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Biocontrol activity of metal tolerant plant growth promoting bacteria isolated from industrial effluent

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Abstract
Plant growth promoting bacteria not only promotes the growth of the plant but it protects the plant from disease causing pathogens by various modes. In this study, previously isolated metal tolerant, plant growth promoting bacterial isolates (R-57 and R-58) were taken for management of the fusarium wilt disease under *in vitro* and *in vivo* conditions. Under *in vitro* conditions, both the isolates R-57 and R-58 were found to effectively inhibit the radial mycelial growth of the pathogen by 44.4% and 55.5% respectively. In *in vitro* study, no wilting was observed in the plans treated with isolate R-57 and R-58 where as wilting incidence was observed in control plant, inoculated with *F.oxysporum*. On the base of present study, the biocontrol agents of plant diseases can be exploited for sustainable disease management and reduce environmental risk.

Keywords: Biocontrol, industrial effluent, plant growth

Introduction
India is primarily an agriculture based country and 58% of India's population derive livelihood from agriculture. Plant pathogens are big threat to agriculture and manufacturing industries during pre-harvest as well as storage, being responsible for great economic loss. Fungal diseases cause heavy yield loss, with decreasing productivity and the quality of the produce. *Fusarium oxysporum* is a ubiquitous soil-borne pathogen that causes vascular wilt on a wide range of plants. Characteristic disease symptoms include vascular browning, leaf epinasty, stunting, progressive wilting, defoliation and plant death (Agrios, 2005) [1]. The *F. oxysporum* infects more than 100 different hosts, provoking severe losses in crops such as melon, tomato, cotton and banana, etc. (Michielse and Rep, 2009) [19].
Tomato (*Solanum lycopersicum*), an excellent source of micronutrients and antioxidants (Lenucci *et al*. 2006, Keswani 2015) [17, 14], is one of the most popular and important commercial vegetable crops grown throughout the globe. Fusarium wilt, one of the most serious diseases affecting tomato plant, reduces greatly to its yield. Pathogenic *Fusarium spp*. can produce a series of toxic secondary metabolites that are a threat to the agriculture bio-safety, food security and health of plants (Berges *et al*., 2013) [5]. *F. oxysporum* are saprophytes and are able to grow on soil organic matter for a prolonged period, thus colonize the plant through the root system and prevent optimal development of the host plant (Gawehns *et al*., 2013) [8]. Control of *Fusarium* with fungicides is an effective measure but its persistent residue cause harmful effects on environment and human health. Alternatively, microorganisms can be effectively employed as biocontrol agents in agricultural crops for sustainable agriculture (Keswani *et al*., 2014, Bisen *et al*, 2015) [13, 6].
On account of that, the present study is designed to investigate the antagonistic characteristics of the metal tolerant plant growth promoting bacteria as a biocontrol agent in tomato plant *invitro* for plant protection.

Materials and Methods
Collection of antagonists and its characteristics
Present interest of bacterial isolates, R-57 and R-58 previously isolated from Rourkela steel plant effluent. The isolates were tolerant to heavy metals like Ni, Cd, Cr, Pb and Hg. Both the bacteria showed plant growth promotion activities such as indole acetic acid (IAA) production, solubilization of inorganic phosphate, ammonia production, siderophore production and HCN production. After performing various morpho-biochemical activities and sugar fermentation test, both the isolates i.e R-57 and R-58 were gram –ve and belong to *Pseudomonas sp*. Bacterial colony of R-57 was metallic bluish green and R-58 was lemon green at 30^{0}C in 48 hr of incubation on luria agar plate.

Antagonistic effect of bacteria against plant pathogen

Fusarium oxysporum ITCC-4998, a causal agent of tomato wilt collected from Department of Microbiology, CBSH, OUAT, BBSR, The antifungal bioassay was carried out *in vitro* by growth inhibition of phytopathogenic fungus in LA-PDA media. The bacterial inoculum was aseptically streaked on two sides of the petridish. A, 7 days old, fungal agar disc (1cm diameter) punched out with sterilised gel puncture from the growing margin of colonies were placed at the centre of each inoculated plates. The plates with phytopathogens alone served as control. Four replicates for each treatment were incubated at 28°C for five days (Altindag *et al*., 2006) [3]. Inhibition of the fungal growth was recorded and the percentage of fungal growth reduction was calculated according to formula adopted by Topps and Wain (1957) [23] as follows:

Percentage of reduction = [(A-B)/A] x 100

Where:
A=diameter of the control hyphal growth
B= diameter of the treated hyphal growth
The antagonistic effect on fungal mycelia were observed under stereo microscope.

Antifungal activity by volatile assay

The two-sealed- base- plate method was used to test the antifungal activity of VOCs from bacterial isolates. One base plate contained 15 ml of Luria bertani agar, and another base plate contained PDA. Bacterial isolates were streaked on the LA plates and a 5 mm diameter of plant pathogenic fungi agar disc was placed on the PDA plate. Both the plates were paired in such a way that the plate containing fungal disc was in the lower position and the bacterium in the upper one and were sealed in the parafilm and incubated at 28^0C for 10 days. The paired plate without bacterial inoculation was treated as control. Every experiment was repeated three times (Gao *et al*., 2017) [7].

Pot culture experiment to study the Biological control of *F.oxysporum* on tomato wilt

The interaction of bacterial isolates R-57 and R-58 with *F.oxysporum* ITCC-4998 were studied *in vivo* by pot culture method.

Collection of seeds and preparation for seedlings

Seeds of tomato (*Solanum lycopersicum*) were obtained from local seed shop of Bhubaneswar. Later the seeds were in potting mixture in a tray, watered in interval of days to obtain seedlings.

Potting mixture for seedlings

Potting mixture (red soil: sand: decomposed FYM at 1:1:1 w/w/w) was prepared and autoclaved one hr for two consecutive days and filled in a tray and in pots. The pots were used to determine the biocontrol potential of selected isolates.

Preparation of growth medium

Potato dextrose broth (PDB) and Luria bertani (LB) broth were prepared and autoclaved at 15 lb pressure and 121^0C temperature for 15 minutes. Autoclaved PDB inoculated with phytopathogen, LB inoculated with isolates R-57 and R-58 separately were incubated at 28^0 C in an incubator shaker.

Pot experiments for biocontrol efficacy

Bioefficacy was studied by following Patil, 2011 and Barari, 2016.*Fusarium* pure culture was grown in 150 ml of potato dextrose broth (PDB) for seven days in an incubator shaker. Twenty-days-old seedlings of tomato were dipped in a beaker containing *F.oxysporum* biomass for 1 hour and then transplanted in pots filled with potting mixture. Before inoculation, the roots were slightly wounded by inserting a sterile needle, 1cm away from the stem. Wound was done to ensure pathogen penetration through roots.

After one day, two days old bacterial culture of R-57 and R-58 were inoculated in to the seedlings by soil drenching method. In the soil drenching method, 10 ml of bacterial suspension (water: bacterial culture, 1:1) was inoculated to each of the seedlings by drenching the soil around the root zone with the help of micropipette. Seedlings only inoculated with *Fusarium* were considered as control for the experiment. Disease incidence (wilt) was recorded at two-days interval based on external symptoms. The experiment performed in triplicates and was repeated twice.

Results

Antifungal effect of isolates on *F.oxysporum*

The two metal tolerant plant growth promoting bacteria (PGPB) evaluated for antifungal activity against *Fusarium oxysporum*, showed antagonistic effect under dual culture assay. Isolates R-57 and R-58 significantly affected the mycelial growth. The colony diameter, 2.8 ± 0.09 with R-57 and 2.0 ± 0.13 with R-58 recorded a decrease of 44.4% and 55.5% over the control (Table-1). The microscopic structure of inhibited mycelia showed changes in colour morphology of fungal mycelia structure (fig.1).

Table 1: Antifungal activity of R-57 and R-58 against *Fusarium oxysporum*

Bacterial Treatments	Colony Diameter of *Fusarium oxysporum*	% of fungal growth reduction
Untreated Control	4.5 ± 0.19 a	0
R-57	2.8 ± 0.09 b	44.4
R-58	2.0 ± 0.13c	55.5

Tested by Duncan's Multiple Range Test with 5% critical range. Means represented by the same letter are not significantly different. Data given in above are average of four replicates, ± standard error of mean (SEM).

Antifungal activity by volatile assay

Paired plate assay was carried out to detect the production of inhibitory volatile antifungal compound against all phytopathogens. There was no significant difference observed in the diameter of fungal growth on control plate and inoculated with isolate R-57 and R-58. The antifungal compound responsible for mycelia growth reduction may not be a volatile compound.

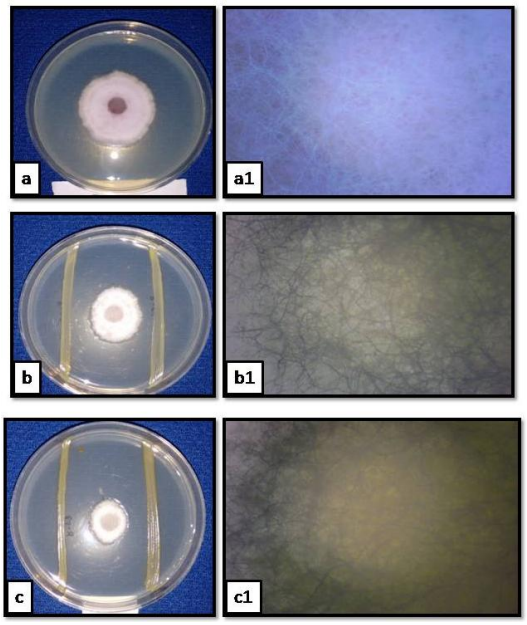

Fig. 1: Antifungal activity of R-57 and R-58 strains against phyto-pathogen *Fusarium oxysporum*
(a) Fungal growth on PDA plate (Control),
(a1) Stereo microscopic structure under 5X (Control);
(b) Coinoculation of *F. oxysporum* with R-57,
(b1) Stereo microscopic structure of coinoculated *F. oxysporum* with R-57 under 5X;
(c) Coinoculation of *F. oxysporum* with R-58,
(c1) Stereo microscopic structure of coinoculated *F. oxysporum* with R-58 under 5X

Pot culture experiment

The application of bacterial isolate R-57 and R-58 on *Fusarium* inoculated tomato plant was effective in suppressing wilt incidence after 8 days of inoculation (Fig.2.). Control, only inoculated with *Fusarium* showed wilting incidence on tomato plant.

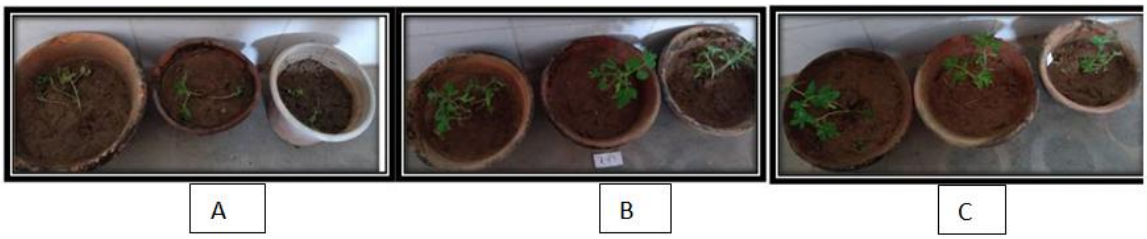

Fig 2: Biocontrol of F. oxusporum in tomato plant
A - Control, Tomato plant inoculated with *F. oxysporum*
B - Pot soil and plant inoculated with *F. oxysporum* + R-57
C - Pot soil and plant inoculated with *F. oxysporum* + R-58

Discussion

Control of the mycopathogen is of great importance because fungi causes a great nuisance in plant growth, sporulate and resist the postharvest treatments of the produce. A number of chemical fungicides are being applied to control the disease and yield loss. However, the regular use of chemical fungicides can potentially pose a risk to the environment, particularly if residues persist in the soil or migrate off-site and enter waterways (Kibria *et al*., 2010; Komarek *et al*., 2010) [15, 16]. This in turn can have adverse effects on soil flora & fauna and move to the human through food chain. It also pose a risk to the long-term fertility of the soil (Wightwick *et al*., 2008; Komarek *et al*., 2010) [26, 16]. This necessitates the search for alternative environmental friendly biocontrol agent.

Several microorganisms/antagonists have been identified and also known for controlling fungal diseases of different fruits and vegetables. Bacterial antagonists such as *Bacillus subtilis* (Jiang *et al*., 2001; Wang *et al*., 2010) [12, 25], *Rhodotorula glutinis*, *Enterobacter aerogenes* (Qin *et al*., 2004) [21] and *Brevibacillus* (Ahmed 2017) [2] were previously reported inhibiting the fungi.

In the present study, bacterial isolates showed a significant inhibition of *F.oxysporum* by dual culture method. *In vitro* dual cultures offer a better method for evaluation of the antagonistic efficiency of the biocontrol agents (Trivedi *et al*., 2008) [24] and may provide a better environment to allow the antagonistic activities from all possible interacting sites. The microscopic observation reveals retardation in the growth of mycelia. Many fungal and bacterial antagonists like *Trichoderma spp*., *Pseudomonas fluorescens*, *Burkholderia cepacia*, non-pathogenic isolates of *Fusarium spp*., etc. have been tested for their efficacy in controlling *Fusarium* wilt of crop plants.

Fusarium oxysporum is an important soil borne fugal pathogen and is able to induce wilt or root rots in variety of vegetable crops (Lopez-Berges *et al*., 2012) [18]. Both the isolates (R-57 and R-58) have shown growth inhibiton of *F.* oxysporum. Reports suggest antifungal activities of bacteria isolated from variety of environments. In this study bacteria

with metal tolerant and plant growth promoting traits, have been previously isolated from industrial effluent. According to Islam *et al*. (2018) [11] cell-free culture filtrate of *Pseudomonas aeruginosa* exhibited significant antifungal activity against *Fusarium oxysporum*. *Bacillus subtillis* isolate (CRB 20) reduced the severity of fusarium wilt under green house condition (Hariprasad *et al*., 2011) [10]. In the present study R-57 and R-58 were applied in the pot soil reduced the wilt incidence. The antagonism effect was observed at the 8th day after inoculation of bacterial culture. Antagonistic effect might be due to production of various antifungal compounds by the bacteria. Microorganisms with stress tolerance capacity and plant growth promotion traits have evidence for being helpful in growth and development of plants under stressed environments (Shrivastava and Kumar, 2015) [22]. The fusarium wilt suppression in this study may involve various mechanisms that include production of antibiotics, lytic enzymes and siderophore production (Harikrishnan *et al*, 2014) [9]. These antagonisms of the isolates indicated potential use for biological control of fusarium wilt in tomato.

Conclusion

The metal tolerant, plant growth promoting bacteria i.e. R-57 and R-58 can be used as a potential biocontrol for fusarium wilt in tomato plant.

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