



Research Paper

**INFLUENCE OF COPPER AND ZINC ON SIDEROPHORE PRODUCTION OF
Fusarium SOIL ISOLATES**

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Abstract

Laboratory experiments were carried out to find the effects of copper and zinc on siderophore production of two *Fusarium* soil species isolated from an agricultural field of Murhidabad district in West Bengal, India. Siderophore is an iron binding compound of microbial origin which creates iron-limiting conditions for competitor microorganisms. Siderophore production is primarily induced in response to iron deficiency, though various ecological factors can directly affect this process. The *Fusarium* fungi were recovered on selective pentachloronitrobenzene (PCNB) medium and subsequent identification was accomplished on the basis of their characteristic conidial morphology. The soil isolates were inoculated in Czapek's dox broth amended with three different concentrations (50 ppm, 100 ppm and 150 ppm) of each of the heavy metals with respect to a control having no heavy metal. Siderophore unit productions were estimated spectrophotometrically using Chrome azurol sulfonate (CAS) reagent. Both the soil species were found to be potential siderophore producers in absence of heavy metals. Adverse effects of both the heavy metals on siderophore production of the tested soil isolates were fairly evident. Copper was found to exert more detrimental effects than zinc on siderophore production with concomitant decrease in mycelial dry weight of both the soil fungi.

Key words: Copper, zinc, siderophore, heavy metal, *Fusarium*, PCNB medium, conidia, CAS reagent etc.

INTRODUCTION

The production of iron binding compound siderophore is the most studied mechanism amongst the various means used by microorganisms. The word siderophore is defined as relatively low molecular weight, ferric ion specific chelating agents produced by bacteria and fungi with a very high affinity for iron. Under conditions of iron starvation, to compete effectively with hydroxyl ions, microorganisms synthesize siderophore. The function of these compounds is to scavenge iron from the environment and to make it available to the microbial cell which is often biologically unavailable in the environment and create iron-limiting conditions for competitors, including pathogenic microorganisms^[1]. Microbial siderophore may also stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens with less efficient iron uptake system. Generally, fungi are stronger

siderophore producers. Most fungi produce a variety of different siderophores, which enable them to adapt different physicochemical conditions^[2]. The metal tolerant siderophore producing fungi play an important role in the successful survival and growth of plants in contaminated soils by alleviating the metal toxicity and supplying the plant with nutrients; particularly iron^[3]. *Fusarium* is primarily a soil borne fungus. Both pathogenic and non-pathogenic species are found in almost all types of agricultural soils. The non-pathogenic or saprophytic *Fusarium* species are of immense importance to the botanists for their ability to improve plant growth by a variety of mechanisms of which siderophore production deserves a special mention.

Copper and zinc are two most common heavy metals found abundantly in agricultural soils. Heavy metals are an essential part of all living organisms and present naturally in trace amount in soil. Heavy metals like Fe, Cu, Zn, Ni and other trace elements are necessary to perform the proper functions of all biological systems. Heavy metals can variously influence soil fungi by changing fungal morphology, physiological activity, growth rate, reproductive process and enzyme production etc. High levels of heavy metals could decrease the rhizosphere microbial metabolic activity, biomass and diversity^[4-5]. Plants grown in metal contaminated soils are often iron deficient^[6].

The economic stability of India is dependent on the agricultural yield. Microbial metabolite production and their applicability in controlling plant diseases is gaining momentum in agriculture. The scope of developing these microbial metabolites for commercial pesticides as an alternative to chemical fungicides which is gaining importance due to increased concerns on environmental pollution, pathogen resistance and high plant protection costs. Siderophores have wide applications in environmental remediation. Siderophores are also able to bind other metal ions, such as magnesium, chromium, gallium, manganese and radionuclide such as plutonium. This property has an influence on metal ion mobility in the environment.

The objective of this study was to evaluate the siderophore producing capability of two different *Fusarium* soil fungi isolated from an agricultural field of Murshidabad district in West Bengal with a view to understand the effects of copper and zinc on their siderophore production which are often found in excess in metal polluted soil. In addition, the role of various edaphic and environmental factors on productions of fungal metabolites can also be assessed.

MATERIALS AND METHODS

Collection of soil sample:

Soil sample was collected from an agricultural field of Bamnabad village located near Indo-Bangladesh boarder region of Murshidabad district in West Bengal. The agricultural field is present in close vicinity of Padma river and cultivated for various crops throughout the year where no *Fusarium* diseases were reported previously. Soil at a depth of 6 cm was collected from the rhizospheric region as the fungus is reported to be present abundantly in the root regions.

Isolation and characterization of *Fusarium* spp.: The *Fusarium* spp. were isolated directly from the soil by dilution plate technique. 1 gm of soil was mixed in 10 ml sterile water to prepare the crude soil suspension. From the crude suspension, 1/10th and 1/100th dilutions were prepared and were subsequently inoculated on potato sucrose agar (PSA) medium [composition (g/l): potato extract 200, sucrose 20, agar 20, pH 6] supplemented with PCNB (0.1%) and chloramphenicol (0.01%) for selective growth of *Fusarium* spp. The plates were incubated at 28°C for 5-7 days until visible sign of colony growth occurred. Later on, the fungal isolates were grown on Czapek's Dox agar (CDA) medium [composition (g/l): Sodium nitrate 2, Di potassium hydrogen phosphate 1, Magnesium sulphate 0.5, Potassium chloride 0.5, Ferrous sulphate 0.01, Sucrose 30] to characterize the *Fusarium* isolates in terms of their growth, colony morphology, sporulation and pigmentation.

Media preparation with the heavy metals and inoculation:

Zinc and copper in the concentration of 10000 ppm were prepared from the zinc and copper as hydrated sulphates (ZnSO₄·7H₂O, CuSO₄·5H₂O). 25 ml of Czapek's dox broths (deficient of iron) were amended with the respective heavy metals separately to prepare broths having 50, 100 and 150 ppm final concentrations of each of the heavy metals. The broths supplemented with these heavy metals at different concentrations were autoclaved and subsequently inoculated with the equal amount of mycelial discs from 7 days old pure cultures of each *Fusarium* soil isolates. The cultures were incubated at 28°C for 14 days.

Detection of siderophore producing *Fusarium* spp.

For qualitative estimation of siderophore production, 0.5 ml pure culture filtrates of 14 days old *Fusarium* soil isolates were taken and 0.5 ml CAS solution was added in two separate 10 ml test tubes. The test tubes were incubated at room temperature and in darkness for 2-3 minutes until color changes were noticed.

Quantitative estimation for siderophore unit production in different heavy metal concentrations:

Spectrophotometric CAS liquid assay was used to determine siderophore unit production of the *Fusarium* isolates^[7]. The pH was adjusted to pH 6.8. 0.5 ml CAS assay solution was added to 0.5 ml culture supernatants of 14 days old pure cultures of both the *Fusarium* soil species amended with different concentrations of copper and zinc. 10 µL of 0.2 M 5-sulfosalicylic acid as shuttle solution was also mixed in separate setup. The mixtures were left for few minutes. The color development was measured by absorbance (A630) for loss of blue color. The sterile culture medium was used as blank and the sterile culture medium plus CAS and shuttle solutions were used as a reference. Siderophores units were calculated as $[(Ar-As)/Ar] \times 100 = \% \text{ siderophores units}$; where Ar is the absorbance reading of the reference, and As is the absorbance reading of the sample.

RESULTS AND DISCUSSION

Isolation and characterization of *Fusarium* spp.

A total of two species of *Fusarium* were isolated from the soil sample studied and their subsequent characterization is depicted in table-1. The soil isolates were named SF 4-5. Isolates were identified based on their recovery on *Fusarium* selective PCNB medium and on the basis of macroscopic viz., colonial morphology, colour, texture, shape, diameter and appearance of colony and microscopic characteristics viz., septation in mycelium, presence of specific reproductive structures e.g. chlamydospore, shape and structure of conidia. Pure cultures of fungal isolates were identified with the help of literature^[8-9].

Fusarium SF-4 was found to be extremely fast growing species on CDA medium among the two soil isolates obtained from soil sample of Bamnabad agricultural field. The colony diameter of SF-4 was 83 mm after 7 days of incubation on CDA medium. On the other hand, *Fusarium* SF-5 was moderately fast growing with a colony diameter of 59 mm after the same days of incubation on CDA medium. Mycelial dry weight of the fungal isolates after 14 days of incubation firmly established the fact that SF-4 was by far the fast growing species among the two soil isolates (table-1). Both the colonies of the *Fusarium* isolates differed to a great extent in terms of colony morphology and pigmentations. Initially, both the fungal colonies were white but after fifth days of incubation faint orange and yellow pigmentations were developed by the colonies of SF-4 and SF-5 respectively. The mycelial colony of SF-4 was more compact than SF-5 but both the colonies have smooth and circular margins without any concentric zonations. The fungal isolates also differed in sporulation pattern to a major extent. Both the fungi showed profuse sporulation on CDA medium. SF-4 produced thick walled chlamydospore in chain and 1-2 celled, slightly curved microconidia. But SF-5 produced typical sickle shaped macroconidia having 3-4 septations in addition to microconidia with prominent degrees of curvature (fig.1). The sizes of microconidia, macroconidia and chlamydospore of the *Fusarium* isolates ranged from 10-15 µ, 30-35 µ and 20-25 µ respectively.

Detection of siderophore producing *Fusarium* spp.

Both the *Fusarium* soil isolates were found positive in the qualitative test for siderophore production. Siderophores withdrew iron from the dye complex, resulting in a reduction in blue color of the solution of CAS reagent. The isolates were strong siderophore producers because the color of the solution turned pink.

Quantitative estimation for siderophore unit production in different heavy metal concentrations:

Siderophore production by the *Fusarium* soil fungi is depicted in table 2. From data, it is clearly evident that both the soil fungi were able to produce siderophore in substantial amount in absence of heavy metals. SF-4 was more potent siderophore producer than SF-5. But when the media were supplemented with different concentrations of Cu and Zn, siderophore productions declined to a considerable extent (Fig. 2).

The specificity of microorganism's metabolic character is dependent upon soil conditions ^[10]. Heavy metals can cause reproduction of soil microorganisms to slow down and consequently decrease biological activity ^[11]. Cu and Zn is essential element required for physiological function and growth of organisms. Also it can modify the fungal morphology and physiological functions in relation to the range of concentrations and fungal strain ^[12]. Both are essential for all organisms, although at higher concentrations they can be toxic. The heavy metal might be directly involved in siderophore biosynthesis pathways or their regulation ^[13]. It was also assumed that the free siderophore concentration in the medium might be reduced by complex formation with heavy metal ions ^[14]. Inhibitory effect of copper on siderophore production was more prominent than zinc in the tested *Fusarium* spp. With the increase in Cu concentrations, mycelial dry weights decreased significantly with simultaneous reduction in siderophore production in both the *Fusarium* soil fungi. Nearly two folds decrease in siderophore production was noticed at 150 ppm Cu concentration with respect to control in both the soil isolates (table-2). Lower mycelial biomass can be attributed to the gradual attenuation in production of siderophore. Though, *Fusarium* SF-4 produced siderophore in quite substantial amount up to 100 ppm Cu concentration. But, in SF-5 siderophore production exhibited steady decrease from 50 ppm Cu concentration onwards. Interesting, both the isolates were found moderately Zn tolerant and siderophore unit production was quite satisfactory in different concentrations of zinc. In SF-4, siderophore production started to fall significantly at 150 ppm zinc concentration only. But SF-5 showed consistent siderophore production in all three different concentrations of zinc. The results were in conformation with the fact that in general, the siderophore production of rhizosphere microorganisms is higher than those isolated from contaminated soils. It has also been seen that the average amount of siderophore produced by fungi isolated from rhizosphere soil was 1.2 fold higher than those in heavy metal contaminated soil.

CONCLUSION

From our experiments, it can be concluded that production of siderophore is largely depended on soil chemical and biological properties, particularly presence of different heavy metals and their concentrations. Both the heavy metals negatively influenced siderophore production to a diverse extent in the tested *Fusarium* spp. Increasing heavy metal concentrations imposed toxic effects on the soil fungi with respect to mycelial dry weight which can be ascribed to the lower production siderophore by the *Fusarium* soil species. Much work on this field is needed to be addressed for finding specific metal tolerant microorganisms from contaminated soils which could be exploited for specific heavy metals mobility and availability to the plant through the release of chelating agents like siderophore.

Table 1: Growth characteristics and sporulation of the *Fusarium* soil isolates on CDA medium

Isolate no.	Colony characters (after 7 days)			Mycelial dry weight after 14 days (gm)	Sporulation
	Diameter (mm)	Morphology	Pigmentation		
SF-4	83	Compact mycelia. circular	Faint orange	0.140	1-2 celled slightly curved microconidia and thick walled chlamydospore.
SF-5	59	Loose mycelia, circular	Faint yellow	0.098	Sickle shaped macroconidia and microconidia with greater degree of curvature

Table 2: Quantitative assay for siderophore unit production by *Fusarium* soil isolates

<i>Fusarium</i> isolate	Name of the heavy metals	Conc. of the heavy metals	Mycelial dry weight after 14 days (gm)	Absorbance at 630 nm	Reference	% of siderophore unit
SS-13/SF-4	Cu	50	0.123	0.251	0.952	73.63
		100	0.109	0.372		60.92
		150	0.095	0.493		48.21
	Zn	50	0.137	0.125		86.87
		100	0.118	0.210		77.94
		150	0.055	0.446		53.15
		Control	0.140	0.118		87.61
	SS-13/SF-5	Cu	50	0.053	0.952	38.55
			100	0.048		37.08
			150	0.033		35.50
		Zn	50	0.065		65.97
			100	0.086		72.48
			150	0.075		67.54
			Control	0.098		78.25



Fig. 1: Chlamydospore (left) and microconidia (middle) of *Fusarium* SF-4, macroconidia and microconidia (right) of *Fusarium* SF-5

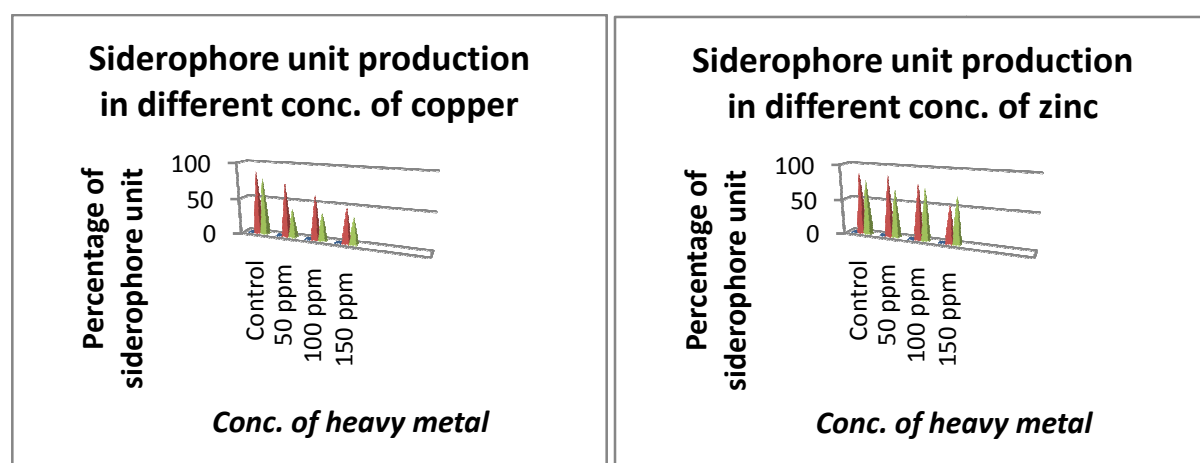


Fig. 2: Siderophore production at different concentrations of copper and zinc by *Fusarium* spp.

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