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Effect of industrial waste water on germination and growth of some fungi

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ABSTRACT

The effect of various concentrations of industrial waste water of the Coke and Chemicals Factory on spore germination, radial growth and biomass of six fungi, isolated from soils contaminated with waste water, were investigated.

Most concentrations (20–100%) of waste water inhibited the germination of spores of *Aspergillus flavus*, *A. niger*, *A. terreus* and *Cladosporium herbarum* but stimulated the germination of spores of *Fusarium oxysporum* and *Stachybotrys atra*. The undiluted waste water inhibited completely the germination of spores of the three *Aspergillus* species. Germ-tube lengths were reduced at higher concentrations of waste water.

Radial growth in all fungal species was inhibited at most concentrations of waste water. The biomass production was nil at the highest experimental concentration in all species. The pH values of the growth media were shifted sharply to the acidic side in the three *Aspergillus* species while no change occurred in the remaining species.

INTRODUCTION

Microorganisms such as bacteria, viruses and fungi have recently been used as sensitive biological indicators of the state of the environment, particularly the degree of pollution (Bagdasar'yan & Geniatulin 1982). Numerous studies have indicated that microorganisms vary in their sensitivity to different heavy metals (Babich & Stotzky 1981; Rodriguez *et al.* 1984; Brown & Wilkins 1985 a, b; Venkateswerlu & Stotzky 1986). Such investigations suggest that fungi are indicators of the severity of pollution in plants and soil.

Helwan district, about 24 km southeast of Cairo, was once famous as a health spa and unique winter resort. In 1954 many industries were established along 20 km of the eastern side of the river Nile in an area of 40 km^2 around Helwan. This led to complicated environmental problems. The Coke and Chemicals Factory drains appreciable amounts of waste water daily which run along a canal and collect in a pool 4 km away. This waste water is a source of pollution by heavy metals and also results in high salinity of the soil causing great damage to vegetation. Fayez & Shahin (1987) reported that microbial numbers increased with

increasing distance from the industrial complex at Helwan, and that this could be attributed to the high level of salinity and total heavy metals near the factories.

The aim of the present study was to collect and analyse a sample of the industrial waste water from the Coke and Chemicals Factory and to investigate its effect on the growth parameters of some fungi isolated from soil sites polluted with this waste water.

MATERIALS AND METHODS

FUNGAL SPECIES

Six fungi were used in the present study: *Aspergillus flavus* Link ex Fr., *A. niger* Van Tieghem, *A. terreus* Thom, *Cladosporium herbarum* (Persoon) Link ex Fr., *Fusarium oxysporum* Schl. ex Fr. emend Snyd. and Hans., and *Stachybotrys atra* Corda. They were isolated from soil sites contaminated with the industrial waste water from the Coke and Chemicals Factory at Helwan, Egypt.

Fungal cultures were maintained on Dox medium (control), consisting of (per litre of distilled water): sucrose (15 g), $NaNO_3$ (2 g), KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0·5 g), KCl (0·5 g), and $FeSO_4$ (0·01 g). For experimental treatments various concentrations of waste water (20%, 40%, 60%, 80% and 100%) were prepared by mixing (v/v) with distilled water.

WASTE WATER ANALYSES

Waste water samples were taken from the tanks inside the factory in sterilized dark bottles and subjected to the laboratory tests immediately. Ten ml of waste water were shaken with 2 ml of extractant consisting of 0·005 M diethylene-triamine pentacetic acid, 0·005 M $CaCl_2$ and 0.1 M triethanolamine, buffered at pH 7·3 (Lindsay & Norvell 1969) for 2 h and filtered. The heavy metal contents (Zn, Cu, Cd, Pb) in the filtrate were determined by atomic absorption spectrophotometry using a Perkin-Elmer model 303 atomic absorption spectrophotometer.

The estimation of chlorides in the sample was carried out according to Golterman (1969). Waste water was titrated against silver nitrate solution using potassium chromate as indicator.

Sulphate estimation was carried out by mixing waste water with sodium chloride-HCl reagent (5 : 1 v/v) and measuring the extinction against an H_2O blank in a colorimeter at 420 μm, then adding 0.5 g $BaCl_2$ crystals and shaking for 5 min. The extinction due to SO_4 was obtained by difference (Golterman 1969).

Nitrate estimation followed the procedure of Peach & Tracey (1956) where a known volume of waste water was evaporated to dryness, then left to cool. One ml of phenol disulphonic acid was added, then shaken for 10–15 min before the addition of 5 ml distilled water followed by 20% NaOH until the mixture turns alkaline. The intensity of the yellow colour which developed was measured at 430 μm.

SPORE GERMINATION

The method of Krause & Weidensaul (1978) was used. Twelve Van Tieghem slides (with central cavity) were used for each fungal species, i.e. two slides for each waste water concentration in addition to two other slides as controls. In the cavity of each

slide, 0·1 ml of the medium with the concentration under test was added. Then a drop of spore suspension from a 7 day old culture slant was placed in each slide cavity. This was carefully prepared to contain a moderate density of spores (25–30 spore in a microscopic field of magnification power $\times 100$). The slides were then incubated at 27°C before being examined at intervals. When germination in the controls reached 50–60%, the treated slides were examined. The germinated spores in four microscopic fields were counted and the percentage spore germination for each treatment was calculated and compared with controls. Reassessment of the percentage germination was then carried out 24 h following the first examination. The lengths of the developing germ tubes were measured using a calibrated occular micrometer.

RADIAL GROWTH

For each fungal species twelve Petri-dishes were supplemented with 20 ml Dox-agar medium of the concentrations under test (20–100% in addition to controls). After solidification, each plate was inoculated with a 5 mm diameter agar disc from the periphery of a 7 day old culture plate. The inoculated plates were then incubated at 27°C and the diameter of the developing colonies was recorded daily over a period of 7 days. The radial growth rate (Kr) was then estimated and compared with that of the control.

BIOMASS PRODUCTION

Conical flasks (250 ml) containing 50 ml Dox liquid medium amended with a range of waste water concentrations as described before were used. Each flask was inoculated with a 5 mm diameter agar disc from the periphery of a 7 day old culture plate, then incubated for 7 days at 27°C. At the end of the incubation period, mycelia were collected by filtration and dried in an oven at 70°C till constant weight. The dry weight of each treatment was compared with that of the control.

pH VALUE

The pH value of each growth medium was determined with a Beckman Zeromatic SS-3 pH meter.

STATISTICAL ANALYSIS

The mean values were tabulated, and the Least Significant Difference (LSD) calculated at the confidence limits of 1% and 5% (Snedecor 1948).

RESULTS

ANALYSES OF WASTE WATER

The results showed that zinc was dominant in the industrial waste water (92 ppm) followed by copper (16 ppm) then lead (7 ppm). A low quantity of cadmium was

detected (0·038 ppm). Appreciable amounts of chlorides were found (204·3 ppm) while lower quantities of sulphates (16·7 ppm) and nitrates (2·6 ppm) were detected.

EFFECT OF WASTE WATER ON SPORE GERMINATION

Table 1 shows the effect of different concentrations of waste water on spore germination. Waste water stimulated the germination of conidia in *Fusarium oxysporum* and *Stachybotrys atra* up to 80% concentration. In *Fusarium oxysporum*, the highest rate was 92% compared with 57% in the control; in *Stachybotrys atra* it was 97% while that of control was 59%. Conversely, the conidial germination of the three *Aspergillus* species and *Cladosporium herbarum* was significantly inhibited by waste water at most concentrations, and the degree of inhibition was directly proportional to the increase in waste water concentration. At 100%, the germination rate was 12% in *C. herbarum*, while that of control was 60%; in *Fusarium oxysporum* it was 16% compared with 57% in the control; in *Stachybotrys atra* it reached 28%, while that of control was 59%. Conidial germination of the three *Aspergillus* species was totally arrested at 100% waste water concentration. Prolonged incubation (24 h after the first reading) resulted in complete recovery and all spores overcame the inhibitory effect up to 40% waste water concentration. At 60% and 80% the recovery was incomplete.

EFFECT OF WASTE WATER ON GERM TUBE ELONGATION

Table 2 shows that industrial waste water retarded elongation of germ tubes of the tested fungi with the exception of *Stachybotrys atra*. In the three *Aspergillus* species all waste water concentrations were inhibitory and the lengths of germ tubes were significantly decreased. On the other hand, waste water had no effect on germ tube development in *Cladosporium herbarum* and *Fusarium oxysporum* up to 80% concentration. The undiluted waste water caused a great reduction in germ tube length from 18 to 6 μm in *Cladosporium herbarum* and from 14 to 5 μm in *Fusarium oxysporum*. In *Stachybotrys atra*, the waste water stimulated the development of germ

Table 1. Effect of industrial waste water on germination (%) of conidia of various fungi. A = First examination when controls reached 50–60%, B = Second examination, 24 h after the first examination. Four microscopic fields were examined (25–30 conidia/field) in each treatment.

Fungal species	Waste water concentration (%)													
	0		20		40		60		80		100		L.S.D.	
	A	B	A	B	A	B	A	B	A	B	A	B	5%	1%
Aspergillus flavus	57	100	56	100	45	100	34	66	19	28	0	0	3·0	4·5
Aspergillus niger	52	100	53	100	28	100	15	28	0	27	0	0	2·8	4·2
Aspergillus terreus	54	100	20	100	12	100	8	31	0	21	0	0	2·2	2·3
Cladosporium herbarum	60	100	60	100	59	100	53	73	41	62	12	31	2·7	4·0
Fusarium oxysporum	57	100	92	100	89	100	83	89	61	83	16	63	3·5	5·1
Stachybotrys atra	59	100	97	100	97	100	91	92	63	63	28	32	3·1	4·6

Table 2. Effect of industrial waste water on germ tube elongation (μm) of various fungi. A = Length of germ tubes at the first examination. B = Length of germ tubes at the second examination. The germ tubes of germinated conidia in four microscopic fields (25–30 conidia/field) were measured for each treatment.

Fungal species	Waste water concentration (%)													
	0		20		40		60		80		100		L.S.D.	
	A	B	A	B	A	B	A	B	A	B	A	B	5%	1%
Aspergillus flavus	81	>250	72	>250	26	>250	15	51	5	13	0	0	2·8	4·1
Aspergillus niger	31	>250	28	>250	26	>250	10	18	0	11	0	0	2·5	4·1
Aspergillus terreus	20	>250	12	>250	9	>250	5	29	0	23	0	0	2·4	3·5
Cladosporium herbarum	18	>250	17	>250	15	>250	15	41	15	22	6	16	5·4	7·2
Fusarium oxysporum	14	>250	14	>250	12	>250	12	72	11	61	5	33	3·2	4·8
Stachybotrys atra	11	>250	18	>250	18	>250	17	82	4	49	4	35	4·2	6·2

tubes up to 60% but when applied at higher concentrations (80–100%) significant inhibition in germ tube elongation was recorded. The length of the developed germ tubes was 4 μm compared with 11 μm in the control.

Prolonged incubation (24 h after the first examination) resulted in unmeasurable (>250 μm) germ tube length of the tested fungi up to 40% waste water concentration. Incomplete recovery was obtained at higher concentrations. The length of the germ tubes was 16, 33 and 35 μm after 24 h at 100% waste water compared to 250 μm in *Cladosporium herbarum*, *Fusarium oxysporum* and *Stachybotrys atra* respectively.

EFFECT ON RADIAL GROWTH RATE (Kr)

Table 3 shows clearly that waste water caused a general decrease in radial growth rate (Kr) in all species except *S. atra*. It is obvious also that an increase in waste water concentration reduced the radial growth rate of all fungi. In *Aspergillus flavus*, *A. terreus* and *A. niger*, the growth rates at 80% waste water concentration were 28%, 23% and 14% of the controls respectively. In *Cladosporium herbarum* and

Table 3. Effect of industrial waste water on the radial growth rate (Kr = mm/h) of various fungi. One developing colony in each of the three replicate plates was measured for each treatment.

Fungal species	Waste water concentration (%)					
	0	20	40	60	80	100
Aspergillus flavus	0·62	0·55	0·56	0·40	0·17	0
Aspergillus niger	0·86	0·68	0·57	0·47	0·12	0
Aspergillus terreus	0·58	0·48	0·43	0·36	0·13	0
Cladosporium herbarum	0·22	0·15	0·16	0·15	0·12	0
Fusarium oxysporum	1·33	0·73	0·73	0·70	0·69	0
Stachybotrys atra	0·31	0·38	0·37	0·35	0·23	0·16

Table 4. Effect of industrial waste water on biomass production (mg dry wt/100 ml medium) and pH value of the growth media of various fungi. The initial pH value of the growth medium was adjusted at 6·5 using 0·1 N HCl and 0·1 N NaOH. The mat developed in each of the three replicate flasks was harvested and its dry wt was determined.

	Waste water concentration (%)															
	0		20		40		60		80		100		LSD biomass		LSD pH	
	Bio-mass	pH	Bio-mass	pH	Bio-mass	pH	Bio-mass	pH	Bio-mass	pH	Bio-mass	pH	5%	1%	5%	1%
Aspergillus flavus	249	4·3	216	3·7	174	3·5	133	3·7	72	3·7	0	2·8	15	25	0·1	0·2
Aspergillus niger	320	3·7	297	1·9	266	1·8	180	2·1	79	2·6	0	2·0	26	42	0·1	0·2
Aspergillus terreus	328	5·6	258	5·1	215	5·4	155	5·7	61	4·3	0	3·3	21	34	0·1	0·2
Cladosporium herbarum	521	7·3	472	6·2	203	6·4	169	6·7	110	6·1	0	6·0	16	26	0·1	0·2
Fusarium oxysporum	174	6·2	123	6·7	146	6·9	153	7·3	80	7·1	0	7·1	17	28	0·1	0·2
Stachybotrys atra	46	6·7	115	6·7	138	6·7	113	6·8	30	7·0	0	7·0	16	27	0·3	0·6

Fusarium oxysporum, application of industrial waste water at 100% concentration caused complete inhibition of radial growth (Kr = zero). In *Stachybotrys atra* a clear stimulation could be observed up to 60% at which Kr was 0·35 mm/h compared with 0·31 mm/h in the control. At the highest concentration (100%) Kr decreased to 52% of the control.

EFFECT ON BIOMASS PRODUCTION AND pH VALUE

Table 4 shows the inhibitory effect of waste water on mycelial growth in liquid cultures. Significant decrease in dry weight was observed after application of waste water at most concentrations, except in *S. atra*. At 100% waste water concentration no biomass was produced in any tested fungus. In *S. atra* an increase in biomass production was observed up to 60%, at which the dry weight gain was 245% of the control. Table 4 also reveals that in the three *Aspergillus* spp the pH value of the growth media was shifted sharply to the acidic side, especially with *A. niger*, at most waste water concentrations. Acidity increased with increase in waste water concentration. In *Cladosporium herbarum*, *Fusarium oxysporum* and *Stachybotrys atra* no significant change in pH of the growth media was observed at any concentration.

DISCUSSION

In a previous study of the effect of waste water contamination on the soil fungal population, Ismail *et al.* (1989) recorded that *Aspergillus flavus*, *A. terreus* and *A. niger* were more dominant in the polluted soil sites and accounted for 12·52%, 10·92% and 6·71% respectively of the total fungal count, while *Cladosporium herbarum*, *Fusarium oxysporum* and *Stachybotrys atra* constituted only 0·05%, 0·54% and 1·56% of the total count respectively.

The present investigation showed a general inhibition in most growth parameters in the presence of waste water in the growth media of the tested fungi. The inhibition was directly proportional to the increase in concentration of waste water. However, fungal species differed in their response to waste water. The differences in response among fungal species could be attributed to the composition of waste water, which reveals a higher level of Cl^- ions and Zn^{2+} ions. Zn^{2+} complexes with Cl^- and depending on the Cl^- concentration, forms $ZnCl^+$, $ZnCl_2$, $ZnCl_3^-$, $ZnCl_4^{2-}$ (Hahne & Kroontje 1973). The divalent Zn^{2+} and its Zn-Cl species may exert differing toxicities on the fungi. This assumption was confirmed by the finding of Babich & Stotzky (1978) who claimed that the toxicity of Zn to fungi was unaffected, lessened or increased by the addition of high concentration of Cl^-. They grouped the fungi tested into three categories. The first category included those fungi whose mycelial growth response to Zn was unaffected by the concentration of Cl^{-1}: *Fusarium solani* and *Cunninghamella echinulata*. The second group included fungi whose mycelial growth sensitivity to Zn^{+2} increased as the concentration of Cl^- increased: *Aspergillus niger* (in these systems the anionic Zn-Cl species were more toxic than was Zn^{+2}). The third category included those fungi whose sensitivity to Zn^{+2} decreased in presence of Cl^-: *Trichoderma viride* and *Rhizoctonia solani*, which indicated a greater sensitivity to Zn^{+2} than to Zn-Cl species.

Another explanation for the various responses of the tested fungi to waste water is the interaction between cationic heavy metals (Zn, Cu, Cd, Pb) present in waste

water which may be antagonistic or synergistic depending on fungal species. Braek *et al.* (1976) reported that Cu and Zn acted synergistically to algae except *Phaeodactylum tricornatum* where an antagonistic effect was observed. Hartmann (1980) found that the heavy metals Cd, Zn, Hg and Pb acted either antagonistically or synergistically on the activity of bacteria in aquatic environment.

The present study revealed that prolonged incubation allowed recovery from the inhibitory effect of waste water in *Cladosporium herbarum*, *Fusarium oxysporum* and *Stachybotrys atra*. Therefore, such inhibition is temporary and waste water only delays germination, i.e. increases the dormancy of the spores. This was nearly the same for the other fungi, but at 100% concentration of waste water conidial germination was totally arrested even with prolonged incubation, indicating a fungicidal effect.

Most of the concentrations used reduced germ-tube length. This suggests that the waste water used in the present study interferes with the biosynthesis of cell components that are required for normal growth. As a result, germ tube development was very slow and germ tubes were thin and malformed in most cases especially at higher concentrations.

It is worth noting that the three *Aspergillus* spp were isolated in higher densities from the waste water-polluted soil sites than *Cladosporium herbarum*, *Fusarium oxysporum* and *Stachybotrys atra*, but they were more sensitive to the presence of this waste water in the laboratory experiments. This sensitivity could be correlated with the drastic drop in the pH value of the media during the growth of the three *Aspergillus* spp, while no such change occurred in the pH of the growth media of the other fungi. Starodub *et al.* (1987) reported that the combined toxicity of Cu, Zn and Pb to fresh water algae was significantly greater at pH 4·5 than at pH 8·5 or 6·5 and that the synergistic effects between the three metals towards algal growth increased at low pH. Wainwright & Grayston (1986) claimed that *Aspergillus niger* oxidized sulphides of Cu, Pb, Zn to sulphate, thiosulphate and tetrathionate and the medium was acidified leading to increased toxicity.

In general, the industrial waste water of the Coke and Chemicals Factory at Helwan significantly inhibited germination, radial growth and biomass production at most experimental concentrations. The undiluted waste water (100% concentration) completely arrested fungal growth. Assuming that fungi are biological indicators of pollution, it may be concluded that the contamination of soil with waste water may be dangerous not only for microorganisms, but for vegetation and human health as well.

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تأثير المياه المتخلفة عن الصناعة على انبات ونمو بعض الفطريات

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خلاصة

استهدف البحث دراسة تأثير المياه المتخلفة عن صناعة الكوك والكيماويات على انبات أبواغ ستة أنواع من الفطريات عزلت من تربة ملوثة بهذه المياه بدرجات متفاوتة .

واتضح أن معظم تركيزات المياه المتخلفة قد ثبطت إنبات الأبواغ لكل من أسپرجللس فلافس ، وأسپرجللس نيجر ، وأسپرجللس تيرياس ، وكلادوسپوريام هيربارم ، بينما نشطت إنبات أبواغ كل من فيوزاريوم أوكسيسپورام وستاكيبوتريس أترا . وحينما استعملت المياه المتخلفة دون تخفيف (أي بتركيز ١٠٠٪) ، فانها أوقفت تماما إنبات أبواغ أنواع الاسپرجللس الثلاثة .

وقد ظهر أيضا أن معدل نمو الهيفات انخفض مع استعمال التركيزات المختلفة من ا لمياه المتخلفة ، كما أن الوزن الجاف الناتج قد انعدم وخاصة عند التركيزات العالية من هذه المياه .

وقد أدى نمو الفطريات في المياه المتخلفة الى زيادة حموضة الوسط في حالة أنواع الأسپرجللس الثلاثة ، بينما لم تتغير درجة الحموضة في وجود الفطريات الثلاثة الأخرى .