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RESEARCH ARTICLE

Effect of different cultural variables on siderophores produced by *Trichoderma* spp.

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Abstract

Siderophore production in *Trichoderma* spp. is a phenomenon which is affected by different environmental variables as pH and available iron concentration in soil. In present communication, we studied the effect of different variables on growth of *Trichoderma* spp. Experiments were conducted in *in vitro*. Among three strains *Trichoderma* MPPLUNS1, *Trichoderma* MPPLUNS2 and *Trichoderma* MPPLUNS3, *Trichoderma* MPPLUNS1 was proved efficient strain, which exhibited higher reaction rate during C.A.S. (Chrome Azurol S) Blue Agar assay for qualitative assessment of siderophore production. Best siderophore production has been shown by *Trichoderma* MPPLUNS1 at 1mM concentration of iron and 4.5 pH.

Introduction

Trichoderma spp. is a group of beneficial microorganisms in rhizosphere which have been considered as plant growth promoter due to nutrient uptake as well as plant disease manager because of phenomenal antimicrobial activity. *Trichoderma* spp. functions as biocontrol agent by depriving the pathogen from iron nutrition, thus resulting in increased yield of crop (O' Sullivan & O'Gara., 1992). Fungi exploit the surrounding soil environment for nutrients primarily by hyphal extension. In this way, fungi secrete siderophores into the soil to chelate or bind tightly iron that is subsequently brought back into the cell by specific uptake mechanisms. This iron will be used for diverse processes that are essential to the organism's survival (Winkelmann, 2007).

Siderophores are classified by the ligands used to chelate the ferric iron. These include the catecholates, hydroxamates, and carboxylates (Miethke and Marahiel. 2007). In an *invitro* experiment culture filtrate of all *Trichoderma* strain has shown the presence of coprogen, coprogen B and ferricrocin whereas T. *longibrachiatum* and *T. pseudokoningii* under iron deficiency condition (Anke *et al.*, 1991). Heidrun *et al*., (1991) reported the production of siderophores by nine *Trichoderma* strains, some with high antagonistic potential towards *Pythium ultimum*. According to them the composition of siderophores of nine different *Trichoderma* strains containing coprogen (A) type ferricrocon type, coprogen (B) type and fusigen type of siderophores. A new type of siderophores from *Trichoderma* spp. is also reported for the first time from any fungi and it is called as pal mitoylcoprogen. The antagonistic of *Trichoderma* are not correlated to the type or that amount siderophores produced (Heidrum *et al.*, 1991).

In soil plant roots normally coexist with bacteria and fungi which may produce siderophores capable of sequestering the available soluble iron and so interfere with plant growth and function. Plant root might be capable of taking up ferric complexes of siderophore and using these as sources of iron. To satisfy iron requirement microorganisms and plants have evolved specific mechanism to chelate insoluble iron through the release of siderophores, which are low molecular weight organic ligands with high affinity and specificity for iron and plant will consume iron- siderophore complexes (Weizhen Qi and Lei Zhao, 2012). Microbial siderophores may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots or indirectly competitively inhibiting the growth of plant pathogens with less efficient iron uptake system (Marek-Kozaczuk *et al.*, 1996).

Studies of siderophores producing microorganism have received much more attention because of the clinical applications and potential utilization of these chelaters in agriculture (Neilands, 1995). Recently, siderophores have been involved in the early stages of the lignocellulose depolymerization in wood cell wall by fungi (Goodell *et al.* 1997).

A vital aspect of any microbe's metabolism is the uptake, storage, and utilization of iron (Fe). Many of the physiological aspects of iron's reduction-oxidation (redox) processes remain unclear. It is, however, well known that Fe (II) acts as an electron source for Fe-oxidizing organisms while Fe (III) acts as an electron sink for Fe-reducing organisms (Weber *et al.*, 2006). Fe is also a cofactor for some enzymes and a component of iron-sulphur proteins (such as protein complexes in the mitochondrial electron transport chain). Additionally, Fe is an essential structural component of proteins, such as ferrichrome, produced by fungi that contain metal-liganded domains (Garrett, 2005).
The role of siderophores in the biocontrol of soilbome phytopathogens is well documented. The low molecular mass biomolecule is known to act as a growth factor, and also exhibits potent antimicrobial activity (Bano and Musarrat, 2004). Several studies have demonstrated that production of siderophores, other secondary metabolites and lytic enzymes by *Pseudomonas* strains was most effective in controlling the plant root (Farah *et al.*, 2006).
The most common detection method for siderophore production is the universal assay of Schwyn and Neilands (1987). This assay is based on a competition for iron between the ferric complex of the dye chrome azurol S (CAS) and a siderophore. The CAS assay can be applied as a liquid test or alternatively the dye can be incorporated in the agar medium for detection of siderophore in solid medium. The CAS assay of liquid supernatants of cultures has been stated to be quantitative; however on the solid medium it is not possible to quantify the CAS reaction (Schwyn and Neilands 1987; Raaska *et al.* 1993). The major works that employ CAS-agar plate assay use the term strong or light and symbols (++) or (+) to evaluate the diameter of coloured halo around the microbial colony (Manninen and Mattila-Sandholm 1994).
The objective of present study to evaluate the potential effects of cultural conditions on growth and siderophore production of *Trichoderma* spp. By employing this methodology, it could be possible to evaluate the effect of the iron concentration and pH of the media on the siderophore production in *Trichoderma* species.

Material and Methods

Fungal species

In the present study *Trichoderma* MPPLUNS1, *Trichoderma* MPPLUNS2 and *Trichoderma* MPPLUNS3 strains were used from the collection of Mycology and Plant Pathology Division, Botany Department, University of Lucknow. The fungal species were maintained on 2 per cent potato dextrose ager slants at 5°C.

Determination of siderophore production in *Trichoderma* Strains

The ability of *Trichoderma* MPPLUNS1, *Trichoderma* MPPLUNS2 and *Trichoderma* MPPLUNS3 to produce iron-binding compounds of siderophore-type were detected in solid medium by universal C.A.S assay as per Schwyn and Neilands (1987). The methodology was slightly modified according to Milagres (1999).

Preparation of the C.A.S. (Chrome Azurol S) Blue Agar

One litre of C.A.S blue agar was prepared according to Schwyn and Neilands (1987) using 60.5 mg C.A.S (chrome Azurol S) dissolved in 50 ml distilled deionized water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$, 10 mM HCI). Under stirring, this solution was slowly added to 72.9 mg H.D.T.M.A. (Hexa decyl tri methyl-ammonium bromide) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved for 20 min. Also autoclaved a mixture of 750 ml water, 15 g agar, 30.24 g Pipes and 12 g of a solution of 50% (w/w) NaOH to raise the pH to the pKa of Pipes (6.8). The dye solution was finally poured along the glass wall and agitated with enough care to avoid foaming. Petri dishes (9.5 cm in diameter) were prepared with 30 ml of appropriate medium for culturing *Trichoderma* MPPLUNS1, *Trichoderma* MPPLUNS2 and *Trichoderma* MPPLUNS3. After solidification, the medium was cut into halves, one of which was replaced by C.A.S. blue agar (15 ml). The halves containing culture medium were inoculated with 5 mm discs of Seven days old culture of *Trichoderma* strains grown on Potato dextrose agar medium. The inoculum was placed as far as possible, from the borderline between the two media. The plates were incubated at growth temperature (28 ± 2°C) of *Trichoderma* MPPLUNS1, *Trichoderma* MPPLUNS2 and *Trichoderma* MPPLUNS3 for 7 days in the dark. The C.A.S. reaction rate was determined by measuring the intensity of color-change in the C.A.S.-blue agar, starting from the borderline between the two media. The C.A.S.-agar colour changed from blue to purple or dark purplish- red (magenta). The experiment was carried out in triplicates. The control plates of C.A.S.-agar uninoculated were incubated under the same conditions as described above and no color change in the C.A.S.-blue agar was observed, even after long incubation periods (10-15 days).

Effect of pH

To study the effect of culture pH on siderophore production, PDA (2% potato dextrose agar) medium was buffered with sodium acetate 0.1 M at pH 4.5 and 5.5. The plates were inoculated and incubated as described above. Growth of species and CAS reaction rate were evaluated daily and compared with the non-buffered PDA medium (pH 6.5).

Effect of iron

To study the effect of iron on siderophore production by the three *Trichoderma* strains, PDA mediums were prepared modified with iron (1mM to 4mM). The plates were inoculated with 5 mm diameter culture discs of three

different *Trichoderma* strains on PDA medium modified with iron source which were incubated at 28±2°C for 7 days. Growth of species and CAS reaction were evaluated daily.

Statistical Analysis

Experiments were carried out in triplicates. Data were expressed as means of five replicates. Statistical analysis was performed with Microsoft excel 2007. Difference on statistical analysis of data were considered Significant at $P<0.05$. ANOVA was made based on the diameter of the radial growth among *Trichoderma* strains.

Result and Discussion

Results were qualitatively described in terms of colour of C.A.S. reaction as presented in Table 1, colour change from blue to purple or pink was observed. *Trichoderma* MPPLUNS1 grew rapidly in the appropriate plate half containing PDA medium but did not grow at all in the plate half containing C.A.S blue agar (Fig 1). Siderophore was derived from a Greek term meaning, iron carrier (Brianne, 2004) under iron-limited conditions, most aerobic and facultative anaerobic microorganisms (bacteria and fungi) release some low-molecular-weight (500-1000 Da) compounds. The siderophore type compounds were excreted by the microorganisms and diffused through the C.A.S blue agar producing a colour change from blue to purple or pink for most *Trichoderma* strains. The colour change was observed in the CAS-blue agar when *Trichoderma* strains started to cover half of the Petri plate (Fig 1a, 1b, 2a, 2b). The production of siderophores in solid medium by *Trichoderma* strains was evaluated as the CAS reaction rate and expressed in mm per day by measuring radial diameter.

The results indicated that *Trichoderma* spp. had shown production of siderophores in *in vitro* conditions but the potential of each strain to produce siderophores in the same conditions was different. The fastest reaction of colour change was observed in *Trichoderma* MPPLUNS1 whereas *Trichoderma* MPPLUNS2 and *Trichoderma* MPPLUNS3 comparatively slow reaction which has indicated the moderate reaction rate. The distinct responses of colour change, C.A.S reaction (purple or pink) observed with the different *Trichoderma* strains could be related to structural differences in the types of siderophores secreted. (Perez-Miranda *et al.,* 2007) from their study, it was concluded that fungi mainly produced hydroxamates type of siderophores, whereas bacteria exclusively produces catecholates type of siderophore. It was previously mentioned in a manuscript that the biosynthesis of siderophores is regulated by the iron content of the medium (Neilands, 1995) As the results revealed *Trichoderma* MPPLUNS1 has given the fastest reaction of colour change which means *Trichoderma* MPPLUNS1 is a good type of siderophores producer instead of *Trichoderma* MPPLUNS2 and *Trichoderma* MPPLUNS3 only recognized as moderate siderophore producer. Microbes produce siderophores; a low molecular weight iron chelating secondary metabolites that can remain intracellular or be secreted into the surroundings (Winkelmann, 2007).

In this manner we can correlate the production of siderophores in solid medium evaluated as CAS-reaction rate (mm/day), which can provide us the intensity of siderophores production in respective of test fungus. Siderophores also have been shown to be useful as a drug when administered to patients combating iron-overload diseases. Iron-overload disease, known as thalassemia, is a major problem in the world, affecting hundreds of thousands of people each year (Brianne, 2004).

Alexander and Zuberer (1991) demonstrated that CAS agar effectively differentiated bacteria that were able to excrete large amounts of siderophore. Frey-Klett *et al.* (2005) used this same method however the CAS media was modified.

The effect of different pH values on the siderophore production both in buffered PDA medium (pH 5.5 and 4.5) and in non-buffered PDA medium (pH 6.5) was studied. Radial diameter of test fungi were measured every day, are shown in Table 2. The most significant influences were observed with *Trichoderma* MPPLUNS1 during CAS-reaction, the reaction rate was altered because of different pH conditions. Maximum radial growth was noticed in *Trichoderma* strains at pH 4.5 whereas a notable decrease was found at pH 5.5.

Siderophore production was affected by iron concentration in growth medium with different degrees. As the iron concentration in medium was increased, siderophores production was repressed. It was clear that increasing iron concentration has stopped the siderophores concentration by decreasing radial growth of *Trichoderma* strains. According to our data 1mM concentration was proved to be most suitable dose of iron which can enhance siderophore reaction in *Trichoderma* strains.

In *in vitro* condition, (Table 2 and 3), the radial growth of *Trichoderma* MPPLUNS 1 was observed in mm/day. Same methodology was followed in respect of iron requirement of micro-organisms, In iron deficient condition, *Trichoderma* MPPLUNS 1 has demonstrated higher reaction rate at 1mM iron concentration which was lowest among all provided iron dose (Table 3) that was also proved in context of *Pseudomonas, N. crassa, F. dimerum* and *Mucor* sp, siderophores production was repressed as at 3 µm Fe (III) (Dave and Dube, 2000).

Table1. Efficacy of *Trichoderma* strains for production of siderophore

BCAs	CAS reaction	CAS blue Ager colour change
Trichoderma MPPLUNS 1	+++	Blue to purple to dark mangeta
Trichoderma MPPLUNS 2	++	Blue to purple to light mangeta
Trichoderma MPPLUNS 3	++	Blue to purple to light mangeta
Control	-	Blue

Each value is mean of three replicates and CAS reaction for
- - No reaction
+ - Slow reaction
++ - Moderate reaction
+++ - Fast reaction

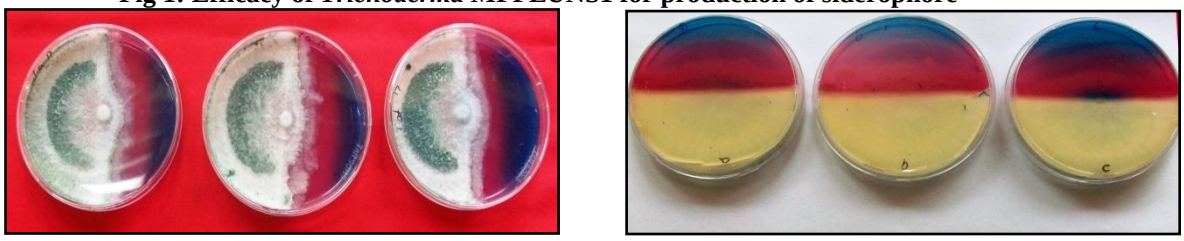
Table2. Effect of the pH medium on the siderophore production by *Trichoderma* strains growing on CAS-agar plates modified

Medium (pH)	Radial diameter (mm/day)		
	*T.*MPPLUNS1	*T.*MPPLUNS2	*T.*MPPLUNS3
4.5	7.90 ± 0.41	5.32 ± 0.45	5.30 ± 0.32
5.5	6.38 ± 0.09	3.10 ± 0.07	5.40 ± 0.12
6.5	6.9 ± 0.7	4.80 ± 0.52	5.63 ± 0.33

Table3. Effect of different iron concentration on siderophore production by *Trichoderma* strains growing on CAS-agar plates modified

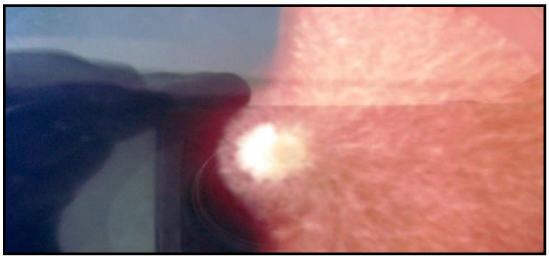
Fe conc. (mM)	Radial diameter (mm/day)		
	*T.*MPPLUNS1	*T.*MPPLUNS2	*T.*MPPLUNS3
control	7.67 ± 0.41	6.32 ± 0.15	5.25 ± 0.12
1	8.38 ± 0.09	7.10 ± 0.07	6.80 ± 0.12
2	7.9 ± 0.7	6.80 ± 0.52	6.63 ± 0.33
3	4.38 ± 0.09	4.10 ± 0.07	4.40 ± 0.12
4	3.9 ± 0.7	2.80 ± 0.62	2.63 ± 0.53

Fig 1: Efficacy of *Trichoderma* MPPLUNS1 for production of siderophore

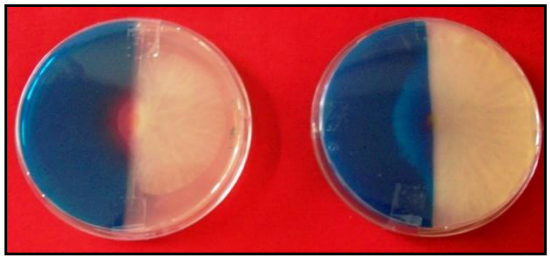


(a. Dorsal view) **(b. Ventral view)**

Fig 2: siderophore production



(a)



(b)

Conclusion

The ecological advantage of these experiments is that as *Trichoderma* is always has been considered best biocontrol, it should be inoculated in soil at certain pH condition which would be beneficial for soil environment, as iron in an aerated environment exists in the ferric form and so is highly insoluble in neutral or alkaline soil (Shenker *et al*, 1995), *Trichoderma* MPPLUNS 1 would be able to utilize a high affinity iron transport system, would produce efficient siderophores which would be able to combat iron deficient condition and also with hazardous pathogens.

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