidence of *Trichoderma* in the woody substrates but reduced *Armillaria* inoculum viability slightly except in potted soils where inoculum viability was higher in the amended soils. It is concluded that the direct application of wheat bran-formulated *T. harzianum* into field soil can significantly suppress *Armillaria* resident in woody substrates and that no further organic amendment to enhance the development of the antagonist is needed.

Introduction

Species of *Armillaria* cause root rot in a range of plant species including tea (*Camellia sinensis*), coffee (*Coffea arabica*), avocado (*Persia americana*), banana (*Musa acuminata*), pine (*Pinus* spp.), eucalyptus (*Eucalyptus* spp.), and cypress (*Cupressus* spp.) in highland areas (altitude > 1500 m) of Kenya. Due to the high economic value of tea, extensive plant mortality often warrants disease control. Primary infection in tea plantations originates from stump and root remnants of forest trees that harbour *Armillaria* (Goodchild, 1960). Leach (1939) recommended ring barking to girdle forest trees one year before felling as a method of reducing longevity of *Armillaria* viability in their stumps and therefore reducing the risk of the disease in tea plantations. Girdling is thought to deplete

root carbohydrate reserves, favouring invasion by saprophytic fungi and making it more difficult for *Armillaria* to access the tissue. Although this method is applied commonly to kill trees prior to planting tea, *Armillaria* remains a major limitation to establishment of the crop on land where stumps of forest trees are not removed thoroughly during land preparation. Remnants of roots in the soil seem to be essential requisites as food bases for ensuring longevity of *Armillaria* inoculum (Munnecke *et al.*, 1981) and their removal is considered to be the single most effective way for minimising the risk of the disease in tea plantations (Goodchild, 1960; Onsando *et al.*, 1997). This can only be done conveniently during primary land preparation but the operation is difficult when it has to be carried out manually to remove potential sources of inoculum occurring deep within the soil or over large areas and complete eradication of *Armillaria* from an infested site by use of this method is difficult to achieve. Alternative methods of destroying *Armillaria* within plant materials in the soil are therefore desirable.

Trichoderma spp. can antagonise plant pathogenic fungi including *Armillaria* (Fox *et al.*, 1994; Nelson *et al.*, 1989, 1995; Munnecke *et al.*, 1981) through mycoparasitism and antibiosis (Elad *et al.*, 1982). Instances of their use in practice to control Armillaria root rot are, however, rare. Garrett (1958) attributed this to the difficulty of achieving sufficiently high population densities of antagonistic strains hindering their ability to colonise woody *Armillaria* inoculum sources in most soils. A successful biocontrol agent should be able to colonise and persist in the soil (Baker and Cook, 1974), hence identifying methods that enable proliferation of *Trichoderma* spp. is key to their successful application. Some studies have shown that the population density of *T. harzianum* added to pathogen-infested soil steadily declines (Elad *et al.*, 1981; 1982; Marois and Mitchel, 1981) suggesting the need to change the soil ecosystem to encourage their persistence or attain their effect on targeted pathogens shortly after application. Factors that have been shown to determine the success of *Trichoderma* include type and age of preparation of the fungus applied to the soil (Lewis and Papavizas, 1984). Onsando and Waudo (1992) reported that soil amendment with coffee pulp, an agricultural waste product from coffee processing, encouraged proliferation of indigenous *Trichoderma* spp. and sustained their population densities above 2.3×10^5 colony-forming units (cfu) g^{-1} soil over a period of 9 months. Some of the *Trichoderma* isolates

obtained from the soil after coffee pulp amendment (Table 4.1) were antagonistic to *Armillaria* in culture (Onsando and Waudo, 1994). However, the effectiveness of the most inhibitory *Trichoderma* isolate in autoclaved tea stems was limited in non-sterile soil (Chapter 4 of this thesis).

The aim of this chapter was to investigate the effect of coffee pulp and soil infestation with isolate T4 of *T. harzianum* on *Armillaria* in woody inoculum sources.

Materials and methods

The experiments were carried out at Timbilil tea estate of the Tea Research Foundation of Kenya, Kericho (located: 0°22′S; 5°21′E, altitude of 2180 m) between April 1998 and July 1999.

Trichoderma harzianum culture

Trichoderma harzianum isolate T4 (chapter 4 table 4.1) was cultured in wheat bran as follows: Five 1-l conical flasks were each filled with 500 g dry wheat bran moistened with 250 ml tap water and autoclaved twice at 121°C for 1 h on two consecutive days. Ten ml of conidial suspension prepared from a 120-h old culture of *T. harzianum* containing 10^4 propagules ml^{-1} was used to inoculate the autoclaved wheat bran in each flask. Inoculated wheat bran was incubated under room conditions (21 – 23°C) and shaken every 24 h to promote uniform colonisation. After 1 wk, the number of propagules in the culture was adjusted to 5×10^4 colony-forming units (cfu) g^{-1} (fresh weight) by adding an equal amount of freshly autoclaved wheat bran and the culture used to infest the soil.

Armillaria inocula

Armillaria inocula used in the pot experiment consisted of artificially inoculated cassava (*Mannihot esculenta* L.) stems (diam. 2.5-3.0 cm). These were cut into 6-cm-long sections, ten of which were placed in each of nine 1-l kilner jars, 300 ml tap water added, and autoclaved for 15 min at 121°C. After cooling to room temperature, a 5-mm-diam. agar disc aseptically removed from a young culture of an *Armillaria* sp. isolate

$13T_1$ (Table 3.1) was placed on the cut transverse surface of each stem section. Inoculated stem sections were incubated in the dark under room conditions (21 – 23°C) for 6 wk. For field experiments inocula were prepared from naturally infected stumps of 4-yr-old tea plants. These were dug out from a single infection site in a commercial field at Timbilil tea estate. Each of these was approximately 25-30 cm long with 5-10 cm collar diam. For Exp. 2 the inocula were prepared by cutting infected stumps into pieces approx. 10-cm long and bulking these together. Inocula for Exp. 3 consisted of whole stumps.

Experiments

Experiment 1: *Effect of Trichoderma harzianum and coffee pulp on Armillaria inoculum in potted soils.*

A sample of the 0 - 30 cm layer of a local nitisol was taken and divided into three equal lots. Coffee pulp was incorporated in the first, the second, and the third lot in quantities of 0, 0.83, and 1.67 kg (dry weight) m^{-3} respectively. Each lot was then divided into three parts and *T. harzianum* culture incorporated in each part in quantities of 0 (control), 10, and 20 g respectively for every 1000 cm^3 of soil resulting in approximate densities of 0, 5×10^2 and 10^3 cfu cm^{-3} respectively. Ten *Armillaria* inoculum pieces were buried in each pot and the pots placed under a concrete bench in the greenhouse. The soil was kept moist by adding 100 ml tap water every week. After 6 months, the inocula were removed and assayed for presence of *Trichoderma* and viability of *Armillaria*.

Experiment 2: *Effect of Trichoderma harzianum and coffee pulp on small inoculum pieces.*

In ploughed plots each measuring 3 x 3 m, coffee pulp was applied by uniformly broadcasting amounts of 0, 0.25, and 0.5 kg m^{-2} on the soil surface. The coffee pulp was then worked in by forking repeatedly to a depth of 30 cm resulting in approximate densities of 0, 0.83, and 1.67 kg m^{-3} respectively. Sixteen 30-cm deep holes were dug in each plot at a spacing of 0.6 x 0.6 m. *Trichoderma harzianum* culture was mixed into the

soil as described for Exp. 1. Ten inoculum pieces were placed in each hole at 15 cm depth, 6-month-old tea plants raised vegetatively in the nursery were planted and the *T. harzianum*-infested soil was used to fill up the planting holes. The tea was maintained under standard agronomic practices (Anon., 1986). After 6 and 12 months, the inocula were assayed for the presence of *Trichoderma* and viability of *Armillaria* as described below.

Experiment 3: *Effect of Trichoderma harzianum and coffee pulp on large pieces of woody Armillaria inocula.*

The procedure followed in this experiment was the same as in Exp. 2. One inoculum piece was placed in the 5 to 30-cm depth of each planting hole during planting of tea and the *T. harzianum*-infested soil was used to fill up the planting holes as described for Exp. 2. After 6 and 12 months four inoculum pieces were taken from each plot and assayed for the presence of *Trichoderma* and viability of *Armillaria* as described below.

Assays of the inoculum pieces

Each of the experiments combined soil organic amendment and soil infestation with *T. harzianum* in a split plot arrangement with three replications. The inoculum pieces from each sub-plot were assayed for presence of *Trichoderma* and *Armillaria* viability as follows. In Exp. 1 the inoculum pieces were split length-wise and ten small pieces of the plant tissue with *Armillaria* mycelium taken from each. These were bulked together for each pot and then ten of them plated in each petri dish. In Exp. 2 and 3 the inoculum pieces were removed from the root zone of four plants in each sub-plot. The bark was lifted off the wood and ten small pieces of plant tissue with *Armillaria* mycelium removed at various points. These were bulked together for the rooting zone of each plant and ten of them plated per petri dish, incubated under room conditions and observed regularly over a period of 7-21 days for growth of *Armillaria* and *Trichoderma*. For *Armillaria,* incubation was done in the dark. All platings were done on Martin's (1950) Rose Bengal (glucose, 10 g; Lab M agar No 1, 17 g; peptone, 0.5 g; yeast extract, 0.5 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $Mg_2SO_4.7H_2O$, 0.5 g; rose bengal, 0.05 mg; streptomycin

sulphate, 40 mg; distilled water, 1000 ml) medium for selective growth of *Trichoderma* and *Armillaria* semi-selective (malt extract, 20 g; glucose, 20 g; Lab M agar No 1, 20 g; peptone, 6.0 g; streptomycin sulphate, 0.1 mg; rose bengal, 0.03 mg; PCNB, 0.01 mg; benomyl, 0.01 mg; distilled water, 1000 ml) medium. Each plating was done in triplicate. The proportions of plated pieces from which *Trichoderma* and *Armillaria* grew were recorded. The data from each treatment combination was expressed as a percentage of the highest value in each block and then arcsine transformed (Snedecor and Cochran, 1989). The transformed data were analysed on a computer using MSTAT (Version 2.10, R. D. Freal, Michigan State University).

Results

The number of isolations that yielded *Trichoderma* and *Armillaria* either alone or together is summarised in Table 5.1. In Exp. 1 only 7.4% of all the isolations had *Armillaria* alone while 18.5% of the isolations had *Trichoderma* alone and 74.1% had both fungi together. In the field experiments there were no instances where *Armillaria* was isolated alone, 44.4 and 33.3% of the isolations made at 6 and 12 months for Exp. 2 had *Trichoderma* alone respectively, and 55.6 and 66.7% had the two fungi together. In Exp. 3, 7.4 and 11.1% of isolations at 6 and 12 months had *Trichoderma* alone and 92.6 and 88.8% respectively had the two fungi together. Overall, the two fungi were isolated together in > 55% of all the isolations for each experiment.

Incidence of *Armillaria* in the inoculum pieces was invariably highest in the control treatment and strongly decreased with increase in the quantity of *T. harzianum* applied to the soil (Table 5.2; Figures 5.1 - 5.3). Reduction of *Armillaria* occurred fast for the small inoculum sources, being already complete after 6 months of incubation. However, the reduction was slower for the large inoculum sources, where decrease in viability continued until 12 months. The treatment with the large inoculum sources was also the single treatment where application of a high dose of *T. harzianum* did not lead to the total elimination of *Armillaria* after 12 months.

Presence of *Trichoderma* in the inoculum pieces increased significantly ($P < 0.001$) with the density of *T. harzianum* applied to the soil but did not change with coffee pulp

Table 5.1: Summary of isolation frequencies of *Armillaria* and *Trichoderma* from the inoculum pieces (cassava stem in Exp. 1 and tea wood in Exp. 2 and Exp. 3) showing outgrowth of *Armillaria* sp. and/or its antagonist *Trichoderma harzianum* as function of addition of coffee pulp and/or the antagonist

Coffee pulp[1]	*T. harzianum*[2]	*Trichoderma* isolated	Exp. 1		Exp. 2-6 months		Exp. 2-12 months		Exp. 3-6 months		Exp. 3-12 months	
			Armillaria isolated		*Armillaria* isolated		*Armillaria* isolated		*Armillaria* isolated		*Armillaria* isolated	
			Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
0	0	Yes	7.4	0.0	11.1	0.0	11.1	0.0	11.1	0.0	11.1	0.0
		No	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0	10	Yes	3.7	7.4	11.1	0.0	11.1	0.0	11.1	0.0	11.1	0.0
		No	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0	20	Yes	0.0	11.1	3.7	11.1	3.7	7.4	11.1	11.1	11.1	0.0
		No	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.83	0	Yes	7.4	0.0	11.1	0.0	11.1	0.0	11.1	0.0	11.1	0.0
		No	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.83	10	Yes	11.1	0.0	7.4	3.7	7.4	3.7	11.1	0.0	11.1	7.4
		No	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.83	20	No	11.1	0.0	0.0	11.1	0.0	11.1	11.1	0.0	11.1	0.0
		No	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.67	0	Yes	11.1	0.0	11.1	0.0	11.1	0.0	11.1	0.0	11.1	0.0
		No	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.67	10	No	11.1	0.0	7.4	0.0	7.4	3.7	7.4	3.7	11.1	3.7
		No	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.67	20	Yes	11.1	0.0	3.7	0.0	3.7	7.4	7.4	3.7	11.1	0.0
		No	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[1] kg m^{-3} of soil.
[2] g l^{-1} soil wheat bran culture.

Table 5.2: Average percentage of plated pieces of plant material (cassava stem in Exp. 1 and tea wood in Exp. 2 and Exp. 3) showing outgrowth of *Armillaria* sp. or its antagonist *Trichoderma harzianum* as function of addition of coffee pulp and/or the antagonist

Treatment combinations		Experiments									
		Exp.1		Exp. 2 (6 months)		Exp. 2 (12 months)		Exp. 3 (6 months)		Exp. 3 (12 months)	
Coffee pulp[1]	*T. harzianum*[2]	*Trichoderma*	*Armillaria*	*Trichoderma*	*Armillaria*	*Trichoderma*	*Armillaria*	*Trichoderma*	*Armillaria*	*Trichoderma*	*Armillaria*
0	0	0.8a[3]	7.2a	0.7a	3.6a	3.3a	1.7a	2.1a	5.0a	9.2a	2.6a
0	10	9.7b	0.1b	8.7b	2.7b	8.1b	0.9b	5.3b	3.5c	10.0a	1.3c
0	20	10.0b	0.0b	9.8b	0.0c	9.5b	0.0c	8.0b	2.8d	10.0a	0.9d
0.83	0	1.3a	3.3c	0.9a	1.5d	3.6a	1.1b	1.7a	3.7b	9.6a	2.0b
0.83	10	8.2b	5.2d	9.1b	0.7e	8.8b	0.4d	5.4b	2.5d	10.0a	1.4c
0.83	20	10.0b	4.5d	9.6b	0.0c	9.4b	0.0c	7.3b	1.4e	10.0a	0.0e
1.67	0	1.3a	4.0d	1.1a	1.6d	4.5a	1.2b	2.4a	3.4b	9.7a	2.3b
1.67	10	9.2b	3.5d	8.7b	0.5e	8.7b	0.2d	5.1b	1.8e	10.0a	0.8d
1.67	20	10.0b	4.0d	9.5b	0.0c	9.3b	0.0c	6.7b	0.0f	10.0a	0.0e

[1] $kg\ m^{-3}$ of soil.
[2] $g\ l^{-1}$ soil wheat bran culture.
[3] In each column, means followed with the same letter are not significantly ($P < 0.01$) different according to Turkey's multiple comparison procedure.

density. In most cases incidence of *T. harzianum* was slightly higher in the inocula that had been incubated for 12 months than for those that had been incubated for 6 months. In Exp. 1 application of *T. harzianum* reduced completely incidence of *Armillaria* in the inoculated cassava stems. However, coffee pulp reduced the effect of *T. harzianum* (Figure 5.1) on *Armillaria* although the incidence of the antagonist in the inocula had not

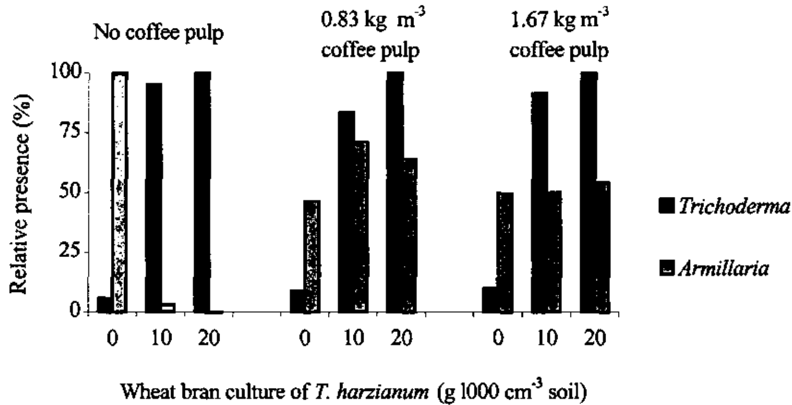


Fig. 5.1: Relative presence of *Trichoderma* and *Armillaria* in inoculum sources (cassava stem sections) in potted soil with varying densities of coffee pulp (kg m^{-3} soil) and *T. harzianum* (Exp. 1). The presence of each fungus is expressed as percentage of the highest value scored. The plotted values are back transformed means of the analysed data

been affected. This effect of coffee pulp did not appear in field experiments where *T. harzianum* was effective at both coffee pulp amendment levels (Figures 5.2 and 5.3). In non-*T. harzianum*-infested soil, coffee pulp also reduced the incidence of *Armillaria* by 12-58% (Table 5.1).

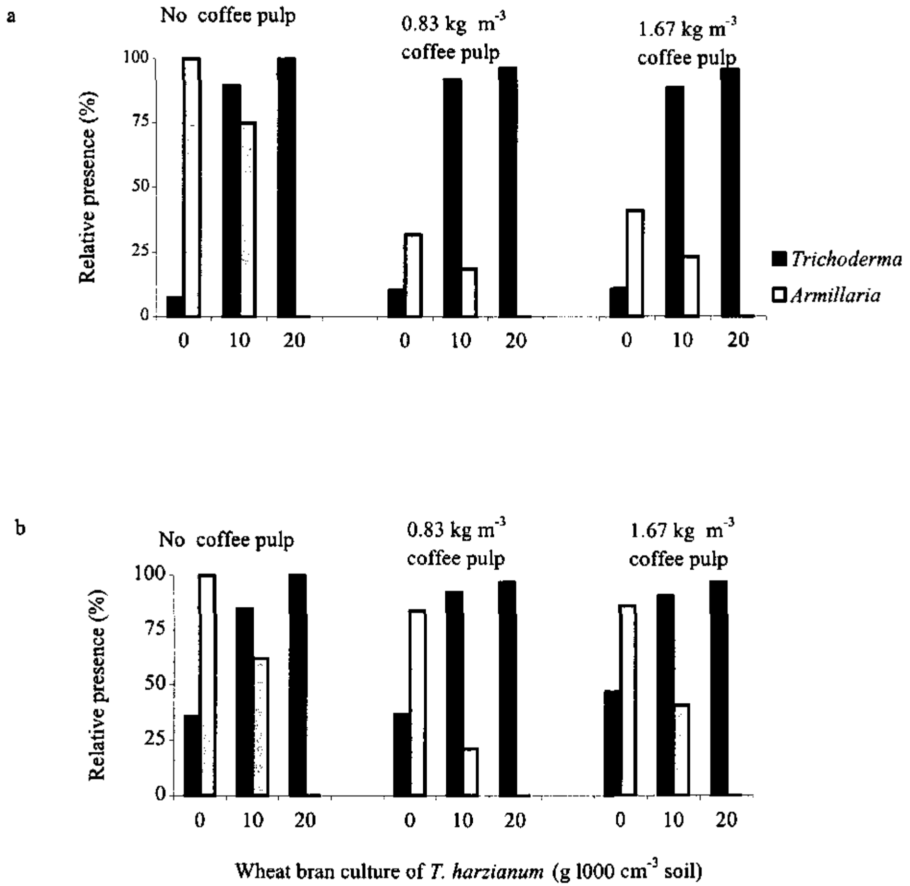
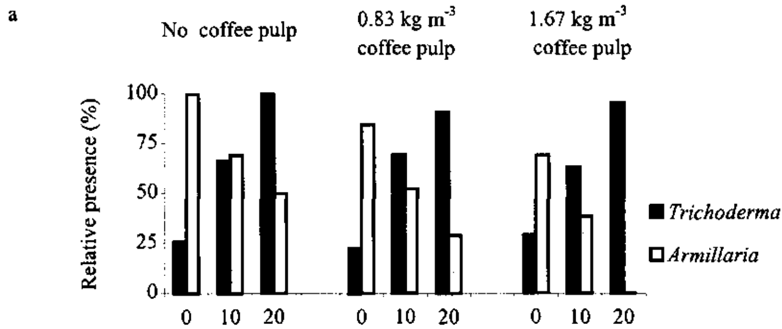


Fig. 5.2: Relative presence of *Trichoderma* and *Armillaria* in small pieces of inoculum sources (tea wood) incubated in soil amended with varying densities of coffee pulp (kg m^{-3} soil) and *T. harzianum* (a) after 6 months and (b) 12 months (Exp. 2). The presence of each fungus is expressed as percentage of the highest value scored. The plotted values are back transformed means of the analysed data



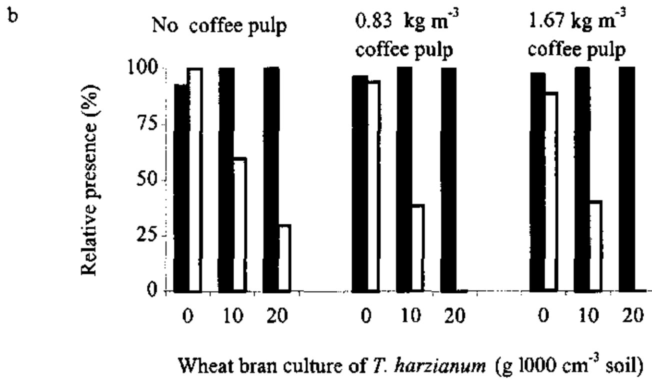


Fig. 5.3: Relative presence of *Trichoderma* and *Armillaria* in large pieces of inoculum sources (tea wood) incubated in soil amended with varying densities of coffee pulp (kg m^{-3} soil) and *T. harzianum* (a) after 6 months and (b) after 12 months. The presence of each fungus was expressed as percentage of the highest value scored. The plotted values are back transformed means from the analysed data

The relationship between incidence of *Armillaria* with that of *Trichoderma* shown in Figure 5.4 indicates higher incidence of *Armillaria* at low incidence of *Trichoderma.* However, high incidence of *Trichoderma* was not necessarily related to low incidence of *Armillaria.*

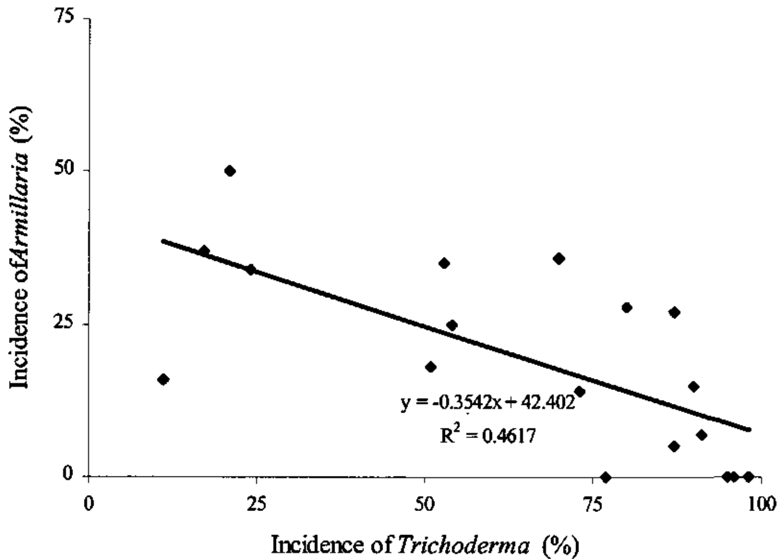


Fig 5.4: Relationship between the presence of *Armillaria* and *Trichoderma* in inoculum pieces from soils with varying densities of coffee pulp and *T. harzianum* at 6 months under field conditions

Discussion

The presence of *Trichoderma* in the inoculum pieces from the control plots can be attributed to colonisation by species resident in the soil. No attempts were made to identify or distinguish the strain isolated from the inoculum pieces from the *T. harzianum* used to infest the soil. The high incidence of *Trichoderma* in the inoculum pieces from infested soils compared to the control suggests colonisation of the plant materials mainly

by the introduced antagonist which must therefore have been predominant among *Trichoderma* isolated from the inoculum pieces during the assays. Although *Trichoderma* population densities in the soil were not monitored in these experiments, the increase in incidence of the fungus in the plant materials as the amount of wheat bran culture of *T. harzianum* applied to the soil increased suggests that colonisation depended on population density of the antagonist in the soil. Soil amendment with coffee pulp, though shown earlier to increase population density of *Trichoderma* spp. resident in the soil (Onsando and Waudo, 1992), did not affect the incidence of these in the inoculum pieces. Perhaps the cellulolytic activity of *Trichoderma* was repressed by the presence of more readily available nutrients (Wood, 1991), which may have been retained in the soil of the pot experiments, thus exerting a relatively long-lasting effect on *Trichoderma*, while they may have leached out into deeper soil layers in the field plots.

Woody materials are essential food bases for survival of *Armillaria* in the soil (Munnecke *et al.*, 1981) particularly in regions where rhizomorphs are scarce. Elimination of the pathogen from these is one option for managing Armillaria root disease. If *Trichoderma* spp. that antagonise *Armillaria* can colonise these inoculum sources when introduced into the soil, they would be useful for biocontrol of the disease. Studies on the effect of *T. viride* on *A. mellea* showed that survival of the pathogen depended on the population density of the antagonist in the soil surrounding its woody inoculum sources (Garrett, 1958). Thus at low population densities antagonistic strains of *Trichoderma* would be limited in their efficacy against *Armillaria*. This may explain why, despite various studies having demonstrated antagonism of *Trichoderma* spp. to *Armillaria*, little is known about their practical use to control Armillaria root disease. The first consideration in attempts to control the disease using antagonistic *Trichoderma* would therefore be to identify methods of increasing their population density in the soil in such a manner that would also improve their colonisation of *Armillaria* inoculum sources. The concurrent application of *Trichoderma* and coffee pulp to the soil apparently does not lead to improved colonization of *Armillaria*-infected wood. Since the effect of coffee pulp was negative on the effect of *Trichoderma* in the pot experiment we would not advise the application of this material as a method of increasing the population density of antagonistic *T. harzianum* introduced to the soil for the control of *Armillaria*.

We conclude that infesting soil surrounding the inoculum sources by applying *T. harzianum* as wheat bran culture has potential to achieve disease control by reducing longevity of *Armillaria* inoculum in the soil through inactivation of the pathogen at pre-infection stage.

Chapter 6

Efficacy of soil solarization, *Trichoderma harzianum*, and coffee pulp amendment against *Armillaria*

Abstract

Soil solarization was evaluated singly or in combination with *Trichoderma harzianum* infestation or coffee pulp amendment for effect on wood-borne inoculum of an *Armillaria* sp. Solarization increased maximum soil temperatures at 10 cm depth by 9-13°C and reduced ($P < 0.001$) viability of *Armillaria* up to 100%. Its efficacy was similar at 5, 10, and 15 cm soil depths. *Trichoderma harzianum* applied to the soil surrounding the inocula also significantly ($P < 0.001$) reduced viability of *Armillaria*. Application of the antagonist at 20 g of the wheat bran culture consequent to solarization for 5 wk caused total loss of *Armillaria* viability and was similar to 10 wk of solarization without application of the antagonist. Coffee pulp amendment reduced inoculum viability slightly though its effect was apparent only in unsolarized soils. Soil solarization had significant ($P < 0.001$) interaction with *T. harzianum* infestation. These observations are discussed in regard to their implications in developing an integrated approach to management of Armillaria root disease.

Introduction

Successful control of Armillaria root rot is hampered by various factors reviewed by Fox (2000). The most important of these is the difficulty of gaining access to the pathogen residing in plant materials that are situated deep within the soil. In tea plantations, infections start from stump and root remnants of previous vegetation. Consequently the fungus spreads rapidly when infected plants are not promptly identified and removed. Removal of plant residues that may harbour *Armillaria* and eradication of diseased plants are recommended for minimizing the risk of the disease (Anon., 1986). Complete

elimination of inoculum by these methods is difficult in established crop stands since their success also depends on early and accurate diagnosis of the disease, which is difficult before the appearance of above-ground symptoms. The limited access to *Armillaria* inside dead plant materials and its extensive rhizomorph systems also limits the efficacy of chemical fumigants such as carbon disulphide (Fox, 2000). Guillaumin (1988) noted that to be effective, the fumigants should be injected at least 60 cm deep in the soil but even this does not completely eradicate the pathogen. However, they disrupt antibiotic production by *Armillaria* and thus increase its susceptibility to infection by mycoparasitic *Trichoderma* spp. (Ohr and Munnecke, 1974; Munnecke *et al.*, 1981). Exposure to heat and desiccation similarly predisposed *A. mellea* to *T. viride* (Munnecke *et al.*, 1973, 1976, 1981). From an environmental point of view, soil heating would be a more acceptable method of enhancing efficacy of antagonistic *Trichoderma* against *Armillaria* than the use of the fumigants.

Soil heating can be accomplished in warm climates by solarization. This involves covering the soil surface with a thin clear polyethylene tarp for several weeks to enhance accumulation of heat. The resulting high soil temperatures inactivate some of the soil microbes and therefore partially disinfest the soil (Davis, 1991). Soil solarization has been used to control *Fusarium oxysporum* f.sp. *conglutinans* (Ramirez-Villapudua and Munnecke, 1988), *Verticillium dahliae* (Melero-Vara *et al.*, 1995; Pinkerton *et al.*, 2000); *Phytophthora cinnamomi* (Pinkerton *et al.*, 2000) and *Macrophomina phaseolina* (Chellemi *et al.*, 1997; Lodha *et al.*, 1997). Although soil temperatures attained by solarization may be sufficiently high to directly kill propagules of some of these pathogens in the top soil, the efficacy declines with soil depth (Katan, 1981). Incorporation of additional suppressive factors is therefore often necessary for improving the efficacy of soil solarization in controlling plant diseases.

The results of the experiments in Chapter 5 of this thesis showed the potential of infesting the soil with *T. harzianum* and amendment with coffee pulp to reduce longevity of *Armillaria* inoculum. In this chapter the antagonist and coffee pulp amendment were tested further, in combination with soil solarization, for the effect on survival of the pathogen in woody substrates.

Materials and methods

The experiments were carried out at Timbilil tea estate (details given in chapter 5) between October 1998 and March 2000. The effect of solarization on survival of *Armillaria* at different soil depths was studied in Exp. 1 while effect of solarization and *Trichoderma harzianum* or solarization and coffee pulp amendment were studied in Exp. 2 and 3 respectively. These factors were evaluated singly or in combination with various durations of soil solarization for effect on survival of *Armillaria* in naturally infected stumps of tea. Each experiment was carried out twice.

Armillaria inocula and Trichoderma preparation

Armillaria inocula were obtained from the same site and prepared as described for Exp. 3 of chapter 5 except for Exp. 1 where the stumps were split longitudinally into two pieces of approximately the same size to provide the inocula. In Exp. 2 and Exp. 3 whole stumps each with collar diameter 5-10 cm were cut to 25-30 cm length and used as inoculum sources. Wheat bran culture of *T. harzianum* was prepared and used as described in chapter 5.

Soil solarization

The land was finely ploughed and the inocula buried as described below for each experiment. The soil was then watered to field capacity (approx. 5-10 l m^{-2}) and plots to be solarized covered with polyethylene tarp (Uni-Plastics Ltd) 0.5 mm thick buried to 30 cm depth at the margins. The plots were exposed to solar radiation for 4 or 8 wk (Exp. 1) and 5 or 10 wk (Exp. 2 and 3). Soil thermometers were placed at 10-cm depth, one in a solarized and the other in a non-solarized plot and temperatures read daily at 9, 12, and 15 h.

Experiments

Experiment 1: *Effect of soil solarization and inoculum depth on survival of Armillaria.*

The inocula were buried 5, 10, or 15 cm deep at spacing of 30 x 30 cm in plots measuring 2 x 4 m. The soil was watered and plots solarized for 0, 4, and 8 wk.

Solarization was carried out from 31 January to 26 March 2000 (Exp. 1a) and 5 February to 31 March 2000 (Exp. 1b).

Experiment 2: *Effect of soil solarization and Trichoderma harzianum on survival of Armillaria.*

The inocula were buried at a spacing of 30 x 30 cm with their collar at the ground level. Sixteen inoculum sources were buried in each plot measuring 1.2 x 2.4 m. The plots were watered and solarized for 0, 5, and 10 wk. At the end of each solarization period, soil was removed from a 10-cm radius around each inoculum and *T. harzianum* culture incorporated into the soil as described for Exp. 2 and Exp. 3 in Chapter 5. The soil – *T. harzianum* mixture was replaced to surround each inoculum piece to a depth of 10 cm. After application of *T. harzianum,* the inoculum sources were left in the soil for 16, 11, and 6 wk in plots solarized for 0, 5, and 10 wk respectively. Solarization was carried out from 23 October to 31 December 1998 (Exp. 2a) and 17 November 1999 to 25 January 2000 (Exp. 2b).

Experiment 3: *Effect of soil solarization and organic amendment on survival of Armillaria.*

Coffee pulp was incorporated in the soil as described in Exp. 2 and 3 of chapter 5. *Armillaria* inocula were then buried in each plot, the soil watered and solarized for 0, 5, and 10 wk. In the treatments where solarization was done for 5 wk, application of the treatment was carried out 5 wk after the application in the 10 wk solarized plots. At the end of the solarization period, 10 g of *T. harzianum* culture was incorporated in the soil surrounding each inoculum piece in all plots as described in Exp. 2 and left for further 11 wk. Solarization was carried out from 5 February to 25 April 1999 (Exp. 3a) and 26 November 1999 to 3 February 2000 (Exp. 3b).

Experimental design

In each of the experiments, duration of soil solarization was combined with levels of the second treatment (soil depth in Exp. 1; *T. harzianum* infestation in Exp. 2; coffee pulp amendment in Exp. 3) in a split plot arrangement. The levels of the second treatment

factor were split over each level of solarization. Each treatment combination was replicated three times in a randomised complete block design.

Assays for viability of Armillaria

At the end of each experiment four inoculum sources were removed from each subplot and assayed for viability of *Armillaria* as follows: the bark was lifted off the wood of each inoculum piece and small pieces of plant tissue containing *Armillaria* mycelium taken from each. These were bulked together for each stump and ten of them plated in each petri dish on *Armillaria* semi selective medium (malt extract, 20 g; glucose, 20 g; Lab M agar No 1, 20 g; peptone, 6.0 g; streptomycin sulphate, 0.1 mg; rose bengal, 0.03 mg; PCNB, 0.01 mg; benomyl, 0.01 mg; distilled water, 1000 ml), incubated under room conditions in the dark and observed regularly over a period of 21 days for growth of *Armillaria*. Each plating was done in triplicate. The proportion of plated materials from which the fungus grew was recorded. The data from each treatment combination was expressed as a percentage of the highest value in each block to give relative survival of the pathogen. The relative survival data were arcsine transformed (Snedecor and Cochran, 1989) and analysed using MSTAT Version 2.10 (R. D. Freal, Michigan State).

Results

Soil temperature characteristics

Soil temperature characteristics in solarized and unsolarized plots in the three experiments are summarised in Table 6.1. The pattern of daily temperature changes was more or less similar in solarized and unsolarized soils but individual temperatures recorded were higher in solarized soils. The difference between maximum temperatures of solarized and unsolarized soils ranged from 9-12°C while the differences between the corresponding mean temperatures ranged from 7-11°C. The maximum absolute temperatures ranged from 44-50°C in solarized soils and from 33-35°C in unsolarized soils.

Table 6.1: Soil temperature characteristics during solarization and difference with unsolarized plots

Exp.	Time of the year[2]	Tsol[1] Max[3]	Mean[4]	Change[5]	Absolute Max[6]	Max (Tsol – Tunsol) Difference[7]
1a	1-3, 2000	39.5 ± 0.4	32.9 ± 0.3	14.8 ± 0.4	48.0	11.4 ± 0.3
1b	2-3, 2000	39.8 ± 0.4	33.2 ± 0.2	15.2 ± 0.4	46.0	12.0 ± 0.4
2a	10-12, 1998	35.9 ± 0.5	30.7 ± 0.4	11.4 ± 0.4	45.0	9.7 ± 0.4
2b	11-1, 1999-2000	37.7 ± 0.5	31.9 ± 0.4	12.3 ± 0.5	44.0	9.6 ± 0.3
3a	2-4, 1999	37.5 ± 0.6	31.1 ± 0.4	13.2 ± 0.6	50.0	10.1 ± 0.4
3b	11-2, 1999-2000	37.0 ± 0.5	31.3 ± 0.4	11.7 ± 0.5	45.0	8.9 ± 0.3

[1] Soil temperatures in solarized treatment.
[2] Months of the year and year indicated.
[3] Mean maximum temperature in solarized treatment (averaged over the experiment).
[4] Mean temperature in solarized treatment (averaged over all recordings done).
[5] Mean change of temperature in the solarized treatment (average of the daily difference between the highest and the lowest recordings) during solarization.
[6] Absolute maximum temperature.
[7] Mean of difference of average maximum temperature between solarized and unsolarized treatment.

Viability of Armillaria

The data on viability of *Armillaria* are presented in Tables 6.2-6.4 for Exp. 1-3 respectively. In all the experiments, the isolation frequency of *Armillaria* was much lower in solarized than in unsolarized treatments (Figures 6.1-6.3). After 4 wk, the effect of solarization on *Armillaria* was nearly as great as after 8 wk. Effect of solarization was more or less the same at 5, 10, and 15-cm inoculum depth. In Exp. 2 and 3 incidence of *Armillaria* was low in all treatment combinations including the control, which was probably due to dry weather prevailing during the time of the year when these experiments were done; this probably desiccated the inoculum sources and adversely affected survival of the pathogen. Nevertheless there was a clear effect of solarization. In Exp. 2 survival of the pathogen in soils solarized for 5 wk was higher than in soils solarized for 10 wk where inoculum viability was 0% while in Exp. 3 inoculum viability was 0% in soils solarized for 5 as well as 10 wk.

Table 6.2: Average number of plated pieces (n=10) of tea wood blocks showing *Armillaria* outgrowth. The tea wood blocks had been incubated at different depths in soils solarized for 0, 4, and 8 wk (Exp. 1)

Treatments			
Solarization duration (wk)	Inoculum depth (cm)	Exp. 1a	Exp. 1b
0	5	9.4a[1]	9.2a
0	10	9.5a	9.3a
0	15	9.8a	9.6a
4	5	0.4b	0.6b
4	10	0.4b	0.6b
4	15	0.4b	0.7b
8	5	0.2b	0.2c
8	10	0.3b	0.5b
8	15	0.3b	0.7b

[1] In each column, means followed with the same letter are not significantly ($P < 0.01$) different according to Tukey's multiple comparison procedure.

Infesting soil with *T. harzianum* affected viability of *Armillaria* inoculum in the same way as in Exp. 2 and Exp. 3 of chapter 5 with incidence of the pathogen decreasing by 26-32% in Exp. 2 between 0 and 10 g of *T. harzianum* wheat bran culture and decreasing by 31-43% between 10 and 20 g of the culture. Effect of *T. harzianum* was clear in the control as well as in the plots solarized for 5 wk (Figure 6.2). Beyond this period, the effect of solarization masked that of the antagonist, rendering *Armillaria* completely non-viable after 10 wk which accounts for the significant ($P < 0.001$) interaction between solarization and *T. harzianum* treatments. Effect of soil amendment with coffee pulp was evident only in unsolarized soil where the incidence of *Armillaria* decreased by 12 and 28% between coffee pulp densities of 0 and 0.83 kg m^{-3} in Exp. 3a and 3b respectively. Between densities of 0.83 and 1.67 kg m^{-3} the incidence decreased by 14 and 44% in Exp. 3a and Exp. 3b. respectively. The effect was however, not apparent in solarized treatments as *Armillaria* could not be recovered from inoculum sources in soils solarized for 5 or 10 wk.

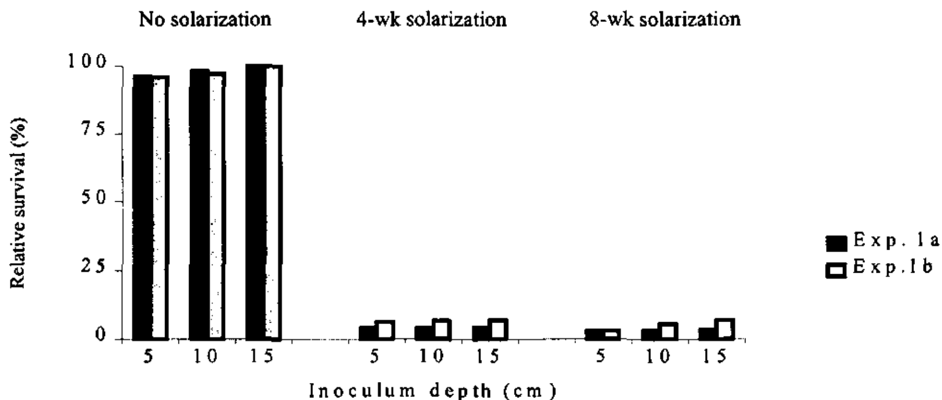


Fig. 6.1: Relative survival of *Armillaria* in inocula of tea wood blocks incubated at different depths in soil solarized for 0, 4, and 8 wk (Exp. 1). Presence of *Armillaria* in the tea wood blocks is expressed as percentage of the highest value scored. The plotted values are back transformed means of the analyzed data

Table 6.3: Average number of plated pieces (n=10) of tea wood blocks showing *Armillaria* outgrowth. The tea wood blocks had been incubated 5 - 30 cm deep in soil and solarized for 0, 5, or 10 wk followed by infestation with *T. harzianum* (Exp. 2)

Treatments			
Solarization duration (wk)	*T. harzianum* culture (g)[1]	Exp. 2a	Exp. 2b
0	0	7.1a[2]	6.9a
0	10	4.8b	5.1b
0	20	3.3c	2.9c
5	0	4.8b	2.7c
5	10	3.8c	1.5c
5	20	3.4c	1.3c
10	0	0.0d	0.0d
10	10	0.0d	0.0d
10	20	0.0d	0.0d

[1] g 1000 cm^{-3} soil of wheat bran culture.

[2] In each column, means followed with the same letter are not significantly ($P < 0.01$) different according to Tukey's multiple comparison procedure.

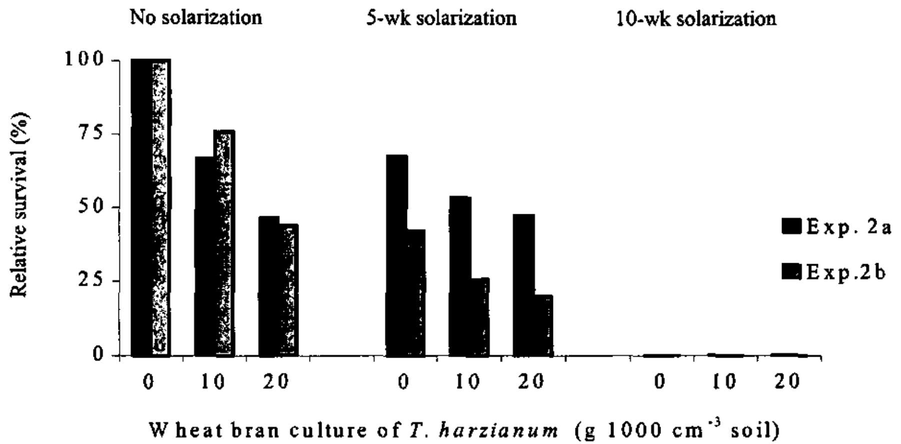


Fig 6.2: Relative survival of *Armillaria* in inocula of tea wood blocks incubated 5 - 30 cm deep in soil, solarized for 0, 5, or 10 wk and/or infested with *T. harzianum* (Exp. 2). Presence of *Armillaria* in tea wood blocks is expressed as percentage of the highest value scored. The plotted values are back transformed means of the analyzed data

Table 6.4: Average number of plated pieces (n=10) of tea wood blocks showing *Armillaria* outgrowth. The tea wood blocks had been incubated 5 - 30 cm deep in soil, solarized for 0, 5, or 10 wk and/or amended with coffee pulp. In all treatment combinations, the soil surrounding each inoculum block was infested with *T. harzianum* after solarization by applying 10 g 1000 cm^{-3} soil of wheat bran culture of the antagonist (Exp. 3)

Treatments			
Solarization duration (wk)	Coffee pulp[1]	Exp. 3a	Exp. 3b
0	0.00	5.6a[2]	5.0a
0	0.83	5.0a	3.6b
0	1.67	4.3b	2.5c
5	0.00	0.0c	0.0d
5	0.83	0.0c	0.0d
5	1.67	0.0c	0.0d
10	0.00	0.0c	0.0d
10	0.83	0.0c	0.0d
10	1.67	0.0c	0.0d

[1] kg m^{-3} soil.

[2] In each column, means followed with the same letter are not significantly ($P < 0.01$) different according to Tukey's multiple comparison procedure.

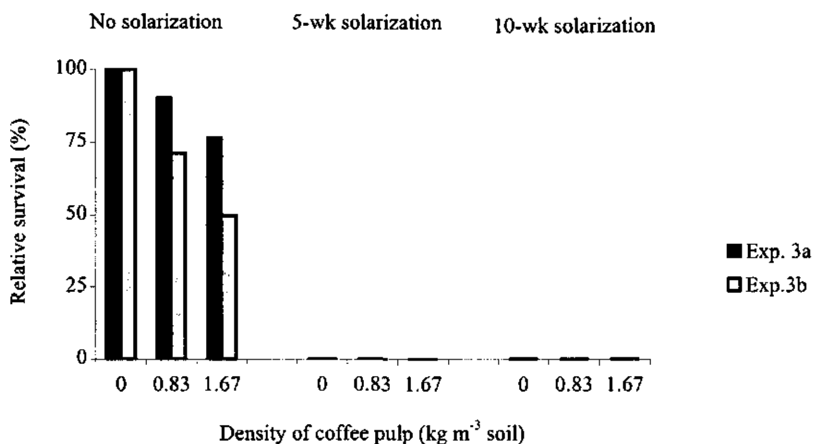


Fig. 6.3: Relative survival of *Armillaria* in tea wood blocks incubated 5 - 30 cm deep in soil, solarized for 0, 5, or 10 wk and/or amended with coffee pulp. In all treatment combinations, the soil surrounding each tea wood block was infested with *T. harzianum* after solarization by applying 10 g 1000 cm^{-3} soil of wheat bran culture of the antagonist. Presence of *Armillaria* was expressed as percentage of the highest value scored. The plotted values are back transformed means of the analyzed data.

Discussion

The rise in soil temperature due to solarization was comparable to what has been observed in similar experiments done elsewhere. Merenco and Lustosa (2000) reported maximum temperatures at 5-cm depth that were about 10°C higher in solarized soils than in the control plots. Bohra *et al.* (1996) also reported temperature increases of 10 and 7°C of solarized dry and wet soils respectively while maximum temperatures were 8-16°C higher in solarized than in non-solarized soils at 5 and 30 cm soil depth (Pinkerton *et al.*, 2000). In the present study, solarization was carried out during the warmest time of the year and it raised temperatures at 10 cm soil depth to daily maxima > 35°C that were, on average, about 10°C higher than at a corresponding depth in unsolarized soils.

The incidence of *Armillaria* was significantly ($P < 0.001$) reduced within 30-cm soil depth when soil was solarized for 4-10 wk indicating that repeated exposure of the inoculum sources to elevated soil temperatures adversely affected survival of the pathogen. The effect was more or less the same at 5, 10, and 15-cm soil depth (Figure 6.1) where the incidence was 3 - 6% of that in unsolarized soil. For inoculum sources situated 0-30 cm deep, the incidence in soil solarized for 5 and 10 wk was 32 - 61% respectively lower than in the control (Figure 6.2).

Sensitivity of *Armillaria* to high soil temperatures has been a subject of only a few studies. A dramatic loss in viability of *A. ostoyae* was observed when high soil temperatures resulting from forest burning were tested for the effect on viability of woody inoculum of the fungus buried in the soil (Filip and Yang, 1997). In solarization, the primary mode of action as a method of disease control is the inactivation of soil borne pathogens by prolonged exposure to high temperatures, leading to significant reduction in disease incidence. Denner *et al.* (2000) observed a 45% reduction in incidence of black dot of potatoes (*Colletotrichum coccodes*) when solarization was done for 8 wk and temperatures in the top 5 cm of soil reached 56°C. Also stalk rot of corn, caused by *Fusarium moniliforme* and *Macrophomina phaseolina* (Ahmad *et al.*, 1996; Pinkerton *et al.*, 2000) and verticillium wilt caused by *Verticillium dahliae* (Lopez-Escudero and Blanco-López, 2001; Melero-Vara *et al.*, 1995) have been controlled effectively using solarization. Unlike *Armillaria*, the pathogens against which solarization has been shown to be effective usually occur in the soil as dispersed propagules. The results presented here suggest a similar effect on *Armillaria* borne in coarse plant materials.

Suppresiveness of solarized soil to some plant diseases has also been attributed to enhancement of the action of antagonists such as *Trichoderma* (Alabouvette *et al.*, 1979; Greenberger *et al.*, 1987). In Exp. 2, *T. harzianum* also caused a significant reduction in *Armillaria* incidence in the inoculum sources in the unsolarized treatment and when solarization for 5 wk was followed with a high density of *T. harzianum* in the soil, inoculum was quickly rendered non-viable (Figure 6.2). However, solarization was more effective than the application of *T. harzianum* as has been reported also by Melero-Vara *et al.* (2000) for the control of *Sclerotium cepivorum*. The efficacy of combining solarization with the antagonist illustrates the possibility of better disease control by

integrating the two treatments. Soil organic amendments may suppress soil borne pathogens and the data presented here and in chapter 5 showed a slight effect of coffee pulp amendment on viability of *Armillaria*. Improvement in efficacy of solarization by using organic amendments such as reported for *Fusarium oxysporum* f.sp. *conglutinans* (Ramirez-Villapudua and Munnecke, 1988) was not observed in Exp. 3 because solarization, after only 5 wk, completely eradicated *Armillaria* (Figure 6.3) and thus did not allow the expression of the effect of coffee pulp in solarized soil. The more dramatic effect observed in Exp. 3 was also due to an additional effect of *T. harzianum* which was applied in all treatments. Through the solarization treatment a partial biological vacuum was created that allowed an efficient establishment of the introduced *T. harzianum*. It has been reported that effects of solarization on the natural population of *Trichoderma* spp. is limited (Sastry and Chattopadhyay, 1999), which may be due to fast recolonization from deeper soil layers (Ristaino *et al.*, 1991) thus explaining the fact that they are frequently observed on sclerotia and other fungal tissues after solarization (Bihan *et al.*, 1997; McLean *et al.*, 2001). In addition, the high temperatures may have weakened the resistance of *Armillaria* to the antagonist. In integrated disease management the effect of one factor may exceed the tolerance limit of a pathogen and thereby weaken it and increase its vulnerability to another factor (Baker, 1987). Application of multiple disease control factors is likely therefore to be more effective as exemplified for the combination of solarization and *T. harzianum* against *Armillaria* in the present study. Our study demonstrates the potential of solarization to control Armillaria root rot and confirmed that efficacy of this treatment is greater when combined with *T. harzianum*, and possibly soil amendment, in an integrated disease management strategy.

Chapter 7

General discussion

Results of the study on aspects of Armillaria root rot of tea in Kenya aiming to identify the causal agent and to develop approaches to disease management are summarized in this chapter. An attempt is made to explain the reasons for slow progress in the understanding of species diversity in the African *Armillaria*. Identification of the environmental factors that influence fructification is suggested as a fundamental pre-requisite for conclusive identification of *Armillaria* species in Africa. Soil solarization and the use of antagonistic *Trichoderma* are presented as viable options for controlling Armillaria root rot through eradicating the pathogen from woody inoculum sources in the soil.

Armillaria in Kenya's tea plantations

Characterization of the pathogen

Armillaria is poorly characterised in Africa because of the rare occurrence of basidiomata and rhizomorphs, the key diagnostic features of the fungus in the temperate regions. Although there is much confusion in the literature on the interpretation of African species of *Armillaria*, we tentatively conclude that part of the confusion was created by a too wide interpretation of *A. heimii* by Heim (1963), which partly included characters specific for *A. fuscipes*. Based on the original descriptions in the literature, we provisionally accept that *A. camerunensis*, *A. fuscipes*, *A. heimii*, and possibly *A. mellea s.s.* have been reported from Africa. Although the name *A. mellea* is used quite frequently for some African isolates, no conclusive descriptions of basidiomata of *A. mellea s.s.* have been reported from this continent.

Whenever basidiomata have been found the majority of the isolates have turned out to be homothallic thus excluding the use of mating tests in taxonomic research and prospects for defining 'biological species' in Africa. Most studies on African *Armillaria*

have used methods that do not depend on the presence of the basidiomata to characterise isolates. In this study, characterisation of *Armillaria* pathogenic on tea in Kenya focused mainly on determination of the number of morphological and genetic groups of the fungus. A survey was carried out during which 47 isolates of *Armillaria* were collected from diseased tea and a few other infected hosts in close proximity to tea plantations. The isolates were characterised according to their cultural morphology and differentiated from one another by somatic incompatibility and DNA-based methods that used RAPD and ISSR markers and, in addition, the RFLP of the ITS and IGS regions. During the survey, basidiomata were found only in one tea estate in Kericho, confirming that natural fructification of *Armillaria* takes place in some of Kenya's tea plantations during wet seasons. The frequency of fructification is apparently too sporadic for basidiomata morphology to be used routinely in taxonomic studies. Examination of morphological features of the basidiomata in relation to the characteristics of species that have been reported from Kenya (Pegler, 1977), suggested close resemblance to *A. heimii.* The haploid colonies obtained by culturing basidiospores characteristically changed with age from fluffy to crustose thus indicating homothallism. Cultural morphology separated 33 isolates including those for which the basidiomata were found into a distinct group (Group I) having crustose rhizomorphic colonies. *Armillaria heimii* can exhibit the characteristic observed on monospore cultures since it consists of both heterothallic and homothallic forms (Abomo-Ndongo *et al.*, 1997) and its diploid cultures characteristically have rhizomorphic colonies (Mwangi, pers. comm). This species is widespread across both western and eastern regions of Africa (Mohammed *et al.*, 1994; Abomo-Ndongo *et al.*, 1997) and may be the predominant group among tea pathogenic *Armillaria* in Kenya. The rest of the isolates were differentiated by their more mycelial colony morphology and were categorised separately as Group II.

Rhizomorphs, the other morphological feature that could be used to characterise the isolates, are not frequently found in Africa where their production is restricted by high temperatures (Rishbeth, 1978) and as such may not be suitable for taxonomic research. Under experimental conditions, the majority of *Armillaria* isolates from tea readily produced rhizomorphs in culture and from woody inocula buried in vermiculite indicating that rhizomorph production is inherently characteristic of most of them. The rhizomorphs

produced by woody inocula were typically of the subterranean form, thus contrasting what is frequently found in nature where rhizomorphs, if present, are predominantly cortical or sub-cortical. Exceptions were found for only two of the isolates, $6M_1$ and $9T_2$ (Table 3.1). However, these isolates hardly produced any rhizomorphs in culture or from woody inocula. It is concluded that rhizomorph characteristics are too plastic to be useful in characterising Kenyan isolates especially when production under experimental conditions are extrapolated to the natural situation. Elucidation of the factors that influence rhizomorph production and growth of African *Armillaria* is necessary before they can be used as a reliable taxonomic tool.

Somatic incompatibility has been used by some researchers to distinguish African *Armillaria* isolates into groups presumed to be different species (Guillaumin *et al.*, 1994; Abomo-Ndongo *et al.*, 1997). It is a fast method of distinguishing isolates by the presence of a black line formed along the demarcation zone between paired colonies, in the case of an incompatible reaction. We were able to reveal clear differences between some of the isolates in Group II only when a modified method described by Hopkin *et al.* (1988) that is devised to increase the intensity of the black line formation between incompatible colonies was applied. It clearly showed the incompatibility reaction between isolates within Group II and separated these into five somatic incompatibility groups. All Group I isolates were intercompatible but were incompatible with the isolates in Group II. Genetic analysis of DNA profiles based on RAPD, ISSR, RFLP of the IGS and ITS regions confirmed the distinction between the groups and conformed with cultural morphology in distinguishing the two groups. For Group I, the molecular method conformed entirely to somatic incompatibility tests and further revealed genetic variability among the 33 isolates, placing these into 3 sub-groups depicted in Figure 3.7. This group constitutes the major *Armillaria* pathogenic on tea in Kenya. Group II is genetically more homogeneous but, unlike cultural morphology and the DNA-based techniques, somatic incompatibility separated the isolates in this group into five sub-groups. The disparity in results of the two methods raises the question of the reliability of somatic incompatibility as a criterion for distinguishing species and suggests that care should be taken in interpreting its results.

In addition to *A. heimii*, isolates of a different *Armillaria* have been reported from the western and the eastern regions of Africa (Mohammed *et al.*, 1994; Chillali *et al.*,

1997). These have been named using the invalidly published taxon *A. mellea* 'spp. *africana*'. However, descriptions of basidiomata of these isolates have not been published. It is therefore difficult to verify their identity. Irrespective of whether these isolates could indeed be named as *A. mellea s.s.*, their comparison with our Group II isolates clearly showed that they are different suggesting the existence of another species. As no basidiomata of Group II isolates have been found, their identification is still impossible. Even if fruiting bodies would be obtained for Group II isolates by *in vitro* induction, it is questionable whether morphology of basidiomata produced would serve as a basis for species description because basidiomata produced *in vitro* often appear atypical. Nomenclature of this taxon is difficult if it has completely lost the ability to form fruiting bodies.

As little seems to have been achieved in nomenclature of African *Armilaria* so far, there is a need to explore various methods for determining species diversity in the continent. Biochemical and molecular methods are likely to remain the preferred tools for accomplishing this. While nomenclature of *Armillaria* species continues to be based solely on basidiome morphology and mating tests, species description in Africa will still be difficult. Evidence from studies based on biochemical and molecular studies point to clear differences of several isolates from the described species in this continent. Further differences between isolates referred to as *A. mellea* 'ssp. *africana*' and the temperate *A. mellea s.s.* may be due to a misnomer for the former, having been described when the temperate *A. mellea s.s* was considered part of an *A. mellea* complex. The observations from this study and from an earlier study (Mohammed *et al.*, 1994) that some Kenyan isolates could belong to a new species distinct from both *A. mellea* and *A. heimii* may indicate the existence of three different *Armillaria* species in Kenya. The situation reported by Coetzee *et al.* (2000) for South Africa implies the distinction of *A. heimii* from *A. fuscipes*. It is probable that species diversity in the African *Armillaria* is larger than currently thought to be the case.

In summary, according to morphological data, basidiomata and colony characteristics in culture, Group I was provisionally identified as *A. heimii* and occurs in all locations where tea is grown. Group II is probably a new *Armillaria* species and has a more restricted distribution. Thus it is concluded that at least two different species of

Armillaria cause root rot of tea in Kenya. Conclusive identification of these groups should be the focus of future research on species diversity in Kenyan *Armillaria*.

Control of Armillaria root rot

The scarcity of basidiomata and rhizomorphs hinders diagnosis of Armillaria root rot in Africa. It is probable that many cases of the disease have regularly gone unreported and its significance overlooked. The current status of knowledge on the disease reviewed in chapter 2 of this thesis shows that *Armillaria* may be causing considerable damage in several economically important crops. Without improving the understanding on the methods for accurate disease diagnosis and appropriate control methods, it is likely that the situation will worsen. Whereas mechanical removal of potential inoculum sources from the infested sites seems to be an effective method of disease control in plantations of commercial crops such as tea, this practice is unpopular with growers who cannot afford the use of heavy machinery and must rely on manual operations. Hence the need to develop methods that are easier to apply.

Three options were considered in this study as alternative methods of controlling Armillaria root rot of tea. These involved attempts to eradicate the pathogen from infected plant materials in the soil at pre-infection stage by using biocontrol agents, soil organic amendment and/or physical soil disinfestation accomplished by solarization. Species of *Trichoderma* antagonise several plant pathogenic fungi including *Armillaria*. Few reports exist on their use to control Armillaria root rot in practice. The failure to achieve biological disease control with *Trichoderma* had been attributed to low population density of strains antagonistic to *Armillaria* in most soils (Garrett, 1958) and to resistance of *Armillaria* to mycoparasitism (Munnecke *et al.*, 1981; Fox, 2000). The performance of *Trichoderma* against *Armillaria* in infected stumps has been evaluated in a few studies with promising results (Rishbeth, 1976; Nelson *et al.*, 1995). In this study artificial introduction into the soil of a *Trichoderma* isolate selected on the basis of the capacity to antagonise *Armillaria* in woody plant materials was evaluated in combination with soil amendment with coffee pulp or solarization for effect on survival of the pathogen.

Isolates of *Trichoderma* were screened for inhibition of *Armillaria* growth in stem sections of tea using the method of Nelson *et al.* (1989). This enabled the selection of one

T. harzianum isolate based on the capacity of the antagonist to inhibit colonisation of plant materials by *Armillaria* and for the effect on the pathogen after it colonised the woody inocula. The approach demonstrated that despite some *Trichoderma* isolates showing antagonism to *Armillaria* in synthetic media, this is not invariably reproducible *in vivo.* However, an isolate selected on the basis of antagonism to the pathogen in the plant materials still antagonised it when introduced into the soil surrounding the inoculum sources as wheat bran culture, and reduced its viability within plant materials. Normally *Armillaria* survives in the soil as rhizomorphs and more commonly as mycelia associated with plant materials that serve as its food bases (Munnecke *et al.*, 1981). The scarcity of rhizomorphs in Africa means that infected wood is the main inoculum source and the application of *Trichoderma* for controlling Armillaria root rot should target the pathogen at this pre-infection stage. The ability of the antagonist present in the soil to colonise *Armillaria* food bases is an essential step in bringing hyphae of the two fungi into physical contact thereby creating the opportunity for parasitism. In addition to or in conjunction with mycoparasitism, individual strains of *Trichoderma* may produce antibiotics which further limit growth and activity of their host fungi (Benhamou and Chet, 1997) and in the soil, production of these close to their site of action may be essential for avoiding their inactivation before they exert the desired effect on the target fungus. Although wheat bran culture of *T. harzianum* has been shown to have high activity of lytic enzymes (Elad, *et al.*, 1982), action of such enzymes against *Armillaria* would be useful only when they are produced within the food bases. Wheat bran was therefore used in the present study mainly because it provided a convenient means of culturing and applying *T. harzianum* to the soil.

Soil amendment with coffee pulp had been shown in an earlier study (Onsando and Waudo, 1992) to encourage proliferation of indigenous *Trichoderma* in the soil and was included in these experiments as one of the treatments. However, it did not affect the presence of *Trichoderma* in woody *Armillaria* inoculum sources but had a suppressive effect on inoculum viability apparently due to a mode of action independent of the action of *Trichoderma*. Earlier studies (Bliss, 1951; Munnecke *et al*, 1973, 1976, 1981) reported improved efficacy of *T. viride* against *A. mellea* after the pathogen had been subjected to sub-lethal levels of chemical fumigants, heat, or desiccation. Munnecke *et al.* (1981)

concluded that this was due to a stressing effect that weakened or destroyed its defence mechanisms of *A. mellea* against *T. viride.* The present study tried to achieve the same effect through soil solarization. This treatment was very strongly suppressive of *Armillaria* viability and the pathogen could not be recovered from inoculum sources after 10 wk solarization. When *T. harzianum* was applied after solarization for 5 wk the degree of inactivation of *Armillaria* was similar to that attained by solarization alone for 10 wk. This confirmed that antagonism of *Trichoderma* is more effective against *Armillaria* when used in conjunction with partial soil disinfestation.

By adversely affecting the inoculum viability, soil solarization and, to a lesser extent, organic amendment with coffee pulp, enhanced the efficacy of *T. harzianum* against *Armillaria.* The mechanisms of *Armillaria* suppression were not investigated but in the case of solarization it can be attributed to inactivation by exposure to high soil temperatures. Solarization, through partial soil disinfestation, may create a (partial) biological vacuum that allows rapid establishment of the introduced *T. harzianum* while exposure of *Armillaria* to high temperatures could also weaken its resistance and make it more vulnerable to *T. harzianum.* Thus better disease control would be attained through the integrated action of the two factors rather than each of them alone.

Application of multiple disease control factors is the foundation of improved integrated disease control strategies and is exemplified here by the combined application of solarization and *T. harzianum* against *Armillaria.* Natural soil population densities of antagonistic *Trichoderma* may not be sufficiently high for effective practical control of Armillaria root rot hence a highly effective biocontrol strain with appropriate mechanisms must be identified by screening using procedures relevant to the ecology of *Armillaria.* In addition, the soil environment should be modified to allow for efficient interaction of the introduced antagonist with the pathogen, which, in the case of *Armillaria*, requires colonisation of the food bases. This approach based first on finding an antagonist and then applying it to a modified soil environment to control the disease, requires production of the biocontrol agent and its application in a formulation that will facilitate competition with indigenous soil microbiota in colonising the niche occupied by the pathogen. The results from experiments presented in chapters 5 and 6 indicate that soil solarization followed by soil infestation with *T. harzianum* presumably can be used

as a partial substitute for mechanical removal of woody inoculum sources to control *Armillaria* in small patches such as those found at the infection centres in tea plantations.

References

Abomo-Ndongo, S. and Guillaumin J.J., 1997. Somatic incompatibility among African *Armillaria* isolates. European Journal of Forest Pathology 27: 201-206.

Abomo-Ndongo, S., Mohammed, C. and Guillaumin, J.J., 1997. Sexual behaviour of *Armillaria heimii* and *A. mellea* isolates from Africa. European Journal of Forest Pathology 27: 207-224.

Agustian, A., Mohammed, C., Guillaumin, J.J. and Botto, B., 1994. Discrimination of some African *Armillaria* species by isozyme electrophoretic analysis. New Phytologist 128: 135-143.

Ahmad, Y., Hameed, A. and Aslam, M., 1996. Effect of soil solarization on corn stalk rot. Plant and Soil 179: 17-24.

Ahmed, A.S., Pérez-Sanchez, C. and Candela, M.E., 2001. Evaluation of induction of systemic resistance in pepper plants (*Capsicum annuum*) to *Phytophthora capsici* using *Trichoderma harzianum* and its relation with capsidiol accumulation. European Journal of Plant Pathology 106: 817-824.

Alabouvette, C., Rouxel, F. and Louvet, J., 1979. Characteristics of Fusarium wilt-suppressive soils and prospects for their utilization in biological control. B. Schippers and W. Gams (Eds.), Soil-Borne Plant Pathogens. Academic Press, London, pp. 165-182.

Anderson, J.B., 1986. Biological species of *Armillaria mellea* in North America: Redesignation of Groups IV and VII and enumeration of Voucher strains of other groups. Mycologia 78: 837-839.

Anderson, J.B., Petsche, D.M. and Smith, M.L., 1987. Restriction fragment polymorphisms in biological species of *Armillaria*. Mycologia 79: 69-76.

Anderson, J.B. and Stasovski, E., 1992. Molecular phylogeny of northern hemisphere species of *Armillaria*. Mycologia 84: 505-516.

Anonymous, 1986. Tea Growers Handbook. 4th Ed. Tea Research Foundation of Kenya, 249 pp.

Arulpragasam, P.V., 1992. Disease control in Asia. In: K.C. Willson and M.N. Clifford (Eds.), Tea: Cultivation to Consumption. Chapman & Hall, London.

Baker, K.F., 1987. Evolving concepts of biological control of plant pathogens. Annual Review of Phytopathology 25: 67-85.

Baker, K.F. and Cook, R.J., 1974. Biological control of plant pathogens. American Phytopathological Society, St. Paul, 433 pp.

Banik, M.T., Paul, J.A. and Burdshall, H.H., 1995. Identification of *Armillaria* species from Wisconsin and adjacent areas. Mycologia 87: 707-712.

Beeli, M., 1927. Contribution à l'étude de la Flore Mycologique du Congo II. Bulletin de la Société Royale de Botanique de Belgique 109 (Série II,9): 101-112.

Benhamou, N. and Chet, I., 1993. Hyphal interactions between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold cytochemistry of the mycoparasitic process. Phytopathology 83: 1062-1071.

Benhamou, N. and Chet, I., 1997. Cellular and molecular mechanisms involved in the interaction between *Trichoderma harzianum* and *Pythium ultimum*. Applied and Environmental Microbiology 63: 2095-2099.

Bihan, B. le, Soulas, M.L., Camporota, P., Selerno, M.I. and Perrin, R., 1997. Evaluation of soil solar heating for control of damping-off fungi in two forest nurseries in France. Biology and Fertility of Soils 25: 189-195.

Bliss, D.E., 1951. The destruction of *Armillaria mellea* in citrus soils. Phytopathology 41: 665-683.

Bohra, M.D., Harsh, L.N. and Lodha, S., 1996. Solar heating for controlling pathogens of jojoba (*Simmondsia chinensis*) in nursery soils. Indian Journal of Agricultural Sciences 66: 679-683.

Carr, M.K.V. and Stephens, W., 1992. Climate, weather and the yield of tea. In: K.C. Willson and M.N. Clifford (Eds.), Tea: Cultivation to Consumption. Chapman and Hall, London.

Cha, J.Y., Sung, J.M. and Igarashi, T., 1994. Biological species and morphological characteristics of *Armillaria mellea* complex in Hokkaido: *A. sinapina* and two new species, *A. jezoensis* and *A. singula*. Mycoscience 35: 39-47.

Chellemi, D. O., Olson, S. M., Mitchell, D. J., Secker, I. and McSorley, R., 1997. Adaptation of soil solarization to the integrated management of soil borne pests of tomato under humid conditions. Phytopathology 87:250-258.

Chillali, M., Idder-Ighili, H., Guillaumin, J.J., Mohammed, C. and Botton, B., 1997. Species delimitation in the African *Armillaria* complex by analysis of the ribosomal DNA spacers. Journal of General Applied Microbiology 43: 23-29.

Coetzee, M.P.A., Wingfield, B.D., Wingfield, M.J. and Coutinho, T.A., 1997. Identification of the causal agent of Armillaria root rot in South African forest plantations. In: C. Delatour, J.J. Guillaumin, B. Lung-Escarmant and B. Marçais (Eds.), Root and butt rots of forest trees. Carcans-Maubuisson (France), 1-7 September 1997, INRA, Paris (Les Colloques no. 89), pp. 49-61.

Coetzee, M. P. A., Wingfield, B. B., Coutinho, T. A. and Wingfield, M. J., 2000. Identification of the causal agent of Armillaria root rot of *Pinus* species in South Africa. Mycologia 92(4): 777-785.

Dadant, R., 1963. Contribution à l'étude du pourridié du caférer causé par le *Clitocybe elegans* Heim à Madagascar. Revue de Mycologie 28: 95-168.

Davis, J.R., 1991. Soil solarization: pathogen and disease control and increases in crop yield and quality: short- and long-term effects and integrated control. In: J. Katan and J.E. DeVay (Eds.), Soil solarization, pp. 39-50.

Denner, F.D.N., Millard, C.P. and Wehner, F.C., 2000. Effect of soil solarization and mouldboard ploughing on black dot of potato, caused by *Colletotrichum coccodes*. Potato Research 43: 195-201.

Duchesne, L.C. and Anderson, J.B., 1990. Location and direction of transcription of the 5S rRNA gene in *Armillaria*. Mycological Research 94: 266-269.

Dumas, M.T. and Boyonoski, W., 1992. Scanning electron microscopy of mycoparasitism of *Armillaria* rhizomorphs by species of *Trichoderma*. European Journal of Forest Pathology 22: 379-383.

Elad, Y., Hadar, Y., Hadar, E., Chet, I. and Henis, Y., 1981. Biological control of *Rhizoctonia solani* by *Trichoderma harzianum* in carnation. Plant Disease 65: 675-677.

Elad, Y., Chet, I. and Henis, Y., 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Canadian Journal of Microbiology 28: 719-725.

Entry, J. A., Martin, N. E., Cromack, K. and Stafford, S. G. 1986. Light and nutrient limitation in *Pinus monticola* seedling susceptibility to *Armillaria* infection. Forest Ecology and Management 17: 189-198.

Entry, J. A., Cromack, K. and Waring, R., 1991(a). Response of western coniferous seedlings to infection by *Armillaria ostoyae* under limited light and nitrogen. Phytopathology 81: 89-94.

Entry, J. A., Cromack, K., Kesley, R. G. and Martin, N. E., 1991(b). Response of Douglas fir to infection by *Armillaria ostoyae* after thinning or thinning plus fertilization. Phytopathology 81: 682-689.

Filip, G.M. and Yang, E.L., 1997. Effects of prescribed burning on the viability of *Armillaria ostoyae* in mixed-conifer forest soils in the Blue Mountains of Oregon. Northwest Science 71: 137-144.

Fox, R.T.V., 2000. Cultural methods to manage *Armillaria*. In: R.T.V. Fox (Ed.), Armillaria root rot: biology and control of Honey Fungus. Intercept, UK, pp. 151-171.

Fox, R.T.V., McQue, A.M., West, J.S. and Raziq, F., 1994. Use of antagonistic fungi to control Armillaria root rot. Brighton Crop Protection Conference, Pests and Diseases 3: 1115-1120.

Garrett, S.D., 1958. Inoculum potential as a factor limiting action of *Trichoderma viride* Fr. on *Armillaria mellea* (Fr.) Quél. Transactions of the British mycological Society 41: 157-164.

Gibson, I.A.S., 1960a. A note on the variation between isolates of *Armillaria mellea* (Vahl ex Fr.) Kummer. Transactions of the British mycological Society 44: 123-128.

Gibson, I.A.S., 1960b. Armillaria root rot in Kenya pine plantations. Empire Forestry Review 39: 94-99.

Gibson, I.A.S. and Goodchild, N.A., 1960. *Armillaria mellea* in Kenya tea plantations. Proceedings of the Commonwealth Mycological Conference, pp. 39-40.

Goodchild, N.A., 1960. *Armillaria mellea* in tea plantations. Journal of Tea Boards of East Africa, pp. 33-45.

Greenberger, A., Yogev, A. and Katan, J., 1987. Induced suppressiveness in solarized soils. Phytopathology 77: 1663-1660.

Grzywacz, A. and Wazny, J., 1973. The impact of industrial air pollutants on the occurrence of several pathogenic fungi of forest trees in Poland. European Journal of Forest Pathology 3: 129-141.

Guillaumin, J.J., 1988. The *Armillaria mellea* complex. In: I.M. Smith, J. Dunez, D.H. Phillips, R.A. Lelliot and S.A. Archer (Eds.), European handbook of plant diseases. Blackwell Scientific Publications, Oxford, pp. 520-523.

Guillaumin, J.J., Anderson, J.B. and Korhonen, K., 1991. Life cycle, interfertility and biological species. In: C.G. Shaw and G.A. Kile (Eds.), Armillaria Root Disease. Agricultural Handbook No 691, United States Department of Agriculture, Washington, D.C., pp. 10-20.

Guillaumin, J. J., Mohammed, C. and Abomo-Ndongo, S., 1993 Vegetative incompatibility and sexual systems of *Armillaria* isolates from tropical Africa. In: Johnsson, M. and Stenlid, J. (Eds.), Proceedings of 8th IUFRO Conference on root and butt rots. Swedish University of Agricultural Science, Uppsala, pp 349-354.

Guyot, J., 1997. *Armillaria* spp., *Havea* root rot in Central Africa: the case of Gabon. Plantations, recherche, developpement, Nov.-Dec. 1997, pp. 416-419.

Harrington, T.C. and Wingfield, B.D., 1995. A PCR-based identification method for species in *Armillaria*. Mycological Research 94: 266-269.

Harris, A.R., 1999. Biocontrol of *Rhizoctonia solani* and *Pythium ultimum* on *Capsicum* by *Trichoderma koningii* in potting medium. Microbiological Research 154: 131-135.

Heim, R., 1963. L'*Armillaria* elegans Heim. Revue de Mycologie 28: 89-94.

Heim, R. and Bouriquet, L., 1937. Maladies des Albizzia à Madagascar. Revue Bot. appl. 17: 405-412.

Henis, Y., Ghaffer, A. and Baker, R., 1979. Factors affecting suppression to *Rhizoctonia solani* in soil. Phytopathology 69: 1164-1169.

Hennings, P., 1895. Fungi camerunensis novi I. Bot. Jb. 22: 72-111.

Hintikka, V., 1973. A note on the polarity of *Armillariella mellea*. Karstenia 13: 32-39.

Hjeljord, L. and Tronsmo, A., 1998. *Trichoderma* and *Gliocladium* in biological control: an overview. In: G.E. Harman and C.P. Kubicek (Eds.), *Trichoderma* and *Gliocladium* Vol. 2. Taylor and Francis Ltd., pp.131-145.

Hood, I.A. and Sandberg, C.J., 1987. Occurrence of *Armillaria* rhizomorph populations in the soil beneath indigenous forests in the Bay of Plenty, New Zealand. New Zealand Journal of Forestry Science 17: 83-99.

Hopkin, A.A., Mallet, K.I. and Blenis, P.V., 1989. The use of L-DOPA to enhance visualization of the 'black line' between species of the *Armillaria mellea* complex. Canadian Journal of Botany 67: 15-17.

Huang, S., 1989. Meteorology of the tea plant: A review. Agricultural and Forest Meteorology 47: 19-30.

Huddack, J. and Wells, R.E., 1974. Armillaria root rot in aphid damaged Balsam fir in Newfoundland. Forestry Chronicle 50: 74-76.

Hulme, M.A. and Stranks, D.W., 1970. Induction and regulation of production of cellulase by fungi. Nature 226: 469-470.

Ivory, M.H., 1987. In: Diseases and Disorders of pines in the tropics - a Field and Laboratory Manual, pp 12-16; Overseas Research Publication, Her Majesty's Stationery Office no 31. Published for the Overseas Development Association by the Oxford Forestry Institute: Oxford.

Jahnke, K.D., Bahnweg, G. and Worral, J.J., 1987. Species delimitation in the *Armillaria mellea* complex by analysis of nuclear and mitochondrial DNAs. Transactions of the British mycological Society 88: 572-575.

Katan, J., 1981. Solar heating (solarization) of soil for control of soil borne pests. Annual Review of Phytopathology 19: 211-236.

Kile, G.A, McDonald, G.E and Byler, J.W., 1991. Ecology and disease in natural forests. In: C.G. Shaw and G.A. Kile (Eds.), Armillaria root disease. Washington D.C, United States Department of Agriculture, pp. 102-121.

Kile, G.A., Guillaumin, J.J., Mohammed, C. and Watling, R., 1993 Biogeography and pathology of *Armillaria*. In: M. Johansson and J. Stenlid (Eds.), Proceedings of the 8th IUFRO Conference on root and butt rots. Swedish University of Agricultural Science, Uppsala, pp. 411-436.

Klein, D. and Eveleigh, D.E., 1998. Ecology of *Trichoderma*. In: C.P. Kubicek and G.E. Harman (Eds.), *Trichoderma* and *Gliocladium*; basic biology, taxonomy and genetics, Vol. 1. Taylor & Francis, London, pp. 57-74.

Korhonen, K., 1978. Interfertility and clonal size in the *Armillaria mellea* complex. Karstenia 18: 31-42.

Leach, R., 1939. Biological control and ecology of *Armillaria mellea* (Vahl) Fr. Transactions of the British mycological Society 23: 320-329.

Lewis, J.A. and Papavizas, G.C., 1984. A new approach to stimulate population proliferation of *Trichoderma* spp. and other potential biocontrol fungi introduced into natural soils. Phytopathology 74: 1240-1244.

Li, Y. and Hood, I.A., 1992. A preliminary study into the biological control of *Armillaria novae-zelandiae* and *A. limonea*. Australasian Plant Pathology 21: 24-28.

Lodha, S., Sharma, S. and Aggarwal, R.K., 1997. Solarization and natural heating of irrigated soil amended with cruciferous residues for improved control of *Macrophomina phaseolina*. Plant Pathology 46: 186-190.

López-Escuredo, F.J. and Blanco-López, M.A., 2001. Effects of single or double soil solarization to control Verticillium wilt in established olive orchards in Spain. Plant Disease 85: 489-496.

Maddison, W.P. and Madisson, D.R., 1992. MacClade: analysis of phylogeny and character evolution. Version 3.0. Sinauer Associates, Saunderland, Massachusetts.

Makambila, C., 1980. Cassava root rot due to *Armillaria tabescens* in Congo. In: Tropical root crops. Proceedings of the First Triannual Root Crops Symposium of the International Society of Tropical Root Crops. Ibadan, Nigeria.

Mallet, K.I. and Hiratsuka Y., 1986. Nature of the 'black line' produced between different biological species of the *Armillaria mellea* complex. Canadian Journal of Botany 64: 2588–2590.

Marin, J.P., 1950. Use of acid, rose bengal, and streptomycin in the plate method for estimating soil fungi. Soil Science 69: 215-232.

Marois, J.J. and Mitchell, D.J., 1981. Effects of fumigation and fungal antagonists on the relationships of inoculum density to infection incidence and disease severity in Fusarium crown rot of tomato. Phytopathology 71: 167-170.

Masuka, A.J., 1993 *Armillaria* occurrence and management in Zimbabwe. In: Johansson, M. and Stenlid, J. (Eds.), Proceedings of the 8th IUFRO Conference on root and butt rots. Swedish University of Agricultural Science, Uppsala, pp. 388-392.

McLean, K.L., Swaminathan, J. and Stewart, A., 2001. Increasing soil temperature to reduce sclerotial viability of *Sclerotium cepivorum* in New Zealand soil. Soil Biology and Biochemistry 33: 137-143.

Melero-Vara, J.M., Blanco-López, M.A., Béjarano-Alcázar, J. and Jiménez-Díaz, R.M., 1995. Control of Verticillium wilt of cotton by means of soil solarization and tolerant ultivars in Southern Spain. Plant Pathology 44: 250-260.

Melero-Vara, J.M., Prados-Ligero, A.M. and Basallote-Ureba, M.J., 2000. Comparison of physical, chemical and biological methods of controlling garlic white rot. European Journal of Plant Pathology 106: 581-588.

Merenco, R.A. and Lustosa, D.C., 2000. Soil solarization for weed control in carrot. Pesquisa Agropecuria brasileira 35: 2025-2032.

Miller, R.N.G., Holderness, M., Bridge, P.D., Chung, G.F. and Zakaria, M.H., 1999. Genetic diversity of *Ganoderma* in oil palm plantings. Plant Pathology 48: 595-603.

Mohammed, C., 1994. The detection and species identification of African *Armillaria*. In: A. Schots, F.M. Dewey and R.P. Oliver (Eds.), Modern assays for plant pathogenic fungi; identification, detection and quantification. Willingford, CABI, pp. 141-147.

Mohammed, C. and Guillaumin, J.J., 1994. *Armillaria* in tropical Africa. In: S. Isaac, J.C. Frankland and R. Watling (Eds.), Aspects of Tropical Mycology. Cambridge: Cambridge University Press, pp. 207–217.

Mohammed, C., Guillaumin, J.J., Botton, B. and Intini, M., 1993 Species of *Armillaria* in tropical Africa. In: M. Johansson and J. Stenlid (Eds.), Proceedings of the 8th IUFRO Conference on root and butt rots. Swedish University of Agricultural Science, Uppsala, pp. 402–410.

Morrison, D.J., 1982. Effects of soil organic matter on rhizomorph growth by *Armillaria mellea*. Transactions of British mycological Society 78: 201-207.

Munnecke, D.E., Kolbezen, M.J. and Wilbur, W.D., 1973. Effect of methyl bromide and carbon disulphide on *Armillaria* and *Trichoderma* growing on agar medium in relation to survival of *Armillaria* in soil following fumigation. Phytopathology 63: 1353-1357.

Munnecke, D.E., Wilbur, W.D. and Darley, E.F., 1976. Effect of heating or drying on *Armillaria mellea* or *Trichoderma viride* and the relation to survival of *A. mellea* in the soil. Phytopathology 66: 1363-1368.

Munnecke, D.E., Kolbezen, M.J., Wilbur, E.D. and Ohr, H., 1981. Interactions involved in controlling *Armillaria mellea.* Plant Disease 65: 384-389.

Mwangi, L.M, Lin, D. and Hubbes, M., 1989. Identification of Kenyan *Armillaria* isolates by cultural morphology, intersterility tests and analysis of isozyme profiles. European Journal of Forest Pathology 19: 399-406.

Mwangi, L., Mwenje, E., Makambila, C., Chanakira-Nyahwa, F., Guillaumin, J.J. and Mohammed, C., 1993 Ecology and pathogenicity of *Armillaria* in Kenya, Zimbabwe, and the Congo. In: Johansson, M. and Stenlid, J. (Eds.), Proceedings of the 8th IUFRO Conference on root and butt rots. Swedish University of Agricultural Science, Uppsala, pp. 34-44.

Mwenje, E. and Ride, J.P., 1996. Morphological and biochemical characterization of *Armillaria* isolates from Zimbabwe. Plant Pathology 45: 1031-1051.

Mwenje, E. and Ride, J.P., 1997. The use of pectic enzymes in the characterization of *Armillaria* isolates from Africa. Plant Pathology 46: 341-354.

Mwenje, E., Ride, J.P. and Pearce, R.B., 1998. Distribution of Zimbabwean *Armillaria* groups and their pathogenicity on cassava. Plant Pathology 47: 623-634.

Nei, M. and Li, W.H., 1979. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

Nelson, E.E., Pearce, N.H. and Malajczuk, N., 1989. Competitive colonization of karri (*Eucalyptus diversicolor*) stem sections by *Armillaria luteobubalina* and *Trichoderma* spp. Proceedings of 7th International Conference on root and butt rots of forest trees, pp. 79-83.

Nelson, E.E., Pearce, M.H. and Malajczuck, N., 1995. Effect of *Trichoderma* spp. and ammonium sulphamate on establishment of *Armilaria luteobubalina* on stumps of *Eucalyptus diversicolor.* Mycological Research 99: 957-962.

Ohr, H.D. and Munnecke, D.E., 1974. Effects of methyl bromide on antibiotic production by *Armillaria mellea*. Transactions of the British mycological Society 62: 65-72.

Onsando, J.M. and Waudo, S.W., 1992. Effect of coffee pulp on *Trichoderma* spp. in Kenyan tea soils. Tropical Pest Management 38: 376-381.

Onsando, J.M. and Waudo, S.W., 1994. Interaction between *Trichoderma* species and Armillaria root rot fungus of tea in Kenya. International Journal of Pest Management 40: 69-74.

Onsando, J.M., Wargo, P. and Waudo, S.W., 1997. Distribution, severity, and spread of Armillaria root disease in Kenya tea plantations. Plant Disease 81: 133–137.

Ota, Y., Fukada, K. and Suzuki, K., 1998. The non heterothallic life cycle of *Armillaria mellea*. Mycologia 90: 396–405.

Ota, Y., Intini, M. and Hattori, T., 2000. Genetic characterization of heterothallic and non-heterothallic *Armillaria mellea sensu stricto*. Mycological Research 104: 1046–1054.

Pearce, M.H. and Malajczuk, N., 1990. Factors affecting growth of *Armillaria luteobubalina* rhizomorphs in soil. Mycological Research 94: 38-48.

Pegler, D.N., 1977. A preliminary agaric flora of East Africa. Kew Bull. addit. Ser. 6: 1-615.

Petch, T., 1909. New Ceylon fungi. Annals of the Royal Botanic Gardens Peradeniya 4: 299-307.

Petch, T., 1923. The diseases of the tea bush. London.

Pinkerton, J.N., Ivors, K.L., Miller, M.L. and Moore, L.W., 2000. Effect of soil solarization and cover crops on populations of selected soil-borne plant pathogens in western Oregon. Plant Disease 89: 952-960.

Pichel, R.J., 1956. Les pourridies de l'Hevea dans la cuvette congolaise. Publication of the Institut National pour l'Etude Agronomique du Congo Belge, Series Techniques No. 49.

Popoola, T.O.S. and Fox, R.T.V., 1996. Effect of root damage on honey fungus. Arboricultural Journal 20: 329-337.

Ramirez-Villapudua, J. and Munnecke, D.E., 1988. Effect of solar heating and soil amendments of cruciferous residues on *Fusarium oxysporum* f.sp. *conglutinans* and other organisms. Phytopathology 78: 289-295.

Raziq, F., 2000. Biological and integrated control of Armillaria root rot. In: R.T.V. Fox (Ed.), Armillaria root rot: biology and control of the Honey Fungus. Intercept, UK, pp. 183-201.

Reaves, J.L., Shaw, C.G. and Mayfield, J.E., 1990. The effects of *Trichoderma* spp. isolated from burned and non-burned forest soils on the growth and development of *Armillaria ostoyae* in culture. Northwest Science 64: 39-44.

Redfern, D.B., 1973. Growth and behaviour of *Armillaria mellea* rhizomorphs in soil. Transactions of the British mycological Society 61: 569-581.

Redfern, D.B., 1978. Infection by *Armillaria mellea* and some factors affecting host resistance. Forestry 51: 120-135.

Rishbeth, J., 1976. Chemical treatment and inoculation of hardwood stumps for control of *Armillaria mellea.* Annals of Applied Biology 82: 57-70.

Rishbeth, J., 1978. Effects of soil temperature and atmosphere on growth of Armillaria rhizomorphs. Transactions of British mycological society 70:213-220.

Rishbeth, J., 1980. *Armillaria* on cocoa in São Tomé. Tropical Agriculture (Trinidad) 57: 155-165.

Ristaino, J.B., Perry, K.B. and Lumsden, R.D., 1991. Effect of solarization and *Gliocladium virens* on sclerotia of *Sclerotium rolfsii,* soil microbiota, and the incidence of southern blight of tomato. Phytopathology 81: 1117-1124.

Rizzo, D.M. and Harrington, T.C., 1993. Delineation and biology of clones of *Armillaria ostoyae*, *A. gemina*, and *A. calvescens*. Mycologia 85: 164–174.

Sambrook, J., Fritsch, F.E. and Maniatis, T., 1989. Molecular cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sastry, R.K. and Chattopadhyay, C., 1999. Effect of soil solarization on *Fusarium oxysporum* f. sp. *carthami* populations in endemic soils. Indian Phytopathology 52: 51-55.

Schoeman, M., Webber, J. and Dickson, D., 1994. A rapid method for screening potential biological control agents of wood decay. European Journal of Forest Pathology 24: 154-159.

Score, A.J. and Palfreyman, J.W., 1994. Biological control of *Serpula lacrymans* by *Trichoderma* species: the effects of complex and synthetic media on interaction

and hyphal extension rates. International Journal Biodeterioration and Biodegradation 33: 115-128.

Seif, A.A. and Whittle, A.M., 1984. Diseases of citrus in Kenya. FAO Plant protection Bulletin 32: 122-127.

Shearer, B.L., Crane, C.E., Fairman, R.G. and Grant, M.J., 1997. Occurrence of *Armillaria luteobubalina* and pathogen mediated changes in coastal dune vegetation of south-western Australia. Australian Journal of Botany 45: 905-917.

Siepmann, R. von, 1987. Kriterien zur Beurteilung der Reaktion haploider Teste mit diploiden Armillaria -Isolierungen. European Journal of Forest Pathology 17: 308-311.

Siestma, J. H. and Wessels, J. G. H., 1979. Evidence for covalent linkages between chitin and β-glucan in fungal cell wall. Journal of General Microbiology 114: 99-108.

Singer, R., 1986. The Agaricales in modern taxonomy. 4th ed. Konigstein. Koeltz Scientific Books, 981 pp.

Singh, P., 1983. Armillaria root rot: influence of soil nutrients and pH on the susceptibility of conifer species to the disease. European Journal of Forest Pathology 13: 92-101.

Smith, M.L and Anderson, J.B., 1989. Restriction fragment length polymorphism in mitochondrial DNAs of *Armillaria*: identification of North American biological species. Mycological Research 93: 247-256.

Smouse, J., Foster, D., Freitag, C. and Morrel, J.J., 1999. Ability of selected *Trichoderma* spp. to inhibit microbial discoloration of ponderosa pine sapwood. Material und Organismen 33:107-118.

Snedecor, G.W. and Cochran, W.G., 1989. Statistical methods, 8th Edition. Iowa State University Press/AMES, 289 pp.

Sreenivasaprasad, S. and Manibhushanrao, K., 1993. Efficacy of *Gliocladium virens* and *Trichoderma longibrachiatum* as biological control agents of groundnut root and stem rot diseases. International Journal of Pest Management 39: 167-171.

Swift, M.J., 1964. *Armillaria mellea* diseases in Central Africa. Report Issued on Behalf of Interested Parties. Harare: University of Zimbabwe, 5 pp.

Swift, M.J., 1968. Inhibition of rhizomorph development by *Armillaria mellea* in Rhodesian forest soils. Transactions of British mycological Society 51: 241-247.

Swofford, D.L., 1998. Phylogenetic analysis using parsimony 4.0b2 version. Sinauer Associates, Saunderland, Massachusetts.

Termorshuizen, A.J. and Arnolds, E.J.M., 1987. On the nomenclture of the European species of *Armillaria mellea* group. Mycotaxon 30: 101-116.

Thrane, C., Tronsmo, A. and Jensen, D.F., 1997. Endo-1,3-β-1,3-glucanase and cellulase from *Trichoderma harzianum*: purification and partial characterization, induction of and biological activity against plant pathogenic *Pythium* spp. European Journal of Plant Pathology 103: 331-344.

Tucker, E.J.B., Bruce, A. and Staines, H.J., 1997. Application of modified international wood preservative chemical testing standards for assessment of biocontrol treatments. International biodeterioration and biodegradation 39: 189-197.

Veigh, I., Foucher, S. and Le-Berre, A., 1994. Phytopathological consequences of pruning planee trees in urban environments and screening for natural antagonists against wood-decay basidiomycetes. Acta Phytopathologica et Entomologica Hungarica 29: 315-328.

Veldman, G.M., Klootwijk, J., Regt, V.C.H.F de and Rudi, R.J., 1981. The primary and secondary structure of yeast 26S rRNA. Nucleic Acids Research 9: 6935–6952.

Volk, J.T and Burdsall, H., 1995. A nomenclatural study of *Armillaria* and *Armillariella* species (Basidiomycotina, Tricholomataceae). Synopsis Fungorum 8. Fungiflora, Oslo, Norway.

Vossen, H.A.M. van der and Wessel, M. (Eds.), 2000. Plant Resources of SE-Asia, No. 16. Stimulants, Backyus Publishers Leiden.

Wargo, P.M., 1977. *Armillarialla mellea* and *Agrilluss bilineatus* and mortality of defoliated oak trees. Forest Science 23: 485-492.

Watling, R., 1992. *Armillaria* Staude in the Cameroon Republic. Persoonia 14: 483-491.

Watling, R., Kile, G.A. and Gregory, N.M., 1982. The genus *Armillaria*: nomenclature, typification, the identity of *Armillaria mellea* and species differentiation. Transactions of the British mycological Society 78: 271-285.

White, T.J., Bruns, T., Lee, S. and Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (Eds.), PCR protocols: A guide to methods and applications. Academic Press, San Diego, pp. 315–322.

Willson, K.C. and Clifford, M.N. (Eds.), 1992. Tea Cultivation to consumption. Chapman and Hall.

Wood, T.M., 1991. Fungal cellulases. In: C.H. Haigler and P.J. Weimer (Eds.) Biosynthesis and Biodegradation of Cellulose. Marcel Dekker, Inc., New York, pp. 491-533.

Summary

Tea is cultivated in Kenya mainly as an export crop and is therefore important to the national economy. Due to the requirements for specific ecological conditions for good growth, increased tea production can only be achieved by improving productivity from the already cultivated areas. This has created the need to intensify production by improving agronomic practices and minimizing crop losses. The small holder sector which produces over 70% of Kenya's tea is directly managed at the production level by small holder farmers who do not have the know-how and facilities to effectively cope with problems that militate against improvement in productivity. Effective management of pests and diseases requires urgent attention in efforts to improve income from tea in this sector. Diseases such as Armillaria root rot, which in the large estate sector is not a constraint to tea production because of efficient removal of inoculum reservoirs by heavy machinery used in land preparation, continue to cause considerable plant mortality and crop loss in several small holder farms.

Armillaria is a primary root rot pathogen of tea and a few other plant species in Kenya. Armillaria root rot is, however, poorly understood because of the rare occurrence of basidiomata and rhizomorphs, making disease diagnosis difficult. There is little conclusive evidence on the identity of *Armillaria* isolates causing the disease on tea in Kenya. No studies have been carried out to explore alternative methods of managing Armillaria root rot on tea. Mechanical removal of potential inoculum sources and infected plants remains the only option available to growers for controlling the disease. Due to the difficulty in diagnosing the disease, prompt removal of infected plants, important for checking the spread of the fungus within a plantation, is often not possible. Hence Armillaria root rot persists in many locations as an important constraint to tea production and also excludes the affected areas from use in production of several other crops susceptible to the disease.

Identity of Armillaria species in Kenya

The existing knowledge on *Armillaria* in Africa reviewed in chapter 2 suggests that most cases of the disease reported from the continent occur as sporadic records with little detail on the identity of the species involved and the magnitude of damage or crop losses. Armillaria root rot is poorly known in Africa due mainly to the above-mentioned rarity of basidiomata and

rhizomorphs, features that are characteristic of the disease in the temperature climate zones. Progress in taxonomy of the African *Armillaria* has thus been relatively slow. Mating tests, which form the foundation of resolving species diversity in the temperate *Armillaria* species in Europe, North America and Australia cannot usually be used for African isolates because most of these are homothallic. Existence of various genetically different isolates of *Armillaria* may therefore have gone undetected in Africa. On the basis of morphology of basidiomata, only four species have been recognized: *A. camerunensis*, *A. fuscipes*, *A. heimii*, and possibly *A. mellea s.s.*

In chapter 3 forty seven isolates of *Armillaria* associated with root rot of tea in Kenya were characterized and distinguished by cultural morphology, basidiome morphology, diploid-diploid somatic incompatibility tests and DNA-based characteristics, where possible establishing their genetic relationship with some of the described morphological species. As a result, the isolates were categorized into two groups one of which was tentatively identified as *A. heimii* while the other was concluded to represent a new species.

Disease management options

Due to the difficulty of eradicating *Armillaria* by mechanical removal of potential inoculum sources from soil, solarization, organic amendment and infestation with antagonistic *T. harzianum* were evaluated for use as alternative disease control methods. These were tested for their effect on *Armillaria* inoculum borne in plant materials. The *T. harzianum* isolate used in these tests was selected on the basis of its performance against *Armillaria* in woody substrates (chapter 4). The effect was studied more extensively in field experiments, with the emphasis placed on the effect of the antagonist applied to the soil alone or in combination with soil organic amendment and soil solarization on viability of *Armillaria* (chapters 5 and 6).

When *T. harzianum* was applied to the soil surrounding *Armillaria* inoculum sources as a wheat bran culture, the incidence of this antagonist in the inoculum sources increased and concomitantly reduced viability of *Armillaria* (chapter 5). Coffee pulp amendment affected the efficacy of *Trichoderma* in a pot experiment negatively but this was not repeated in field experiments where the organic amendment reduced slightly the viability of *Armillaria* inoculum. It was concluded that the application of wheat bran-formulated *T. harzianum* into the soil can reduce viability of *Armillaria* resident in woody inoculum sources and thus no further organic

amendment to enhance multiplication of the antagonist in the soil is needed for achieving its suppression of the pathogen.

Solarization for 10 wk resulted in increases in soil temperatures that reduced viability of the pathogen by up to 100%. Application of *T. harzianum* to the soil surrounding *Armillaria* inocula consequent to solarization for 5 wk caused total loss of *Armillaria* viability. These observations demonstrated the potential of solarization to directly eradicate *Armillaria* from woody inoculum sources and, in addition, to enhance the efficacy of *T. harzianum*. It is concluded that the two methods can be integrated into a feasible approach for managing Armillaria root rot, especially targeting the pathogen in small holder tea plantations in Kenya.

Samenvatting

Thee wordt in Kenia vooral geteeld als een exportgewas en levert daarmee een belangrijke bijdrage aan de nationale economie. Gezien de condities waaronder thee kan groeien is verdere uitbreiding van het areaal niet meer mogelijk, maar kan toename in de productie nog wel bereikt worden door intensivering van de teelt. Ongeveer 70% van de teelt van thee in Kenia is in handen van kleine boeren die in het algemeen niet de kennis en machines hebben voor een optimale productie. Effectieve bestrijding van ziekten en plagen is een belangrijke voorwaarde voor verbeterde productie. In Kenia veroorzaakt honingzwam (*Armillaria*) veel schade, met name in theeplantages. In de grote theeplantages wordt inoculum meestal zodanig efficiënt weggehaald gedurende de bereiding van voormalig bos voor de teelt van thee dat daar nauwelijks problemen met honingzwam voorkomen.

Honingzwam is in Kenia een primaire wortelpathogeen van thee en enkele andere plantensoorten. In tropisch Afrika is honingzwam slecht bekend vanwege het zeldzame vóórkomen van zowel vruchtlichamen (paddestoelen) als rizomorfen (zwarte, vetervormige structuren die de schimmel in de grond vormt), waardoor diagnose in het algemeen moeilijk is. Door de zeldzaamheid van vruchtlichamen is bij een aantasting door honingzwam meestal niet vast te stellen om welke soort het gaat. Afgezien van het verwijderen van aangetaste planten is er geen methode bekend om honingzwam in thee te bestrijden. Doordat de ziekte zo moeilijk is vast te stellen in een vroeg stadium, is ook de verwijdering van inoculum moeilijk als de ziekte al verder is voortgeschreden.

Identiteit van honingzwamsoorten in Kenia

De bestaande kennis over honingzwammen in Afrika is samengevat in hoofdstuk 2. De kennis over de verspreiding van honingzwammen in Afrika is beperkt; zeer weinig is bekend over de identiteit van de soorten en de omvang van de geleden verliezen.

In Europa en Noord-Amerika zijn alle isolaten van honingzwammen die houtrot kunnen veroorzaken heterothallisch. Van dit kenmerk is gebruik gemaakt bij de ontrafeling van het soortencomplex, doordat homothallisch mycelium een ander uiterlijk heeft dan heterothallisch mycelium. Dit is een hulpmiddel bij identificatie, omdat als

twee haploïde, onderling compatibele, isolaten elkaar op een voedingsbodem ontmoeten, zij diploïd worden, hetgeen gepaard gaat met een aanzienlijke gedaanteverandering. Door het homothallische karakter van de meeste Afrikaanse isolaten van honingzwam kon dit kenmerk niet gebruikt worden voor de isolaten die thee in Kenia aantasten. Op basis van beschrijvingen van vruchtlichamen kon worden vastgesteld dat drie tot vier soorten honingzwammen in Afrika beschreven zijn: *A. camerunensis*, *A. fuscipes*, *A. heimii* en mogelijk *A. mellea s.s.*

In hoofdstuk 3 werden 47 isolaten van honingzwam uit vruchtlichamen, rizomorfen en (vooral) aangetast plantenmateriaal op verschillende wijzen gekarakteriseerd: de morfologie van de cultures en de vruchtlichamen (indien aanwezig) werd beschreven, resultaten van confrontaties van diploïde isolaten op voedingsbodems werden geëvalueerd en verscheidene DNA-technieken werden gebruikt om de isolaten te karakteriseren. De resultaten hiervan wijzen erop dat in de teelt van thee in Kenia twee soorten honingzwam actief zijn, *A. heimii* en mogelijk een nieuwe soort, waarvan geen vruchtlichamen gevonden zijn. Ook konden van isolaten van deze mogelijk nieuwe soort geen vruchtlichamen in reincultuur verkregen worden.

Opties voor de bestrijding van honingzwam

Omdat het voor kleine boeren zeer lastig is om inoculum uit de grond te verwijderen, werden verscheidene andere opties verkend: solarisatie van de grond, al dan niet in combinatie met toediening van koffiepulp en/of de antagonist *Trichoderma harzianum*. Het isolaat dat uiteindelijk gebruikt werd in veldproeven bleek het beste in staat zowel hout geïncubeerd in niet-steriele grond te koloniseren als honingzwam in dat hout te reduceren (hoofdstuk 4). Als de antagonist werd aangebracht in de vorm van een tarwezemelenculture rondom hout waarin honingzwam aanwezig was dan was honingzwam na 6-12 maanden sterk gereduceerd (hoofdstuk 5). In een potexperiment was de effectiviteit van *T. harzianum* geringer bij toediening van koffiepulp aan grond. In het veld bleek echter bij herhaling het effect van koffiepulp niet negatief te werken op de effectiviteit van *T. harzianum*, en bleek toepassing van uitsluitend koffiepulp zelfs in lichte mate honingzwam te reduceren.

Solarisatie, de afdekking van bevochtigde grond met dun en doorzichtig plastic, resulteerde na 10 weken in volledige inactivering van de honingzwam. Als *T. harzianum* werd toegediend na solarisatie dan werd volledige doding al bereikt na 5 weken. De eindconclusie is dat de twee methoden, solarisatie gedurende 5 weken gevolgd door toepassing van *T. harzianum*, kunnen worden geïntegreerd in een doeltreffende methode om honingzwam in houtig materiaal te bestrijden.

List of publications

Otieno, W. 1992. Armillaria root rot of tea: inoculum sources and disease management. ***Tea 13(2): 134-137, 1992.***

Otieno, W. 1993. Hypoxylon wood rot of tea (*Camellia sinensis* (L.) O. Kuntze.): the casual agent, symptoms and control. ***Tea 14(1): 61-64.***

Otieno, W, Sudoi, V. and Othieno, C. O. 1993. A note on fungicide trials in management of branch die-backs on moribund tea. ***Tea 14(1): 5-6.***

Otieno, W, Onsando, J. M. O, Lang'at, J. K. 1994 .Management of Hypoxylon wood rot of tea (*Camellia sinensis* (L.) O.Kuntze) using sanitation practices and fungicides. ***Tea 15(2): 110 - 112.***

Otieno, W. 1995. Vesicular-arbuscular mycorrhiza in roots of tea (*Camellia sinensis* (L.) O.Kuntze). ***Tea 16(2):115-118.***

Otieno, W. 1996. Hypoxylon wood rot of tea: pathogenesis and control. ***Tea 17(1): 5-6.***

Otieno, W. 1997. A selective medium for studying in vivo interaction of *Armillaria* with *Trichoderma* spp. ***Discovery and Innovation 9: 157-159.***

Otieno, W. 1997. Epidemiology and management of Hypoxylon wood rot of tea. ***Tea 18(2):175-183.***

Otieno, W. 1998. *Armillaria - Trichoderma* interactions and management of Armillaria root rot of tea (*Camellia sinensis* (L.) O. Kuntze. ***Tea 19(1): 11-16.***

Otieno, W. 1999. Competitive colonization of root segments of tea (*Camellia sinensis* (L.) O. Kuntze by *Armillaria* and *Trichoderma* spp: effect on viability of the pathogen. ***Tea 20 (2):76-79.***

Otieno, W., Termorshuizen, A. J. 2001. *Armillaria* in Africa. ***Submitted to Forest Ecology and Management.***

Otieno, W., Perez-Sierra, A., Termorshuizen, A. J. 2001. Characterization of *Armillaria* spp. pathogenic on tea in Kenya. ***Submitted to Mycologia.***

Otieno, W., Termorshuizen, A. J., Jeger, M. J. 2001. Effect of *Trichoderma harzianum* and soil amendment with coffee pulp on survival of *Armillaria*. ***Submitted to Biological Control.***

Otieno, W., Termorshuizen, A. J., Jeger, M. J. 2001. Efficacy of soil solarization, *Trichoderma harzianum*, and coffee pulp amendment against *Armillaria*.***Submitted to Crop Protection.***

Sierra A. P., Otieno W. and Termorshuizen A. J 2001. Characterization of *Armillaria* isolates from tea (*Camellia sinensis*) in Kenya. ***IUFRO Conference, Canada.***

Curriculum vitae

Washington Otieno was born on 5 January 1959 in Kenya. After completing high school education he was a teacher for two years before joining the University of Nairobi where he graduated in 1986 with B.Sc. degree in agriculture. The same year he was awarded a University scholarship for graduate study and graduated with M.Sc. degree in plant pathology in 1989. Between August 1988 and November 1992 he worked as an assistant lecturer and lecturer in the faculty of agriculture, University of Nairobi. In December 1992 he joined the Tea Research Foundation of Kenya as a research scientist. During his tenure as a lecturer and research scientist he has attended several courses on diagnostic techniques in plant pathology. In 1997 he started his PhD study at Wageningen University on Armillaria root rot of tea in Kenya of which this thesis is the final result.