

RESEARCH ARTICLE

Metabolic Diversity of Rhizospheric *Pseudomonas* species of Bt Cotton Plant

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Abstract

A total 20 *Pseudomonas* species were isolated from BT cotton rhizosphere of 3 different regions of Nanded city. All these isolates were evaluated for plant growth promoting functional traits like plant hormones production, Phosphate solubilisation, Siderophore production and Silver nanoparticles production. The experimental results were analyzed using MINITAB (ver. 17) for paired t test ($p < 0.05$) indicate positive co-relationship between produced metabolites, microorganism and silver nano particles in plant growth promotion activity.

Keywords: Cotton rhizospheric *Pseudomonas* species, antagonism, metabolic diversity.

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(Received: 23 September 2018; accepted: 09 November 2018)

Citation: Abhijeet S. Joshi and M.P. Chitanand, Metabolic Diversity of Rhizospheric *Pseudomonas* species of Bt Cotton Plant, *J Pure Appl Microbiol.*, 2018; Vol. **12(4)**: doi: 10.22207/JPAM.12.4.

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INTRODUCTION

Cotton belongs to the family Malvaceae and produces a natural type of fibre that grows around the seeds of the cotton plant. World wide India and China are largest producers of the cotton and the economy of many farmers of these countries were depends on production of cotton. The cotton dependent textile industry of the India is one of the leading industries in the world and the Indian government earns a lot of foreign exchange through this. Economic Survey of India shows that 16% of total world production of cotton was supplied by India¹.

In 1904 Hiltner first time coined a term rhizosphere which later modified by Hartmann et al. (2008) as the area around a plant root that is inhabited by microorganisms and have 3 zones as endorhizosphere, rhizoplane and ectorhizosphere. Roots released secretory and diffusive substances plays an important role in propagation of roots, soil adhering and different rhizospheric interactions respectively². Some microorganisms are capable to colonize rhizosphere and shows different types of interactions as pathogenic and beneficial interaction. The beneficial interaction of bacteria involves plant growth promoting rhizobacteria (PGPR) which can colonize roots in competitive environment and shows beneficial effect on plant growth³. In pathogens attacks plants will recruits the protective microorganisms and enhance the activity of metabolites for suppression of plant pathogens in rhizosphere⁴. There are various ways like production of antibiotics, induction of systematic resistance, production of various enzymes and peptides through which some bacterial species can show bio control activity. The high microbial diversity, density, metabolic activity, way of introduction of inoculums, some abiotic factors and competition occurring in the rhizosphere environment represents biological buffering that generally limits the establishment of new microorganism into the rhizosphere for root colonization^{6,7,8}. *Pseudomonas* is a highly diverse species among all the available bacterial species. It is not that it has much special molecular machinery adopt in given niche but it is that just simple mutation in regulatory pathways that affect the expression of surface polymer and it may be spatial or temporal⁹. The current work is just an attempt to look inside the metabolic diversity

of isolates and its application over plant growth promotion.

MATERIAL AND METHODS

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, packed and transported up to laboratory in UV sterilized plastic bag. The root adhered soil sample was used for physicochemical analysis and bacterial isolation purpose.

Serial dilutions of soil samples were carried in sterile saline and (10⁻³) dilution was streaked on the edge of PDA plates and incubated at 30°C for 24 hours. A 9 mm plug of *Fusarium oxysporium* and *Aspergillus niger* was taken from the 72 hours grown PDA plate and placed at the centre of previous plate. Plates were incubated at 30°C for 6 days. The bacterial culture that inhibits the fungal growth was isolated¹⁰. The morphologically similar colonies were selected and purified by repeated streaking^{11,12}.

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.

Plant growth promoting metabolites

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 determined¹³.

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°C for 48 hours followed by centrifugation at 8000 rpm for 10 minutes. The 1 ml supernatant was added in 1 ml (1mM) Silver nitrate (AgNO₃) solution and incubated at 300C for 24 hours for change in color from yellow to brown. The lambda max was observed at 420 nm. The vacuum dried sample was analyzed for SEM analysis¹⁴.

Determination of IAA Production

Isolates were screened for Indole Acetic Acid production, grown on Tryptic soy broth (1% Extra supplemented Tryptophan) incubated at 30°C for 72 Hrs¹⁵. After incubation, medium (5 ml) was centrifuged at 7000 rpm for 5 min and supernatant was used for IAA detection¹⁶. The isolates which give cherry red color after addition of Salkowski reagent were used for IAA production.

For IAA production 1% extra Tryptophan supplemented tryptic soya broth was used. The inoculums were prepared and inoculated flasks were incubated at 30°C for 72 Hrs. followed by its estimation as per the method suggested by Gordon and Weber (1950). The confirmation of Indole Acetic acid was carried out by HPLC method¹⁷.

Test for Phosphate Solubilization

Isolates were screened for their Phosphate solubilising activity. The isolates were grown on Pikovaskaya's agar, incubated at 30°C for 120 hrs. The isolate which shows clear zone around them were further used for quantitative assay¹⁸.

For production Pikovaskaya's medium was prepared with 5% Tri-calcium phosphate addition, incubate at 30°C, 120 rpm for 336 hrs. After incubation the medium was filtered through Whatmann's filter paper no.1. and the supernatant was used for the assay purpose. Phosphate solubilization assay was carried out as per King method (1932).

Antifungal activity

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

Percentage of Inhibition Radial Growth (PIRG) = $[1 - (\text{length of fungal growth near to bacterial isolate} / \text{length of fungal growth other side at the same plate as control})] \times 100\%$ (20)

Microcosm assay

The potential isolate combinations for microcosm assay were prepared. In microcosm the surface sterilized Bt cotton seeds were used¹⁹ and assay was carried out by two methods.

Effect of metabolites on germination of cotton seeds

The metabolites produced by isolates were partially purified by centrifugation process. After the centrifugation the supernatant with estimated concentrations were used for pot assay purpose. Approximately 2 ml of metabolite was added in every pot with different combinations.

Effect of metabolite producer

The metabolites producing isolates were enriched in King's B broth at 30°C for 24 hours for 0.5 OD at 600 nm. The tested fungi enriched in Potato Dextrose broth at 30°C for 48 hours for 108 spores/ml concentration. The 10 ml of fungal spore suspension and 10 ml of bacterial suspension was mixed in sterilized flask with various combinations and 3 seeds were allowed to soak within it for 10-12 hrs. After the incubation the seeds were air dried and sown in 250 gm of soil. The seeds were observed initially for germination and after 20 days analyzed for root, shoot length and dry mass of plant.

In control non-inoculated seeds were used.

Table 1. Physico chemical analysis of soil samples

S. No.	Parameter Sample	pH	Electric conductivity MS/cm	Organic carbon %	Phosphorus kg/h	Potassium kg/h	Ferrous ppm	Magnese ppm	Zinc ppm	Copper ppm
1	Agricultural College Nanded	7.27	0.22	0.57	17.5	454.15	4.42	2.20	0.80	0.40
2	Cotton Research Center Nanded	7.38	0.27	0.43	10.21	422.46	4.22	2.10	0.64	0.36
3	Dharma-bad	7.26	0.57	0.59	52.01	443.59	4.76	2.40	0.83	0.38

Statistical Analysis

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

PCR reaction for 16S rRNA sequencing

The isolation of genomic material of potential isolates was carried out and the amplification of 16S rRNA gene was performed by using the universal bacterial primers 27F and 1492R. The genes were sequenced on ABI Prism and BLAST was performed for construction of phylogeny using MEGA 6 software. (Fig. 5)

RESULTS AND DISCUSSION

The major characteristics of rhizosphere like physical, biochemical and ecologic characteristics are dependent on the balance between different compounds 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 bacteria²¹. This study is focused on the role of rhizospheric pseudomonas and its metabolic 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 5 Gram –ve, rod shaped; motile bacteria were selected and studied for 16S rRNA sequencing. A morphological, biochemical and molecular characterization of isolates allow the identification as *Pseudomonas* group. (Table 2 & 3).

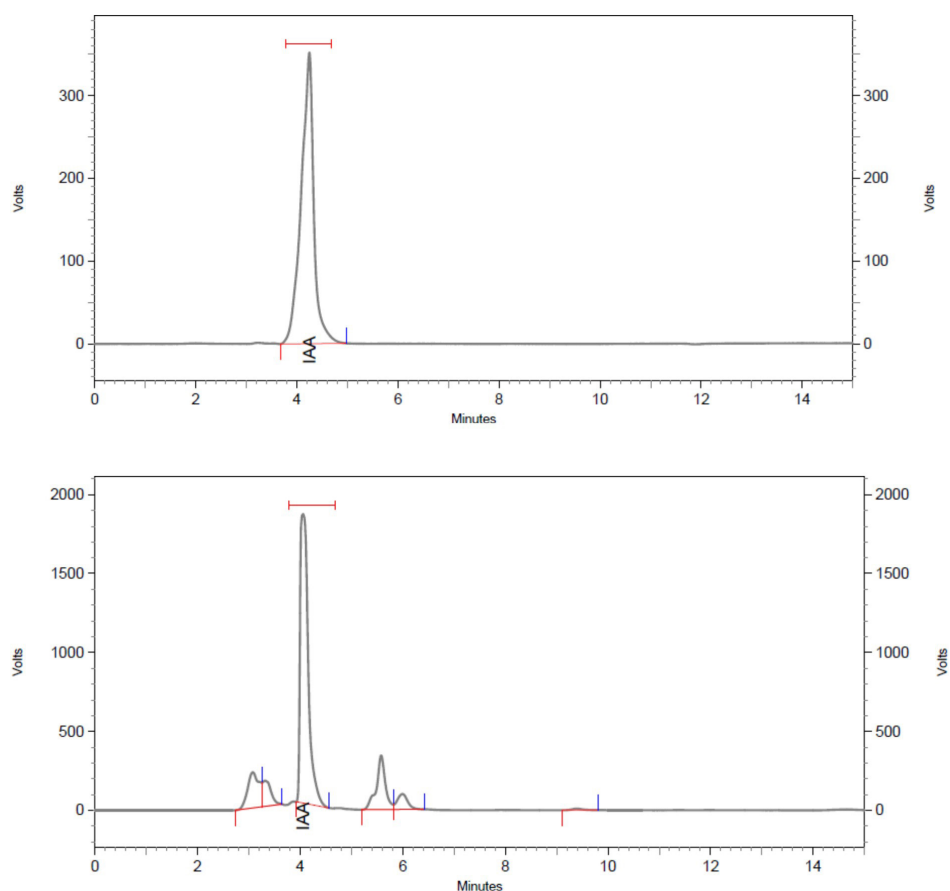


Fig. 1. HPLC analysis of Indole Acetic acid.

Table 2. Morphological characteristics of primary isolates

S. No.	Isolate	Colony Colour	Cellshape	Gram'snature	Motility
1	AJ A 1	White	Rod	Gram -ve	+
2	AJ A 2	Pink	Rod	Gram -ve	+
3	AJ A 3	White	Rod	Gram -ve	+
4	AJ A 4	White	Rod	Gram -ve	+
5	AJ B 1	Light green	Rod	Gram -ve	+
6	AJ B 2	Bright green	Rod	Gram -ve	+
7	AJ B 3	White	Rod	Gram -ve	+
8	AJ B 4	Light green	Rod	Gram -ve	+
9	AJ C 1	Pink	Rod	Gram -ve	+
10	AJ C 2	Light green	Rod	Gram -ve	+
11	AJ C 3	Light green	Rod	Gram -ve	+
12	AJ C 4	Pink	Rod	Gram -ve	+
13	AJ D 1	White	Rod	Gram -ve	+
14	AJ D 2	Pink	Rod	Gram -ve	+
15	AJ D 3	Bright green	Rod	Gram -ve	+
15	AJ D 4	white	Rod	Gram -ve	+
17	AJ E 1	white	Rod	Gram -ve	+
18	AJ E 2	Light green	Rod	Gram -ve	+
19	AJ E 3	Pink	Rod	Gram -ve	+
20	AJ E 4	white	Rod	Gram -ve	+

Table 3. Biochemical characterization of primary isolates

S. No.	Isolate	Biochemical Characterization													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	AJ A 1	+	+	-	-	+	+	+	+	-	-	+	-	-	+
2	AJ A 2	+	+	-	-	+	+	+	+	-	-	+	-	-	+
3	AJ A 3	+	+	-	-	+	+	+	+	-	-	+	-	-	+
4	AJ A 4	+	+	-	-	+	+	+	+	-	-	-	-	-	+
5	AJ B 1	+	+	-	-	+	+	+	+	-	-	+	-	-	+
6	AJ B 2	+	+	-	-	+	+	+	+	-	-	+	-	-	+
7	AJ B 3	+	+	-	-	+	+	+	+	-	-	+	-	-	+
8	AJ B 4	+	+	-	-	+	+	+	+	-	-	+	-	-	+
9	AJ C 1	+	+	-	-	+	+	+	+	-	-	+	-	-	+
10	AJ C 2	+	+	-	-	+	+	+	+	-	-	+	-	-	+
11	AJ C 3	+	+	-	-	+	+	+	+	-	-	+	-	-	+
12	AJ C 4	+	+	-	-	+	+	+	+	-	-	+	-	-	+
13	AJ D 1	+	+	-	-	+	+	+	+	-	-	+	-	-	+
14	AJ D 2	+	+	-	-	+	+	+	+	-	-	+	-	-	+
15	AJ D 3	+	+	-	-	+	+	+	+	-	-	+	-	-	+
15	AJ D 4	+	+	-	-	+	+	+	+	-	-	+	-	-	+
17	AJ E 1	+	+	-	-	+	+	+	+	-	-	+	-	-	+
18	AJ E 2	+	+	-	-	+	+	+	+	-	-	+	-	-	+
19	AJ E 3	+	+	-	-	+	+	+	+	-	-	+	-	-	+
20	AJ E 4	+	+	-	-	+	+	+	+	-	-	+	-	-	+

1 : catalase test 2 : Oxidase 3 : Acid & gas production 4 : Starch hydrolysis 5 : Gelatin liquefaction 6 : Arginine hydrolysis 7 : Levan production 8 : casein hydrolysis 9 : Lipid hydrolysis 10 : Urease test. 11 : Indole production 12 : Methyl Red test 13 : Vogus Proskour test 14 : citrate utilization test

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. 2). *Pseudomonas* species was also able to synthesise silver nanoparticles^{22,23}.

Plant growth promoting activity

95% of isolates were capable to produce IAA within a range of 13 - 70 µg/ml. The isolates AJ D 2 and AJ D 3 were highest (70 µg/ml) producers. Produced IAA was initially identified by TLC and

confirmed by HPLC. 90% isolates were capable to solubilised phosphorus, among them an isolate AJ A 2 shows highest (13.35 mg/lit) solubilisation activity. Phosphate solubilisation efficiency of isolated *pseudomonas* was found to be higher as compared to *Bacillus species*²⁴.

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

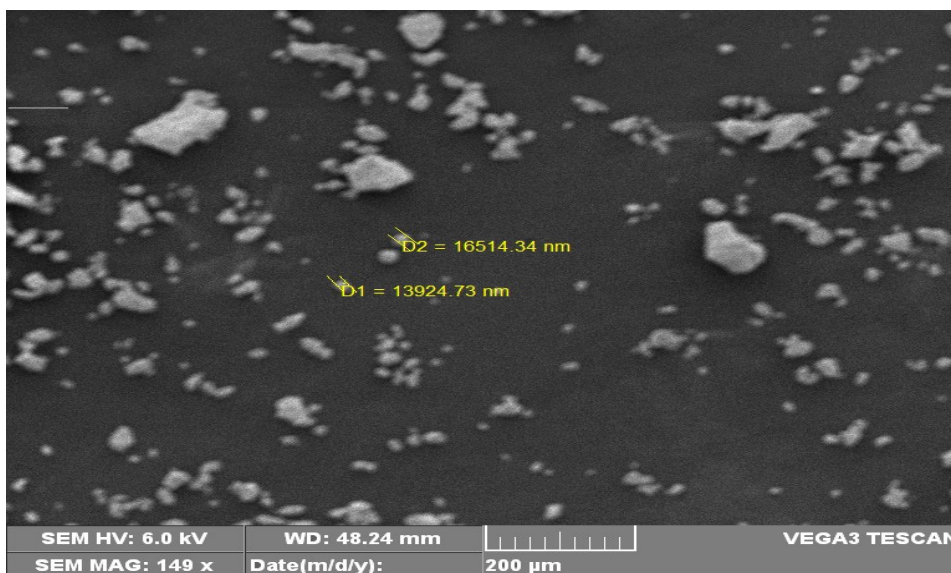
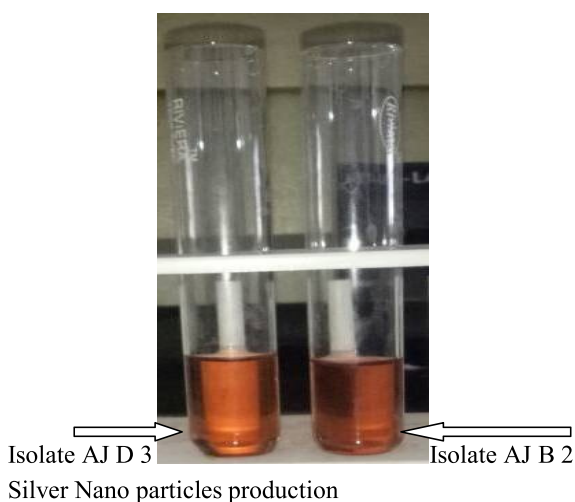


Fig. 2. Electron micrograph of silver nanoparticles

Fusarium oxysporum in monocropped soil²⁵. The effect of metabolite producers were separately evaluated and analyzed by paired t test ($p < 0.05$). A significant positive co relationship was found. An isolates AJ D 3 and AJ B 2 shows highest antifungal activity against *Fusarium oxysporum* and *Aspergillus niger* respectively.

Microcosm assay

When cotton seeds were challenged with pathogenic fungi, the isolate AJ D 3 was found to be most prominent organism. The plant growth promoting metabolites like IAA and phosphate solubilisers were found to be stable with silver

nanoparticles and it exhibits good plant growth promoting activity in presences of fungi also. As compared to IAA, solubilised phosphate shows more plant growth promotion activity. (Fig. 3 & 4)

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 the direct plant growth promoting diversity of culturable *Pseudomonas species* and find out role of silver nano particle as a tool in plant growth promotion.

Table 4. Main characteristics of selected isolates

Metabolite/ Isolatecode	AJ B 2	AJ D 1	AJ D 2	AJ D 3	AJ E 3	AJ A 2
Indole Acetic Acid(µg/ml)	44	62	70	70	25	38
PhosphateSolubilisation (mg/lit)	ND	ND	ND	ND	ND	13.25
Chitinase (IU)	ND	ND	170	710	870	ND
Bacitracin (µg/ml)	ND	11	7	6	ND	ND
Pyocyanin (µg/ml)	ND	ND	NS	3.15	ND	ND
Siderophore production(in %)	ND	69.33	ND	ND	68.83	ND
Silver nanoparticles	Y	ND	ND	Y	ND	ND
ND = Not detect	NS = Not significant		Y = Able to produce			

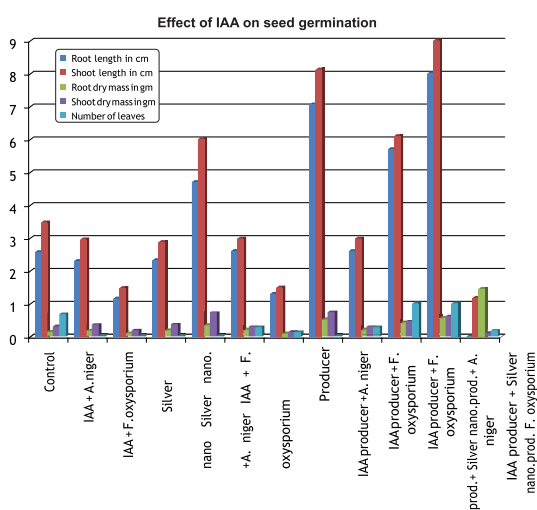


Fig. 3. Effect of Indole Acetic Acid on seed germination

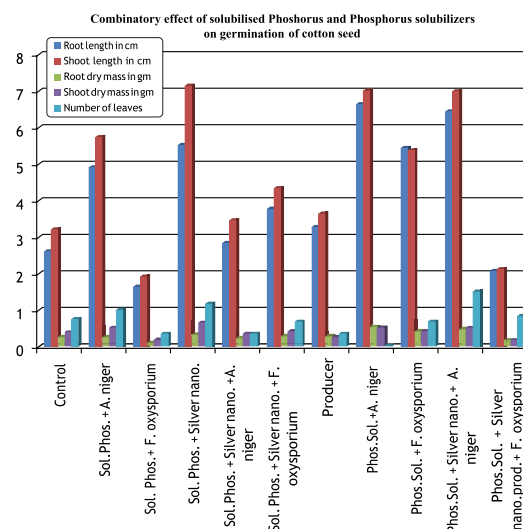


Fig. 4. Effect of phosphate solubilization on seed germination

Table 5. Effect of metabolite, isolates and fungi on germination of cotton seeds

S. No.	Combination	Root length in cm	Shoot length in cm	Root dry mass in gm	Shoot dry mass in gm	No. of leaves	No. of seed germinates
1	Control	1.86 ±0.90	8.06 ±1.76	0.44±0.14	0.75±0.16	1.33±0.57	1.00±0.00
2	Effect of Metabolites	2.48 ±1.59	3.32 ±2.14	0.15±0.09	0.31±0.19	0.40±0.24	0.40±0.24
3	Effect of metabolites with AgNP's	2.60 ±1.11	3.33 ±1.42	0.11±0.05	0.27±0.11	0.50±0.23	0.33±0.14
4	Effect of metabolites and <i>A. niger</i>	3.32 ±1.63	3.80 ±1.87	0.22±0.11	0.28±0.14	0.62±0.32	0.37±0.18
5	Effect of metabolites, AgNP's and <i>A. niger</i>	4.22 ±1.28	5.49 ±1.66	0.22±0.06	0.47±0.14	0.37±0.26	0.62±0.18
6	Effect of metabolites and <i>F. oxysporium</i>	5.29 ±1.22	6.61 ±1.50	0.29±0.07	0.55±0.12	0.50±0.26	0.75±0.16
7	Effect of metabolites, AgNP's and <i>F. oxysporium</i>	5.58 ±1.39	6.62 ±1.62	0.36±0.09	0.52±0.11	1.00±0.26	0.75±0.16
8	Effect of isolates	9.74 ±0.41	10.74±1.45	0.79±0.04	0.70±0.03	1.20±0.49	1.00±0.00
9	Effect of isolates and <i>A. niger</i>	4.24 ±2.60	4.52 ±2.77	0.33±0.20	0.28±0.17	0.40±0.24	0.50±0.07
10	Effect of isolates, AgNP's producer and <i>A. niger</i>	6.99 ±1.10	7.41 ±1.17	0.51±0.08	0.53±0.08	1.12±0.22	0.87±0.12
11	Effect of isolates and <i>F. oxysporium</i>	5.45 ±1.25	5.79 ±1.37	0.39±0.08	0.42±0.09	1.25±0.31	0.75±0.16
12	Effect of isolates AgNP's producer and <i>F. oxysporium</i>	7.10 ±0.80	7.60±0.86	0.49±0.05	0.53±0.05	1.50±0.28	1.00±0.00

± = Mean + SE mean

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