1. Entry and spread of the Fungus

The source of infection of the root is always by hyphae from the fungal mycelium, sclerotia and/or occasionally chlamydospores which are found in the root surface and rhizosphere. There appears to be, however, some difference in the loci of entry of hyphae into the host. Both protocorms and roots have been studied from this point of view, the former more commonly under laboratory conditions. Early colonisation of the developing embryo/protocorm was always reported to be through the basal or suspensor end of the embryo/protocorm and progresses towards the apex (Bernard 1909; Burgeff 1959; Clements 1988; Peterson and Currah 1990; Richardson et al. 1992; Peterson and Furquar 1994). However Rasmussen (1990) had observed that in Dactylorhiza mejalis, the hyphae entering the suspensor region were inhibited from further development and that colonisation of protocorm depended on fungal ingress through rhizoids (epidermal hairs). In roots, the fungal entry is always confined to that part of the root which has root hairs and no other region of the root (Burgeff 1959).

The moot point, however, is whether the fungus enters the host through root hairs or through epidermal cells. In
protocorms, Peterson and Currah (1990), have shown that hyphae were frequently present at the apex of epidermal hair initials of protocorm and around elongating hairs; Williamson and Hadley (1970) and Rasmussen (1990) have reported entry through epidermal hairs of protocorm. In the roots, of some species of orchids, the root hairs serve as the sites of hyphal entry (Burgeff 1959; Currah et al. 1988; Vij and Sharma 1988). The present study also indicates that the entry of the fungus is always through the root hairs. There are reports of entry of the hyphae through ordinary rhizodermal cells (Burgeff 1936; 1959; Alconero 1969). Entry through root hairs as well as rhizodermis has also been reported by a few investigators (Burgeff 1936; see also Burgeff 1959; Katiyar et al. 1985; and Peterson and Furquar 1994). Entry exclusively through the root hairs or through rhizodermis or through both these routes is likely to be dependent on the structure of the involved root, especially the loci of exodermal passage cells. In those species, like Spathoglottis plicata investigated at present, where the passage cells are located directly beneath the root hairs, entry is always through the root hairs. In other species, where entry is through rhizodermis or both root hairs and rhizodermis, either the locations of passage cells and root hairs have obviously no relation with each other or there must be a well-developed multilayered velamen in between
exodermis and rhizodermis. In the latter instances, the fungal hyphae may traverse various layers of velamen before entering into exodermal passage cells.

Like in many other infections involving rhizobia and root parasitic microbes, the mycorrhizal infection of root hairs also promotes the distortion and bending of root hairs after infection. In fact the distorted appearance of the root hairs may form one of the best indications of those hairs that were already penetrated by the fungus (Hadley and Williamson 1972; Vij and Sharma 1988; see also Peterson and Farquar 1994). In fact the present study indicates that the distortion is often restricted to that part of the root hair where the hyphae had actually penetrated.

Irrespective of whether the entry of the fungus from the soil is through rhizodermal cells or root hairs, the hyphae do not settle down in these cells or form coils or pelotons; they only serve as transitory points for the hyphae, which finally settle down only in the root cortical cells. In the material studied at present, as well as in those studied in the past by other investigators, colonisation of epidermis or root hairs by fungal pelotons was never observed (Hadley 1975; Clements 1988; Peterson and Currah 1990). It is likely that, as Peterson and Currah (1990) had suggested that these cells may not have the appropriate
physiological conditions necessary for coil formation and digestion. The only instance of colonisation of epidermal cells by the fungus is reported in *Goodyera repens* by Peterson and Currah (1990); but this is in the protocorm. However, even in this case, it was not clear whether the epidermal cells were colonised by fungus from the soil or from adjacent protocorm parenchyma cells.

The entry from the rhizodermal layer into the cortex is always through the passage cells of the exodermis. This is noticed not only in the orchid studied at present but also in several others investigated earlier (Alcanero 1969; see full literature in Esnault et al. 1994). In fact the exodermal passage cells do have a control of entry of fungi into the cortex (see also Peterson 1988), which remain the only living cells of the exodermis and whose cell walls do not have lignin and suberin, at least to the extent that other exodermal cells have (Esnault et al. 1994). Like the rhizodermal and hypodermal cells, even in the passage cells of the exodermis the fungal hyphae do not settle down or form coils, again indicating that these cells are meant only as a channel for hyphal entry into the cortex (and out of it also) and do not have the appropriate physiological conditions necessary for coil formation and hyphal degradation, although are more active metabolically (Alcanero 1969; Esnault et al. 1994).
2. Infection

The degree of infection by the fungus is invariably estimated by calculating the infection density as per formula developed by Hadley and Williamson (1972) and mentioned in the materials and methods section of this thesis. Based on their results Hadley and Williamson (1972) reported that out of ten orchids studied by them five showed an infection density of more than 60%. These cases were classified by them under the dense infection category and those that showed very little infection were classified under spasmodiac or intermittent infections (see also Alexander 1987). Katiyar et al. (1985) studied 12 species of terrestrial orchids of north-eastern India and recorded an infection density of 68.9% to 97% depending upon the orchid. One of the orchids studied by them was Spathoglottis pubescens with an infection density of 84.5%. The present study on Spathoglottis plicata showed an infection density of 37.5% to 100% with a mean of \( 65.824\% \pm 16.19\% \).

The degree of infection was reported to vary in the same orchid in different seasons of the year by some of the earlier investigators. Hadley (1969) mentioned that the degree of infection was extensive in saprophytic species to virtually free of infection in some green terrestrial orchids, either free for a greater part of the year or to
have many uninfected roots (see also Alexander 1987). In *Goodyera repens* (Alexander and Alexander 1984), a Scotland evergreen orchid, there was a relatively constant amount of root infection throughout the year. Hadley and Williamson (1972) also speak of spasmodic or intermittent infection in some orchids, implying difference in infection percentage in different periods of the year. Benzing (1982) also reported that all roots which are in contact with the substrate were infected in an epiphytic orchid of Florida. The present study on *Spathoglottis plicata* which was carried out for three calendar years showed that infection was seen in all the roots all through the year, but in each of these roots, there was not only small segments free of infection but also the intensity of infection in the infected segments varying from 37.5% to 100%. In other words, heterotrophy or otherwise of the orchid species, do not appear to have a deciding effect on the periodicity of infection or on the intensity of infection.

As in many other previously studied orchids, colonisation of the fungus in *Spathoglottis plicata* was restricted only to the root cortex. Penetration of hyphae into endodermis and central cylinder, as observed by Ruinen (1953) in a species of *Dendrobium*, was never observed in the present taxon. However, infection could be seen upto that layer of the cortex adjoining the endodermis. This is in
variance to observation of Majstrik (1970) in *Dendrobium cunninghamii* who reported the absence of infection in the deeper layers of the cortex. In other words, the host appears to control the growth of the fungus through its cells and restricts it to certain region of the cortical tissue, an observation also shared by Burgeff (1959), Hadley (1969) and Hadley et al. (1971).

In *Spathoglottis plicata*, the fungal mycelium, after entry into the root cortex through passage cells, initially grows intracellularly towards the interior layers of the cortex. Consequently, the first colonisation of cortical cells was noticed in the deeper layers of the cortex and the subsequent ones gradually towards the periphery of the cortex. However, since infection is a continuous process in this orchid and since all the cells of the cortex are not colonised at the same time, in a mature root there was often a mixup of younger and older colonisations across the entire cortex. In other words, in older roots, there is no spatial restriction of younger and older colonisations in the cortex. However, Magnus (1900) (see also Burgeff 1959) reported that the so called "digestion cells" which are equivalent to cells with older colonisation were present in the inner cortex and the so called "host cells" which are equivalent to cells with younger colonisations were peripherally located. Probably Magnus had come to this
conclusion after observing roots at a particular stage of early infection. Hadley (1975) had also come to a conclusion similar to one arrived at by the present worker when he reported that in many orchids a clear spatial separation into "digestion" and "host cells" could not be seen and that all infected cells will at one time or the other would be converted into the so called "digestion cells".

3. The pelotons

In roots of orchids, as in protocorms, the fungal hyphae form coiled complexes called pelotons in cortical cells (see Peterson and Farquar 1994). These coils are typically formed in the root cortical cells of the presently studied orchid, Spathoglottis plicata. Like in other orchids, these pelotons are intracellular coils of branched anastomosing hyphae (see Hadley et al. 1971).

One of the most striking events in the orchid mycorrhizal association (in protocorms as well as roots) is the lysis of the pelotons (see Peterson and Currah 1990). These cells, where lysis of the fungus pelotons takes place, as already stated, were designated as "digestion cells" by Burgeff (1959). The oldest colonised cells were the first to act as digestion cells, and this was followed by cells with subsequent colonisations. In other words,
between colonisation and the process of onset of digestion there was a definite interval. Purves and Hadley (1975) observed digestion after 48 hours of infection while Burgeff (1939) observed digestion a month after infection in *Dactylorhiza incarnata*; other workers have described biannual periods of digestion (Vermeulen 1946) in yet other species. Although in *Spathoglottis plicata* the present worker has not studied the time of digestion after infection, it was noticed that digestion occurred throughout the year in sequence from first infected to subsequently infected cells. Since infection becomes random in cortical cells at later stages of root development, digestion was also randomly observed in the cortex and not in a defined zone of cells, designated as the "digestion layers" which has been recognised in some species in the inner cortex (see Burgeff 1959; Williamson and Hadley 1970; Hadley and Williamson 1971; see also Hadley 1982). Alconero (1969), working with *Rhizoctonia solani* and *Vanilla* orchid, also found that hyphal digestion occurred in cells scattered throughout the root. But interestingly he observed that digestion was most frequently seen in the peripheral cortical cells. A gradient of fungal digestion with more intensity to less intensity was also observed from outer to inner cortical cells in some South east Himalayan orchids by Katiyar et al. (1985).
One of the very significant confirmatory observations made in this work pertains to the separation of lysing pelotons from the rest of the host cell cytoplasm by a distinct layer of material. This material was first reported by Nieuwdorp (1972) and was designated as "cellulose slime layer". This separation layer has also been reported by Peterson and Currah (1990) in the protocorms of the orchid Goodyera repens. These latter two authors reported that this layer was negative to cellufluor and positive to Aniline blue and could be seen as a fluorescing layer with this dye under UV incident light. This pattern of fluorescence and also the electron-lucent nature of this layer in TEM prompted them to suggest that this material was callose. They also stated that callose was induced as a wound response to isolate the degenerate hyphae and presumably a high concentration of a lytic enzymes from the host cell cytoplasm. They further suggested that this could explain how cells can contain degenerating hyphae and at the same time retain (the) cytoplasmic integrity. They also cited instances in the plant body where B-1,3-linked glucans (callose) are known to occur in situations where an impermeable barrier is being laid down (Heslop-Harrison and Heslop-Harrison 1970; Knox and Heslop-Harrison 1973; Peterson and Rauser 1979). A similar layer positive to aniline blue was subsequently reported either continuously or
discontinuously in the lysing pelotons of the orchid *Platanthera hyperborea* infected by *Rhizoctonia cerealis* (Richardson *et al.* 1992). Controlled histochemical studies made at present in *Spathoglottis plicata* have categorically shown that this layer, to start with, was discontinuously initiated and subsequently invested all around the lysing pelotons. This layer is not only Aniline blue positive but also very positive to Lacmoid blue which is a standard unquestionable dye to locate callose (Krishnamurthy 1988) and the purpose of this formation was to effectively isolate and insulate the lysing pelotons from the host cell cytoplasm (See Krishnamurthy 1977). This callosic material was evident till very late stages in the lysis of the pelotons.

It is not very clear from the present study whether the enzymes required for the lysis of the hyphae are produced by the host cell, as has been claimed by Gallaud (1905), Bernard (1909), Burges (1939) and Burgeff (1959), or by the fungus or by both (see Williamson 1973). However, it is more likely that the host cell controls the digestive process rather than the fungus. This can be surmised from the fact that like in many other nutritively efficient cells such as endosperm haustoria, embryo suspensor cells etc., the peloton-containing host cell nucleus, undergoes extensive enlargement with increased DNA content (polytenic). The
nucleus also gets enclosed along with a greater part of host cytoplasm within the callosic barrier. Very often the nucleus also becomes irregularly shaped. These nuclear changes in the peloton-containing host cell have been reported earlier (Bernard 1909; Burgeff 1936; Burges 1939; Campbell 1964; Dorr and Kollmann 1969; Williamson and Hadley 1969; Mejstrik 1970; Williamson 1970; Werner 1992).

Detailed structural and cytochemical studies have been carried out on the lysing pelotons by a number of investigators (see Burges 1939; Peterson and Currah 1990). A number of these observations are confirmed by the present study also. These include: change from the loose arrangement of fungal hyphae to compact arrangement of the hyphae in the pelotons, loss of identity of individual hyphae, from densely cytoplasmic more or less non vacuolated hyphal cytoplasm to highly vacuolated poorly cytoplasmic nature etc. Peterson and Currah (1990) discussed the nature of the fungus host interface including the fungal cell wall. They reported that the mass of collapsed hyphae during hyphal lysis reacted very strongly with acriflavine-HCl indicating that at least some of the materials forming this interface was polysaccharide in nature. The fact that the degenerating hyphal wall, although reacted very strongly to cellufluor in contrast to intact pelotonic hyphal walls, had promoted them to conclude that the walls of intact hyphae
contained some substance that blocked the staining of chitin and that this was presumably removed after lysis. They also questioned the claim of Barroso and Pais (1985) that cellulose formed a part of matrix material. The present study on *Spathoglottis* has indicated that in fresh pelotons the walls contain chitin; although there was intense staining with Chlorazol black E and Celluflour the hyphal wall poorly responded to the IKI-\(\text{H}_2\text{SO}_4\) treatment indicating the paucity of cellulose in the hyphal cell walls. In other words, the hyphal cell wall has both chitin and cellulose but both of them gradually declined in quantity during the lysis of the pelotons. Werner (1992) also reported a gradual digestion of chitin from the fungal cell walls. The present study also indicates that in the interface of fresh pelotons there is a good amount of structural proteins, esterified and non-esterified pectins but no phenolics, but during lysis there was a gradual loss of structural proteins; non-esterified pectins increase with lysis for some time but these could not be detected in the very late stages of peloton degradation.

The present study has indicated that there is a fairly good amount of lipids, especially acidic lipids in the cytoplasm of fresh pelotons; during hyphal lysis there is an increase in lipid content especially in phospholipids and neutral lipids for some time and these could not be detected
in late stages. These lipids, however, were seen in the cytoplasm of host cells indicating that they are released into host cells (see also Peterson and Currah 1990). Richardson et al. (1992) have stated that in Platanthera, during peloton degradation lipids and polyphosphates were observed in the degenerating hyphae. However, they were not sure whether these polyphosphates and lipids were drawn by the host cell cytoplasm as has been demonstrated by the present study. Barroso et al. (1986) reported that in the infected cells of Ophrys lutea they are a number of steroidal substances, they may also perhaps give the positivity that was observed for lipid stains used in the present investigation.

There were not many attempts in the past to study the enzymology of the pelotons before and during lysis (see, however, Williamson 1973; Pais and Barroso 1982; Barroso et al. 1986; Werner 1992). Williamson (1973) reported a marked increase in acid phosphatase activity in the lysing pelotons while esterase was active during the intact stage of the peloton especially at the hyphal tips. In the present study it was recorded that there is a moderate activity of acid phosphatase and ATP-ase in the fresh pelotons while peroxidase and esterase could not be detected in the young pelotons. However, during lysis of pelotons the activity of acid phosphatase continued to be moderate, of ATP-ase and
peroxidase increased while esterase could not be detected. A very high activity of malate dehydrogenase, starch phosphorylase, succinate dehydrogenase, glucose-6-phosphatase and β-glucosidase was observed in the cytoplasm of fresh pelotons; the activity of these enzymes was gradually lost during lysis.

A very interesting aspect of orchid mycorrhiza is the repeated formation of fungal coils or pelotons in the same host cell. A maximum of four generations of pelotons are digested in the same host cell in the orchid studied at present during the life span of a root. Successive peloton formation has been reported already by Hadley et al. (1971), Hadley (1982), Katiyar et al (1985), Peterson and Currah (1990). A number of workers have correlated such repeated reinfections and peloton formations with periods of active growth of the orchid (see Hadley 1982). However, this has probably nothing to do with the growth periods of the orchids because in our material, all through the year, till the life of the root, digestion of pelotons and relaying of pelotons were observed. The number of times the pelotons are reformed depends on the life span of the root as well as on the availability of fungus for recolonisation.

Details of renewed peloton formation in the same host cell have not been worked out in the past in any orchid.
The present study indicates the method by which successive pelotons may be organised. The same host cell shows both pelotonic and non-pelotonic hyphae, the former forming bundles and occupying the major part of the host cell while the latter forming a lining layer around the host cell wall, the two being connected by cross connections. In certain cells, some of the peripherally located hyphal filaments get segregated from the rest of the peloton spatially and become non-pelotonic. Distinct cytochemical differences between these two types of hyphae have already been recorded under the observation section (see page 21). In view of the facts that the non-pelotonic hyphae are separated from the pelotonic hyphae undergoing digestion by an impermeable callose barrier and the significant presence of phenolics in the cell walls of these non-pelotonic hyphae, help them in retaining their integrity and their subsequent involvement in organising fresh pelotons in the same host cell. In those cells where the entire hyphae are pelotonic, fresh pelotons are organised by hyphae that may subsequently find entry into host root cortex.

4. Role of Root hairs in fungal reproduction

One of the interesting observations made in the present thesis is the dual role of root hairs. Not only are they involved as the entry points for the mycorrhizal fungus into
the host root both during primary and secondary infections, but also they serve as venues for the reproduction of the fungus as well as for their exit from the root to the rhizosphere. During the exit process, they do not go out as hyphae but they produce chlamydospores and occasionally selerotia. Chlamydospores are formed in plenty within the root hairs in clumps or in short or long chains terminally or intercalarily. Such chlamydospore-containing root hairs undergo very characteristic spiral dehiscence to release the spores into the rhizosphere. To the best of his knowledge, the present author is not aware of any report of chlamydsospore formation in the root hairs, and the dehiscence of the root hairs to release these in orchids, although in parasitic species of *Rhizoctonia* chlamydospores and sclerotia have been reported occasionally in the epidermal cells of the host (Sneh *et al.* 1991). The present author is also not in a position to give a satisfactory explanation as to why the fungus is not able to form chlamydospores or sclerotia in the root cortex.