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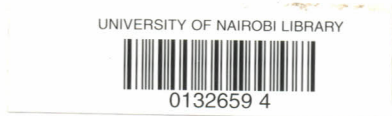# MYCORRHIZAL FUNGI ASSOCIATED WITH KENYAN TERRESTRIAL ORCHIDS AND THEIR ROLE IN ORCHID SEED GERMINATION "

BY

MUGAMBI GEORGE K. (B.Sc. Hons)

A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE OF MASTER OF SCIENCE AT THE UNIVERSITY OF NAIROBI

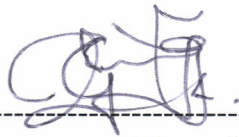COLLEGE OF BIOLOGICAL AND PHYSICAL SCIENCES

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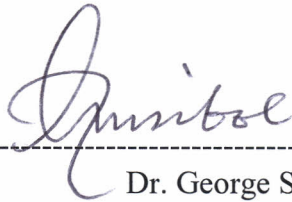DEPARTMENT OF BOTANY

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MAY 1997

DECLARATION

I, Mugambi George K. hereby declare that this thesis is my original work and has not been presented for a degree in any other University.
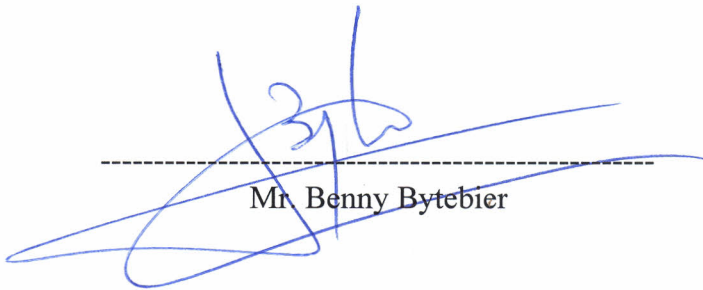
Mugambi George K.

This Thesis has been submitted with our approval as supervisors:

Dr. George Siboe

Mr. Benny Bytebier

DEDICATION

DEDICATED TO DAD AND MUM (MR. AND MRS. FREDRICK MUGAMBI) AND OTHER FAMILY MEMBERS.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

BOM   Basic Oats Medium

FIM   Fungal Isolating Medium

PM   Phytamax™ Orchid Maintenance Medium

MS   Murashige and Skoog Medium

IMI   International Mycological Institute

PDA   Potato Dextrose Agar

µg   Micrograms

µm   Micrometers

EA/ PCP   East African Herbarium/ Plant Conservation Programme

ABSTRACT

Terrestrial orchids are threatened with extinction due to loss of their habitat. This has created awareness towards their *in situ* and *ex situ* conservation. Orchids form mycorrhizal association and their seeds rely on this association for germination in nature. For the successful *in vitro* seed germination, inoculating with an appropriate mycorrhizal fungus has proved necessary.

Kenyan terrestrial orchids were assessed for mycorrhizal fungi and the role the fungi play in seed germination. Isolation was carried out on 14 orchid species namely; *Eulophia stenophylla*, *E. montis-elgonis*, *E. orthoplectra*, *E. horsfallii*, *E. petersii*, *E. streptopetala*, *Bonatea steudneri*, *Liparis bowkeri*, *Satyrium crassicaule*, *Disa stairsii*, *Habenaria petitiana*, *Satyrium corriophoroides*, *Cynorkis anacamptoides* and *Satyrium sacculatum*. The species were collected from different localities in Kenya and Fungal Isolating Medium (FIM) was used for the isolation of the mycorrhizal fungi.

Out of the 24 fungal isolates obtained, eight were found to belong to the form-genus *Rhizoctonia* DC., the group which is known to form mycorrhizal association with orchids. Specific identification was not possible due to lack of conclusive identification keys for this group of fungi. A taxonomic description of the mycorrhizal fungal isolates is given.

Symbiotic germination tests were carried out on five orchid species namely; *Bonatea steudneri*, *Liparis bowkeri*, *Eulophia horsfallii*, *Satyrium crassicaule* and *Cynorkis anacamptoides*. Out of these, three species were stimulated to germinate symbiotically namely; *L. bowkeri*, *S. crassicaule* and *C. anacamptoides*. *Satyrium crassicaule* was

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stimulated to germinate by *Rhizoctonia* spp. Nos. 494/95/1010, 520/95/1018, 515/95/1017 and 536/95/1020. *Cynorkis anacamptoides* was stimulated to germinate by *Rhizoctonia* spp. Nos. 494/95/1010 and 520/95/1018. *Rhizoctonia* sp. No. 494/95/1010 was the most efficient in stimulating the germination of *C. anacamptoides* and *S. crassicaule*. *Liparis bowkeri* was stimulated to germinate by *Rhizoctonia* sp. No. 515/95/1017 only.

Isolation and identification of orchid mycorrhizal fungi and symbiotic germination technique were a prerequisite for the propagation of endangered temperate terrestrial orchid species. The work presented here suggests that the symbiotic germination assay can also be applied on endangered Kenyan terrestrial orchids.

CHAPTER ONE

1.0   INTRODUCTION

1.1   STATEMENT OF THE PROBLEM.

During the last couple of decades, there has been an increased loss of biodiversity due to increased human population and activities. A number of plant species are on the brink of extinction due to pressure on the ecosystems they occupy. This has created concern towards the conservation of biodiversity, through active programmes for both *in situ* and *ex situ* protection of endangered species. To achieve this the Plant Conservation and Propagation Unit (PCPU) was established at the National Museums of Kenya to conserve and propagate threatened Kenyan plant species. The Orchidaceae is one of the families of high interest, not in the least because of their horticultural value. For this purpose an active orchid conservation programme was set up.

The Orchidaceae is a large family consisting of over 20,000 species (Hadley, 1980). The family holds a considerable fascination by virtue of the complexity and diversity of its floral development. Members of the family also form symbiotic association with fungi in their roots. Their seeds rely on this fungal association for germination in nature (Arditti, 1982).

In general the family is very vulnerable to changes in the ecosystem because of its highly specialised habitat requirements. Although difficult and specialised, orchid propagation from vegetative material is well understood. Propagation from seed however is difficult and requires specialised laboratory techniques. For most epiphytic orchids, a defined medium with a carbon source is sufficient for seed germination (asymbiotic germination). This does not

work with many terrestrial orchids (Arditti *et al.*, 1990). Here, it seems as though fungal association is a prerequisite for germination to take place (Arditti *et al.*, 1990), and symbiotic germination techniques are now being developed for their propagation (Warcup, 1973, 1975, 1981). In this method a fungus is introduced into the culture medium together with the seeds. The fungus is said to be mycorrhizal if it infects the roots of the growing orchid seedlings or mature plants and form symbiotic association with them.

The germination and survival rate of orchid seeds in nature is very low. However, with careful control of conditions and isolation of the right symbiotic fungus, uniform germination of orchid seeds can be obtained under *in vitro* conditions (Harley and Smith, 1983). The isolation of the correct symbiotic fungi therefore proves to be essential for the propagation of temperate terrestrial orchids.

Therefore, the isolation of mycorrhizal fungi from Kenyan orchids will provide additional data and, is a prerequisite for the propagation and consequently, the conservation and management of Kenyan terrestrial orchids. It will also help to make available more orchid species for horticultural improvement.

The biology and taxonomy of orchid mycorrhizal fungi is poorly understood in general. This study will therefore contribute to the knowledge of this poorly understood group of fungi.

1.2   LITERATURE REVIEW.

The family Orchidaceae is cosmopolitan except for the deserts and arctic zones but has higher diversity of species in the tropical regions. It has a varied range of life forms which

include terrestrial, epiphytic, and saprophytic (achlorophyllous) species. They have highly developed flowers with complex pollination mechanisms and they produce seeds that are extremely small. Orchidaceae seeds are wind dispersed, and therefore have been considerably reduced in size through evolution. The largest seed is about 14 micrograms in weight (Harley and Smith, 1983). They comprise of a thin testa of a single layer of transparent cells enclosing an embryo of as few as 100 - 200 cells (Smreciu and Currah, 1989). They contain no endosperm which is the food reserve needed to support germination. Energy for germination is produced through an intimate symbiosis between an orchid and an appropriate fungus that breaks down organic matter and avails nutrients to the germinating embryo (Harley and Smith, 1983).

For orchid seeds to germinate in nature, they depend on the presence of a suitable fungus in their neighbourhood (Arditti, 1967; Arditti, 1982). However, when the seeds are dispersed, they are not in contact with the suitable fungus. They depend on chance to land where an appropriate fungus is growing, which is either in the soil, bark of trees or in orchid roots. The orchid-fungus interaction leading to germination seems to be specific in some terrestrial orchids (Clements, 1982). Others are capable of utilising a wide range of fungi for growth (Harley & Smith, 1983).

In Kenya, orchids are disappearing at a very fast rate due to habitat loss and ecosystem degradation caused by the extensive destruction of indigenous habitats. This has been caused by increased agricultural activities, cutting down of trees for woodfuel and timber trade, including burning and overgrazing in grasslands. In some cases, they are further threatened by collection for local and international trade. An example in Kenya is *Anselia africana* Lindl., which the local people at the coast have collected in large quantities for trade such that most

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of the population there has been depleted (Patel, 1992).

1.2.1 Symbiotic fungi

The majority of fungi associated with orchids are considered to be members of the form-genus *Rhizoctonia* DC (Warcup, 1971). The genus *Rhizoctonia* is distinguished from other taxa of Mycelia sterilia primarily by vegetative characteristics (Mordue *et al.*, 1989). On culture media young hyphal branches are inclined in the direction of growth. The branches are constricted at the point of union with the main hyphae. A septum is formed in the branch near the constriction. As hyphae mature they become uniform and rigid and the branches arise at right angles from the main hyphae (Duggar, 1915). Branches also arise at acute angles (Butler and Bracker, 1970).

Species distinction in the genus *Rhizoctonia* presents an extremely difficult taxonomic problem due to lack of stable morphological characters on which to base a definitive classification of the taxa and strains assigned to it (Mordue *et al.*, 1989). The traditional range of taxonomic data has been drawn heavily from cultural and morphological characteristics, rate and appearance of growth in culture, pigmentation, dimensions and branching patterns of hyphae, septal pore structure, number of nuclei, size and shape of chlamydospore (monilioid) cells and sclerotia (Mordue *et al.*, 1989). These characters have been useful to a limited extent but are difficult to quantify and can yield inconsistent results (Mordue, *et al.*, 1989).

Sclerotia are very variable in colour, shape and size depending largely on the substrate. The structure is therefore not considered to be of great taxonomic value. Chlamydospore characters are consistent within a species irrespective of the substrate, and are considered

reliable for specific differentiation (Hadley, 1980). Chlamydospores are formed in chains by the inflation or rounding up of the cells of the monilioid filaments which arise in the manner of the vegetative hyphae (Mordue *et al.*, 1989). The chains may also grow in length by budding from the apical cell and by basipetal abscission. Lateral branch chains develop from the pre-existing branches of monilioid filaments and from side buds produced by cells of the main chain. In some forms, the mature chlamydospore are separated from each other by slender, hyaline, pad-like connection, like the disjunctions of *Albugo*. Chlamydospore chains usually break into smaller lengths when disturbed, but rarely do mature chlamydospore fall apart as single cells (Mordue *et al.*, 1989). The chlamydospores germinate within 5 to 8 hours at 25^0C when floated on tap water on a slide, producing 1 to 3 germ tubes per chlamydospore (Mordue et al., 1989).

Some isolates have been induced to form sexual stages in culture. This has made it possible to classify them into Basidiomycete genera *Ceratobasidium* Olive, *Sebacina* Oberwinkler, *Tulasnella* Schroet, and some species of *Thanatephorus* Donk. For example the endophyte *Rhizoctonia repens* Bernard, is the imperfect state of *Tulasnella calospora* (Boudier) Juel (perfect state). However, it has been difficult or even impossible, to induce perfect states in some *Rhizoctonia* spp. such as *Rhizoctonia solani* Kühn (Stretton *et al.*, 1964).

Several fungal symbionts to both tropical and temperate orchid species are recognised as Basidiomycetes by the presence of clamp connexions   or fruiting bodies. These are usually associated with achlorophyllous orchids and include *Armillaria mellea* Agg., which is symbiotic with species of the genus *Gastrodia* Blume species (Kusano, 1911; Campbell, 1962).

The optimal time for isolating mycorrhizal fungi from most orchids is during the normal rapid vegetative growth period prior to flowering. The fungal isolates do not exhibit extreme sensitivity to cultural conditions and many of them grow at reasonably fast rate (Harley and Smith, 1983). Their requirement for nutrients is generally unspecialised, and most can use a wide range of carbon and both organic and inorganic nitrogen sources (Arditti, 1979). They can be isolated by direct plating of surface sterilised root fragments (Light, 1992). However it has been found necessary to reduce the chances of isolating root surface contaminants by cutting away infected cells which are then used as inocula. In some other cases, coils of fungal hyphae have been hooked out of infected host cells with sterilised needles and inoculated into media (Mitchell, 1989).

Isolated pure cultures can be confirmed as orchid endophytes by back-inoculating them with sterilised orchid seeds. Stimulation of seed germination and development, and the occurrence of seedling infection, has been taken as evidence for the isolate to be an endophyte.

Some fungi isolated from adult orchid plant species do not form symbiotic relationships with germinating seeds of the same species (Arditti, 1982; Clements, 1981, 1982). This means different mycorrhizal fungi might be present at different growth stages of the plant and the required properties of the fungus in the two cases may be different. On the other hand, the outcome of the relationship between the partners is a delicate balance and many fungi isolated from orchid mycorrhizas may after a period in culture become incompatible with or even pathogenic to orchid protocorms. Orchid mycorrhizal fungi are extremely variable and relatively few root inhabitants are true mutualistic symbionts. Therefore, as mentioned previously, identification of the true orchid mycorrhizal fungi is necessary.

Fungi found in, and isolated from, the root tissue of orchids do not always encourage germination and growth of seedlings. This may be due to several reasons:

(a) Roots may harbour a wide range of fungi, and isolation method used may yield surface contaminants as well as fungi from within the tissue.

(b) More than one fungus may be associated with the pelotons of a single root.

(c) Even if a fungus is symbiotically efficient with an adult root, it may not be equally efficient in improving growth of the seedling. Different mycorrhizal fungi might be present at different growth stages of the orchid. This is because the required properties of the fungus in the two cases may be different (Muir, 1989).

There is some evidence of direct effect of soil conditions on the distribution of some of the mycorrhizal fungi of orchids (Curtis, 1939; Downie, 1943). Curtis (1939) observed that *Rhizoctonia sclerotica* Burgeff and *R. stahlii* Burgeff were found in neutral or slightly alkaline wet soils. *R. borealis* Curtis occurred in acidic soils such as those of coniferous woodlands. However, Curtis (1939) also showed that *Rhizoctonia repens*, *R. lanuginosa* Bernard and *R. mucoroides* Bernard, were not greatly affected by soil conditions and were isolated from orchid roots in all kinds of soils. Mackenzie (1964) isolated two typical orchid endophyte and a strain of *R. solani* from the soil where *Coeloglossum viride* (L.) Harm. was growing. However, it is not certain whether these isolates were wide-spread and efficient soil saprophyte or merely poor competitors restricted to the rhizosphere. Smith (1966) reported that *R. repens* and unidentified *Rhizoctonia sp.* both endophytes of orchids, can decompose cellulose both in pure culture and in competition with other organisms in soils. It seems likely, therefore that typical orchid endophytes as well as potential symbionts such as *R. solani* do grow freely in the soil.

Orchid fungi isolated from roots and capable of stimulating seedling growth, differ from other mycorrhizal fungi. They are capable of rapid growth in soluble carbohydrates as well as in insoluble resistant carbon polymers such as cellulose (Harley and Smith, 1983).

From the above evidence, I conclude that, since not all fungi isolated from adult orchids have any symbiotic properties, particularly for stimulating seed germination, it is important to screen as many fungi as possible to attain more effective strains.

1.2.2 Specificity of the orchid-fungus relationship

Initially it was believed that symbiosis between orchids and their mycorrhizal fungi was always highly specific, but this is now known not to be so (Harley & Smith, 1983). Curtis (1939) showed that some orchids harbour more than one fungus, meaning that the relationship is not species specific. He also found out that the distribution of mycorrhizal fungi was related to habitat rather than host. *Goodyera repens* (L.) R. Br. for example, is a species which in root isolation studies is usually found to be infected with one fungus, *Rhizoctonia*

goodyerae-repentis Constantin & Dufour [*Ceratobasidium cornigerum* (Bourdot) Rogers] yet in seed germination experiments forms mycorrhizal relationships with several different fungal isolates (Hadley, 1970). In this case, the orchid will only be infected by one fungal species under natural conditions but can undergo associations with several fungal species under *in vitro* conditions. This means factors other than specificity are in play in this case, since under *in vitro* conditions the species can form mycorrhizal association with different fungi. Harvais and Hadley (1967) supported the idea of non-specificity, and showed that *Dactylorhiza*

purpurella (T. & A. Stephenson) SOÒ, was symbiotic with nearly all tested isolates.

In an extensive series of cross-inoculation experiments using orchids and fungi of world-wide distribution, it was found that some orchids, particularly *Dactylorhiza purpurella*, became infected by many strains of fungi from a variety of habitats (Hadley, 1970). However evidence of possible specificity has been obtained for some orchids. Warcup (1971) found that wild plants of five species of *Diuris* Smith, yielded only *Tulasnella calospora* (*Rhizoctonia repens*) as the symbiotic fungus. In seed germinating tests, two *Diuris* spp. were stimulated only by *Tulasnella calospora*, suggesting a strong degree of specificity. Many species of *Caladenia* R. Brown and related genera yielded almost always (94%) *Sebacina vermifera* Oberwinkler isolates. Further studies found that two species of *Pterostylis* R. Brown were stimulated to germinate only by *Ceratobasidium cornigerum* (Warcup, 1973). However it is still argued that the concept of specificity is clearly related more to ecological characteristics than the original one host/ one fungus theory.

Relatively few orchid fungi are known, and knowledge regarding specificity is largely restricted to a small number of Australian terrestrial fungus-orchid combinations. Therefore, more research is necessary to confirm or refute these ideas regarding the specificity of the orchid-fungus interaction.

1.2.3   Infection and the role of mycorrhizal fungi in orchids

There are two stages of infection in the life cycle of many orchids. The primary infection of germinating seedlings, and the re-infection of the new roots of the adult. The latter is especially important in those species which perennate as uninfected tubers or

rhizomes and form a new root system when dormancy is broken. The source of infection may be the soil, or the tubers, where the fungi may persist on the surface or in the tissue (Warcup, 1971).

For orchid seeds to germinate in nature, they have to be infected by an appropriate mycorrhizal fungus. If a suitable fungus is present, a single fungal hypha penetrates the wall of either the epidermal hairs or epidermal cells near the suspensor of the embryo (Hadley and Pegg, 1989). It is not known whether cellulolytic or pectolytic enzymes are involved in this process. If they are, their action must be very localised because usually minimal disruptions of the cells occur. However, there are cases where disruption of the tissue has been reported, such as infection of *Gastrodia eleta* Blume by *Armillaria mellea.* The key to the range of host-fungus interaction may lie in the mechanism which permits or prevents penetration of the host cells by the fungus. Williamson and Hadley (1970) have shown that eight fungi initiated a compatible infection of protocorms of *Dactylorhiza purpurella* in a similar manner and there is no evidence of a selective mechanism in the penetration phase, irrespective of fungal species or host. This argues for a specific functional role of the fungi, for their infection was controlled, and caused minimal damage to the germinating protocorms (Hadley, 1982).

The rate of growth for asymbiotically grown seeds and protocorms is less than for symbiotic material, regardless of the nutrient status of the medium (Hadley, 1970). It has been shown that protocorms of *Dactylorhiza majalis* (Reichenb.) P.F.Hunt & Summerhayes increased in length or volume one to two days after penetration by the fungus (Hadley & Williamson, 1971). Hadley, (1982) demonstrated that orchid growth and development of protocorm was enhanced by mycorrhizal fungi infection in temperate species. For example *Spathoglotis plicata* Blume, which contains chlorophyll from early stages of growth, grows

fast after infection, while non-infected protocorms of the same species grow slowly, regardless of nutritional and environmental factors.

The orchid mycorrhizal fungi have simple nutrient requirements, and need only a cellulose carbon source (Hadley, 1980). For this reason symbiotic germination is normally carried out in simple nutrient medium with cellulose as a carbon source, unlike asymbiotic germination which requires a complex nutrient combination. Translocation of metabolic materials from fungus to host may take place through two processes, biotrophic and nectrotrophic processes. It is still unclear whether the symbiotic growth stimulus results from either biotrophic or nectrotrophic translocation of metabolites, or whether both processes work hand in hand. Cultures of *Orchis purpurea* Hudson, inoculated with *Rizoctonia solani* showed that stimulation of growth occurred following infection but before any fungal digestion (Harvais and Hadley, 1967). This agrees with the finding that *Goodyera repens* is stimulated by infection of *Rhizoctonia goodyera-repentis* before any sign of digestion of the pelotons (Mollison, 1943). In this case intracellular lysis (digestion) appears to operate as a defence mechanism and not as a means of obtaining nutrients, except perhaps under extreme starvation (Hadley and Williamson, 1971). The catabolites are the primary source of nutrients for the orchid during heterotrophic phases of development. In *Goodyera repens*, carbon transfer from the fungus switches off as photosynthetic activity becomes enhanced following leaf production (Alexander & Hadley, 1985). Provided that mineral nutrients are adequate, the seedlings may become largely independent of symbiotic fungi. The same is probably true for adult plants.

There is a major problem in identifying the contribution of mycorrhizal fungi to the germination of orchid seeds. Experiments with both epiphytes and terrestrial orchids have

shown that several substances from the fungi are taken by the orchids. They include sugars, amino acids, proteins and peptides, vitamins, phosphates and probably a number of other compounds (Smith, 1973; Mead and Bulard, 1975; Hijner and Arditti, 1973; Alexander *et al*., 1984). Use of radioactive tracers has demonstrated the translocation of carbon and phosphorus compounds (Smith, 1966). The substances are provided before, and, or after lysis of the hyphae. Fungal extract, filtrates from media which supported fungi, vitamins, amino acids, hormones, complex additives or any other substances can not replace fungi for some temperate terrestrial orchids (Warcup, 1971). All efforts to isolate and identify specific substances which might support germination of epiphytic and terrestrial orchids on sugar free media have failed (Warcup, 1971). This then means that, apart from nutrient supply, the fungi must be triggering some metabolic pathways. It seems likely that fungi transfer more specific metabolites such as enzyme precursors (Hadley, 1982). For example asymbiotically grown protocorms, slowly accumulate starch but appear unable to metabolise it for growth (Hadley, 1982). Chlorophyll content and photosynthetic activity are enhanced in some tropical orchids grown symbiotically (Hadley, 1982). This suggests processes associated with gene amplification or yet unknown mechanisms triggering latent metabolic activity may be involved. Photosynthetically active seedlings and mature plants however, may be quite independent of their fungal partners (Harley and Smith, 1983). Evidence suggests that in conditions of stress, the fungal partner may mediate in the movement of phosphate and or nitrogen compounds, as in other mycorrhizal systems (Harley and Smith, 1983). The role of the orchid-fungi relationship in the nutrition of orchids in nature could therefore be critical in relation to ecological competition, and survival in conditions of stress.

1.2.4   Control of fungi by orchids.

One of the most important aspect of the orchid-fungus symbiosis, is the mechanism of recognition of compatible fungi. There is inhibition of incompatible or partially pathogenic strains of similar species. Little is known about recognition mechanism. Resistance to phytoalexin-like antifungal substances has been implicated in the establishment and localisation of infection. Resistance to pathogenic fungi is carried out by phytoalexins. The role of phytoalexins in resistance mechanisms to some pathogenic fungi has been well documented (Bailey & Mansfield, 1982). In orchids, the phenanthrene antifungal compounds, orchinol, loroglossol, and hircinol show many properties of phytoalexins (Hadley and Pegg, 1989). They were characterised as intermediate metabolites of tubers, bulbs and presumably roots. Their precise role in the exclusion of non-symbionts or tolerance of symbionts by orchids in general is not clear. Work on mycorrhizal and non-mycorrhizal tissue of cultured *Dactylorhiza majalis* showed that the orchid most probably plays an active role in continuous restriction and elimination of the symbiont. Non-mycorrhizal protocorms were shown to contain the constitutive enzymes, $1,3\beta$-glucanase endochitinase and N-acetylglucosaminidase capable of hydrolysing the fungal cell walls of *Thanatephorus, Ceratobasidium,* and *Tulasnella.* When protocorms were infected with these symbionts, the level of the enzymes rose substantially (Harley and Smith, 1983). The tendency for certain fungi to pass into a post-symbiotic phase of parasitism is seen in some host-fungus combinations. In prolonged experiments this trend becomes more pronounced, presumably as nutrients are depleted (Harvais and Hadley, 1967).

1.3   RATIONALE

This study will help to improve the knowledge of the poorly known orchid-fungus mycorrhizal relationship which is vital for *in vitro* orchid propagation. The symbiotically effective fungi will then be identified and used in the propagation and conservation of threatened terrestrial orchid species of Kenya.

1.4   AIMS AND OBJECTIVES

- To isolate endophytic fungi that are associated with Kenyan terrestrial orchids.

- To identify the isolated orchid mycorrhizal fungi

- To find out whether the endophytic orchid fungi are specific to orchid species they infect.

- To stimulate orchid seed germination by the use of symbiotic fungi

1.5 HYPOTHESES OF THE STUDY

- H_0: Orchids form endophytic mycorrhizal association with a wide range of fungal taxa.

  H_1: Alternative.

- H_0: All endophytic fungi will stimulate orchid seed germination.

  H_1: Alternative.

- H_0: The orchid-fungus is highly specific to the orchid species with which it forms the mycorrhiza association.

  H_1: Alternative.

CHAPTER TWO

2.0   MATERIALS AND METHODS

2.1   STUDY SETTING

2.1.1   Sample collection areas

The orchid species were collected from forest, grassland, swamp, and bushland habitats. The following areas were sampled: Sasamua dam, Saiwa Swamp National Park, Aberdare National Park Elephant Peak, Nairobi Arboretum, Mount Nyiru and Kerita forest. The choice of these collection sites was based on previous information on orchid collection (EA/ PCP data base).

2.1.2   Nature of the study.

(a) Orchid sample collection and preparation.

(b) Laboratory analysis of the root samples. This involves isolation of the mycorrhizal fungi from the root samples.

(c) Identification of the isolated fungi.

(d) Testing the isolated fungi for their effectiveness to stimulate orchid seed germination.

2.1.3   Sample size and sample size determination.

A sample size of 14 orchid species was collected. The sample size was determined by their

availability. Most terrestrial orchids perennate during the dry season and mostly survive as underground bulbs. They are also difficult to locate among other vegetation unless they are in flower.

2.2 *SAMPLE COLLECTION AND PREPARATION*

The orchid plants were dug out with their roots intact and transported to the laboratory in polythene bags. A voucher specimen was always made and deposited at the East African herbarium. After the root samples were collected, the plants were potted and kept at the Plant Conservation and Propagation Unit (PCPU) nursery of the National Museums of Kenya. Fungal isolation from the roots was carried out as soon as the samples reached the laboratory. The root samples from which mycorrhizal fungi could not immediately be isolated, were wrapped in wet tissue paper and then covered with aluminium foil and stored at 4^0C for later isolation. In cases where seeds were available, they were harvested and transported in small paper bags. Once in the laboratory, the seed samples were put in a desiccator to lower their moisture levels in order to impede fungal spore germination and seed infection, and also to enhance seed longevity. The seed samples were then put in small vial bottles and stored at 4^0C for later use in symbiotic germination tests.

2.3 *LABORATORY ANALYSIS.*

2.3.1 Isolation of mycorrhizal fungi

.   The method employed here is the one described by Mitchell (1989) with some few modifications. Root samples were initially surface sterilised (Katherine *et al.*, 1993), a step

not used by Mitchell. Several stages were followed in fungal isolation process:

Stage 1. Pieces of infected root (recognisable by the yellowing of the tissue) from an adult plant were excised and washed gently under running water to remove all lose soil. To sterilise the root pieces they were put into a beaker containing a diluted sodium hypochlorite (20% JIK®) solution in which a detergent (one drop per 100ml final solution of Triton X-100) had been added to increase wetting, and agitated for two minutes. The beaker was then transferred to a laminar flow cabinet and from this stage onwards aseptic techniques were followed. The roots were removed from the bleach and rinsed three times in sterile, distilled water. Even with this surface sterilisation, it was not possible to get rid of all the root surface contaminants but it helped to reduce them to a minimum. Small lengths of roots, about 5 mm long, were cut from the root pieces using a sterile razor blade.

Stage 2. The pieces were sectioned longitudinally to give thin slices of root cortex. These sections were then examined under a binocular microscope to check for the presence of pelotons. When found the remaining epidermis was removed from the tissue and discarded.

Stage 3. The cleaned sections of cortical cells were then transferred to a sterile petri dish and floated in several drops of sterile distilled water. The cortical tissue containing pelotons was teased under a microscope and any excess plant tissue was removed from the plate. The plates were then covered with Fungal Isolating Medium (FIM), (Clements *et al.*, 1986) which comprised of 0.5g of $Ca(NO_3)_2 . 4H_2O$, 0.2g of KH_2PO_4, 0.2g of $MgSO_4.7H_2O$, 0.1g of KCl, 0.1g of Yeast extract (Difco®) and 8.0g of agar, contained in one litre of distilled water. The media was poured at the stage where the agar was still liquid but just at the point of setting. Once the agar had set the plates were sealed with parafilm and incubated at room temperature (23 ± 1^0C). The plates were observed under a microscope at regular intervals from

approximately 12 hours after isolation, for any fungal growth.

Stage 4. As soon as the pelotons begun to grow (those pelotons growing while still in some plant tissue were considered to minimise the chance of isolating a contaminant) they were cut out of the isolation medium in small agar cubes and placed on a clean plastic surface of a 'window' plate (a petri dish that has been partially covered with FIM, leaving a clean edge of agar on the plastic surface). The cubes were placed within 5 mm of the agar edge.

Stage 5. The fungal hyphae grew across the gap between the cube and the fresh agar, leaving behind the contaminating bacteria. Once the hyphae reaches the agar, a cube of medium was taken from the leading edge of the mycelium and placed into fresh FIM to attain a pure culture. The process was repeated several times when contamination was observed or suspected.

2.3.2 Storing the fungi.

Fungi were maintained in FIM slants in screw top test tubes and stored at 4^0C, a method used by Mitchell (1989). The orchid-fungi relationship is a delicate balance and growth of the fungus in pure culture over a period can make it lose its symbiotic ability. The fungi are therefore grown on poor nutrients medium and at low temperature where they develop very slowly.

2.4   *IDENTIFICATION OF THE MYCORRHIZAL FUNGI*

2.4.1 Procedure for microscopic examination.

Clear lactophenol was used for pigmented hyphae and chlamydospores while

lactophenol in cotton blue was used for hyaline ones. A small amount of hyphae was picked from a fungal colony using a mounting needle and placed on a slide on which a drop of the appropriate stain had been placed. A cover slip was then placed on top. Semi-permanent slides were prepared by sealing these slides with clear nail vanish.

2.4.2 Identification procedure

For the purpose of identification, the fungal isolates were grown on 2% PDA. Cultural and morphological characters were recorded.

(a) Cultural characters that were studied included: growth form, colony colour and growth rate. The growth rate was recorded as radial extension in mm h^{-1} at room temperature on PDA. Measurements were recorded once every 48 hours for two weeks. Average radial extension per hour was then calculated.

(b) The morphological characters included:

- Branching form, size and pigmentation of the hyphae.

Branching form was assessed on young and old hyphae. Measurements of hyphal diameter were made on young primary hyphae at the advancing edge of the colony. Fifty measurements were carried out and the average size was calculated. The smallest and largest measurements recorded were also noted.

- Size, shape, pigmentation and the presentation of chlamydospores.

To avoid the risk of assessing immature chlamydospores, the cultures were incubated long enough so that all chlamydospores were mature. Measurements (length and width) were randomly taken for 50 chlamydospores. The average size of the chlamydospores was then

calculated. The size range (the smallest and largest measurement recorded) was also noted.

An attempt was made to stimulate the isolated fungi to produce teleomorph stages. Three methods were used:

(i) Incorporating sterilised grass straws in their growing colonies.

(ii) Incubating the fungal colonies over prolonged time.

(iii) Use of slide culture method. Agar block with fungus hyphae was placed on a microscope slide and then incubated in a moist chamber. The fungal hyphae grew on the glass surface and it was expected that the harsh conditions would cause the fungus to sporulate.

Identification of the fungi was attempted using keys by Currah and Zelmer (1992) and Sneh *et al*. (1991).

2.5   *SYMBIOTIC ORCHID SEED GERMINATION*

The choice of orchid seeds was determined by their availability. The orchid plants from which the seeds were obtained are listed in Table 1. The methods of seed sterilisation and seed sowing are as per Mitchell (1989) and are described bellow.

2.5.1   Sterilisation of seeds

The seed samples were cleaned of debris, and then placed in a packet made from filter paper (Whatman No.1, 5.5 cm) and secured by stapling. Care was taken not to put too many seeds into one packet as the sterilising agent may not penetrate the dense mass of seeds. The packets were then transferred to a beaker containing diluted sodium hypochlorite (10% JIK®) solution in which one drop per 100 ml final solution of detergent (Triton X-100) had been

added to increase wetting of the seeds. The seed packets were squeezed to expel any trapped air. The beaker was then placed on a magnetic stirrer and the seed packets allowed to agitate for 10 minutes, after which it was transferred to a laminar flow cabinet. From this point onwards aseptic techniques were followed. The packets were removed from the sterilant and rinsed three times in sterile distilled water.

2.5.2   Seed Inoculation

(i) Symbiotic Inoculation

The packets were removed from the beaker and squeezed to expel excess water. The stapled part of the packet was cut off and the filter paper pealed off to expose the seeds. The seeds were then transferred to the medium and spread out as evenly as possible. A small cube of agar containing fungal hyphae from the inoculum plate was cut and placed on the agar surface. The plates were then sealed and incubated at room temperature in the plant culture room under light for germination and early protocorm development. Germination was assessed once every week, from 50 randomly counted seeds from each plate. Plates inoculation was done in triplicates. Some plates were left uninoculated to serve as controls. This means that seeds were spread on BOM and no fungal isolate was introduced into the plate.

A fungal species *Ceratobasidium cornigerum* (IMI No. 123665) which has been reported to stimulate germination of a number of temperate terrestrial orchids such as *Dactylorhiza fuschii* (Druce) SOÒ, *D. elata* (Poiret) SOÒ , *D. praetermissa* (Druce) SOÒ, *Orchis coriophora* subsp. *fragrans* Poll. Sudre, *O. laxiflora* Lam., *O. militaris* L., *O. Morio*

L., *O. sancta* L., O. *Simia* Lam., *Serapias lingua* L. and *S. parviflora* Parl. (Rasmussen, 1995; Miur, 1989; Ramsay, pers. comm.) was used as a standard in symbiotic germination tests. This isolate was obtained from the International Mycological Institute, Egham, UK

(ii) Asymbiotic Inoculation

For the purpose of comparison, germination was also carried out on asymbiotic media. Two types of asymbiotic media (commercial brands) were used, MS (Murashige & Skoog, 1962) and PM (Phytamax™ orchid maintenance). The procedure was exactly as in symbiotic germination except that no fungus was introduced.

2.5.3   Germination media

Seeds were germinated symbiotically on Basic Oats Medium (BOM) (Clements *et al.*, 1986) (whose composition was 3.5g of powdered oats, 0.1g of Yeast extract (Difco[®]), 6.0g of agar and the mixture was then made up to one litter with distilled water and asymbiotically on PM (Phytamax™ orchid maintenance, Sigma) and MS (Murashige and Skoog, 1962) media.

Phytamax orchid maintenance medium (PM) contains:

	mg/l
Sucrose	2000.000
Peptone type I	2000.000
Charcoal	2000.000
MES (Free acid)	1000.000
Amonium nitrate	825.000
Potassium nitrate	950.000
Myo-Inositol	100.000
Calcium chloride anhydrous	166.000
Disodium EDTA dihydrate	37.240
Ferrous sulphate heptahydrate	27.850
Magnesium sulphate anhydrous	90.350
Potassium phosphate monobasic	85.000
Thiamine hydrochloride	10.000
Magnesium sulphate	8.450
Zinc sulphate heptahydrate	5.300
Boric acid	3.100
Nicotinic acid (Free acid)	1.000
Pyridoxine hydrochloride	1.000
Sodium molybdate dihydrate	0.125
Potassium iodide	0.415
Cobalt chloride hexahydrate	0.013
Cupric sulphate pentahydrate	0.013

Murashige and Skoog (MS) contains:

	mg/l
Potassium nitrate	1900.000
Amonium nitrate	1650.000
Calcium chloride anhydrous	332.200
Magnesium sulphate anhydrous	180.700
Myo-Inositol	100.000
Potassium phosphate monobasic	170.000
Disodium EDTA dihydrate	37.260
Ferrous sulphate heptahydrate	27.800
Magnesium sulphate monohydrate	16.900
Boric acid	6.200
Glycine (free base)	2.000
Zinc sulphate heptahydrate	8.600
Potassium iodide	0.830
Pyridoxine hydrochloride	0.500
Thiamine hydrochloride	0.100
Nicotinic acid (free acid)	0.500
Sodium molybdate dihydrate	0.250
Cobalt chloride hexahydrate	0.025
Cupric sulphate pentahydrate	0.025

Table 1. Orchid plant sources of seeds used in this study.

Source of seeds	PCPU Accession No.	Accession date	Locality
Liparis bowkeri	359/94/494/0176	Nov. 1994	Saiwa Swamp National Park
Satyrium crassicaule	476/95/1055/0187	Nov. 1995	Sasamua dam
Cynorkis anacamptoides	475/95/1056/0188	Nov. 1995	Sasamua dam
Bonatea steudneri	513/95/1179/0196	July 1995	Sagana
Eulophia horsfallii	56/93/183/0056	Oct. 1993	Saiwa Swamp National Park

2.5.4   Assessment of seed germination

The germination of the orchid seeds is often erratic hence difficult to evaluate in numerical terms (Hadley, 1970). The term germination here was used to describe sequential stages in the process of growth of protocorms. As a means of quantifying the assessment of such studies, germination was conveniently categorised into stage 0 - 4, culminating in the development of leaf and root initials.

0   No germination.

1   Swelling of the embryo and bursting of the seed coat.

2   Production of rhizoids

3   Production of shoot primordium

4   Production of root initial.

Swelling of an embryo and bursting of the seed coat (stage 1) without any subsequent development, was not considered as germination since this could be as a result of the embryo imbibing water.

CHAPTER THREE

3.0   RESULTS

3.1   ISOLATION OF MYCORRHIZAL FUNGI.

Fourteen terrestrial orchid species collected from various localities in Kenya were investigated for the presence of mycorrhizal fungi. Table 2 shows the 24 fungal isolates obtained from the 14 orchid species. Eight of the isolates belong to the form-genus *Rhizoctonia*.

Isolation from *Habenaria petitiana* was carried out at three stages of growth (pre-flowering, flowering and post flowering stages) and in all the three stages *Rhizoctonia* sp. No. 501/95/1013 was constantly isolated. This comparison was possible because the orchid species was available in the field in all the three growth stages.

Isolation attempts from *Eulophia streptopetala*, *E. horsfallii*, *E. orthoplectra*, *Satyrium coriophoroides* and *Disa stiarsii* did not yield any mycorrhizal fungi. Isolation from *Satyrium crassicaule* was carried out from two samples from two different localities and at different stages of growth, however no mycorrhizal fungi was realised. The few pelotons found in the roots of this orchid species were yellowish in colour and appeared as irregular masses of tissue showing no individual hyphae when observed under a microscope. One of the non-mycorrhizal fungi isolated from this species is shown (Plate 9). Isolation attempts from *Cynorkis anacamptoides* did not yield any fungus.

The other orchid species, *Eulophia stenophylla*, *E. montis-elgonis*, *E. petersii*, *Bonatea steudneri*, *Liparis bowkeri* and *Satyrium sacculatum* all yielded mycorrhizal fungi which

belong to the form-genus *Rhizoctonia*. The pelotons in the roots of these orchid species comprised of hyaline healthy hyphae (Plate 12).

Table 2. Fungi isolated from orchid species at different growth stages

Fungal isolate No.	Fungus	Host	Orchid growth stage
513/95/1015	*Rhizoctonia* sp.	*Bonatea steudneri*	Flowering
513/95/1016	Unidentified	*B. steudneri*	Flowering
513/95/1014	*Rhizoctonia* sp.	*B. steudneri*	Flowering
----------------	No isolate obtained	*Cynorkis anacamptoides*	Flowering
491/95/1007	Unidentified	*Disa stairsii*	Flowering
491/95/1008	,,	*D. stairsii*	Flowering
492/95/1009	,,	*D. stairsii*	Flowering
491/95/1006	,,	*D. stairsii*	Flowering
450/94/1001	,,	*Eulophia horsfallii*	Post-flowering
520/95/1018	*Rhizoctonia* sp.	*E. montis-elgonis*	Pre-flowering
520/95/1019	Unidentified	*E. montis-elgonis*	Pre-flowering
454/94/1003	,,	*E. orthoplectra*	Post-flowering
454/95/1004	,,	*E. orthoplectra*	Post-flowering
144/95/1023	*Rhizoctonia* sp.	*E. petersii*	Post-flowering
494/95/1011	Unidentified	*E. stenophylla*	Flowering
494/95/1012	,,	*E. stenophylla*	Flowering
442/94/1000	,,	*E. streptopetala*	Post-flowering
494/95/1010	*Rhizoctonia* sp.	*E. stenophylla*	Pre-flowering
501/95/1013	*Rhizoctonia* sp.	*Habenaria petitiana*	All the three stages
515/95/1017	*Rhizoctonia* sp.	*Liparis bowkeri*	Pre-flowering
537/95/1021	Unidentified	*Satyrium coriophoroides*	Pre-flowering
537/95/1022	,,	*S. coriophoroides*	Pre-flowering
451/94/1002	,,	*S. crassicaule*	Flowering
476/95/1005	,,	*S. crassicaule*	All the three stages
536/95/1020	*Rhizoctonia* sp.	*S. sacculatum*	Pre-flowering

Key:

Example 494/95/1010

494   Collector's number
95   Year of isolation
1010   PCPU accession number

3.2 IDENTIFICATION OF MYCORRHIZAL FUNGI

All the descriptions of the isolates were made from cultures grown on 2% PDA (Difco®).

Non of the fungal species was stimulated to produce a teleomorph stage. Therefore the descriptions made were based on the anamorph states. One of the non Mycorrhizal fungi isolated from *Satyrium crassicaule* is shown (Plate 9).

3.2.1 *Rhizoctonia* sp. No. 494/95/1010 (Plate 1)

The mycelia are cream turning golden brown with age. The reverse colour is the same as front. The isolate forms no aerial hyphae and colonies appear waxy and have a growth rate of 0.33mm h^{-1}. The hyphae are septate, branching often at right angles and septa forming on the branches immediately after branching. There is often a constriction of the hyphal branch at the point of origin from the main hyphae. Hyphae are hyaline and septate with a diameter range of 2.5 -6.0μm and a mean size of 4.3μm. Chlamydospores (monilioid cells) are hyaline and the shape ranges from sub-globose to ellipsoid. They occur in simple or in branched chains. Their size range from 8.5 - 20.5 X 8.5 - 17.5μm with mean size of 14.9 X 11.8μm. Sclerotia were not formed even after prolonged incubation.

Host: *Eulophia stenophylla*, a forest species collected from Nairobi Arboretum, at flowering stage.

3.2.2 *Rhizoctonia* sp. No. 513/95/1014 (Plate 2)

Young colonies are cream, turning brown with prolonged incubation. The reverse colour is same as front. Aerial hyphae are absent and the colonies appear waxy. The growth rate of the isolate is 0.39mm h^{-1}. Hyphae are hyaline and septate with a diameter range of 2.0 - 4.5μm and a mean size of 3.5μm. The hyphal branches are constricted at the point of origin and septa formed immediately after on the branch. There is coiling and twisting of the hyphae in culture. Hyphal branches arise from the main hyphae at right angles. Chlamydospores are hyaline, sub-globose to elliptical in shape and are broadly attached. They range in size from 9.5 - 19.0 X 7.0 - 12.0μm with a mean size of 11.2 X 9.6μm. The chlamydospores occur in simple or in branched chains. The chains are mostly curved, rarely straight. Sclerotia were not observed even after prolonged incubation.

Host: *Bonatea steudneri*, a scrubland species collected from Sagana at flowering stage.

3.2.3 *Rhizoctonia* sp. No. 515/95/1017 (Plate 3)

The mycelia are cream with no aerial hyphae. The reverse colour is same as front. The colonies appear waxy and have a growth rate of 0.34mm h^{-1}. The hyphae are hyaline and septate with a size range of 2.5 - 4.5μm and a mean size of 3.5μm. Branching often occurs at right angles while constriction of the hyphal branches often occurs at the point of origin and septa formed on the branches immediately after branching. Chlamydospores are hyaline sub-globose to elliptical in shape occurring in branched or unbranched long chains. Numerous chlamydospores are produced and they range in size from 9.5 - 16.5 X 9.5 - 14.0μm with a mean size of 12.7 X 11.2μm. No sclerotia were produced even after prolonged incubation.

Host: *Liparis bowkeri*, a forest species collected from Saiwa Swamp National Park at pre-flowering stage.

3.2.4 *Rhizoctonia* sp. No. 520/95/1018 (Plate 4)

The mycelium are cream and waxy in appearance with a growth rate of 0.39mm h^{-1} and produces no aerial hyphae. The reverse colour is same as front. Hyphae are hyaline and septate with constriction of the hyphal branches at the point of origin from the main hyphae. A septum is formed on the branch immediately after branching. Branches arise more or less at right angles in older mycelia. The hyphae diameter range from 2.5 - 4.5µm with a mean size of 3.3µm. Chlamydospores are hyaline and pyriform in shape. They occur in simple or in branched short chains and range in size from 9.5 - 16.5 X 9.5 - 12.0µm with a mean size of 13.1 X 10.6µm. No sclerotia were observed even after prolonged incubation.

Host: *Eulophia montis-elgonis,* a forest species collected from Saiwa swamp National park at pre-flowering stage.

3.2.5 *Rhizoctonia* sp. No. 536/95/1020 (Plate 5)

The colonies are cream and waxy in appearance, produce no aerial hyphae and have a growth rate of 0.31mm h^{-1}. The reverse colour is same as front. Hyphae are hyaline and septate and forms hyphal coils. There is constriction of the hyphal branches at the point of origin from the main hyphae. Septa are formed on the hyphal branches immediately after branching. In older colonies, hyphal branches tend to arise from the main hyphae at right angles. Chlamydospores are hyaline, burrel shaped and occur in simple or in branched

chains. They range in size from 9.5 - 26.0 X 7.0 - 9.5µm with a mean size of 18.4 X 7.9µm. No sclerotia were produced even after prolonged incubation.

Host: *Satyrium sacculatum,* a grassland species collected from Saiwa Swamp National Park at pre-flowering stage.

3.2.6 *Rhizoctonia* sp. No. 513/95/1015 (Plate 6)

The mycelium is yellowish-brown becoming brown with age. Colonies show colour zonation and have a growth rate on PDA of 0.50mm h^{-1}. Brown aerial hyphae present, while sub-merged hyphae are hyaline. The hyphal branches show constriction at the point of origin from the main branch. A septum is formed on the branch immediately after branching. The hyphal diameter range 2.5 - 4.5µm and has a mean size of 3.42µm. Chlamydospores are sub-merged, hyaline and burrel shaped occurring in simple or in branched chains. Their size range from 19.0 - 28.0 X 7.0 -12.0µm with a mean size of 22.7 X 10.0µm.

Host: *Bonatea steudneri* a scrubland species collected from Sagana at flowering stage.

3.2.7 *Rhizoctonia* sp. No. 144/95/1023 (Plate 7)

The mycelium is yellow with no aerial hyphae. The reverse colour is same as front. The isolate has a growth rate of 0.18mm h^{-1}. The hyphae are hyaline and septate with a constriction of the hyphal branch at the point of origin from the main hyphae. A septum is formed on the branch immediately after branching. No chlamydospores were formed even after prolonged incubation. However some hyphae became enlarged in size after prolonged incubation.

Host: *Eulophia petersii,* a dry scrubland species collected from Mt. Nyiru at post-flowering stage.

3.2.8 *Rhizoctonia* sp. No. 501/95/1013 (Plate 8)

The mycelium is yellowish-cream turning brown with age and the reverse colour is same as front. The colonies show zonation occurring in concentric rings. Aerial hyphae present and the isolate has a growth rate of 0.45mm h^{-1}. There is often a constriction of the hyphal branch at the point of origin from the hyphae. Septum is formed on the branch immediately after branching and branching often occurs at right angles. Chlamydospores are submerged, hyaline and burrel shaped occurring in simple or in branched chains. Their size range between 18.0 - 27.0 X 8.5 - 11.5µm with a mean size of 21.8 X 10.6µm.

Host: *Habenaria petitiana*, a grassland species collected near Kerita forest at pre-flowering, flowering and post-flowering stages.
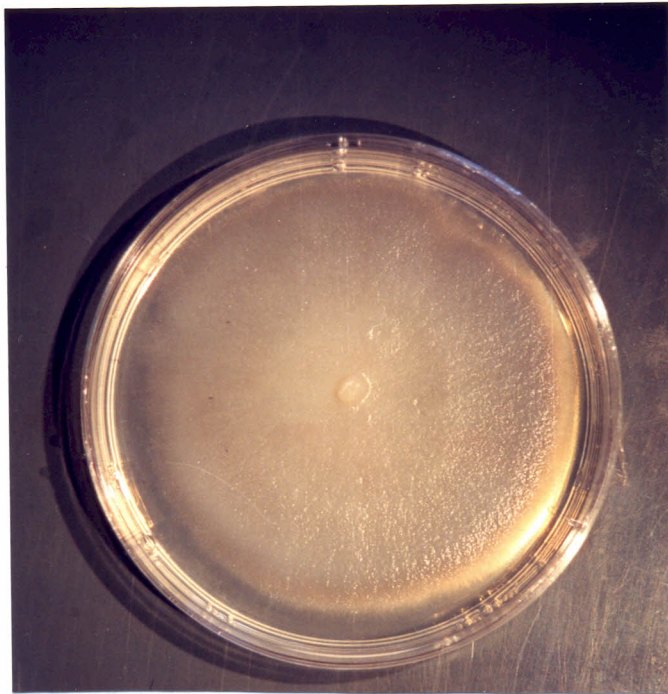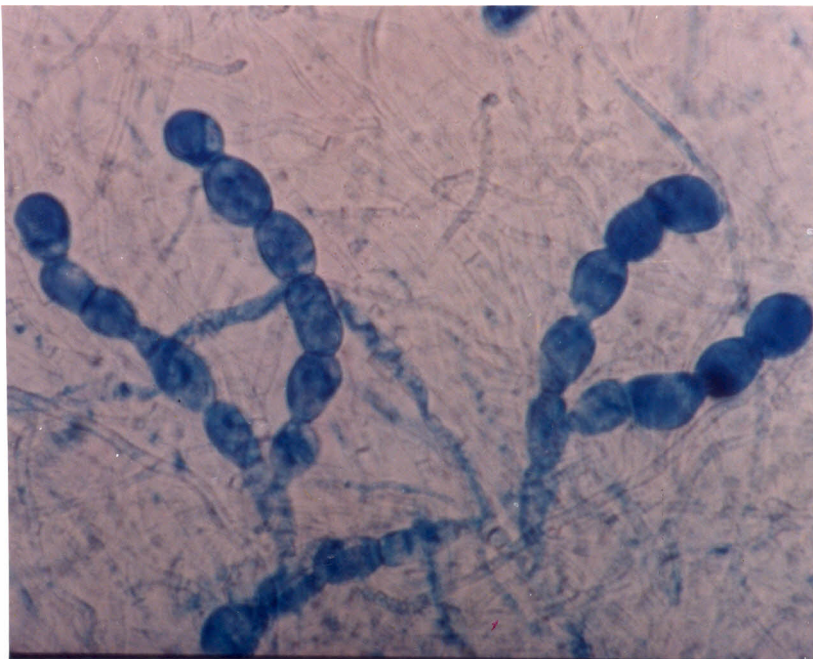
PLATE 1: (A) TWO WEEK OLD COLONY OF *RHIZOCTONIA* SP. NO. 494/95/1010 ON PDA
(B) CHLAMYDOSPORES OF THE SAME ISOLATE (X 500)

PLATE 2: **(A)** TWO WEEK OLD COLONY OF *RHIZOCTONIA* SP. NO. 513/95/1014 ON PDA.
(B) CHLAMYDOSPORES OF THE SAME ISOLATE (X 500)
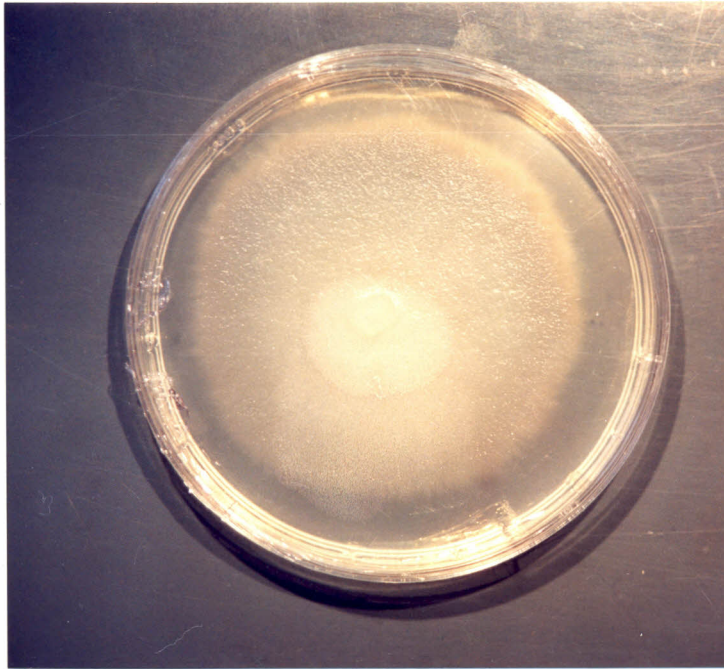
PLATE 3: **(A)** TWO WEEK OLD COLONY OF *RHIZOCTONIA* SP. NO. 515/95/1017 ON PDA
(B) CHLAMYDOSPORES OF THE SAME ISOLATE (X 500)
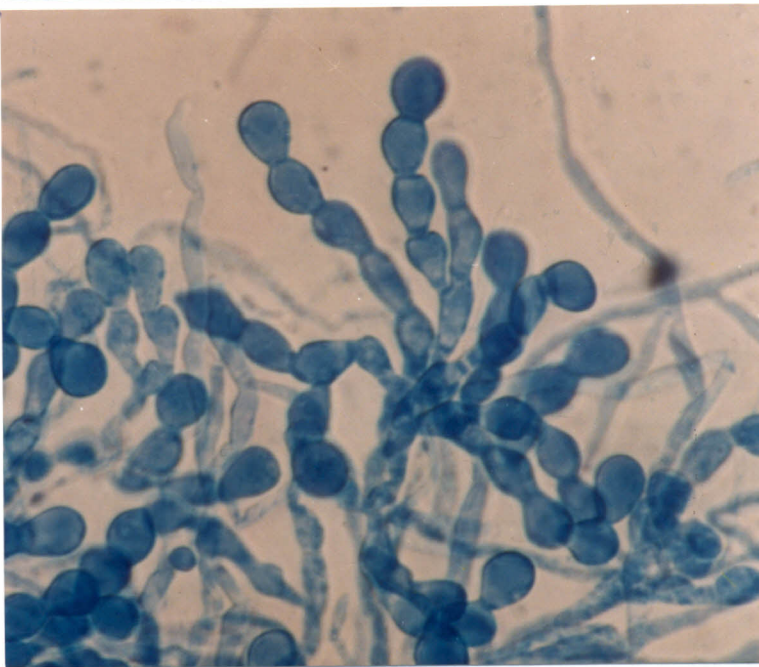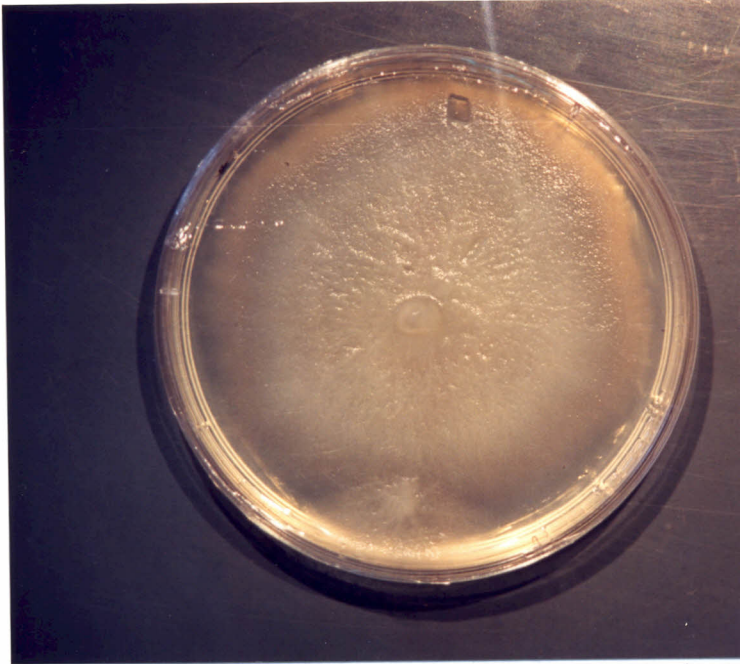
PLATE 4: **(A)** TWO WEEK OLD COLONY OF *RHIZOCTONIA* SP. NO. 520/95/1018 ON PDA.
(B) CHLAMYDOSPORES OF THE SAME ISOLATE (X 500).
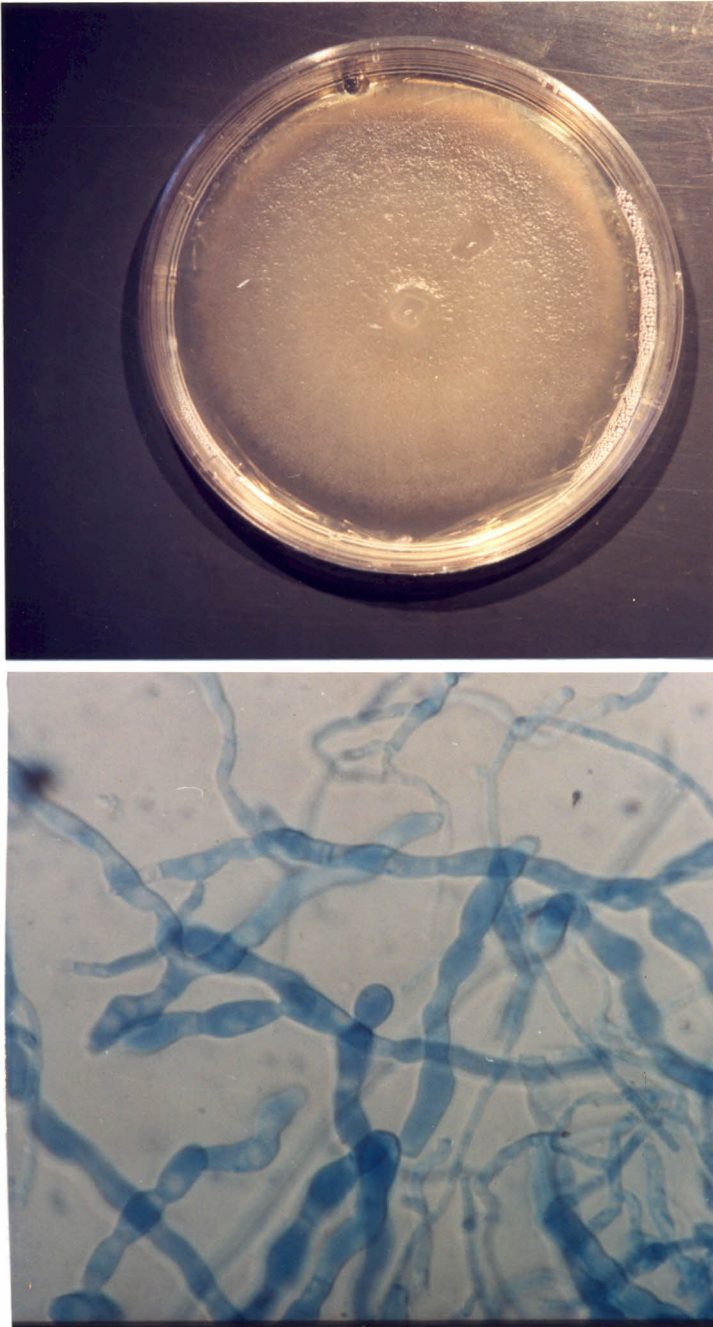
PLATE 5: **(A)** TWO WEEK OLD COLONY OF *RHIZOCTONIA* SP. NO. 536/95/1020 ON PDA
(B) CHLAMYDOSPORES OF THE SAME ISOLATE (X 500)
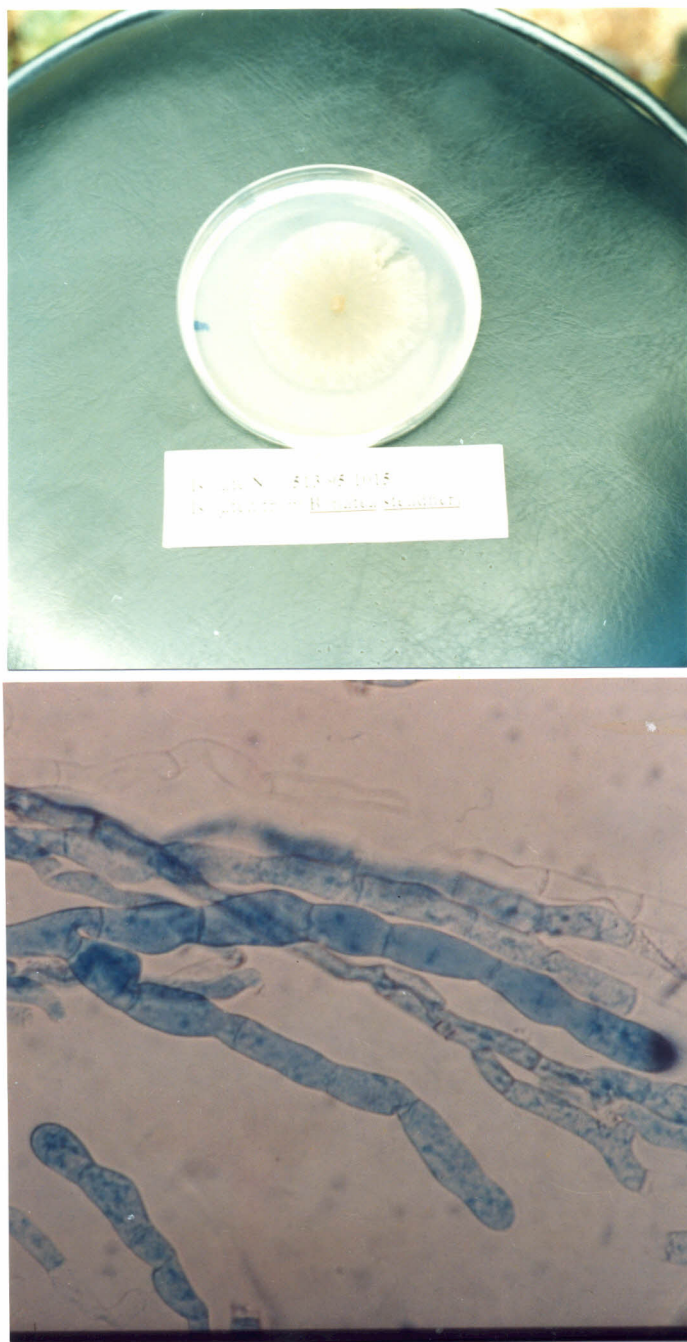
PLATE 6: **(A)** TWO WEEK OLD COLONY OF *RHIZOCTONIA* SP. NO. 513/95/1015 ON PDA.
(B) CHLAMYDOSPORES OF THE SAME ISOLATE (X 500).
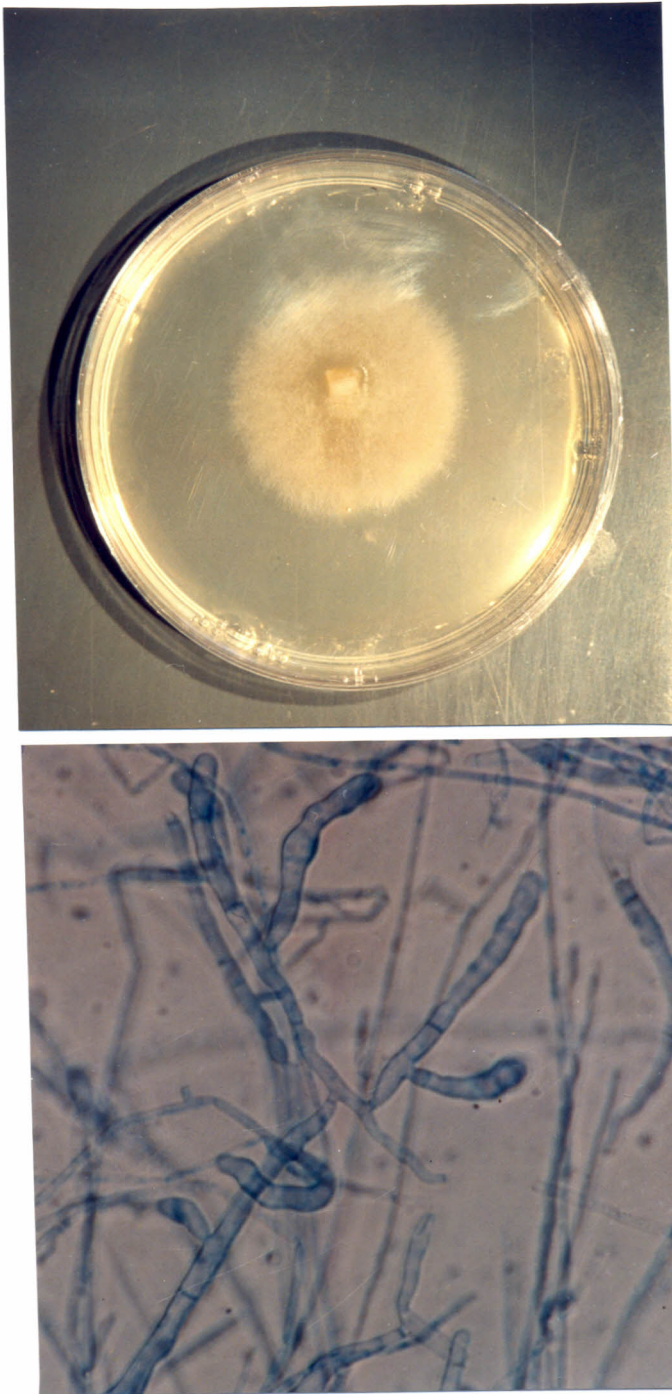
PLATE 7: (A) TWO WEEK OLD COLONY OF *RHIZOCTONIA* SP. NO. 144/95/1023 ON PDA.
(B) MYCELIA OF THE SAME ISOLATE (X 500).
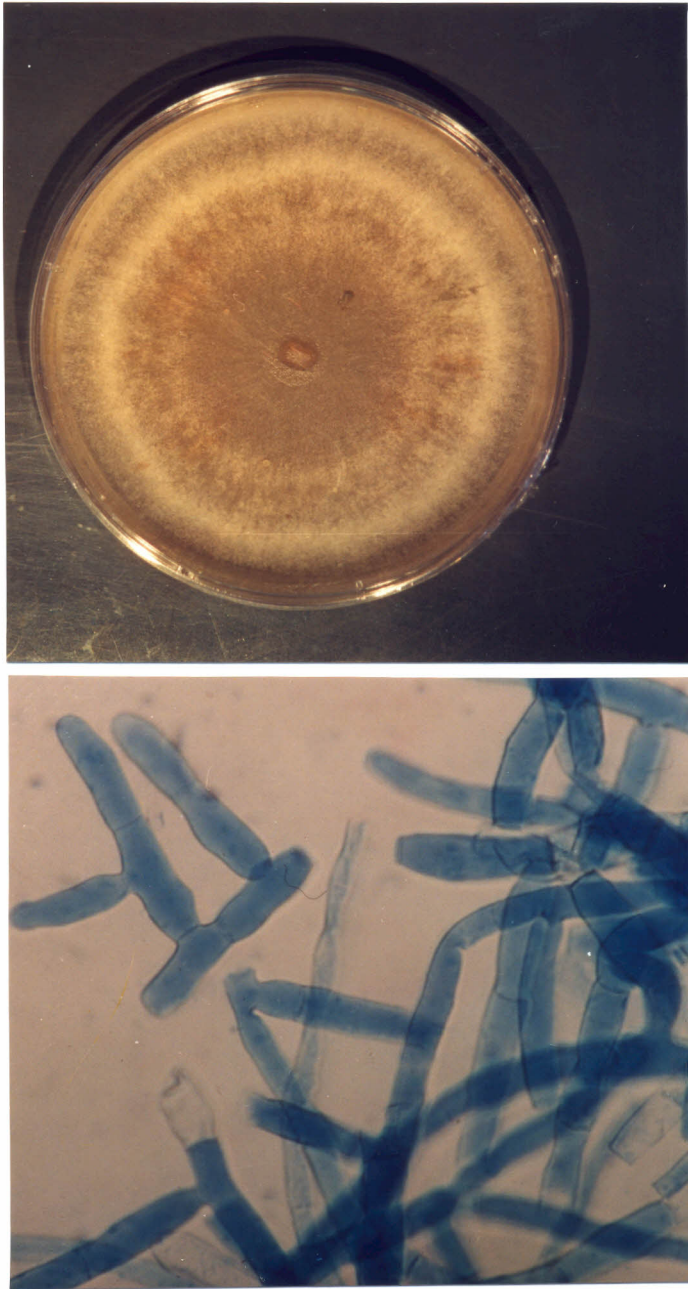
PLATE 8: (A) TWO WEEK OLD COLONY OF *RHIZOCTONIA* SP. NO. 501/95/1013 ON PDA
(B) CHLAMYDOSPORES OF THE SAME ISOLATE (X 500)
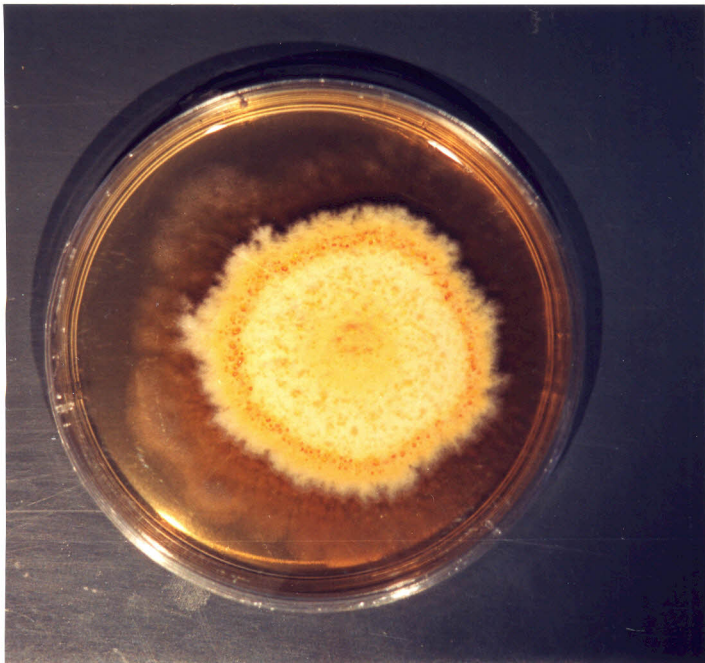
PLATE 9: ONE WEEK OLD COLONY OF ISOLATE No. 451/94/1002 ISOLATED FROM *SATYRIUM CRASSICAULE.*

3.3   SYMBIOTIC GERMINATION

The fungal isolates were tested for mycorrhizal activity with five terrestrial orchid species namely: *Bonatea steudneri*, *Liparis bowkeri*, *Satyrium crassicaule*, *Cynorkis anacamptoides* and *Eulophia horsfallii*. Out of these three species namely, *S. crassicaule*, *L. bowkeri* and *C. anacamptoides* were stimulated to germinate symbiotically. *S. crassicaule* was stimulated to germinate by *Rhizoctonia* spp. Nos. 494/95/1010, 520/95/1018, 536/95/1020 and 515/95/1017. *L. bowkeri* was stimulated by *Rhizoctonia* sp. No. 515/95/1017 only, while *C. anacamptoides* was stimulated by *Rhizoctonia* spp. Nos. 494/95/1010 and 520/95/101. These two *Rhizoctonia* species supported complete germination of the orchid species. They also germinated asymbiotically on PM (Phytamax™ orchid maintenance) and MS (Murashige and Skoog, 1962) media. The other two, *B. steudneri* and *E. horsfallii* were not stimulated to germinate by any fungi and neither did any of them germinate asymbiotically on PM or MS media. *Rhizoctonia* sp. No. 144/95/1023 did not grow on BOM [Basic Oats Medium, (Clements *et al.*, 1986)], the medium used for symbiotic seed germination.
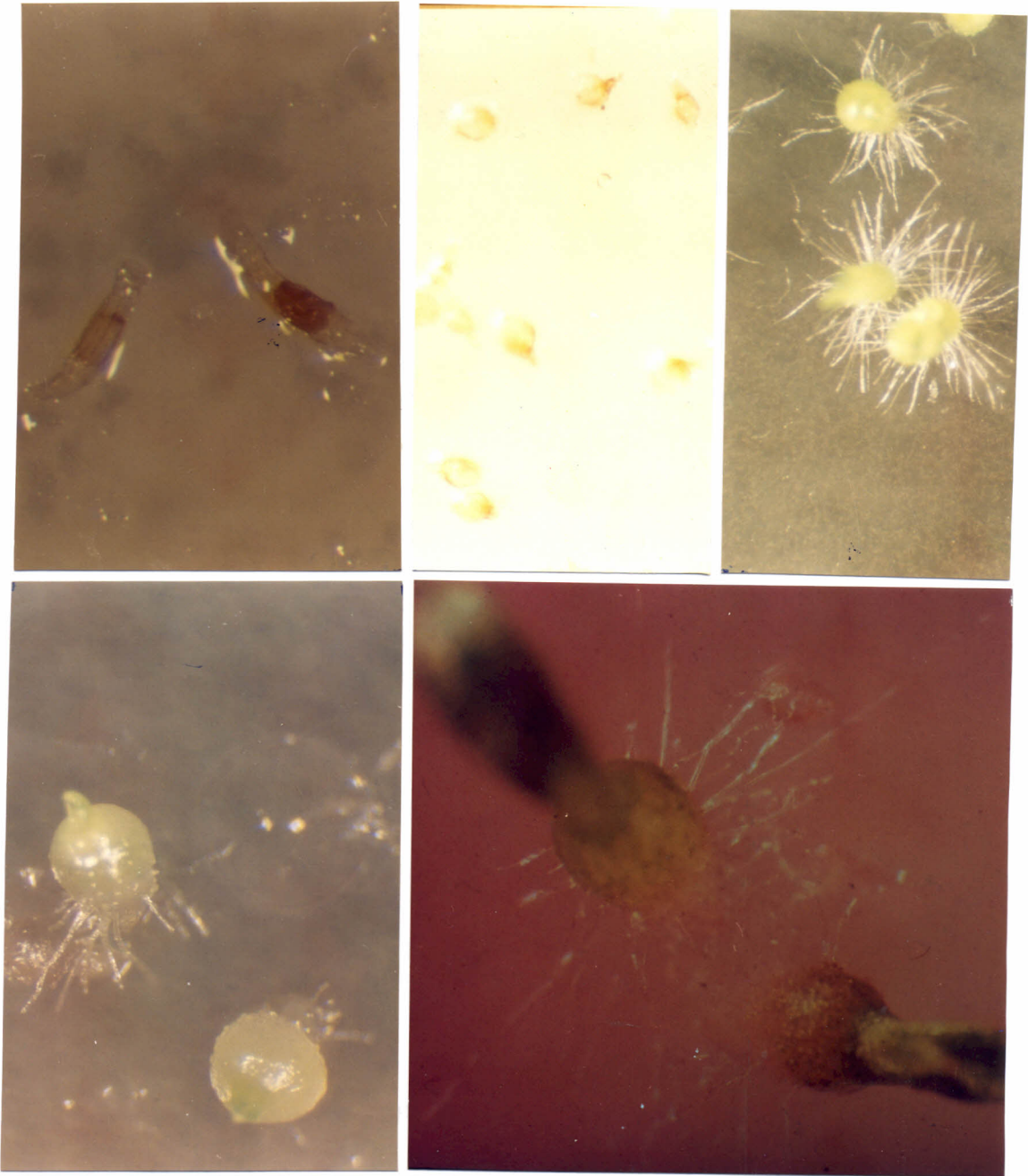
PLATE 10: GERMINATION STAGES OF AN ORCHID SEED.
    (a) Ungerminated seed (**stage 0**)
    (b) Swelling of the embryo and bursting of the seed coat (**stage 1**)
    (c) Production of rhizoids (**stage 2**)
    (d) Production of shoot primordium (**stage 3**) [Arrow]
    (e) Production of root initial (**stage 4**) [Arrow]

3.3.1   Symbiotic germination of *Satyrium crassicaule* seeds

Four fungal isolates stimulated the germination of this orchid species and they belong to the form-genus *Rhizoctonia*. *Rhizoctonia* sp. No. 494/95/1010 stimulated 84.7% of the protocorms to stage four of the germination process after 36 days of incubation (Figure 1). *Rhizoctonia* sp. No. 520/95/1018 stimulated 15.3% protocorms to stage four of the germination process within the same time (Figure 1), while *Ceratobasidium cornigerum* had stimulated 45.3% of the protocorms to stage one. Tests using isolates Nos. 451/95/1002 and 476/95/1005 obtained from the same species, showed no germination of seeds. Isolates 520/95/1019, 537/95/1021 and 537/95/1022 were parasitic to the seeds. Seeds germinated on the two asymbiotic media (PM and MS) used. PM medium showed germination capabilities than MS and had stimulated 28.7% of the protocorms to stage four after 36 days of incubation. The control plates showed no germination, they had 6.7% seeds at stage one after 36 days but no further development was observed in these protocorms. All the plates inoculated with other fungal isolates and the control plates showed no germination.

3.3.2   Symbiotic germination of *Cynorkis anacamptoides* seeds

Germination was recorded in all symbiotic and control plates except those inoculated with fungal isolates Nos. 520/95/1019, 537/95/1021 and 537/95/1022 which were parasitic to the seeds. However, growth in others including the control plates never progressed beyond stage two of the germination process, except for the plates inoculated with *Rhizoctonia* spp. Nos. 494/95/1010 and 520/95/1018. *Rhizoctonia* sp. No. 494/95/1010 stimulated 68.7% of the protocorms to stage four of the germination process after 36 days of incubation (Figure 2).

Plates inoculated with *Rhizoctonia* sp. No. 520/95/1018 had 16% of the protocorms at stage three after 36 days (Figure 2). *Ceratobasidium cornigerum* stimulated 24% of protocorms to stage two after incubating for the same period but growth did not progress beyond this stage. The seeds of this orchid species also germinated on the asymbiotic media (PM and MS). PM showed better germination capabilities and had stimulated 2.7% of protocorms to stage three after 36 days of incubation.

3.3.3   Symbiotic germination of *Liparis bowkeri* seeds

This orchid species was stimulated to germinate symbiotically by *Rhizoctonia* sp. No. 515/95/1017 only, isolated from this same species. After 67 days of incubation 4.7% of protocorms had reached stage three of the germination process (Figure 3). However the protocorms did not develop beyond this stage, even after prolonged incubation. *Ceratobasidium cornigerum* did not stimulate germination of this orchid species. Germination was successful with both asymbiotic media (PM and MS). PM which showed better germination effects than MS, had 18% of the protocorms at stage four after 67 days (Figure 3). No germination was recorded on the control plates.

3.3.4   Symbiotic germination of *Bonatea steudneri* seeds

This orchid species was not stimulated to germinate by any *Rhizoctonia* species or any other fungal isolate, not even the *Rhizoctonia* spp. Nos. 513/95/1014 and 513/95/1015 which were isolated from this species. The seeds were also not stimulated to germinate by *Ceratobasidium cornigerum* or by the asymbiotic media.

3.3.5   Symbiotic germination of *Eulophia horsfallii* seeds

This orchid species was not stimulated to germinate by any *Rhizoctonia* spp. or any other fungal isolate used. The seeds also did not germinate on any of the asymbiotic media use.
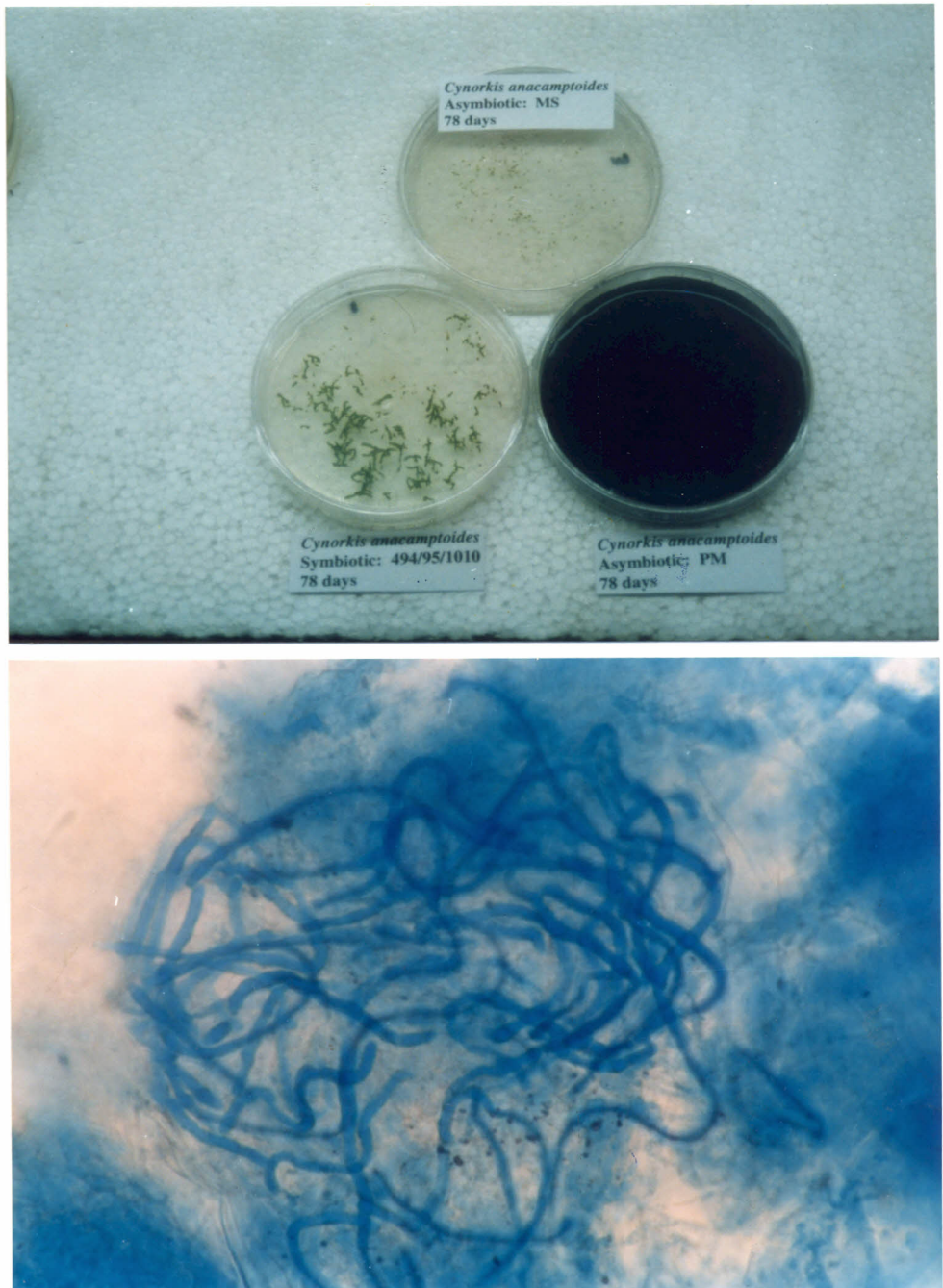
PLATE 11: (A) 78 DAYS OLD SEEDLINGS OF *CYNORKIS ANACAMPTOIDES* INOCULATED WITH *RHIZOCTONIA* SP. NO. 494/95/1010 AND ON ASYMBIOTIC MEDIA (PM AND MS). **(B)** SQUASHED PROTOCORM OF *C. ANACAMPTOIDES* AFTER INFECTION SHOWING A PELOTON OF *RHIZOCTONIA* SP. NO. 494/95/1010 (X 500).

PLATE 12: ROOT HAIR OF *EULOPHIA STENOPHYLLA* INFECTED BY MYCORRHIZAL FUNGI (X 500).
[**H** - HYPHA; **R** - ROOT HAIR]
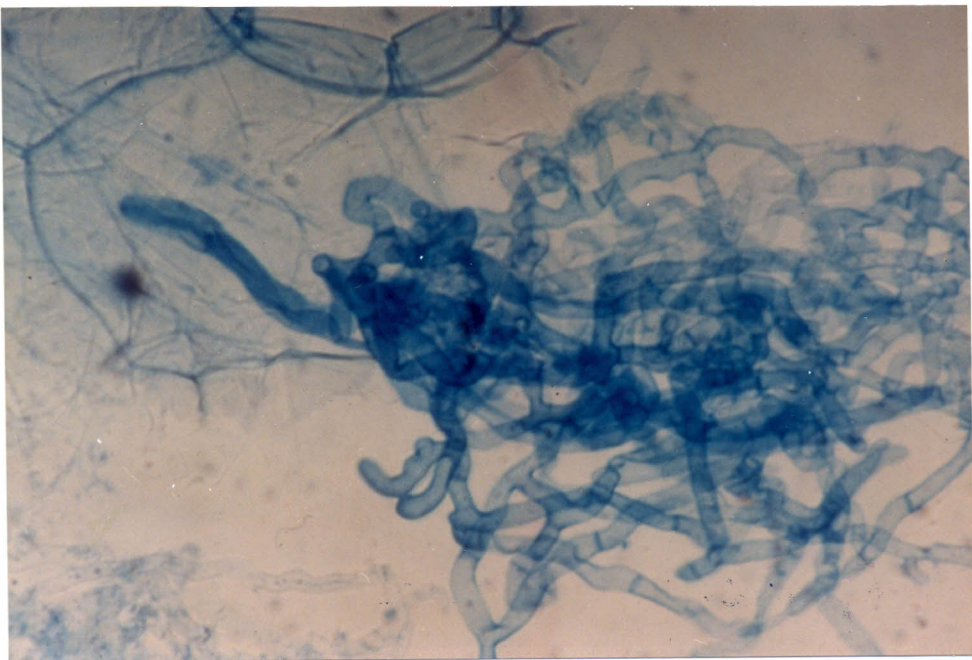
PLATE 13: A HEALTHY PELOTON IN THE ROOT CORTEX OF *EULOPHIA STENOPHYLLA* (X 500).

Fig. 1 The percentage of protocorms of *Satyrium crassicaule* at various stages of germination at the 36th day after inoculating with *Rhizoctonia* spp.
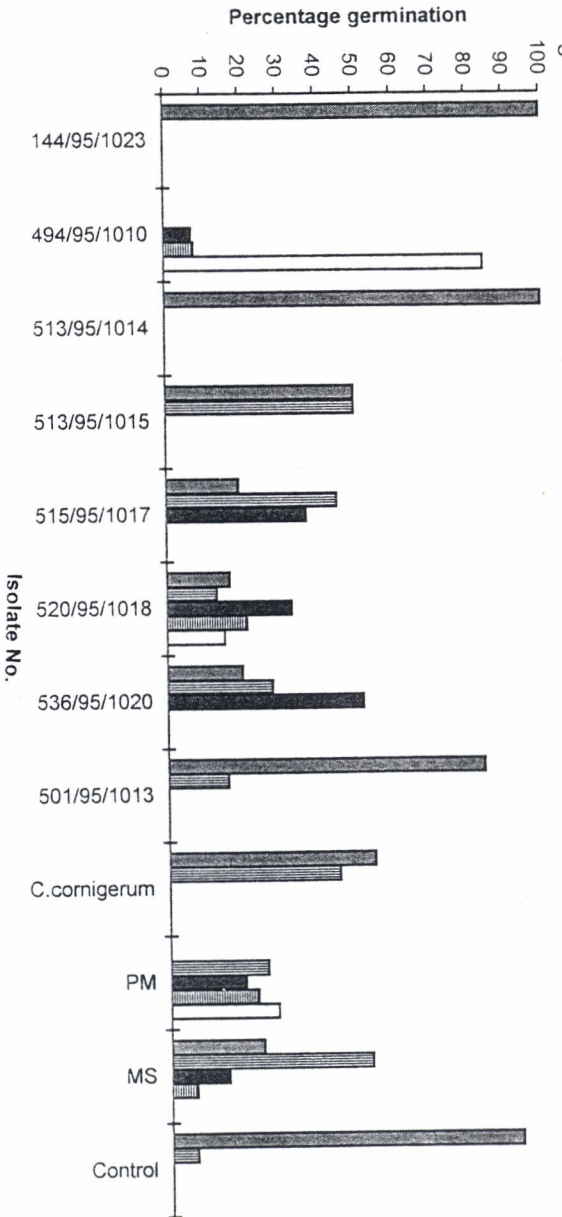
Fig. 2 The percentage of protocorms of *Cynorkis anacamptoides* at various stages of germination at the 36th day after inoculating with *Rhizoctonia* spp.
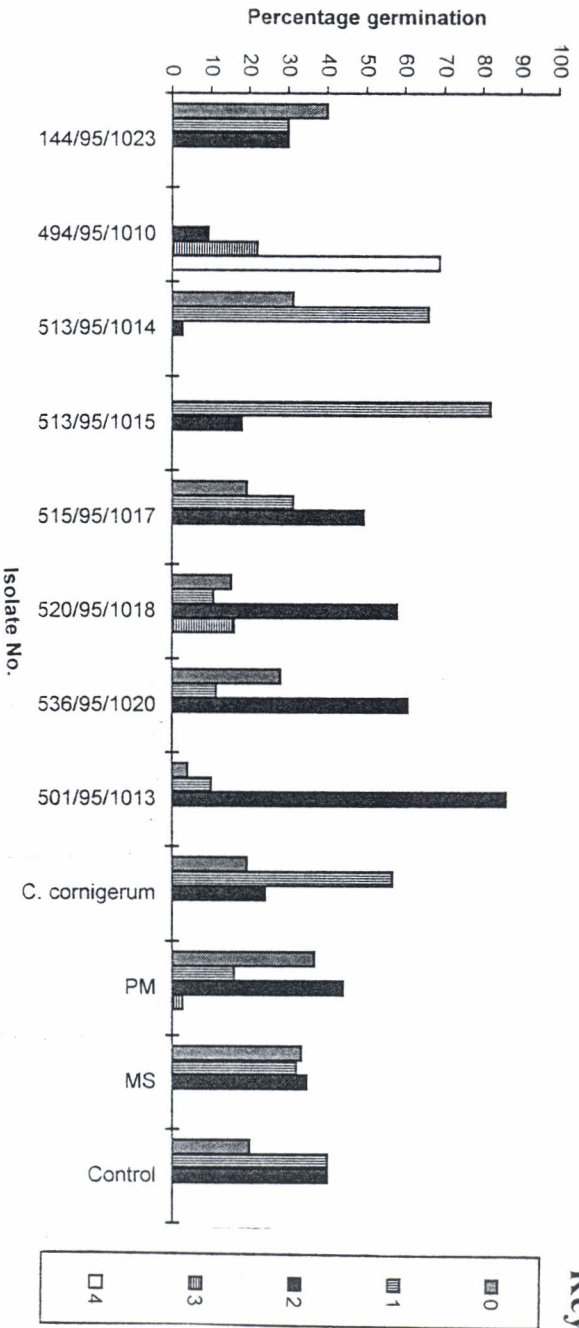
Fig. 3 The percentage of protocorms of *Liparis bowkeri* at various stages of germination at the 67th day after inoculating with *Rhizoctonia* spp.

Key

☐ 4   ▦ 3   ■ 2   ▤ 1   ▥ 0
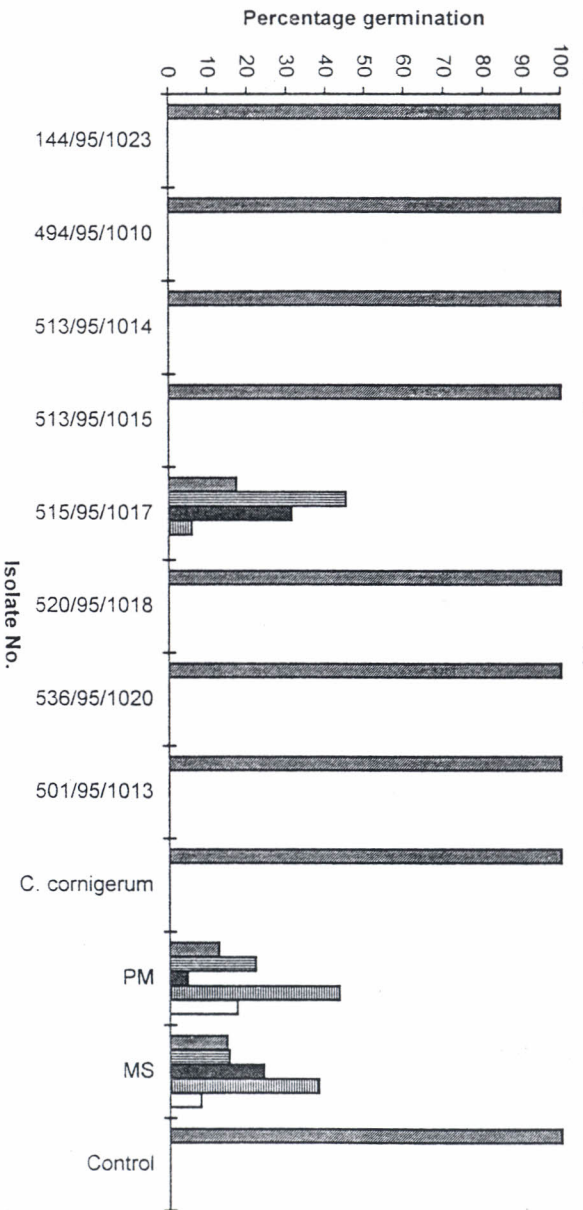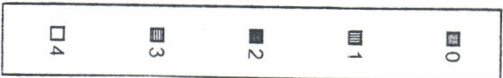
CHAPTER FOUR

4.0 DISCUSSION

4.1 ISOLATION OF MYCORRHIZAL FUNGI.

The method for the isolation of the mycorrhizal fungi involves Sterilisation of the root material to get rid of soil contaminants. This method however, is not full proof because a harsh root Sterilisation procedure might also destroy the endophytic fungi (mycorrhizal fungi). This means that a number of fungal isolates are inevitably contaminants. To supplement Sterilisation procedures, isolation of fungi was carried out from pelotons which were still in root tissue. Harvais and Hadley (1967) showed that this method increases the chance of isolating mycorrhizal fungi and cuts down the probability of isolating contaminants. The ratio of Rhizoctonias to non-Rhizoctonias obtained from the study is high (Mordue, Pers. Comm.). This means the method of isolation is reliable.

The timing for the isolation of mycorrhizal fungi is crucial. *Eulophia stenophylla, E. montis-elgonis, E. petersii, Satyrium sacculatum, Liparis bowkeri, Habenaria petitiana* and *Bonatea steudneri* all yielded mycorrhizal fungi. They were all collected at pre-flowering stage, except for *B. steudneri* and *E. petersii* which were collected at flowering and post-flowering stages respectively. These orchid species were found to possess pelotons in their roots which comprised of hyaline and distinctly healthy hyphae (Plate 12). The other orchid species namely, *Eulophia streptopetala, E. horsfallii, E. orthoplectra, Cynorkis anacamptoides, Satyrium coriophoroides* and *Disa stairsii* were collected at flowering or post-flowering stages except *S. crassicaule* which was collected at all the three stages of growth (Table 2). These orchid species did not yield any mycorrhizal fungi. The pelotons

in these species were yellowish in colour and appeared as a mass of irregular tissue showing no individual hyphae when observed under a microscope, suggesting that the pelotons were already in the digested state. This confirms that the best time to isolate orchid mycorrhizal fungi is in the early stages of growth. This conclusion supports Hadley's (1980) observation that the optimal time for the isolation of mycorrhizal fungi is during the rapid vegetative growth prior to flowering, and by Harvais and Smith (1983) who showed that most pelotons in the roots are in the digested state at the late stages of growth of orchids. This study confirms that selecting the proper growth stage of the plants is crucial for isolation of mycorrhizal fungi.

Despite being available at all the three stages of growth (pre-flowering, flowering, and post-flowering), isolation from *Satyrium crassicaule* did not yield any mycorrhizal fungi. Most of the roots obtained from this species were uninfected. Few roots showed sparse pelotons and when observed under a compound microscope the pelotons appeared as a mass of homogeneous tissue showing no individual hyphae. This suggests that they were probably in the digested state. This observation indicates that this species may be largely independent of mycorrhizal fungi at adult stage since it seems to be poorly infected at all the adult stages observed. Harley and Smith (1983) showed that photosynthetically active seedlings and mature plants of some orchid species may be quite independent of their fungal partners. *S. crassicaule* could be falling under this category, hence the poor infection if any at all. Isolation of mycorrhizal fungi from *S. crassicaule* probably need to be done at very early stages of growth, before the establishment of the photosynthetic phase.

4.2 IDENTIFICATION OF MYCORRHIZAL FUNGI.

Eight fungal isolates were found to be mycorrhizal and they all belong to the form-genus *Rhizoctonia*, the group that is known to form mycorrhizal association with orchids (Table 2). The non-*Rhizoctonia* isolates obtained belong to two families; Moniliaceae and Dematiaceae. Most members of these two groups of fungi are commonly found growing in the soil saprophytically and are therefore common contaminants during mycorrhizal fungal isolation. They produce conidia, hyaline or coloured from unorganised conidiophores or directly from the hyphae, unlike the members of the form-genus *Rhizoctonia* which reproduce by chlamydospores.

Out of eight *Rhizoctonia* species isolated during the study, five bear close resemblance. These are *Rhizoctonia* spp. Nos. 494/95/1010, 520/95/1018, 536/95/1020, 515/95/1017 and 513/95/1014. They have similar cultural characters of colonies that are cream and waxy in appearance, no aerial hyphae and have growth rate on PDA ranging 0.31-0.39 mm h^{-1}. They have hyaline hyphae and chlamydospores but the size, shape and presentation of the chlamydospores vary among the isolates. Chlamydospore characters are consistent within a species irrespective of the substrate and are considered reliable for specific differentiation (Hadley, 1980). This chlamydospore and hyphal characters were used to separate these fungal isolates.

Rhizoctonia sp. No. 494/95/1010, has a hyphal diameter range of 2.5 - 6.0μm and a mean size of 4.5μm. The chlamydospores are sub-globose to elliptical in shape and have a mean size of 14.9 x 11.8μm (Plate 1). *Rhizoctonia* sp. No. 520/95/1018 has hyphal size range of 2.5 - 4.5μm and a mean of 3.3μm. The chlamydospores are pyriform in shape and have a mean size of 13.1 x 10.6μm (Plate 4). Both species stimulated the germination of *Satyrium*

crassicaule and *Cynorkis anacamptoides*. However, *Rhizoctonia* sp. No. 494/95/1010 was more efficient in stimulating germination than *Rhizoctonia* sp. No. 520/95/1018 (Figure 1 and 2). *Rhizoctonia* sp. No. 520/95/1018 differs from *Rhizoctonia* sp. No. 494/95/1010 in that it produces pyriform chlamydospores, while *Rhizoctonia* sp. No. 494/95/1010 produces sub-globose to elliptical ones.

Rhizoctonia spp. Nos. 513/95/1014 and 515/95/1017 have chlamydospores mean sizes of 11.2 x 9.6 and 12.7 x 11.2μm respectively. They show same chlamydospore shapes of sub-globose to ellipsoid. However, *Rhizoctonia* sp. No. 513/95/1014 chlamydospores are broadly attached and occur in short chains (Plate 2), while *Rhizoctonia* sp. No. 515/95/1017 chlamydospores are narrowly attached and occur in long chains (Plate 3). On the other hand, *Rhizoctonia* sp. No. 513/95/1014 did not stimulate germination of any orchid species tested but *Rhizoctonia* sp. No. 515/95/1017 stimulated the germination of *Liparis bowkeri* (Figure 3). *Rhizoctonia* sp. No. 536/95/1020 differs from all the rest by producing burrel shaped chlamydospores (Plate 5).

Identification was attempted using two keys, one by Currah and Zelmer (1992) for temperate orchid mycorrhizal fungi, and the other by Sneh *et al*. (1991) for *Rhizoctonia* species. Specific identification was not possible because non of the species fitted those in the keys.

The five *Rhizoctonia* species (*Rhizoctonia* spp. Nos. 494/95/1010, 513/95/1014, 515/95/1017, 520/95/1018 and 536/95/1020) resemble *Epulorhiza repens* (Bernard) Moore (Sneh *et al*., 1991), in producing colonies that are waxy in appearance and having hyaline hyphae and chlamydospores. *E. repens* on PDA produces colonies with aerial mycelium in irregular low cream to yellowish patches. It also produces sclerotia and chlamydospores that

are hyaline, ellipsoidal to nearly spherical, 13-18 x 8-17μm in short branched or unbranched chains. However non of the five fungal isolates produced aerial hyphae or sclerotia on PDA. Apart from the lack of the aerial hyphae and sclerotia, *Rhizoctonia* spp. Nos. 494/95/1010 and 513/95/1014 appear more closely related to *E. repens* than the others, with chlamydospore size ranges of 8.5-20.5 x 8.5-17.5 μm and 9.5-19 x 7.0-12.0 respectively. The chlamydospore shape for both species are also similar to that of *E. repens* and they occur in branched or unbranched short chains just as in *E. repens*. However *Rhizoctonia* sp. No. 494/95/1010 has a hyphal diameter range of 2.5 - 6.0μm and a mean size of 4.5μm unlike that of *E. repens* which is 2.5 - 3.5μm. On the other hand *Rhizoctonia* sp. No. 513/95/1014, chlamydospores are broadly attached and has an hyphal diameter range of 2.5 - 4.5μm.

The other three *Rhizoctonia* species vary more from *E. repens*. *Rhizoctonia* sp. No. 520/95/1018 produces chlamydospores that are pyriform in shape. *Rhizoctonia* sp. No. 515/95/1017 chlamydospores occur in long chains (Plate 3), unlike those of *E. repens* which are short.

Rhizoctonia spp. Nos. 513/95/1015 and 501/95/1013 resemble *Ceratorhiza goodyerae-repentis* (Costantin and Dufour) Moore, (=*Rhizoctonia goodyerae-repentis* Costantin and Dufour). They produce burrel shaped chlamydospores, have high growth rates and the colony colour (yellowish-cream to brown) are all characters similar to those of *C. goodyerae-repentis*. However the aerial mycelia of the *Rhizoctonia* isolates are scanty and show no sclerotia, while *C. goodyerae-repentis* shows abundant cottony aerial mycelium and the colonies are glabrous due to numerous sclerotia. Colonies of *Rhizoctonia* sp. No. 501/95/1013 show colour zonation occurring in concentric bands, while *Rhizoctonia* sp. No. 513/95/1015 colour zonation is due to age, with the older parts of the mycelium turning brown

while the periphery or the young mycelium remains yellowish-cream.

Rhizoctonia sp. No. 144/95/1023 varied from all the rest. Apart from having a very slow growth rate, it did not produce chlamydospores or sclerotia. It was placed to the form-genus *Rhizoctonia* purely on mycelial characteristics (branching of the hyphae, constriction of hyphal branches at the point of origin and formation of septa immediately after branching).

Identification of the isolated mycorrhizal fungi using the available keys was hampered due to several reasons:

(a) The characters used in the keys such as colony colour, texture, growth rate, sclerotia and hyphal characters all may change depending on the culture media used and other growth conditions. These characters are therefore not stable and can not be adequately used to describe a species. Although chlamydospore characters have been reported to be stable, when employed alone it is difficult to describe a species because their similarity may not be due to their relatedness.

(b) The number of species described in these keys are few as compared to the number of the reported species. The key by Currah and Zelmer (1992) describes 29 species only. This is inclusive of the *Rhizoctonia* species whose teleomorph stages are known. The key by Sneh *et al*. (1991), describes only 12 species of the form-genus *Rhizoctonia*, the other species described are the teleomorph states of the genus *Rhizoctonia*.

(c) Identification was even made more difficult because attempts to stimulate the isolated fungi to produce teleomorph stages failed. Specific identification would have been easy had the species produced teleomorph states. However it is difficult to induce teleomorph states in the form-genus *Rhizoctonia* (Stretton *et al*., 1964).

The fungal isolates were also sent to International Mycological Institute, Egham, UK

to confirm the identification, but was done to the genus level, emphasising the difficulty in the identification of this group of fungi.

Identification of this group of mycorrhizal fungi has generally been seen as problematic. Out of the 23 orchid mycorrhizal fungi isolated from various orchid species by Warcup in 1973, he identified four to the species level, four to the genus level and the rest remained unidentified. Benzing and Friedman (1981) and Benzing (1982) isolated mycorrhizal fungi from 14 orchids native to the Costa Rica rain forest, and Goh *et al*. (1992) surveyed the mycorrhizal fungi of 20 terrestrial and epiphytic orchid species of Singapore. Neither of these workers even attempted to identify the fungi. This is because proper keys are globally not available for this group of fungi.

The lack of conclusive identification keys for orchid mycorrhizal fungi, has resulted in a confused state of the taxonomy for this group of fungi. Andersen and Stalpers (1994) have shown that most of the specific names, even those that are in frequent use, are illegitimate since there is neither a selected nomenclatural type specimen nor a Latin description. Out of the 119 epithets published within the *Rhizoctonia* group, 48 were found to constitute a *nomina nuda* (not effectively published), 30 to belong to taxa that should be excluded from *Rhizoctonia* and nine to be designated to taxa whose identity is uncertain since there is no extant type material and the diagnoses are vague. The remaining, 25 were regarded as taxonomic or nomenclatural synonyms, thus reducing the number of the distinguishable taxa with validly published names to seven. This confused state of taxonomy should make it clear that any discussion of specificity based on tentatively named strains is futile.

The whole group is therefore in need of taxonomic revision, making use of the hyphal characters available combined with modern methods of analysis such as ultrastructure, DNA

analysis and anastomosis testing. The isolates of *Rhizoctonia* can be assigned to anastomosis groups by pairing the isolates with the "tester" strain and observing the hyphae for fusion (Parmeter et al., 1969; 1979; Hyakumachi and Ui, 1987). The type of septa

formed and the number of nuclei per hyphal cell can be a good source of characters. Next to this, DNA base sequence homologies can also be used to assess genotypic relationships among these species (Vilgalys, 1988).

4.3 SYMBIOTIC GERMINATION

Symbiotic germination tests were carried out on five orchid species. Three species namely: *Satyrium crassicaule*, *Cynorkis anacamptoides* and *Liparis bowkeri* were stimulated to germinate symbiotically. However not all mycorrhizal fungi formed association with these orchid species. *S. crassicaule* was stimulated to germinate by *Rhizoctonia* spp. Nos. 494/95/1010, 520/95/1018, 536/95/1020 and 515/95/1017 and the asymbiotic media. In the other symbiotic plates only swelling of the embryos was noted which could be due to the seeds imbibing water. Out of the four *Rhizoctonia* spp. which stimulated germination of *S. crassicaule*, *Rhizoctonia* sp. No. 494/95/1010 was found to be most efficient (Figure 1). It was also more efficient than both asymbiotic media. Plates inoculated with this *Rhizoctonia* sp. had 84.7% of the protocorms at stage four of the germination process after 36 days of incubation as compared to 28.7% on PM. Though the asymbiotic media is made up of complex nutrient formulations, symbiotically germinated seeds on poor nutrient media (FIM) gave better germination percentage. This agrees with Harvais and Hadley (1967) findings that mycorrhizal seedlings grow more rapidly than the asymbiotic ones. The control plates (seeds

spread on FIM but no fungal isolate introduced) showed no germination. This observation indicates that for germination of *S. crassicaule* to take place under *in vitro* conditions, an appropriate fungus is needed or some nutrient requirements met.

Cynorkis anacamptoides, a swamp species, germinated in all symbiotic plates except those inoculated with fungal isolates Nos. 520/95/1019, 537/95/1021 and 537/95/1022 which were found to be parasitic to the seeds. The seeds also germinated in asymbiotic and control plates. However, except for the plates inoculated with *Rhizoctonia* spp. Nos. 494/95/1010 and 520/95/1018 and the asymbiotic plates, the protocorms in the other symbiotic and control plates did not develop beyond stage two. The fact that *C. anacamptoides* can develop to stage 2 of the germination process in the control plates (seeds incubated on FIM only), shows that mycorrhizal fungi or complex nutrient formulation may be playing little role if any in the early germination process of this species under *in viro* conditions. However, successful germination was only achieved on seeds inoculated with *Rhizoctonia* spp. Nos. 494/95/1010, 520/95/1018 and the asymbiotic media. Hadley and Williamson (1971) showed that seeds of some orchid species, but not the ones used in study, when spread on the moist substratum, take up water, swell, rupture the testa and produce few rhizoids. The embryos do not develop further unless they receive at least an exogenous supply of nutrients or are infected by a compatible fungus. *Cynorkis anacamptoides* may not rely on complex nutrients or mycorrhizal fungi at the very early stages of germination under *in vitro* conditions, but for successful completion of the germination process, an appropriate mycorrhizal fungi has to be present or complex nutrient formulations made available.

Rhizoctonia sp. No. 494/95/1010 was more efficient than *Rhizoctonia* sp. No. 520/95/1018 and the asymbiotic media in stimulating germination of *C. anacamptoides*

(Figure 2). The isolate had stimulated 68.7% of protocorms to stage four after 36 days of inoculation while *Rhizoctonia* sp. No. 520/95/1018 and PM had stimulated 16% and 2.7% to stage three respectively.

Bonatea steudneri and *Eulophia horsfallii* were not stimulated to germinate either symbiotically or asymbitically. The two most possible reasons for this could either be the seeds had lost viability or the right conditions were not provided. Loss of viability for *B. steudneri* is unlikely as the seeds had only been stored for one week when germination was attempted. This species was not stimulated to germinate, not even by *Rhizoctonia* spp. Nos. 513/95/1014 and 513/95/1015 which were isolated from it. However studies have shown that isolates from one species of orchid do not necessarily stimulate seed germination or protocorm development of the same species (Hadley, 1967). There is a possibility that there are different fungi associated with various stages of orchid development. Therefore a fungus may be an effective mycorrhizal partner in the mature plant but may not be effective during early stages of orchid development. Another possible reason for the failure of the species to germinate could be due to seed dormancy as stated by Stoutamire (1974). In such a case the seeds have to be subjected to a specific treatment for them to germinate. For *Eulophia horsfallii* the seed batch used, had been reported to germinate in an earlier test on the asymbiotic media (PM and MS) used (Bytebier, pers. comm.). Its failure to germinate therefore indicates a loss of viability of this seed batch.

Ceratobasidium cornigerum which has been reported to stimulate germination of a wide range of temperate terrestrial orchids and was included as a standard, did not stimulate the germination of any orchid species used in the study. This might indicate that the fungi forming mycorrhizal association with tropical orchids differ from their temperate

counterparts.

Orchid mycorrhizal fungi have been found not to be species specific at least to *Cynorkis anacamptoides* and *Satyrium crassicaule*. *Rhizoctonia* spp. Nos. 494/95/1010 and 520/95/1018 successfully stimulated germination of two orchid species, *Cynorkis anacamptoides* and *Satyrium crassicaule*. If each of the two orchid species could be stimulated to germinate by the two *Rhizoctonia* species, then it means that they are not strictly specific to the fungal species they form association with and the vice versa. This agrees with Curtis (1939) findings that orchid mycorrhizal fungi are not species specific.

Liparis bowkeri was stimulated to germinate by *Rhizoctonia* sp. No. 515/95/1017 only (Figure 3), a fungus isolated from this particular species. This may represent a case of species specificity. The isolate however did not support development beyond stage three. Specificity as such, can not be concluded easily since there might be a follow up of different mycorrhizal fungi in the development of the plant. On the other hand this orchid species was tested with only eight *Rhizoctonia* species. However wide spread tests with a wide range of *Rhizoctonia* species should be carried out before a firm conclusion can be reached on orchid-fungus specificity. Some degree of specificity in some species has been reported by Warcup (1973) who claimed that two species of *Pteristylis* were stimulated to germinate by *Ceratobasidium cornigerum* only.

5.0   SUMMARY

The study has confirmed that timing for isolation of orchid mycorrhizal fungi is crucial. Isolation should be carried out from actively growing plants prior to flowering. The living pelotons are loose aggregates of almost transparent fungal hyphae that eventually become digested, forming irregular, often yellowish clumps towards the end of the growing season of the orchid plant.

Isolation processes yielded eight *Rhizoctonia* species, five of these (*Rhizoctonia* spp. Nos. 494/95/1010, 513/95/1014, 515/95/1017, 520/95/1018 and 536/95/1020) bear close resemblance. They were separated by the use of chlamydospore and hyphal characters. These five fungal isolates resemble *Epulorhiza repens*, but are not close enough in some characters to warrant them to be placed under this species. *Rhizoctonia* spp. Nos. 513/95/1015 and 501/95/1013 resembles *Ceratorhiza goodyerae-repentis*, although they also did not fit exactly. *Rhizoctonia* sp. No. 144/95/1023 did not resemble any *Rhizoctonia* species in the keys.

Symbiotic germination was successful on three out of the five orchid species tested. *Satyrium crassicaule* was stimulated to germinate by *Rhizoctonia* spp. Nos. 494/95/1010, 520/95/1018, 536/95/1020 and 515/95/1017. *Rhizoctonia* sp. No. 494/95/1010 was the most efficient in stimulating germination. *Cynorkis anacamptoides* was stimulated to germinate by *Rhizoctonia* sp. No. 494/95/1010 and 520/95/1018. *Rhizoctonia* sp. No. 494/95/1010 was the most efficient in stimulating germination of this orchid species as well. *Liparis bowkeri* was stimulated by *Rhizoctonia* sp. No. 515/95/1017 only. Data from the study confirms that orchids form mycorrhizal associations with one fungal taxa (form-genus *Rhizoctonia*). Mycorrhizal fungi have not been found to be species specific to two orchid species (*Satyrium*

crassicaule and *Cynorkis anacamptoides*). *Liparis bowkeri* shows specificity, but further tests with more *Rhizoctonia* species should be carried out before a firm conclusion is made.

Specific identification of the orchid mycorrhizal fungi has been shown to be difficult owing to the confused state of the current taxonomy of this group. The whole group is therefore greatly in need of taxonomic revision.

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7.0. APPENDICES

Appendix 1

The locality and habitat of the orchid species used in the study

SPECIES	NO	LOCALITY	HABITAT	ALT. (M)
Bonatea steudneri (Reichb. F.) Th. Dur. & Schinz	513	Kirinyaga (Sagana)	Bushland	1200
Cynorkis anacamptoides Kraenzl.	475	Nyandarua (Sasumua Dam)	Aquatic	2340
Disa stairsii Kraenzl.	492	Nyandarua (Elephant Peak)	Grassland	3200
Disa stairsii Kraenzl.	491	Nyandarua (Kinangop Peak)	Grassland	3400
Eulophia horfallii (Batem) Summerh	450	Trans Nzoia (Saiwa Swamp National park)	Semi-aquatic	1800
Eulophia montis-elgonis Summerh	520	Trans Nzoia (Saiwa Swamp National Park)	Grassland	1800
Eulophia orthoplectra (Reichb. F) Summerh	454	Trans Nzoia (Saiwa Swamp National Park)	Grassland	1800
Eulophia petersii Reichb. F.	144	Samburu (Mount Nyiru)	Forest	1600
Eulophia stenophylla Summerh	494	Nairobi (Nairobi Aboretum)	Forest	1700
Eulophia streptopetala Lindl.	442	Trans Nzoia (Saiwa Swamp National Park)	Riverine Forest	1820
Habenaria petitiana (A. Rich.) Th. Dur. & Schinz	501	Kiambu (Kerita Forest)	Grassland	2330
Liparis bowkeri Harv.	515	Trans Nzoia (Saiwa Swamp National Park)	Forest	1800
Satyrium coriophoroides A. Rich.	537	Trans Nzoia (Saiwa Swamp National Park)	Grassland	1800
Satyrium crassicaule Rendl	476	Nyandarua (Sasamua Dam)	Aquatic	2340
Satyrium crassicaule Rendl.	500	Kiambu (Kerita Forest)	Semi-aquatic	2300
Satyrium sacculatum (Rendl) Rolfe	536	Trans Nzoia (Saiwa Swamp National Park)	Grassland	1800

Appendix 2

Chlamydospore measurements (length x width in μm) for *Rhizoctonia* sp. No. 494/95/1010

13.0 x 11.5	14.5 x 11.5	14.5 x 11.5	14.0 x 12.0	14.5 x 14.5	14.5 x 14.5
14.5 x 11.5	17.5 x 11.5	17.5 x 11.5	16.5 x 9.5	10.0 x 8.5	17.5 x 17.5
14.5 x 11.5	13.0 x 11.5	14.5 x 8.5	14.0 x 14.0	11.5 x 11.5	20.5 x 11.5
11.5 x 8.5	14.5 x 13.0	14.5 x 11.5	14.0 x 12.0	19.0 x 12.0	14.0 x 12.0
8.5 x 8.5	17.5 x 14.5	17.5 x 11.5	16.5 x 12.0	19.0 x 14.5	14.0 x 12.0
14.5 x 11.5	11.5 x 11.5	17.5 x 11.5	16.5 x 12.0	19.0 x 11.5	16.5 x 10.0
1.6 x 11.5	14.5 x 11.5	17.5 x 14.5	16.5 x 12.0	17.5 x 14.5	14.0 x 12.0
14.5 x 11.5	11.5 x 11.5	14.5 x 11.5	16.5 x 14.0	14.0 x 9.5	13.0 x 11.5
14.5 x 11.5	14.5 x 11.5				

N 50   Size range 8.5 - 20.5 x 8.5 - 17.5 μm   Mean size 14.9 x 11.8 μm

Hyphal diameter in μm for *Rhizoctonia* sp. No. 494/95/1010

3.0	6.0	4.5	6.0	3.0	3.0	4.5	3.0	4.0	3.0	6.0	5.0	5.0	6.0
3.0	4.5	4.5	4.5	5.0	5.0	2.5	5.0	4.5	3.0	4.5	4.5	4.0	4.5
3.0	5.0	4.5	3.0	5.0	4.0	4.5	4.5	6.0	4.5	3.0	2.5	5.0	
5.0	5.0	4.0	4.5	6.0	5.0	5.0	6.0	4.5	4.5	3.0	3.0	5.0	

N = 50   Size range = 2.5 - 6.0 μm   Mean = 4.3 μm

Appendix 3

Chlamydospore measurements (length x width in μm) for *Rhizoctonia* sp. No. 513/95/1014

9.5 x 9.5	9.5 x 9.5	12.0 x 9.5	9.5 x 8.0	12.0 x 9.5	9.5 x 9.5	12.0 x 9.5
12.0 x 9.5	12.0 x 9.5	9.5 x 9.5	9.5 x 8.0	12.0 x 7.0	12.0 x 9.5	9.5 x 9.5
9.5 x 9.5	9.5 x 9.5	9.5 x 7.0	12.0 x 9.5	12.0 x 9.5	9.5 x 9.5	9.5 x 9.5
12.0 x 9.5	12.0 x 9.5	12.0 x 9.5	14.0 x 12.0	12.0 x 9.5	12.0 x 9.5	9.5 x 7.0
12.0 x 12.0	12.0 x 12.0	12.0 x 12.0	12.0 x 9.5	9.5 x 9.5	12.0 x 12.0	9.5 x 9.5
9.5 x 9.5	19.0 x 9.5	9.5 x 9.5	9.5 x 9.5			

N 50.   Size range 9.5 - 19.0 x 7.0 - 12.0 μm.   Mean 11.2 x 9.6 μm

Hyphal diameter in μm for *Rhizoctonia* sp. No. 513/95/1014

4.5	3.5	3.5	4.5	4.5	3.5	3.5	4.5	4.5	4.5
4.5	2.0	3.5	4.5	4.5	2.0	3.5	4.5	3.5	3.5
4.5	3.5	2.5	3.5	4.5	3.5	2.5	3.5	2.5	2.5
4.5	3.5	2.5	2.5	4.5	3.5	2.5	2.5	3.5	3.5
2.5	2.5	3.5	3.5	2.5	2.5	3.5	3.5	4.5	4.5

N = 50
Size range = 2.0 - 4.5 μm
Mean = 3.5 μm

Appendix 4

Chlamydospore measurements (length x width in μm) for *Rhizoctonia* sp. No. 515/95/1017

12.0 x 12.0	9.5 x 9.5	12.0 x 9.5	14.0 x 12.0	12.0 x 9.5	12.0 x 12.0
14.0 x 12.0	12.0 x 12.0	14.0 x 12.0	14.0 x 14.0	14.0 x 9.5	14.0 x 12.0
14.0 x 12.0	12.0 x 9.5	14.0 x 14.0	14.0 x 14.0	12.0 x 9.5	14.0 x 12.0
14.0 x 12.0	12.0 x 12.0	12.0 x 12.0	12.0 x 9.5	14.0 x 12.0	14.0 x 12.0
14.0 x 10.0	14.0 x 12.0	14.0 x 12.0	12.0 x 12.0	12.0 x 12.0	14.0 x 10.0
12.0 x 9.5	12.0 x 9.5	12.0 x 12.0	12.0.0 x 9.5	9.5 x 9.5	
14.0 x 12.0	12.0 x 12.0	12.0.0 x 9.5	12.0 x 12.0	12.0 x 12.0	
14.0 x 12.0	16.5 x 14.0	14.0 x 9.5	12.0 x 9.5	14.0 x 9.5	
12.0 x 12.0	12.0. x 12.0	12.0 x 12.0	12.0 x 12.0	14.0 x 12.0	

N 50   Size range   9.5 - 16.5 x 9.5 - 14.0μm   Mean size   12.9 x 11.3 μm

Hyphal diameter in μm for *Rhizoctonia* sp. No. 515/95/1017

2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	4.5	4.5	4.5	4.5
3.5	2.5	2.5	4.5	3.5	2.5	2.5	4.5	2.5	2.5	2.5	2.5
4.5	4.5	2.5	3.5	4.5	4.5	2.5	3.5	3.5	4.5	3.5	4.5
3.5	4.5	4.5	2.5	3.5	4.5	4.5	2.5	4.5	3.5	4.5	2.5
3.5	2.5										

N = 50   Size range = 2.5 - 4.5 μm   Mean size = 3.4 μm

Appendix 5

Chlamydospore measurements (length x width in μm) for *Rhizoctonia* sp. No. 520/95/1018

12.0 x 9.5	14.0 x 12.0	14.0 x 12.0	12.0 x 12.0	14.0 x 12.0	14.0 x 9.5	14.0 x 12.0
12.0 x 9.5	9.5 x 9.5	14.0 x 9.5	14.0 x 12.0	14.0 x 12.0	14.0 x 12.0	12.0 x 9.5
14.0 x 9.5	14.0 x 12.0	14.0 x 12.0	12.0 x 9.5	12.0 x 12.0	12.0 x 9.5	14.0 x 12.0
12.0 x 9.5	12.0 x 12.0	14.0 x 12.0	12.0 x 9.5	12.0 x 9.5	12.0 x 9.5	
12.0 x 9.5	12.0 x 9.5	14.0 x 9.5	14.0 x 12.0	12.0 x 12.0	12.0 x 12.0	
16.5 x 12.0	14.0 x 12.0	14.0 x 9.5	14.0 x 9.5	12.0 x 9.5	14.0 x 9.5	
12.0 x 9.5	14.0 x 9.5	14.0 x 12.0	14.0 x 12.0	14.0 x 12.0	12.0 x 9.5	

N 50   Size range 9.5 - 16.5 x 9.5 - 12.0.0 μm   Mean size 13.1 x 10.6 μm

Hyphal diameter in μm for *Rhizoctonia* sp. 520/95/1018

2.5	2.5	3.5	3.5	2.5	2.5	3.0	2.5	2.5	2.5	3.5	3.5	3.5
4.5	3.5	3.5	3.5	3.5	4.5	4.5	3.5	4.5	3.5	3.5	3.5	4.5
3.5	2.5	3.5	3.5	4.5	2.5	2.5	2.5	3.5	2.5	3.5	3.5	
2.5	2.5	3.5	3.5	4.5	3.5	4.5	4.5	2.5	2.5	3.5	3.5	

N = 50   Size range = 2.5 - 4.5 μm   Mean size = 3.3 μm

Appendix 6

Chlamydospore measurements (length x width in μm) for *Rhizoctonia* sp. No. 536/95/1020						
19.0 x 7.0	16.5 x 7.0	14.0 x 9.5	14.0 x 9.5	19.0 x 9.5	19.0 x 7.0	14.0 x 9.5
9.5 x 7.0	23.5 x 9.5	21.0 x 7.0	21.0 x 7.0	16.5 x 7.0	9.5 x 7.0	16.5 x 7.0
16.5 x 7.0	21.0 x 9.5	21.0 x 9.5	19.0 x 7.0	19.0 x 9.5	16.5 x 7.0	18.5 x 7.0
16.5 x 7.0	14.0 x 7.0	26.0 x 9.5	21.0 x 7.0	26.0 x 7.0	16.5 x 7.0	19.0 x 7.0
23.5 x 7.0	16.5 x 7.0	16.5 x 7.0	21.0 x 7.0	21.0 x 9.5	23.5 x 7.0	21.0 x 7.0
19.0 x 9.5	16.5 x 7.0	19.0 x 7.0	21.0 x 7.0	14.0 x 9.5	19.0 x 9.5	19.0 x 9.5
19.0 x 9.5	16.5 x 7.0	19.0 x 9.5	16.5 x 7.0	23.5 x 9.5	21.0 x 9.5	14.0 x 7.0
16.5 x 7.0						

N 50   Size range 9.5 - 26.0 x 7.0 - 9.5 μm   Mean size   18.4 x 7.9 μm

Hyphal diameter in μm for *Rhizoctonia* sp. No. 536/95/1020													
3.0	6.0	4.5	6.0	3.0	3.0	4.5	3.0	4.0	3.0	6.0	5.0	5.0	6.0
3.0	4.5	4.5	4.5	5.0	5.0	2.5	5.0	4.5	3.0	4.5	4.5	4.0	4.5
3.0	5.0	4.5	3.0	5.0	4.0	4.5	4.5	6.0	4.5	3.0	2.5	5.0	
5.0	5.0	4.0	4.5	6.0	5.0	5.0	6.0	4.5	4.5	3.0	3.0	5.0	

N = 50   Size range = 2.5 - 6.0 μm   Mean = 4.3 μm

Appendix 7

Chlamydospore measurements (length x width in μm) for *Rhizoctonia* sp. No. 513/95/1015

23.5 x 12.0	23.5 x 9.5	21.0 x 9.5	23.5 x 12.0	21.0 x 9.5	19.0 x 12.0
21.0 x 12.0	19.0 x 12.0	21.0 x 12.0	21.0 x 12.0	21.0 x 12.0	28.0 x 12.0
28.0 x 9.5	28.0 x 12.0	23.5 x 16.5	28.0 x 9.5	23.5 x 12.0	19.0 x 9.5
19.0 x 7.0	19.0 x 9.5	16.5 x 9.5	19.0 x 7.0	23.5 x 7.0	23.5 x 9.5
19.0 x 9.5	23.5 x 9.5	21.0 x 9.5	19.0 x 9.5	21.0 x 12.0	23.5 x 12.0
23.5 x 7.0	23.5 x 12.0	23.5 x 9.5	23.5 x 9.5	23.5 x 9.5	
23.5 x 9.5	26.0 x 7.0	23.5 x 9.5	23.5 x 9.5	26.0 x 9.5	
23.5 x 9.5	23.5 x 9.5	23.5 x 9.5	23.5 x 7.0	21.0 x 9.5	
23.5 x 9.5	23.5 x 7.0	23.5 x 9.5	23.5 x 9.5	23.5 x 9.5	

N 50   Size range 19.0 - 28.0 x 7.0 - 12.0.0 μm   Mean size 22.7 x 10.0 μm

Hyphal diameter in μm for *Rhizoctonia* sp. No. 513/95/1015

2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	4.5	4.5	4.5	4.5
3.5	2.5	2.5	4.5	3.5	2.5	2.5	4.5	2.5	2.5	2.5	2.5
4.5	4.5	2.5	3.5	4.5	4.5	2.5	3.5	3.5	4.5	3.5	4.5
3.5	4.5	4.5	2.5	3.5	4.5	4.5	2.5	4.5	3.5	4.5	2.5
3.5	2.5										

N 50   Size range 2.5 - 4.5μm   Mean size 3.4μm

Appendix 8

Results of the different symbiotic germination tests

Fungal isolate No.	Fungus	Orchid species				
		Satyrium crassicaule	*Cynorkis anacamptoides*	*Liparis bowkeri*	*Eulophia horsfallii*	*Bonatea steudneri*
494/95/1010	*Rhizoctonia* sp.	+	+	-	-	-
513/95/1014	*Rhizoctonia* sp.	-	+	-	-	-
515/95/1017	*Rhizoctonia* sp.	+	+	+	-	-
520/95/1018	*Rhizoctonia* sp.	+	+	-	-	-
536/95/1020	*Rhizoctonia* sp.	+	+	-	-	-
513/95/1015	*Rhizoctonia* sp.	-	+	-	-	-
501/95/1013	*Rhizoctonia* sp.	-	+	-	-	-
513/95/1016	Unidentified	-	+	-	-	-
450/94/1001	,,	-	+	-	-	-
451/94/1002	,,	-	+	-	-	-
454/94/1003	,,	-	+	-	-	-
454/95/1004	,,	-	+	-	-	-
476/95/1005	,,	-	+	-	-	-
491/95/1006	,,	-	+	-	-	-
491/95/1007	,,	-	+	-	-	-
491/95/1008	,,	-	+	-	-	-
492/95/1009	,,	-	+	-	-	-
494/95/1011	,,	-	+	-	-	-
520/95/1019	,,	-	-	-	-	-
537/95/1021	,,	-	-	-	-	-
537/95/1022	,,	-	-	-	-	-
442/94/1000	,,	-	+	-	-	-
144/95/1023	*Rhizoctonia* sp.	-	+	-	-	-
494/95/1012	Unidentified	-	+	-	-	-
C. cornigerum		+	+	-	-	-
PM		+	+	+	-	-
MS		+	+	+	-	-
Control		-	+	-	-	-

Key

+   Germination

-     No germination

Appendix 9

Results of the germination tests on *Satyrium crassicaule* seeds

Fungal Isolate No.	Number of protocorms per each germination stage														
	27/6/95					10/7/95					18/6/95				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
491/95/1006	40	10	0	0	0	36	14	0	0	0	28	22	0	0	0
454/94/1004	45	5	0	0	0	38	12	0	0	0	45	5	0	0	0
450/94/1001	40	10	0	0	0	45	5	0	0	0	36	14	0	0	0
476/95/1005	49	1	0	0	0	48	2	0	0	0	40	10	0	0	0
451/94/1002	48	2	0	0	0	47	3	0	0	0	45	5	0	0	0
501/95/1013	22	28	0	0	0	18	32	0	0	0	42	8	0	0	0
494/95/1011	30	20	0	0	0	8	42	0	0	0	11	39	0	0	0
491/95/1008	45	5	0	0	0	40	10	0	0	0	46	4	0	0	0
494/95/1012	45	5	0	0	0	40	10	0	0	0	38	12	0	0	0
454/94/1003	38	12	0	0	0	47	3	0	0	0	44	6	0	0	0
491/95/1007	46	4	0	0	0	47	3	0	0	0	41	9	0	0	0
492/95/1009	40	10	0	0	0	44	6	0	0	0	42	8	0	0	0
513/95/1016	43	7	0	0	0	37	13	0	0	0	39	11	0	0	0
442/94/1000	46	4	0	0	0	40	10	0	0	0	42	8	0	0	0
537/95/1022	N/A														
520/95/1019	N/A														
537/95/1021	N/A														
513/95/1015	15	35	0	0	0	23	27	0	0	0	25	25	0	0	0
494/95/1010	0	10	40	0	0	0	0	6	44	0	0	0	4	5	41
513/95/1014	50	0	0	0	0	47	3	0	0	0	50	0	0	0	0
515/95/1017	8	29	13	0	0	19	24	7	0	0	10	22	18	0	0
520/95/1018	10	9	31	0	0	13	4	25	6	0	8	7	17	11	8
536/95/1020	12	18	20	0	0	13	18	19	0	0	10	14	26	0	0
144/95/1023	48	2	0	0	0	50	0	0	0	0	49	1	0	0	0
PM	11	34	5	0	0	6	18	19	76	0	0	0	7	23	20
MS	35	15	0	0	0	23	24	3	0	0	11	28	8	3	0
C. cornigerum	27	23	0	0	0	28	22	0	0	0	27	23	0	0	0
Control	50	0	0	0	0	48	2	0	0	0	47	3	0	0	0

Key: Stage 0 = Ungerminated
Stage 1 = Swelling of the embryo and bursting of the seed coat
Stage 2 = Production of rhizoids
Stage 3 = Production shoot primordium
Stage 4 = Production of root initial
N/A   = The seeds were overgrown by the fungus and killed

Appendix 10

Results of the germination tests on *Liparis bowkeri* seeds

Fungal Isolate No.	Number of protocorms per each germination stage														
	6/9/95					13/9/95					20/9/95				
	0	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**
491/95/1006	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
454/94/1004	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
450/94/1001	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
476/95/1005	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
451/94/1002	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
501/95/1013	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
494/95/1011	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
491/95/1008	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
494/95/1012	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
454/94/1003	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
491/95/1007	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
492/95/1009	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
513/95/1016	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
442/94/1000	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
537/95/1022	N/A														
520/95/1019	N/A														
537/95/1021	N/A														
513/95/1015	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
494/95/1010	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
513/95/1014	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
515/95/1017	10	24	11	5	0	7	15	22	6	0	8	23	14	5	0
520/95/1018	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
536/95/1020	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
144/95/1023	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
PM	6	12	11	21	0	12	8	9	15	6	11	10	2	22	5
MS	8	13	24	5	0	9	12	12	17	0	8	6	11	19	6
C. cornigerum	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0
Control	50	0	0	0	0	50	0	0	0	0	50	0	0	0	0

Key: Stage 0 = Ungerminated
Stage 1 = Swelling of the embryo and bursting of the seed coat
Stage 2 = Production of rhizoids
Stage 3 = Production shoot primordium
Stage 4 = Production of root initial
N/A   = The seeds were overgrown by the fungus and killed

Appendix 11

Results of the germination tests on *Cynorkis anacamptoides* seeds

Fungal Isolate No.	Number of protocorms per each germination stage													
	27/6/95					10/7/95					18/7/95			
	0	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**
491/95/1006	6	31	13	0	0	5	14	31	0	0	0	13	37	0
454/94/1004	7	42	1	0	0	2	26	22	0	0	5	45	0	0
450/94/1001	7	40	3	0	0	3	40	7	0	0	0	30	20	0
476/95/1005	10	39	1	0	0	5	40	5	0	0	5	20	25	0
451/94/1002	13	29	8	0	0	20	30	0	0	0	10	37	3	0
501/95/1013	1	20	29	0	0	3	15	32	0	0	2	5	43	0
494/95/1011	12	31	17	0	0	1	35	14	0	0	7	13	30	0
491/95/1008	5	43	2	0	0	0	19	31	0	0	0	7	43	0
494/95/1012	11	24	15	0	0	1	22	27	0	0	0	30	20	0
454/94/1003	10	20	20	0	0	3	23	24	0	0	0	11	39	0
491/95/1007	15	23	12	0	0	4	13	33	0	0	0	2	48	0
492/95/1009	15	30	5	0	0	5	31	14	0	0	0	40	10	0
537/95/1022	N/A													
520/95/1019	N/A													
537/95/1021	N/A													
513/95/1015	0	24	26	0	0	0	15	35	0	0	0	41	9	0
513/95/1016	30	20	0	0	0	15	31	4	0	0	7	20	23	0
494/95/1010	0	20	30	0	0	0	12	38	0	0	0	0	5	10
513/95/1014	20	27	6	0	0	16	28	6	0	0	16	33	1	0
515/95/1017	7	20	23	0	0	9	17	24	0	0	10	15	25	0
520/95/1018	15	12	23	0	0	10	12	24	4	0	8	5	29	8
536/95/1020	9	11	30	0	0	12	15	23	0	0	14	6	30	0
144/95/1023	2	48	0	0	0	6	44	0	0	0	5	40	5	0
442/94/1000	40	10	0	0	0	30	15	5	0	0	31	13	6	0
PM	39	10	1	0	0	29	5	16	0	0	20	5	23	1
MS	37	13	1	0	0	22	23	5	0	0	15	14	21	0
C. cornigerum	10	28	12	0	0	12	30	8	0	0	15	20	15	0
Control	4	40	6	0	0	2	30	18	0	0	5	20	25	0

Key: Stage 0 = Ungerminated
Stage 1 = Swelling of the embryo and bursting of the seed coat
Stage 2 = Production of rhizoids
Stage 3 = Production shoot primordium
Stage 4 = Production of root initial
N/A   = The seeds were overgrown by the fungus and killed

Appendix 12

The percentages of protocorms of *Satyrium crassicaule* at various stages of germination at the 36th day after inoculating with various *Rhizoctonia* spp.

Rhizoctonia sp. No.	Percentage germination per stage				
	0	**1**	**2**	**3**	**4**
494/95/1010	0	0	7.3	8.0	84.7
520/95/1018	16.7	13.4	33.3	21.3	15.3
536/95/1020	20.0	28.0	52.0	0	0
515/95/1017	19.3	45.3	35.3	0	0
513/95/1014	100.0	0	0	0	0
513/95/1015	50.0	50.0	0	0	0
501/95/1013	84.0	16.0	0	0	0
144/95/1023	100.0	0	0	0	0
Ceratobasidium cornigerum	54.7	45.3	0	0	0
PM	0	27.0	20.0	24.3	28.7
MS	24.7	53.3	15.3	6.7	0
Control	93.3	6.7	0	0	0

Key: Stage 0 = Ungerminated
       Stage 1 = Swelling of the embryo and bursting of the seed coat
       Stage 2 = Production of rhizoids
       Stage 3 = Production shoot primordium
       Stage 4 = Production of root initial

Appendix 13

The percentages of protocorms of *Cynorkis anacamptoides* at various stages of germination at the 36th day after inoculating the seeds with various *Rhizoctonia* spp.

Rhizoctonia spp.	Percenatge germination per stage				
	0	**1**	**2**	**3**	**4**
494/95/1010	0	0	9.3	22.0	68.7
515/95/1017	19.3	31.3	49.3	0	0
520/95/1018	15.3	10.7	58	16	0
536/95/1020	28.0	11.3	60.7	0	0
513/95/1014	31.3	66.0	2.7	0	0
513/95/1015	0	82.0	18.0	0	0
503/95/1013	4.0	10.0	86.0	0	0
144/95/1023	40.0	30.0	30.0	0	0
Ceratobasidium cornigerum	19.3	56.7	24.0	0	0
PM	36.7	16.0	44.0	2.7	0
MS	33.3	32.0	34.7	0	0
Control	20	40	40	0	0

<u>Key:</u> Stage 0 = Ungerminated
Stage 1 = Swelling of the embryo and bursting of the seed coat
Stage 2 = Production of rhizoids
Stage 3 = Production shoot primordium
Stage 4 = Production of root initial

Appendix 14

The percentage of protocorms of *Liparis bowkeri* at various stages of germination at the 67th day after inoculating the seeds with various *Rhizoctonia* spp.

Rhizoctonia spp. No.	Percentage germination as per stage				
	0	**1**	**2**	**3**	**4**
515/95/1017	17.3	45.3	31.3	6.0	0
494/95/1010	100.0	0	0	0	0
513/95/1014	100.0	0	0	0	0
513/95/1015	100.0	0	0	0	0
501/95/1013	100.0	0	0	0	0
520/95/1018	100.0	0	0	0	0
536/95/1020	100.0	0	0	0	0
144/95/1023	100.0	0	0	0	0
Ceratobasidium cornigerum	100.0	0	0	0	0
PM	12.7	22.0	4.7	43.3	17.3
MS	14.7	15.3	24.0	38.0	8.0
Control	100.0	0	0	0	0

Key: Stage 0 = Ungerminated
Stage 1 = Swelling of the embryo and bursting of the seed coat
Stage 2 = Production of rhizoids
Stage 3 = Production shoot primordium
Stage 4 = Production of root initial