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Mycotoxin Production in *Cladosporium* Species Influenced by Temperature Regimes

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Cladosporium **is a mycotoxin secreting and potentially pathogenic fungus frequently occurring in outdoor environments. In this study prevalence of *Cladosporium* species in the atmosphere within and around Riyadh city was monitored and production of mycotoxin by the isolates was assessed at different incubation temperatures under in vitro conditions. Two hundred air samples were collected from twenty locations of Riyadh. Only 20 samples were found to carry *Cladosporium* inoculum, belonging to two species, namely, *C. cladosporioides* and *C. sphaerospermum*. Crude extracts of the fungal cultures in acetone were scanned by spectrophotometry for presence of mycotoxins. It was noticed that cultures grown at lower temperature (10 and 15°C) yielded higher amount of mycotoxins as compared to cultures incubated at higher temperatures (20-30°C). HPLC assays of the extracts revealed five compounds corroborating with spectrophotometry findings of higher levels at low temperature. LC/GC-MS analysis revealed several compounds known for diverse activities.**

Key words: Airborne fungi; *Cladosporium*; Environment; Mycotoxin.

Cladosporium is a ubiquitous fungus prevailing at a comparatively high frequency in the outdoor environment[1, 2,3]. Habit of this fungus is largely saprophytic inhabiting dead organic matter and often contaminating the food material; but it may be pathogenic as well causing clinical conditions of diverse nature in humans[1]. *Cladosporium* species are known to secrete mycotoxins which are believed to be causal agents for allergies[4], Cutaneous/sub-cutaneous infections[5, 6, 7, 8], Pulmonary mycosis [4, 5, 9], phaeohypho mycosis[10] etc. Some of these manifestations may be life threatening. As such, this airborne fungus has significant repercussions for human health.

Pathogenicity and virulence of *Cladosporium* species, like other pathogenic fungi, owe their degree mainly to the nature and level of mycotoxins produced by these organisms in combination with the response of corresponding host[11, 12, 13]. Major mycotoxins produced by *Cladosporium* species are cladosporin[14], isocladosporin[15],emodin[16], epi- and fagi-cladosporic acid[17], and ergot alkaloids[18]. Besides these, over a dozen molecules have been detected as secondary metabolites of *Cladosporium* species; many of which have shown toxigenic activity[19]. Apart from their involvement in pathogenic conditions of humans, animals, and plants, some of the mycotoxins have been found to possess significant pharmaceutical properties

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as well[14, 20, 21, 22]. Their dual role as agents of pathogenesis and pharmaceuticals makes the mycotoxins interesting entities to study in greater details and from different viewpoints.

Present study was focused on assessing the ability of air borne *Cladosporium* species to produce different secondary metabolites under in-vitro conditions at variable temperature.

MATERIALS AND METHODS

Collection of samples and preparation of pure cultures

In all, 200 air samples were collected from 40 sites transecting through northern, southern, eastern, western, and central region of Riyadh city during summer season. For each sample of air, single plate of potato dextrose agar (PDA) medium was inserted into the air sampler (SAS HiVac Petri-17407). The instrument was set for suction at 80 cuft minute^{-1} and air was sucked for 1 minute for each sample. Mean temperature was 44.2 °C and mean relative humidity (RH) was 23 during the collection of samples.

Plates taken directly from the air sampler were incubated at 28°C for 48 hours. During this period many colonies appeared in the plates. Immediately after the appearance, tiny portion of each colony was transferred to fresh medium on a separate plate. Procedure was repeated till individual pure colonies were obtained.

Identification of *Cladosporium* isolates

Sixteen cultures tentatively identified as *Cladosporium* on the basis of microscopic examination were sent to Assiut University Mycological Centre (AUMC) at Assiut, Egypt for confirmation and species identification; where ten were identified as *C. sphaerospermum* and six as *C. cladosporioides*. Single most actively growing isolate of each species was selected for onward experimentation.

Incubation of *Cladosporium* isolates at different temperatures

Pure cultures of *C. cladosporioides* and *C. sphaerospermum* were incubated on potato dextrose agar (PDA) medium at 10, 15, 20, 25, 30°C for 2 weeks.

Extraction of mycotoxins

Two weeks after incubation at respective temperatures, mycotoxins were extracted from the fungal mat using a procedure modified from [23, 24, 25]. Mycelial mat along with the underlying medium measuring 1.5 cm in diameter was punched out with a piece of steel tube, taking care to maintain uniformity of the quantity of medium attached with cultures. The collected mass was homogenized in 70% methanol and volume was made up to 5 ml. After stirring the homogenate for 2 hours at room temperature, it was filtered through Whatman filter No1. Methanol was evaporated under vacuum and the volume was made up to 5 ml with 0.1M phosphate buffer (pH 8.5). After shaking for two minutes, the crude extract was partitioned with 50 ml ethyl acetate in a separating funnel. Partitioning was done three times. Ethyl acetate phase was removed by vacuum and pH of the aqueous phase was adjusted to 2.5 with 1N HCl. The remaining aqueous solution was partitioned with 50 ml diethyl ether three times. Diethyl ether phase was collected and dried over sodium sulfate. After evaporating diethyl ether completely under vacuum, the residue was dissolved in 1 ml methanol and was stored at 4°C.

Spectrophotometric assays

Spectrophotometric assays were done to verify the presence of mycotoxins before separation of molecules by high performance liquid chromatography (HPLC) and their characterization by liquid chromatography-mass spectrophotometry (LC-MS) and gas chromatography-mass spectrophotometry (GC-MS). Extracts of the isolates selected as above were used for spectrophotometric assays.

Since the known *Cladosporium* toxins were not available in the market which could be used as standards, quantification of individual toxins by spectrophotometry was not possible. In the absence of standards, only a comparative assessment for the level of total toxins in the extracts under different treatments was made in terms of absorbance values.

Methanol extracts were dried under vacuum and the residue was re-dissolved in 1 ml of acetone. For detecting the presence of toxins acetone samples, were scanned for absorbance at 540 nm in a spectrophotometer (Gene Quant Pro, Amersham Biosciences, USA). Solvent acetone was used as blank.

Separation of mycotoxins by HPLC

In the absence of standards, only a

comparative analysis of number and level of mycotoxins produced at different temperatures of incubation was conducted by HPLC using previously tested methods[23, 24, 25]. Extracts of the same isolates as used for photometry were used for HPLC analysis. Analysis was performed on Finnigan Surveyor Plus (Thermo Scientific, USA) HPLC system. An octadecylsilane (C-18) column of dimension: 250 x 4.6 mm with 3µm particle size (Thermo Scientific) was used for separation of constituent molecules. Methyl alcohol-water (45:55 – pH 2.5) was used as mobile phase and 5µl of sample was injected into the system for each run. Column temperature of 25°C and flow rate of 0.8 ml/min were maintained throughout the analysis. The eluate was scanned at a wave length of 265 nm with a UV detector.The analysis was performed thrice and each set of data was treated as a replicate.

LC-MS analysis

Following Pais and Knize[26], Pais*et al.*[27], and Galceran *et al.*[28], LC-MS analysis of samples was done on a triple–quadrupole mass spectrometer (3U, Waters Corp. USA) in electro-spray negative ionization mode. Other conditions were as follows: voltage (capillary: 3.5 kV, cone: -40 V, and extractor: -3 V); temperature (source: 120°C, desolvation: 350°C); gas flow (desolvation: 600 lh^{-1}, cone: 60 lh^{-1}). Data acquisition was done with MassLynxV4.1 software.

GC-MS analysis

For non-polar and volatile compounds, gas chromatography-mass spectrometry (GC-MS) is a better suited detection technique, which ideally combines the advantages of the high separation efficiency of capillary GC with high sensitivity and selectivity of MS detector.

Following Pais and Knize[26], a GC unit (model Trace GC Ultra, Thermo Scientific Co.) with auto sampler (AI3000) and MS unit (TSQ Quantum GC, Thermo Scientific Co.) was used with a column of dimensions: 60 m x 0.25 mm x 0.25 µm film (Thermo TR-1). Following **i**nstrument parameters were set for the analysis: inlet temperature: 275°C, split flow:50 ml min^{-1}, injection volume: 0.5 µl, carrier gas: helium, carrier gas flow: 1.0 ml min^{-1}, and MS transfer line temperature: 250°C. Data was acquired with Xcaliber software.

Statistical procedures

During spectrophotometry, five sets of absorbance readings were recorded for each treatment and variation was estimated by standard deviations of the means. HPLC analysis was conducted in three replicates and variance was calculated by F test following which, means were separated by least significant difference (LSD) at $t_{5\%}$.

RESULTS

Most of the samples developed fungal colonies on PDA within 48-72 hours and pure cultures could be obtained after a few subcultures. Only 16(8.0%) air samples out of 200 collected from all over the city of Riyadh developed *Cladosporium* colonies on PDA plates, besides other fungi. Seven of these samples carried *C. sphaerospermum* Penzig only and three developed only *C. cladosporioides* (Fresenius) deVries, while remaining six showed both these species.

Mycotoxin level at variable temperature detected by spectrophotometry

Considering absorbance to be a function of mycotoxin concentration, it was noticed (Table1) that *C. cladosporioides* cultures incubated at lower temperatures of 10 and 15 °C produced toxins in greater quantity (absorbance: 0.703 and 0.739 respectively) as compared to cultures grown at higher temperatures of 20, 25, and 30°C

Table 1. Absorbance values of crude extracts of *Cladosporium* isolates grown at variable temperature

Incubation		Absorbance (540 nm)	
Medium	Temperature(°C)	*C. cladosporioides*	*C. sphaerospermum*
PDA	10	0.703±0.002	0.659±0.002
	15	0.739±0.003	0.702±0.002
	20	0.049±0.001	0.710±0.002
	25	0.065±0.001	0.061±0.001
	30	0.053±0.001	0.090±0.001

Table 2. Peak characteristics of the five compounds detected in HPLC analysis of crude extracts of *Cladosporium* isolates grown at variable temperature

Fungal genotype	Incubation temp.(°C)	1st peak			2nd peak			3rd peak			4th peak			5th peak		
		Ret. Time (min)	Height (mAU)†	Area (mAU.S)‡	Ret. Time (min)	Height (mAU)†	Area (mAU.S)‡	Ret. Time (min)	Height (mAU)	Area (mAU.S)‡	Ret. Time (min)	Height (mAU)	Area (mAU.S)‡	Ret. Time (min)	Height (mAU)	Area (mAU.S)‡
C. cladosporioides	10	6.32	9.21	106.84^{b}	7.36	98.58	1662.48^{a}	9.24	38.78	123.13^{b}	ND	ND	ND	14.48	10.82	207.53a
	15	6.34	10.49	126.15^{a}	7.36	98.62	1662.31^{a}	9.25	49.84	158.24^{c}	ND	ND	ND	14.36	9.85	188.92b
	20	6.31	9.56	112.81^{b}	7.33	76.52	1124.36^{b}	9.28	50.23	159.48^{c}	13.22	11.82	211.58^{a}	14.29	10.91	209.25a
	25	6.32	7.52	88.74^{c}	7.34	75.65	1096.92^{b}	9.22	53.63	170.26^{a}	13.64	12.02	215.05^{a}	14.29	11.06	212.19a
	30	6.34	7.54	87.46^{c}	7.35	68.47	814.54^{c}	9.27	56.22	162.64^{ac}	13.23	12.09	216.41^{a}	14.19	11.36	217.88a
			LSD $t_{5\%}$	8.42		LSD $t_{5\%}$	68.65		LSD $t_{5\%}$	9.25		LSD $t_{5\%}$	6.28		LSD $t_{5\%}$	10.90
C. sphaerospermum	10	6.34	11.23	139.25^{a}	7.35	81.46	1569.24c	9.23	42.26	134.18d	13.21	12.84	229.84^{a}	14.22	9.68	185.67b
	15	6.32	11.17	138.51^{a}	7.37	92.34	1662.12b	9.27	46.52	147.70c	13.18	12.35	221.06^{b}	14.18	10.25	196.60ab
	20	6.31	8.45	100.56^{b}	7.37	97.57	1786.70a	9.24	54.23	172.19b	13.22	12.86	230.19^{a}	14.26	10.84	207.92a
	25	6.32	7.21	81.75^{c}	7.31	78.65	1245.14d	9.29	56.15	178.27b	13.21	10.21	182.76^{c}	ND	ND	ND
	30	6.31	7.24	83.98^{c}	7.33	78.42	1186.56d	9.22	59.22	188.02a	13.24	10.32	184.72^{c}	ND	ND	ND
			LSD $t_{5\%}$	7.86		LSD $t_{5\%}$	76.54		LSD $t_{5\%}$	8.82		LSD $t_{5\%}$	7.35		LSD $t_{5\%}$	9.86

Values followed by the same superscript are not significantly different from each other at P≤0.05 † Mille absorbance units Height x ½ width of peak base in seconds ND: Not detected

(absorbance: 0.049, 0.065, and 0.053 respectively). Similarly, *C. sphaerospermum* cultures also produced higher levels of mycotoxins at 10, 15, and 20°C (absorbance: 0.659, 0.702, and 0.710 respectively) as compared to cultures grown at 25 and 30°C (absorbance: 0.061 and 0.090 respectively).

Mycotoxins at variable temperature detected by HPLC

HPLC analysis revealed five compounds in the extract of the two *Cladosporium* species. Figure 1 shows the chromatograph for the extract from *C. cladosporioides* cultures incubated at 25°C. First compound showed a retention time of 6.31-6.34 minutes; the peak attained a height of 7.52 to 11.23mAU (mille absorbance units) with corresponding peak areas ranging from 81.75 to 139.25 mAU-seconds for the two species under different regimes of incubation (Table 2). Second, third, fourth, and fifth peaks appeared between 7.31-7.37, 9.22-9.29, 13.18-13.24, and 14.18-14.48 minutes respectively. Ample variability was noticed in the height and area of the peaks representing different incubation temperatures. Comparatively larger peak areas symbolizing greater quantities were noticed for first and second compounds at 10 and 20ºC. Conversely, third compound showed larger peak areas at higher temperatures. Fourth peak was absent in extracts of *C. cladosporioides* incubated at 10-15ºC; while fifth peak did not appear in extracts of *C.sphaerospermum* incubated at 25-30ºC. Cumulatively, incubation at 20°C supported the production of all the five compounds by both the species. In the absence of standards for known mycotoxins produced by *Cladosporium* species, identity and concentration of these compounds could not be ascertained by the HPLC procedures.

Molecules detected by LC/GC-MS

Seven molecules with known anti-fungal activity were detected in LC/GC -MS analysis of different *Cladosporium* samples (Table 3). Of these, cladosporin, isocladosporin, cladosporid A, and pentanorlanost-3β-diolwere present in samples of both the species incubated at all temperatures. Mycoversillin and epidechloro griseoful-vin were produced by both the species of *Cladosporium* only when incubated at low temperature of 10-15°C and 10-20°C respectively. Octaketideacetate diol, on the other hand, was not

Table 3. Molecules detected by LC/GC–MS at variable incubation temperature

Molecules with Mass (Arranged alphabetically)	*C. cladosporioides* Incubation temperature (°C)					*C. sphaerospermum* Incubation temperature (°C)					Known activity			
	10	15	20	25	30	10	15	20	25	30	Anti fungal	Anti bacterial	Other toxicities	Plant growth regulators
Altenuisol (278)	+	+	+	+	+	+	+	+	+	+	-	-	+	-
Calphostin A (310, 311)	+	+	+	+	+	+	+	+	+	+	-	-	+	-
Calphostin C ((310, 311)	+	+	+	+	+	+	+	+	+	+	-	-	+	-
Calphostin D (310 (311)	+	+	+	+	+	+	+	+	+	+	-	-	+	-
Cladospolid A (308)	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Cladospolid B ((308)	+	+	+	+	+	+	+	+	+	+	-	-	-	+
Cladospolid C (309)	+	+	+	+	+	+	+	+	+	+	-	-	-	+
Cladosporol (307)	+	+	+	+	+	+	+	+	+	+	-	-	+	-
Cladosprin (314)	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Collectodiol (296-297)	+	+	+	+	-	+	+	+	-	-	-	-	+	-
Deacetylyanuthone A (123)	+	-	-	-	-	+	+	+	-	-	-	+	-	-
Emodin (86)	+	+	+	+	+	+	+	+	+	+	-	-	+	-
Epidechlorogriseofulvin (318)	+	+	-	-	-	+	+	+	-	-	-	-	+	-
1-Hydroxyyanuthone A (124)	+	-	-	-	-	+	+	-	-	-	-	+	-	-
Isocladosporin (313)	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Isoharzandione (240)	+	-	-	-	-	+	-	-	-	-	+	-	-	-
Koninginin A ((241-242)	+	+	+	+	-	+	+	+	+	-	-	-	-	+
Mollicellin C (302)	-	-	-	+	-	-	-	-	+	-	-	+	-	-
Mycoversillin (97-99)	+	+	-	-	-	+	+	-	-	-	+	-	-	-
Octaketide-acetat diol (322)	-	+	+	+	+	-	-	+	+	+	+	-	-	-
Ophibolin K (102)	+	-	-	-	-	+	+	-	-	-	-	+	-	-
Pentanorlanost-3β-diol (312)	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Pergillin (114)	-	-	-	+	+	-	-	-	+	+	-	-	-	+
Ustic acid (119-120)	-	+	+	+	+	-	-	+	+	+	-	-	+	-

produced by either of the species at 10°C and appeared only at higher temperatures.

Four known anti-bacterial compounds were found in the samples of *Cladosporium* extracts (Table3). Out of these, deacetyl-yanuthone A, 1-hydroxyyanuthone A, and ophiobolin K were produce only at lower temperatures; while mollicellin C appeared at moderate incubation temperature of 25°C in cultures of both the species.

Besides anti-fungal and anti-bacterial compounds, some molecules known to possess other types of toxicities were also detected in GC/LC-mass analysis of the samples (Table3). In all 9 such molecules were noticed in the samples. Of these, altenuisol, calphostin A, C, D, cladosporol, and emodin, all inhibitors of protein kinase C, were produced at all incubation temperatures by both the species. Collectodiol and epidechlorogriseofulvin showed a tendency of production at lower temperatures; and conversely, ustic acid appeared at higher temperatures only.

Some compounds which are known to have plant growth regulatory activity were also found in the samples (Table 3). Four of these compounds, namely cladosporin, isocladosporin, koninginin A, and pergillin fall in the category of plant growth inhibitors; while three forms of cladospolid (A, B, and C) found in our samples are known to have plant growth regulatory activity. These three forms of cladospolid, cladosporin,and isocladosporin were produced by both the species of *Cladosporium* at all temperatures of incubation. Koninginin was absent at 30°C and pergillin did not appear at lower temperatures of 10-20°C.

DISCUSSION

Occurrence of *Cladosporium* in only 8% of the samples collected from all over the city indicates that this fungus occurs at a low frequency in the atmosphere of Riyadh. In a similar study elsewhere, Abdel-Hameed *et al.*[29] isolated many fungi, including *Cladosporium cladosporioides* from the atmosphere and have evaluated production of mycotoxins by these fungi using thin layer chromatography (TLC) and HPLC. They found *Cladosporium* to be one of the dominant genotypes in the air. Shelton *et al.*[2] and Lee *et al.*[30] also recorded *Cladosporium*at high frequency inoutdoor air in several regions of the US during all the seasons. Reason for sparse occurrence of *Cladosporium* in Riyadh may be high temperature and low relative humidity (RH) conditions prevailing at the time of sample collection.Comparatively lower concentration of fungi including *Cladosporium* during summer was recorded by Shelton *et al.*[2] as well. Occurrence of only two species in our samples points to low genotypic diversity of *Cladosporium* in the area of collection; in the other study[29], *cladosporioides* was the only species of *Cladosporium* recovered from the air samples.

Since all the cultures were incubated under identical water activity conditions and for equal length of time, fungal genotype and temperature were the only functional variables for mycotoxin production. Considering absorbance to be a function of mycotoxin concentration, it was noted that *C. cladosporioides* cultures incubated at lower temperatures of 10 and 15°C produced more toxins (absorbance: 0.703 and 0.739 respectively) as compared to cultures grown at higher temperatures of 20, 25, and 30°C (absorbance: 0.049, 0.065, and 0.053 respectively). Similarly, *C. sphaerospermum* cultures also produced higher levels of mycotoxins at 10, 15, and 20°C (absorbance: 0.659, 0.702, and 0.710 respectively) as compared to cultures grown at 25 and 30°C (absorbance: 0.061 and 0.090 respectively).

Production of mycotoxins in culture is known to be influenced by water activity (a_w) and temperature [31, 32, 33]. However, few studies have investigated this aspect in *Cladosporium.* In the case of *Alternaria*, level of alternuene (AE), alternariol (AOH), and alternariol monomethyl ether (AME) produced was markedly different at variable incubation temperatures, the optimal being 25°C. Gqaleni *et al.*[34] studied the effect of temperature, water activity and incubation period on production of aflatoxin (AF) and cyclopiazonic acid (CPA) by *Aspergillus flavus* and found that 30°C and 25°C were the optimal temperatures for AF and CPA respectively. Optimal temperature for production of *Fusarium* mycotoxins was also recorded to be 25-30°C [35]. It is interesting to note that optimal temperature for production of mycotoxins in *Cladosporium* is lower than reported for other fungal genotypes. Association of temperature with mycotoxin production acquires enhanced

relevance in the context of growing concerns of climate change [36, 37].

HPLC procedures carried out without standards have shown the presence of five compounds in the extracts. Effect of incubation temperature on production of mycotoxins is apparent in HPLC analysis. Disappearance of the fourth peak at lower temperature of 10 and 15°C in *C. cladosporioides* and absence of the fifth peak at higher temperature of 25 and 30°C in *C. sphaerospermum* suggest that effect of temperature on mycotoxin production may be coupled with that of the genotype. Some previous studies also reported such interaction[31, 32, 33].

HPLC assays corroborate with the results of spectrophotometry, indicating higher level of mycotoxins at lower temperatures. Arranz *et al.*[38] have emphasized suitability and authenticity of HPLC procedures for identification of *Fusarium* mycotoxins, which supports our choice of this method for analyzing mycotoxins produced by *Cladosporium*. However, Jing *et al.*[39] have cautioned that mycotoxins detected by HPLC need to be verified by mass spectroscopy procedures. They detected ochratoxinA supposedly produced by contaminating fungi in longan fruit pulp with HPLC procedures; but failed to verify it by electron spray ionization-mass spectrometry (ESI-MS).

Sforza *et al.* [40] have convincingly elaborated the advantages of using LC-MS and GC-MS techniques for detecting mycotoxins at very low concentrations. Plattner[41] has used this technique successfully for estimating production of fumonisins and deoxynivalenol by *Fusarium graminearum*, cultures. Analysis of our samples by LC-MS has revealed much higher number of molecules as compared to only three detected by HPLC. This shows very high level of sensitivity of this technique. Using LC-MS,Richard *et al.*[42] have also reported precise detection of a large number of mycotoxigenic molecules in mature corn silage. However, unlike in our study, they have used standards for several known mycotoxins of other fungi and could link the production to their sources. Several mycotoxins have been detected in food items also with the help of this technique[43].

GC-MS analysis in the present study has revealed several molecules in the fungal extracts of the two species of *Cladosporium*. Some of these compounds seem to be at a very low concentration; but were detected due to high sensitivity of the GC-MS system. These findings point out that fungi release a large number of molecules with diverse activities, which may help them not only in growth and survival but in protecting themselves and modifying the biological activities of the host to perpetuate their own life cycle. Using GC-MS, Bloom *et al.*[44] detected presence of several mycotoxins in air with in fungus-infested buildings. They have shown that GC-MS is a highly sensitive technique and can detect molecules even at very low concentrations. Edwards[45] reported the analysis of 300 samples of wheat grains and detected a large number of known mycotoxins in the samples. Similar to our study he has also noticed presence of several molecules at very low concentration. Abdel Hameed *et al.* [29] found *Cladosporium cladosporioides* in air samples collected from the industrial surroundings at a frequency next only to *Aspergillus* species.

The present study provides an idea about mycotoxigenic potential of airborne fungi and the level of health risk they pose to the residents of a given area. This study shows that prevalence as well as genotypic diversity of *Cladosporium* is low in the atmosphere of Riyadh city. Major mycotoxins of *Cladosporium* (cladosporin, isocladosporin, and emodin) were produced by both species at all the incubation temperatures. Our results also indicate that the level of total toxins as well as the number of molecules produced was higher at temperatures of 10-20°C as compared to incubation at higher temperatures.

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