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SPORES OF MYCORRHIZAL *ENDOGONE* SPECIES EXTRACTED FROM SOIL BY WET SIEVING AND DECANTING

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(With 6 Text-figures)

Extra-matrical spores of *Endogone* were found to be common in cultivated soils in Scotland. Six types of spores are described which are believed to represent distinct species. In preliminary inoculation experiments 4 of the 6 formed endotrophic mycorrhiza: three of these formed arbuscules and vesicles, and one formed only arbuscules. In field soil extra-matrical spores are much more abundant than spores formed in sporocarps.

There is increasing evidence to indicate that species of *Endogone* are frequent as mycorrhizal associates. Workers who have studied the external mycelium of such endophytes have stressed the *Endogone* characteristics (Butler, 1939; Mosse, 1959*b*; Nicolson, 1959; Kubíková, 1961), and *Endogone* fruiting-bodies have been associated with naturally infected material (Peyronel, 1924, 1937; Dowding, 1959). The capacity of *Endogone* spp. to form such mycorrhizal infections has been amply confirmed experimentally when sporocarps or spores have been used as inoculum (Gerdemann, 1955, 1961; Mosse, 1956; Meloh, 1961; Koch, 1961). The use of surface-sterilized spores precludes the possibility that such infections arise from contaminating fungi (Mosse, 1956, 1959*a*, 1962).

Mosse (1959*b*), Nicolson (1959) and others have noted that the hyphae of vesicular–arbuscular endophytes occur in great abundance on the surfaces of plant roots from many habitats, and their profuse occurrence can be seen when soil is examined directly (Warcup, 1957). Indeed, there is little doubt that these are among the most prevalent fungi in soils which support plant growth. Despite their prevalence they have not been isolated from soil and attempted isolations from infected roots have given only limited success. Only Barrett (1961) has reported successful isolation of an *Endogone*-type (*Rhizophagus*) endophyte from infected roots.

Most techniques used to examine soil fungi depend to some degree on the ability of the organisms to grow on artificial media (Warcup, 1960). Such techniques do not reveal the presence of mycorrhizal *Endogone* spp. since they appear to be obligate or near-obligate symbionts, and other methods must be sought to demonstrate them.

Using Morgan's flotation method, Triffitt (1935) reported that 'micro-

cysts', which were often confused by nematologists with cysts of *Heterodera*, were common in various English soils. She concluded that they did not represent a stage in the life cycle of *Heterodera*, and from her description it seems that these bodies were *Endogones* pores. Using the technique of wet sieving and decanting, Gerdemann extracted two types of mycorrhizal spores from soil in Illinois. In inoculation tests the larger of these, with a bulbous basal attachment (B spores), gave phycomycetous mycorrhizal infection; arbuscules but not vesicles were produced and clumps of echinulate spores (C spores) were formed on external hyphae (Gerdemann, 1955). Surface-sterilized spores of this fungus germinated and gave limited growth on artificial media but it could not be subcultured and maintained. The second, smaller spore was extracted from maize soil by the same method. In inoculation tests in sterilized soil it induced typical vesicular-arbuscular mycorrhiza and, in addition, *Endogone* sporocarps and free extra-matrical chlamydospores were formed on hyphae attached to roots. These chlamydospores were identical with those extracted from the field soil (Gerdemann, 1961). Similar methods have been used to culture fungi from soil debris (Chesters, 1948) and to extract hyphal fragments and sclerotia from soil for culturing (Warcup, 1959).

Recently six different types of *Endogone* spores have been found in Scottish soils (Gerdemann & Nicolson, 1962). This paper describes the technique used for obtaining spores from soil, gives descriptions of the spores found, and the results of preliminary inoculation tests.

EXTRACTION OF SPORES FROM SOIL

Soil samples were obtained during the autumn of 1961 from fields containing wheat, barley, oats, potatoes, and strawberries, and from pastures containing a mixture of grasses and legumes, all in the area west of Dundee, Scotland. All the samples contained large quantities of root material.

Spores were obtained by wet sieving and decanting, a technique commonly employed by plant nematologists to extract nematode cysts and larvae from soil. Approximately 250 ml. of soil were suspended in 1 l. or more of water. Heavier particles were allowed to settle for a few seconds and the liquid was decanted through a sieve fine enough to remove the larger particles of organic matter, but coarse enough to allow the desired spores to pass through. The suspension that passed through this sieve was saved and stirred to re-suspend all particles. The heavier particles were allowed to settle for a few seconds and the liquid decanted through a sieve fine enough to retain the desired spores. In order to remove colloidal material, the debris retained on the sieve was either thoroughly washed under a stream of water or re-suspended and decanted again through the sieve. Small amounts of debris were transferred from the sieve to a shallow layer of water in a Petri dish. A dish should contain only enough debris to form a single layer of particles. The debris was examined with a stereoscopic microscope ($\times 25$ and $\times 50$) with strong direct lighting. The *Endogone* spores were discernible in the mixture of organic debris and root fragments and were picked out individually with a flattened needle and placed in a small concave dish of water. They were transferred through

one or two changes of water in order to free them from as much soil and organic material as possible. For measurement and detailed observation under higher magnification, spores were placed in water in cavity slides. Crushed specimens were used to observe internal details and wall thickness.

In the initial phases of the study the following fractions were examined: 1 mm. to 710 μ, 710 to 420 μ, 420 to 250 μ, 250 to 149 μ, 149 to 105 μ, 105 to 74 μ and 74 to 44 μ. The majority of the desired spores fell within 420 to 149 μ and this fraction was used in most routine work. Other smaller spore types and spore aggregates were observed but were not included in the present study.

DESCRIPTION OF SPORES

The six types of spores found appear to belong to different species. Some of them probably represent new species; however, further details in life cycles are required before names are given.

Spore type 1 (for figure, see Gerdemann, 1961). Spores of this type were found in only one field in the Dundee area. They are, however, very similar to the extra-matrical chlamydospores produced by a mycorrhizal species of *Endogone* recently found in cultivated soils in Illinois (Gerdemann, 1961). The spore walls are bright yellow with a somewhat shiny water-repellant surface, 2–4 μ thick and consist of a single layer only. The spores are generally spherical and measure 136–273 μ in diameter. The attached hypha enlarges into a distinct funnel shape at the base of the spore and the spore wall thickening extends into the funnel and forms a thicker ring at the point of expansion. A plug or septum separates the spore contents from the hypha in this region. Occasionally, a short hypha extends from the funnel shaped base towards the spore. In general, the hyphae attached to spores of this type are longer and often more branched than the hyphae attached to other spores described here. One spore with two attached hyphae was found. A few small sporocarps containing 2 or 3 dead spores were found in soil from the field containing this spore. They were very similar to sporocarps produced by the Illinois *Endogone* with similar extra-matrical chlamydospores.

Spore type 2 (Fig. 1). This was the most common and the largest of the spores found during the study. It occurred in most of the sampled soils and in considerable abundance in some of them. The wall consists of 2 distinct layers, a thin colourless outer layer approximately 2 μ thick, which is easily removed with a needle, and a light yellow or light brownish yellow inner wall about 4 μ thick. Sand and soil particles tend to adhere to the spore surface. The spores are spherical, ellipsoidal or irregular in shape and 124–391 μ diam.

The attached hypha is generally quite short and does not significantly increase in diameter at the base of the spore. The thick inner wall extends into the hypha for a short distance. The spore contents are separated from the empty hypha by a basal plug just below the point of attachment.

Spore type 3 (Fig. 2). Spores of this type were found in soil from a number of fields. They were, however, much less abundant than type 2. The wall is brown and consists of a single layer 6–12 μ thick. The spores are

spherical or ellipsoidal and measure 91–318 μ diam. The thick wall extends into the attached hypha for some distance, and the spore contents are separated from the empty hypha by a plug near the base of the spore.

Spore type 4 (Fig. 3). This spore was found in soil from one site only. The most conspicuous feature is its black colour. The black wall consists of a single layer 5–6 μ in thickness which completely obscures the contents.

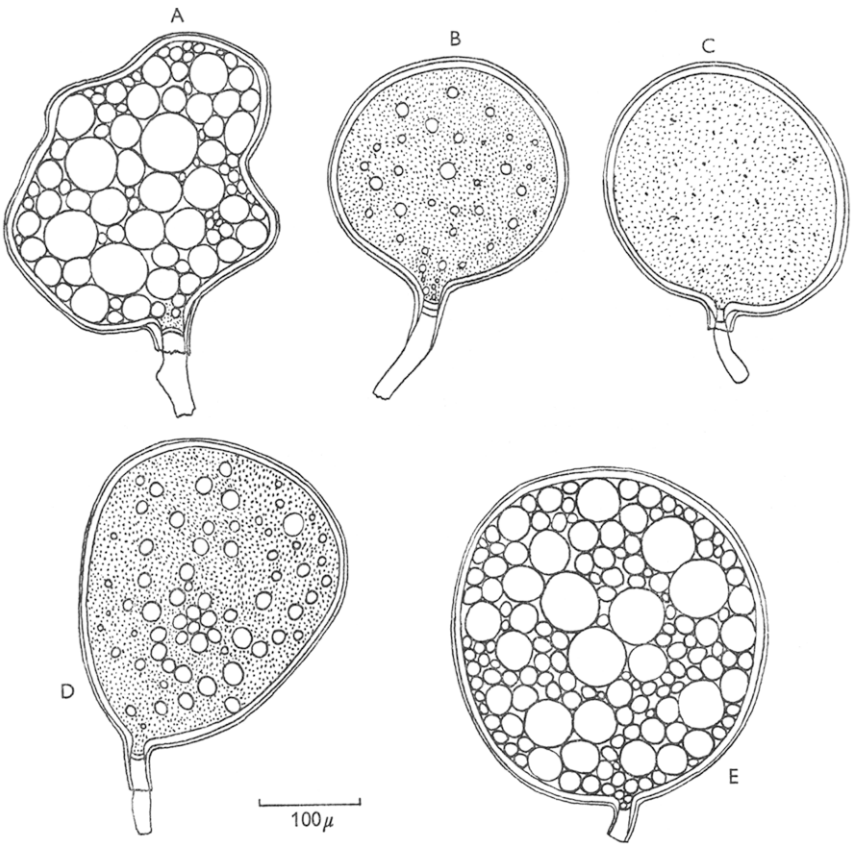

Fig. 1. Spore type 2, representative specimens showing typical attachment and range in size and shape; A, B, D, E with oil globules.

The spores are spherical to slightly ellipsoidal and measure 145–236 μ diam. The wall thickening extends into the attached hypha for some length (Fig. 3). This spore is most like type 3, differing primarily in its striking colour. The size also tends to average somewhat smaller and it has a thinner wall.

Spore type 5 (Fig. 4). This spore was found in many soil samples but was never abundant. The wall consists of two layers, a light yellow outer layer 3–4 μ thick and a very thin membrane-like inner wall. The shape varies from spherical to cylindrical. Since the hyphal attachment may occur either at the end or in the middle of cylindrical spores there is an extremely

wide range in both width and length. Dimensions could be misleading even if the range in length and width were both given since large measurements in both directions do not occur in any one specimen. The smallest spore observed was $126 \times 90\,\mu$ and the largest $111 \times 511\,\mu$ (Fig. 4F).

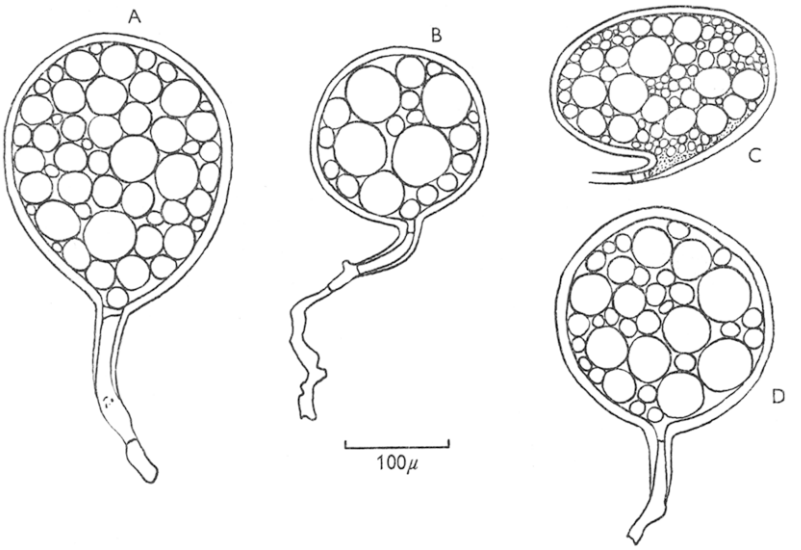

Fig. 2. Spore type 3 showing type of attachment and oil globules.

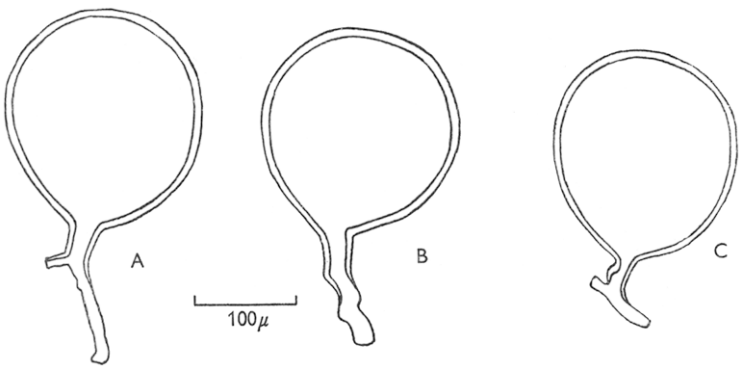

Fig. 3. Spore type 4, showing type of attachment. The black wall obscured the contents.

Cylindrical spores were often enclosed in dead roots. Spherical types appear to form free in the soil. The attached hypha enlarges into a distinct bulbous swelling at the base of the spore. The spore wall thickening does not extend into the bulbous swelling and the spore opening is nearly occluded, with only a fine line being visible.

Spore type 6 (Fig. 5). This spore was found in soil from only one site and relatively few specimens were obtained. The wall consists of a brown outer

layer approximately 3 μ thick and 2 thin colourless inner membranes. The spore is usually spherical and 136–227 μ diam., though one unusual specimen (Fig. 5 B) 191 × 345 μ was found. Many of the spores did not have a hyphal attachment and the spore opening was completely occluded. Others were found that were attached laterally to a coarse hypha with an empty vesicle at one end. Spores attached terminally were never seen. In its manner of attachment this spore differs from all described species of Endogonaceae.

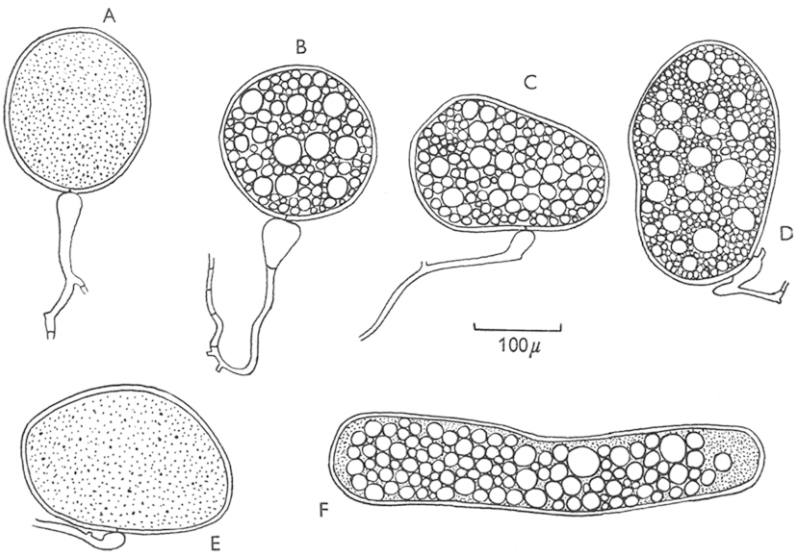

Fig. 4. Spore type 5, showing bulbous hyphal attachment (A–E) and oil globules (B, C, D, F); a hyphal attachment was not present on the specimen drawn in F but its general appearance placed it in this group. This spore was probably formed with a root.

Spore types 2, 3, 4 and 5 were occasionally found to contain other smaller spores (Fig. 6), which are thin-walled, usually spherical, and of quite variable numbers. Spores containing spores usually appear old and often the wall is partially collapsed. It is not known if the small spores represent a part of the life cycle of these species or if they are produced by a parasitic fungus.

Inoculation tests

The funnel inoculation technique (Gerdemann, 1955) was used to determine whether the 6 types of spores would produce endotrophic mycorrhizal infection. Small funnels were made by pressing a sheet of aluminium foil around a glass funnel. A cotton-wool plug was placed in the end of the aluminium funnel and the neck was filled with washed, autoclaved sand. Spores were carefully examined to ensure that they were of the same type and washed to remove as much soil and organic debris as possible. They were then poured over the surface of the sand at the top of the funnel neck and the funnel was filled with sand and planted with

red clover (*Trifolium*). Funnels were watered from above until the seed germinated, after which they were watered from below by placing the funnel in an Erlenmeyer flask of water. Approximately 20 days after

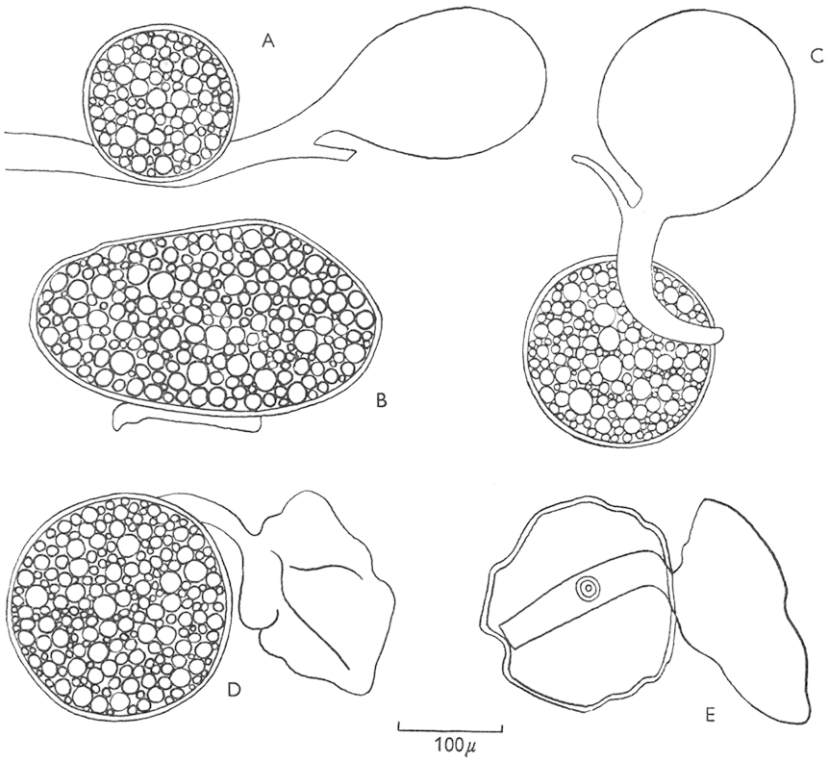

Fig. 5. Spore type 6, showing attached empty vesicles (A, C, D) and spore without an attached vesicle (B). E shows the base of spore D viewed from within with the lateral attachment and occluded opening.

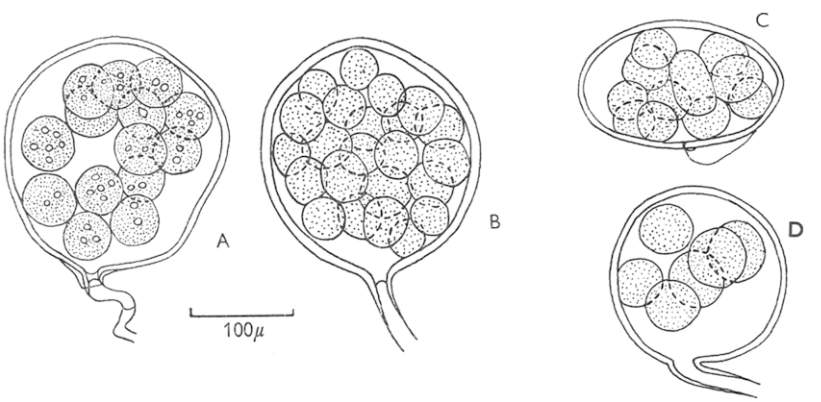

Fig 6. Spores containing smaller spores. A, spore type 2; B, spore type 3; C, probably spore type 5; D, spore type 4.

sowing 4 ml. of Knop's nutrient solution was added to each funnel, and 1 week later an additional 2 ml. were added. After 35–41 days the roots were examined by opening the funnels and gently washing away the sand. Roots were first examined in water under a dissecting microscope. They were then cleared and stained by boiling for a few minutes in lacto-phenol cotton blue and re-examined in clear lacto-phenol.

Spore types 1, 2 and 3 germinated by the production of a germ tube from the broken end of the attached hypha as described by Godfrey (1957) and Mosse (1956, 1959*a*) for species of *Endogone*. Germinated spores were easily observed and it was possible to trace hyphae from spores directly to the points of entry into the roots. These 3 spore types produced typical vesicular-arbuscular mycorrhiza like that described and illustrated by Mosse (1956, 1962). Round the roots extensive hyphal growth developed, on which small spherical thin-walled vesicles on short lateral branches were produced. Further inoculations conducted over a longer period of time will be required to determine whether these vesicles remain small or whether they develop into the large thick-walled spores.

Spore type 5 germinated by the production of a number of germ tubes through the spore wall in the area round the base of the spore. The hyphae infected the roots to produce endotrophic mycorrhiza. Arbuscules but not vesicles were formed. Small spherical to irregular spores were produced singly at the end of short coiled branches on the external hyphae.

Infections have not yet been obtained with spore types 4 or 6. Because of the small number of spores found inoculation tests were limited.

DISCUSSION

It is surprising that spores as large and as abundant as these have been reported by so few investigators. From observations both in Scotland and in Illinois it appears that mycorrhizal species of *Endogone* are a normal component of the soil microflora. The abundance of extra-matrical spores indicates that they are of greater importance as a source of infection than spores produced in sporocarps. Similar spores will probably be found in most soils but further examination of samples from different regions, habitats and soil types is desirable.

Spore type 1 is very similar to the chlamydospores obtained from Illinois soil and is probably the same species (Gerdemann, 1961). Similar spores collected in Illinois produced sporocarps in pot culture. More extensive inoculation studies are required to determine whether the Scottish type produces a similar sporocarpic stage.

Spore types 2, 3, and 4 would also appear to be chlamydospores. Their distinct morphology and apparent lack of intermediate types strongly suggest that they represent 3 distinct species. The black colour of spore type 4 distinguishes it from all described species of *Endogone* (Thaxter, 1922). Although two of these are definitely mycorrhizal, further inoculation studies are necessary to determine whether they produce sporocarpic stages.

Spore type 5 closely resembles the large-spored mycorrhizal species found in Illinois (Gerdemann, 1955) in its bulbous hyphal attachment,

germination, infection, and in the accessory spores (C spores) on the external hyphae. The spore found in Scotland differs mainly in its much smaller size, and although the C spores produced by the two species are analogous they differ considerably from each other. While these 2 species differ from previously described species of *Endogone*, their general appearance suggests a close affinity with the genus. It would seem desirable to formally describe and name them as soon as their life cycles are more fully understood.

Spore type 6 was the most unusual spore found and has been noted only in samples from one site. Further, it has never been extracted from the soil in large numbers. In two main features, namely the lateral position of the spore on its attachment and the presence of an associated empty vesicle, it is completely different from any of the other spores observed. Certain characteristics, such as size and contents, suggest a relationship with the Endogonaceae. However, a similar spore has not been described for any of the named species in this family (Thaxter, 1922; Hawker, 1954; Godfrey, 1957).

It is now apparent that a number of *Endogone* species form endotrophic mycorrhiza. The wet sieving and decanting technique provides a method for isolation and obtaining spores that can be utilized in a wide variety of investigations.

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