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Production of siderophores by the antagonists *Trichoderma* spp. and the pathogen *Fusarium oxysporum* causing wilt disease of a medicinal plant *Coleus forskohlii*

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Coleus forskohlii (Willd.) Briq. [synonym *C. barbatus* (Andr.) Benth.] is a member of the mint family Lamiaceae. In India, the chief medicinal species of *Coleus* is the tuberous *C. forskohlii.* The wilt disease of *C. forskohlii* caused by *Fusarium oysporum*, the isolation of which has been confirmed by IARI (Indian Agricultural Research Institute), New Delhi, is so severe that it poses a total crop loss in the fields under cultivation. In the present study all the antagonists *viz.* five species of *Trichoderma* were subjected to give trial for verification of their efficient efficacy to check the growth of test-pathogen. The production of iron-chelating compounds, siderophores by the pathogen as well as by the antagonists was evidenced by their positive responses to $FeCl_3$ test, Chrome Azurol Sulphonate (CAS) assay and CAS agar plate test assay. The result demonstrated that the siderophore produced by *T. harzianum* was most promising as exhibited highest CAS activity than the test pathogen *Fusarium oysporum* and other species of *Trichoderma.* It concludes that *T. harzianum* shows higher degree of antagonism by producing siderophore that can bind with iron in a high specificity and affinity thus making the iron unavailable for other microorganisms thereby limiting the growth of other microbes.

Keywords : siderophore production, *Trichoderma* spp., *Fusarium oxysporum,* wilt, *Coleus forskohlii*

INTRODUCTION

Coleus forskohlii (Willd.) Briq. [synonym *C. barbatus* (Andr.) Benth.] is indigenous to India (Khatun *et al.* 2011a) and is recorded in Ayurvedic *Materia Medica* under the Sanskrit name 'Makandi' and 'Mayani'. The plant, *Coleus forskohlii* suffers from wilt disease has been reported (Khatun *et al.* 2011b).

Biological control of wilt disease of *Coleus forskohlii* caused by *Fusarium oxysporum* was attempted earlier with the application of antagonistic agents like *Trichoderma harzianum*, *T. viride, T. reesei, T. lignorum and T. hamatum* and found that *T. harzianum* showed maximum growth inhibition (86.16%) of the pathogen through mycoparasitism (Khatun, 2020). This result may be correlated by finding of Vinale *et al.* (2013). Iron is an essential nutrient for nearly all organisms because it serves as an obligate component of many indispensable enzymes and other proteins (Worrall and Michael, 2022). Iron is needed in relatively small amount but is essential as a donor and acceptor of electrons in cellular processes including cytochrome system in aerobic respiration (Deacon, 2006). The optimum requirement of iron for the normal growth and metabolism of soil microorganisms ranges from 10^{-8} to 10^{-6} M, but Fe (III) in aerobic aqueous environment is limited to an equilibrium concentration of 10^{-7} M which is much below than its optimum value. Iron normally occurs in the ferric (Fe^{3+}) form, insolubilized as ferric oxides or hydroxides at a pH above 5.5. Microbial pathogens have collectively evolved a diverse repertoire of highly effective iron acquisition mechanisms, some of which appear to be specially designed to defeat the iron withholding system or to target iron-rich niches of the host (Klebba *et al.* 2021). A more commonly used strategy for microorganisms to acquire iron is the secretion

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of high affinity iron chelators called siderophores (Page, 2019). Siderophores (Greek: sideros=iron, phores=bearers) are low molecular weight iron chelating compounds produced by microorganisms under iron stress condition (Passari *et al.* 2023). Thus stress condition of iron is a decisive factor for the biosynthesis of siderophores (Dutta *et al.* 2006). No systems analogous to siderophores have been found for any other metal ion and thereby making iron unique in requiring such specific ligands (Miethke and Marahiel, 2007). Reports are available that large number of fungi and bacteria produce siderophores under iron-limiting conditions in the soil (Chincholkar *et al.* 2006). Siderophores chelate a ferric ion (Fe^{3+}) then they reabsorbed through a specific membrane protein and Fe^{3+} is reduced to Fe^{2+} within the cell, causing its release because the siderophore has a lower affinity for Fe^{2+} than for Fe^{3+} and finally the siderophore is exported again to capture a further ferric ion (Deacon, 2006).

As siderophore produced by a microorganism can bind with iron in a high specificity and affinity thus making the iron unavailable for other microorganisms and thereby limiting the growth of the other microbes, the strategy may certainly be involved in the biological control of plant diseases. Competition for iron by siderophore production has long been recognised as an important antagonistic trait of many biocontrol agents against plant pathogens (de Boer *et al.* 2003). Growth promotion of crop plants followed by higher yield by using siderophore producing microorganisms has been reported by different workers (Singh *et al.* 2022). Some *Trichoderma* spp. produce highly efficient siderophores that chelate iron and stop the growth of other fungi (Tycękiewicz *et al*. 2022). Swayambhu *et al.* (2021) recorded hydroxamate type of siderophore type of siderophore production by different species of *Trichoderma. Trichoderma* spp. are also capable of producing phenolate type siderophores and production of such metabolites by *Trichoderma* may contribute to the biological control of different fungi (Yao *et al.* 2023). Therefore, siderophore producing microorganisms may promisingly be used in biological control of plant pathogens. Pseudobactin-siderophore producing pseudomonads can suppress germination of the chlamydospores of *Fusarium oxysporum* on low-iron media, whereas mutant pseudomonads, deficient in siderophore production are ineffiective (Deacon, 2006). Du and Li (2024) had successfully employed siderophoregenic biocontrol microbes in control of ground nut root pathogen *Fusarium oxysporum.* Production of siderophores was attempted by the antagonists *Trichoderma* spp. and the pathogen *Fusarium oxysporum* causing wilt disease of the medicinal plant *Coleus forskohlii*.

MATERIALS AND METHODS

Pathogens and Biocontrol agents

Isolation of the pathogen (*Fusarium oxysporum*) from the diseased plants and its identification has been confirmed by IARI (Indian Agricultural Research Institute), New Delhi (ITCC No.6933.08). Among the five species of *Trichoderma, T. viride, T. lignorum and T. reesei* were isolated from Mycology and Plant Pathology laboratory, Burdwan University, West Bengal and the remaining two antagonists viz. *T. harzianum* and *T. hamatum* were procured from Indian Agricultural Research Institute (IARI), New Delhi. The pathogen as well as *Trichoderma* strains were grown on potato dextrose agar (PDA) plates for a week at 28° ± 1°C.

Detection of siderophores

For detection of siderophores production by the test fungi i.e. antagonists and pathogen in liquid culture, low nutrient medium (LNM) without the iron component was used. The glasswares were washed with HCl (6M) to remove traces of iron and rinsed with distilled water. Double glass distilled water was used for medium preparation. Aliquots (50 ml) of the medium was taken in each conical flask (50 ml) of the medium was taken in each conical flasks (100 ml) and autoclaved at 15 lbs. p.s.i. Flasks were inoculated separately with *F. oxysporum, T. viride, T. harzianum* and *T. hamatum* from their actively growing cultures on PDA medium respectively. After 10 days, cultures were centrifuged at 10,000 rpm and the cell-free supernatants were examined for extracellular siderophores following standard protocols.

$FeCl_3$ Test

The test was based on the method of Jalal and Helm (1990). 0.5 ml of 2% aqeous $FeCl_3$ solution was added to 0.5 ml of cell free supernatants. Presence of siderophore was indicated by the appearance of reddish brown colour.

Chrome Azurol Sulphonate (CAS) assay

CAS assay was performed following the method of Schwyn and Neilands (1987). 0.5 ml of cell free culture supernatant was added to 0.5 ml of CAS assay solution. The change in colour of blue dye to orange indicated the presence of siderophores.

CAS assay solution was prepared in the following manner:

6 ml of hexadecyl trimethyl ammonium bromide (10 mM, HDTMA) was taken in a 100 ml volumetric flask and diluted with water. A mixture of 1.5 ml of iron (III) solution (1 mM $FeCl_3$, $6H_2O$ in 10 mM HCl) and 7.5 ml of 2 mM aqueous CAS dye solution were slowly added under stirring. Anhydrous piperazine (4.304 g) was dissolved in distilled water and 6.25 ml of HCl (12M) then added to get a buffer solution of pH 5.6. It was again added to the above volumetric flask and made to 100 ml with distilled water to prepare the CAS assay solution.

CAS activity

To determine the concentration of siderophore, cell-free supernatants of the cultures were assayed by measuring CAS activity (OD at 630 nm) following the method of Alexander and Zuberer (1991). 0.5 ml cell free culture filtrate was added to 0.5 ml of CAS assay solution and its OD was recorded at 630 nm in a spectrophotometer.

CAS agar plate test

Production of siderophore was determined by a detection method based on their high affinity towards Fe (III) metals (Schwyn and Neilands, 1987). The ternary complex chrome azurol sulphonate (CAS)/ iron III/ hexadecyl trimethyl ammonium bromide serves as an indicator. When a strong chelator such as siderophore removes the iron from the dye, its colour turns from blue to purple-pink or orange. When this CAS complex was incorporated into agar plates, halos around the colonies are indicative of siderophore excretion. Low nutrient medium (LNM) omitting the iron component was used for siderophore production. The glasswares were washed with HCl (6 M) to remove traces of iron. CAS agar plates were prepared as CAS (60.5 mg) was dissolved in 50 ml distilled water and mixed with 10 ml of iron (III) solution (1 mM $FeCl_3$, $6H_2O$ in 10 mM HCl) with stirring. This solution was slowly added to 72.9 mg of hexadecyl trimethyl-ammonium bromide (HDTMA) dissolved in 40 ml of distilled water. The resultant dark blue liquid was autoclaved at 15 lbs. p.s.i. pressure. Also a mixture of 750 ml of LNM (containing ingradients of LNM omitting the iron component), 15 g agar, 30.2 g pipes (12.0 g of 50% W/W NaOH solution to raise the pH of pipes to 6.8) were also autoclaved. The dye solution was finally added to this sterile solution, along the glass wall with enough agitation to achieve mixing without generation of foam. Each plate contained 30 ml of agar. Plates of CAS agar (Schwyn and Nelands, 1987) were inoculated separately with core of each of *Trichoderma* species and *F.oxysporum* and finally incubated at 25^0C.

Statistical analysis

Data were expressed as mean±standard deviation. Significant differences among the means were determined by Fisher's least-significant difference test after one-way analysis of variance. Significance of between-treatment means was tested at the 0.05 level of probability using Stat Plus Version 4.8, 2007 software.

RESULTS AND DISCUSSION

The results (Table 1) showed the production of iron-chelating compounds, siderophores by all the test fungi as evidenced by positive responses to different biochemical tests like $FeCl_3$ test, CAS assay and CAS agar plate assay. In CAS agar plate test, different sizes and colours of halo zones were found when the test fungi were subjected to grow on CAS. The CAS activity was further strengthened by measuring the OD values

Table. 1: Siderophores production by the *Trichoderma* spp. and the pathogen.

Fungi	$FeCl_3$	CAS assay	CAS agar plate test	CAS activity (OD at 630 nm)
T. hamatum	+	+	+	0.74^{b}±0.03
T. lignorum	+	+	+	0.68^{b}±0.00
T. reesei	+	+	+	0.62^{b}±0.00
T. harzianum	+	+	+	0.91^{b}±0.00
T. viride	+	+	+	0.86^{b}±0.00
F. oxysporum	+	+	+	0.15^{a}±0.00

Data shows mean ± standard deviation of 5 replicates; Difference in CAS activity between pathogen and other antagonists significant at 5% level (denoted by superscripts a & b)

of cell-free supernatant of fungal cultures. The test fungi expressed their CAS activity (Table 1) in the order of *T. harzianum* > *T.viride* > *T. hamatum* > *F. oxysporum*. Production of siderophores happened to be highest in *T. harzianum* followed by *T.viride* and *T. hamatum* and the lowest in *F. oxysporum.* Statistically the results remained highly significant. Different fungi under consideration showed distinct responses to change of colour of modified CAS-agar blue. It was recorded that the changes of colour pattern was from blue to purple or purplish red in all the tested fungi. It is apparent from the result (Table 1) that both antagonistic species of *Trichoderma* and the pathogen *F. oxysporum* are capable of producing siderophores as the fungi showed positive responses to $FeCl_3$ test, CAS assay and CAS agar plate assay. Reports are available that a large number of fungi and bacteria produce siderophores under iron-limiting conditions (Timofeeva *et al.* 2022). The above findings are corroborative with the present investigation. Iron is an essential nutrient for nearly all organisms because it serves as an obligate component of many indensable enzymes and other proteins (Worrall and Michael, 2022). Iron is needed in relatively small amount but is essential as a donor and acceptor of electrons in cellular processes, including cytochrome system in aerobic respiration (Deacon, 2006). No systems analogous to siderophores have been for any other metal ion and thereby making iron unique in requiring such specific ligands (Miethke and Marahiel, 2007). Siderophores chelate a ferric ion (Fe^{3+}) then they reabsorbed through a specific membrane protein and Fe^{3+} is reduced to Fe^{2+} within the cell, causing its release because the siderophores has a lower affinity for Fe^{2+} than for Fe^{3+} and finally the siderophore is exported again to capture for further ferric ion (Deacon, 2006). From the result (Table 1) it is evident that production of siderophore was appeared to be highest in *T. harzianum* followed by *T. viride* and *T. hamatum* and lowest in *F.oysporum*. It implies that *T. harzianum* shows higher degree of antagonism by producing siderophore that can bind with iron in a high specificity and affinity thus making the iron unavailable for other microorganisms thereby limiting the growth of other microbes. This strategy may certainly be involved in biological control of plant diseases (Haque *et al.* 2023; Kredics *et al.* 2024). Competition for iron by siderophore production has long been recognized as an important antagonistic trait of many biocontrol agents against many plant pathogens (Yu *et al.,* 2011). *Fusarium* spp. showed positive responses towards siderophore production (Sun *et al*. 2022) that corresponds with the present findings. Fungal siderophores are classified as ferrichromes, coprogens, rhodotorulic acid, fusarimines and rhizoferrins (Chincholkar *et al.* 2006). According to their chemical nature, siderophores may be grouped into three classes like hydroxymate type, catecholate type and carboxylate type (Timofeeva *et al.* 2022). *Trichoderma* species are capable of producing both the phenolate and hydroxamate types of siderophores and production of such metabolites may contribute their efficiency to control the different pathogenic fungi biologically (Tycækiewicz *et al.* 2022). The colour of the CAS-blue agar was changed by the test fungi from blue to purple or purplish-red which is the typical colour described by the authors (Louden et al., 2011) for the reaction to the removal of iron from CAS by the siderophores. The distinct responses of colour change in CAS reaction observed with the microorganisms could be related to structural differences in the types of siderophores secreted (Srivastava *et al.* 2013). The availability of iron for microbial assimilation in environments such as rhizosphere is extremely limiting. Iron starvation, due to competition between the pathogen and the antagonists, prevents the germination of spores of fungal pathogens in rhizosphere as well as rhizoplane (Tycækiewicz

et al. 2022). Role of siderophores in biological control of plant-root infections depends exclusively on environmental conditions. It is speculated that siderophores can act as biocontrol molecules only under soluble iron scared environment and when pathogen fails to utilize those ferrated siderophores. At this juncture, other secondary metabolites like antibiotics definitely help to suppress pathogens (Singh *et al.* 2022).

DECLARATION

Conflict of interest. Author declares no conflict of interest.

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