EFFECT OF METAL IONS ON *TRICHOPHYTON RUBRUM* CULTURE

by A. K. CHANDRA *and* A. B. BANERJEE*, *Department of Botany, Calcutta University, Calcutta-700019*

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The mycelial growth of *Trichophyton rubrum* in a synthetic medium comprising of glucose, glutamic acid and salt mixture, could be correlated with the concentration of Fe^{++}, Zn^{++}, and Mn^{++} up to the concentration 2.5 µg, 0.5 µg, and 0.05 µg/ml respectively. At higher concentration these metal ions are growth inhibitory.

In Zn^{++} and Mn^{++} deficient media, *p*H was reduced during the growth of the organism. Lactic acid was identified and found responsible for this shift. The culture of the organism under mineral deficiency is characteristically devoid of differentiated reproductive bodies.

Ag^{+}, Bi^{+++}, Ba^{++} and Hg^{++} are not growth inhibitory for *T. rubrum* in complex medium up to a dose level of 20 ppm. The organism is very sensitive to the toxic action of Pb^{++}, Cu^{++} and Cd^{++}. The culture characters of the organism are changed in the presence of heavy metals even at subtoxic dose.

INTRODUCTION

For the pathogenico rganism, the trace element metabolism poses an interesting but complicated problem. The requirement level of a particular micro-element may be different for the host and the parasite. So the availability of trace element in host system may influence the host-pathogen interaction by regulating the growth and metabolism of pathogen in host tissue. Similarly the effect of 'toxic' metal ions on the pathogen and its host may also vary qualitatively and quantitatively. In the present study the trace element requirements of *Trichophyton rubrum* in a synthetic medium are described. Elements tested were iron, zinc and manganese, which bear general importance in the physiology of fungi. The effects of different toxic metal ions on the growth of this dermatophyte were determined.

MATERIALS AND METHODS

Strain and media—The strain of *Trichophyton rubrum*, obtained from the School of Tropical Medicine, was maintained in Sabouraud dextrose agar. For trace element requirement studies, synthetic medium of the following composition was used throughout : $MgSO_4$, $7H_2O$ (0.1 g), KH_2PO_4 (2.0 g), glutamic acid (2.5 g), glucose (4.0 g). water (1 litre). The final *p*H of the medium was adjusted to 5.6. Glucose solution was sterilised separately and added just before inoculation.

**Permanent address* : Department of Biochemistry, Calcutta University, Calcutta-700019.

The medium was provided with other metal salts at concentration over the threshold level. The completed medium contained (per ml) iron as $FeSO_4.7H_2O$, 1.25 μg; zinc as $ZnSO_2.7H_2O$, 0.5 μg; manganese as $MnCl_2.4H_2O$, 0.5 μg; calcium as $Ca(NO_3)_2.4H_2O$, 2.0 μg; Copper as $CuSO_4.5H_2O$, 0.4 μg. The test element was omitted from the completed medium or added at graded dose. The primary medium was first purified free of metal ions by the procedure described below. Inhibitory effect of toxic metal ions were studied in Sabouraud's dextrose broth without purification.

Removal of minerals from medium and glass wares—Borosilicate glass ware was used throughout the experiments. These were rinsed with 6N nitric acid to remove surface contaminants and washed several times with distilled water and finally with deionised double distilled water, till the wash water was free from minerals. The final wash water was tested for possible presence of metal ions with 0.001 per cent solution of dithiozone in carbon tetrachloride at *p*H 6.5. The presence of any metal dithizonates would give a colour change from the green to red.

The medium was made free of metal ions by alumina treatment as described by Donald *et al.* (1952).

Preparation of inoculum—The spores of *T. rubrum* were transferred from one month old agar slant culture to a 100 ml conical flask containing 25 ml purified synthetic medium supplemented with salt solution and allowed to germinate by incubation at room temperature under shaking for one week. The germinated mycelia were harvested by centrifugation and washed six times with 0.05 per cent KCl solution in distilled water and finally suspended in 25 ml of purified synthetic medium without salt solution. The experimental flasks were inoculated with 0.1 ml of this suspension.

Incubation and measurement of growth—Flasks were incubated in a rotary shaker at 30°C for 30 days. Dry mycelial weight was taken as index of growth which was determined by filtering the entire mycelia in the flasks followed by thorough washing with cold water and drying at 105°C to constant weight.

Morphology of cells in mineral deficient media—A small fragment of mycelium was mounted on glass slide, stained with lactophenol and cotton blue, and observed at 450X magnification.

Detection of organic acids in the medium—Culture filtrates free from cell material were extracted with peroxide free-diethyl ether repeatedly. The pooled ether extracts were evaporated to dryness under reduced pressure. The dry residue was dissolved in a small volume of water. For identification of organic acids the solution was fractionated by two dimensional descending chromatography on Whatman No. 1 paper using ethanol : ammonia : water (80 : 5 : 15, V/V) and ethyl cellosolve : eucalyptol : formic acid (50 : 50 : 20, V/V) as solvent systems (Cheftel *et al.* 1952). The dried chromatogram was sprayed with 0.04 per cent ethanolic bromocresol blue for localisation of organic acid spots.

Minimum inhibitory concentration (MIC) of metal ions—The relative toxic effect of different metal ions was calculated on the basis of concentration of metal ion necessary to inhibit the growth of the organisms. Sabouraud's dextrose broth of double concentration was dispensed in culture tubes in 5 ml quantities. Different salt solutions were added to the tubes at desired graded concentration and the final

volume was adjusted to 10 ml with distilled water. The tubes were inoculated with *T. rubrum* and incubated as described above.

RESULTS

The mycelial growth of *T. rubrum* could be correlated with the concentration of all the three micronutrients tested, viz., iron, zinc and manganese, up to certain limit (Figs. 1 and 2). The maximum growth attained at concentrations, 2.5 μg/ml, 0.5 μg/ml and 0.05 μg/ml of iron, zinc and manganese respectively. At higher concentrations, these metal ions exhibited inhibitory effect on the growth. Complete inhibition of growth was noticed with 5 μg/ml of iron. Toxic effect at level beyond 0.5 μg/ml for zinc and 0.05 μg/ml for manganese are also characteristic (Figs. 1 and 2).

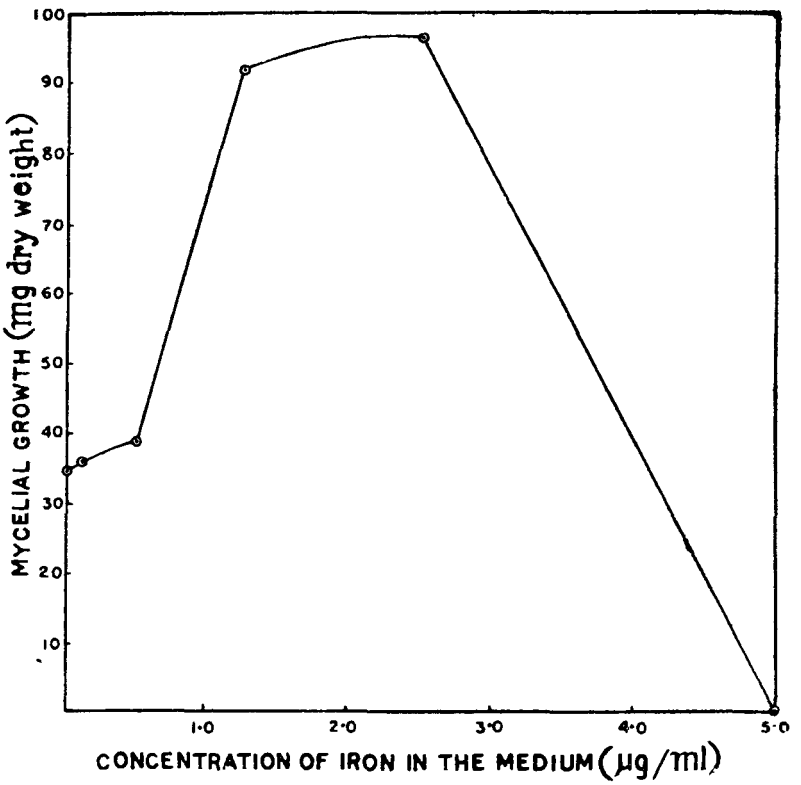

FIG. 1. Effect of iron on *T. rubrum* culture

In zinc and manganese deficient media, *p*H was reduced by one unit after 30 days' growth and this was found due to an accumulation of lactic acid (Table I). The results are noteworthy as no such accumulation of lactic or any other organic acid occurs when the organism was cultivated in the presence of sufficient amounts of minerals.

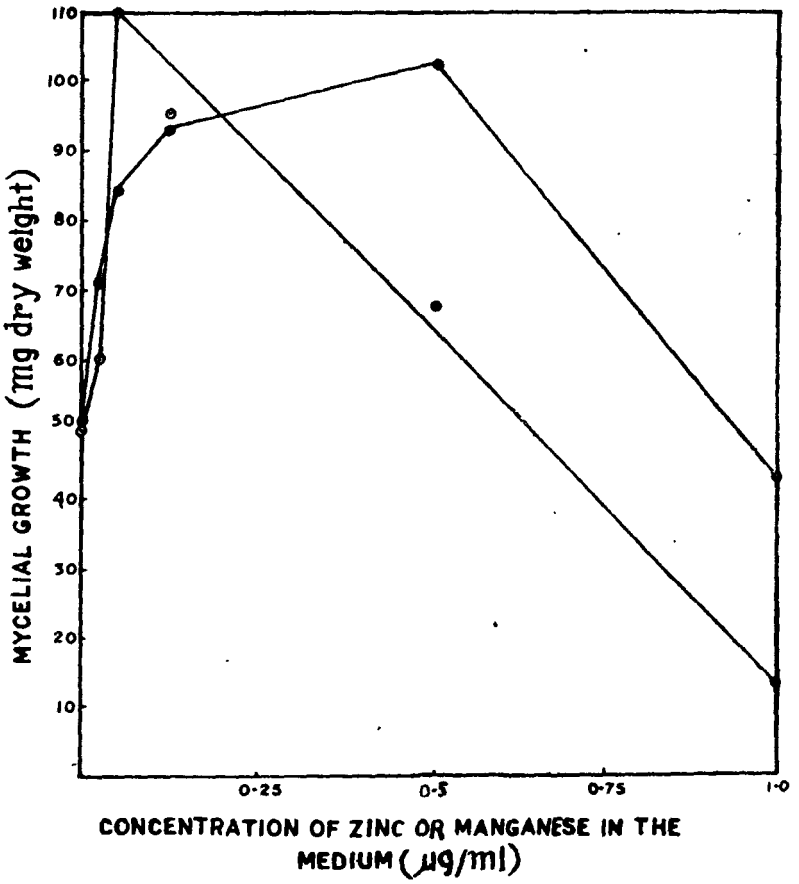

FIG. 2. Effect of zinc and manganese on *T. rubrum* culture. ⊙ Manganese; ● Zinc

Neither chlamydospores nor hyphal swelling could be traced in the *T. rubrum* culture grown in iron deficient medium. Almost same results were obtained with the culture grown in zinc and manganese deficient media. At optimal and also at supra-optimal concentration of these minerals chlamydospores and hyphal swellings are noticeable. Chlamydospores were both terminal and intercalary, solitary or in chains.

It was observed that Ag^{+}, Bi^{+++}, Ba^{++}, and Hg^{++} ions were not growth inhibitory for *T. rubrum* (Table II) in complex medium up to a dose level of 20 ppm. Among the toxic heavy metals tested, Pb^{++} seems to be most toxic, as MIC for this metal ion was found to be 2 ppm. For Cu^{++} and Cd^{++}, MIC value came to about 8 and 15 ppm respectively. The morphological and cultural characteristics of *T. rubrum* in the presence of different heavy metals also changed. In absence of these metal ions, the growth was profuse, and submerged with abundant number of chlamydospores. In the presence of Cu^{++}, Ag^{+}, Cd^{++} and Ba^{++}, the organism grew only on the surface and with few reproductive bodies.

TABLE I

Change of pH *and acid accumulation in mineral element deficient or sufficient culture medium after the growth of* T. rubrum

Treatment	Concentration of ion µg/ml	Medium Initial *p*H	Final *p*H	Net difference	Organic acid detected
Control		5.6	5.2	—0.4	None
Fe^{++}	5.0	,,	5.6	0	,,
	2.5	,,	5.8	+0.2	,,
	1.25	,,	5.6	0	,,
	0.5	,,	5.6	0	,,
	0.1	,,	5.6	0	,,
	0.0	,,	5.6	0	,,
Zn^{++}	1.0	,,	5.6	0	,,
	0.5	,,	5.6	0	,,
	0.125	,,	5.2	—0.4	,,
	0.05	,,	4.6	—1.0	Lactic
	0.025	,,	4.6	—1.0	,,
	0.0	,,	4.6	—1.0	,,
Mn^{++}	1.0	,,	5.6	0	None
	0.5	,,	5.6	0	,,
	0.125	,,	5.6	0	,,
	0.05	,,	5.8	+0.2	,,
	0.025	,,	4.6	—1.0	Lactic
	0.0	,,	4.6	—1.0	,,

TABLE II

Minimum inhibitory concentration of heavy metal ions against T. rubrum

Metal ion tested & the salt used	Concentration (in ppm) of the metal ion									Comments
	1	2	4	6	8	10	12*	15	20	
Copper as $CuSO_4.5H_2O$	+	+	+	+	Trace growth	Trace growth	—	—	—	Only surface growth
Silver as $AgNO_3$	+	+	+	+	+	+	+	+	+	Both submerged and surface growth
Bismuth as $Bi(NO_3)_3.5H_2O$	+	+	+	+	+	+	+	+	+	Mostly submerged
Cadmium as $3CdSO_4.8H_2O$	+	+	+	+	+	+	+	—	—	Mainly surface growth
Barium as $(CH_3COO)_2Ba$	+	+	+	+	+	+	+	+	Trace growth	Surface growth in all tubes
Mercury as $HgCl_2$	+	+	+	+	+	+	+	+	+	Growth mostly submerged
Lead as $(CH_3COO)_2Pb.3H_2O$	+	—	—	—	—	—	—	—	—	A few chlamydospores with 1 ppm concentration

+ & — indicate growth and growth inhibition respectively.

Discussion

The trace metal requirements of dermatophytes was studied by Mosher *et al.* (1936), Stockdale (1953) and English and Barnard (1955). Only the last two workers attempted detailed and systematic studies on copper, iron, zinc and manganese requirements of *T. mentagrophytes* and *T. rubrum* following modern analytical methods. They established that optimal dose of copper, iron, zinc and manganese requirement of a *T. rubrum* strain is 1.3, 5.8, 8.1, 1.01 μg/ml respectively in medium containing complex nitrogen source. Interpretation of the values obtained in complex media are difficult due to the fact, that the amount of the metal ions removed by the medium components by complex formation could not easily be accounted for. So in the present study we used chemically defined medium and found that the values obtained are less than those recorded by English and Barnard (1955). These authors have shown that when there was poor growth, specilaised organs developed were fewer. In our study we find that for the development of chlamydospores, the presence of optimal amounts of trace elements is mandatory and also in the mineral deficient medium there is less hyphal swelling.

Zinc deficiency causes distortion of carbohydrate and protein metabolism in many fungi. A shift of medium *p*H towards acidity was noticed when *T. rubrum* was grown in zinc deficient media. The accumulated acid was identified to be lactic. Lactic acid was also identified in the medium when the organism was grown in Mn^{++} deficient condition.

General biological toxicity of heavy metal ions is also manifested in *T. rubrum.* However, characteristically, the organism can tolerate some toxic metals including Hg and Ag to a relatively high dose. This observation is interesting as it was reported earlier that these metal ions are partially inhibitory for *in vitro* respiratory oxygen uptake in this organism (Nickerson and Chadwick 1946).

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