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MYCORRHIZAE IN THE PAKISTAN ERICALES

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Abstract

Ectendomycorrhizae of the Pakistan Ericales viz., *Rhododendron lepidotum* Wall. ex D. Don., *R. afghanicum* Aitch. & Hemsl., *Gaultheria trichophylla* Royle., *Cassiope fastigiata* D. Don., *Pyrola rotundifolia* Michx., and *Monotropa hypopitys* L., were described. Microscopic examination showed typical endophyte in and on the roots and its absence from aerial or subaerial parts. This was supported by attempted isolation experiments.

Morphological and anatomical studies suggest a series of increasing saprophytism and its correlated characters from Ericaceae, through Pyrolaceae to Monotropaceae.

Endogone spores, responsible for the formation of vesicular-arbuscular mycorrhiza in the majority of plants, were extracted from heath rhizospheres, except that of *Monotropa hypopitys*, but no vesicular-arbuscular infections in roots of Ericales studied were observed.

The endophyte isolated from *Rhododendron afghanicum* lacked any reproductive bodies and resembled *Mycelia radicis myrtillii*, isolated by other workers. A clamp-bearing basidiomycetous fungus emerged from root pieces of *Monotropa hypopitys*.

Introduction

Ericaceous plants were recognised as having their roots invested with fungal hyphae as early as 1881 by Kamienski. Frank (1882), who coined the name 'mycorrhiza', described and figured the fine hair-like rootlets of certain heath plants, the cells of which were filled with fungal hyphae. Present information suggests that most ericaceous plants possess mycorrhizae instead of roots which function in a manner similar to that in ectomycorrhizae of forest trees, as efficient salt absorbing organs on nutrient deficient soils (Brook, 1952; Morrison, 1957).

Two morphological types of mycorrhiza, namely ericoid, characteristic of Ericaceae and Epacridaceae, and arbutoid, characteristic of Arbuteae, Pyrolaceae and Monotropaceae, are known to occur and according to Henderson (1919) a series of increasing saprophytism could be arranged from ericoid to arbutoid through many intermediates. Moreover, the resemblance between ectomycorrhizae of forest trees on one hand and endomycorrhizae of Orchidaceae on the other hand indicates that they form a sort of link between the two types (Harley, 1969).

In Pakistan himalayan Ericales have been shown to possess mycorrhizae typical of the heaths (Khan, 1972). In the present investigation a detailed account of these mycorrhizae is given. Attempts were also made to isolate the endophyte from *Rhododendron afghanicum* and *Monotropa hypopitys*.

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Material and Methods

(a) *Anatomical Investigations:*

Roots and shoots of the following species were collected from Hazara Hills (Himalayan Range). Three to five examples of each host species were examined.

Ericaceae: *Rhododendron lepidotum* Wall. ex D. Don.; *R. afghanicum* Aitch. & Hemsl.; *Gaultheria trichophylla* Royle; *Cassiope fastigiata* D. Don.

Pyrolaceae: *Pyrola rotundifolia* Michx.

Monotropaceae: *Monotropa hypopitys* L.

Plant material was fixed in formalin-acetic acid-ethanol and embedded in paraffin wax (mp 56° C). Transverse and longitudinal sections, 10-12/μm thick, were cut with a rotary microtome. Paraffin wax was removed with xylol, and the slides were stained with safranin and fast green (Johansen, 1940). The diagrams of anatomy of heath mycorrhizae were based on sections of many roots from 3-5 host plants in each case.

Whole roots, leaves, flowers and hand sections of branches were also stained with 0.05% trypan blue in lactophenol, after clearing them in 10% KOH at 90°C for 24 hours (Phillips & Hayman, 1970).

(b) *Extraction of Endogone spores:*

Heath soils were examined for the presence of *Endogone* spores according to the technique previously described (Khan, 1971).

(c) *Isolation of Endophyte:*

Roots, leaves, sections of stems and flowers from *Rhododendron afghanicum* and *Monotropa hypopitys* were washed, surface-sterilized in 10% solution of sodium hypochlorite for 30 minutes, washed several times with sterilized distilled water, and crushed with sterile instruments. The crushed material was transferred aseptically to malt-marmite, potato-dextrose and heath-soil extract-agar slopes and the cultures were incubated for three weeks at 24°C.

Results

Morphology of Mycorrhizae

i) *Mycorrhizae in Ericaceae:* Pakistan Ericales are mostly alpine, evergreen, erect or prostrate shrubs or shrublets found from 3000 to 4000 m in Swat, Kaghan, Kashmir and Hazara, extending eastwards through the himalayas to Bhuttan (Ali, 1971).

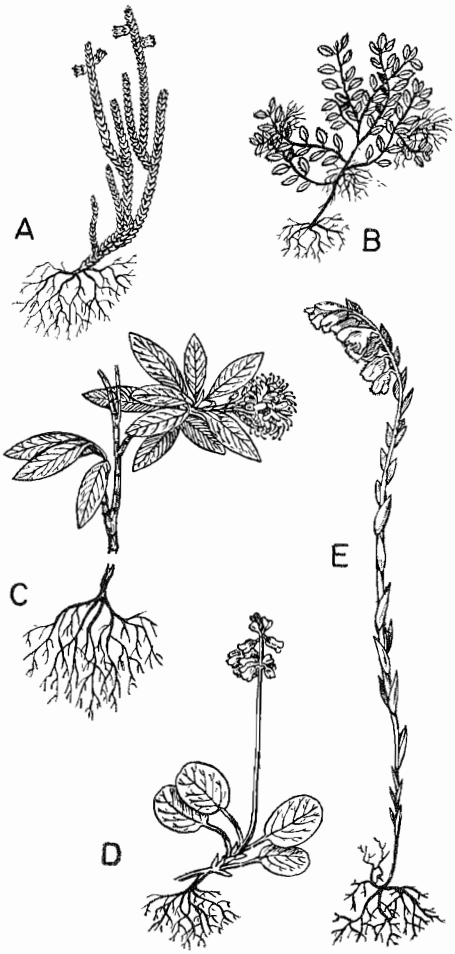

Fig. 1. Plants of the Pakistan Ericales. X $\frac{1}{4}$.
A. *Cassiope fastigiata.*
B. *Gaultheria trichophylla.*
C. *Rhododendron afghanicum.* (Branch and roots).
D. *Pyrola rotundifolia.*
E. *Monotropa hypopitys.*

Gaultheria trichophylla is a prostrate to procumbent shrublet often found in association with mosses on earth, moist rocks and tree stumps, giving off adventitious roots at points touching soil (Fig. 1, B). *Cassiope fastigiata*, a shrub 5-30 cm tall, is characterised by its crowded fastigiate branches covered by 4 rows of small thick leaves appressed to the stem. (Fig. 1, A). *Rhododendron* species are tall evergreen and aromatic shrubs with large leathery leaves crowded towards the ends of the branches (Fig. 1, C). Despite these morphological and habitat diversities among members of Ericaceae, they all possess a root system in which roots are branched into long, thread-like rootlets, 40—90μm in diameter, and devoid of root hairs, with a white glistening appearance to the naked eye. Uniformity in their structure is so great that a generalization can be made. The histological studies bear this out.

Observations of whole root, cleared and stained, showed that numerous sparsely septate and slender fungal hyphae of 0.5—0.7μm in diameter ramify loosely over them. There was no dense mantle nor dot he roots exhibit heterorrhizic habit, comparable to that of ectomycorrhizae of forest trees.

Anatomically the fine rootlets had a very reduced cortex only one cell thick and a small central stele (Fig. 2, A). No epidermis (piliferous layer) was present. Hyphal branches from the exterior passed through the outer walls into the cavity of cortical cells where they formed many short curved and densely interwoven segments, resembling with pelotons of orchid mycorrhiza rather than arbuscules of VA mycorrhizae. They were frequently in close association with the nucleus of the host cell (Fig. 2, B). Sometimes the hyphae were seen passing between the radial walls of two adjoining cells without actually entering these cells. In such cases, hyphae might force their way inside the cells through their lateral walls. The penetrating branch or haustorium was encapsulated, except for its small tip, by a callose sheath (Fig. 2, C).

The endophyte did not penetrate into or beyond the endodermis, which appears to form a 'barrier', similar to that noted by Clowes (1951) in the mycorrhizal roots of *Fagus sylvatica* and by Khan (1971a) in gymnosperms with vesicular-arbuscular mycorrhizae. The apical meristem of rootlets also remained free from external as well as internal hyphae. Rarely extension of fungal hyphae from one cell to another may be seen, but the fungus does not seem to spread very far in this way from the point of entry; the source of infection for the majority of the cortical cells appears to be superficial hyphae which had direct connections with external hyphae. Later the hyphae became thick-walled and wider and contained oil globules. They soon lost their contents but the moribund empty hyphae remained as irregular and inflated clumps which were also finally digested. The aged roots shed their cortical coverings and developed a corky bark which was not invaded by hyphae.

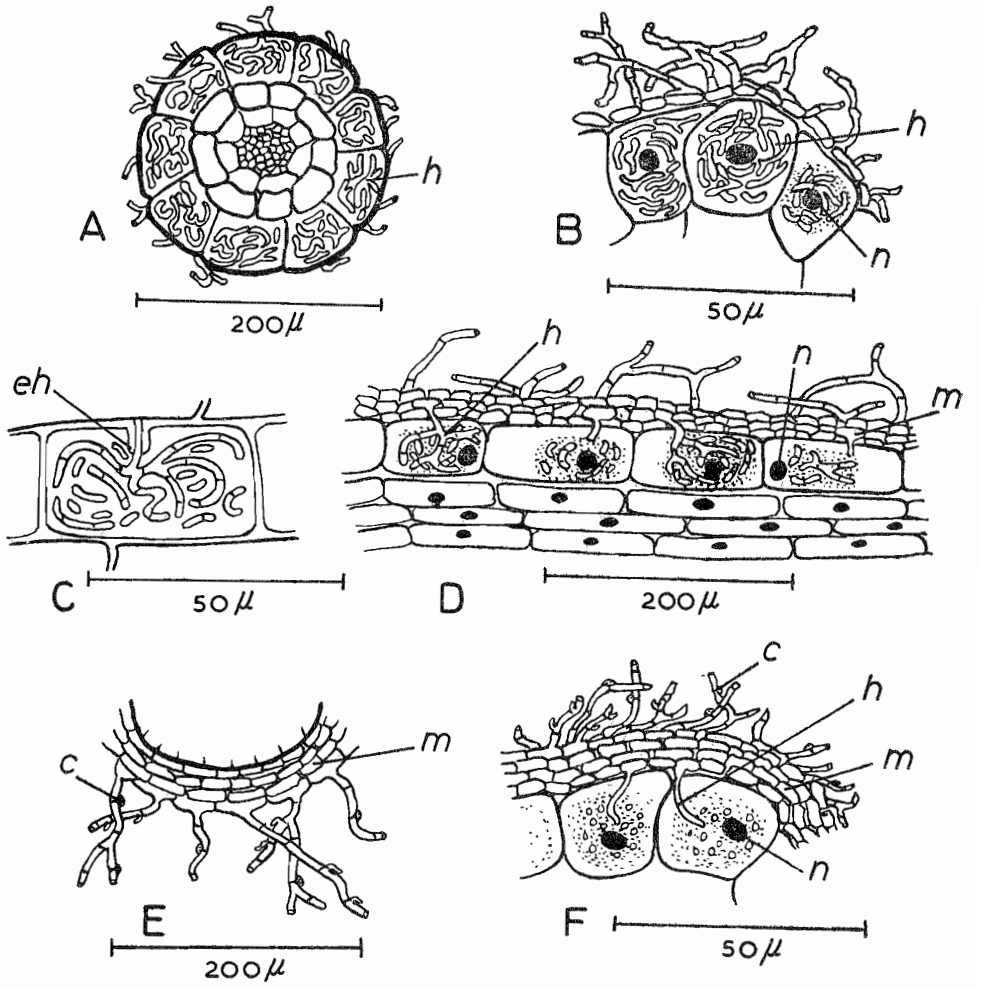

Fig. 2. Anatomy of mycorrhizae of the Pakistan Ericales.

SYMOBLS: c, Clamp connection; h, Haustorium; eh, encapsulated penetrating branch of haustorium; m, fungal mantle; n, host cell nucleus.

A. T.S. *Rhododendron afghanicum* rootlet, showing one-celled cortex and intracellular hyphal complexes or haustoria resembling pelotons.
B. T.S. infected cortical cells of *R. afghanicum*. Note the haustoria in intimate association with the host cell nucleus.
C. L.S. of infected cortical cells of *R. afghanicum* showing encapsulated penetrating branch of haustorium.
D. L.S. of infected cortical cells of *Pyrola rotundifolia* showing various stages in haustorial digestion and a fungal mantle on their surfaces.
E. L.S. of root apex of *Monotropa hypopitys* overarched by fungal mantle with many radiating hyphae.
F. T.S. of infected cortical cells of *M. hypopitys*. Note simple haustoria and many-layered fungal mantle.

No abnormal cytological effect of invasion by the fungal endophyte could be seen. The cytoplasmic contents, however, appeared denser as compared to those of older roots which, although retaining the external fungal network, are usually not colonized by the endophyte. The nuclei of invaded cells were slightly enlarged after digestion of the intracellular hyphal complexes.

The endophyte was confined to the roots only and did not infect the aerial or subaerial parts of the plants. It was never found in sections of healthy leaves, stems and floral parts including ovaries, suitably stained.

ii) *Mycorrhizae in Pyrolaceae: Pyrola rotundifolia* (Fig. 1, D) is an alpine creeping perennial with a flowering scape growing from a rosette of orbicular dark green leaves and a creeping rootstock possessing ectendomycorrhizae. The rootlets are enveloped by a thick and compact fungal sheath or mantle. Considerable penetration into enlarged epidermal and sub-epidermal cells by haustoria, encapsulated by callose sheath similar to that in Ericaceae, occurred. Various stages in disintegration of the intracellular hyphal complexes were observed (Fig. 2, D). There was a loosely woven mass of hyphae round the root tip, though no penetration into meristematic cells was observed. The root diameter increased partly due to an extensive covering of fungal mantle and partly to the enlargement of epidermal cells, a situation comparable with ectomycorrhiae of forest trees. However, a Hartig net, so typical of ectomyorcrhizae, waw not present, though many intercellular mycelial threads were present. No evidence of systemic infection in organs other than roots was obtained.

iii) *Mycorrhizae in Monotropaceae: Monotropa hypopitys*, a saprophytic or epiparasitic herb 10-40 cm tall, with a flowering scape covered with brownish yellow scales (Fig. 1, E), is distributed in Kashmir, Kaghan, Swat and Hazara forests rich in moist humus. Its roots are invested with a 4-5 layered dense mantle of fungal hyphae, which also ramify in the surrounding humus and are seen in direct connection with the fruiting bodies of *Boletus* species and with fungal hyphae radiating from the fungal mantles of ectomycorrhizae of *Quercus*, *Abies*, *Picea*, *Pinus* and *Cedrus* species under which *Monotropa* grows. The cells in the majority of root apices were uninfected, although they were completely overarched by fungal hyphae which were closely appressed to their outer surfaces, growing as a 2-4 layered mantle with many radiating hyphae (Fig. 2, E). Branches from the exterior passed through the outer walls into the cavities of epidermal cells or in between the radial walls of two adjoining cells to form a Hartig net. The intracellular branches or haustoria were simple in form, encapsulated except at the apex with a callose sheath and they grew towards the host nucleus (Fig. 2, F). The disintegration of haustoria occurred in the region of the nucleus, which was large and conspicuous. In the deeper seated layers of the cortex the hyphae were occasionally attenuated and were seen running between the longitudinal walls and they rarely entered the cavities of these cells.

Infection by the endophyte was not observed in the flowering scape or scale leaves. The endophyte remained confined to the roots.

Endogone spores in Heath Rhizospheres

Almost all the rhizospheres of Ericales examined, contained *Endogone* spores, except that around the roots of *Monotropa hypopitys*, which was growing in association with puffballs and mushrooms under oaks, blue pines, deodars and yews, the forest trees with ectomycorrhizae. The spores belonged to either yellow vacuolate non-endosporic or bulbous reticulate types of *Endogone* spores (Mosse & Bowen, 1968; Khan, 1971). These types of spores are very common in the soils of Western hilly regions of Pakistan (Khan, 1971).

Attempts to Isolate Endophytes

Since the root cortex in ericaceous plants is very much reduced, it was not possible to tease out single cells or cell groups for isolating the endophyte. Instead small pieces of washed surface-sterilized roots, crushed and plated aseptically on various culture media, were employed. In all about 300 slopes were inoculated, 70% of which remained sterile after incubation and 25% of which contaminated with common laboratory moulds. From the remaining 5% of root pieces of *Rhododendron afghanicum* several slow growing septate and sterile fungi emerged. The isolates did not produce any reproductive bodies even when sub-cultured on fresh media where they continued their slow growth amounting to less than one mm a day.

Mature colonies were dark brown with white margins. The mycelium was mainly submerged, with aerial hyphae regularly septate when young and becoming irregularly septate with age. Because of lack of reproductive bodies the exact taxonomic position of the endophytes could not be determined. No attempts to synthesize mycorrhiza with the isolates were made.

A fast-growing, white and clamp-bearing basidiomycetous fungus emerged from root pieces of *Monotropa hypopitys*.

No fungi developed from surface-sterilized healthy leaves, slices of stems and flowers of ericaceous plants on any of the media used.

Discussion

The typical, naturally developed mycorrhizae of the Pakistan Ericales described above agree with the main features described by other workers (Christoph, 1921; Rayner, 1925; Luck, 1940, 1941; Lihnell, 1942; Brook, 1952; McNabb, 1961). In

contrast with the observations of Rayner (1915, 1922, 1925, 1927, 1929), Lewis (1924), Addoms & Mounce (1931, 1932) and Barrows (1936, 1941), the endophytes in the present study were found to be confined to the roots only and did not penetrate the shoot, as determined by microscopic examination, supported by attempted isolations. Absence of systemic infection in organs other than roots of evergreen *Rhododendron* species to chlorophylless *Monotropa* is in agreement with the observations of other workers (Christoph, 1921; Doak, 1928; Knudson, 1933; Freisleben, 1933; McLennan, 1935; Gordon, 1937; Bain, 1937; and Brook, 1952), who were also unable to find systemic infection in heath mycorrhizae.

The complete absence of root hairs, reduced amount of cortical tissues and lack of infection in the apical meristems of most ericaceous species studied are in accord with the observations of previous workers. Burgeff (1961), on the other hand, noted a direct attack on the apical meristem and its destruction in *Calluna* by the endophyte.

While it is not known whether the fungus developing in cultures, apparently free from contaminants, from some of the surface-sterilized root pieces of *Rhododendron* spp. was the endophyte, it agreed closely in its colour, rate of growth and lack of reproductive bodies with *Mycelia radicis myrtillii*, isolated by other workers (Freisleben, 1933, 1934; Bain, 1937; Brook, 1952; McNabb, 1961). However, it may well be pointed out that slowness of growth is not a good criteria; species of *Ceratobasidium* and *Thanatephorus* which are common mycorrhizal fungi, are septate, sterile in culture (unless efforts are made to induce them to fruit), and very fast-growing. Furthermore, *Mycelia radicis myrtilli* is a meaningless name, despite its use in literature and could apply to many different sterile mycelia isolated from roots. One should be extremely cautious about putting names to sterile mycelia as so many different fungi can look alike in the sterile mycelial state.

The presence of direct connections of fungal hyphae of puff-balls and mushrooms with the ectomycorrhizae of forest trees on one hand and with the ectendomycorrhizae of *Monotropa hypopitys* on the other hand indicate the possibility that these mycorrhizae belong to basidiomycetous fungi. The endophyte isolated from *M. hypopitys* roots supports this contention. This is in accord with the conclusions of Kamienski (1881), Francke (1934) and Bjorkman (1960). Bjorkman (1960) synthesized mycorrhizae in pine, in culture, using a fungal isolate from *Monotropa* roots.

In the present study a series of increasing saprophytism and its correlated characters is evident from Ericaceae through Pyrolaceae to Monotropaceae, a situation previously noted by Henderson (1919). The following series can be proposed:

1. Gradual increase in amount of hyphal investment from roots of *Rhododendron* through *Pyrola* to *Monotropa*, together with a gradual decrease in the number of layers in the root cap.

2. Gradual decrease in amount of wood formed from typical Ericaceae, with very woody stems, through *Pyrola* which is less woody to *Monotropa* which has very limited wood formation, correlated with gradual increase in phloem, and together with reduction from shrubs to herbs.

3. Gradual reduction in size and structure of leaves from evergreen leathery in Ericaceae, through less leathery in *Pyrola rotundifolia* to yellow brown scales in *Monotropa hypopitys*.

4. Gradual reduction in number of stomata from very numerous in *Rhododendron*, through less numerous in *Pyrola* to very few in scales of *Monotropa*.

5. Gradual reduction in size and increase in number of seeds.

All the above changes are correlated with increasing saprophytism, but physiological evidence is required to support such 'arrangements'.

The endophyte of potted plants of *Pernettya macrostigma* is reported to produce spore-like bodies or sporangia within the cortical cells of older infected rootlets (Brooke, 1952; Morrison, 1957; McNabb, 1961). The function of these sporangia is not known neither their germination was reported. Brook (1952) critically ruled out the similarity of these sporangia with the vesicles of vesicular-arbuscular mycorrhizal fungus *Endogone* because of their much smaller size and absence of arbuscules in infected rootlets. No sporangia were found in the present specimens taken from their natural habitats. Presence of *Endogone* spores in rhizospheres of these plants is quite an interesting observation but the absence of a typical vesicular arbuscular infection in their rootlets indicate either a host specificity of *Endogone* or the need for a stimulus responsible for ericaceous endophyte(s) to associate closely with the roots of susceptible hosts and/or an antagonism between the ericaceous endophyte and *Endogene* in heath soils. Handley (1963) has suggested that the endophyte of *Calluna* exerts an antagonistic effect on the ectotrophic mycorrhizal associates of coniferous trees.

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