Antagonistic potential of Silver nanoparticles with ectorhizospheric *Pseudomonas species* of Bt-cotton.

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Abstract

A metabolic diversity in rhizospheric microorganisms plays a major role in plant pathogen suppression and plant growth promotion.To be active,the microorganism secrets the diverse metabolites in minor quantity inpico or nano gram. In this study antifungal metabolites and silver nanoparticles producing *Pseudomonas species* were isolatedfrom rhizosphere of Bt cotton plant. A combinatory effect of silver nanoparticles, *Pseudomonas species*/metabolites was evaluated against two cotton plant pathogens*Fusarium oxysporium* and *Aspergillus niger*.The experimental results were analysed using MINITAB (ver. 17) for ANOVA ($p < 0.05$) indicate that the addition of silver nanoparticles increases fungal growth inhibition and plant growth promotion efficiency.

Key words: Antagonistic potential, silver nanoparticles, ANOVA.

Abbreviations:
AgNP's = Silver Nanoparticles
HPLC = High Performance Liquid Chromatography
UPLC = Ultra Performance Liquid Chromatography
SEM = Scanning Electron Microscopy
TLC = Thin Layer Chromatography

1. Introduction

Before the introduction of BT technology more land comes under cotton butyield of cotton wasfound to be low in the world. Introduction of BT technology was meant to reduce losses to pests and improving harvested yields. The reduction in pesticide use may result in improvement of farmers' incomes and reduces environmental damage. After the introduction of BT hybrids cotton yields have increased significantly, along with aggregate production. (Herring and Rao, 2012, Frisvold and Reeves, 2007). But in a decade the yield of cotton depleted because of attack of BT non targeted arthropods, continuous exposure of cry proteins and environmental conditions (Tian *et al.*, 2018). Fungal attacks are also responsible for significant

crop loss in cotton (Patel *et al.,* 2014). The *Fusarium species* were found to be more dominant within them and cause Fusarium wilt disease to the cotton plant (Shirsath and Patil, 2016). The bacterial species like *Pseudomonas, Bacillus species* have ability to produce antifungal compounds and suppress the growth of plant pathogenic fungi (Waller, 2007). In nature the antifungal compounds are not uniformly distributed in soil, rather they are present in nutrient rich microhabitats (Waller and Thomashow, 1993). In this study we focused on the isolation and identification of indigenous antifungal and silver nanoparticles producing bacteria from rhizosphere of cotton plants. The combinatory effect of bacterial synthesised Silver nanoparticles and antifungal compounds was evaluated against *Fusarium oxysporium* and *Aspergillus niger* and try to find out the possible role of nano particles in plant pathogen suppression.

2. Material and Methods

2.1 Sampling, isolation and identification of bacteria

Roots were collected from cotton plants growing in the mono cultivated 3 fields of Maharashtra (77.319717- 19.169815) India. The soil adhering with roots were collected and treated as rhizosphere soil samples. The collected soil samples were analysed for its physicochemical properties like pH, electric conductance capacity, Organic content, and for available some major and minor elements (Methods Manual soil testing in India, 2011).

The antagonist bacteria were isolated from serially diluted soil sample as per the method described in Montelegrae *et al.,* 2003.The morphologically similar colonies were selected and purified by repeated streaking (Sharma and Kour, 2008, Hu *et al.,* 2009).

The selected isolates were turn into metabolically active by maintaining its optical density 0.5 at 620 nm, and then screened for biochemical tests, production of hydrolytic enzymes and plant growth promotion activities. The characterizations of isolates were carried out as per the Bergey's Manual of Systematic bacteriology.

Antifungal metabolites

The indirect plant growth promoting activities were evaluated which includes production of chitinase, antibiotics like bacitracin, water diffusible pigment pyocyanin,siderophore and silver nanoparticles (Kumar G.*et al.,* 2012).

Detection of chitinase

Bacterial chitinolytic activity was tested by plating bacterial isolates on sterile chitin agar medium as describe by Renwick *et al.,* (1991). The positive isolates were used for chitinase production and enzyme assay was performed (Ramyasmruthi*etal.,* 2012).

Detection of Bacitracin

The screening of isolates was carried out by dual culture method (Chen and Echandi, 1982).The extraction of Bacitracin was carried out by solvent extraction method(Bishat, 2011). The sample was initially analyzed by TLC with butanol: water (8:2) solvent system(Al-janabi, Ali Abdul HusseinS, 2006)and confirmed by UPLC (D'Hondt *et al.,* 2015).

Detection of pyocyanin

The water soluble (green – blue) pigment producing isolates were used for production of pyocyanin. The extraction of pigment was carried out by organic solvent method and primarily confirmed by TLC (T. Sudhakar *et al.,*2013). The further confirmation of pigment was carried out by HPLC (El-fouly M Z*et al.,*2014). The quantification of pyocyanin was carried out by the formula

Concentration of pyocyanin(µg/ml) = Optical density at 520 nm x 17.072(Essar*et al.,*1990)

Detection of siderophore

The screening and confirmation of siderophore was carried out over universal CAS medium. The isolates shows holo orange colour zone surrounding them was used for production and siderophore concentration was determined.(Tailor and Joshi, 2012)

Detection of Silver nanoparticles

The screening of silver nanoparticles producers were carried out according to the method of Jo Young-ki*et al.,*(2009) with slight modification.The isolates were inoculated in king's B medium and incubated at 150 rpm, 37^0C for 48 hours followed by centrifugation at 8000 rpm for 10 minutes. The 1 ml supernatant was added in 1 ml (1mM) Silver nitrate ($AgNO_3$) solution and incubated at 30^0C for 24 hoursfor change in color from yellow to brown. The lambda max was observed at 420 nm. The vacuum dried sample was analyzed for SEM analysis (Patel N. *et al.,* 2014).

Antifungal activity

The combinatory effect of metabolites and silver nanoparticles was carried out against *Fusarium oxysporium* and *Aspergillus niger* by the poison food method (Balouiri *et al.,* 2016) and the antifungal activity was measured as -

Antifungal activity (%) = ((Dc −Ds) / Dc) ×100

Where Dc is the diameter of growth in control plate and Ds is the diameter of growth in the plate containing tested antifungal agent.

The effect of isolate on fungi was carried out by dual culture method.

Percentage of Inhibition Radial Growth (PIRG) = [1- (length of fungal growth near to bacterial isolate/length of fungal growth other side at the same plate as control)] x 100% (Dikin *et al.*,2006)

Co Culture and Microcosm assay

The potential isolate combinations for co culture and microcosm assay was prepared. In co culture assay the metabolically active (18 hrs grown culture of bacteria with 10^8 cfu/ml concentration and 10^8 fungal spore/ml) isolates were grow together and after 24 hours of incubation assayed for growth of fungi (Schrey *et al.,* 2012). In microcosm the surface sterilized Bt cotton seeds were used (Rathore *et al.*,2006) and assay was carried out by two methods.

Statistical Analysis

The correlation analysis of data was carried out by using MINITAB software (ver.17) Pennysylvania State University, USA.

PCR reaction for 16S rRNA sequencing

The genomic material of potential isolates were carried out and the amplification 16S rRNA gene was performed by using the universal bacterial primers (5'- AGA GTT TGA TCC TGG CTC AG- 3') and (5'- AAG GAG GTG ATC CAG CCG CA- 3').The PCR amplifications of 16S rRNA gene of bacterial isolates were performed and the sequencing of amplified DNA was carried out in ABI prism 3100 Genetic Analyzer (Applied Biosystems).The BLAST was performedand sequences submitted in Gene bank.

Results and discussion

The major characteristics of rhizosphere like physical, biochemical and ecologic characteristics are dependent on the balance between differentcompounds released, with timing of release and production of any unique substances.The characteristics of soil and plant species exudates play a major role in selection of rhizospheric bacteria (Randy Ortíz-Castro*et al.,* 2009).This study is focused on the role of rhizospheric pseudomonas and its functional diversity on cotton plant.

Isolation and identification of bacteria

A primary selection for the antagonism test plates helps for detection of bacteria. Pure bacterial cultures isolated for those isolates were screened for production of different metabolites as mentioned in material and methods. The 40 isolates were rescreened for morphological characterization and only 5Gram –ve, rod shaped; motile bacteria were selected and studied for 16S r RNA sequencing. A morphological, biochemical and molecular characterization of isolates allow the identification as Pseudomonas group.**Gene bank accession numbers**

The 16S rRNA sequences of isolates were deposited in Gene bank and accession numbers retrieved. (**Table 1**)

Antifungal metabolites

The production of secondary metabolites including antibiotics, pigments and certain enzymes can suppress the activity of plant pathogenic fungi (Hass and Defago, 2005).**Table 2** summarizes the results for antifungal metabolite production.

An enzyme like chitinase plays a foremost role in controlling of plant pathogenic fungi. 20% isolates shows chitinase production ability. The isolates AJ E 3(780 EU) and AJ D 3(710 EU) were prominent producers. The isolated *Pseudomonas species* were also evaluated for Bacitracin production and identified on the basis of Rf value of TLC and UPLC **(Fig. 1)**. An isolate AJ D 1 shows significant production(11µg/ml)**.**As like antibiotic and enzyme pigments also shows antimicrobial activity. The isolates were screened for blue green pigment pyocyanin and 30% isolates shows water soluble pigment pyocyanin production. The isolate AJ D 3 produces maximum quantity of pyocyanin and confirmed by HPLC (Jayaseelan and *et al.,* 2013) **(Fig. 2).** The iron scavenger siderophore activity of isolates was evaluated and only 35% of isolates were able to synthesise. An isolates AJ C 2(75.11%) was found to be significant siderophore producer thanAJ D 1(69.33%) and AJ E 4(68.88%).A nanoparticles synthesis ability of isolates was evaluated for enhancing antimicrobial activity. Only two isolates AJ B 2 and AJ D 3 were able to produce silver nanoparticles and production is characterized by UV Visible spectrophotometer and SEM **(Fig. 3).***Pseudomonas species* was also able to synthesise silver nanoparticles (Abdallah*et al.*, 2016, Quinteros *et al.,* 2016).

Antifungal activity

Haas and Defago (2005) reported that by various mechanisms pseudomonas control soil borne pathogens. The cotton rhizospheric microorganisms dominantly *Pseudomonas species* suppress the attack of root rot causing pathogen *Fusarium oxysporum* in monocropped soil (Li *et al.,*2015). The effect of metabolites and metabolite producers were separately evaluated and analyzed by paired t test ($p < 0.05$). A significant positive co relationship was found. The metabolite producers were found to be less significant (46% of inhibition) than metabolites (71%of inhibition). It may because of change in media composition or environmental conditions. The combinatory study of metabolites shows a significant effect against *Fusarium oxysporium* (76% inhibition) as compared to *Aspergillus niger*(66% inhibition). When metabolites were combined with silver nanoparticles their efficiency was increased by 3%. Jo and *et al.,* (2009) states that, a direct introduction of silver with fungal spores or germ tubes found to be critical to resist the fungal disease development. Hence combinatory effect was required and the experimental results indicate that addition of silver nanoparticles withchitinase (AJ E 3) and bacitracin (AJ D 1) increases their potential.

All the metabolites other than pyocyanin show high activity against *Fusarium oxysporium*as compared to *Aspergillus niger*.

An isolates AJ D 3 and AJ B 2 shows highest antifungal activity against *Fusarium oxysporium* and *Aspergillus niger* respectively. **(Fig. 4, 5 &6)**

Co Culture and Microcosm assay

The co culture of metabolite producer, tested fungi and silver nanoparticles shows 40% effectiveness i.e. survival of metabolite producer and killing of tested fungi. The co culture study also supports that it was more homicide over *Fusarium oxysporium* (62.5%) than *Aspergillus nigher* (37.5%).

The every combinatory activity was evaluated in triplicates and correlated for correlation coefficient(r) analysis. The r value comes positive and near to 1, fortify the data.

The goal of this study is just to find out silver nano particle as a tool for controlling root rot fungi. The molecular mechanism may give new insight for plant pathogen suppression.

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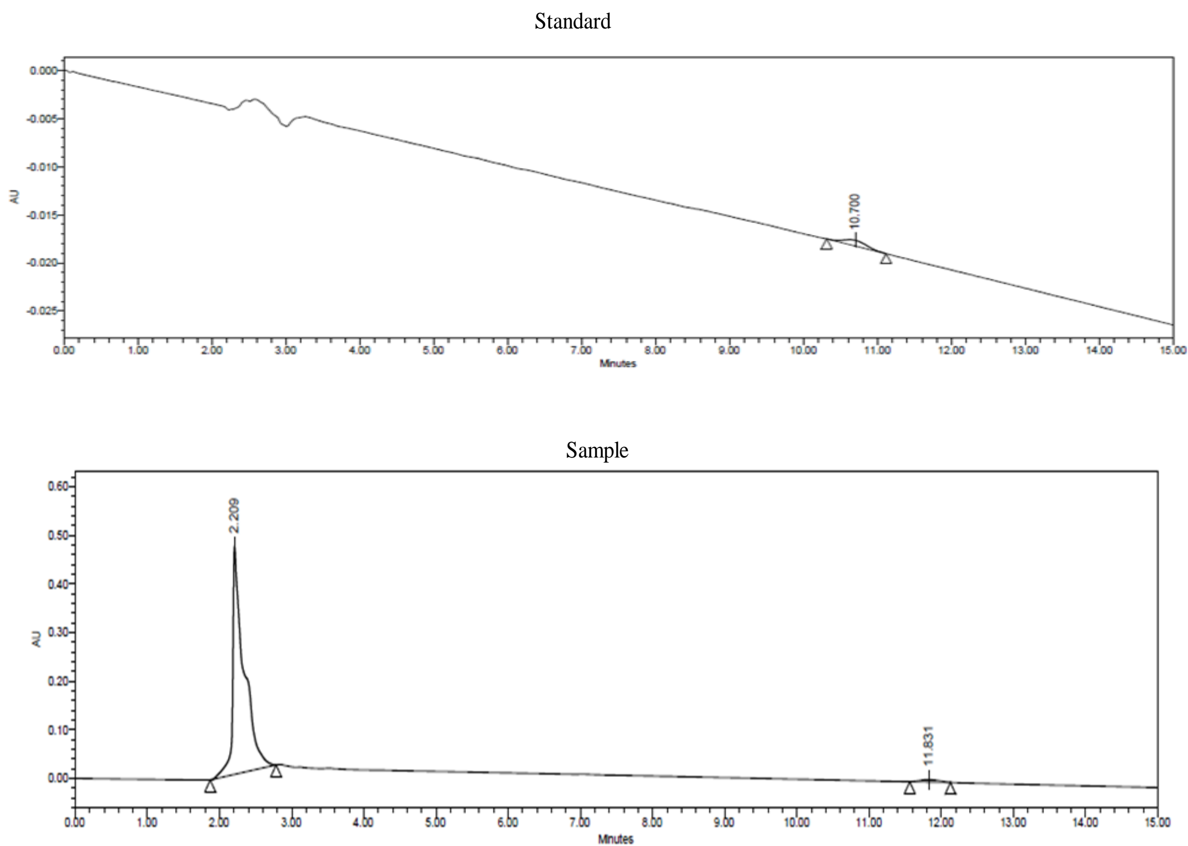
Table-1: Gene bank accession number

Sr. No.	Isolate code	NCBI Accession number	Name of organism
1	AJ D 1	MG234530	*Pseudomonas fluorescence*
2	AJ A 2	MG230527	*Pseudomonas aeruginosa*
3	AJ A 3	MG238544	*Pseudomonas fluorescence*
4	AJ B 2	MG230469	*Pseudomonas fluorescence*
5	AJ D 2	MG234531	*Pseudomonas aeruginosa*
6	AJ D 3	MG234532	*Pseudomonas fluorescence*
7	AJ E 3	MG234527	*Pseudomonas aeruginosa*

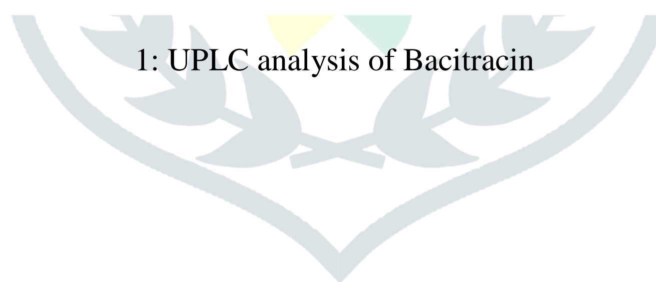
Table-2 : Main characteristics of selected isolates

Metabolite/ Isolate code	AJ B 2	AJ D 1	AJ D 2	AJ D 3	AJ E 3
Chitinase (IU)	ND	ND	170	710	870
Bacitracin (µg/ml)	ND	11	7	6	ND
Pyocyanin (µg/ml)	ND	ND	NS	3.15	ND
Siderophore production (in %)	ND	69.33	ND	ND	68.83
Silver nanoparticles	Y	ND	ND	Y	ND
ND = Not detect	NS = Not significant		Y = Able to produce		



Bacitracin UPLC peaks comparison with standard

Figure 1: UPLC analysis of Bacitracin



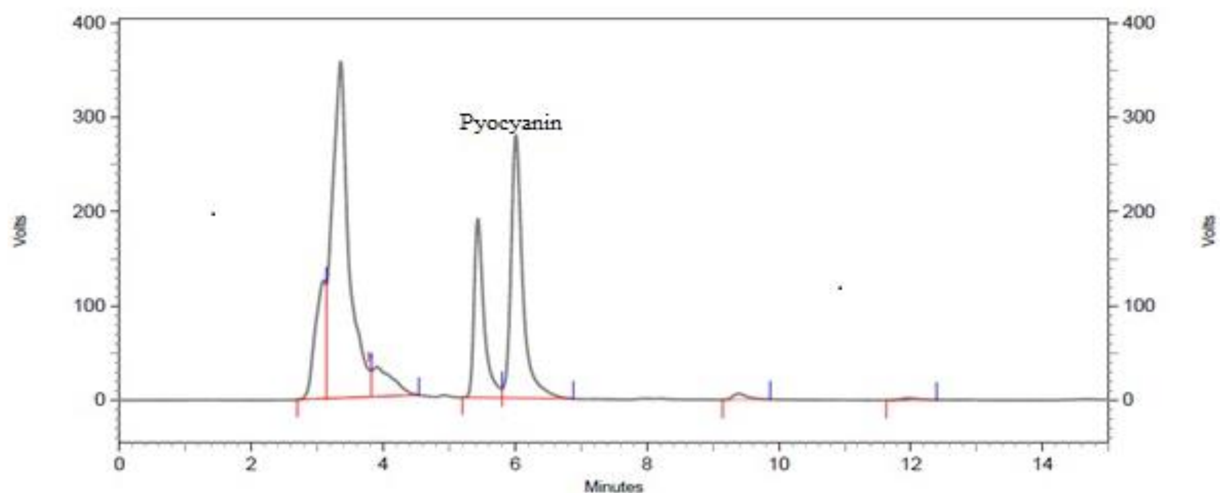
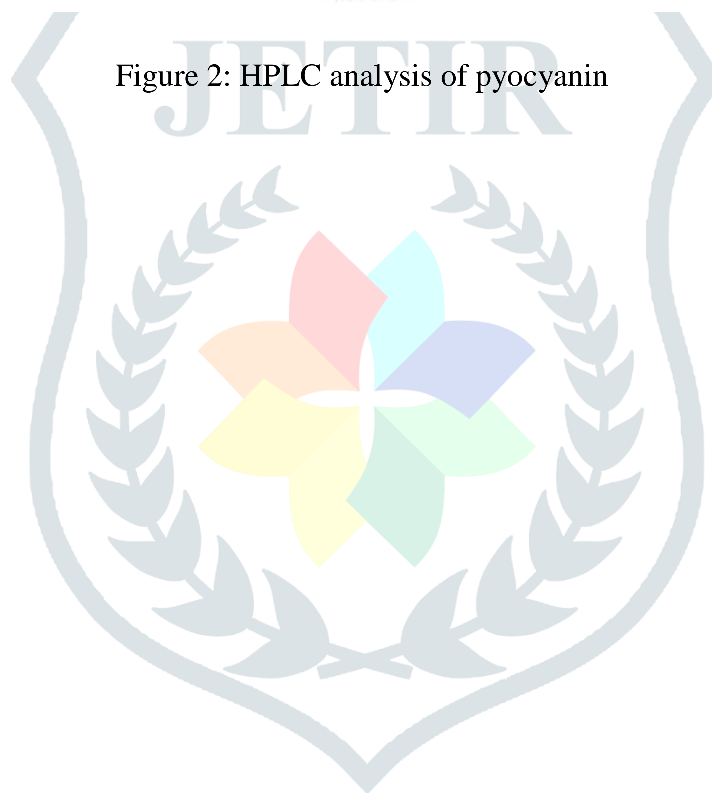


Figure 2: HPLC analysis of pyocyanin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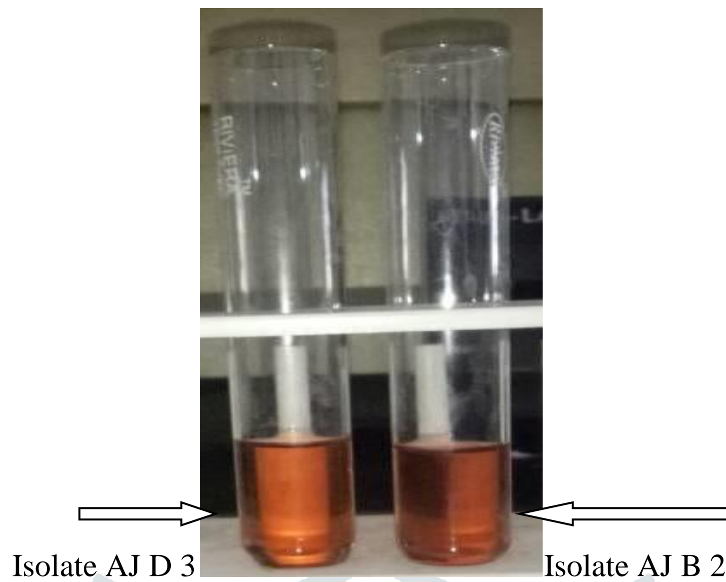

Silver Nano particles production

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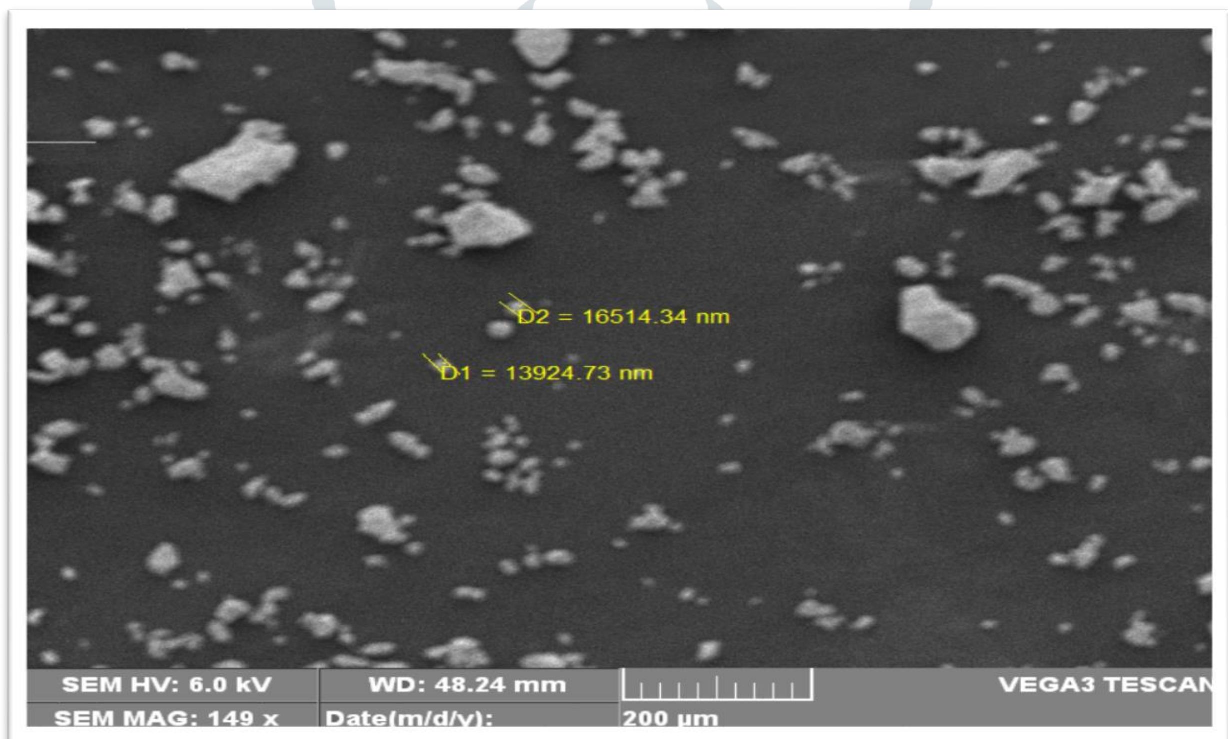

Figure 3: Electron micrograph of silver nanoparticles

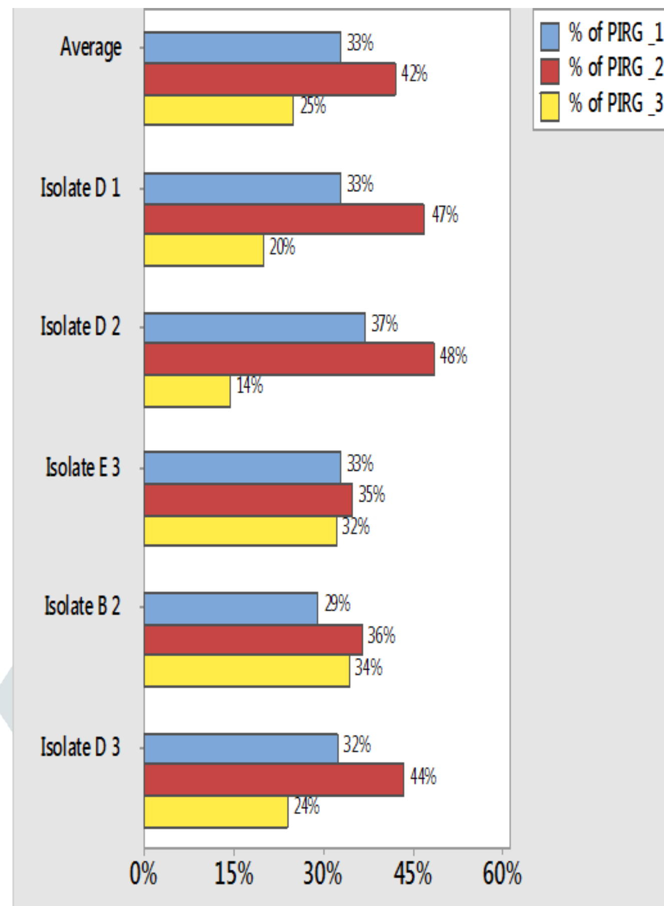

Figure 4: Percentage of Inhibition Radial Growth (PIRG) effect of isolates on *Fusarium oxysporium*

PIRG 1 = zone of inhibition after 24 hours,

PIRG 2 = zone of inhibition after 48 hours,

PIRG 3 = zone of inhibition after 72 hours.

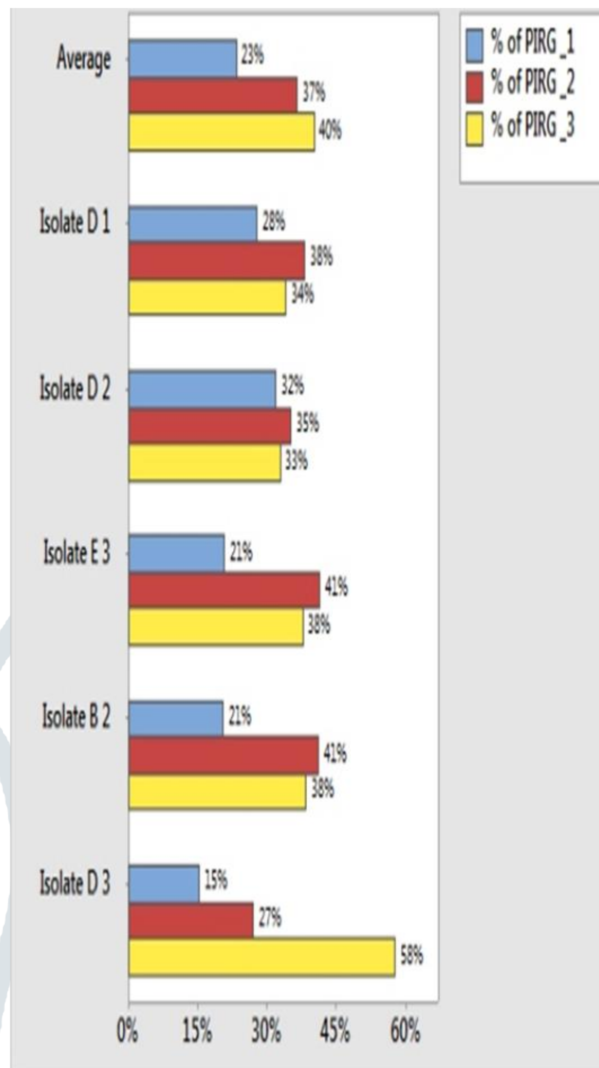

Figure 5: Percentage of Inhibition Radial Growth (PIRG) effect of isolates on *A. niger*

PIRG 1 = zone of inhibition after 24 hours,

PIRG 2 = zone of inhibition after 48 hours,

PIRG 3 = zone of inhibition after 72 hours.

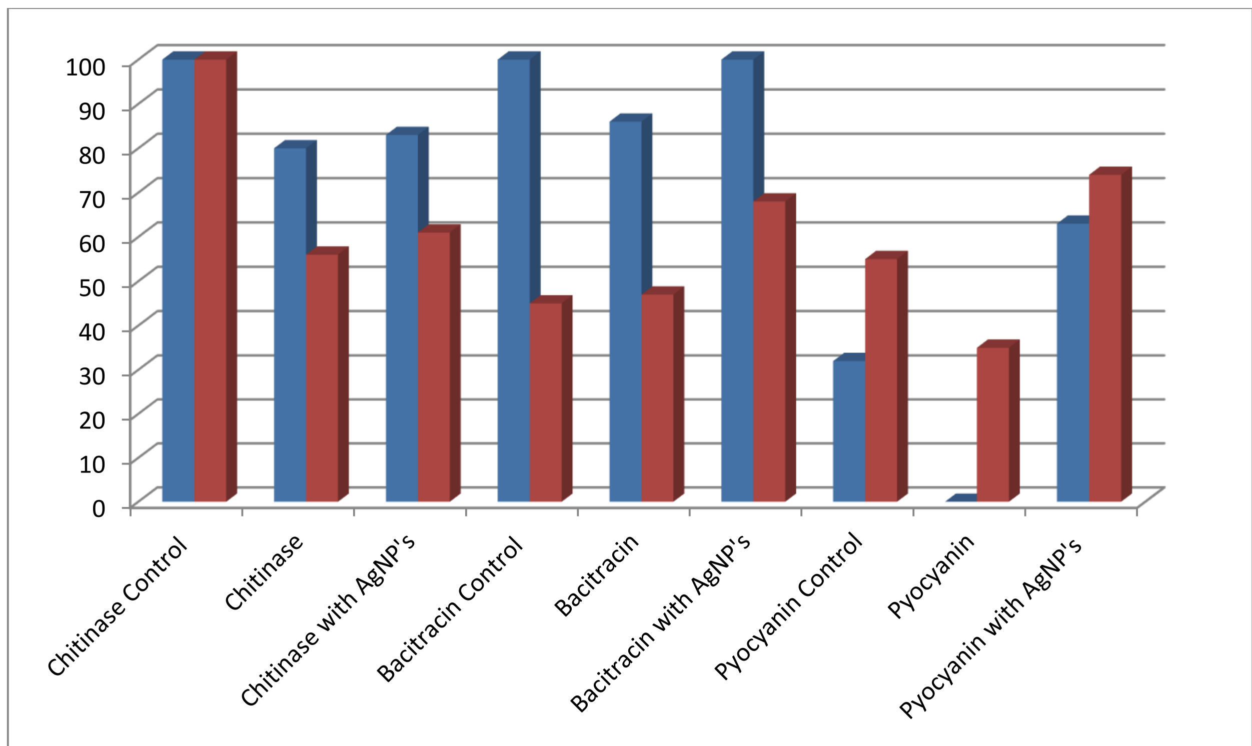

Figure 6: Effect of metabolites on fungi

In control commercially available metabolites were added.