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Research Article

Antifungal activity of *Streptomyces fradiae* JT13 isolated from rhizosphere of *Gloriosa superba* Linn.

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Abstract: Rhizosphere is the intense zone of microbial diversity and activities. The associated microflora of rhizosphere is found to exhibit strong antagonistic activities against phytopathogens which can cause enormous loss to crop plants. A bacterial strain was isolated from the rhizosphere of *Gloriosa superba* Linn., commonly known as glory lily or 'agnisikha' a potent medicinal plant belonging to the family Colchicaceae. The strain showed antifungal activity toward a number of fungal plant pathogens as tested *in vitro*. The bacterial strain was characterized through biochemical tests and identified as *Streptomyces fradiae* (NCBI accession no. MG679514) by16s rRNA gene sequence analysis and molecular phylogeny. The bacterial strain produces broad spectrum antifungal metabolite as it inhibited the growth of eight fungal pathogens, tested *in vitro*. However, the antifungal spectrum is varying with different fungal pathogens and is maximum toward *Pyricularia oryzae* and *Acremonium* sp whereas minimum toward *Penicillium* sp and followed by *Fusarium oxysporum*. The antifungal metabolite of *S. fradiae* JT 13 was extracted in ethyl acetate and examined in FT-IR and Uv-Vis spectroscopy. FT-IR spectrum of the metabolite suggested the presence of hydroxyl groups, hydrocarbon chassis and also indicated a double bond in the compound. Uv-vis spectrum exhibited characteristic peaks between 200-400 nm also suggested a high polyenic nature of the compound.

Key words: Antifungal metabolite, *Streptomyces fradia,* FT-IR, Fungal pathogen

Introduction

Rhizosphere microorganisms are immensely important as it produce broad spectrum of secondary metabolites like antibiotics, siderophores, plant growth regulators etc. A large community of microorganisms is accumulated in rhizosphere region due to suitable nutrient environment. The microorganisms of this region have been isolated and exploited by different scientific communities for plant growth promotion, disease suppression and for pharmaceutical activities. The genus *Streptomyces* is a major soil bacterium, containing a number of species which produce characteristics antimicrobial metabolites. They are of special interest for their ability to produce bioactive secondary metabolites, such as antifungal, antibacterial, antiviral, antitumor, anti-hypertensive, and especially antibiotics (Rudi *et al.,* 2012, Apichaisataienchote *et al.,* 2006). A number of clinically useful antibiotics have been obtained from *Actinomyces*, such as chlooramphenicol, deptomycin, fosfomycin, linomycin, neomycin, aminoglycoside, anthracyclines, β-lactams, macrolides, tetracyclines etc. Large number of species of this genus have been isolated so far, accounting 70%-80% of relevant secondary metabolites available commercially (Baltz 2008). A number of species of Streptomyces has been isolated and identified by different researchers, producing bioactive metabolites having plant disease suppression and pharmaceutical interest (Berdy 2005, Zhang *et al.*, 2005, Takahashi and Omura, 2003, Patzer and Volkmar, 2010, Sajid *et al.*, 2011, Bignell, 2013). Works of different researchers focus that this group of bacteria still remains a rich source of narrow as well as broad spectrum of antibiotics (Iwai and Omura, 1982). Fourati-Ben Fuguira *et al.,* (2005) isolated and identified three antifungal and antibacterial metabolites irumamycin (1a), X-14942B (1b), and 17-hydroxy-venturicidin A from a newly isolated *Streptomyces* sp. strain US80. El-Naggar (2007) isolated kosinostatin, a quinocycline group of antibiotic from *Streptomyces violaceusniger* strain HAL64 which is strong inhibitor of gram positive bacteria but is less antagonistic to gram negative bacteria. Reddy Shetty *et al.,* (2015) reported the production of polypeptide antibiotic (Actinomycin) by *Streptomyces parvulus* RSPSN2 isolated from marine soil sediment of Visakhapatnam sea coast having antibacterial activity against bacterial pathogens including streptomycin resistant bacteria like *Pseudomonas mirabilis*, *Pseudomonas putida* and *Bacillus cereus*. Future screening of different species of *Streptomyces* may lead to the discovery of nearly 100,000 new compounds with pharmaceutical intrest (Williams *et al.*, 1983). Wang *et al.,* (2013) reported antifungal activity of a volatile compound of *Streptomyces alboflavus* TD-1 against a number of storage fungi *in vitro*. They have identified 27 volatile compounds from *S. alboflavus* TD-1, among which dimethyl disulfide showed antifungal activities against *Fusarium moniliformae* by fumigation *in vitro*. Wang *et*

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al., 2015 reported the antifungal activities of *Streptomyces aureoverticillatus* HN6 against *Fusarium* wilt of banana caused by *Fusarium oxysporum* f. sp. They also demonstrated that the extract of HN6 have broad spectrum antifungal activity as it inhibits a number of eight indicator fungal plant pathogens.

The aim of this study is to isolate and characterize bacterial strain from rhizosphere soil of medicinal plant *Gloriosa superba* Linn. and to investigate its antifungal property against some plant pathogenic fungi. Furthermore, investigation of antifungal metabolite and its partial characterization is also another approach of this study.

Materials and Methods

Isolation and identification of bacterial strain:

Collection of soil sample: Soil samples were collected from the rhizosphere of glory lily in sterile polythene bags from the Sonapur area of Kamrup district, Assam, India. The samples were air dried and sieved and isolation was done using standard serial dilution method and spread plate technique on nutrient agar medium.

Fungal pathogens: The fungal pathogens *Fusarium oxysporum* MTCC-284, *Scerotinia minor* MTCC-1125, *Ustilago maydis* MTCC-1474, *Pyricularia oryzae* MTCC-1477, *Alternaria solani* MTCC-2101, *Rhizoctonia oryzae-sativa* MTCC-2162 were obtained from Institute of Microbial Technology, Chandigarh, India. *Penecilium* sp and *Acremonium* sp were isolated from infected orange and infected cucurbita leaves respectively.

Bioassay: Screening for *in vitro* antagonism was done by dual culture method (agar diffusion) according to Utkhede and Rahe (1983) by placing the agar block with actively growing fungal mycelium in petridishes containing nutrient agar media and inoculated the bacterial culture at a distance from the pre-inoculated fungal pathogens. Antimicrobial spectrum was evaluated by measuring the zone of inhibition (in mm) created by the isolates after 7 days of incubation.

Characterization and identification of bacterial isolate:

The isolates showing maximum zone of inhibition against the test organisms were characterized based on morphological and biochemical attributes according to Cappuccino and Sherman (1983) and Bergy's Manual of Systematic Bacteriology (1984). Further identification was done through PCR amplification and 16s rDNA sequence analysis as per standard protocol and through molecular phylogenetic analysis by using bioinformatics tools. DNA was isolated from the nutrient broth culture. Its quality was evaluated on 1.0% Agarose Gel, revealing a single band of high-molecular weight DNA. Fragment of 16S rDNA gene was amplified by PCR. A single discrete PCR amplicon band of 1100 bp was observed when resolved on Agarose. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 27F and 1115R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programme Clustal W. Distancematrix was generated using RDP database and the phylogenetic tree was constructed using MEGA4.

Extraction and purification of antifungal metabolite

The isolates were incubated in nutrient broth for 96 hours. Culture was centrifuged at 10,000 rpm for 20 min. and bioactive metabolite was extracted in ethyl acetate. The ethyl acetate fraction was dried in vacuum. The dried residue was dissolved in hexane-chloroform (50:50) and partially purified through column chromatography (Merck silica gel 60, 35-70 mesh) by eluting in chloroform. The concentrated chloroform extract was subjected to thin layer chromatography using petroleum ether: chloroform (1:1) as mobile phase. The separated band was visualized in iodine vapor, and Rf value was calculated.

Analytical study

The TLC purified metabolite was further assayed through UV-Vis and FT-IR spectroscopy. The absorption spectrum of the active extract was determined in the UV region (200-400 nm) by using a Carry-60 UV-VIS spectrophotometer. The spectra were recorded at 200-400 nm range. For FT-IR analysis, a small amount of KBr was mixed with the dried sample and pellets were made to record the spectra of the metabolites. Spectrum was detected by using Cary-630 FT-IR. The spectra were scanned in the 500 to 4500 cm^{-1} range. The spectra were plotted as intensity versus wave number.

Results

Characterization of bacterial isolate:

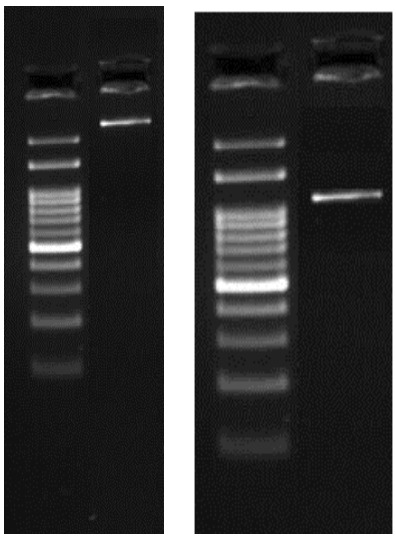
A biochemical test of the bacterial strain JT 13 showed positive reaction for Catalase, oxidase, nitrate reduction, starch and casein hydrolysis and was able to ferment sugars like Glucose and Dextrose. However, it showed negative reaction for Methyl Red, Vogues Proskeur, Coagulase, Gelatin liquefaction, motility, urease, ammonia production, citrate utilization and acid fast reaction (Table 1).

Table 1. Biochemical characteristics of isolate JT13

Biochemical test	Results
Gram staining	Positive
Indole test	Positive
Catalase test	Positve
Citrate utilization test	Negative
Urease test	Negative
Lactose fermentation test	Negative
Sucrose fermentation	Negative
Glucose fermentation test	Positive
Urease test	Negative
H_2S production test	Negative
Casein hydrolysis	Positive
Methyl Red test	Negative
Vogues Proskeur test	Negative
Oxidase test	Positive
Amonia production	Negative
HCN production	Negative
Triple sugar iron	Positive
Starch hydrolysis test	Positive
Nitrate reduction test	Positive
Gelatin liquifaction test	Negative
Motility	Non-motile
Acid Fast Stain	Negative

Identification by 16S rDNA sequence analysis

Amplification of the fragment of 16S rDNA was done by 27F and 1115R primers using BDT v3.1 (Fig.1). From forward and reverse sequence data consensus sequence of 16S rDNA gene was generated which was used to carry out BLAST with the NCBI genebank database. First ten sequences were selected based on maximum identity score and aligned using multiple alignment software programs (Clustal W) and the phylogenetic tree was constructed using MEGA 4 (Fig. 1b).



gDNA **16S PCR amplicon**

Figure 1. 16S rDNA amplicon of strain JT13

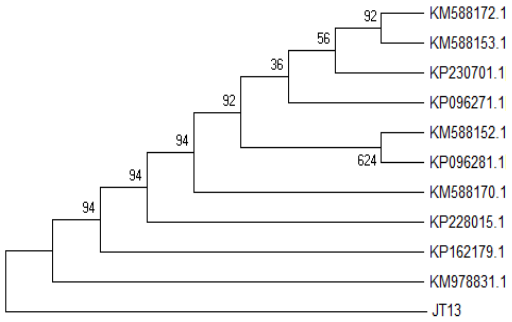


Figure 2. Phylogenetic tree of *Streptomyces fradiae* JT 13

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.02177660 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates is shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There was a total of 879 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

***In vitro* antibiosis study**

A total of thirteen isolates were obtained from the rhizosphere of glory lily and designated as JT 1 to JT 13. All the isolates were screened for their antagonistic property against the test fungal pathogens. The isolate designated as JT13 was found to have more antagonistic activity against the phytopathogens as tested *in vitro* and thus selected for the further study. The isolate JT 13 showed the maximum zone of inhibition against *Pyricularia oryzae* (20 mm plate 1) and *Acremonium* sp. (20 mm) followed by *Alternaria solani* (18mm) and *Ustilago maydis* (17mm). The inhibition was minimal toward *Penicillium* sp. (11 mm) and followed by *Fusarium oxysporum* (14 mm). Zone of inhibition toward *Scerotinia minor* and *Rhizoctonia oryzae-sativa* were 15 mm each respectively (table 2).

Table 2. Bioassay of *S. fradiae* JT13 against fungal pathogens

Fungal pathogens	Zone of inhibition (mm)
Fusarium oxysporum MTCC-284,	14.0
Scerotinia minor MTCC-1125	15.0
Pyricularia oryzae MTCC-1477	20.0
Alternaria solani MTCC-2101	18.0
Rhizoctonia oryzae-sativa MTCC-2162	15.0
Ustilago maydis MTCC-1474	17.0
Acrimonium sp	20.0
Penicillium sp	11.00



Plate 1. Antibiosis against *Pyricularia oryzae* MTCC-1477

Thin Layer Chromatography

The crude ethyl acetate extract of *Streptomyces fradiae* JT13 was separated by thin layer chromatography using Petroleum ether: chloroform (1:1) yield a single yellow band with R_f factor 0.7.

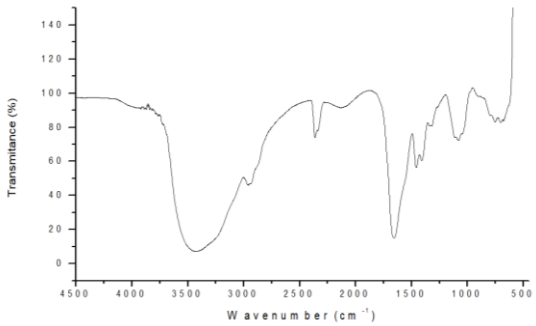


Figure 3. FTIR spectrum of metabolite of *S. fradiae* JT 13

Analytical study of the antifungal metabolite:

Analytical study

FT-IR analysis of antifungal metabolite of JT 13 exhibited peaks at 3411 cm^{-1} and 3422 cm^{-1} indicates the presence of hydroxyl groups, 2372cm^{-1} indicating hydrocarbon chassis and the absorption at 1629 cm^{-1} indicating a double bond of polyenic compound (Fig.3). The UV-VIS spectral analysis of (Fig.4) the extracted metabolites exhibited prominent sharp peaks at 209.5 nm, 293.1 nm and 372.9 nm.

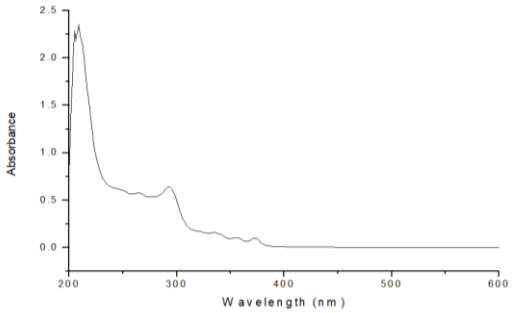


Figure 4. Uv-vis spectrum of metabolite of *Streptomyces fradiae* JT 13

Discussion

The bacterial strain JT 13, isolated from *Gloriosa superba* Linn. was characterized as gram positive, non-motile and exhibited positive reaction for Catalase, oxidase, nitrate reduction and was able to ferment sugars like Glucose and Dextrose. The isolated strain has been finally identified as *Streptomyces fradiae* through 16srDNA sequence alignment, homology modeling and phylogenetic analysis with 98% identity. The sequence having 1036 base pair submitted in the Gene Bank of NCBI database with accession no. MG679514 (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=1906). Antagonistic activity of *Streptomyces* against fungal pathogens has been studied extensively (Ahmed *et al.,* 2016, Crawfold 1993, Lim *et al.,* 1993, Reddy Shetty 2014, Kumar *et al.*, 2014) by a number of authors. Usually the organisms exhibiting zone of inhibition more than 10 mm are termed as 'effective', while lesser than it is signified as "non-effective" (Kathiresan *et al.*, 2005). Taechowisan *et al.,* (2012) isolated an "effective" antagonist *Streptomyces* sp. LJK109 from root tissue of *Alpina galangal* which exhibited over 18 mm zone of inhibition against all the test fungal pathogen *Alternaria porri*, *Colletotrichum gloeosporioides*, *Colletotrichum musae*, *Curvularia* sp., *Drechslera* sp., *Exserohilum* sp., *Fusarium oxysporum*, *Verticillium* sp. and *Sclerotium rolfsii*. In our study the bacterial strain *S. fradiae* JT13 is an effective antagonist as it exhibited a zone of inhibitions more than 10 mm against all the test fungal pathogens. Anitha and Rebeeth (2009) reported high antagonistic effect of *Streptomyces griseus* against *oxysporum* f. sp. *lycopersici* showing 20 mm zone of inhibition. They concluded that the inhibitory effect of this antagonist is due to presence of some inhibitory substance, antibiotics and other enzymes such as glucanases, protease essential for complete cell-wall lysis. Hadi and Omid (2011) reported different spectrum of antibacterial activity of *Streptomyces* G614C1against bacterial pathogens such as *Yrwinia. enterocolitica* (58%), *L. monocytogenes* (42%), *Klebsilla. pneumoniae* (37%), *Bacillus cereus, Stephyllococcus. aureus,* and *Escherisia. coli* (33%), *Stephyllococcus. flexneri* (17%and 24%). In our study the zone of inhibitions showed by *Streptomyces fradiae* JT 13 are above 10 mm against

all the test fungal pathogen, however, it is significantly more against *Pyricularia oryzae, Acremonium sp, Alternaria solani, and Ustilago maydis* (20 mm to 17 mm) and therefore it can be concluded that the bacterial strain is more "effective" against these fungal pathogens.

The ethyl acetate extract of *S. fradiae* JT 13 exhibited a single yellow band in Thin Layer Chromatography when run with petroleum ether-chloroform (1:1) with Rf value 0.7. Ahmed *et al.,* (2016) when purified the ethyl acetate extract of secondary metabolites of *Streptomyces* strain PS1 and PS28 through thin layer chromatography using petroleum ether and chloroform (1:1), recovered antibacterial metabolites with Rf value=0.7 and 0.6 for strains PS1 and PS28 respectively. This finding suggested the similarity of antifungal metabolite of our strain JT13 with the antibacterial metabolite of that of *Streptomyces* PS1. The UV spectra of the ethyl acetate extract is similar to the findings as obtained by Swaadoun *et al.,* (1999) who found that the UV spectral data for the ethyl acetate extract of the selected strains exhibited a maximum absorbance peaks range between 215 to 320 nm and the characteristics of absorption peaks indicate production of highly polyenic naturel of the compounds. The bioactive compound exhibited a maximum UV absorption at 217–221 nm in ethyl acetate extract. Therefore these strains produced a broad-spectrum of anti-microbial compound of several compounds with different activities. Maximum absorbance peaks observed at around 240 nm suggest the compound to show a high polygene nature. The spectral data are consistent with those obtained by Slavic *et al.,* (2005) who reported that the maximum absorbance peaks of UV spectral data ranged between 215 and 270 nm of *Streptomyces* isolates from the soil samples of South-eastern Serbia. Augustine *et al.,* (2005), examined the ethyl acetate extract of culture filtrate of *Streptomyces albidoflavus* PU23 under FTIR and exhibited absorption bands at 3296 and 1031.8 cm^{-1}, indicating presence of hydroxyl groups and absorption at 1639 cm^{-1} indicating double bonding in the compound.

Similarly, the FTIR spectrum of the ethyl acetate extract of bacterial strain *S. fradiae* JT 13 exhibited peak at 3411 cm^{-1} and 3422 cm^{-1} which suggests the presence of hydroxyl groups, 2372 cm^{-1} indicating hydrocarbon chassis and the absorption at 1629 cm^{-1} indicating a double bond of polyenic compound (Fig.1). More or less similar finding was also attributed from the works of Hadi and Omid (2011). The analytical study of FTIR spectrum of ethyl acetate extracts of *Streptomyces* G614C1exhibited absorption at 3411 cm-1, which indicates hydroxyl groups, the absorption at 2856 and 2915 cm-1 indicating hydrocarbon chassis and the absorption at 1649 cm-1 indicating a double bond polyenic compound.

Conclusion

A bacterial strain isolated from rhizosphere of *Gloriosa superba* Linn has been characterized and identified as *Streptomyces fradiae* through biochemical tests, 16s rDNA sequence analysis and molecular phylogeny. The bacterial strain produces an effective fungicidal metabolite, showing antagonism against eight fungal phytopathogens as tested *in vitro*. The fungicidal metabolite extractable in ethyl acetate is purified through column chromatography and separated through Thin Layer Chromatography using chloroform-petroleum ether (1:1) exhibited a single yellow band with Rf value 0.7. FT-IR and Uv-vis spectroscopy suggested the metabolite is a polyenic compound having double bond with hydroxyl group and hydrocarbon chassis.

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