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ARTICLE

Ascospore discharge, germination and culture of fungal partners of tropical lichens, including the use of a novel culture technique

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Abstract: A total of 292 lichen samples, representing over 200 species and at least 65 genera and 26 families, were collected, mainly in Thailand; 170 of the specimens discharged ascospores in the laboratory. Generally, crustose lichens exhibited the highest discharge rates and percentage germination. In contrast, foliose lichen samples, although having a high discharge rate, had a lower percentage germination than crustose species tested. A correlation with season was indicated for a number of species. Continued development of germinated ascospores into recognizable colonies in pure culture was followed for a selection of species. The most successful medium tried was 2 % Malt-Yeast extract agar (MYA), and under static conditions using a liquid culture medium, a sponge proved to be the best of several physical carriers tested; this novel method has considerable potential for experimental work with lichen mycobionts.

Key words:
Ascomycota
colony development
mycobiont
seasonality
Thailand

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INTRODUCTION

The highest species diversity for most groups of organisms lies in the tropics. Lichenized fungi do not appear to be an exception, as Sipman & Aptroot (2001) estimated that between one-third and one-half of the world's lichen diversity occurs there, and suggested that 50 % of the tropical lichen biota remained unknown. Yet there have been few experimental studies on ascospore discharge, germination, development of mycelia, and physiology of the fungal partners (mycobionts) of tropical lichens compared with those on temperate species. This is a major gap in our understanding of even basic aspects of the biology of tropical lichens.

The first cited studies on the isolation of lichen-forming fungi are generally those of Töbler (1909) and Thomas (1939), although Töbler was primarily interested in the re-synthesis of lichens from their individual symbionts (Turbin 1996). However, Werner (1927), innovatively examined the effect of different media and additions on the growth of selected mycobionts from a range of lichens. Subsequent workers have concentrated on the development of methods for lichen re-synthesis (Ahmadjian 1964), and later Ahmadjian *et al*. (1980) and Ahmadjian & Jacobs (1981) produced the two most successful protocols (Bubrick 1988). Crittenden *et al*. (1995) were the first to attempt the isolation of a wide range of fungal partners of lichens, and also lichenicolous fungi, on a worldwide basis, although their material was predominantly from non-tropical regions. More recently, Yoshimura *et al.* (2002) reviewed the protocols available for isolation and cultivation of fungal and algal partners of lichens, emphasising studies by Japanese researchers, but again based largely on non-tropical material. A brief synopsis of methods used is provided by Stocker-Wörgötter & Hager (2008), with an emphasis on the production of extrolites ("lichen substances" or "secondary metabolites").

The lack of basic information on the isolation and growth of the fungal partners of tropical lichens provided the rationale for the present study. We investigated ascospore discharge from a wide range of tropical lichens in order to make a preliminary assessment of the conditions under which discharge occurred, and whether there could be any seasonal correlations. Observations on factors affecting germination and subsequent development on solid, or in liquid, growth media are also reported, since these are virtually undocumented for the fungal partners of tropical lichens. Our studies were carried out to identify apparent trends and issues that merited in-depth investigations, as well as testing the efficacy of alternative culture methods.

Table 1. Lichen collections according to locality and growth form type.

Location	Code	Number of samples	Crustose	Foliose	Erect shrubby or pendent
Thailand					
Chiang Mai Province	CM	27	15	11	1
Chiang Rai Province	CR	5	5	–	–
Kanjanaburi Province	KJB	8	8	–	–
Nakon Sithammarat Province	TS, RPB	6	6	–	–
Ratchaburi Province	SP	7	7	–	–
Khao Yai National Park	KY	208	140	60	8
Phu Kradueng National Park	PKD	5	4	–	1
Sakaeraj Research Station	SKR	2	2	–	–
Huai Kha Khaeng Wildlife Sanctuary	HKK	4	4	–	–
Cambodia	CAM	5	4	1	–
Vietnam	VN	15	15	–	–
Total		292	210	72	10

MATERIALS AND METHODS

Taxon sampling

The collection of samples began in 1998, and concentrated on Khao Yai National Park (KY), central Thailand. The remainder were collected during field surveys to Doi Suthep (18°49' N, 99°53' E) and Chiang Dao (l9°40' N, 99° E) in Chiang Mai Province (CM), Mae Fah Luang Arboretum (20° N, 99.5° E) Chiang Rai Province (CR), Sai Yok District (14° N, 99° E) Kanjanaburi Province (KJB), Khao Sok National Park (8° N, 99.5° E) Nakon Srithammarat Province (TS and RPB respectively), Phu Kradueng National Park, (16.8° N, 101.8° E) Loei Province (PKD), Suan Phueng District (13.5° N, 99° E) Ratchaburi Province (SP), and Sakaeraj Research Station (14° N, 102° E) Nakhon Ratchasima Province (SKR). Some collections from Huai Kha Khaeng Wildlife Sanctuary Kanjanaburi Province, Vietnam (VN) and Cambodia (CAM) were donated by colleagues and friends. Khao Yai National Park was visited monthly during one year (1999–2000) for seasonal observations and experiments to explore the development of thalli, and also to ascertain if there were seasonal differences in ascospore discharge and spore viability

Samples were cut into pieces, wrapped in tissue paper, and placed in individual strong brown paper bags. These were then returned to a survey house workroom and cleaned of attached soil or other extraneous material. Each sample was given a collection number, and information on the locality, substratum, and collection details were recorded. Then. if the specimens could not be immediately transferred to the laboratory for pre-isolation treatment, they were either kept in a cool place, or (where available) a domestic refrigerator, until they could be transferred. In the laboratory, samples were air-dried at room temperature (30 °C) overnight, and then transferred to new paper envelopes with identification labels and stored in a domestic refrigerator at 4 °C until the isolation protocol had been completed.

Specimens were identified as precisely as possible on the basis of their morphology, anatomy, and chemical constituents (determined by standard thin-layer chromatographic methods; Orange *et al.* 2001). In many cases it was not possible to fully determine the samples to species as identification remains a major problem in tropical lichenology. The basic monographic treatments required to provide a sound taxonomic basis for studies of lichen distribution, ecology, and physiology are still lacking for most lichen families and genera in the tropics. Species that have not previously been described are also likely to be found; Homchantara & Coppins (2002) described 26 species of *Thelotremataceae* as new to science from Thailand[1], and Aptroot *et al*. (2007) added 300 tropical species to the national list, of which 12 were new to science.

The number of collections made for each morphological type of lichen, together with their geographical locations, are summarized in Table 1, while full details of selected collections for which positive results were obtained are given in Table 2. A list of the material collected is included as Supplementary Information (Table S1, online only) and in Sangvichien (2005). Voucher specimens are maintained in The Lichen Herbarium, Ramkhamhaeng University, Bangkok (RAMK).

Spore discharge and germination

The specimens were removed from storage, and surface-cleaned with air from an aerosol camera blower to remove any remaining soil and debris. A sterile surgical blade (Gowlands No. 11) was used to dissect specimens to obtain small portions with ascomata, and the remainder of the samples were then returned to storage. The process was repeated if the first isolation attempts were unsuccessful. The portions of lichen with ascomata, or occasionally only a single ascoma, were attached with a small quantity of petroleum jelly onto the inverted lid of a 9 cm diam Petri dish (Sterilin). The spores were allowed to shoot upwards onto an overlying layer of Tap Water Agar (WA; Booth 1971). The agar surface was examined daily with a stereozoom binocular microscope (Olympus SZ11), and once ascospores had been discharged,

[1]Eleven of these have since proved to be synonyms of previously described species (Papong *et al.* 2010).

small agar blocks (3–5 mm^2) with ascospores on the surface were excised and transferred to Malt-Yeast extract Agar (MYA; Merck or Oxoid). Germination of ascospores was assessed under the stereozoom microscope; observations were made daily for 7 d, and subsequently twice weekly. If no germ tubes had been observed after six weeks, then "no-germination" in that collection was recorded. Germinated ascospores were maintained at room temperature for further studies on growth and colony morphology, or were used as inoculum for liquid media.

In order to investigate the seasonality and discharge of ascospores, thalli were collected each month from the same trees in Khao Yai National Park over a one year period, and their discharge patterns and rates of germination were determined for each monthly sample following the protocol described above.

The distance to which ascospores were discharged was studied in a representative sample of 15 species. Clear plastic boxes 18 x 7.5 x 5 cm were used with a layer of tap water agar in the lid of the box. Ascomata samples, approx. 0.3 mm diam, were attached to one vertical microscope slide (2 x 4 cm) which was shallowly immersed in the agar layer. The box was then incubated on the laboratory bench at an ambient temperature of 25–30 °C, with approximately 12 h of daylight. The agar surface was examined under an Olympus stereozoom microscope (Model SZ 11) daily for 5 d. If no spores were discharged within 3 d, the procedure was repeated, and then, if after a second 3 d period no discharge was observed, the protocol was repeated for a third and final time.

We also investigated the effects of relative humidity by incubating Petri dishes in plastic moist chambers containing different saturated solutions to maintain the relative humidity at particular levels, following Kaye & Laby (1966): potassium nitrate (92 %), ammonium sulphate (80 %), and sodium nitrate (65 %).

We tried, but did not adopt, the surface sterilization protocol of Crittenden *et al*. (1995) as we found it to be detrimental to ascospore discharge in the tropical lichens tested; in consequence, untreated lichen samples were used throughout.

Fungal culture on solid media

MYA (see above) was the medium of choice for all cultures of the fungal partners, but Potato Dextrose Agar (PDA), Corn Meal Agar (CMA), Oatmeal Agar (OMA), and Czapek-Dox Agar (CDA), were also used to determine the optimum medium for growth. For recipes see Booth (1971).

Fungal culture in liquid media

Malt Yeast Extract Broth (MYB) was selected as the standard medium for studies of the isolated fungi in liquid culture, since good growth rates of several fungal partners had been observed on solid MYA. MYB has also been favoured by previous researchers (e.g. Hamada 1989, Honegger 1990, Yamamoto *et al*. 1998). Static culture was most frequently used, and following initial trials with direct inoculation of pieces of the fungal cultures into the liquid medium, different types of physical support for the fungi on the surface of the liquid were tested.

Four types of material were evaluated: (1) Stacked Membrane filters (pores 0.22 µm diam; polyvinylidene fluoride, PVDF) were promising when tested first, but the slippery surface when floating on the liquid rendered them difficult to inoculate. (2) Whatman No.1 filter papers were tested in order to overcome the problem of stacked layers. (3) Kraft paper was tried as an alternative to Whatman No.1. And (4), synthetic sponge (polystyrene) pieces 2.5 x 2.5 x 0.3 cm, together with pieces of fungal colonies cut from solid media of 0.4 x 0.4 cm, placed on the surface of these materials, and floated on the surface of 50 ml MYB in 250 mL Erlenmeyer flasks. Observations were made twice daily and, at the same time, the flasks were gently swirled for 10–15 s to circulate the medium.

Since poor aeration could be a factor limiting growth, the effect of increased aeration was tested in two ways. First, air was supplied by an aquarium air pump and passed through a sterile filter (Sartorious, Sartofluor®; pores 0.2 µm diam) to prevent contamination. Second, flasks were placed on an orbital shaker (Innova 4230, New Brunswick). Shake cultures were prepared using inocula produced in the same way as for static cultures, and transferred to 250 mL Erlenmeyer flasks containing 50 mL of MYB. The orbital shaking incubator was set at a speed of 200 rpm, and at a temperature of 30 ± 0.5 °C.

Scanning electron microscopy

Specimens for scanning electron microscopy (SEM), either intact ascomata or cultures of the isolated fungal partners, were fixed in 5 % glutaraldehyde and dehydrated in a graded ethyl alcohol series. The specimens were then attached to aluminium stubs using either Dag metallic paint or adhesive carbon pads to prevent electron charging of the specimens. The samples were gold-coated using a Sputter Coating Unit (Polaron RU-SC7620) and examined either with a Jeol 840 SEM, a Jeol SEM5410LV, or a Leo 1455VP scanning electron microscope. Digital images were produced using an image-capture system (Röentec) or with accessories of Leo 1455 VP.

RESULTS AND DISCUSSION

This study employed a large number of samples in order to gain an impression of possible general features of ascospore discharge and development in tropical lichens to provide a basis on which to determine directions future investigations might take. As replicates were not used for most of the species, the conclusions must be viewed as preliminary and treated with caution. Nevertheless, some indications of trends emerged, although we recognize that further work may require their modification or refinement. This caveat must be borne in mind with respect to this discussion of our results.

The number of crustose lichen collections made was much larger than that of foliose lichens (Table 1). Crustose

Table 2. Lichen collections, ascospores discharged, germination (%), and colony development in selected species. The classification follows Lumbsch & Huhndorf (2010).

Taxon	Collection number	Ascospores discharged	Germination (%)	Colony development
ARTHONIALES				
Arthoniaceae				
Arthothelium sp.	KY175	11	100	2
LECANORALES				
Cladoniaceae				
Cladonia submultiformis	KY117,118,119	> 500	100	1
Haematommataceae				
Haematomma puniceum	KY107	NA	ND	0
Haematomma sp.	VN3	NA	ND	0
Lecanoraceae				
Lecanora cenisia	SP4	47	0	0
Lecanora intumescens	CM27	NA	ND	+
Lecanora leprosa	SP6	310	0	0
Lecanora polytropa	KY177	47	92	0
Pyrrhospora sp. 1	CR8	NA	ND	+
Parmeliaceae				
Parmelina sp.	CM33	50	0	0
Relicinopsis sp.	KY81	NA	ND	0
Relicinopsis sp.	CM32	191	0	0
Usnea complanata	CM12	> 500	0	0
Pilocarpaceae				
Sporopodium argillaceum	VN16	2	50	+
Ramalinaceae				
Bacidia subannexa	SP10	> 900	89	0
Bacidia sp. 1	SP12	190	21	0
OSTROPALES				
Graphidaceae				
Cyclographina sp. 2	KY390	23	57	0
Glyphis cicatricosa	SP7	12	100	3
Glyphis cicatricose	KY231	NA	ND	+
Graphina cleistoblephara	KY129	96	98	+
Graphina hiascens	KY160	69	99	3
Graphina sp. 2	KY104	NA	ND	+
Graphina sp. 5	KY157	124	91	+
Graphina sp. 9	KY124	10	90	+
Graphina sp.18	KY171	28	100	3
Graphina sp. 19	KY91	NA	ND	+
Graphina sp. 20	KY180	186	90	+
Graphis afzelii	CR5	NA	ND	+
Graphis albocolpata	KY147	NA	ND	+
Graphis analoga	HKK7	> 1000	100	0
Graphis apertella	HKK4	255	100	0
Graphis apertella	SP2	307	259	0
Graphis elegans	KY162	73	99	3
Graphis kakaduensis	TS3	435	100	0
Graphis librata	RPB1	760	100	0

Table 2. (Continued).

Taxon	Collection number	Ascospores discharged	Germination (%)	Colony development
Graphis rigidula	KY165	> 500	100	3
Graphis rimulosa	CR3	5	100	3
Graphis xanthospora	TS2	56	100	0
Graphis sp. 10	KY148	111	94	3
Graphis sp.	KY133	5	0	0
Graphis rimulosa	SP1	180	100	0
Graphis rimulosa	KY133	NA	ND	+
Graphis sp.	SP9	> 1000	100	3
Gyrostromum sp.	KY161	26	96	+
Ocellularia s. lat. sp.	KY173	129	89	3
Phaeographina caesioradians	HKK1	450	100	0
Phaeographina quassiaecola	VN6	1	100	3
Phaeographina sp.	CAM5	1	100	3
Phaeographina sp. 4	HKK2	> 1000	100	3
Pheopgraphis melanostalazans	KY144	113	88	3
Phaeographis melanostalazans	KY121	NA	ND	+
Phaeographis pyrhochora	PKD4	42	100	3
Phaeographis sp. 27	KY229	NA	ND	+
Sarcographa actinobola	KY205	NA	ND	+
Sarcographa labyrinthica	KY240	NA	ND	+
Thelotrema s. lat. sp. 3	KY233	NA	ND	+
Thelotrema sp. 4	VN9	1	0	0
Thelotrema s. lat. sp. 5	KY245	NA	ND	+
Thelotrema sp. 6	VN10	1	100	+
PELTIGERALES				
Nephromataceae				
Nephroma sp.	CM30	15	67	0
PERTUSARIALES				
Pertusariaceae				
Pertusaria sp. 4	PKD2	18	100	2
PYRENULALES				
Pyrenulaceae				
Pyrenula sp.	KY208	NA	ND	+
Pyrenula sp.	KY230	NA	ND	+
Pyrenula sp.	KY249	NA	ND	+
Pyrenula sp.	KY95	NA	ND	+
TELOSCHISTALES				
Physciaceae				
Rinodina sp.	KY169	NA	ND	+
Teloschistaceae				
Caloplaca sp.	CR6	2	0	0
TRYPETHELIALES				
Trypetheliaceae				
Campylothelium sp.	SKR2	NA	NA	+

Table 2. (Continued).

Taxon	Collection number	Ascospores discharged	Germination (%)	Colony development
Laurera benguelensis	KY61	197	99	3
Laurera madreporiformis	KY14	NA	ND	+
Laurera megasperma	KY238	NA	NA	3
Laurera meristospora	KY195	NA	NA	3
Laurera subdiscreta	SKR1	NA	NA	+
Pseudopyrenula diluta	KY113	NA	ND	+
Trypetheliaceae sp.	KY135	39	100	3
Trypetheliaceae sp.	KY131	63	87	+
Trypethelium eluteriae	KY66	82	100	3
Trypethelium ochroleucum	KY235	NA	ND	+
INCERTAE SEDIS				
Unidentified	KY141	46	83	+
Unidentified	KY143	6	50	+

NA = not available, ND = not detected.

Colony development : 0 = none, 1 = poor, 2 = moderate, 3 = good , + = germination but with premature cessation of growth.

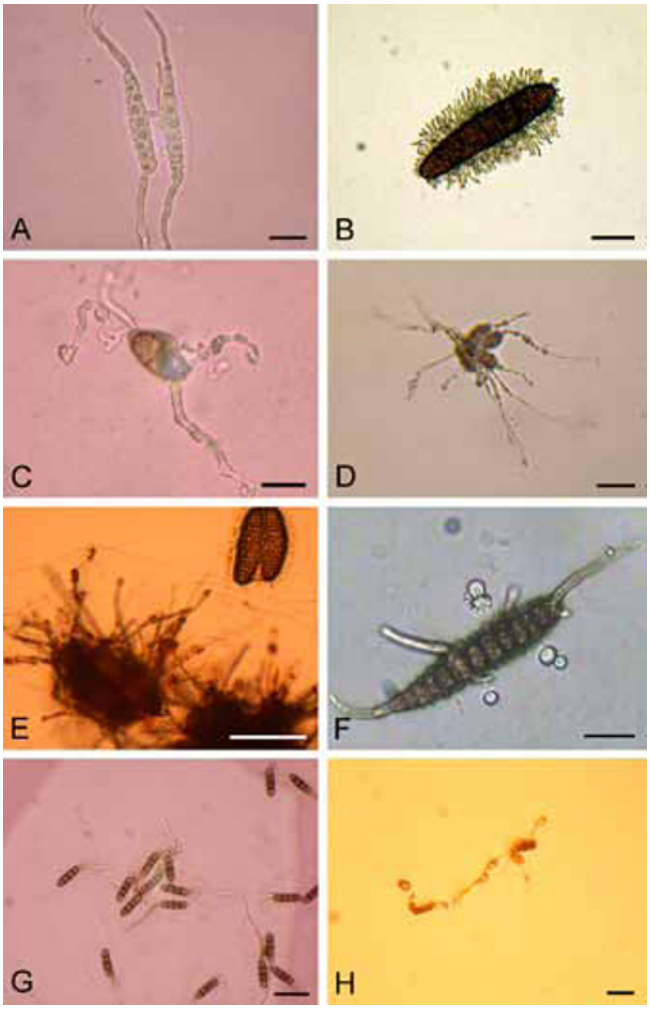

Fig. 1. Ascospore germination in selected species. **A.** *Glyphis cicatricosa* (KY231). **B.** *Phaeographina montagnei* (KY263). **C–D.** *Pyrenula* sp. (KY95 and 208). **E–F.** *Trypethelium eluteriae* (KY66). **G-H.** *Trypethelium tropicum* (KY131).

lichens were preferentially selected for experimentation as preliminary studies suggested that their ascospores germinated more readily on artificial media. Ascomata of erect shrubby (fruticose) and pendent lichens were much less common than crustose or foliose ones, especially at Khao Yai National Park, and so could not be investigated further.

Of the 292 lichen samples collected (Table 1), 170 samples liberated ascospores in the laboratory, and in several instances successive samples of the same lichen exhibited high percentage germination rates (Table 2, Fig.1). Crustose lichens exhibited the highest rate of spore discharge, and also subsequent germination. In contrast, foliose lichen species (e.g. *Heterodermia diademata*) exhibited a high discharge rate, but only a low percentage of ascospores germinated.

Seasonal influences on ascospore discharge and germination were explored in selected species (Fig. 2). In *Trypethelium eluteriae* (KY 66), *Graphis elegans* (KY162),and *G. rigidula* (KY165), ascospores were discharged readily each month, and spores from each monthly sample also germinated. However, in contrast, *Cladonia submultiformis* (KY117) discharged ascospores only towards the end of the winter (January-February), and in the summer (April-June) none were discharged (Fig. 2).

Following germination, the ability of the isolated fungi to continue to develop and form colonies was investigated. In some common crustose lichen species, the fungal partners grew well and produced small colonies within a few months (Fig. 3). Species of *Trypethelium* and *Laurera* developed colonies readily, while in *Haematomma wattii* and *Lecanora intumescens* the spores germinated but growth was either very slow or soon ceased. In addition to growth on solid media, liquid culture under static growth conditions was tried, but generally growth was slow. Further, when the fungi were inoculated onto the surface of membrane filters floating on the surface of MYB, this was not satisfactory as

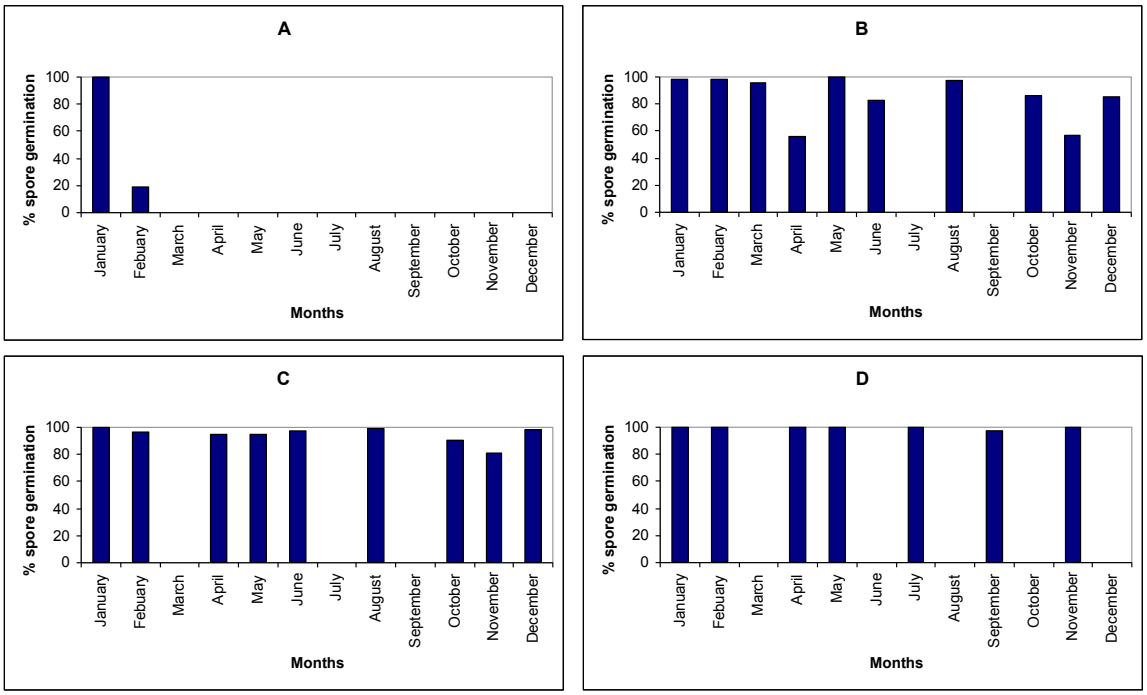

Fig. 2. Apparent seasonal effect on ascospore discharge and germination on selected species. **A.** *Cladonia submultiformis* (KY117). **B.** *Graphis elegans* (KY162). **C.** *G. rigidula* (KY165). **D.** *Trypethelium eluteriae* (KY66).

the membranes often sank following inoculation, or tended to collapse after a period of growth. Amongst the other physical carriers tested, were segments of unbleached Kraft paper or Whatman filter paper several layers thick. Growth occurred on the surface of these, but it was only possible to assess this visually as it proved impossible to physically separate all the fungal material from their surfaces. In contrast, the sponge pieces tested as alternative carriers facilitated growth after incubation in the fungal partners tested; in most cases, sponge proved to be superior to the other carriers tried (Fig. 3).

No contamination by spores from other fungi during discharge were encountered; the distinctive ascospores from the lichens were deposited, either as small packets of spores or as single spores, and could easily be recognized for subculturing using a stereozoom microscope.

Our results suggest that high spore discharge rates are correlated with the freshness of the samples and season of collection, as well as the state of maturity of the ascomata. Spore germination also appeared to be correlated with species distributions. Widely distributed species, such as *Trypethelium eluteriae, Laurea bengualensis*, and most *Graphidaceae* studied, exhibited relatively high rates of germination.

There was, however, considerable variation in ascospore discharge between the species tested, and, also between different collections of the same species. In *Laurera bengualensis* and *L. madreporiformis*, spores were readily discharged in all of the collections examined, but in *L. meristospora*, although discharge occurred, it was at a much lower rate. In *Trypethelium eluteriae,* spore discharge occurred throughout the year, but in *Cladonia submultiformis,* although ascospores were also readily discharged, only those from the end of the winter season (February) germinated. This suggests that in some lichen-fungi, seasonality is important even in the tropics. These observations of differences in spore discharge between species are in agreement with those of Crittenden *et al*. (1995), based mainly on samples from non-tropical regions.

The distance of discharge of ascospores is important for the dissemination of the species (Table 3). Of the 15 species tested, those of *Graphina* sp. 9 (KY104) were discharged the furthest, to 63 mm. This figure compares with the maximum of 45 mm obtained for the temperate *Rhizocarpon umbilicatum* (Bailey & Garrett 1968). However, in that study no information was given as to the distance attained by the majority of spores, which is perhaps the most pertinent parameter in relation to effective dispersal and establishment in nature. There was a wide variation even within the 75 % ranges of projection in many cases, and we speculate that this could be an adaptation to increase the probability of contact with a suitable new substrate. In nature, air turbulence currents and wind would also be expected to influence the final distance travelled.

Environmental conditions also appeared to influence ascospore discharge. At 15 °C any discharge was rare, occurring only in *Arthopyrenia* sp. (PKD3) and *Laurera subdiscreta* (SKR1), while at 45 °C no discharge was observed in any species tested (Table 4). As might have been expected for tropical species, most species discharged at 30 °C and 35 °C, but the mean optimum discharge temperature for all species was close to 25 °C. Relative humidity also appears to be important, with three of the four species investigated discharging at relative humidities from 65 %

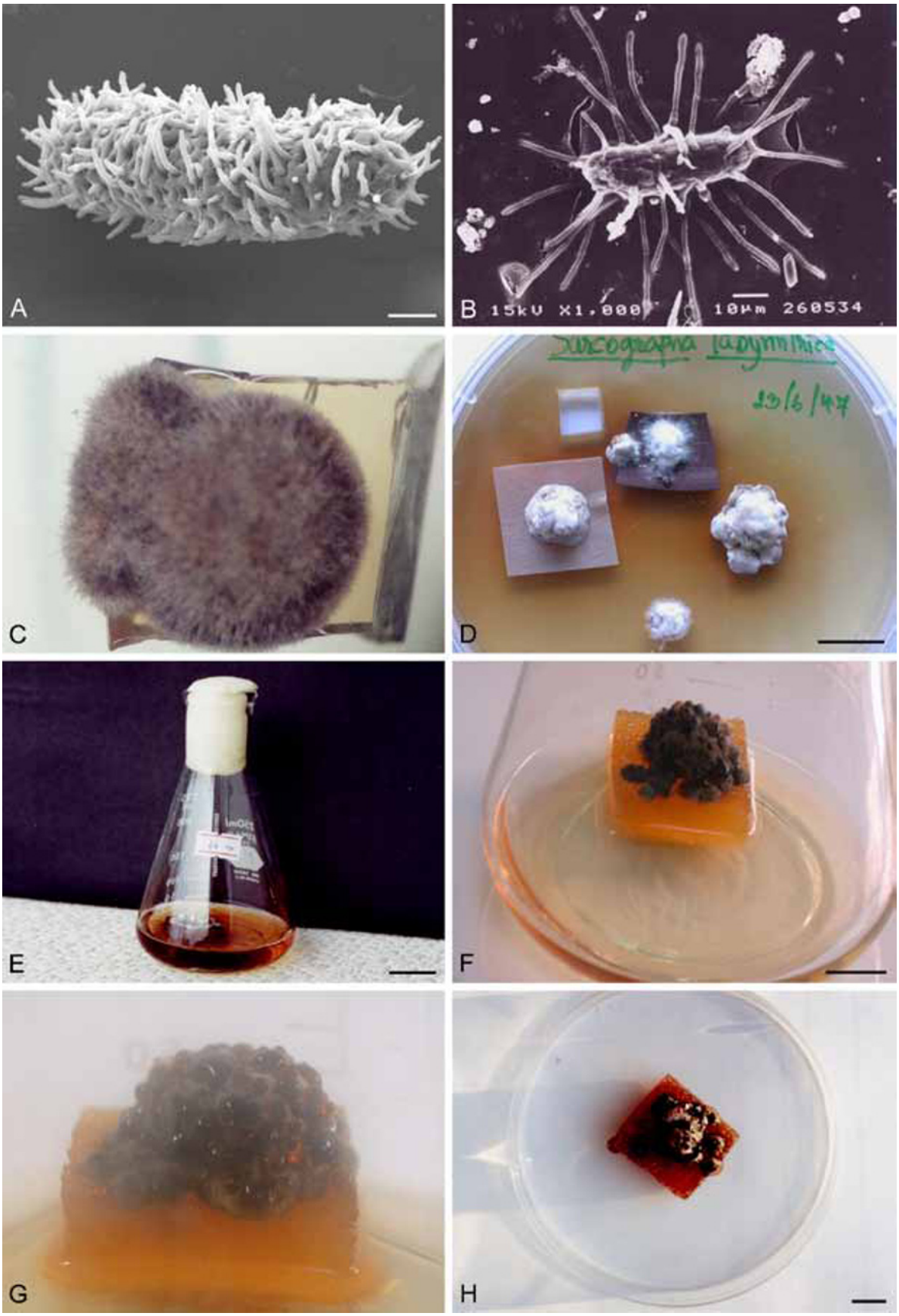

Fig. 3. Development and growth of selected species. **A.** SEM of germinating ascospore of *Cyclographina platyleuca* (KY390). **B.** SEM of germinating ascospore of *Phaeographina chapadana* (KY474). **C.** Colony development of *Thelotrema* sp. on 2 % MYA agar (VN10). **D.** Colony development of *Sarcographa labyrinthica* (KY240). **E.** 2 % MYB broth showing colony development of *Trypethelium eluteriae* (KY131) under static conditions. **F–H.** Colony of *T. eluteriae* (KY131) developing on sponge in 2 % MYB broth under static conditions. Bars: A = 10 µm; E = 3 cm; F, H = 1 cm.

Table 3. Ascospore discharge and distance of projection from selected lichens studied (arranged in descending order).

Species	Collection number	Number of ascopores discharged	Distance ascospores projected Minimum – (average range of 75 % spores) – Maximum (mm)
Graphina sp. 2	KY104	52	5–(10–50)–63
Thelotrema s. lat. sp. 3	KY233	114	7.5–(10–29)–40
Graphina sp. 20	KY180	78	2.5–(3.5–20)–38
Graphina hiascens	KY160	38	14.5–(15–21.5)–36
Graphina sp. 10	KY217	57	15–(15–31)–34.5
Sarcographa actinobola	KY205	62	2.5–(5–25)–26
Graphis elegans	KY162	154	3–(6–15)–24.5
Glyphis cicatricosa	KY231	183	3.5–(7.5–21)–24
Pyrenula sp. 6	KY206	81	8–(10–15)–21.5
Pheographis sp. 27	KY229	67	5–(9–15)–21.5
Graphina sp. 9	KY124	4	12.5–20
Trypethelium ochrolecum	KY235	19	4–(4–10)–18
Sarcographa labyrinthica	KY240	102	3.5–(3–15)–18
Graphis albocolpata	KY147	95	5–(5–9.5)–14.5
Buellia sp. 5	KY220	31	1–(1–9.5)–12.5

Table 4. Ascospore discharge from selected species over the temperature range 15–45 °C.

Species	Collection number	15 °C	20 °C	25 °C	30 °C	35 °C	45 °C
Arthopyrenia sp.	PKD3	+	+	+	+	+	–
Graphina sp. 9	KY124	–	+	+	+	–	–
Graphis albocolpata	KY147	–	–	+	+	+	–
Haematomma puniceum	KY108	–	+	+	–	+	–
Laurera subdiscreta	SKR1	+	+	+	+	–	–
Pertusaria sp. 4	PKD2	–	+	+	–	+	–
Phaeographina pyrrhochroa	PKD4	–	+	+	–	+	–
Phaeographina sp.	CAM5	–	+	+	+	–	–
Pyrrhospora sp. 1	PKD1	–	+	+	+	+	–
Trypethelium eluteriae	KY79	–	+	+	+	–	–

to 100 % (Fig. 4). However, *Laurera subdiscreta* (SKR1), *L. benguelensis* (KY 61), *Pyrenula* sp. (KY95), and *Graphis* sp. 3 (KY260), discharged only at 100 % relative humidity. These results suggest that both temperature and relative humidity, which will vary with habitat and season, influence ascospore discharge in tropical lichens to different degrees, something that would be a major factor in their performance and occurrence in nature.

Ascospore germination appeared also to be linked to species distributions. Widely distributed species such as *Trypetheium eluteriae* and *Laurera bengualensis*, together with most *Graphidaceae* tested, exhibited relatively high rates of ascospore germination. Ascospores of crustose lichens generally germinated readily, whilst those from shrubby and pendent species were much more difficult, or failed to germinate.

Ascospores of the different fungi exhibited several distinctive germination patterns (Fig.1A–H): (1) multiple germination tubes developing from different regions of the spore (e.g. *Pyrenula* and *Arthopyrenia* species, *Graphis cicatricosa*, *Laurera benguelensis, Graphina irabensis*; (2) bipolar germination (e.g. *Trypethelium tropicum*); and (3) multiple germination tubes developing all over the spore from individual segments within them (e.g. *Thelotremataceae*, *Cyclographina platyleuca* KY390/RPB3).

When germination was successful, fungal partners of most crustose species tested grew well on solid media, with small colonies developing within a few months. *Trypethelium* and *Laurera* species generally grew well, but *Haematomma wattii* and *Lecanora intumescens* developed very slowly and growth often ceased – even though the ascospores germinated readily. Growth in liquid culture was generally very slow, and static culture was found to be superior to shake culture for all species tested. However, growth on static liquid culture was much enhanced by the use of a physical carrier. While segments of Kraft paper or Whatman filter paper proved to be successful carriers, sponge pieces were superior in relation to visible enhanced growth. We consider that sponge pieces used as a carrier have a wide potential for studies on the physiology and development of lichen fungi as the colonies can be transferred without disruption

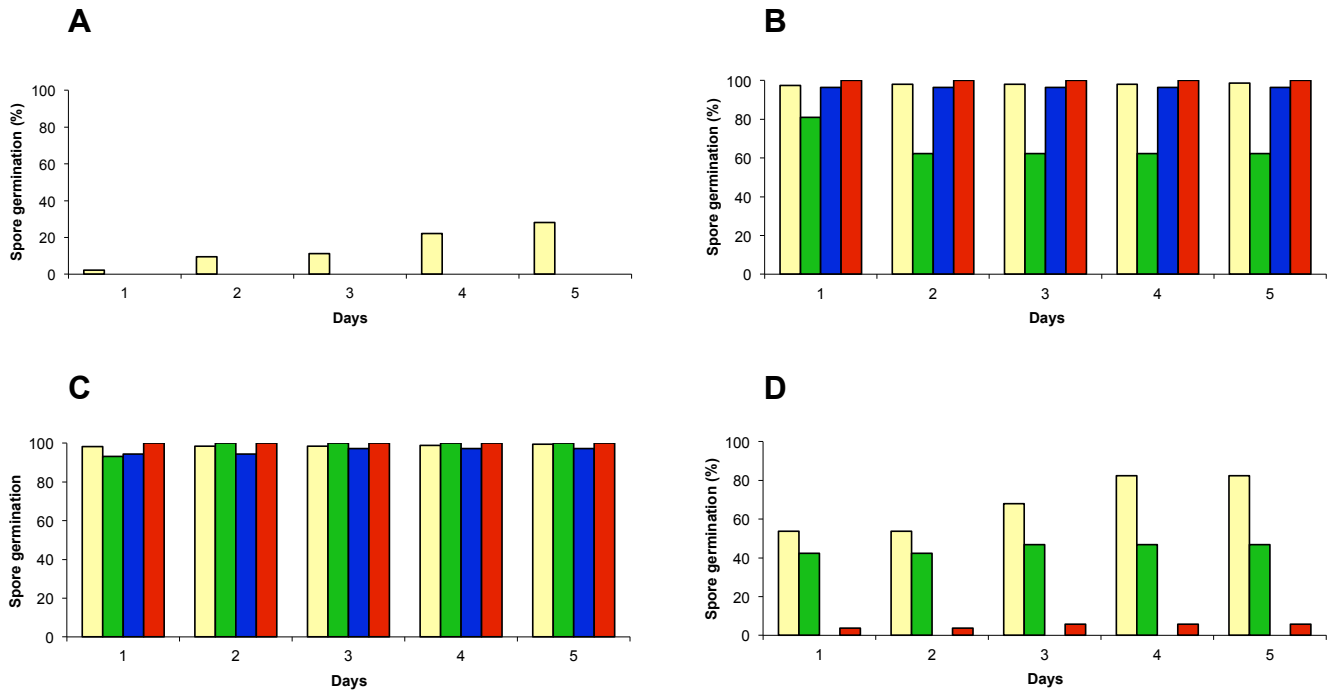

Fig. 4. Ascospore discharge and germination in selected species as influenced by percentage humidity in the experiments. **A.** *Laurera subdiscreta* (SKR1). **B.** *L. benguelensis* (KY61). **C.** *Graphis* sp. 3 (KY 260). **D.** *Pyrenula* sp. (KY95).

to different liquid media. This means that, for example, the effect of different nutrients in the medium on the production of extrolites could be explored. Culberson & Armaleo (1992), in their investigation of *Cladonia grayi*, previously concluded that the production of compounds concentrated in the naturally occurring lichen was linked to the aerial growth habit. Their conclusion was based on the finding that, following the transfer of lightly fragmented mycelia from liquid to solid media, there was a subsequent proliferation of aerial hyphae and extrolite production. Although only a limited investigation of the chemical products of the isolated fungal partners of the tropical lichens was undertaken in our study, comparison of extrolites from the whole lichen thallus with those produced by the fungal partner alone indicated that in some cases more compounds were produced by the whole thallus than in the isolated fungal cultures. This conforms to the findings of a previous investigation (Leuckert *et al.* 1990). However, in *Graphidaceae* little difference between the two was observed. There were also few differences between the compounds produced by the fungus cultured under static conditions compared to those grown in shake culture. In a few cases, however, some additional compounds were detected in the shake culture extracts.

Our results, and those of Crittenden *et al.* (1995) in particular, demonstrate that, contrary to a general belief of recalcitrance to grow on artificial media, it is possible to obtain many lichen-forming fungi in isolated culture – provided that recently collected material is used. Further, our results on ascospore discharge show that the seasonal behaviour and discharge distances of the ascospores of tropical lichens recalls that of those in temperate regions. We also suspect that the short distances over which ascospores are discharged, especially where these are multicelled and large, contributes to the inability of many to spread into secondary environments from old-growth native forests and so facilitates their utility as bioindicators of ecological stability (Wolesley *et al.* 1994).

We hope that this preliminary study will encourage more experimental work on the factors affecting the reproductive biology of tropical lichens, which are crucial to an understanding of their ecology and distribution – especially at local scales.

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