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Studies on the Aquatic Hyphomycetes of a Sulfur Spring in the Western Ghats, India

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Abstract. Studies on the occurrence of aquatic hyphomycetes were carried out in Panekal sulfur spring in the Western Ghats, India by incubation of leaf litter and analysis of natural foam and of induced foam. Sampling was done once every three months over a period of two years from September 1989 to June 1991. The temperature, pH, dissolved oxygen, and sulfide content of water were also measured. No fungi were observed within the spring, whereas 16 species belonging to 13 genera were isolated from two outflow sites of the stream. The percent frequency of *Triscelophorus monosporus* was high (24.0%). The temperature of water in the spring ranged between 30.0 and 38.5°C and the sulfide content between 3.2 and 4.3 mg l^{-1}. Studies showed that sulfide water (4.0 mg l^{-1}) from the spring inhibited the growth of the colonies of *Dactylella aquatica, Phalangispora constricta, Tetracladium setigerum, Vermispora cauveriana,* and *Wiesneriomyces laurinus*. When the leaves colonized by aquatic hyphomycetes were incubated at different temperatures in sulfur-spring water and stream water separately, sporulation was not observed in any of the fungi at and above 35°C except *Phalangispora constricta,* which could sporulate at 35°C. At lower temperatures (15–30°C) relatively fewer species were found to sporulate in sulfur-spring water than in stream water.

Introduction

Aquatic hyphomycetes have been reported from various aquatic habitats and terrestrial regions such as tree hollows, stem flow, aqueous films, etc. [11]. Most of the studies on these fungi are restricted to aquatic systems where the temperature of waters ranged between 0 and 26°C [2, 3, 9, 12]. However, recently Chandrashekar et al. [4] reported the occurrence of aquatic hyphomycetes in the out flow region of a sulfur spring in the Western Ghats where the temperature of the water ranged between 26.5 and 36.5°C.

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In this paper, we report the occurrence of aquatic hyphomycetes in another sulfur spring, Panekal, in the Western Ghats. The objectives of this study were to find out their seasonal occurrence, the effect of spring water on the growth and sporulation, and the characteristics of water that correlated with frequencies of species.

Materials and Methods

Location

The Panekal sulfur spring originates at Panekal (12° 54.40′ N lat, 75° 17.50′ E long) in Dakshina Kannada District of Karnataka and flows through paddy fields for a distance of about 0.5 km before joining River Nethravathi. Three sampling sites were selected for the study. At the first site, the spring proper, water emerges from the crevices of rocks and collects to form a small pond. The surrounding vegetation was sparse and consisted of trees including *Artocarpus heterophyllus* Lam., *Ficus benghalensis* L., *Mangifera indica* L., *Macaranga peltata* Muell.-Arg., *Bambusa arundinacea* Willd. Two other sites in the outflow region were at about 20 and 40 m from the spring. There was no prominent vegetation surrounding this region except a paddy crop during the monsoon.

Isolation

Three methods of analysis were employed to study the mycoflora: leaf litter incubation, and analysis of natural foam and of induced foam [3, 6, 8, 10]. For leaf litter analysis about 10 submerged leaves of different species collected in sterile polythene bags from each of the three locations were brought to the laboratory and washed several times in water to remove extraneous materials. The leaves were cut into small pieces (3 × 1.5 cm) and incubated (at 28 ± 2°C) separately in Petri dishes containing 20 ml of distilled water. The leaf pieces were examined on alternate days under a low power microscope (×125) to detect the conidia of hyphomycetes. Simultaneously, water in the Petri dishes was replaced by fresh water to minimize the growth of bacteria and other organisms. This process was repeated up to 60 days and the fungi were identified on the basis of morphology of their conidia.

For natural foam analysis about 10 ml of foam was collected from the sampling sites in plastic bottles and mixed with 1 ml of formalin-acetic acid-alcohol (FAA) to fix the biological materials including conidia in the foam. The samples were examined under a microscope for the presence of conidia. For analysis of induced foam about 20 leaves collected from each sampling site were cut into small pieces of approximately 3 × 1.5 cm and incubated in a large tray. At the end of 48 h, the tray was shaken gently and 200 ml of incubated water was collected in a beaker. To this, 100 mg of sodium dodecyl sulphate (SDS) was added and aerated to induce foam. Two milliliters of the foam was collected in a Petri dish and examined under a microscope for the presence of conidia. After every collection, the water in the tray was replaced by fresh water to reinduce growth and sporulation of hyphomycetes colonized on the leaves. This process was repeated for 20 days. Sampling was done once every 3 months for 2 years from September 1989 to June 1991.

The frequency of occurrence of each species was calculated on the basis of its presence or absence in 72 samples (3 techniques × 3 sampling sites × 8 collection dates).

For the estimation of dissolved oxygen and sulfide content, water was collected in 5-liter plastic cans from each sampling site. The dissolved oxygen content was determined by following Winkler's titration method [1], the sulfide content was estimated by the Iodometric method [1], and the pH was measured at time of sampling with a portable pH meter. Water temperature was measured with a mercury thermometer at the sampling site.

Effect of Sulfur Spring Water on Growth

The effect of sulfur spring water on the growth of aquatic hyphomycetes was studied by a poisoned food technique [8]. For this, water from the spring was transported in sealed polythene containers

and the sulfide content was determined in the laboratory. Malt extract agar (MEA) medium was prepared by dissolving 15 g of agar in a small quantity of spring water in an autoclave under 1.41 kg cm^2 pressure for 15 min and filter-sterilized spring water was later added to make 1 liter of medium. Media with two other dilutions, i.e., equal parts of spring and distilled water (1:1) and 1:2 ratio of spring and filtered stream water were prepared in the same way as above.

Single spore isolates of the species *Dactylella aquatica* (Ingold) Ranzoni, *Phalangispora constricta* Nawawi et Webster, *Tetracladium setigerum* (Grove) Ingold, *Vermispora cauveriana* Rajeshekhar, Bhat et Kaveriappa and *Wiesneriomyces laurinus* (Tassi) P.M. Kirk obtained on 1% MEA medium were used for the study. Mycelial discs (1 mm diam) from 10-day-old cultures of these fungi were placed at the center of the Petri dishes containing the sulfide medium. The mycelial discs inoculated into MEA medium prepared in distilled water served as control. The cultures were subjected to natural daylight and darkness at room temperature (26 ± 2°C) for seven days. The extension of colonies was measured daily along two diameters at right angles to each other. The experiment was conducted in triplicate and averages of the data were calculated.

Effect of Temperature on Sporulation

To study the sporulation of hyphomycetes at different temperatures, water from the sulfur spring and from a freshwater stream near Mangalore University campus were collected in plastic cans and filtered through Millipore filters (Millipore Corp., Bedford, MA) before use. The hyphomycetes colonized on submerged broad leaves, such as *Holigarna ferruginea* Marchand, *Lophopetalum wightianum* Arn., and *Vateria indica* L. collected from a freshwater stream were used as test organisms. Twenty submerged leaves were collected, washed thoroughly in tap water and rinsed in distilled water. From each leaf, 36 segments (5 × 5 mm) were cut, along the two sides of the midrib and grouped separately. From each group, the segments were randomly distributed into two sets each of 18 Petri dishes. To one set of Petri dishes sulfur spring water was added and to the other set stream water was added. The dishes were incubated at 15, 20, 25, 30, 35, and 40°C and were examined on alternate days under a low power (×125) microscope to detect the spores of hyphomycetes. Water in the dishes was replaced after each observation. The observation was carried out up to 30 days.

Statistical Analyses of the Data

The correlation analysis [13] was applied to test the significance of some physico-chemical factors of spring water on the occurrence of hyphomycete species. The Chi-square test was done for analyzing the relationship between the fungal species and temperature and sulfide content of spring water.

Results

The temperature of water at site I was high (38.5°C) and remained constant during the sampling period. It varied between 30.0 and 34.2°C at the other two sites. The pH of water in the three sites ranged between 8.4 and 9.2. The dissolved oxygen content of waters at the three sites varied between 3.1 and 4.0 mg l^{-1}. Sulfide content of waters was higher at site I (4.0–4.3 mg l^{-1}) than at the other two sites (3.2–3.8 mg l^{-1}). A total of 16 species belonging to 13 genera was recorded from the two outflow sites (Table 1). *Triscelophorus monosporus* was the most frequent organism (24.0%). Some fungi like *Isthmotricladia laeensis* and *Speiropsis hyalospora* were found at low frequencies (1.4%). None of these fungi was recorded from the spring itself (site I). A seasonal record of the fungi at the three sites is given in Table 2. The number of species collected in September samplings was the highest.

Table 1. Aquatic hyphomycetes collected from Panekal sulfur spring during 1989–1991

Fungus	Site[a]	I			II			III			Total	Percent
	Technique[b]	1	2	3	1	2	3	1	2	3	frequency[c]	frequency
Triscelophorus monosporus Ingold		−	−	−	+	−	+	+	−	+	17	24.0
Flabellospora verticillata Alasoadura		−	−	−	+	+	+	+	−	+	11	15.3
Helicomyces sp.		−	−	−	+	−	+	+	+	+	11	15.3
Speiropsis pedatospora Tubaki		−	−	−	+	−	−	+	−	+	9	13.0
Lunulospora curvula Ingold		−	−	−	+	−	+	+	−	−	7	10.0
Phalangispora constricta Nawawi ex Webster		−	−	−	+	−	+	+	−	−	7	10.0
Vermispora sp.		−	−	−	+	−	+	+	−	+	7	10.0
Wiesneriomyces laurinus (Tassi) P.M. Kirk		−	−	−	−	−	−	+	−	+	6	8.3
Helicosporium sp.		−	−	−	+	−	+	+	−	−	5	7.0
Triscelophorus acuminatus Nawawi		−	−	−	+	−	+	+	−	+	5	7.0
Anguillospora longissima (Sacc. & Syd.) (Ingold)		−	−	−	+	−	−	+	−	−	4	6.0
Beltrania rhombica Penzig		−	−	−	+	−	+	+	−	−	4	6.0
Flabellospora multiradiata Nawawi		−	−	−	+	−	−	+	−	+	4	6.0
Camposporium antennatum Harkness		−	−	−	−	−	−	+	−	+	3	4.2
Isthmotricladia laeensis Matsushima		−	−	−	−	−	−	−	−	+	1	1.4
Speiropsis hyalospora Subramanian ex Lodha		−	−	−	−	−	−	+	−	−	1	1.4

[a] I, Sulphur spring; II, about 20 m from the spring; III, about 40 m from the spring

[b] 1 = Leaf litter analysis; 2 = natural foam analysis; 3 = induced foam analysis

[c] Out of a total of 72 samples (3 sampling sites × 3 techniques × 8 collection dates)

Table 2. Number of species of aquatic hyphomycetes recorded in Panekal sulfur spring during 1989–1991

	Total number of species recorded							
	Sept.		Dec.		Mar.		Jun.	
Site[a]	1989	1990	1989	1990	1990	1991	1990	1991
II	6	6	4	4	3	2	3	4
III	6	7	4	2	5	2	5	4

[a]For site I (the spring itself) the record was nil

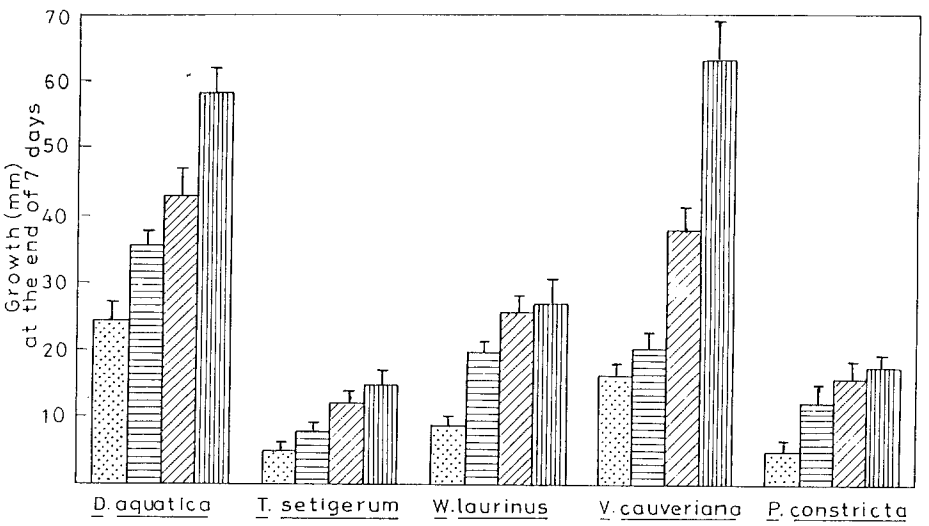

Fig. 1. Growth of five species of aquatic hyphomycetes on malt extract agar (in sulfur spring water) medium; $n = 3$, bars represent standard error of the mean. Dilution of sulfur spring water (sulfide: 4.0 mg l^{-1}: ⊡ , 1; ▤ , 1:1; ▨ , 1:2; ▥ , control

Percent inhibition of growth of the five species of fungi cultured in sulfur spring water decreased with increased dilution. The sulfide content of the filtered spring water was 4.0 mg l^{-1} and in the filtered freshwater was only a trace (0.00015 mg l^{-1}). Inhibition of growth was maximum in undiluted spring water and correspondingly less in the other two dilutions. The percent inhibition of growth was maximum in *V. cauveriana* and minimum in *T. setigerum*. As shown in Fig. 1, in all the species, overall growth in treated cultures was less compared to the control.

The data on sporulation of fungi on the leaves incubated at different temperatures in spring water and stream water are given in Table 3. The sulfide content of sulfur-spring water was 4.2 mg l^{-1} and in the fresh water there was only a trace (0.00018 mg l^{-1}). In both the waters more fungi sporulated between 20.0 and 30°C. But, the record of fungi was less on the leaves incubated in spring water than in stream water. At 35.0°C, only one species, *Phalangispora constricta,* sporulated in stream water and at 40.0°C; no sporulation was observed in either spring or stream.

Table 3. Sporulation of aquatic hyphomycetes colonized on submerged leaves incubated in stream water and sulfur spring water at different temperatures

	Incubation temperature (°C)							
	15		20		25		30	
Fungus	St[a]	Sp[b]	St	Sp	St	Sp	St	Sp
Actinospora megalospora Ingold	–[c]	–	+[d]	–	+	–	–	–
Anguillospora crassa Ingold	+	–	+	–	+	–	–	–
Bacillispora aquatica Nilsson	+	–	+	+	+	–	–	–
Beltrania rhombica Penzig	+	–	+	+	+	+	+	–
Camposporium antennatum Harkness	–	–	+	+	+	+	+	–
Campylospora chaetocladia Ranzoni	–	–	+	+	+	–	–	–
Campylospora filicladia Nawawi	–	–	+	+	+	–	–	–
Codinaea sp.	+	–	+	–	+	–	–	–
Dendrosporium lobatum Plakidas & Edgerton	+	–	+	–	+	–	–	–
Diplocladiella scalaroides Arnaud ex Ellis	–	–	+	–	+	–	+	–
Flabellospora crassa Alasoadura	–	–	+	–	+	–	–	–
Ingoldiella hamata Shaw	+	–	+	–	+	–	–	–
Isthmotricladia gombakiensis Nawawi	–	–	+	–	+	–	+	–
Isthmotricladia laeensis Matsushima	–	–	+	–	–	+	–	–
Jaculispora submersa Hudson & Ingold	+	–	+	–	–	–	–	–
Laridospora appendiculata (Anasta.) Nawawi	–	–	+	+	+	+	–	–
Lunulospora curvula Ingold	+	–	+	+	+	+	+	–
Phalangispora constricta Nawawi & Webster	–	–	+	–	+	–	+	+
Speiropsis pedatospora Tubaki	–	–	+	–	+	+	–	+
Triscelophorus monosporus Ingold	–	–	+	–	+	+	+	–
Triscelophorus sp.	+	–	+	–	+	–	–	–
Total	9	–	21	7	19	7	7	2

[a] St, stream water
[b] Sp, spring water
[c] –, absent
[d] +, present

The correlation of fungal species versus temperature shows that temperature is highly significant with respect to species ($r = -0.8669$; $P < 0.0001$). Further, the correlation analysis based on number of fungal species versus sulfide content is also highly significant ($r = -0.8531$; $P < 0.0001$). Chi-square test indicates high significance between species and temperature ($P < 0.0001$) and also between log species and sulfide ($P < 0.001$).

Discussion

This is the second report on the occurrence of aquatic hyphomycetes in a sulfur spring. Chandrashekar et al. [4] reported the occurrence of 20 species of aquatic hyphomycetes in the outflow sites of a sulfur spring near to the current study site. When the mycoflora of the two sulfur springs were compared, there were ten species common to both the springs and ten others found in only one spring. In both the studies, no fungus was recorded in the spring proper where the sulfide content (3.1 and 4.0–4.3 mg l^{-1} in Bendrethirtha and Panekal, respectively) and the temperature (36.5 and 38.5°C in Bendrethirtha and Panekal, respectively) of waters were higher than the outflow sites. In both the springs, sites nearer to the spring contained fewer species of fungi than the sites away from the spring. Records of temperature and sulfide content of water at the sites away from the spring were correspondingly lower. Since the upward and outward flow of water in the spring was very slow the force of emerging water in the spring might not have been responsible for the differences in the number of species of aquatic hyphomycetes recorded in the three locations of the spring. Therefore, it was thought that some factor in the spring water might be affecting the aquatic hyphomycetes population. The pH and dissolved oxygen of waters at the three sites of Panekal sulfur spring did not show much variation. This discounted the possibility of these factors affecting the occurrence of hyphomycetes. However, the temperature and sulfide content of waters at the three sites varied significantly, indicating the possibility of their interference with the occurrence of hyphomycetes.

In the leaves colonized by hyphomycetes incubated separately in sulfur-spring water and stream water at different temperatures, the number of species recorded in the stream water incubated leaves was more than those incubated in spring water. In both the types of waters, the number of species recorded at different temperatures varies, with a maximum at 20.0°C and 25.0°C. Further, studies on the effect of sulfide-containing water on the growth of five species of hyphomycetes showed that the percent inhibition of growth increased with an increase in the concentration of sulfide. These results confirmed that high sulfide content together with higher temperature of water were responsible for the total absence of hyphomycetes in the sulfur spring proper and corresponding reduction in the number of species at the two outflow sites. However, Chandrashekar et al. [4] indicated that higher temperature was responsible for variation in the mycoflora in the three locations of sulfur spring investigated by them.

Field and Webster [5] have suggested a tolerance limit of 5 mg l^{-1} of sulfide for the growth of aquatic hyphomycetes. However, the results of this study show that inhibition of growth could take place at lower levels.

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