Ingoldian Fungi: some field and laboratory techniques

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Some preparatory techniques for the taxonomic, chorological and preliminary ecological study of Ingoldian fungi (i.e. fungi with conidial shapes adapted for anchorage in running waters) are discussed and detailed step by step; suggestions are also made for their improvement. Three introductory topics concerning field collection are first discussed, i.e.: criteria for selecting sites and timing sampling procedures, the recording of complementary field data and the planning of field trips involving long-distance air travel. The two main sections cover preliminary studies on species richness (by means of foam surveys), and preparatory techniques for description and herbarium preservation. The core of this contribution discusses pure culture: 1- isolation techniques, 2- recording colony characters, and 3- sporulation induction techniques. Isolation starts with asexual or sexual spores, either concentrated in stream foam or produced on natural substrates. Isolation may be done in two basic ways depending on spore size and abundance: 1- very large spores occurring infrequently are detected and identified under the dissecting microscope and lifted with the aid of mounted hairs or micropipettes; 2- spores of any size may be spread on an antibiotic medium, allowed to adhere to agar-based isolation media, identified under a compound microscope, relocated under the dissecting scioe with the aid of a finder slide and lifted manually. Anamorph induction is in contact with free water, i.e. in/on unchanged water (either standing, aerated or agitated), or in changed water (periodically or continuously) Alternatively, anamorph induction may be by means of moist incubation. Herbarium preservation relies mainly on microscope preparations, complemented by the use of preserved mycelial portions as well as of dried cultures.
Keywords: *Ingoldian Fungi, field techniques, laboratory techniques.*

FONGS INGOLDIANS: ALGUNES TÈCNIQUES DE CAMP I LABORATORI. Algunes tècniques preparatòries per a l'estudi taxonòmic, corològic i ecològic dels fongs ingoldians (fongs amb formes conidials adaptades a l'ancoratje en aigües corrents) són detallades i se suggereixen possibles millores d'aquestes. Se comenten tres temes introductoris: criteris per a la selecció de localitats i temporades de recol.lecció, el registre de dades de camp complementàries i la planificació de campanyes de recol.lecció incloent vols a llarga distància. Les dues seccions principals cobreixen l'estudi preliminar de la riquesa específica en aigües naturals (exploració a base d'escumes), i tècniques preparatòries per a la descripció en cultiu pur i herborització. Se discuteixen tres aspectes fonamentals del cultiu pur: 1-tècniques d'aïllament, 2- caracterització de colònies, 3- tècniques d'inducció de l'esporulació. L'aïllament se fa a partir de espores asexuals o sexuals, ja estiguin concentrades en escumes de rius o produïdes sobre substrats naturals. L'aïllament és de dos tipus depenent de la mida i abundància de les espores: 1- les espores de gran envergadura i poc freqüents són localitzades i identificades sota lupa binocular i aïllades manualment amb l'ajuda de cabells emmangats o micropipetes; i 2- les espores de qualsevol mida, un cop espargides sobre un medi antibiòtic, són incubades per induir la seva adherència sobre el medi; després són localitzades i identificades sota el microscopi compost, i tot seguit són relocalitzades sota lupa amb l'ajuda d'un porta localitzador i aïllades manualment. La inducció de l'anamorf se fa posant en contacte el miceli amb aigua, és a dir: en aigua estanca, amb o sense aireació o agitació, o bé amb canvi (periòdic o continu) de l'aigua. Alternativament se poden fer incubacions humides.

L'herborització se basa en: 1- col.leccions de preparacions microscòpiques, suplementades amb porcions de miceli esporulant guardades en conservant; i 2- colònies en cultiu pur dissecades.
Paraules clau: *fongs ingoldians, tècniques de camp, tècniques de laboratori.*

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I- Introduction

The Ingoldian fungi (Descals, 1978), inaccurately referred to as the "aquatic hyphomycetes" (Ingold, 1942), are a loose assemblage of fungi bearing relatively **large, modified conidia** found in **continental waters and humid habitats**. Such conidia are known to occur especially in the hyphomycetes, but can also belong to ascomycetes, basidiomycetes, coelomycetes and even amphibious Entomophthorales (although the latter will be left out of this contribution because they are insect parasites and require different techniques for their study). Ingoldian fungi typically live in or close to streams (less so in lentic habitats). Some may also colonize non-aquatic habitats (forest litter, plant canopies, etc...) and reproduce here as, anamorphs and/or teleomorphs. Most are known to be saprotrophic, but some may be plant parasites. Others may behave as endophytes in wood (Fisher & Petrini, 1989). The Ingoldian fungi are thus not a taxon nor an ecological group, but they do have in common what is believed to be a **morphological adaptation** of their **conidia** to **colony establishment** in fast-running waters (Webster, 1959).

Conidial morphology will in most cases allow us to use **a short-cut** approach to the study of these fungi, i.e.: instead of **identifying** fungi after **blind isolation** and culture of large numbers of them (as is generally done for example with soil and other aquatic fungi), Ingoldian conidia are typically **species-diagnostic**, i.e.: pure culture is needed only in some cases for **confirmation of identification to species. Selective isolation** of conidia recognizable in many cases to species greatly simplifies pure culture work, and the implications are discussed below.

By simply collecting and observing conidia in water or foam, or from submerged substrates, one can perform **chorological and ecological studies** on the Ingoldian component in stream communities (Bärlocher, 1992), i.e.: 1- **Seasonality of sporulation** can be estimated by identifying conidia trapped in foam or in water. 2- A number of **ecological parameters** can be correlated with conidial numbers in stream water. 3- **Saprotrophic relationships** between Ingoldian fungi and their substrates (e. g. substrate preference and decomposition rates) can be determined by recording sporulation levels on the substrates after controlled submersion (i.e. baiting) followed by *in vitro* induction of conidiation. 4- **Fungus-plant-invertebrate interactions** of various kinds may also be analyzed and quantified at the species level without culture (e.g.: Suberkropp *et al.*, 1983). (Some of the under 3 and 4 studies, however, may presume a not fully proven direct correlation between conidial numbers and mycelial abundance or activity).

The study of the Ingoldian fungi is becoming increasingly popular since their discovery by Ingold (1942). There are now well over a thousand publications on these fungi, and a steadily growing number of mycologists are recording and isolating them worldwide due to their relevance to stream ecology and, lately, for their possible pharmaceutical applications.

Techniques for their isolation, pure culture, *in vitro* conidiation and preservation are often unique to the group, but have so far not been compiled in detail. The main purpose here is a description of techniques which have been tested for a number of years and which may prove useful to other workers in the field. Many of those techniques have been learned or developed in Prof. J. Webster's laboratories at the University of Exeter (UK). A second aim is to highlight still unsatisfactory techniques and to suggest means for improving or replacing them.

It must be emphasized that many of the techniques discussed below are also applicable to hundreds of other aquatic as well as terrestrial fungi producing species-diagnostic conidia.

A-CRITERIA FOR SELECTING AND TIMING SAMPLING PROCEDURES

Current aspects of interest in the study of the Ingoldian fungi are: 1- description and classification of anamorphs, 2- studies on life cycles and teleomorph connections, 3- preliminary studies on species richness, biogeography and conservation, 4- ecology (substrate decomposition and invertebrate relationships) and 5- pharmaceutical and other industrial uses.

1- **Description and classification of anamorphs**: Hundreds of Ingoldian anamorphs await description, and a number of the close to **300 known** ones need redescription because morphological characters were not properly applied

in the past, because new characters based on ontogeny or even molecular aspects are being introduced, or because herbarium material is scanty, poorly preserved or altogether missing.

In some temperate streams, up to 80 or more taxa may be collected in a few drops of foam (e.g. Regelsberger *et al.*, 1987). Habitats in **warm climates**, however, have been much less studied. Foam is easier to find in **soft or neutral to mildly acid waters**, and possibly for this reason these have been more intensively explored than **hard, alkaline or saline streams**. But the latter may bear different mycotas. Undisturbed habitats rich in riparian plant species yield more Ingoldian fungi; but more extreme habitats, often with low plant species diversity, may support a significant number of undescribed fungi. Such is the case with acid moorlands.

2- **Life cycles**: Only about **27 teleomorphs** are known among the Ingoldian fungi (Webster 1992, Descals ined.), but it is believed that more will be found if we concentrate on isolating from sexual spores. For this purpose, one should search for streamside habitats with abundant and varied long-lasting **woody substrates** (and possibly sometimes decomposing leaves) where the teleomorphs can complete their development. This takes place in most cases out of water. In cold and temperate climates, teleomorphs seem to be more abundant in the warmer seasons.

3- Biodiversity and conservation: These are hardly explored fields, but Ingoldian fungi may eventually have an **indicator value** for monitoring the effects of Mankind and/or of environmental (e.g. climatic) changes on freshwater habitats. Either **endangered** or **well-preserved habitats** could be the chosen object of study in order to justify their preservation.

If conidial abundance and species diversity are the aims for studying any of the three aspects discussed above, the **ideal collecting sites** are small streams flowing over rocky beds (in mountainous or hilly areas), with foam accumulation, upstream from any source of organic urban and agricultural waste. The catchment area should have a rich and varied, undisturbed native vegetation. In temperate climates, **deciduous angiospermous trees** are richer sources than conifers, although these usually bear characteristic mycota. A good clue to adequate collecting areas is the presence of trout and salmon **angling sites** along streams.

Conidial abundance is normally associated with availability of decaying submerged substrates, such as fallen leaves, and therefore the most productive collecting season in streams flowing through deciduous woods in cold and temperate climates tends to be in the **autumn**, although minor peaks may occur at other times. Very few critical seasonality studies have been carried out in streams flowing exclusively through conifers or in tropical latitudes, where leaf fall patterns are less marked.

Casual observations suggest that conidial numbers in stream foams rise drastically after heavy showers, as much riverbank litter (and to a lesser degree grassland) is also colonized by Ingoldian fungi (Webster, 1977). Their conidia may be produced aerially but dispersed in flood waters.

4- **Ecology**: The relevance of higher fungi capable of underwater substrate colonization (among which are the Ingoldian fungi) and as intermediaries in energy and food webs associated with running waters is well documented

(Bärlocher, 1992). For such studies, the choice of site is subject to selected experimental criteria rather than those based on fungal species abundance or variety.

If the goal is to discover new plant substrates for particular species one should obviously collect in first-order streams, not far from the source of the conidia.

5- **Pharmaceutical and other industrial uses**: Metabolites produced by the Ingoldian fungi are being studied at the present by the industry, mainly because these fungi are still relatively unknown. By now, most known species from temperate habitats have been screened. However, large culture collections are not often maintained; and, as techniques and target substances in the search of bioactive compounds keep changing, it is likely that well-known sites will have to be repeatedly tapped in the future. The choice of sites here depends on the specific needs of the industry.

B- COMPLEMENTARY FIELD DATA

These are geographical, climatological, vegetational and physio-chemical parameters (Table 1) which should be recorded for: 1- a more complete characterization of fungal species, and 2- a better understanding of the environmental conditions needed for *in vitro* reproduction.

Other valuable information may be obtained from land survey maps (1: 25,000 to 1: 50,000, and including vegetation types) as well as from yearly public records kept for larger river basins, such as patterns of rainfall, water temperature, chemical parameters and flow rate.

C- SUGGESTIONS FOR FIELD TRIPS INVOLVING LONG-DISTANCE AIR TRAVEL

Due to the specialized equipment needed, which is often not available at destination, long-distance air travel presents special problems for mycologists wanting to isolate Ingoldian fungi. What little experience has been gained by the author from such trips may be of use to some readers.

Ideally one should plan **cooperative work** with a local mycologist, who may have much to contribute; e.g.: suggesting collecting sites, arranging for transport and accomodation, translating, providing necessary basic gear, etc. If time allows, such trips are also an excellent opportunity for training local postgraduate students.

The equipment and material must be carefully selected and packed. If time is a limiting factor, or if working conditions are inadequate, one should aim for **self-sufficiency**. **Media**, for example, should be prepared prior to travelling.

-**Hand luggage** should be reserved for the more delicate and expensive

Table 1. Complementary field data.
Taula 1. Dades de camp complementàries.

water temperature	riparian vegetation on site
" conductivity	" " upstream
" pH	altitude
flow characteristics	latitude
rock type	longitude
" pH	name of stream

compound and dissecting microscope equipment (e.g. nosepieces, objectives, condenser lenses, eyepieces, filters), bulbs and cameras, as well as for cultures on the return trip.

-**Flammable items** such as alcohol, propane cartridges, etc., are not usually allowed on airplanes.

-**Agar media should not be exposed to freezing**. Large, modern airplanes normally have temperature-controlled luggage compartments, and poured agar media can be packed with the check-in luggage. But this may not be so in smaller aircraft on inland flights.

-For **check-in luggage**, canvas travelbags with an additional thick, polystyrene foam lining should be more shock-proof than hard-walled, Samsonite-type suitcases. Water tightness is a further aspect to consider.

II- PRELIMINARY STUDIES ON SPECIES RICHNESS: FOAM SURVEYS

An idea of **species richness** of waterborne fungi may be obtained by examining natural foams, although the technique is not quantitative, as Ingoldian conidia are trapped with higher efficiency than others (Webster, 1959; Iqbal & Webster, 1973a) and information on foam trapping dynamics is lacking. Foam surveys fulfil an added useful purpose: **selecting sites and seasons** for later isolation of interesting species.

RECOMMENDED GEAR

-**Foam jars**: wide-mouthed (ca. 5 cm diam.) screw-capped jars. Plastic is preferred for lightness and strength, but conidia may adhere less to glass.

-A **spoon** or kitchen **skimmer**.

-**Extensible rod** for foam jars: foam is sometimes in awkward spots out of arm's reach. It is relatively easy to fit a ring at the end of a rod, onto which one may screw a foam jar. The ring may just be a perforated lid of a same-sized jar.

-An **inflatable boat** may be handy for collecting foam in larger bodies of water.

-**Fixative** for foam. A few drops of 4% formalin or 90% methyl alcohol appear to be adequate for short-term preservation, and formalin-acetic-alcohol (FAA, Anon. 1968) may be used if storage is for longer periods. The fixative should not affect later treatments in slide preparation.

-**Waders**: thigh-high gum boots. A repair kit for punctures should be kept handy.

-**Rucksack** with general purpose gear (see below).

-A couple of **buckets**.

-A **container** (e.g. a large tin can), with the bottom cut off.

-**Field notebook**.

PROCEDURE FOR FOAM SAMPLING

-**Label jars** with a felt pen before wetting.

-Look for clean, thick **scum** below waterfalls, along rapids, on the downstream side of any obstacles (e.g. boulders and woodpiles), along lakeshores where drift collects through wave and wind action, etc.

Foam that breaks down quickly in the jar will have trapped few conidia. Muddy scums harbour too many bacterial contaminants and debris.

-**Scoop up** the foam with the jar itself or with the lid or spoon, and **IMMEDIATELY DECANT ALL EXCESS WATER**, as the conidial concentration in water is much lower than in foam and this will have a strong diluting effect.

Foam is often scarce (especially when submerged leaves are not abundant) and several subsamples per site may be needed. Some of the foam will

have liquefied between scoops, and when decanting, spores in this water from previous scoops will be lost. Therefore use a **fresh jar for every scoop** and compound the samples after the last scoop for the site.

For collecting foam along lakeshores, where it is often scanty and trapped among pebbles and vegetation, one may slightly dig an open-ended can into the ground and then pour some lake water into the can. This will raise the water level long enough for the foam to be collected. The use of an inverted funnel may even help concentrate the foam within.

-**Do not liquefy the foam** (by shaking the jar) if it is meant for spore isolation.

When you have returned to the vehicle:

-**Fix some foam** in a labelled vial as a voucher specimen.

-**Note** the collection number and complementary field data in the field notebook.

PREPARING SPORE DEPOSITS ON MICROSCOPE SLIDES

MATERIALS

-**Mountant: conc. lactic acid with some acid fuchsin** (with or without phenol) are of standard use. However, semipermanent mountants may cause a significant loss of optical resolution. This is especially noticeable with differential interference contrast (DIC) optics. It is therefore preferable to do all the descriptive work from temporary **water mounts**, and only add chemicals afterwards for preservation. On the other hand, if one is doing large batches time is short, and there may be no alternative but to first fix the specimens.

The use of phenol is discouraged by safety regulations in some countries as it is carcinogenic, but contaminant fungi seem to be able to grow in lactic acid within microscope preparations, and a general biocide will have to be added.

Cotton blue is claimed to often result in serious crystal precipitation.

The synthetic resins **DPX 8711** (Difco) or Merckoglas (Merck code 11-20 UN-1866/3.2 IMDG WGK2) have not proven satisfactory in recent trials, as the spores are severely distorted and standard stains are not readily mixed. (Although a stain may not be needed for phase or DIC optics). PVA is currently being used in certain laboratories for mounting myxomycete and other spores (Pando, pers. comm.), and may be worth trying.

-**Sealant**:

Semipermanent liquid mounts need to be sealed, but there is so far no satisfactory sealant in the market. The commercial resin "Glyceel", which had become of widespread use (but no longer produced) will eventually shrink, and cracks appear precisely at the margin of the coverslip, allowing the evaporation of the mountant. The preparation will then need repairing, which is time-consuming and often damages the contents. To correct this, Gams (ined., Ananet Newsletters 10:3, 17:7, accessible through Internet) suggests applying a second layer of Glyceel soon after the first. But if this is done too late, it will set unevenly and wrinkle severely.

It is claimed that more elastic nail polish brands containing nylon are more satisfactory.

Volkmann-Kohlmeyer & Kohlmeyer (1996, and *in litt.*) recommend the preparation of permanent slides by means of a "double-coverglass" technique, which might be adapted for Ingoldian fungi.

-Large **coverslips** (22x22, or 20x20 mm), grease-free **slides** in slide boxes and **slide labels**.

PROCEDURE FOR PREPARING SPORE DEPOSITS

-**Air-dry** 3-4 drops of foam placed on the centre of a slide. This may be done in the laboratory, where gentle heat may be applied for faster drying (e. g. under a table lamp or with a fan-heater or hot plate).

-Drying **a known volume** of liquefied foam on each slide may allow the information to be at least partially comparable with other foam samples.

-If the stream water is alkaline or saline, **salt crystallization** will take place, and when an acid mountant is added (e.g. lactic acid), alkaline deposits will release abundant CO_2 under the coverslip, spoiling the preparation.

In this case, add a drop of the mountant to the deposit and heat gently to release the gas before covering. Alternatively, the water in the spore suspension may be acidified prior to mounting. Another option may be to test Waterman's ink diluted to approx. 1/10 (an aqueous solution used by lichenologists for observing asci, and successfully used by us for staining conidia on membrane filters). This would not have an acid reaction. The preparation would not be permanent, however.

-For a very thin preparation, and also to reduce gas release, **remove the larger debris** (sand grains, organic matter, insect parts, etc.) with a needle or forceps under the dissecting microscope prior to applying the coverslip.

-Add a small drop of **mountant** to a coverslip.

The drop is not placed on the slide to avoid touching any fungal material (including spores in foam) with the tip of the dropper because this might get contaminated and transfer the spores to slides with other samples.

-**Invert the coverslip and, to avoid trapping air, rest one side** on the preparation with the aid of a needle and slowly lower over the dried spot.

-**Heat** gently over the Bunsen burner and, if necessary, apply slight **pressure** with a needle to release trapped gas bubbles.

-**Seal** and **label** the slide, **noting** down the collection code, date and mountant used. The latter record will be useful for reflooding if the slide needs repairing.

LIQUEFYING FOAM

If an even spore concentration is needed in the foam, for example for quantitative studies, or for spreading conidia on agar media for isolation, liquefy it by:

-**Shaking** the closed foam jar violently for a few times.

Shaking is not always effective, especially with some very thick scums. Gentle **heat** (applied close to the foam, for example with a hot metal) is effective but it may be detrimental to spore viability. Applying alcohol, or freezing, as well as anti-foaming agents, may be worth testing.

ARTIFICIAL FOAM

If stream foam cannot be readily found, it may be obtained artificially by **reducing the surface tension** of stream water with commercial detergents or wetting agents (e.g. Tween-80), resulting in foam production. A technique involving digging up a small reservoir under a waterfall is detailed in Iqbal (1983; 1995).

An alternative (but only briefly tested) way of concentrating spores in artificial foam would be the following:

Sample a known volume of stream water in a bucket, add a drop of dil. Tween-80 to a second bucket, pour the water into this from a certain height (to create turbulence and hence foam), and then back into the first. Do this several times with the same water, scooping up the foam as it builds up and placing it in a jar, where it may then be processed (either for isolation or for preservation).

If the number of conidia of specific Ingoldian species trapped in the artificial foam in relation to that in the sample of stream water is proven to be constant, the above technique might allow for quantification.

Preliminary trials carried out in this laboratory by A. Díaz (unpubl.) on conidia from pure culture suggest that Tween- 80 does not affect viability.

III- PREPARATORY TECHNIQUES FOR FUNGAL IDENTIFICATION, DESCRIPTION AND PRESERVATION

The source of the material to be studied may be: a- fungi naturally colonizing and sporulating on the substrates, either as conidiogenous structures or as free conidia in water or foam; b- the same fungi, but after having induced their sporulation in the laboratory; c- fungi grown on artificial media but induced to sporulate either in the field (never done) or under controlled conditions in the laboratory.

III-A: STUDYING FIELD MATERIAL

Especially when describing new species, one should test that the morphology of conidiogenous structures and of free conidia produced in the laboratory (cases 2 and 3 above) is not different from that in nature. This may be done by **fixing field material immediately after sampling**, comparing fungal morphology with that produced in the laboratory, and, if differences are significant, illustrating and preparing voucher specimens.

Conidiophores are normally produced over the entire surface of leaves or wood, but these are usually opaque, and leaf clearing techniques, for example, are too severe on such delicate fungal material. Conidiophores must then be seen along the leaf margins and on the sides of woody tissues and petioles, or by examining **scrapings** mounted on slides. (Preparation of free conidia, e.g. in foam, has already been discussed).

There is a need for developing an efficient, preferably quantitative microtechnique for sampling conidiophores from natural substrates. Collodion peels have proven successful with plant material and are worth testing.

III-B: LABORATORY SPORULATION OF FUNGI ON FIELD MATERIAL

-**Rinse** field material in abundant water.

Tapwater will have fungal contaminants (occasionally even Ingoldian conidia) but may be used for short incubations if the aim is induction of conidiogenous structures.

The basic steps for submerged incubation are explained under anamorph induction below.

So far, natural substrates have been submerged in standing or aerated, often unchanged distilled water (DW). These conditions may not be ideal, as there is presumably a buildup of staling compounds interrupting or distorting sporulation processes. More natural continuous water flow or drip systems such as those applied to pure culture (see below) are now being tested.

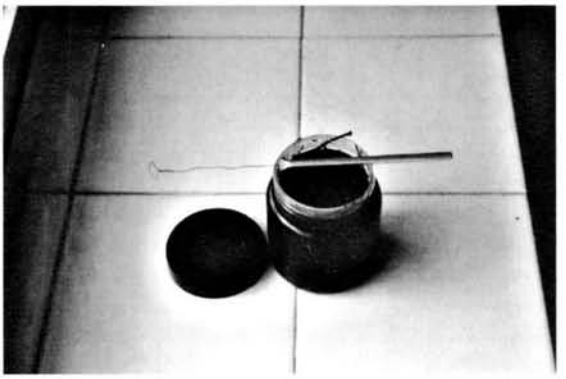

Fig. 1. Harvesting surface spores from moist-incubated substrates: the container is slowly flooded with distilled water, and the water surface touched with a broad loop, which may then be spread on a slide or on antibiotic agar.
Fig. 1. Recol·lecció d'espores superficials a partir de substrats incubats amb humitat; l'aigua destil.lada es deixa entrar en el recipient lentament i per la superfície de l'aigua es passa una ansa de sembra ampla que després es frega sobre una preparació d'agar antibiòtic.

Field material (as well as pure cultures) should **always** be subjected to **moist incubation** too, as not all Ingoldian fungi are aquatic sporulators (Fig. 1).

1- **STANDING WATER** in Petri dishes:

-Use a **low substrate/water ratio** because of rapid build-up of bacterial and yeast populations, as well as of staling compounds from both fungi and bacteria. For example, place one small leaf, or portions of a larger one, in a 9 cm diam. Petri dish containing 30 ml water.

-**Filtered stream water** may in some cases induce more sporulation than DW, and should be tested.

-**Changing the water** regularly should increase spore production and/or lengthen the sporulation phase.

Incubation in water containing antibacterial antibiotic solutions has not been tested, but may improve sporulation.

-**Cool temperatures** around 15°C for cold-temperate species should be used to slow down bacterial growth.

-Incubating in **glass** Petri dishes may reduce the number of conidia adhering to the dish walls, but does not allow for near- ultraviolet (NUV) irradiation, unless the glass is Pyrex.

The effect of NUV on fungal sporulation on natural substrates has not been critically tested.

-**Check** for sporulation after one day, and then every 2-3 days for at least 10 days.

The maximum time for incubation to allow all resident species to sporulate under the above conditions is not known, but certainly two days, as reported in many publications, may not be sufficient.

AERATED WATER:

-**Place substrate** in a glass container, e.g. a measuring cylinder or conical flask.

-**Add DW** without filling up (as turbulence may cause some spilling).

-**Force air** into the system, to just create gentle mixing of the water. An aquarium pump system such as described under pure culture, but ignoring precautions for sterile technique, is suitable (Fig. 2).

Excessive bubbling will thrust spores out of the suspension and these will be lost into the air or remain tapped on the walls.

-**Incubate** and check for sporulation as for standing water.

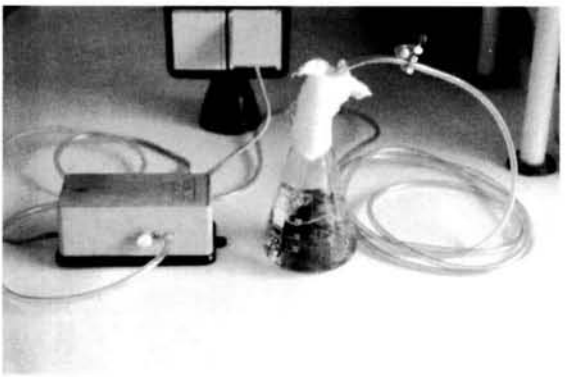

Fig. 2. Induction of conidiation on leaves with aerated water: air is bubbled from an aquarium pump into water containing leaves in a 250 ml conical flask. Air flow is regulated with a wheel clamp.

Fig. 2. Inducció de conidiació sobre fulles vegetals amb aigua orejada, l'aire és pompeja des d'una pompa d'aquari fins a un matrau cònic de 250 ml, on hi ha fulles vegetals amb aigua. El flux de l'aigua es controla amb una grapa.

-For **concentrating** conidia, artificial foam may be induced (see above).

III-C: **PURE CULTURE STUDIES**

For the taxonomist, pure culture is needed for: 1- characterizing the morph which has been isolated, 2- providing irrefutable proof of pleomorphism (i.e. a genetic connection between the anamorph isolated and the resultant teleomorph and/or synanamorphs), 3- studying vegetative characters (otherwise mostly concealed in the colonized substrate), 4- increasing the number of diagnostic characters available for study, some of which may even be artefactual (e.g. colony zonations, aerial mycelium, pigmentation, etc.); 5- increasing the material available for identification, description and preservation; 6- studying any other characters, e.g. genetic, etc.

Pure culture may be broken down into the following activities:

a- **Field work**

1- collection of fungal specimens, substrates and/or water

2- preparation for isolation (if the finder slide technique explained below is used)

3- initial steps in preservation of voucher specimens from nature.

b- Work to take place in the improvised **field laboratory** (when on major collecting trips):

1- isolation into pure culture

2- initial description, especially recording characters (e.g. through microphotography) that may be lost in later processing

3- further preservation of voucher specimens from nature.

c- Work to take place in the **main laboratory:**

1-emulation of field conditions for inducing:

a-spore germination and vegetative growth

b-the anamorph(s)

c-the teleomorph

2-observing and describing the above events

3-preservation of voucher specimens (from pure culture).

FIELDWORK AIMED AT PURE CULTURE: SAMPLE COLLECTION

Field samples may be of three possible types:

1- **the fungi** or parts thereof: these can be **propagules** (i.e. conidia in water or foam) or the **thallus** itself, e.g.: conidiogenous structures on, or mycelium in/on the substrate;

2- the presumed **natural substrates**, which may be used for pre-

paring natural media or for chemical analyses;

3- the medium in or on which fungi sporulate in nature, i.e.: **water.** This may be needed for e.g. chemical analyses, or for replacing DW in media preparation or as an *in vitro* sporulation medium.

Soon after collecting, stream water should be filtered through 0.2 - 0.45 µm pore membranes: 1- to eliminate propagules which would mix with those produced later in pure culture, and 2- to stop microbially induced chemical changes (which could presumably affect sporulation). Autoclaving often causes serious salt precipitation, and hence an alteration in the chemical composition of water. Membrane filtration is therefore preferred, although it is slower and costlier.

SOURCES OF INGOLDIAN FUNGI

1- **conidia or sexual spores in foam** (see foam surveys, above)

2- **conidia or sexual spores in water**

Although this has not been done, water samples could presumably be directly plated onto isolation media if spore loads are in the many thousands per liter. The technique would allow for quantitative sampling.

3- **conidia on plant substrates.** The conidiophores may already be present on the substrates at collection, but usually not in large quantities, due to invertebrate grazing and/or possibly to some form of chemical inhibition. However, a short but spectacular outburst of sporulation is typically observed after laboratory submersion for one to a few days.

It is not known if this artefactual sporulation peak also occurs in the case of moist incubation.

Plant substrates are particularly useful: 1- for detecting fungi producing few conidia, as these would be too highly diluted in the stream and would not appear often enough in foam; 2- because, conidiogenous structures being present, species identification can be more accurate.

There is normally not enough time during collecting trips for incubation of natural substrates, and this will have to be done in the main laboratory. The material may therefore have to be stored for several days, or even weeks, and it is not known to what degree transport conditions affect sporulation. Yeast and bacterial populations (and probably also other fungi) are certainly known to build up quickly, interfering with isolation.

The effect of freezing or drying of plant substrates during transport on subsequent sporulation has not been tested.

4- **the thallus in plant tissues.** Substrates may be decomposing (the fungi acting as saprotrophs on leaves, twigs, dead tips of trailing submerged vegetation, as in *Juncus* and grasses, etc.), or alive (the fungi acting as parasites or endophytes, as mentioned above).

Plating out plant matter from streams onto isolation media (i.e.: **particle plating** techniques) will yield colonies of Ingoldian as well as of other fungi, both of which may be active underwater (see Bärlocher & Kendrick, 1974; Cooke, 1974; Schoenlein-Crusius & Milanez, 1995). If the sampling procedure is quantitative and surface sterilization does not damage superficial mycelia, one may obtain a more accurate picture of the species composition and biomass of the submerged saprotroph community by particle plating than by any of the spore-collecting techniques. A critical comparison of these two approaches has not yet been made, but, as in any other sampling procedure for fungi, it is probably necessary to combine both.

Particle plating is a well-known general sampling technique (see for example Kirby, 1987) and will not be dealt with here, except remind one that the isolation media should be dilute and supplemented with broad-spectrum antibacterial antibiotics, and that incubations should be in cool temperatures.

5- **the thallus in FPOM** (fine particulate organic matter) suspended in water. It is most probable that Ingoldian fungi are also dispersed as minute mycelia in FPOM, but isolation might not prove easy as surface sterilization is not possible and there might be too strong competition from faster-growing mycelia or spores of contaminant fungi.

ISOLATING FROM SPORES: PLANNING THE TRIP AND BASIC FACILITIES AND EQUIPMENT

-Vehicle: a 4-wheel drive may be necessary for fieldwork on unpaved roads. Otherwise, a vehicle with softer suspension is preferred for transporting delicate laboratory equipment. This should rest on a spring-loaded seat or other cushioned surface, and not directly on the floor.

It seems most efficient to select a base (a hotel room or field centre) with several streams within roughly an hour's drive, and work in that area for at least 3 days, in order to secure interesting isolates.

If time is limited long walks should be avoided, and collecting routes on mountainous areas should be planned on roads running more or less along contour lines crossing various streams.

-The **field laboratory** should be kept cool to check contaminations from airborne fungi. In warm weather, a **portable air conditioner** might be useful. The room should be draught- and dust-free. In small rooms contaminations may be a serious problem.

-The design of a portable, collapsible horizontal **air flow cabinet** could substantially reduce the levels of aerial contaminants.

-A **solid table**, with a minimum of ca. 2 sq. m surface, is advisable to avoid water rocking in Petri dishes, if isolation is done from spore suspensions.

-A **dissecting microscope** reaching 100x magnification (e.g. with 20x eyepieces) with transmitted light and preferably an adjustable substage mirror, is necessary for manual spore isolation.

The **adjustable mirror** can create a partial dark field against which the illuminated, mostly hyaline, conidia stand out.

If the transmitted light base does not have an adjustable mirror, or if one wants to save weight when travelling by air, it is easy to improvise a base with an inverted plastic box (e.g. a rigid lunch or biscuit box) containing a small mirror fixed on a sled. The light may be projected onto the mirror from a table lamp or even from a battery-operated torch behind the box.

Supplementary high-power objectives are commercially available for dissecting microscopes (e.g. Leica). But the working distance for magnifications above 200x is uncomfortably short for micromanipulation.

-A routine **compound microscope** with mechanical stage, bright field optics and 10, 20 and 40x objectives, preferably with a built-in transformer to save space. The nosepiece should preferably come off for transport in hand luggage. Remember to take **extra bulbs**. Some microscopes have an extra adjustable mirror at the base which may be directed at an external source of light, in case of power failure.

Ideally one should always carry microphotographic equipment and a drawing tube on field trips, where fresh specimens will be available from nature.

But costlier, more delicate microscopes would then be needed. Microscopic and electronic equipment should in any case be adequately **insured** for travel.

-A portable **ice chest** (or cool box) (ca. 50x30x40 cm), preferably with a draining tap, and **crushed ice**. The ice chest should be sufficiently insulated so that ice can last in it for 24-36 h, in case of overnight travel. Ice blocks may be crushed by hitting them in a canvas sack against a hard floor. Alternatively one may use commercially available freezer packs.

-A **water-tight container** in the ice chest: 1- to protect **"foam plates"** (i.e. Petri dishes in which foam is spread for spore isolation) from being flooded in melting ice; and 2- to avoid direct contact of the ice with the agar in the Petri dishes. Frozen agar does not reconstitute, and the foam sample would thus be ruined. A **max./min. thermometer** in the ice chest is advisable.

There is no experience on low-temperature storage techniques for foam, but it has been possible to isolate viable conidia from icicles in streams. These had formed by slow freezing as air temperatures dropped through the night.

-A **portable refrigerator** (gas -or mains and car battery- operated) may be necessary on long trips if lodging conditions are primitive and ice is not available.

-A generous supply of media slopes poured in disposable sterile vials (e.g. Sarstedt Corp., Nürnbrecht, Germany, Code 55.526.006), where cultures can be kept until one returns to the main laboratory. These isolates can survive in cold storage for months if necessary, although it is not known how this affects sporulating capacity and metabolite production for possible industrial (e.g. pharmaceutical) applications. Slopes are prepared with an **agar dispenser**.

Slopes may be neatly arranged in portfolio-type folders lined with thin foam. This will save much time in sorting them out when isolating, and also reduces chances of mis-labelling vials.

If the purpose of the trip is large-scale non-selective isolation and foam samples are rich and varied, a 2-week excursion involving ca. 6-8 h daily culture work could easily yield ca. 500 cultures per person. For highest efficiency there should be two people alternating field and laboratory work during daylight, and, if the days are short, both should be fully equipped for joint laboratory work at night.

If space or weight are not a limiting factor, one can save time by subculturing directly onto Petri dishes instead of vials. In this case one should take extra precautions to reduce aerial contaminations, which are much more likely than with vials.

GENERAL ISOLATION MEDIA

An agar-based isolation medium facilitates **spore attachment** onto its surface, which is believed to be a prerequisite for germination. Because transmitted light is needed, the medium should also be clear. Some cheaper brands of agar may contain much particulate matter and need filtration, or they may set poorly. Although malt extract seems to supply all the growth factors needed for most known Ingoldian fungi, the choice of isolation media and of their concentrations has not yet been carefully tested. A significant percentage of conidia do not germinate when plated on 0.1% MA (malt extract agar) with standard antibiotics. This may be due to ageing, an inadequate nutrient or vitamin supply and/or antibiotic toxicity. The addition of some yeast extract may provide a richer vitamin complement for germination. This would have to be added after autoclaving, or else filter-sterilized.

-**Antibacterial antibiotics** are not always necessary. Ingold, for instance, never used them. But, if added to the isolation medium, one need not clean the isolates from bacteria as frequently or thoroughly, and one can also work with dirtier sources (e.g. foams, or spore suspensions from longer-standing leaf or wood incubations), or incubate the isolation plates for longer periods. The antibiotics normally used are cheap, readily obtained and conveniently added to the media. Sometimes, however, bacterial colonies will spread rapidly and can be difficult to control, even with standard antibiotics. In this case, a concentrated solution of the same antibiotic, or of a different one, could presumably be poured over the spore spread on the isolation medium.

The following medium proved satisfactory for many years, both in temperate as well as tropical latitudes: one million IU **penicillin** plus 1 g **streptomycin sulphate** in 1 litre of warm **0.1% MA** after autoclaving. The medium should then be swirled, but without too much agitation, as this forms bubbles which will leave craters on the agar plates after setting.

-One g **chloramphenicol** added to 1 liter 0.1% MA **before this is autoclaved**, is simpler to use and seems to be equally effective, although, as with streptomycin, it is nowadays difficult to find in some countries due to its health hazard.

-**9 cm diam. polystyrene Petri dishes** have always been used by us, but some mycologists might prefer 5 cm dishes.

-Prepare the isolation plates a few days before travelling (or in any case before starting isolations) for two reasons:

1- the agar medium will have dried up slightly, thus absorbing more rapidly the water drops added at isolation (see below) than if these are placed on freshly poured media.

(It is not known how changes in the osmotic potential of drying agar media may affect spore germinability).

2-contaminations, either due to insufficient autoclaving or from air spora at pouring, can be detected in time and fresh plates prepared.

Label and stack the poured dishes in closed polythene bags.

-If, due to an accident, fresh antibiotic media have to be prepared during the field trip, it is advisable to take along:

-**extra 0.1% MA** in test tubes, or better still in flat-based screw- capped bottles (10-20 ml per bottle), (e.g. McCartney bottles).

-a **pan** for melting the media in boiling water,

-a **source of heat** (e.g. a camping stove, or a small electric cooker),

-spare sterile **polystyrene Petri dishes**,

-a concentrated solution of the **antibiotic(s)**, e.g. 1 g chloramphenicol in 100 ml water (see below),

-spare antibiotics (as powder). Pack them as exact doses rather than in larger containers, because it may not be possible to weigh with sterile technique,

-sterile plastic **10-ml pipettes** graduated into 1 ml sections.

-Some sterile distilled water (**SDW**) in McCartney bottles as well as in 100 ml bottles may be needed for various other purposes (e.g. rinsing mounted hairs and micropipettes, rinsing foam plates prior to scanning with the finder, making up fresh antibiotic concentrate, hydrating media, etc...).

The **procedure** is as follows:

Prior to the field trip prepare the antibiotic concentrate:

-Autoclave 100 ml DW (distilled water) in a glass bottle (with autoclavable screw-cap).

-Add the antibiotic(s) (full strength) with sterile technique into this water.

-Keep the antibiotic concentrate in cold storage.

Penicillin and streptomycin will keep at least for several months in solution. However, after long storage always check for presence of contaminant mycelia before using.

-When needed:

-Melt the media in boiling water in the pan. (Remember to first loosen the screw caps slightly to avoid explosions, or the sucking in of the rubber seals at cooling).

-Pipette 1 ml aliquots of the concentrate using sterile technique into the polystyrene Petri dishes.

-Add 10 ml 0.1% MA to each dish (after slight cooling).

-Immediately mix the antibiotic solution with the medium by slightly tilting each plate clockwise and anticlockwise a few times.

-Allow the medium to set on a horizontal surface.

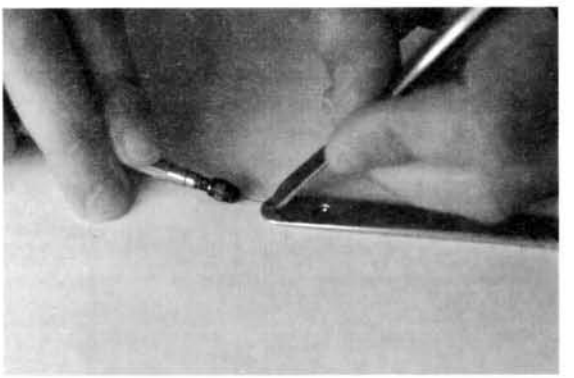

Fig. 3. Technique for sharpening the microscalpel: the needle is laid on a handle and the tip scraped downwards with the edge of another handle.

Fig. 3. Tècnica per afilar el microescarpell: l'agulla es posa sobre el mànec d'una espàtula i el seu extrem fregat suaument amb la vorera d'un altre mànec.

INSTRUMENTS FOR MICROTECHNIQUE

-**Microscalpel**:

Use a **fine insect pin** (size 00). Cut or burn off the head. Secure the base of the pin onto a handle.

Flatten the tip of the pin with a small hammer. Alternatively, lean the tip at a 30º angle on a metal surface (e. g. the flat face of the handle of a forceps or scalpel) (Fig. 3). With the edge of another metal tool (e.g. any metal handle) scrape the tip of the needle firmly against the base and downwards for several times. Check under the dissecting microscope. Wipe off metal bits and, with forceps, slightly arch the distal end of the microscalpel to facilitate scooping up the agar blocks with the spores.

Flame the tip of the microscalpel between manipulations, but only for an instant to reduce heat corrosion.

Scrape the microscalpel every now and then to remove rust and to re-flatten the surface. Do this under the dissecting scope to ensure that the tip lies flat on the surface.

-Fine **watchmakers' forceps**.

-A mounted **needle**.

-A standard broad-bladed **scalpel** (with spare blades).

-A mounted **surgical scalpel** (with spare blades).

-**Table cloths**, **towelling** and **tissue** for benchwork.

SPORE ISOLATION TECHNIQUES: INTRODUCTION

This section is mostly applicable to conidia, as sexual spores are seldom

recognizable to species. Conidia are by far the main constituents of the Ingoldian spora in water or foam, although sigmoid ascospores, for example of some discomycetes, may also be present.

It is presumed that the Ingoldian fungi may be found in the following situations, which will have a bearing on the isolation technique(s) adopted (unproven cases are queried):

Conidiophores, in relation to water, may be:

1- **aerial** (in humid air, on partly submerged substrates), and/or

2- **aquatic** (in contact with free water), and then

a- **superficial** (e.g. the *five-armed* morphotype of *Articulospora tetracladia* Ingold), and/or

b- **submerged**.

Surface conidiophores release their conidia on water, and these mostly remain superficial. Bandoni (1974) discussed the dynamics of spore dispersal on surface films. Spore surface properties may affect the sampling technique. For example, when pouring spore suspensions, surface spores may flow backwards and thus be left behind.

Depending on where the substrate is in relation to water, conidiophores may form:

1-on **aerial** plant parts which may be:
living (phylloplane fungi, e.g. *Tripospermum myrti*)
dead (e.g. saprotrophs in trunk holes, forest litter, etc.)

2-on **submerged** plant parts, which may be
2a-in **open water**
living? (submerged roots of *Alnus*)
dead (decaying leaves, twigs, etc.)
2b-in **hyporrheic habitats?**
living? (roots)
dead? (buried decaying plant matter)

The dispersal of Ingoldian conidia produced in hyporrheic habitats, i.e. those presumably produced on underground dead or living plant matter and dispersed in the liquid phase of soils underlying freshwater bodies, has not been studied.

Conidia will be passively dispersed in contact with water:

A- from **aerial conidiophores**, whose conidia will be:

1- **introduced into water bodies** by rain splash, dew drops, etc., and either
a- remain on the surface or
b- enter into suspension

2- **dispersed aerially** in/on spray droplets caused by wind, and either:
a- land on **aerial plant surfaces** (and germinate?) or
b- land on **water**

B- from **surface conidiophores**, and then:
1- remain on the **surface**
2- enter into **suspension**
-(and possibly **re-surface** by turbulence)

C- from **submerged conidiophores**, from where they may:
1- remain **underwater** and:
a- **settle**, become anchored and start germination
b- remain **suspended** for longer periods (and eventually settle and germinate)

2- **re-surface** through turbulence, and then:
a- remain on the **surface** or
b- be dispersed **aerially** in spray droplets

Conidia of Ingoldian fungi may **adhere** readily to walls and instruments, and should thus be manipulated as little as possible. This adhesiveness is especially noticeable when suspensions (e.g. liquefied foam) are examined a couple of hours after being kept at room tem-

perature. Large tangled masses of conidia mixed with debris will have formed, and recognition, let alone isolation, become practically impossible. It is not known whether such a flocculation process intervenes in natural freshwater purification.

PROCEDURE FOR ISOLATION

Spores dispersed in water (as those in air, but unlike those in soil) normally need to be **CONCENTRATED** for efficient observation, description, counting or isolation. Natural spore accumulations in foams and scums are especially abundant in turbulent waters with a presumably lower than normal surface tension. High concentrations may also be obtained by laboratory incubation of submerged substrates. Otherwise, spores have to be concentrated artificially (see p. 175).

Spores may be **ISOLATED** in two basic ways: as spores **SUSPENDED** in water, or as spores previously spread and starting to germinate (**ANCHORED**) on agar. In the first case (efficient for species producing **FEW, LARGE** spores, such as conidia of *Actinospora megalospora* Ingold) these are detected and identified under the dissecting microscope, which has a poor optical resolution but covers a broad field of view. With the second method (much more efficient for smaller spores and also for large ones if these are more frequent) the spore suspension is placed on an agar medium and the spores detected and identified with the higher-resolution optics but small field of view of the compound microscope.

Both techniques have the limitation that the spore has to then be **manually removed** and, if necessary, also **manually cleaned of contaminants.** This is normally done under the dissecting microscope.

A third limitation is that neither observing spores on or in water, nor on the surface of agar, whether with the dissecting or compound microscope, gives the degree of resolution obtained in a water mount on a slide (unless a water immersion lens is used in the anchored-spore technique, as explained below). A number of conidia may thus still be misidentified at the isolation stage, and we therefore need to confirm identification after *in vitro* sporulation.

If spore isolation is carried out carefully (without the aid of a sterile airflow cabinet) and the suspension is not too contaminated with small-spored fungi or yeasts, one should expect to lose no more than 5% of the isolates.

For a better comparison of the two above techniques before describing them in detail, the steps will be outlined below.

1- ISOLATION OF SUSPENDED SPORES

The whole procedure has to take place in the field laboratory.

a-**LIQUEFACTION** of the spore suspension (i.e. foam) is needed.

b-**SETTLING** of the suspended fraction in a Petri dish should not exceed a few minutes in order to avoid spore adherence to the bottom.

Floating spores may not settle for a long time, and will then have to be isolated from the surface.

c-**SCANNING** of the suspension with the dissecting microscope at up to 100 power. Scanning is mainly done at the water surface and at the bottom, but conidia of some species may also remain suspended in the water column for longer periods (e.g. *Tetrachaetum* elegans Ingold).

d-**IDENTIFICATION TO SPECIES** (often only approximate).

e-**TRANSFERRING** (at lower magnification) onto the isolation medium, where **germination** will take place.

f-**SUBCULTURING** of part of the incipient colony to a fresh medium.

Steps (c) and (d) could be performed under the compound microscope with a water immersion lens, which would improve optical resolution and facilitate identification. But there would still be the inconvenience of transferring the spore manually onto the isolation medium. Transferring the spore suspension to the dissecting scope and relocating the selected spore is not easy because the spore is not fixed onto a substrate. An alternative worth testing could be the Leica Kombi stereomicroscope, which is a dissecting microscope with a supplementary compound objective on the same nosepiece. It would be worth testing an immersion objective here.

An inverted microscope would provide relatively high optical resolution for identification, and would also enable direct isolation, but this has not been tested. It would also be costly, as two microscopes would be needed.

2-ISOLATION OF SPORES ANCHORED ON AGAR

In the field:

a-**LIQUEFACTION** of the spore suspension is not needed, although it may provide a more even distribution of spores over the isolation medium.

-If the scum does not liquefy easily, it may be **SPREAD** over the isolation medium with a bent glass rod or wide loop. Spreading has a diluting effect.

b-**FLOODING** of the isolation plate with the spore suspension.

c-**SETTLING** of spores, to increase concentration on the isolation medium, but only if the sample is very poor.

d- A short incubation to allow **ANCHORING** of spores to the medium, probably through mucilage production and/or incipient germ tube formation.

Germination happens very soon after sampling. It will also take place in the ice chest while *en route* to the field laboratory. The germ hyphae must not be allowed to grow to the point of being confused with conidial branches, which would impair spore identification. This problem might occur with some species within 3 or 4 h of collecting if incubated at ca. 20-25°C, but if kept at ca. 4°C, spores of some Ingoldian species may still be efficiently isolated after even 36 h.

In the laboratory:

e-**SCANNING** of spores on the isolation medium (under low power with the COMPOUND microscope), part of which will have been previously cut off and placed on the finder slide.

f-**SPECIES IDENTIFICATION** (under higher power of the compound microscope, which will be much more accurate than under the dissecting microscope).

g-Recording the **LOCATION** of the spore by means of a reference code on a finder slide.

h-**RELOCATION** of the spore under the dissecting microscope.

i-**TRANSFER** of the spore to another Petri dish with isolation medium.

j-**SUBCULTURE** from the incipient colony to the growth medium.

It is possible to isolate spores spread on agar without the aid of a finder. The spores are then scanned, identified and removed under the compound microscope (Marvanová, pers. comm.). But this requires a steady hand and great concentration, as the image on the lens is inverted and the working distance is very small. The use of optics

which do not invert the image, such as those available for the Olympus BX series, may obviate this problem.

The stepwise procedures for the isolation techniques are as follows:

a- **ISOLATION OF SUSPENDED SPORES**

Reminder: only efficient for **large spores appearing infrequently**.

If adhered onto the bottom of the dish, the spore, or part of it, may be **detached** with a mounted hair before transferring. (It is not known to what degree this may impair germination).

Spores may be removed with a **MICROPIPETTE** from inside water, or from any level, with a mounted **HAIR**.

The hair technique is fast and clean, and especially effective for branched spores, but needs testing as it has been noticed that conidia, mainly sigmoid ones, collected from the water surface often will not germinate. This may be because dead conidia float.

MATERIALS

-Both for rinsing the mounted hair as well as for manipulating the micropipette, place near the dissecting scope a **small volume of SDW** (e.g. in a 5-cm Petri dish), which may be left uncovered during isolation. The SDW should be changed every few hours to reduce airborne contaminations.

For the hair technique:

-A mounted **hair**: **glue** the base of a slightly arched, uncut hair (from the eyebrow, eyelashes, arm, etc.) onto the tip of a **needle**, for example with nail polish, and mount the needle on a **dissecting handle**. Alternatively, one may use a bristle from a toothbrush (J. Webster, pers. comm.).

To remove debris on the hair, this may be wiped or dug into the agar medium in a clean area.

Sterilization of the hair is not normally needed, but to achieve this one may dip it in alcohol and rinse in SDW.

For the micropipette technique:

-A pulled **Pasteur pipette** (Fig. 4), prepared as follows:

-**hold** one end in each hand,

-**roll** the Pasteur pipette over a gas burner or alcohol lamp. Apply the heat near the base of the thinner portion of glass (Fig. 5).

-When the glass softens, **take it out** of the flame and

-quickly but gently **pull** the two ends a few cm apart.

-**Break off** leaving ca. 2 cm of fine tubing. The length can be adjusted by nipping off bits from the end with forceps.

-With a hot needle **bore** a tiny hole through the top of the teat to allow wa-

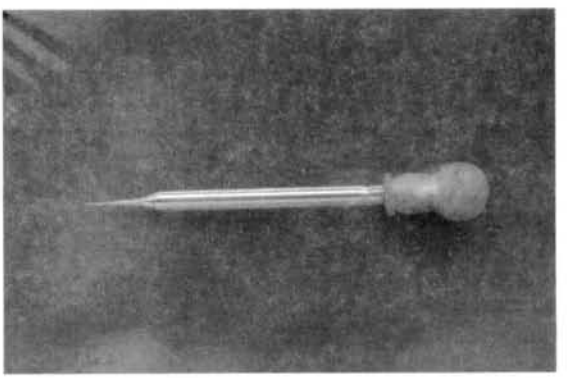

Fig. 4. Pulled Pasteur pipette.
Fig. 4. Pipeta Pasteur estirada a la flama.

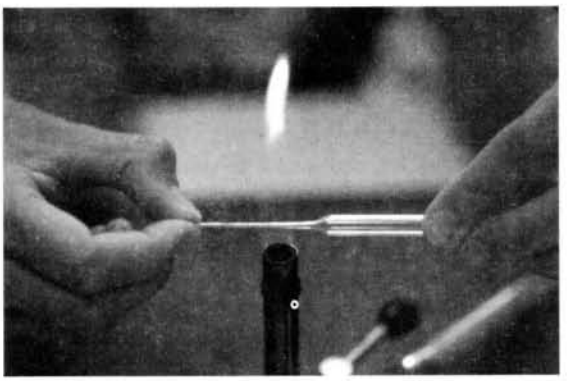

Fig. 5. Pulling a Pasteur pipette: the thin part is rolled back and forth over the flame of a bunsen burner. When the glass is soft, the pipette is moved away from the flame and quickly pulled.

Fig. 5. Com estirar una pipeta Pasteur: l'extrem fi es gira damunt de la flama d'un encenedor Bunsen. Quan el vidre està fluix la pipeta es retira de la flama i s'estira ràpidament.

ter to flow into the pipette by capillary action.

-**Test** the micropipette with SDW. This should only suck up a drop large enough to transport the spore onto the isolation medium. If the water flows in too slowly, with the forceps gradually **nip off** the tip of the micropipette. If too much water flows in, **soften** the glass and pull again to obtain a thinner tube; or else start with a fresh pipette. Alternatively, reduce the width of the opening by quickly passing it over a small flame (J. Webster, pers. comm.)

If the pipette is too thin, the spores may either not enter (some are over a mm in span) or they may attach to the inner walls. It is better to secure the spore by using a wider bore, even if a larger volume of suspension (with contaminants) flows in. This isolation procedure will anyhow need a second (cleaning) step whereby the spore is dragged out of its drop on the isolation medium.

-Flush out the water drop from the micropipette by covering the teat hole with the thumb and squeezing gently.

If handled with care and properly stored (e.g. in a plugged test tube), the pulled micropipette may last indefinitely. Nevertheless, when on field trips have some spares available.

CLEANING THE MICROPIPETTE

The micropipette will eventually accumulate debris on its inner walls, which interferes with isolation. To remove it:

-**Draw in** some water.

-**Boil** the upper part of this water over the pilot flame of a Bunsen burner, while blocking the hole on the teat with your thumb. There will be a tiny explosion as the vapour shoots out of the tip dragging along all debris but without damaging the glass.

Do not flame the tip itself, as it will melt and block the exit. If this happens, pince off the melted end with the fine forceps.

PREPARING THE ISOLATION PLATE

Marking out compartments with a felt pen on the reverse of the plate and placing one spore in each facilitates their relocation and saves space and isolation medium.

Two designs are suggested, which enable isolation of 12 spores per plate. The first one (the **ladder design**) (Fig. 6), consisting of 4 ladders delimiting 6 squares each (total: 24 squares, 2 per spore), has been used for many years. The second design (which may be

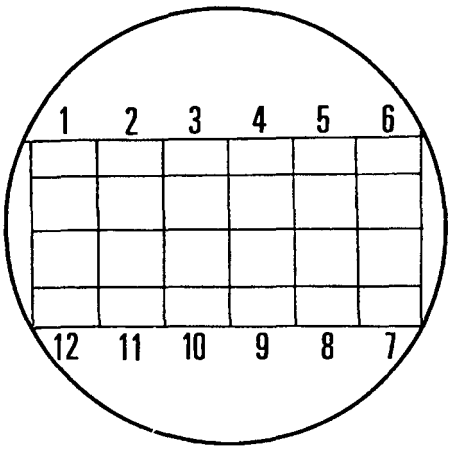

Fig. 6. Example of ladder-like line grid for isolation of spores in suspension.
Fig. 6. Exemple d'entramat lineal, del tipus escala, per aïllar espores en suspensió.

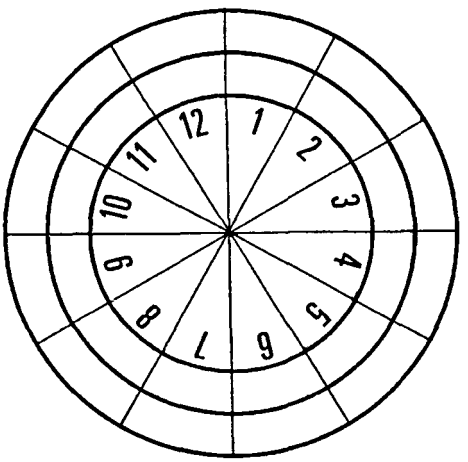

Fig. 7. Example of spoke-like design for isolation of spores in suspension.
Fig. 7. Exemple de disseny amb forma de raig per aïllar espores en suspensió.

called the **spoke design**) (Fig. 7), consisting of 24 radial sectors (also 2 per spore) enclosed in concentric circles, has not been tested, but should give each spore a larger surface area for growth, thus reducing chances of contamination from neighbouring sectors.

When using the ladder design a spore is first placed on a square in one of the two inner ladders, allowed to germinate and then transferred onto the neighbouring square on the outer ladder. The area of agar medium enclosing the two inner ladders (which will bear contaminants) can eventually be cut off with a flamed scalpel as a single block and discarded.

When using the spoke design each spore is placed in an outer sector, allowed to germinate and then transferred to the neighbouring inner sector. The outer ring of agar can likewise be eventually discarded to reduce the risk of contamination.

CONTROLLING UNICELL CONTAMINANTS (bacteria, yeasts)

Unicell contaminants lying near the spores sometimes develop into colonies which swamp the isolate and interfere with or even interrupt germination or growth. In order to avoid this, one may carry out the following simple operation, which is especially useful for slow-growing colonies:

-Before isolating, punch the centre of each of the outer squares (in the ladder design) or of the inner sectors (in the spoke design) on the isolation plates with the tip of a glass tube at least 2 mm wide (e.g. an unpulled Pasteur pipette). Take out the resulting cylinders to a depth of about 2-3 mm. The agar blocks with the germinating spores will be placed in these wells. If contaminant unicell colonies develop within, they will normally remain trapped long enough to allow the hyphae to grow out into the clean agar, from where they may be subcultured.

If wells have not been dug, and a conidium is being swamped by a unicell colony, it may be saved by transferring to a freshly dug well in a clean area.

Alternatively, apply a small glass ring on the contaminated spore. The ring will act as a barrier for the unicell colony, while the hyphae grow underneath and away from the ring.

The procedure would be:

-Cut 5 mm sections of thin glass tubing (ca. 3 mm diam.).

-Holding a ring with the forceps, dip in alcohol and light this over a flame.

-After it has cooled down, and still holding it with the forceps, slightly dig the ring end-on into the agar encompassing the contaminated spore.

PROCEDURES FOR ISOLATING SUSPENDED SPORES:

-Pour liquefied foam into a **glass Petri dish**, to a depth of 3-4 mm. If not enough, add SDW.

If the suspension in the dish is too shallow, convection currents will not allow the spores to settle. It may also be impossible to break the air/water boundary layer with the micropipette, because this cannot go deep enough.

-Allow spores to **settle** for a few min. on the bench, avoiding heat sources under the Petri dish, which will also create convection currents.

THE HAIR TECHNIQUE

-With the flamed top of a needle handle, touch the surface of SDW and place the resulting drop on the centre of each inner square (or outer sector) in the isolation plate (see above).

-Place this plate on the side of the dissecting microscope.

-With the dissecting microscope, **scan** the spore suspension for spores.

-Swing to highest power to **identify** the spores.

-If necessary, **push away** with the mounted hair any spores or debris nearby.

-**Transfer** the spore with the tip of the mounted hair.

Spores settled on the bottom or suspended in water may be easily stroked up to right under the water surface, from where they may be lifted.

-Quickly **place** the spore in the drop on the isolation plate. Uncover the plate as little as possible.

Ensure that the spore comes off by wiping the tip of the hair a few times over the agar surface, inside the drop.

-In a notebook write down the **isolate code** and **sketch** the spore to represent shape and approximate size.

-If the drops of SDW in each compartment have dried up on the agar before or after a spore has been placed in them, **add more**. (This step should be avoided).

-When all transfers have been made for that dish **relocate** the first spore under the dissecting scope.

(This could be done under the compound microscope to better observe and identify the spore and to detect possible contaminants, but it would imply leaving the dish open for too long and exposed to contaminations, unless one is working in a sterile air-flow cabinet).

If a spore cannot be immediately spotted in its drop, do not assume that it is lost. Adjust the light beam with the sub-stage mirror to **enhance contrast** with the background. If it still has not been found, it will appear on the agar surface after the drop dries up. (But avoid this, as the spore will still rest among the debris and contaminants).

-With the mounted hair, **drag** the spore to the edge of the drop. The dragging action helps detach debris and contaminants off the spore surface.

Fig. 8. Single-sporing: once the spore (arrow) has been dragged with the mounted hair onto a clean area, two marks on either side will aid in its location.

Fig. 8. Aïllament monospòric una vegada que l'espora s'ha granat amb el pèl sobre una superficie neta, es fan dues marques a bandes oposades per facilitar la seva localització.

Especially if the foam is very rich, old or dirty, the drop may be heavily contaminated and one should always **move the spore** out of it:

-With the mounted hair, pull a short, slender channel of water out of the drop.

-Sweep the spore to the end of this channel.

-With smaller spores, **mark** their location by lightly piercing holes in the medium with the flamed microscalpel on either side (Fig. 8).

-**Repeat** the above steps for all other spores on the dish.

-Label the dish.

-**Incubate** the dish at room temperature.

-**Check** daily for germination and contaminants without opening the dish. The most dangerous fungi are some of the airborne Mucorales, as their colonies may swamp a plate in a couple of days.

-If the underside of the lid is covered with condensation droplets, **replace** the lid with another clean, dry one during observation.

(Condensation may be serious when dishes have been recently taken out from the cold).

Some germ hyphae are difficult to detect, and one must again adjust the orientation of the sub-stage mirror for contrast.

-With a flamed scalpel, **remove contaminant colonies** as they appear.

If small contaminant colonies (usually *Cladosporium, Penicillium* or *Aspergillus*) have started to sporulate:

-**Fix** them with a drop of 90% alcohol before removing them, to avoid further contamination. Alternatively, one may burn them off with a needle, or with the commercially produced Pyropen (Webster, pers. comm.)

Surface hyphae of some contaminants (e.g. mucoraceous ones) overgrowing a selected spore can sometimes be neatly lifted with the needle and discarded.

-As soon as a spore has produced a short germ hypha **check** under highest power with the dissecting scope for contaminations.

-With the flamed microscalpel, **cut** into the medium around the spore, and as close as possible to it.

If the conidium is large and much-branched (e.g. *Dendrospora*) and has germinated from various cells, it is safer to transfer only a small portion of it, or of the germ hyphae.

-**Transfer** the resulting agar block with the microscalpel and place it in the well in its neighbouring (smaller) compartment.

When all spores have been transferred:

-under the dissecting microscope, and with the surgical needle, **cut out** the inner two ladders (or the outer ring in the spoke design) as a single piece, flaming always between cuts. Take care not to cut through any colonies, contaminant or not, as you will sow new colonies throughout the dish.

-**Turn** the dish over some tissue paper and

-**discard** this piece of medium by flicking it off from one end with the scalpel.

Note: the above steps require opening the isolation plate several times. This will normally result in some airborne contamination. It is thus important to carry out the motions swiftly.

-When the spores have developed into distinct microcolonies (this happens in one to several days), with the surgical needle **transfer** a small block of agar from their margin to fresh medium.

-**Label** the new container with the three basic data: isolate code, date of transfer and habit sketch of the spore.

-**Incubate** these cultures at room temperature, or in the refrigerator if they grow quickly, until you have returned to the main laboratory.

THE MICROPIPETTE TECHNIQUE

-Sterilize the micropipette.

-Allow **SDW** from the 5-cm dish to flow by capillarity into the micropipette.

-Withdraw the micropipette from the water, cover the teat hole with the thumb and squeeze to **force out** some of this water. (This same volume will later flow in with the spore). Release pressure on the teat slowly to keep air bubbles from being drawn in.

-Under the dissecting microscope, **locate** the spore you want, which may be settled or in suspension.

-**Dip** the tip of the micropipette onto the selected spore, and watch it flow in.

If it flows in too slowly (e.g. the micropipette had too much water to start with), it will be left behind when you withdraw the pipette from the suspension. To avoid this, start with less water in the pipette.

-**Withdraw** the micropipette as soon as the spore has been sucked in.

-You may then dip in for some **more SDW**, as a larger volume helps eject the spore onto the medium and dilutes possible contaminants.

-Lift the lid of the isolation plate slightly, cover the teat hole with your thumb and **squeeze** the water with the spore onto the appropriate compartment.

As the drop flows out, it sometimes rolls up onto the outside of the micropipette wall and the spore is lost. To avoid this:

-make the drop **touch the agar** surface as it flows out. Do not scrape the agar surface as the spore might get lost in the scars.

-Close the dish and draw in SDW for the next spore **before** the micropipette dries up. This keeps any remaining debris from drying up and adhering to the inner wall.

-**Check** regularly that the spores are being isolated. If not so, flush the micropipette with boiling SDW, as explained above.

-Proceed with the rest of the spores as above.

ISOLATION OF SPORES ANCHORED ON AGAR

Most Ingoldian fungi are now being isolated by us with this technique, as it offers several important advantages over the spore-suspension technique:

1- Spores can be identified under the compound microscope with much

greater accuracy. 2- Relatively small spores, down to 5 μm in span or less, can also be isolated (as long as one can relocate them, or their more visible colonies, under the dissecting microscope in the transfer stage). 3- The spores on the "foam plates", if these are kept refrigerated, may remain recognizable even many hours after collecting (depending on the species), thus allowing for much more collection and/or isolation time; i.e.: one can collect much further away (even after overnight trips); or, alternatively, rich samples can be scanned more thoroughly. 4- The isolation plate is left open much less time, chances for contamination thus being minimized. 5- Isolating a germinating spore greatly increases the chances of establishing the culture. 6- The technique is more rapid. 7- Less skill is required.

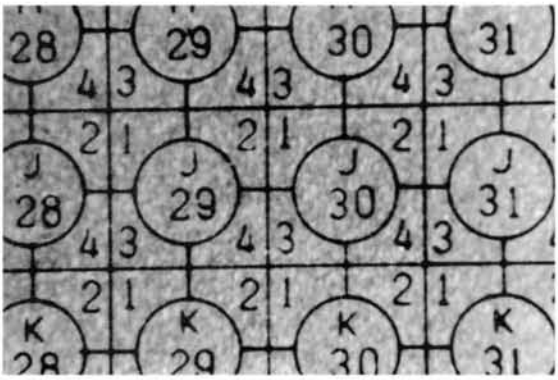

Fig. 9. Single-sporing with the England finder: detail of the letter-number pattern engraved on the slide. Letters are sequentially arranged vertically and numbers horizontally: each resulting circle is enclosed in a square itself subdivided into subsquares 1-4.

Fig. 9. Aïllament monospòric amb el localitzador England: detall del patró gravat sobre la porta. Les lletres es graven seqüencialment en sentit vertical i la numeració horitzontal: cada cercle resultant s'inclou en un quadre que, a la vegada, es subdivideix en 4 subquadres 1-4.

MATERIALS

The **slide finder**: This is a standard-sized microscope slide bearing a photographically reduced grid pattern, each compartment being identified with letter and/or number coordinates arranged sequentially along the horizontal and vertical axes. A piece of agar medium from the "foam plate" is placed on the finder, the spores are located individually under the compound microscope, the coordinates directly underneath each spore are noted and the spores relocated under the dissecting scope for manual lifting.

A commercially produced device, the "New England" finder, is available as item code S7 from Graticules Ltd., Morley Rd., Tonbridge, Kent TN9 1RN (UK) (Fig. 9). But one may build a much cheaper and equally useful finder by printing a similar template on paper and photographing it on a 35 mm diapositive film. The frame on the film may then be cut up and glued with nail polish on a microscope slide under a coverslip.

As the finder slide may break and is indispensable for this technique, it is advisable to have some **spares**.

-**Isolation plates**: With a fine-pointed felt pen, draw a 6 x 6 grid pattern (Fig. 10) on the reverse of an isolation plate. Each of the squares on the grid can be identified by its two coordinates.

-A small central well may be plugged out in each square for controlling unicell contaminants (see above).

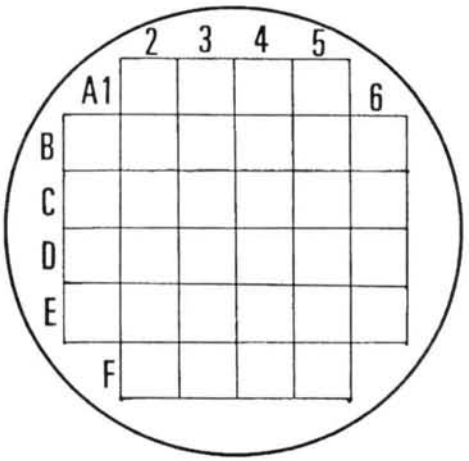

Fig. 10. Example of line grid for spore isolation with finder.
Fig. 10. Exemple d'entramat lineal per aïllar espores amb el localitzador.

-**Field dissecting microscope or pocket compound microscope** (optional):

Either of these is advisable for quickly deciding whether the foam samples need diluting. Valuable time and effort may thus be saved. High-quality portable compound microscopes with battery-powered built-in lamps are commercially available (e.g. Nikon), but these are costly and delicate. Alternatively, use a spare dissecting microscope with transmitted light. (A transmitted-light base may be improvised, as explained above). The spore concentration in the foam can thus be quickly judged, even at 25 x.

-**Isolator** (optional):

This device replaces an objective on the nosepiece of a compound microscope. At its bottom end is a tiny, sharp-edged tube which is used to mark the location of the spore: it is dug into the agar enclosing the spore by racking the nosepiece down a few mm and then racking it up again. The resulting cylinder is then relocated under the dissecting scope, transferred with a microscalpel onto fresh isolation medium.

The isolator may be useful because: 1- time is saved in transferring the marked spores; 2- a less steady hand is required for manipulating the spore. On the other hand one may have less control over contaminant spores in the vicinity. It is also somewhat cumbersome if one needs to flame the isolator tip between spores.

The Keyworth isolator (Ivor Saint Co., 50 Clarendon Ave., Leamington Spa, England) (Keyworth 1959) (Fig. 11) may be used to replace the objective lens next to the 10x one (e.g. the 100x); not the 20x objective, because this will be needed for observing spores in more

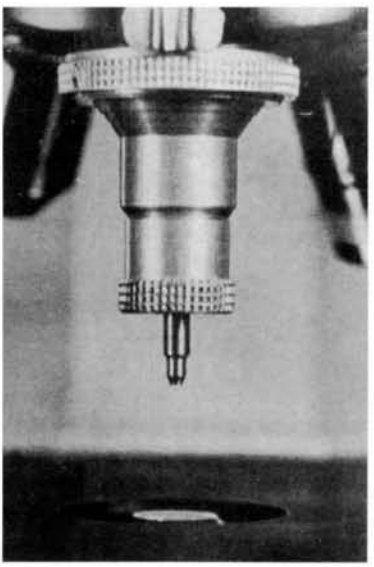

Fig. 11. Keyworth isolator screwed onto the nosepiece in place of the oil immersion objective. (Reproduced from Tuite, 1969).
Fig. 11. Aïllador Keyworth situat en el lloc de l'objectiu d'immersió d'oli (reproduït de Tuite, 1969).

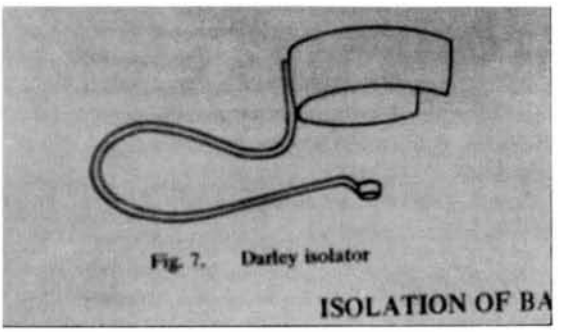

Fig. 12. Darley isolator: the cutter is mounted on a flexible ring which fits on the objective (Reproduced from Tuite, 1969).

Fig. 12. Aïllador de Darley: l'anell tallador es monta sobre un anell flexible que abraça l'objectiu (reproduït de Tuite, 1969).

detail. When a spore has been located with the 10x objective, the isolator is swung down and used as detailed above.

The Darley isolator (Tuite 1969) (Fig. 12) is a 1-mm diam. metal biscuit cutter held on a clamp which embraces the same low-power objective used for spotting the spore. It has the great advantage over the Keyworth isolator that one can see the spore while marking it. The mechanical stage can then be steered to keep the spore in the middle of the field as the biscuit cutter is lowered onto it, and thus reduce the chances of damaging or losing it.

PROCEDURE FOR ISOLATING SPORES ANCHORED ON AGAR

- As the foam sample may need diluting, put two **"foam plates"** per planned sample in the box in the ice chest. No more than three or four samples should suffice for one day's isolation work, even if they are not rich.

- **Collect** the foam in a jar and take it to the vehicle.

- Also collect a small amount of stream water for diluting the foam sample, if necessary.

- **Shake** the jar firmly several times to help liquefy the foam.

- Label a plate with the isolation medium.

- Pour some of the suspension into this.

- **Swirl** the plate gently to wet the entire surface.

- **Decant** all the excess spore suspension back into the jar, and use this for preparing a voucher specimen.

- If the foam is scanty, pour the excess directly into a small labelled vial, fix and keep as voucher specimen.

-If the suspension is not rich, do not decant, and let it **settle** on the surface of the agar medium to increase spore density.

-If the spore load is rich or suspected to be rich, the remaining suspension may be **diluted** with the same stream water or with SDW and poured as above into a different, also pre-labelled dish.

-Scums that do not break down readily may be **spread** over the agar medium with a bent glass rod or wide loop.

-**Store** the inoculated plates in the box within the ice chest.

-**Transport** to the field laboratory, keeping the ice chest as horizontal as possible to maintain spore suspensions on agar evenly spread on the medium.

-In the laboratory, most of the spores should by now be anchored to the agar. (If not, incubate at room temperature for an hour or so).

-**Store** the foam plates in the refrigerator.

It may be wise to put a **max./min. thermometer** alongside the plates in the refrigerator, as this will often operate at higher temperatures (especially those in hotel kitchens, which are opened frequently). If so, it may be advisable to keep the foam plates in the ice chest, or in a portable refrigerator.

-When isolating, bring out the foam plates from the refrigerator (one at a time to retard germination).

-Once most spores have germinated, you may gently **rinse** the surface of the agar in the foam plate with excess SDW to eliminate some debris. However, this may cause some valuable, slow-germinating spores to be lost when decanting.

Do not use DW from a wash bottle, as this is often contaminated with yeasts.

If you use the isolator, it may not be advisable to add SDW, as contaminants may flow into the cuts in the agar.

Note (The following approach has not been tested): If the agar surface is rinsed at a fixed time after applying the spore suspension and the washings decanted onto another isolation plate, one may be able to select for slow-germinating spores; i. e.: spores of weed species (such as *Heliscus lugdunensis* Sacc. & Thérry) usually become anchored and germinate faster than rarer, more interesting species. At rinsing, the former should remain adhered onto the medium, while the latter ungerminated ones are washed onto the antibiotic medium in the second plate, where they may be incubated for longer periods.

-**Examine** the foam plate and look for an area where the spores are conveniently spaced for isolation.

-With a flamed scalpel, **cut out** a rectangular slab of agar medium slightly narrower than the printed area on the finder slide. The length of the slab will depend on the richness of the sample. About 3 cm would be adequate in most cases.

Make vertical cuts so that the margins of the slab can be examined without difficulty.

-**Dig** under this slab with a spatula and transfer it onto the finder slide.

-Return the "foam plate" to cold storage for later sampling, if it were to prove interesting.

-You may want to keep the agar surface of the slab **wet** by regularly adding drops of SDW.

This is done: 1- to improve optical resolution. (The meniscus surrounding spores lying on agar tends to impair vision and thus species recognition). 2- To keep the agar surface from drying, as it would then become gummy and difficult to cut. It will also shrink unevenly, and you will need to refocus often as you scan. Agar dries up faster on the edges of the slab. You may want to transfer valuable spores from here first. 3- To keep contaminant airborne spores from germinating on the agar.

-With the 10x objective of the compound microscope, **scan** the entire surface of the slab using the mechanical stage.

-**Identify** the selected spores under the highest possible power (usually 20-25x). The objective should have enough working distance so that it will not touch the agar or become misty with condensation.

-Check for nearby contaminant spores.

-Swing back to the 10x objective and rack down the nosepiece to read the finder **coordinates** immediately underneath it. Record these in the laboratory notebook. Sketch the spore shape and note presence of nearby contaminants that may need removing.

-To save time, **transfer** all spores only after you finish scanning the whole agar slab.

-If an isolator is used, **punch out** the spores as you find them, and transfer all of them afterwards.

If a contaminant is present nearby, do not use the isolator and lift the spore with the microscalpel, as explained below. The contaminant spore or microcolony may also be burnt off with a "Pyropen" (J. Webster, pers. comm.).

-**Transfer** the finder slide to the dissecting microscope. If the image under the compound microscope is inverted, first turn the slide around 180º.

-Rack down to the level of the grid and **relocate** the coordinates, rack the nosepiece up and you should find the selected spore on the agar surface. (If in doubt, check the spore shape with your habit sketch in the notebook).

-**Remove** contaminants and debris, if necessary, with the mounted hair or the flamed microscalpel, or burn them.

-To clean around the spore, with the microscalpel plough up three **moats** around it. The agar should be lifted from underneath and the resulting flaps turned over and AWAY from the spore. The flaps need not be removed (Fig. 13).

In this way the contaminants will have been neatly separated and the spore left in the middle of a tiny peninsula.

-**Flame** the microscalpel every time after it touches the agar medium.

-Finally, finish cutting out the agar block by making a deep **cut** in the agar joining the ends of the U-shaped moat (Fig. 13). Do not lift a flap here.

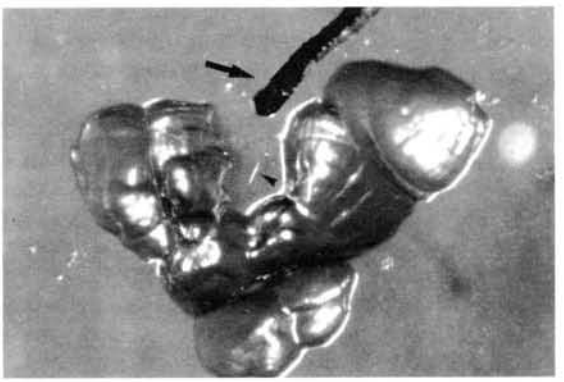

Fig. 13. Single-sporing: detail of the U-shaped moat plowed out around the spore. The piece of agar supporting this is then lifted from underneath with the tip of a microscalpel.

Fig. 13. Aïllament monospòric: detall del canal amb forma d'"u" aixecat enrevoltant l'espora (punta de la fletxa). El bocí d'agar que el sostén s'eleva després empenyent des d'abaix amb la punta del microescarpell (fletxa).

-**Lift** the resulting agar block with the microscalpel by piercing it underneath from the middle moat (the bottom of the U) and sliding the block upwards while leaning against the opposite wall (i.e. the fourth cut).

-**Transfer** the agar block with the spore to a well in the isolation plate.

It does not matter in which position the agar block is laid on the agar medium.

(Between transfers of the agar blocks to the isolation plate, it is advisable to place this inverted on the bench. The bottom of the dish can then be lifted and turned over in one step, without there being a need to first uncover it. It is also easier to locate the correct square for transfer).

-When all transfers have been made, **incubate** the isolation plate and proceed as above.

The agar slab on the finder slide will obviously be exposed to some aerial contamination by fungal spores during isolation, but this does not pose problems, as only a minute portion of the agar medium is sampled.

ISOLATING SPORES FROM TELEOMORPHS ON NATURAL SUBSTRATES

This highly interesting aspect in the study of Ingoldian fungi is being sadly neglected, although it should provide valuable information on systematic affinities and ecological aspects.

Teleomorphs (e.g. **discomycetes**, such as *Miladina, Hymenoscyphus, Hyaloscypha, Mollisia, Orbilia*, etc., and *Lophiostoma*- and *Massarina*-like **Loculoascomycetes**, or even **corticiaceous** and **gelatinous basidiomycetes**) are often present on decaying wood from aquatic habitats. Alternatively teleomorphs may be induced after longer incubation in moist chambers under daylight + NUV at cool temperatures. (Ideal conditions are unkncwn in most cases). Smaller fruit bodies may possibly be also found on leaves, although these usually are anamorphic (pycnidia, spermodochia, etc.).

RECOMMENDED FIELD GEAR

-Pocket knife (preferably chained to trousers), rucksack with : chisels and mallet, folding handsaw, lightweight toolbox with compartments for individual collections, large polythene bags for bulky wood, smaller polythene bags for leaves, paper bags for Pyreno- and Loculoascomycetes, specimen vials and plastic jars for delicate apothecia, felt pens, altimeter, field notebook.

An ice chest will be needed in warm weather.

LABORATORY MATERIAL

-A **wide loop** (ca. 1.5 cm diam.). This can be made by coiling a bacterial inoculation needle or any soft wire around a cylindrical object e. g. a felt pen.

PROCEDURE

-**Collect** twigs, surface portions of branches and trunks in various stages of decomposition. Exposed but wet substrates, such as half-buried trunks along riverbanks, or wood trapped in large piles amongst boulders, in gorges or below waterfalls are often well colonized by Ingoldian fungi.

-**Chisel** or **peel off** surface wood, or **cut off** twigs with pruning shears. If delicate apothecia are present, store in labelled vials (where they can be secured by adding a green leaf or some moss; but not litter, as this may contaminate the sample with other saprotrophs), or in compartments of the toolbox.

-Muddy samples may be rinsed *in situ* in polythene bags by adding some stream water and shaking vigorously. Repeat until washings are clear. (It may be worth examining the washings for Ingoldian conidia).

-Keep the specimens **damp** but not wet (as this will increase unicell contaminations) until they are further processed in the laboratory. Storing perithecia or pseudothecia for a few days does not seem to affect ascospore germination. This may also be true for some apothecia, although they may be then invaded by other fungal mycelia.

-**Insecticides** and **acaricides** may be sprayed on during storage to stop invertebrates from feeding on fruit bodies and mycelia.

Two basic techniques may be used for isolating ascospores (or any other spores, whether sexual or asexual, and whether produced in or on fruit bodies): 1- in **"hymenial squashes"**, normally used for perithecia or pseudothecia and for very small apothecia, a portion of the

hymenium is squashed and spread on the isolation medium, whence the germinating ascospores will be transferred to clean medium; 2- in "**ascospore deposits**", normally used for apothecia, ascospores are allowed to shower over the isolation medium and, once germinated, some are transferred to clean medium.

Spore isolates from a single fruit body obviously belong to a single species. But nearby fruit bodies may however belong to different species, and their isolates should bear separate codes.

HYMENIAL SQUASHES

MATERIALS

-Prepare an **isolation plate** by drawing a line pattern on the reverse of a polystyrene Petri dish poured with isolation medium (Fig. 14).

The two halves of the dish may be used for isolating from two different fruit bodies. The central swath keeps the material from the two fruit bodies from mixing. The two swaths on either side are meant for spreading the corresponding squashed centrum contents. Upon germination, single ascospores and/or asci will be transferred to the squares in the corresponding ladder for further growth and subculturing.

PROCEDURE

-Dip a microscope slide held with forceps in 90% alcohol, and **flame**.

-Place several **drops of SDW** in a row from one end of the slide to the centre. The central drop, where the centrum will be squashed, should be larger.

-Under the dissecting scope, **scan** the substrate for fruitbodies.

-There should preferably be a troop of **similar fruitbodies** for later descriptive purposes.

-Check that the contents have not been ejected or eaten by arthropods by slightly piercing the wall with the microscalpel.

-With a needle or microscalpel remove a **single fruitbody**, avoiding as much substrate as possible.

-Place it in the **outer drop** on the slide.

-With two needles or microscalpels, **detach debris** from the fruit body.

-When isolating from perithecia or pseudothecia, if spores are seen oozing out of the ostiole or forming a dried-up mass on it, transfer them en masse directly to the central drop.

-**Lift** the fruitbody to the next drop. (Do not drag it across, as you will carry contaminants along).

-**Clean** off more debris if necessary, always flaming instruments between transfers.

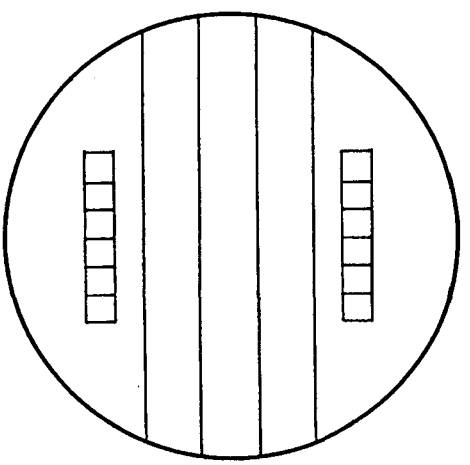

Fig. 14. Example of line grid for isolation of sexual spores from a hymenial squash.
Fig. 14. Exemple d'entramat lineal per aïllar espores sexuals d'una maceració himenial.

-Repeat until the fruitbody is in the central drop. Carefully **wipe out** the other drops.

-**Drag** the fruitbody to the edge or even out of the drop, where it will sit on the glass and be easier to handle than if lying in deeper water.

-**Squash** the centrum or hymenium with the needle and microscalpel, or with the flattened tip of a thick copper wire.

-Check that **free ascospores** are present, as the fruitbody may be immature.

-Drag the **fruitbody wall** out of the drop. This may be mounted separately for descriptive purposes.

-**Stir** the macerate in the drop to obtain an even suspension.

-**Spread SDW** with a flamed wide loop over one of the swaths on the isolation plate. (If water is absorbed quickly by the agar, keep wetting). Do not scrape the agar surface.

-Depending on the ascospore concentration, **transfer** one or more loopfuls of the macerate and spread evenly over the swath. The transfer may also be done with a micropipette.

-**Check** that the ascospore or ascus concentration on the medium is adequate for comfortable isolation.

-**Incubate** the isolation plate overnight or longer at room temperature. Keep it in a closed polythene bag to reduce evaporation.

-Under the highest power of the dissecting microscope, or preferably by means of the finder slide under the compound microscope (see above), **scan** for germinating ascospores and for asci containing germinating ascospores.

If the fruit body is heterothallic, a single-ascus isolate (if at least half + 1 ascospores grow) should provide the two mating types. The single-ascospore isolates may produce somewhat less vigorous colonies than single-ascus or multi-ascospore isolates, but they are useful for confirmation of pure culture.

-With the microscalpel, **transfer** tiny agar blocks with germinating material to individual squares on the ladder, labelling these accordingly (e.g. "a" for single ascospores, "A" for single asci, "ma" for more than one ascospore).

-With the flamed surgical needle or scalpel, cut off and **discard** the piece of agar medium containing the swath with the spore spreads, which will have contaminants.

-**Incubate** at room temperature until colonies are large enough for **subculturing** from the margin onto growth media. Colonies that look different may be contaminants.

For preservation of voucher specimens:

-The remains of the squash on the slide should be air-dried and mounted, and kept as the "**isolation slide**". The wall material of peri- or pseudothecia may be mounted on a different slide to keep the centrum squash preparation as thin as possible.

-Also **preserve** the dried substrate with the remaining fruitbodies.

-Label all specimens and slides individually.

ASCOSPORE DEPOSITS

(These are meant mainly for apothecia, but may be used for perithecia or pseudothecia too, if large enough.)

-Invert an isolation plate and **draw a line** across the centre of the base with a felt pen. On one half will be deposited the spore cloud from the apothecium, and on the other will be the ascospore transfers. For this step draw laddea (Fig. 15a).

-**Label** the dish.

Build an **agar support** for the apothecium (Fig. 15b), so that it dries

up less quickly and its hymenium can be conveniently oriented towards the agar:

-With a flamed scalpel cut out an **agar block** ca 5 mm square from the isolation plate and lay it on the underside of its lid, off centre, e.g. 1-2 cm from the margin. If the apothecium is small it may be necessary to superimpose agar blocks to bring it closer to the agar surface.

-In the opposite half of the lid lay a small wad of **wet paper** tissue.

This keeps the isolation plate damp while being used. SDW may be added to the wad regularly for longer incubations.

-Select an apothecium that looks ripe.

-Record macroscopic characters. Do a **habit sketch** with the dissecting microscope.

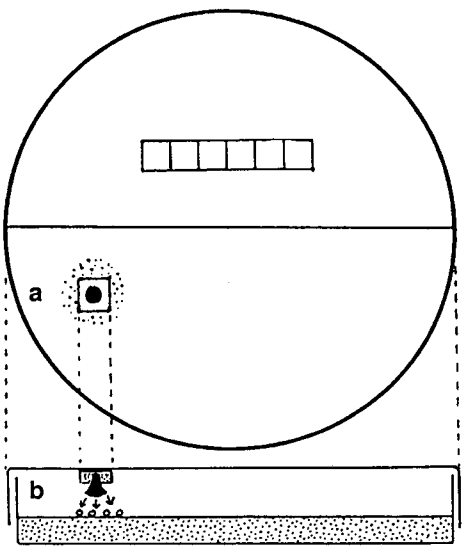

Fig. 15. Example of line grid for self-isolation of sexual spores. a: top view; b: side view.
Fig. 15. Exemple d'entramat lineal per a l'autoaïllament d'espores sexuals. a: visió frontal, b: visió lateral.

-**Lift** the apothecium (if stipitate, slice it off its base, which may be dirty) and with forceps place it vertically on the agar support, with the disc facing the underlying medium.

-**Replace the lid**.

-Under the plate, draw a **circumference** with a felt pen, enclosing the area where the ascospores are expected to land.

-**Incubate** the dish inverted for larger apothecia, or upright if these are small (under 2 mm diam.), or if a poor deposit is expected.

Ascospores landing on the agar will form a "cloud" or deposit. To allow the ascospores to be spread out, the hymenium should not be too close to the agar.

Incubate the isolation plate at room temperature and exposed to daylight to induce ascospore ejection, and keep inside a polythene bag to retain moisture and protect from contaminants.

-In many cases an **ascospore deposit** should be visible after overnight incubation.

-It may be very tenuous and require careful **examination** with the dissecting microscope, either inverting the plate and looking through the agar with a dissecting scope (which would require a clear, filtered medium), or through the lid of the upright dish after rotating this a few degrees to get the apothecium out of the line of view.

-When the ascospores have started germinating, **isolate** 2 or 3 of them by cutting out small cubes with the microscalpel.

At the edge of the deposit they should be further apart.

-**Place** the isolated material individually in the squares of the neighbouring ladder.

-Also do a couple of **multi-ascospore transfers** by taking larger

agar blocks from the centre of the cloud. (These may include contaminants from the surface of the disc; it is thus important to do parallel single-ascospore isolates and compare colony morphologies).

-If one should want to obtain further isolates from the same fruitbody, **rotate** the lid some 45º and re-incubate to obtain a new spore deposit.

-**Remove** the apothecium from the isolation plate and

-Prepare an "**isolation slide**" with some of the remaining hymenium (see above).

-**Air-dry** the apothecium as soon as possible and

-**Preserve** for descriptive and herbarium purposes, in case the culture eventually produces an Ingoldian anamorph.

For Corticiaceae and other basidiomycetes commonly associated with the Ingoldian fungi, use similar techniques. Because basidiospore ejection may not be as efficient as with ascospores, the isolation plate should probably be kept upright to allow them to fall on the plate.

WORKING IN THE MAIN LABORATORY

This section will discuss the processing of material for **observation**, **description** and **identification**, as well as the **preservation** for herbarium purposes.

Description and **identification** procedures for Ingoldian fungi are specialized activities which need discussing separately (Descals ined.). Line drawing techniques have been suggested by Seifert, Gams, Descals, etc. (see various issues of the Ananet Newsletters, e.g. 15:9, accessible through Internet).

BASIC FACILITIES AND EQUIPMENT

Ideally there should be: 1- a preparation room; 2- a sterilization room; 3- a general purpose laboratory; 4- cool-temperature room(s); 5- a microscopy room and 6- a working herbarium.

It is assumed that there will be an office and taxonomic library, as well as adequate storage facilities.

1- **Preparation room**: this should preferably be at some distance from the laboratory and other facilities, to reduce chances of mite infestation.

Besides the usual biology preparation room requirements, there should be:

-Air-conditioning with adequate temperatures throughout the year for incubation of field material.

-Window(s) not exposed to direct sunlight (north-facing in the northern Hemisphere), for room-temperature induction of fruiting in field material.

-Refrigerator(s) for field material and antibiotics.

-A precision balance for media preparation.

-pH meter and conductimeter, for determinations on water and special media.

-Membrane filtration equipment with vacuum pump.

-Cupboards for storing dry media and nutrient salts, as well as autoclaved media and SDW in bottles and conical flasks.

2- **Sterilization room**:

-autoclave, with capacity for at least a 10 liter bottle if a continuous water-flow system is to be used for anamorph induction (see below),

-a source of DW,

-a safety cabinet for solvents and flammables.

3-**The general-purpose laboratory:**

This should be draught- and dust-free and have:

-Year-round air conditioning (as for the preparation room).

-Window(s) with indirect daylight, for fruiting induction of pure cultures.

-A sterile air-flow cabinet for subculturing.

-A microwave oven for melting agar media, which will have been stored in bottles or flasks. Plug them with cottonwool and then cover with aluminium foil. Before putting in the microwave oven, remove the foil or cover this with more cotton.

-A hot-water bath for keeping the melted media warm, if pouring large batches into plates.

-If only one cool-temperature room is available, a glass-fronted cool cabinet (as used in supermarkets) in case the machinery in the cool-temperature room breaks down while incubations are underway. The cabinet should also be equipped with NUV and daylight lamps.

-Two refrigerators (one for media poured in plates and slopes, and the other for a working culture collection). Both should have freezer compartments, in case one breaks down. It is convenient to have a third refrigerator for field material, unless this has been treated against mites.

-A lyophilizer may be useful if a working culture collection is to be maintained.

4- **Cool-temperature room(s)**

-There should ideally be two such rooms, 1- to enable different incubation regimes; 2- for keeping field material separate from pure cultures, thus reducing the risk of mite infestations; 3- as a precaution for possible breakdowns.

For inducing sporulation in cold-temperate species, set one room at 15ºC. Ventilation should be minimal, as this will increase air contaminations and dry up the incubating material.

-A mechanical shaker may prove useful for anamorph induction by agitation (see below).

-A sink or drain, for the continuous water-flow system.

-a ca. 2 x 1.5 m workbench, on which there should be:

-a cool-white and **NUV** light frame: often needed either before or during sporulation. The plates are spread underneath.

As this takes up much space, it may be worth testing if lateral irradiation of plates is effective. The light tubes could then be placed vertically and the dishes stacked up on the sides.

Two smaller frames would allow for different light regimes run simultaneously.

-A source of pressurized air-flow for anamorph induction (see under "aeration" below).

-Extra space and sockets will be needed in the room for wheeling in either the dissecting or microscopy benches, or the continuous water flow module (see below), for which there should be a ramp instead of a step, and a wide door.

4- **The microscopy room**:

-One U-shaped station per researcher, made up of three separate modules: the material is first taken to a **dissection bench**, then to the **microscopy bench** and finally to the **preservation bench**. The benches should preferably be on wheels for ease of transport for example into the constant-temperature rooms, or into teaching laboratories for demonstrations. The seat should be height-adjustable and wheeled.

1- The **dissection bench** (on the right for right-handed workers) should have

-the **dissecting microscope** within

-a horizontal **air flow cabinet** (without a front shield). There should be enough depth and height for installing optical fibre lighting for the dissecting

scope and photographic and drawing equipment.

Underneath the bench:

-a **drawer unit** for dissecting equipment.

2- The central **"microscope bench"**, which should be vibration-free for microphotography. On it will be:

-The **compound microscope,** equipped with phase-contrast, DIC and fluorescence optics, and microphotographic and drawing equipment, all of it necessary for descriptive purposes.

3- The **"preservation bench"** is meant for preparing material for herbarium use after having been observed.

The space underneath these two benches is available for:

-**cabinets** and **drawer units** for microscopy accessories, films, etc.

-**Cupboards** for:

-taxonomic literature

-working collections (microscope preparations kept in slide boxes)

-and reference material: photograph, negative, contact and colour slide collections, photocopies of published figures and of original drawings.

5- **The herbarium**, with:

-Light- and preferably fire-proof cabinets for storing microscope slide boxes, specimens in FAA (formalin-acetic- alcohol), dried culture collections and field material.

The herbarium should be damp- and pest-proof.

PURE CULTURE

Taxonomic characters are derived from: the **vegetative phase** and/or the **anamorph(s)** and/or the **teleomorph.** Some aspects will be briefly discussed before detailing the procedures.

1-VEGETATIVE GROWTH. Spore germination and colony growth requirements need not be the same, and should be considered separately.

Germination requirements are generally met with seminatural media such as 0.1% MA (with or without antibiotics), or possibly with water agar, although conidia of a number of species consistently fail to germinate on this, and others do so only occasionally (e.g. *Flabellospora acuminata* Descals). It is possible that some antibacterial antibiotics may be toxic at the concentrations normally used, or that MA lacks some nutritional requirement(s).

Colony characters are mostly obtained under artificial conditions, i.e. on semisolid (agar-based) media in closed vessels (Petri dishes) which impede gas exchange and elimination of noxious metabolites. But such characters are nevertheless of taxonomic use at the present.

Some of the more commonly recorded ones are colony growth rate and topography, morphology of the aerial mycelium, margin outline, mycelial or diffusible pigments, concentric zonations affecting vegetative or reproductive processes, surface matting, sclerotia and other survival structures, odours, crystals, exudations, etc.

Although Ingoldian fungi are assumed to be saprotrophic, there is ample evidence for substrate preferences in nature. Furthermore, a number of species remain undescribed in pure culture because of excessively slow and restricted growth, at least in some cases due probably to nutritional deficiencies in the media. There is here much scope for research.

Ingold (1942 and later papers) grew his "aquatic hyphomycetes" on **2% MA**, and this still seems to be a standard growth medium for description. Some colony pigments, such as the melanins

of numerous Ingoldian fungi, are not produced if the malt extract concentration is reduced. A number of other taxonomically useful pigments, as well as other characters, are also better expressed on 2% MA than on weaker media, and it should be used if only for this purpose.

Maintaining fertile cultures of Ingoldian fungi is cumbersome and labour-intensive because subculturing must be with conidia. Consequently culture collections are not normally maintained, even in most of the larger public institutions.

2-ANAMORPH INDUCTION

Cultures of Ingoldian fungi, in contrast with those of most other culturable fungi, seldom produce conidia without being placed in contact with water, except in some cases when weaker sporulation media, e.g. 0.1% MA, are used. If conidia happen to be formed (e.g. in *Heliscus lugdunensis* Sacc. et Sydow, *Lemonniera terrestris* Tubaki, etc.), conidia may be of different morphology than when submerged. Portions of the colonies therefore need to be transferred to other containers (e.g. Petri dishes or conical flasks) and partly or wholly submerged in free water. The various procedures currently used are time-consuming and subject to contamination.

An ideal sporulation chamber for Ingoldian (and possibly many other) fungi still has to be designed. It should at least meet the following requirements: 1- its walls should be transparent to the light wavelengths required for sporulation; 2- staling compounds (both volatile and soluble) should be readily removed; 3- it should allow for aerial and aquatic (superficial or submerged) sporulation; 4- sporulating material (i.e. free conidia or whole conidiogenous structures, as well as any fruit bodies) should be easily sampled without the chamber becoming contaminated.

A simple polystyrene or Pyrex container, such as a small tissue culture flask, with inlets and outlets for continuous water and gas flow should meet most of the above requirements.

An improvement of the continuous water flow chamber used by Descals *et al.* (1976), which also enables microscopic observation, would be of great value for monitoring conidiogenesis, an important source of taxonomic information. For this purpose, at least part of the chamber should provide good optical resolution (i.e.: be less than 1 mm deep).

3- Even after longer incubation, **TELEOMORPHS** seldom develop in culture, although microconidia presumed to have a spermatial role are seen more often. The cause(s) for failed or incomplete sexual reproduction may be genetic (the required mating types may not be present in single-spore isolates) or environmental (e.g. light requirements), and need intensive study. Multiascospore isolates of several Ingoldian species have sometimes produced ascomata after extended incubation of semisubmerged colony portions exposed to NUV + cool white light (Fig. 16).

The techniques for preparing teleomorphs obtained in pure culture for taxonomic study are those detailed above for natural substrates.

RECORDING TAXONOMIC CHARACTERS

A simple card file, or. alternatively, a database operated from a lap-top computer beside the microscopes, are necessary for recording the progress of cultures through the vegetative and reproductive growth phases. For example: on each card write the isolate code, the

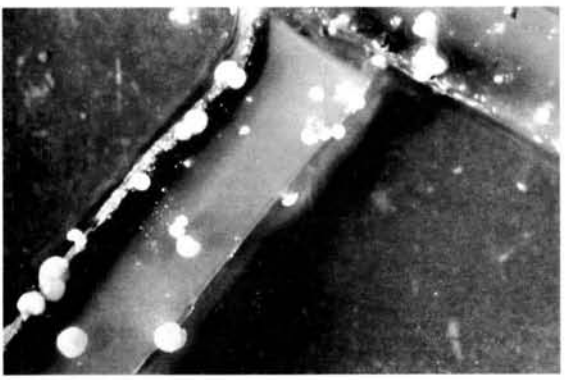

Fig. 16. Apothecia of *Mollisia* sp. developing on slowly drying slice of monoconidial isolate of *Filosporella* cf. *annelidica*.

Fig. 16. Apoteci de Mollisia *sp. desenvolupant-se sobre una llesca prima d'un cultiu monoconidial de* Filosporella *cf.* annelidica..

much cheaper paper tape (used by painters) may also be used. The latter has the disadvantage of being opaque, and thus contaminant colonies along the periphery of the dish are not so quickly spotted. Parafilm needs to be cut into strips before spreading along the rim of the dish.

Sealing with adhesive tape can be done quite expediently and neatly in the following way:

-place the inverted plate on the bench.

-A stretch of tape somewhat longer than the perimeter of the dish is pulled out (without cutting). (The length of tape needed may be marked on the bench).

-The tape roll is laid on the bench on one side of the dish.

-The tip of the tape is affixed on its side

-and the dish, resting on the bench, is rolled around the tape and this then cut off.

dates and the various operations or observations, e.g.: pigment development, radial growth rate, conditions of incubation for both vegetative growth and reproduction (asexual or sexual), location of conidiophores and conidia with respect to water, presence of secondary conidia and herbarium information, such as the number of microscope slides prepared and their contents (e.g. whether developing stages or detached conidia).

CONTAINERS FOR PURE CULTURE

-Conical **flasks** (of mostly 1 and 2 liter volumes).

-Polystyrene **Petri dishes**.

Adhesive tape is needed for sealing Petri dishes, as cultures of most Ingoldian fungi grow relatively slowly and would otherwise dry up or become contaminated.

The use of a 2.5 cm wide transparent tape is probably the fastest way of sealing dishes neatly, but Parafilm or

1- THE VEGETATIVE PHASE

PROCEDURE

-With a felt pen, **draw** a diametric line, or a radius of standard length (e.g. 5 or 2.5 cm respectively) on the underside of the Petri plate. This may be done to record colony growth rate (see below).

-**Point-inoculate** centrally onto the plate.

(Mass inoculation, done by spreading a conidial suspension or mycelial macerate over the surface of the medium, may be convenient for obtaining fully grown colonies in a very short time. This technique is commonly used when carrying out physiological experiments).

-**Seal** the plate.

-**Label** the lid of the plate with the isolate code, the date and a tiny habit sketch of the source spore, to give you an idea of its shape and size.

This is useful for selecting the magnification at which conidia will be searched for. Smaller Ingoldian conidia may only be detected with 100x magnification.

-**Incubate** the cultures under diffuse light and at room temperature or, if characters have to be recorded more accurately, under controlled temperature and light conditions.

-**Check** every other day first, and then weekly, for fungal contaminants (especially near the edge of the dishes).

Some fungal contaminants may not be detected until much later (e.g. when cultures are submerged and one then finds different kinds of conidia). It is conceivable that a contaminating sterile mycelium may never be detected.

-In the sterile air cabinet, **subculture** contaminated plates, or cut out contaminated portions of the medium if still small.

Some yeasts and bacteria are difficult to remove, as they follow hyphal growth close behind the tips. Other antibiotics should then be tested.

-Record **radial growth rate**: e.g. the diameter (or the radius) of the colony when this has covered the line drawn under the dish, or after a fixed number of days. Note the date and incubation temperature along this line.

-Check for **fruit bodies**: teleomorphs, pycnidia/spermodochia, etc.

-Record **pigment** development (on 2% MA).

-Scan carefully for **mycophagous mites.**

These can easily infest plates, even if sealed. Mites seem to prefer dematiaceous cultures. They may leave very conspicuous meandering tracks among the condensation droplets under the lids of the dishes or contaminant colonies on the agar medium. Mites will quickly lay eggs, which can be detected on the colony under the dissecting microscope. If a single mite is found, the whole collection must be checked and the incubation rooms and cultures fumigated or sprayed with acaricides. (Mite control should in any case be done routinely).

2- ANAMORPH INDUCTION

Miura & Kudo's (1970) medium has been suggested as an alternative to 0.1% MA for sporulation, but further work is needed.

Two methods of induction are currently used (i.e.: unchanged standing and aerated water), and three more are suggested (agitated water and continuous water renewal, either as flowing or as dripping water). All involve putting the mycelium in contact with free water to partly simulate natural conditions. Below is a summary of the five techniques, for easier contrast.

1- **Standing unchanged water**: a relatively small portion of the stock culture on agar medium is placed in SDW (20-30 ml) in a 9 cm diam polystyrene Petri dish.

Exposure of the cultures to cool-white and NUV lights may be important for inducing conidial production, one of the most striking responses to NUV being that of *Mycocentrospora acerina* (Hartig) Deighton.

The standing water technique is popular because: 1- it is simple; 2- it allows for detecting conidia with the dissecting microscope and sampling without too much damage to the culture; 3- it gives the culture a chance to sporulate above or at the water surface, and 4- if

done in a microscope chamber (e.g. the hanging drop technique) it allows for direct monitoring of conidiogenous processes. But a significant proportion of species sporulate poorly this way, if at all, most probably because staling compounds are not eliminated from the water or colony. In most cases, as with natural substrates, sporulation levels reach a pronounced peak within roughly a couple of days, followed by an irreversible decline and eventual interruption. In fewer cases, conidia develop slowly for several weeks (e.g. *Anavirga dendromorpha* Descals & Webster, *Casaresia sphagnorum* G. Fragoso).

2- **Aerated unchanged water**: if the culture does not sporulate in standing water, a portion of the colony may be submerged in aerated water, e.g. in 250 ml SDW in a glass conical flask connected to a forced sterile-air system. This enhances sporulation in some species, and apparently even triggers it in others. The main objections are that conidial development cannot be followed *in situ* because of turbulence, the staling effect is present, preparing the flasks and sampling for conidia is cumbersome the contents of the flask often becoming contaminated with bacteria. The effect of aeration has not yet been explained, although it may help disperse sporulation inhibitors from the hyphal milieu through turbulence.

3- **Agitated water**: this technique has not been tested critically. It seems to have the same effect as aeration in some species, but the set-up (a bench shaker) and manipulations are simpler. However, there will still be a staling effect.

Conidial production would probably improve with the above three techniques if water were replaced periodically. The following technique is promising and deserves further testing:

4- The **continuous water renewal** technique, by means of a flow system (Descals, 1978; Descals *et al.*, 1976) has proven successful with a number of Ingoldian species (Sanders & Webster, 1980). If incubation is done in an observation chamber (Fig. 17), it allows for direct observation of conidiogenesis. A continuous supply cf SDW may be ap-

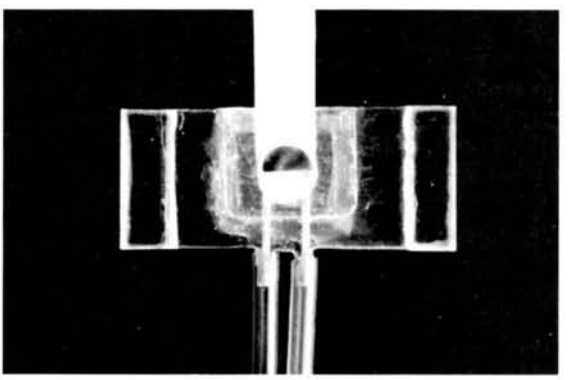

Fig. 17. Induction of conidiation in continuously changing water: a coverslip is placed on the bottom of the observation chamber and sealed with hot paraffin; half a disc from an agar colony is placed on this coverslip, a second coverslip is placed above the half-disc and sealed in the same way. The observation chamber is placed under the objective and water is then allowed to flow slowly in and out of the chamber. *Fig. 17. Inducció de conidiació en aigua amb renovació contínua es col.loca un cubre objectes a la base de la cambra d'observació i es segella amb parafina calenta; la meitat d'un disc d'una colònia d'agar es posa sobre el cubre i un segon cubre es col.loca sobre el mig disc i es segella de la mateixa manera. La cambra d'observació es posa sota l'objectiu i després es deixa fluir l'aigua lentament dintra i fora de la cambra.*

plied through gravity flow or by means of a peristaltic pump.

5- If the cause of optimal sporulation is simply the elimination of staling compounds, it may suffice to just apply a **continuous drip** system (Kegel, 1906).

The last three techniques will not be discussed further.

Synanamorphs, as already mentioned, are known in a number of Ingoldian fungi, and may be easily confused with **Penicillium-like** contaminants. But they develop mostly underwater (although some may form aerially). They may appear in small, scattered patches and may thus be difficult to detect.

PROCEDURE FOR ANAMORPH INDUCTION:

A- STANDING WATER

If one only intends to confirm identifications of known species, one may submerge batches of up to **50 cultures,** as most likely not all will sporulate at the same time. However, for careful, time-consuming taxonomic work (describing, illustrating, preserving, etc.), one should submerge less cultures.

-Fill 1-litre conical flasks or bottles up to about 3/4 with DW and autoclave. (Larger containers may be too heavy for pouring comfortably).

-In the **air flow cabinet** of the dissection bench: put the cultures to be sampled for submersion, an equal number of empty sterile polystyrene Petri dishes, SDW (calculate ca. 0.5 liter per 15 plates), 90% alcohol for flaming, a broad-bladed scalpel, a spatula and a felt pen.

-Condensation droplets under the lid of the Petri dish bearing the culture may interfere with vision. They may be **shaken off** by knocking the dish sideways firmly several times, for example against one's free hand.

-Alternatively, **replace** the lid during observation with a dry one. A glass lid is recommended, as it may be flamed after each use to avoid cross contaminations.

-First scan the plates under a dissecting microscope and record the presence of **fruit bodies** (apothecia, perithecia, pycnidia, spermodochia, synnemata, etc).

Do not describe material from these cultures until you are sure that the above fruit bodies do not belong to contaminants.

-**Unseal** the culture dish: run a hot scalpel along the tape, underneath and around the rim of the lid. Hold the scalpel perpendicularly to the side of the dish as you slice around the tape, to keep the blade from pushing slivers of tape onto the agar and contaminating the culture.

-Look for **conidiophores** or **conidia** (e.g. *Heliscus lugdunensis, Lemonniera terrestris*, etc.). produced prior to submersion. Also look **inside the agar.** *Goniopila monticola* (Dyko) Marvanová & Descals, for example, may produce clusters of conidia here, especially in media with high water activity (e.g. 0.1% MA).

Conidiogenous structures of Ingoldian fungi produced aerially may need to be described, as they are often morphologically distinct from aquatic ones.

-If the colony has developed much aerial mycelium, which is often the case on 2% MA, with the flamed scalpel first **scrape** some off the area which will be sampled.

Cottony aerial mycelium traps air and the sampled portions of culture do not sink easily in water. Nevertheless, in some species sporulation takes place on aerial mycelium.

-With the flamed scalpel, **slice across the colony** down to the base of the dish and from the centre to the edge. Cut a large piece and at least one very thin (Fig. 18).

Sampling from across the colony radius is important, as sporulation sometimes is better on either older or younger mycelium, depending on the species. Sporulation mostly occurs on the cut surfaces. This wounding effect needs to be studied.

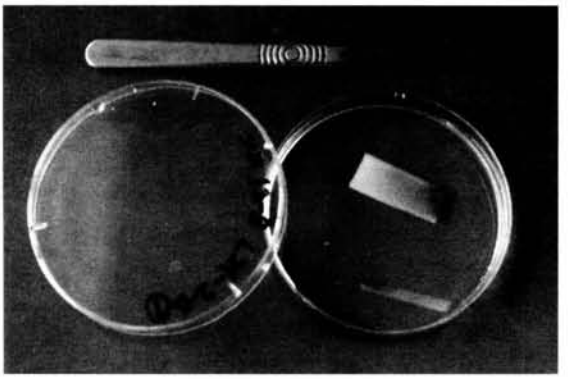

Fig. 19. Thick slab and thin slice of colony, placed in standing water.
Fig. 19. Llesques gruixada i prima d'una colònia col.locades dins aigua estanca.

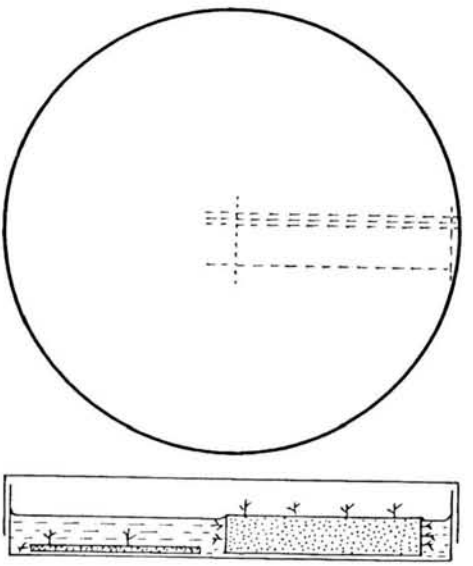

Fig. 18. Induction of conidiation in standing water: the colony is sliced more or less radially with a flamed scalpel, the thin colony slice settles on the bottom of the dish, while the broad one sits semisubmerged.
Fig. 18. Inducció de conidiació en aigua estanca: la colònia es talla més o manco radialment amb un microescarpell prèviament flamejat. Les llesques primes de la colònia s'esfonsen mentres la llesca gruixada roman semisubmergida.

It is possible that some species need to reach **physiological maturity** for sporulation. In others, however, sporulation occurs even a couple of days after conidial germination.

-With the scalpel, **transfer** the colony slices and large piece of the colony (preferably in one operation) to an empty sterile Petri dish (Fig. 19).

-**Add SDW** to this dish.

To do this, stack the Petri dishes in sets of 10 and start pouring SDW from the bottom plate upwards. Put a few dummy dishes underneath the stack, which makes it easier for pouring water into the lower plates.

When placed in water, the thin colony slices should sink and allow for underwater sporulation. The large piece should remain **exposed** at the top, to also enable sporulation at the water surface or aerially.

A number of Ingoldian fungi do not sporulate underwater but do so at the surface, at least under the described

conditions. There is a need to study these aspects.

-One could **adjust the pH and conductivity** of the SDW to approach those of the source stream, especially if this is strongly alkaline, acid (moorland water pH may be very low) or saline (e.g. high in sulphates). The pH should then be monitored during incubation, as it may change drastically.

-**Label** the lid of this "sporulation plate" with the three usual data: isolate code, date of submersion and a sketch of the inoculum spore.

It is worth testing **filtered stream water**, as it may stimulate sporulation.

-Before incubation, with the dissecting microscope briefly **scan** the sporulation plates for presence of aerially produced conidia, as they may have been missed on the original plate, especially if small or produced in low numbers.

-**Incubate** the dishes at 15 - 20°C (for cold-temperate species) in the cool-temperature room, under NUV + cool white light.

Higher temperatures may be needed for species from warmer climates. 15ºC may be below the optimum even for temperate species, but it slows down sporulation processes allowing more time for working with cultures. (It is not known whether this slowing down of the sporulation rate may significantly affect mode of conidiogenesis and/or anamorph morphology in some species).

-**Check** the plates under the dissecting microscope for sporulation. This may be necessary in some species even after only 24 h (e.g. *Anguillospora rosea* sp. ined.).

It is suspected that a significant change of temperature (going from 15ºC in the cool-temperature room to for example 25ºC in the microscopy room) may upset sporulation at least in some species, and possibly irreversibly. If this is the case, wheel the dissection bench into the constant-temperature room and observe there or, alternatively, induce sporulation at room temperature.

-First **scan the colony** for possible presence of teleomorph initials.

If present, they should be allowed to continue development under the same environmental conditions.

-**Scan the water** from surface to bottom for free conidia, using the appropriate enlargement on the dissecting microscope.

-If there are none or only a few, proceed with the incubation.

-Compare conidial dimensions and degrees of branching between the time of release and at later stages, if there is any suspicion that these may vary.

-**Scan** the edges of the submerged slices and the edges and exposed surface of the large semi-exposed piece of colony for conidiogenous structures.

-**Sample, describe** and **preserve** the specimens (see below).

The conidiophores should also be described after **conidial proliferation**, (which may take a few days), as this is an important diagnostic character.

Some species require many days to initiate sporulation. Others seem to sporulate only on new mycelium produced in contact with water, and this may also take some time to develop and mature.

B- AERATED WATER

MATERIALS

-Pressurized air flow system in the constant-temperature room.

If pressurized air ducts are not available in the cool- temperature room, an air pump may be used. Small commercial aquarium pumps (Fig. 2) will

supply enough pressure for several aeration flasks.

-Connect the pump to rubber or latex tubing with T-connectors. The free ends of the tubes are then connected to the air filters on the aeration flasks. There should be a clamp for each flask, to regulate air flow.

-Aeration flasks:

-Attach ca. 10 cm of latex tubing to one end of a commercially produced **air filter or cartridge**. A cheap substitute is a 10 x 2 cm glass tube stuffed with cottonwool and with both ends flame-pulled to produce necks that will allow connecting to the latex tubing.

-Connect a piece of **glass tubing** (which may be a Pasteur or a volumetric pipette) to the other end of the latex tubing. The glass tube should be inserted almost to the bottom of the aeration flask.

-Add 150-200 ml **DW** to a 250 ml conical flask.

-**Plug** the flask by rolling a strip of cottonwool around the top of the glass tube and fit the cotton plug snuggly into the neck of the flask. You should be able to lift the flask from the air-filter without the plug coming off.

-Prepare as many flasks as cultures will be submerged.

-A piece of **aluminium foil** wrapped over the cottonwool plug after this has been fitted in the neck of the flask helps prevent aerial contamination when longer incubations are foreseen. But remember that incoming air will have to get out.

-**Autoclave** flasks for 30 min. at the standard 121°C.

Store flasks in a dust-free cabinet and allow water to cool before introducing the culture pieces.

PROCEDURE

-In the air-flow cabinet, **lift** the lid of the culture dish and

-**cut** several thin, radial slices from the centre to the margin of the colony.

-**Open** the aeration flask and flame the neck.

-**Transfer** the colony pieces.

-Again **flame** the neck and

-**replace** the cotton plug.

-When all flasks have been inoculated, **connect** them to the pressurized air system in the cool-temperature room.

-Switch on the pump and **regulate** the rate of forced air flowing into the flasks.

Water should bubble very gently. Excessive bubbling will cause too much evaporation, damage the mycelium, wet the cottonwool plug (on which contaminants will grow), and project conidia onto this plug. Bubbling rates should be checked regularly, as they tend to vary with time.

-**Incubate** as for standing water.

-To check for sporulation **decant** a few ml of suspension into a clean glass Petri dish.

Floating conidia may stay behind, and therefore some swirling immediately prior to decanting will bring them into suspension.

When sporulation is heavy, conidia tend to accumulate on the inner walls just above the level where the bubbles break. Resuspend by swirling the flask before decanting.

-Allow a few minutes for suspended conidia to **settle** in the dish, for easier localization.

-Proceed as for standing water.

Alternatively, conidial production in the flask may be monitored through an inverted microscope.

If sterile conditions are not needed, as for aeration of leaves or wood from streams, a simple and inexpensive sys-

tem may be devised: use 250 ml soft-drink plastic bottles with screw caps. Perforate these and introduce the tubing from the aquarium pump. Seal around the hole with a quick-setting cement. The outgoing air flow is regulated by tightening or loosening the screw cap.

CONCENTRATING SPORES IN WATER

Conidia may not be in high enough numbers for observation, description, identification or counting. This is the case with stream water samples, and often also after laboratory incubation.

There is therefore a need for **concentrating** spores, and this may be done by at least one or any combination of the following techniques:

- membrane filtration
- sedimentation
- evaporation by means of:
  - heat
  - ventilation
  - vacuum
- centrifugation
- impaction
- foaming by means of detergents

Membrane filtration is practically the only technique used by mycologists working with Ingoldian fungi. It is accurate, simple and fast and may be carried out in the field with stream water.

Its handicaps are: 1- conidia have to be stained (phase contrast or differential interference contrast optics (DIC, e.g. Nomarsky) do not work) and sit on an opaque or granular background; the optical resolution can be seriously affected and detail needed for critical identification may be lost. This is the case even with transparent filters produced by Millipore; 2- if too much vacuum is applied, the conidia may be mangled and thus more difficult to identify; 3- in many cases conidia do not lie flat on the filter, which also impairs identification; and 4- it is an expensive technique for many countries.

The other techniques, or combinations of them, would seem to be cumbersome at first thought, but do not appear to have been thoroughly tested and therefore optimized.

Sedimentation is a standard technique used on phyto- and zooplankton by limnologists. Its main handicap is that the sedimented samples have to be observed with an inverted microscope, where resolution is not the best. But this could possibly be solved if sedimentation is carried out in a burette and the bottom portion collected and observed.

If water samples have to be large in order to be representative (e.g. half to one liter), as is the case in field studies, **evaporation** would seem too slow a technique.

But it could be combined with sedimentation as follows: the bottom volume (e.g. 50 ml) of the spore suspension collected from a burette could be placed on a disc of cellophane attached by its margins onto a Petri dish (for example with some silicone rubber) and air dried in front of a fan heater (this would take ca. 2 h). The spores adhered to the cellophane could then be mounted under coverslips placed directly on it and observed under the microscope, or the cellophane disc could be lifted from the Petri dish and pieces of it cut up and mounted on slides.

Centrifugation is possible with even a simple table centrifuge. The sample would then have to be resuspended (this might cause damage), dried on slides and mounted as above.

Impaction onto an adhesive surface, for example by placing an adhesive-coated slide in an aerated or agitated water sample, deserves testing.

The use of **artificial foam** has been discussed above, although the technique does not seem easily quantifiable.

The above techniques would need **pre-treating** the conidia for the following reasons:

Some conidia may be **floating**, and will need sinking if a representative sample is to be taken. Sinking techniques have not been studied, although the use of heat, possibly in a microwave oven, might be successful. Anti-foaming agents might also be effective.

Conidia often have mucilaginous ends which render them **adhesive**. The percentage conidia that may be lost through manipulation is not known, but could be significant. Techniques for neutralizing the mucilages have not been worked out, but KOH (added as pellets) appears to be effective. This treatment should of course not precede the impaction technique mentioned above.

A third problem is rapid conidial **germination**. But this can be easily controlled by chemical fixation.

The membrane filtration technique will be detailed below, as it is the standard one used by ecologists for sampling stream water.

MEMBRANE FILTRATION

MATERIAL

-filter membranes: 5-μm pore membranes are traditionally used.

-Stainless steel **filtration equipment** (e.g. Millipore) is expensive but preferred over more delicate glass for field work.

-A **hand-operated pump**: this is commercially produced by various companies. A simpler, less costly model is used in hospitals for body fluid extraction (Fig. 20).

-**Lactofuchsin**

-Waterman's or other **water-soluble ink**, diluted.

-**Immersion oil.**

-Polystyrene Petri dishes (as many as samples), preferably stacked in

-**acarrier rack**.

-Half- and one-liter plastic **measuring cylinders.**

Conidia are supposedly less prone to adhere to glass walls, but this needs further study.

PROCEDURE

-Collect stream water in the measuring cylinder and filter a representative volume with its replicates.

-**Filter** with the minimum necessary vacuum to avoid deforming the more delicate, long-limbed spores.

-Place 2-3 drops of **lactofuchsin** on the base of a clean, empty Petri dish.

-With forceps, **lay the membrane** upright on the mountant drops. These will eventually soak through. (Do not place the mountant on the membrane, as spores may be washed away).

Fig. 20. Hand-operated pump (without manometre) available from hospital suppliers.
Fig. 20. Pompa manual (sense manòmetre) que proporcionen els distribuïdors als hospitals.

-**Cover** the dish, **label** and place in the carrier rack.

In the laboratory:

-**Cut a piece** of the membrane filter, the size of a coverslip, with scissors. Wash the scissors between samples.

-Gently lower a **coverslip** on the membrane, without trapping air.

-Infiltrate from the side of the coverslip a small amount of mountant if the membrane appears dry.

-**Scan** a number of randomly selected fields (which will depend on the conidial concentration).

-Convert the **spore concentration** for this known surface to that per liter stream water. Do this by measuring the diameters of the field of view and of the portion of the membrane filter containing the conidia (Iqbal & Webster 1973b).

If the membrane filter is to be rendered transparent:

-Instead of lactofuchsin, use dil. Waterman's ink.

-Air-dry.

-Soak in immersion oil.

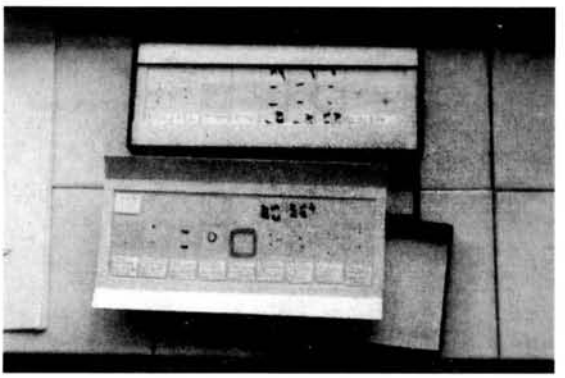

Fig. 21. Microscope slides arranged on flat trays in herbarium box.
Fig. 21. Portaobjectes ordenats sobre palanganes planes en una caixa d'herbari.

IV- HERBARIUM PRESERVATION

MATERIALS

-20x20 or 22x22 mm **coverslips**.

-**Slide boxes**: semipermanent slide mounts are normally kept on commercially produced flat trays in rigid cardboard slide boxes (Fig. 21).

If the preserved material has been allowed to air-dry on the slide prior to mounting, and thus become affixed to the glass, the slides could be kept vertically in standard slotted slide boxes, which save space and are cheaper. But they would then not be as easily accessible and rearranged.

-**Vials** with screw caps for preserving spore suspensions (e.g. liquefied foam).

The ideal container should be gas- and liquid-tight, with rustproof screw-caps which should be easy to reopen.

-**Paper envelopes** (or "crystal bags") for storing dried cultures. The envelopes should be long enough to allow the open end to be folded over the dried culture (Fig. 22).

-**Cardboard boxes** for storing **dried cultures**.

-**Herbarium cabinets**.

-**Pesticides**.

PRESERVING LIQUEFIED FOAM

Foam samples are normally preserved as microscope mounts (see above), but extra samples may be kept in FAA or other preservative, or possibly even as dried up deposits.

PRESERVING DRIED CULTURES

Because unsubmerged colonies of Ingoldian fungi do not normally

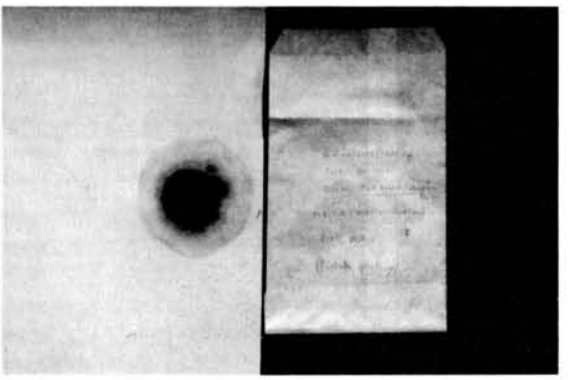

Fig. 22. Dried culture and negative envelope in which it will be placed.
Fig. 22. Cultiu secat i sobre de negatius on es guarda.

sporulate, they are only useful for their vegetative characters. Dried cultures, however, if protected from dust, may eventually become valuable for DNA-based taxonomic techniques.

-After having been sampled for sporulation, the left-over agar culture is **air-dried** by opening and placing the Petri dish (preferably in a vertical position to reduce dust deposition) in a clean, ventilated room. Drying may take several days. Eventually the dry colony will be reduced to a thin gelified film which will readily detach itself from the base.

Heat or forced ventilation should not be used for speeding up the drying process, especially at its end, as the agar film will curl up severely.

Alternatively glue and dry the culture on a cardboard square, which may be labelled on the reverse.

-**Store** the dried culture in a labelled negative bag or crystal envelope.

-**Arrange** these in boxes in the herbarium cabinet and protect chemically from mycophagous weevils.

PRESERVING CONIDIA

Conidia produced in culture may be preserved as semipermanent slide mounts, or in FAA, or air-dried in the same sporulation dishes.

1- Preserving **conidia on slides**:

Conidia should always be sampled while the material in the sporulation chamber is intact, i.e. prior to sampling for developing stages. This is especially important when conidia are large and delicate because, when removing conidiogenous structures from mycelium (see below), some of the material may be damaged; e.g. conidial branches may be broken off, or conidia may be ripped off their conidiogenous cells, thus erroneously appearing to be seceded rhexolytically.

1a- If conidia are not very sparse:

-With a felt pen, **mark** with a circumference (roughly the width of the coverslip) the area where the conidia will be placed on the slide. (This may not be necessary if the spore concentration is high, as the dry deposit will be visible).

-**Place** the slide on the bench with the circumference underneath.

-**Transfer** a few drops of the conidial suspension onto the enclosed area. Use a wide loop for floating conidia and a Pasteur pipette for submerged ones. Alternatively, first sink floating conidia to obtain a representative suspension (see above) and sample only once.

-**Air-dry** the slide with gentle heat from a table lamp or fan heater.

-**Mount**, **seal**, **label** and **store** the slide as usual.

1b- If only very few but valuable conidia are present, one can save much scanning time as follows:

-With a felt pen, **mark** a small circumference (ca. 3 mm diam.) underneath a slide.

-With the flamed upper tip of a handle, place a tiny drop of **water** on the circle.

-**Lift** conidia with a mounted hair and place in the drop.

-**Air-dry** and **preserve** as above.

-Alternatively, suspended conidia may be transferred individually with a capillary pipette on the slide and air-dried as above.

-The exact location of smaller conidia may be marked later by inking dots on the coverslip around them with a fine felt pen or with a drawing pen.

2- **Preserving conidia in FAA**

There does not seem to be published information on this, but presumably conidia from pure culture could be fixed and kept indefinitely in vials with FAA or a stained mountant, as for stream foam.

3- **Preserving air-dried conidia**

Excess conidia may be preserved by air-drying them on the inside of the inverted dish lid (which is shallower than the dish base and therefore takes up less space in negative envelopes). For this:

-First **treat** conidia to induce sinking, to neutralize their mucilages and to fix them (see above).

-If necessary, **concentrate** conidia (see above).

-**Place** some of the spore suspension in the inverted lid of the Petri dish.

-**Air-dry** with the fan heater.

-For later observation directly on the lid:

-**Locate** an area with conidia.

-Place a small drop of **mountant** on a coverslip.

-**Turn over** the coverslip, rest one end and lower gently over the conidial deposit to avoid trapping air.

-Remove the **mechanical stage** on the compound microscope.

-**Observe** conidia on the microscope stage.

Note: Differential interference contrast optics may not be satisfactory when observing through polystyrene Petri dishes, as evidenced by the strong colour aberrations when the polarizer is rotated.

If conidia have become anchored onto the base of the dish, it is difficult to release them without damage. Also, when there are very few conidia it may be too time-consuming to transfer them individually to slides. One can then dry the material in the sporulation dish base itself, and preserve it as follows:

-**Pre-treat** the conidia as above.

-**Uncover** the dish, nesting the base on the inverted lid to retain the isolate data written on the latter.

-**Air-dry** as above.

-**Preserve** the dish in a negative envelope.

-To save space, the lateral walls of the dish may be **trimmed off** with strong scissors, or with a hot scalpel or wire.

PRESERVING CONIDIOGENOUS STRUCTURES

-Place a large drop of **DW** on the centre of a clean slide.

-Under the dissecting microscope, **locate** an area of the colony piece with conidiogenous structures.

When sporulation is at the surface, e.g. on the large colony piece, and if the water level is slightly too low, the resulting meniscus around this may interfere with vision, and one should then add some more water.

-With a microscalpel, and holding the colony piece with a needle or forceps, **detach** the conidiophore(s) with a minimal amount of mycelium.

a- If the sporulating material is made up of dense forests of **small conidiophores**:

-**Transfer** the material onto the drop of DW on the slide.

-With lintless tissue paper, **draw off** excess water in the drop.

The material will then sit on the glass surface and not slide away when manipulated.

-Secure it on the slide with a needle.

-With microscalpel and needle, **slice** the colony piece into several smaller pieces.

-**Spread** these over an area slightly smaller than that of the coverslip.

-**Rest** the coverslip over this material.

-To **flatten** the preparation, you may need to apply slight pressure, but taking care not to displace the coverslip laterally, as this may upset the natural arrangement and branching patterns of the conidiophores and/or conidia.

Alternatively **heat** gently over a flame. The agar will melt suddenly and the mount will flatten itself. This technique works well, but sometimes the conidiogenous structures will have been distorted and mangled by the hot melting agar as it spreads. The heat may in some cases damage or shrink the cell contents.

Another way of applying gentle heat to a slide preparation is by rubbing the underside with a second heated slide (J. Webster, pers. comm.).

-When all the observations in water have been completed, the slide may be preserved by:

-infiltrating a small drop of **mountant** from the side of the coverslip and

-**warming** gently over a flame.

-Excess liquid is **drawn** from the opposite end with tissue paper.

-**Wipe** the edges of the coverslip, **seal** and **store** as usual.

b- If conidiophores are very long and delicate, they become badly tangled when manipulated. Instead:

-**Transfer** the previously detached material to the slide with an unpulled (wide-mouthed) Pasteur pipette.

-The excess liquid on the slide will roll off by slightly **tilting** the slide.

-This is then **wiped off** with some tissue paper.

-With two needles, **spread out** the conidiophores very gently.

This is often tedious, as conidiophores may adhere to each other and to the instruments.

Alternatively, conidiogenous structures may be **removed** from the sporulation chamber thus:

-Hold a coverslip with **forceps**.

-**Submerge** the coverslip slowly under the previously detached sporulating material, which will be suspended in the water.

-**Secure** this material on the coverslip with a needle while slowly lifting it from the water.

-**Turn over** the coverslip and lower onto a microscope slide.

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