Jahangirnagar University J. Biol. Sci. **6**(2): 1-9, 2017 (December)

Optimization of culture condition, phytoconstituents and cytotoxicity of *Xylaria hypoxylon* (L.) Grev.

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Abstract

Experiments were carried out to determine the optimum culture condition, phytochemical constituents as well as cytotoxic activity of the endophytic fungus *Xylaria hypoxylon* (L.) Grev. grown on rotten bamboo poles at Jahangirnagar University campus. The fungus was first identified up to the species level, then suitable growth medium under laboratory condition was ascertained. Among the different types of culture media, Potato dextrose agar (PDA) medium was found to be the most suitable for optimum growth of this fungus at pH 6 and 30ºC. Phytochemical study of fruit body extract showed substantial presence of terpenoids, steroids, phenolic compounds, and weak presence of glycosides, falvonoids, and saponins. Brine shrimp lethality assay of the extract showed moderate cytotoxicity with LC_{50} value 327.00 µg/ml after 6 hours.

Key words: Bioactivity, culture condition, phytoconstituents, *Xylaria hypoxylon*.

INTRODUCTION

Fungi of the genus *Xylaria* are known to be a rich source of secondary metabolites namely, succinic acid derivatives (Anderson and Edwards, 1985), cytochalasin (Dagne *et al.*, 1994) and the more common melleins (Whalley & Edwards, 1995). The production of antifungal antibiotic Griseofulvin and dichlorogriseofulvin from *Xylaria* sp. strain F0010 (Park *et al*., 2005) were great discovery for medical science. *Xylaria hypoxylon* (L.) Grev., the "candle snuff fungus", is one of the most acquainted fungi of Xylariaceae family; first reported by Linnaeus (1753) with basionym *Clavaria hypoxylon* (Stadler *et al.*, 2014). This fungus is widely known for causing white rot in wood and commonly found on dead wood, often on stumps or woody material buried in the soil, always in humid environments in temperate regions of the world (Chen *et al.*, 2013). The studied fungus *X. hypoxylon* has been reported to produce novel secondary metabolites viz., Cytochalasins, epoxycytochalasin Q and R (Espada *et al*., 1997), α pyrone derivatives (Schüfflera *et al*., 2007) and tetralone derivatives (Gu & Ding, 2008). Some of these important bioactive compounds have shown significant toxic activity against human cancer cell lines (Schüfflera *et al*., 2007).

There is a very few report on *Xylaria* in Bangladesh. Siddiqui *et al*. (2007) reported *X. hypoxylon* from the forests of Dhaka, Chittagong, Sylhet as well as in the village grooves of Bangladesh. Till now, no report emphasizing on cytotoxicity of secondary metabolites

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of this fungus in Bangladesh came up to view. Therefore, laboratory experiments were carried out to determine the ideal culture condition followed by assessment for phytochemicals and cytotoxicity of the fruiting body extract of *X. hypoxylon*.

MATERIALS AND METHODS

Fresh fruiting bodies of *X. hypoxylon* were collected from rotten bamboo poles from Jahangirnagar University campus, Savar, Dhaka and representative voucher specimen was properly processed and deposited at the Phytochemistry and Herbal medicine research facilities of Botany Department of Jahangirnagar University with accession no. Jahan1. The taxonomic study and identification of this species was carried out following Perŝoh *et al.* (2009), Fournier *et al.* (2011) and Stadler *et al.* (2014). Observation was carried out on dry material. Length and width of asci, ascospore, paraphysis were measured in teleomorphic stromata using ocular micrometer. Ascospores were examined in distilled water, and ascus plugs were stained in Melzer's reagent. Photomicrographs were taken by stereomicroscope OLYMPUS (DP72) under magnification 10x, 20x and 100x at Wazed Mia Science Research Center, Jahangirnagar University, Savar, Dhaka.

Different culture media were prepared following Koley & Mahapatra (2015). Potato dextrose agar (PDA), Corn meal agar (CMA), Yeast-extract malt glucose agar (YMGA), Oatmeal agar (OA) and Bamboo shoot extract agar were tested to select the appropriate growth medium for the fungi. Experiments on culture condition establishment were carried out following Ahmed *et al.* (2013). Besides, the test fungus was grown on PDA with varying pH and temperature to assess its biological features. PDA medium was adjusted to different pH levels viz., 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with 1N NaOH or 1N HCl by using pH meter. Lower and higher temperature was provided by ice box and incubation chamber (Jouan Quality System) respectively. Methanolic crude extract was obtained by powdering 36 grams of fruit body of the fungus and soaking in a clean, flat bottom glass container in 100 ml of 100% methanol. The mixture was kept for 4 days in dark place with occasional stirring. The eluent was filtered with Whatman no.1 filter paper and the extract was concentrated at 45°C temperature under reduced pressure using a rotary evaporator and resultant residue was stored under refrigerated conditions until further studies.

A portion of the crude extract was subjected to different qualitative tests to find out the presence of secondary metabolites referring to characteristic color changes using standard procedures (Trease & Evans, 1989; Ghani, 1998; Dev, 2002). The cytotoxicity assay was carried out through brine shrimp lethality assay at 10, 20, 40, 80, 160, 200 µg/ml concentrations following Meyer *et al*. (1982). Duncan's Multiple Range Test (DMRT) was performed following the method of Steel and Torrie, (1960) using SPSS-16.0 software to weigh up the differences in growth performances of *X. hypoxylon* under variable conditions.

RESULTS AND DISCUSSION

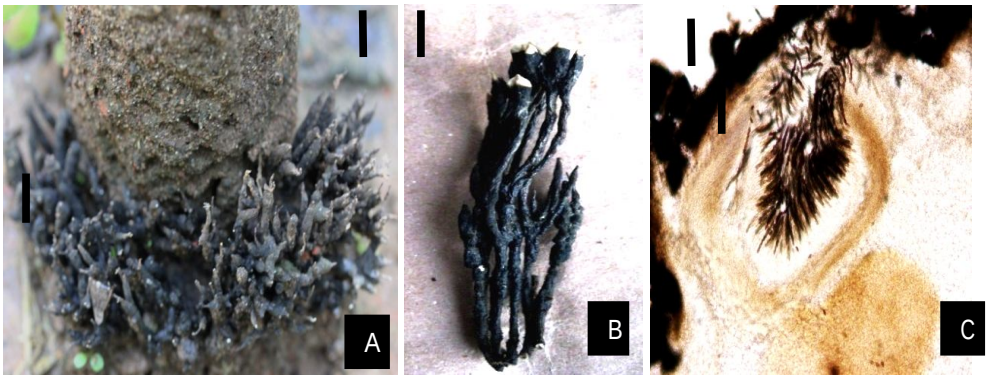
Xylaria Hill ex Schrank is a complex genus. Detailed descriptions on cultures as well as morphological characters of the stromata and teleomorphs are necessary to identify *X. hypoxylon*.

Xylaria hypoxylon (L.) Grev., Flora Edinensis, pp. 355 (1824). Plate 1
Basionym: *Clavaria hypoxylon* L., *Species Plantarum*. **2**: 1182 (1753).

Height of stromata ranged between 60-85 mm, fertile part 1-2.5 mm broad and (6.5-)10-25 mm long. Morphologically the stromata cylindrical to slightly fusiform, terete to flattened, branched from the base and middle, arising from medium lengthed stipes (35-60 mm high and 1-3 mm broad). The apex of stromata always flattened to mucronate and sterile. Whitish stromata at immature state, gradually turning to dark brown to black at matured fertile state. In well-developed stromata the outer crust peeling, furrowed, leathery, black covering creamy, cheesy, solid interior.

Asci cylindrical, long stipated, unitunicate in structure, 8 spored. Spore bearing part 69-88.32 µm long × 4.6-8.2 µm broad. The apical apparatus or operculum tubular in shape with slightly wide apex bluing in Melzer's iodine reagent. Paraphysis slender, sterile, filamentous structure that surround the fertile spore bearing apparatus. In the studied sample paraphysis were sparse, hyaline, thread like with oil droplets, 90.3-102.75 µm long × 0.75-2 µm broad.

Ascospores ellipsoid-inequilateral with narrowly to broadly rounded ends, uniseriate overlapping in ascus, olivaceous to medium brown, 11.8-16.4 µm long × 4.6-5.52 µm broad, two guttules at matured spore, conspicuous straight to slightly curved germ slit 4/5 to nearly spore length on the flattened and rarely on the convex side. Mycelial colony appeared after 4 days incubation in PDA medium at 30ºC showed characteristic white colony, lannose to filiform, with fine fibrous appearance in the middle and sparse cottony appearance at the edge.



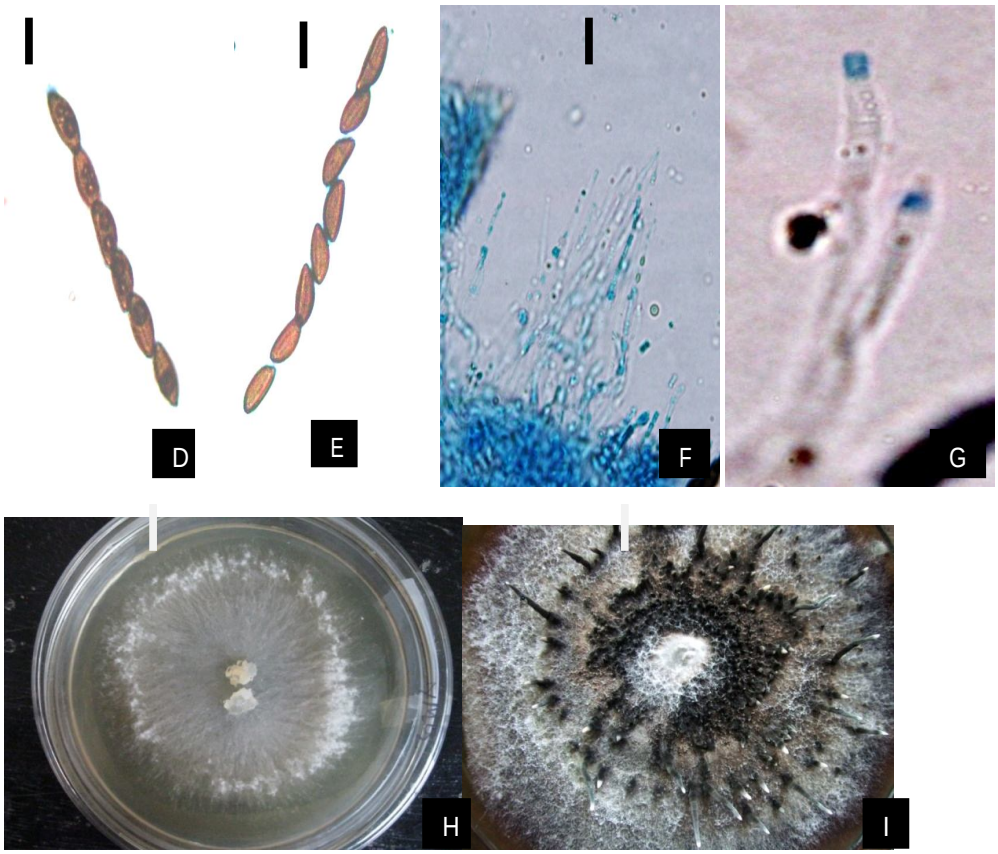


Plate 1. ***Xylaria hypoxylon.*** **A. fruit body in natural habitat B. fruiting bodies with white sterile apices (6.5-) 10-30 mm high C. subglobose perithacia (253-402.5µm) containing ascospore. D. biguttulate ascospores (11.8-16.4 µm long × 4.6-5.52 µm broad) in ascus (69-88.32 µm long × 4.6-8.2 µm broad). E. straight germ slit up to spore length. F. paraphysis (90.3-102.75 µm long × 0.75-2 µm broad). G. Tubular operculum in immature ascus. H. Fungal colony in PDA medium (9 cm plate). I. Anamorphic stromata. Bars: A, B=5 mm; C= 40 µm; D, E= 7.74 µm; F=14.39 µm; H, I=1.5 cm.**

After 3 weeks of culture, abundant small stromata developed in a circular manner holding sinnematal conidiomata on the top. *X. hypoxylon* could easily be confused with its three closely related species *X. longipes* Nitschke, *X. longiana* Rehm and *X. multiplex* (Kunze) Fr. (Fournier *et al.*, 2011). *X. longiana* is a scrawny species of *Xylaria,* virtually indistinguishable from *X. hypoxylon*, except for having smaller spores (9-11 x 4-5 µ) (Kuo, 2008). Conversely stromata of *X. hypoxylon* and *X. longipes* are identical but the ascospores of *X. longipes* are (11-)13-15(-17) × (5-)6(-7) µm, with spiraling germ slits. Despite the similarities with *X. hypoxylon* in appearance of ascospores and germ slit, stromata of *X. multiplex* first appear as dark brown to blackish with paler brown shredding outer layer in contrast to whitish stromata with black, leathery and peeling furrowed outer layer of *X. hypoxylon*, consistent with Rogers *et al*. (2008).

Determination of laboratory culture condition for *X. hypoxylon*: Malt extract agar, corn meal agar, potato dextrose yeast extract agar and oat meal agar are frequently used media for *X. hypoxylon* culture (Peršoh *et al.*, 2009, Fournier *et al.*, 2011 and Stadler *et al.*, 2014). Vegetative growth of *X. hypoxylon* was investigated using some common culture media at pH 6 and 30ºC and has been presented in Table 1.

Table 1. Effect of different culture media on growth of the *X. hypoxylon*

Culture medium	Mycelial growth (cm) at 30°C temperature and pH 6.0						
	2nd day	4th day	6th day	8th day	10th day	12th day	14th day
Potato dextrose agar (PDA)	1.63a	2.54a	4.21a	6.35a	7.21a	7.51a	8.11ab
Corn meal agar (CMA)	1.00c	1.22d	1.43e	2.37e	4.85d	5.17c	6.37c
Yeast extract malt glucose agar (YMGA)	1.25b	1.43c	2.97bc	4.69b	6.37b	6.85b	7.80b
Oat meal agar (OA)	0.78d	1.58b	2.61bc	3.97c	5.92c	6.11d	7.96ab
Bamboo shoot extract agar	0.48e	0.97e	1.58d	3.13d	4.26e	5.25c	5.98d

Note: Data are averages of three replications. In a column means followed by the same letter are not different significantly at 5% level by DMRT.

Significant variation was observed during optimization of laboratory culture condition. After two weeks of incubation, maximum mycelial growth was observed in PDA medium (8.11 cm) followed by Oat meal agar medium (7.96 cm), yeast extract malt glucose agar medium (7.8 cm), corn meal agar (6.37 cm) and bamboo shoot agar (5.98 cm). Oat meal and yeast malt glucose agar (YMGA) medium showed a moderate response to the mycelia growth of the fungus (Table 1). In contrast, Callan & Rodgers (1993) reported on growth of this fungus on 2% oat meal agar (OA) medium and mentioned that *Xylaria* cultures can differ considerable in appearance on various media. Laessøe & Lodge (1994) reported that oat meal agar, 2% malt agar and potato dextrose agar to describe the mycelial features of *Xylaria axifera*.

Effect of temperature on growth of fungus was determined by growing the fungus on PDA medium at pH 6 with varying temperature ranged between 10-35°C. The fungus started responding on the 2nd day of incubation. It grew 8.36 cm diameter at 30°C in 2 weeks followed by 7.35 cm (35°C), 6.41 cm (25°C), 4.97 cm (20°C). The lowest growth was observed at 15°C and no response at 10°C. So optimum temperature for fungal growth ranged between 30-35°C (Table 2).

Table 2. Effect of temperature on mycelial growth of *X. hypoxylon*

Temperature	Mycelial growth (cm) at pH 6.0 in PDA medium						
	2nd day	4th day	6th day	8th day	10th day	12th day	14th day
10°C	0.00d	0.00d	0.00f	0.00e	0.00f	0.00f	0.00f
15°C	0.00d	0.13cd	0.42e	1.21d	1.68e	2.33d	2.54e
20°C	0.31c	0.67c	1.56d	2.75c	3.09d	3.74d	4.97d
25°C	0.69b	1.66b	2.42c	3.13bc	4.73c	5.79c	6.41c
30°C	1.52a	2.47a	4.36b	6.40a	7.29ab	7.68a	8.36a
35°C	1.83a	2.89a	5.78a	6.19a	6.96b	7.11b	7.35b

Note: Data are averages of three replications. In a column means followed by the same letter are not different significantly at 5% level by DMRT.

During two weeks of incubation, *X. hypoxylon* showed consistent growth from pH 5 to 10. The fungus started responding after 2 days of inoculation. Statistically significant growth was observed in pH 6 (8.80 cm), 5 (8.50 cm) and 7 (8.60) followed by pH 8 (7.80 cm), 9 (7.30 cm) and 10 (7.20 cm) respectively (Table 3). The optimum pH for growth ranged between 5 and 7. Thus the optimum culture condition for *X. hypoxylon* may be PDA medium or oat meal agar with pH 5 to 7 and 30° to 35°C temperature.

Table 3. Effect of pH on growth of *X. hypoxylon* on PDA medium

pH of medium	Mycelium growth (cm) at 30°C						
	2nd day	4th day	6th day	8th day	10th day	12th day	14th day
4	0.00c	0.00e	0.00d	0.00d	0.00d	0.00d	0.00c
5	1.63b	2.54cd	4.21b	6.35a	7.21ab	7.51b	8.50a
6	2.12ab	3.47a	5.67a	6.63a	7.45ab	7.90a	8.80a
7	1.98ab	2.92b	5.11a	6.48a	7.30ab	7.75ab	8.60a
8	1.60b	2.37c	3.95b	5.12b	6.00c	6.22c	7.80b
9	1.54b	2.25c	3.35c	4.52c	5.94c	6.45c	7.30b
10	1.12ab	2.20b	3.28a	4.48a	5.30ab	6.25ab	7.20a

Note: Data are averages of three replications. In a column means followed by the same letter are not different significantly at 5% level by DMRT.

Phytochemical screening: Crude methanolic extract of *X. hypoxylon* showed strong presence of terpenoids, steroids and phenolic substances and weak presence of carbohydrates, tannins and flavonoids. Alkaloids and saponins were moderately present in the extract (Table 4). Adeduntan & Adeniyi (2014) reported a weak presence of tannin in *X. hypoxylon*. Moderate presence of alkaloids was supported by the findings of Espada *et al* (1997). Strong presence of terpeniods in the fruit body extract of *X. hypoxylon* can be correlated with the findings of Deyrup *et al.* (2007) where he mentioned the isolation of triterpenoid glycosides from *Xylaria* sp.

Brine shrimp lethality bioassay was carried out to determine the cytotoxicity of fruit body extract of *X. hypoxylon* at different concentration.

Table 4. Qualitative chemical screening of fruiting body extract of *X. hypoxylon*

Sl. No.	phytochemicals	Name of the test	Extract of *X. hypoxylon*
1	Carbohydrates	Molish's test	+
		Fehling reagent	+
2	Alkaloids	Wagner's reagent	++
		Mayer's reagent	+
		Dragendorff's reagent	++
		Tannic acid solution 10%	-
3	Glycosides	$FeCl_3$ test	+
	a. Cardiac glycosides	Keller Killiani's test	-
	b. Anthraquinone glycosides	Borntrager's test	+
4	Flavonoids	Lead acetate test	-
		Alkali test	-

		Conc. H_2SO_4	+
5	Tannins	$FeCl_3$+ MeOH extract	+
6	Terpenoids		
	a. Terpene	$CHCl_3$+H_2SO_4	++
	b. Triterpene	$CHCl_3$+Acetic anhydride	+++
7	Steroids	Salkowski test	+++
8	Phenols	$FeCl_3$ test	+++
		Ammonia test	+
		Lead acetate test	+++
9	Saponins	Foam test	++

Note: +++ = strong reaction, ++ = moderate reaction, + = weak reaction, - = nil/no reaction

Table 5. Brine shrimp lethality assay of crude extract of *X. hypoxylon*

Counting time	Sample concentration (µg/ml)	Log conc.	Mortality (%)	LC_{50} µg/ml	LC_{90} µg/ml	LC_{50} of vincristine sulphate
6 hrs.	Control	0	0	327.00	572.49	0.0699
	10	1	0			
	20	1.301	0			
	40	1.601	3.33			
	80	1.903	6.66			
	160	2.203	23.33			
	200	2.301	30			
12 hrs.	Control	0	0	107.28	191.49	0.031
	10	1	0			
	20	1.301	3.33			
	40	1.601	20			
	80	1.903	46.66			
	160	2.203	80			
	200	2.301	86.66			
24 hrs.	Control	0	0	85.09	165.72	0.009
	10	1	3.33			
	20	1.301	10			
	40	1.601	26.66			
	80	1.903	76.66			
	160	2.203	83.33			
	200	2.301	100			

Kamuhabwa *et al.* (2000) and Schüfflera *et al.* (2007) reported the production of tetralone derivative xylariol A, B and xylarone in *X. hypoxylon* respectively which indicated cytotoxicity against HepG2 and various cancer cell lines. As crude methanolic extract contain most of the compounds thus the cumulative effect of all the compounds might have shown comparatively higher cytotoxicity. Cytochalasins, cytochalasin Q; 19, 20 epoxycytochalasin Q and R (Espada *et al.*, 1997), tetralone derivatives xylariol A and B (Gu *et al.*, 2008), α pyrone derivatives xylarone and 8, 9-dehydroxyxylarone (Schüfflera *et al.*, 2007) have been isolated from different strains of *X. hypoxylon*. Secondary

metabolites from *Xylaria* proved to be valuable drugs for human health worldwide (Ramesh *et al*., 2012). Bangladesh drug industries may provide prospective facilities for intensive research in this aspect and may become benefited with the findings. So there is a large room for extensive research on this fungus and its allied species.

Acknowledgment: This research work was supported by the University Grants Commission (UGC) research fund in 2015-2016 fiscal year, which is gratefully acknowledged. Authors also wish to concede the laboratory support given by the Plant Pathology and Plant Protection laboratory, Department of Botany, JU in terms of research facilities.

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