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## A method to promote sporulation in palm endophytic fungi

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Guo, L.D., Hyde, K.D. and Liew, E.C.Y. (1998). A method to promote sporulation in palm endophytic fungi. *Fungal Diversity* 1: 109-113.

A method to promote sporulation in palm endophytic fungi is introduced. Mycelia sterilia isolated from the palm *Livistona chinensis* were inoculated into flasks containing malt extract agar and a sterilised petiole fragment of palm *Livistona chinensis*. Flasks were then incubated under 12 h alternating near UV-light and darkness. After three months, some isolates formed fruiting bodies on the surface of petioles. Some endophytic mycelia sterilia can be identified using this method. A comparison of this method with more conventional methods is also presented.

### Introduction

The most common technique employed in the detection of endophytic fungi in plants involves three steps: (i) surface sterilisation of the plant tissue; (ii) incubation of discs cut from plant tissue on agar and isolation of endophytes which grow out; and (iii) identification of the sporulating cultures by traditional methods. Many endophytes isolated from plant tissues sporulate on different artificial media, under different cultural conditions. Many, however, will not sporulate and are known as mycelia sterilia, and cannot be identified. Petrini, Stone and Carroll (1982) reported that approximately 15 % of endophytes isolated from evergreen shrubs in western Oregon did not sporulate. Espinosa-Garcia and Langenheim (1990) found that about 26.9 % of endophytes isolated from coastal redwood trees were mycelia sterilia. Fisher *et al.* (1994) pointed out that there were various isolation frequencies of mycelia sterilia from *Quercus ilex* from different sites and different tissues. Eighteen percent of endophyte isolates from leaves in Switzerland were mycelia sterilia, while 17.5 % were isolated from leaves in Spain. An even higher frequency of mycelia sterilia (41.3 %) were isolated from twigs of *Q. ilex* in Switzerland. Fröhlich (pers. comm.) found that 12.9 % of endophytes isolated from the palm *Licuala* sp. were mycelia sterilia, while Taylor (pers. comm.) found about 11.2 % of endophytes isolated from palm *Trachycarpus fortunei* were mycelia sterilia. It is therefore extremely important that methods are developed to promote sporulation of

mycelia sterilia for identification purposes. Mycelia sterilia can be identified by promoting sporulation by various means or by using molecular techniques. In this paper we report on a modification of a method introduced by Whalley (pers. comm.) to promote sporulation of mycelia sterilia.

### Materials and methods

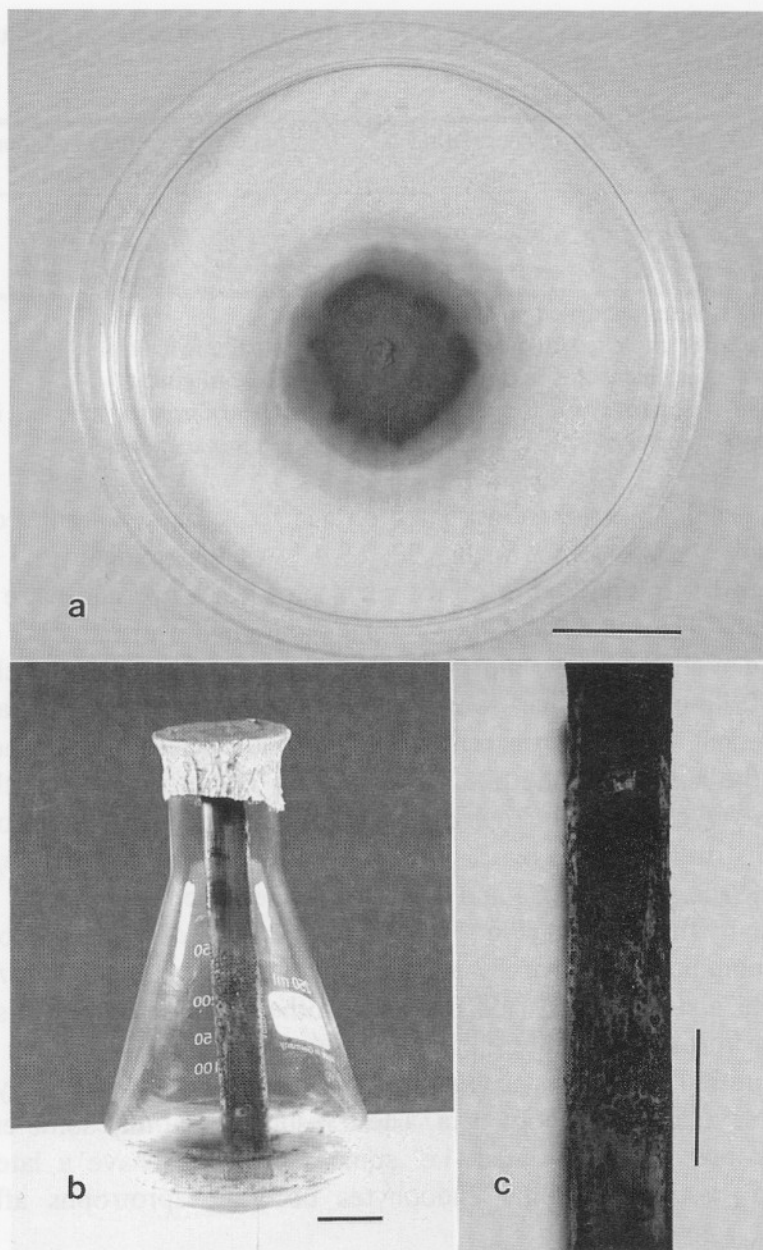
Enophytic fungi were isolated from the chinese fan palm *Livistona chinensis* in Hong Kong. These isolates were first incubated on MEA (malt extract agar) at 25 C, under 12 h alternating near UV-light and darkness. This is because MEA was considered suitable for promoting sporulation in endophytic isolates (Bills and Polishook, 1992). Some of the isolates sporulated and were identified.

Isolates that did not sporulate on MEA agar were then incubated by a second method. Healthy fronds of *Livistona chinensis* were collected in the field, and leaves (inter-vein) were cut into strips (20 × 20 mm) and sterilised in an autoclave at 121 C for 20 min. The isolates were then transferred onto agar plates and a sterilised leaf strip was simultaneously added onto the surface of the agar in each plate, and incubated under the same conditions as the first method. In this case, some isolates produced fruiting bodies on the surface of the leaf strips and were identified.

The remaining mycelia sterilia were divided into 40 "morphospecies" based on their cultural characters, such as colony colour and growth rates, and incubated on 90 mm Petri dishes with MEA at 25 C in darkness. A representative isolate from each of the 40 "morphospecies" was then incubated by a third method. Healthy fronds of the palm *Livistona chinensis* were collected, and petioles were cut into 12 cm lengths and sterilised in an autoclave at 121 C for 20 min. The 1 cm block containing the growing edge from the colonies of each of the 40 "morphospecies" was then transferred into 250 ml flasks containing 50 ml solid MEA (sterilisation at 121 C for 20 min), and a fragment of sterilised palm petiole was simultaneously placed in each flask. These cultures were incubated at 25 C under 12 h alternating near UV-light and darkness and examined periodically. Isolates were identified when they produced fruiting bodies.

### Results and discussion

A comparison of the endophytic isolates of the three incubation methods is given in Table 1. Of the 778 fungal endophytes isolated from the palm *Livistona chinensis*, 372 (47.8 %) sporulated on MEA and were identified. The remaining 406 (52.2 %) isolates did not sporulate and were recorded as mycelia sterilia.



**Fig. 1.** Method to promote sporulation of the endophyte *Astrosphaeriella bakeriana*. **a.** Colony on MEA. **b.** Culture in 250 ml flask with a sterilised petiole fragment of *Livistona chinensis*. **c.** Ascomata on the surface of petiole. Bars = 20 mm.

**Table 1.** Numbers of endophytic fungi isolated from *Livistona chinensis* sporulating using three incubation methods.

Method	No. of isolates	No. of sporulating isolates	Cumulative percentage of sporulating isolates <sup>a</sup> (%)
A	778	372	47.8
B	406	90	59.4
C	316 (40 )	188 <sup>b</sup> (20)	83.5

A = endophyte isolates incubated on MEA agar.

B = mycelia sterilia incubated on agar plates containing a palm leaf strip.

C = "morphospecies" incubated in flasks containing a petiole fragment.

<sup>a</sup> = percentage of isolates sporulating out of the total number of isolates obtained, i.e. 778.

<sup>b</sup> = extrapolated number of isolates from the sporulating "morphospecies".

These 406 isolates were then incubated on agar containing a sterilised leaf strip of *Livistona chinensis* and a further 90 (59.4 % of the original total number of isolates) formed fruiting bodies on the surface of leaf strips and were identified. The remaining 316 (40.6 % of the original total number of isolates) of mycelia sterilia were divided into 40 "morphospecies" based on their cultural characters.

The 40 "morphospecies" were then incubated in flasks with a sterilised petiole fragment, and 20 of the remaining "morphospecies" formed their fruiting bodies on the surface of the petiole fragments. Therefore, half of the species recorded as "mycelia sterilia", which did not sporulate in the first two methods, formed their fruiting bodies by this third method. For example, *Astrosphaeriella bakeriana* (Sacc.) K.D. Hyde and J. Fröhl. formed ascomata on the surface of palm petiole by this method (Fig. 1). *Astrosphaeriella bakeriana* has been found to be very common in the survey of saprophytes of *Livistona chinensis* in Hong Kong (Hyde and Fröhlich, 1998). Several other mycelia sterilia also formed fruiting structures when cultured in this way, including *Guignardia cocogena* (Cooke) Punith. (Hyde, 1995), *Myelosperma tumidum* Syd. and P. Syd. (Hyde, 1993), and several xylariaceous taxa. These results reveal that some endophytes and saprotrophs are interrelated, i.e. some saprotrophs have a latent period inside plant tissues, or some endophytes become saprotrophs after plants senesce.

It is not clear why fungi sporulate more easily on the palm petioles. It may be that the mycelia sterilia can grow up along the petiole, away from the medium in the flask. The mycelia do not grow well on the media, but grown well on the petioles. This method appears to provide a more natural condition for mycelia sterilia to grow and sporulate.



## Acknowledgements

We would like to thank Professor A. Whalley for providing some ideas, which lead to the development of this protocol. L.D. Guo would like to thank The University of Hong Kong for the award of a Postgraduate Studentship. E.C.Y. Liew would like to thank The University of Hong Kong for the award of a Post Doctoral Fellowship. Thanks are also extended to H. Leung for photographic and technical assistance.

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