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Optimal Growth Conditions for *In Vitro* Cultures of Plant Parasitic Algae *Cephaleuros* Kunze ex E. M. Fries

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The genus *Cephaleuros* comprises plant parasitic algae distributed in tropical and subtropical zones globally. This study aimed to select culture media and optimize the culture conditions for *Cephaleuros* species. An extensive survey was conducted in southern Thailand and five species were found, namely, *Cephaleuros diffusus* on baegu leaves, *C. expansa* on orange jasmine leaves, *C. karstenii* on cacao leaves, *C. pilosa* on mangosteen leaves, and *C. virescens* on soursop leaves. To select suitable culture media, the five *Cephaleuros* species were cultured on Bold's basal medium (BBM), bristol medium, high salt medium (HSM), trebouxia medium, trebouxia bristol medium, and modified BBM. All the *Cephaleuros* species grew well in BBM, followed by HSM and bristol media. Bold's basal medium amended with indole-3-acetic acid (IAA) was found to be the most suitable for growth of all these *Cephaleuros* species when compared with plain BBM. Furthermore, culture under 24 h light maximized growth of the five *Cephaleuros* species. The results showed that BBM amended with IAA was the most suitable medium for *Cephaleuros* species cultures under incubation with 24 h light. Gametangia-like body and sporangia were formed on BBM after 3 mo of incubation. Further research is needed regarding cultures of pure *Cephaleuros* species on synthetic media in genetic studies and in tests of pathogenicity in plants.

Key Words: algal culture, *Cephaleuros*, growth condition, parasitic algae

Abbreviations: BBM – Bold's basal medium, HSM – high salt medium, IAA – indole-3-acetic acid, PCR – polymerase chain reaction

INTRODUCTION

The *Cephaleuros* genus of algae belongs to Division Chlorophyta, Class Ulvophyceae, Order Trentepohliales, and Family Trentepohliaceae (Guiry and Guiry 2017). Algae in this genus are subaerial and cause obvious orange to dark brown velvety lesions on several herbaceous and woody plants (Joubert and Rijkenberg 1971; Thompson and Wujek 1997; Suto and Ohtani 2009; Suto et al. 2014). Among the 16 species that have been included in this genus, four species grow intercellularly (*Cephaleuros biolophus*, *C. minimus*, *C. parasiticus*, and *C. pilosa*), whereas the remaining 12 species grow beneath the host cuticles. *Cephaleuros virescens* is the most frequently reported species worldwide and has a wide range of hosts (Joubert and Rijkenberg 1971; Marlatt and Alfieri 1981; Chapman and Good 1983; Holcomb 1986; Thompson and Wujek 1997; Suto and Ohtani 2009; Sunpapao et al. 2015). *Cephaleuros* growth on the upper leaf surfaces of a host plant causes depletion of water and mineral nutrients from the host tissues (Wolf 1930) and secretes harmful algal metabolites (Joubert and Rijkenberg 1971). *Cephaleuros* species have been recorded in tropical and subtropical zones worldwide (Chapman and Good 1983). *C. solutus* was first characterized in durian plantations of southern Thailand (Pitaloka et al. 2014). Later studies in Thailand have reported on species composition of *Cephaleuros* and their hosts (Pitaloka et al. 2015; Sunpapao and Pitaloka 2015; Sunpapao et al. 2015, 2016a, 2016b, 2017).

Several studies have focused on the culture of *Cephaleuros* on various synthetic media (Mann and Hutchinson 1907; Wolf 1930; Suématu 1957; Joubert 1969; Chowdary 1970; Joubert et al. 1975; Jose and Chowdary 1977, 1978; Ponmurugan et al. 2009, 2010; Suto and Ohtani 2011a). Culturing a pure *Cephaleuros* species on synthetic media was necessary for DNA extraction and PCR

amplification in a study of genetic relationships (Pitaloka et al. 2014; Sunpapao et al. 2015). Pure culture of *Cephaleuros* species is needed for the inocula in verification of Koch's postulate requirements of a plant pathogen (Suto and Ohtani 2011a, 2011b), and in the conduct of karyological studies (Jose and Chowdary 1977; Suto and Ohtani 2011a). This study was conducted to select a synthetic medium for *Cephaleuros* growth, and to optimize the culture conditions most favorable for *Cephaleuros* species.

MATERIALS AND METHODS

Sample Collection and Morphology Identification

Leaf samples containing algal thalli were collected from baegu (*Gnetum gnemon*), cacao (*Theobroma cacao*), mangosteen (*Garcinia mangostana*), orange jasmine (*Murraya paniculata*) and soursop (*Annona muricata*) as host plants in Hatyai City, Songkhla province, southern Thailand. During collection from June to August 2016, the temperature range was 27–30 °C, with 80–83% relative humidity and 10–15 mm rainfall (Regional Meteorological Center, Hatyai, Thailand, 2016). Algal thalli were removed from the upper leaf surfaces with a razor blade and placed on glass slides for morphology identification. Macroscopic and microscopic features of the algal specimens were observed under a stereomicroscope (Leica S8AP0, Leica, Germany) and a compound microscope (Leica DM750, Leica, Germany), and were identified based on the key to species by Thompson and Wujek (1997).

Isolation and Culture

Algal thalli isolations were conducted according to the method previously described by Suto and Ohtani (2011a). Leaf samples containing algal thalli were surface cleaned with running tap water for 1 h, wiped with sterilized paper, and dipped in 70% ethanol for 30 s, in 5% NaClO for 30 s, and in sterilized distilled water three times. Algal thalli were removed from leaf surfaces with a sterilized razor blade as a thin layer (2–3 mm), and were placed on synthetic media. The algal portions were transferred to algal culture broth since this is an autotrophic medium (Chapman and Henk 1985). The medium alternatives tested were Bold's basal medium (BBM) (Bischoff and Bold 1963), bristol medium (Bold 1949), high salt medium (Sueoka 1960), trebouxia medium (Atlas and Parks 1997), trebouxia bristol medium (Keller et al. 1987), and modified BBM (plus peptone 1.5 g L^{-1}, dextrose 10 g L^{-1}, and yeast extract 0.5 g L^{-1}), with incubation at 28 ± 2 °C, and 12/12 h dark/light regime for 3–6 mo. Colonies of algal growth on the synthetic media were measured, and morphologies of the algal filaments were observed.

Effects of Growth Hormone (Indole-3-acetic Acid) and Light on Algal Growth

To investigate the effects of indole-3-acetic acid (IAA) on algal growth, BBM and BBM amended with 2 mg L^{-1} were prepared. Each colony of *Cephaleuros* species, sampled from the various host plants, was transplanted on BBM and on BBM amended with 2 mg L^{-1}, and incubated at 28 °C, with 12/12 h dark/light regime for 1 mo. Growth of the algal colonies on the synthetic media was measured.

Light is the main physical factor required for photosynthesis by several plants and green algae. To test the effects of light period on algal growth, each colony of *Cephaleuros* species was transplanted onto BBM and incubated at 28 ± 2 °C with alternative light and dark cycles of 6:18, 12:12 and 24:0 h for 1 mo. Algal growth was measured afterwards.

Statistical Analysis

The effects of culture media, light and growth hormone were normalized in terms of percentage relative to the control. Algal growth diameter and dimensions were measured on each medium. Data on colony size were analyzed using SPSS software version 15 for Windows. Statistical significance was evaluated using Tukey's HSD test at $P < 0.05$.

RESULTS AND DISCUSSION

To identify the algae causing leaf spot disease on several host plants, lesions with algal thalli were collected from baegu, cacao, mangosteen, orange jasmine and soursop leaves. The most obvious symptom was orange tufts found on upper leaf surfaces, consisting of *Cephaleuros* thalli. The lesions varied from small spots that were more or less circular and raised, to flat discs with entire or crenate margins. The general characteristics of *Cephaleuros*, including growth habit and the dimensions of filaments, setae and sporangia, were observed under stereomicroscope and compound microscope (Fig. 1). Based on the key to species used by Thompson and Wujek (1997), the algal morphospecies were identified as *C. diffusus* on baegu, *C. expansa* on orange jasmine, *C. karstenii* on cacao, *C. pilosa* on mangosteen, and *C. virescens* on soursop (Table 1). The growth habit of *C. pilosa* was intercellular, while the growth habit of the other four species was subcuticular.

Among the media alternatives tested for isolation and culture of the five *Cephaleuros* species, Bold's basal medium (BBM) was found to be the most suitable, whereas high salt medium (HSM) supported moderate

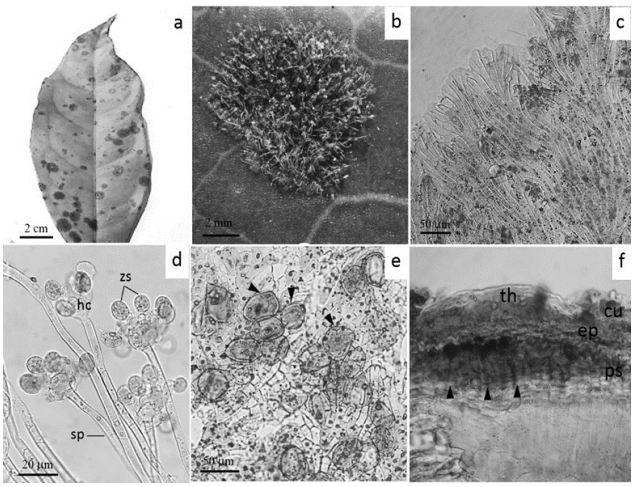

Fig. 1. General characters of *Cephaleuros* species: a) orange to dark brown velvety spot lesions on leaf, b) sporangiophores project from upper leaf surface, c) filamentous cells of *Cephaleuros virescens*, d) asexual reproductive structures composed of sporangiophores (sp), head cells (hc), and zoosporangia (zs), e) gametangia, sexual reproductive structures (arrows), and f) subcuticular growth habit of *Cephaleuros* species, showing thallus (th), cuticle (cu), epidermis (ep), and palisade cells (ps), and necrotic plant tissue beneath the algal thallus (arrows).

growth (Table 2). Algal *C. diffusus*, *C. expansa* and *C. pilosa* could grow on bristol medium, whereas the other species could not. The thalli of all five *Cephaleuros* species on any of the culture media were soft and floccose with aerial greenish filaments (Fig. 2). The colony margin was initially entire and became irregular in later stages (Fig. 2). The algal filaments were straight in all colonies of *C. diffusus*, *C. expansa*, *C. karstenii*, *C. pilosa* and *C. virescens* (Fig. 2e). Depending on the culture medium, the filamentous cells sometimes swelled into globose to ellipsoid shapes resembling gametangia (Fig. 2f), known as gametangia-like bodies; sporangia formations also developed in the greenish filaments (Fig. 2g). No gametes were produced by the gametangia-like bodies. Hematochrome was observed in the filaments and gametangia-like bodies appeared as small droplets (Fig. 2f & 2g). These observations are in agreement with those of Suto and Ohtani (2011a), who cultured five *Cephaleuros* species (*C. aucubae*, *C. biolophus*, *C. japonicus*, *C. microcellularis*, and *C. virescens*) without production of gametes or zoospores, on BBM and CA media.

BBM was the most suitable for culturing the five *Cephaleuros* species and was therefore selected to further test the effects of growth hormone and light on algal growth. Bold's basal medium was amended with 2 mg L^{-1} IAA and its effects were compared with the effects of using BBM alone. BBM amended with IAA was found to give maximum growth of the five *Cephaleuros* species (Fig. 3A, Table 3) compared with use of BBM alone. Mean colony sizes of the five *Cephaleuros* species were 4.6–6.3 mm on BBM amended with 2 mg L^{-1} IAA, and 1.6–2.6 mm on BBM alone (Table 3). No reproductive structures (gametangia or sporangia) developed in the algal colonies cultured on either medium (Fig. 3B). To test the effects of light on algal growth, the five *Cephaleuros* species were cultured on BBM and incubated at 28 ± 2 °C with alternative light and dark cycles of 6:18, 12:12 and 24:0 h. The results showed that 24 h light resulted in maximum growth of the five *Cephaleuros* species, with mean colony sizes of 3.0–3.6 mm at 1 mo of incubation, whereas the

Table 1. *Cephaleuros* species detected from leaf samples of select host plants.

Host		Location	*Cephaleuros* Species
Common Name	Scientific Name		
Baegu	*Gnetum gnemon*	Songkhla, Thailand	*Cephaleuros diffusus*
Cacao	*Theobroma cacao*	Songkhla, Thailand	*C. karstenii*
Mangosteen	*Garcinia mangostana*	Songkhla, Thailand	*C. pilosa*
Orange jasmine	*Murraya paniculata*	Songkhla, Thailand	*C. expansa*
Soursop	*Annona muricata*	Songkhla, Thailand	*C. virescens*

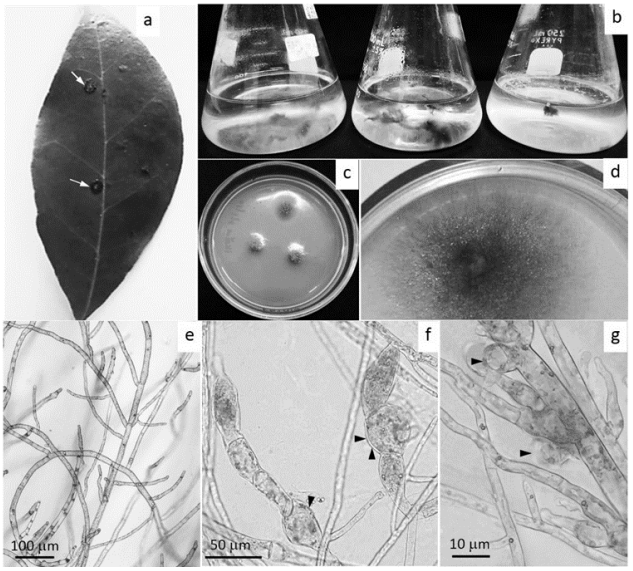

Fig. 2. Culture characteristics of *Cephaleuros* species: a) algal leaf spot lesions on a mature leaf (arrows), b) growth of algal colonies on high salt medium broth, c) growth of algal colonies on Bold's basal medium, d) magnified view of a greenish colony of a *Cephaleuros* species, e) greenish filaments of a *Cephaleuros* species, f) gametangia-like bodies (arrows), and g) sporangium formation on a greenish filament (arrows).

Table 2. Growth of *Cephaleuros* on synthetic media.

Culture Media	*Cephaleuros* Species				
	C. diffusus	***C. expansa***	***C. karstenii***	***C. pilosa***	***C. virescens***
Algal culture broth	–[1, 2]	–	–	–	–
Bold's basal medium	+++	+++	+++	+++	+++
Bristol medium	+	+	–	+	–
High salt medium	+	++	+	++	++
Trebouxia medium	–	–	–	–	–
Trebouxia Bristol medium	–	–	–	–	–
Bold's basal medium (modified)	–	–	–	–	–

[1] +++ Prominent growth, ++ moderate growth, + weak growth, – no growth

[2] Colony size > 2 mm = high growth, 1–2 mm = moderate growth, ≤ 1 mm = low growth at 3 mo of incubation

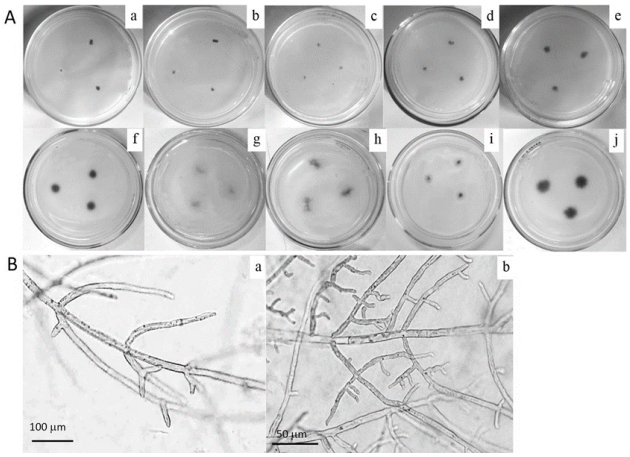

Fig. 3. A: Colonies of *Cephaleuros* species on Bold's basal medium (BBM) (a–e), and on BBM + indoleacetic acid (IAA) 2 mg L^{-1} (f–j). The species are *Cephaleuros diffusus* (a & f), *C. expansa* (b & g), *C. karstenii* (c & h), *C. pilosa* (d & i), and *C. virescens* (e & j). B: No reproductive structures on filamentous cells of *Cephaleuros* spp. without IAA (a) and with IAA (b).

light and dark cycles 6:18 and 12:12 h resulted in mean colony sizes of 1.6–2.6 and 2.0–2.8 mm, respectively (Table 4). Since *Cephaleuros* species are distributed in tropical and subtropical zones worldwide, these experiments were done at ambient room temperature (28 ± 2 °C) without testing for effects of temperature on algal growth.

C. virescens cultured on agar medium containing mineral salts and dextrose showed cushion-like filament mass (Wolf 1930). Furthermore, cultures of *C. solutus* and *C. virescens* on agar medium containing mineral salts, ferric citrate, citric acid, and micronutrient solutions have

Table 3. Growth of *Cephaleuros* species on Bold's basal medium (BMM) and indoleacetic acid (IAA).

Cephaleuros Species	Mean Colony Size (mm)	
	BBM	**BBM + 2 mg L^{-1} IAA**
C. diffusus	2.33 ± 0.57[a]	6.33 ± 1.15[b]
C. expansa	1.66 ± 1.15[a]	6.00 ± 1.73[b]
C. karstenii	2.66 ± 1.52[a]	5.66 ± 2.08[b]
C. pilosa	2.33 ± 1.15[a]	6.00 ± 1.00[b]
C. virescens	2.33 ± 1.52[a]	4.66 ± 0.57[b]

Different superscripts within a row indicate significant difference by Tukey's HSD test ($p < 0.05$)

Mean +/– standard deviation from three replicates

shown fluffy types of thalli that are quite different from those found on plant leaves (Jose and Chowdary 1977). Reproductive structures such as gametangia-like bodies and sporangia were formed by the five *Cephaleuros* species, but zoospores or gametes were not observed on agar media. *C. virescens* formed gametangia on Benecke's agar medium but no gametes were observed (Suématu 1957). Furthermore, gametangia and sporangia were formed when *C. virescens* was cultured on nutrient agar amended with growth hormones including auxin, indolyl-3-acetic acid, indolyl-3-butyric acid, and indolyl-3-propionic acid (Chowdary 1969).

Recently, algae in the genus *Cephaleuros* were considered novel as plant pathogens (Brooks et al. 2015). That study on *Cephaleuros* focused particularly on biology, host range, species identification, and seasonal developments. Selection and optimization of the synthetic medium for culturing *Cephaleuros* species may be useful

Table 4. Effects of light on growth of *Cephaleuros* species on Bold's basal synthetic medium.

Cephaleuros Species	Mean Colony Size (mm)		
	L:D 6:18[1]	L:D 12:12	L:D 24:0
C. diffusus	1.6 ± 0.55[a]	2.0 ± 0.00[a]	3.4 ± 0.54[b]
C. expansa	2.6 ± 0.54[a]	2.2 ± 0.84[a]	3.6 ± 0.54[b]
C. karstenii	1.6 ± 0.00[a]	2.8 ± 0.55[a]	3.4 ± 0.89[b]
C. pilosa	1.8 ± 0.45[a]	2.0 ± 0.00[a]	3.0 ± 1.00[b]
C. virescens	2.2 ± 0.44[a]	2.4 ± 0.00[a]	3.4 ± 1.00[b]

[1] Hours of light and dark in one cycle (L: D)

Different superscripts within a row indicate significant difference by Tukey's HSD test ($p < 0.05$)

Mean +/– standard deviation from three replicates

because i) a pure *Cephaleuros* culture may be subjected to DNA extraction and PCR amplification for genetic relationship studies, and ii) inoculation by algal fragments from a pure culture is required to verify Koch's postulates of a plant pathogen (Suto and Ohtani 2011b). Recently, Ratanapaiboonkit and Wannathong (2015) reported that cultures of *Cephaleuros* on HSM and Bristol media accumulated high levels of β-carotene, and might be used as a new β-carotene source for the food industry.

Our study indicates that BBM amended with 2 mg IAA and incubated under 24 h light may be used as a suitable medium and condition, respectively, for culturing *Cephaleuros* species. Recently, Ponmurugan et al. (2010) revealed that some amino acids found in *C. parasiticus* filaments may relate to pathogenicity of these algae to host plants. A knowledge of biochemistry may be useful in understanding the physical relations between the host and the parasite in nature (Ponmurugan et al. 2007). Further studies are still needed on useful and other biochemical components accumulated in plant parasitic green algae *Cephaleuros* spp. when cultured on synthetic media.

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