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Antibiofilm Activity of Iron Nanoparticles Synthesised from *Camellia sinensis* against Bacterial and Fungal Pathogens

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ABSTRACT

New and resistant pathogenic bacterial and fungal strains in agricultural field have raised the need to develop new compounds with broad range of antimicrobial activity. Therefore, present study was aimed for green synthesis of iron nanoparticles and testing their efficacy against phytopathogeic bacteria and fungi. The bacterial isolates (TBT, TBK, TBC, TBO, TBA, TBL and TBR) were obtained from rotten plants of *Solanum lycopersicum*, *Citrus limon L*, *Citrus reticulata cv.* Kinnow Blanco, *Malus pumila*, *Brassica rapa subsp. rapa*, *Allium cepa*, *Zea mays* while fungal isolates were obtained from rotten *Citrus limon L*, *Citrus reticulata cv. Kinnow Blanco* and *Prunus dulcis.* Bacterial isolates showed similarity to *Bacillus* species while fungal isolates (TFA and TFK) showed similarity to *Penicillium* sp. and TFL to *Cladosporium.* The percentage yield of iron nanoparticles from tea extract was 25 %. Scanning electron microscopy showed round particles of size 70 nm. FTIR of synthesized nanoparticles showed the presence of different functional groups i.e., C=C, O-H, C-H and C-O-C. The antibacterial activity of nanoparticles showed increase in bacterial cell densities, while no significant inhibition on fungal isolates Antibiofilm effect of iron nanoparticles on pathogenic bacterial and fungal isolates was found to be significant at concentration 250 µg/ml as compared to control treatment. Microscopy of bacterial and fungal biofilms development trend was corresponding to the normalized values. Twitching motility was highest in non NP treated plates as compared to NP treated plates. In general, there was a significant effect of nanoparticles on *Triticum aestivum* Var. (Lasani 08) growth and resulted in significant increment in shoot length (cm), root length (cm), fresh weight and percentage germination of seedlings. These findings showed promising potential of iron nanoparticles on phytopathogens in protecting fruit plants.

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Key words
Biofilm, FTIR, Iron nanoparticles, Scanning electron microscopy, Twitching motility

INTRODUCTION

Due to extreme worldwide public health issues of resistant bacterial and fungal strains to antibiotics and antifungals (Ferri *et al*., 2017), nanoparticles have raised a need to be introduced as new antimicrobial compounds against bacteria and fungi. Previous investigations are supporting the antimicrobial approach (Rajakumara *et al*., 2012; Wang *et al*., 2017) and are contributing greatly in the development of novel and innovative applications in antimicrobials/biocides sector like biocoatings and biopolymers tissue engineerings (Aschberger *et al*., 2015). The virulence of phytopathogenic bacteria results in blockage of xylem vessels, increased resistance to plant antimicrobial compounds, and enhanced colonization of specific habitats due to biofilm formation (Mansfield *et al*., 2012; Dwivedi *et al*., 2017).

Iron oxide nanoparticles are seen to be involved in showing the antibiofilm effect by causing oxidative stress induction as antibacterial effect (Wang *et al*., 2017). Moreover, electrostatic interactions between nanoparticles and bacterial cell membranes or cell membrane proteins result in physical damage, which ultimately leads to bacterial cell death (Sathyanarayanan *et al*., 2013). The need of efficient synthetic techniques, that are ecofriendly and economically favorable have compelled the researchers to go for green synthesis of iron oxide nanoparticles (Hoag *et al*., 2009; Cai *et al*., 2010; Lu *et al*., 2010; Chrysochoou *et al*., 2012; Venkateswarlu *et al*., 2013). One of such most simple, inexpensive, least toxic

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and environmental friendly approach involves the usage of phytochemicals for nanoparticles synthesis. The effects of iron nanoparticles on biofilm forming pathogens have been greatly explored. According to Sathanarayanan *et al*. (2013) higher concentration of iron oxide nanoparticles were found to be effective in the reduction of biofilm growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Iconaru *et al*. (2012) showed that iron nanoparticles inhibited the ability of *P. aeruginosa* to develop biofilms on the inert substratum. Superparamagnetic iron nanoparticles are also been seen to inhibit biofilms of MRSA at very low concentrations (Taylor *et al*., 2012). Another study showed the antibiofilm effect of super paramagnetic nanoparticles on gentamycin resistant *Staphylococcus aureus* (Subbiahdoss *et al*., 2012). The biological assays revealed that the newly fabricated nanobiocoating exhibited antimicrobial properties, rendering the wound dressings fibers more resistant to fungal cells adherence and biofilm development (Anghel *et al*., 2012). Previous study proved that the nano biocoating combined the excellent properties of iron oxide nanoparticles and the essential oils having antimicrobial properties. This approach is a successful alternative for inhibiting fungal adhesion and biofilm formation on medical devices and other clinically relevant materials and any surfaces (Chifiriuc *et al*., 2012). Iron nanoparticles show various applications in agricultural field either in the form of fertilisers, pesticides or growth stimulators as reported previously (Aleksandrowicz-Trzcińska *et al*., 2019). Iron nanoparticles have been involved in influencing the rhizosphere of plants by their toxicity on the *Trifolium repens* mychorrhizal microbes either by reducing the glomalin content or reducing the acquisition of root nutrient of AMF but this depends on the concentration of nanoparticles (Feng *et al*., 2013). The bsAgNPs have been reported to show strong antifungal activity against the causative agent of spot blotch pathogen *Bipolaris sorokiniana* of wheat (Mishra *et al*., 2014). The diverse applications of iron nanoparticles has led to their usage as antimicrobial compounds in agriculture fighting with the resistance of emerging microbes (Rani *et al*., 2015). Studies have shown that direct application of nanoparticles significantly suppressed the tested plant pathogenic fungi (*Candida albicans*, *C. krusei*, *C. tropicalis*, *C. glabrata* and *Aspergillus brasiliensis*) and bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* (Khan and Rizvi, 2014). Present study is aimed at green synthesis of iron nanoparticles and their ability to inhibit the biofilm formation of phytopathogens.

MATERIALS AND METHODS

Isolation and purification of bacterial and fungal isolates from rotten fruits and vegetables

The bacterial samples of rotten fruits and vegetables (*Solanum lycopersicum*, *Citrus limon L. mandarin*, *Malus pumila*, *Brassica rapa subsp. rapa*, *Allium cepa*, *Zea mays*) and fungal isolates from spoiled *Citrus limon* L. *Citrus reticulata cv.* Kinnow Blanco and *Prunus dulcis* were serially diluted (10-5) and spread on LB agar plates, further purified by quadrant streaking method to get pure individual colonies. These isolates were named as TBT, TBK, TBC, TBO, TBA, TBL and TBR on the basis of their isolation source tomato, kinnow, corn, onion, apple, lemon and turnip, respectively and stored in the form of glycerol stocks prepared in LB broth (Gerhardt *et al*., 1994) at -80 ˚C freezer (Avasonic MDF-C8V1-PE ultratemperature freezer). Fungal isolates were obtained by picking spores with the help of sterile needle directly from the infected fruits or vegetable surfaces and inoculating in the potato dextrose agar (Cappucino and Sherman, 2005) plates. The isolated fungal colonies were named as TFA, TFK, and TFL isolated from almond, kinnow and lemon fruits, respectively and stored in glycerol stocks at -80˚C in refrigerator (Avasonic MDF-C8V1-PE ultra-temperature freezer) prepared in PDA broth.

Green synthesis of iron nanoparticles from green tea (Camellia sinensis) *leaves extract and their characterization*

Green synthesis of iron nanoparticles were done by the same method as described by Gottimukkala (2017) using green tea leaves. The synthesis of iron nanoparticles as immediate black color was done by adding 0.01 M ferric chloride in green tea leaves extract at 1:1 ratio in sterilized flask. The yield of nanoparticles was calculated by the following formula:

Yield = (obtained weight/total culture) × 100

One percent solution of nanoparticles was made for further use in antimicrobial and biofilm assays. Characterization of nanoparticles was carried out by documenting the nanoparticles spectra at 400–4,000 cm^{-1} range using flourier transform infrared spectroscopy (FTIR) (Bruker). Scanning electron microscopy (SEM) (TESCAN Vega-LMU–Variable pressure Scanning Electron Microscope) was done to observe morphology of nanoparticles.

Antimicrobial activity of iron nanoparticle

The antibacterial activity of iron nanoparticles were determined by turbidity assay (Lourenço and Pinto, 2011) for the bacterial isolates while agar well assay for fungal isolates.

Bacterial cultures were adjusted at equal cell densities in LB broth medium (OD adjusted to 0.5 at 600 nm: 10^8 cfu per ml). The nanoparticles were supplemented at increasing concentration from 50 µg/ml to 250 µg/ml concentrations in the broth medium along with the inoculum. For control plates, an antibiotic ciprofloxacin (0.5 µg/ml) was added instead of iron nanoparticles. Cultures were then incubated at 37˚C for 24 h. Finally, bacterial growth was determined in terms of optical density using spectrophotometer at 600 nm (Specord 200 plus Germany). The experiment was done in replicates and the values were then taken in average. For fungal growth experiments, the PDA medium was prepared and supplemented with different concentration of iron nanoparticles. After sterilization, the agar media was poured in petri plates. Solidified agar plates were inoculated by fungal isolates with the help of sterile needle and plates were incubated at 30 ˚C for 4 days. Diameter of fungal colony was scored. For control fungal plates, antifungal Axicon (500 µg/ml) was added in sterilized PDA agar plate instead of iron nanoparticles.

Antibiofilm activity against bacterial and fungal pathogens

The biofilm assay of bacterial cultures was determined by following method of Qurashi and Sabri (2012). Briefly, the cultures were inoculated (cell densities adjusted at 0.5 at 600 nm) and incubated at 37 °C for 144 hours in sterile L broth medium (Gerhardt *et al*., 1994) without shaking in test tubes. Tubes were supplemented with sterile coupons (to observe adhered biofilm) and nanoparticles (50 µg/ml to 250 µg/ml). Bacterial growth in test tubes after 144 hours of incubation was determined at 600 nm. To check the adherence of bacterial cells, the medium was removed, and the biofilms were stained with aqueous crystal violet for 20 min. Biofilm formation was determined by taking OD 570 nm using solubilized crystal violet in 70 % ethanol as previously described. Fungal biofilm formation was determined by following Pierce *et al*. (2008). Fungal isolates were inoculated at 30 °C for 3 days in sterile PDA broth medium. Inoculum was given according to 0.5 McFarland in biofilm culture tubes supplemented with sterile coupons to observe adhered biofilm and nanoparticles (50 µg/ml to 250 µg/ml) and incubated at 30 °C for 144 optimized hours without shaking. Fungal planktonic cells were washed with PBS and tightly bound cells were stained with safranine. The dye was solubilized in 30 % glacial acetic acid and was quantified by measuring the absorbance at 490 nm. All the experiments were carried out in replicates. Microscopic analysis of bacterial and fungal biofilm developed in the presence and absence of nanoparticles on the polystyrene coupons, was done using microscope (Model No: MT4300H, Meiji Techno Co, LTD) attached with camera at 40X.

Effect of nanoparticles on bacterial motility

To check the effect of nanoparticles on bacterial motility response, twitching assay was performed following the assay of Deziel *et al*. (2001). For this bacterial cells were picked and were stab inoculated with a sterile needle in a thin layered LB agar (1% agar) supplemented with 250 µg/ml of iron nanoparticles, at the bottom of the petri dishes. A control without the iron nanoparticles was also checked. Hazy zones of growth at the interface between the agar and glass surface were observed after incubation for 24 to 48 h at 37°C. The ability of bacteria to strongly adhere and form a biofilm on the glass surface was then examined by removing the agar, washing unattached cells with a stream of tap water, and staining the attached cells with crystal violet (1% wt/vol solution) and then measuring the hazy zone diameter (cm) of the bacterial growth in the both the control and nanoparticles supplemented plates.

Effect of iron nanoparticles on the growth of bacterial, fungal isolates and seeds of Triticum aestivum *(wheat)*

Plant growth experiment was performed as described by Qurashi and Sabri (2012). Briefly, healthy and certified, disinfected seeds (using 0.1 % mercuric chloride) of *Triticum aestivum* Var. Lasani 08 obtained from the Punjab Seed Corporation Lahore were grown in the plastic pots containing 200 g sterile soil in the presence of pathogenic inoculum (OD of fresh cultures adjusted to 0.5 at 600 nm) and nanoparticles. Seeds were soaked in iron nanoparticles for 20 min before soaking in the pathogenic strains. Regular watering of potted plants was done using 20 ml sterile water at room temperature. Plant growth was determined in terms of protein contents of plants. Protein analysis was done following Lowry *et al*. (1951).

Statistical analysis

All experiments were performed in triplicates and the values were expressed as average values. Standard errors were calculated from the mean values and shown in bars of each figures. Analysis of variance (ANOVA) was performed to record pairwise comparisons of means and calculating differences in each experiments for least significant difference (LSD) method at $p < 0.05$ level of significance.

RESULTS

Bacterial and fungal isolates

For bacterial isolation rotten tomatoes (*Solanum lycopersicum*), lemons (*Citrus limon* L.), kinnow (mandarin), apples (*Malus pumila*), turnip (*Brassica rapa subsp. rapa*), onion (*Allium cepa*), corn (*Zea mays*) were collected in the month of July. Isolates were named as TBT, TBK, TBC, TBO, TBA, TBL and TBR isolated form

tomato, kinnow, corn, onion, apple, lemon and turnip, respectively (Supplementary Table I). Selected bacterial isolates were characterized through cultural characteristics, morphologically and biochemical testing following Cappuccino and Sherman (2005) techniques. Results showed that isolates TBT, TBC, and TBR showed off-white to yellow colored with irregular form, undulate margins and flat colonies on N agar medium, while TBK, TBO, TBA, and TBL showed off-white colored with circular form, entire margins and raised colonies (Supplementary Table I). Morphological characterization showed that all isolates showed positive results for G staining and appeared as rods under microscope except isolate TBL that were cocci. Isolates TBT, TBK and TBCwere positive for capsule staining while negative results for TBO, TBA, TBL and TBR. With the exception of TBT, all isolates showed negative results for Spore staining. All bacterial isolates showed positive results for catalase, methyl red (except TBA), voges-proskauer (except TBT), citrate utilization test, and motility test. The negative response of these isolates was recorded towards Urease (except TBO and TBL) and sulphide production assay (Supplementary Table I). Three isolates TBT, TBK, TBL and TBA, showed yellow colonies on mannitol salt agar. TBC, TBO and TBR showed no growth on mannitol salt agar.

The isolated fungal colonies were named as TFA, TFK, and TFL from almond, kinnow and lemon fruits (Supplementary Fig. 1). The fungal isolates were observed using light microscope and were found to have different morphologies. Results showed that fungal isolates TFA and TFK had greenish blue colonies. Under the microscope, conidiophores and conidia and a septate mycelium were observed thus indicating *Penicillium* sp. (Cappuccino and Sherman, 2005) which is usually found in citrus fruits and soil, while isolate TFL showed greenish black powdery colonies, conidia present at the terminal end of conidiophores with septate mycelium and showing similiarity to *Cladosporium* (Cappuccino and Sherman, 2005).

Characteristics of iron nanoparticles

Iron nanoparticles were synthesized from green tea extract as black powder settled in the form of pellet after centrifugation. Figure 1A shows the black coloured pellet of nanoparticles obtained after centrifugation. The percentage yield obtained form 100 ml of tea extract was 25 %.

FT-IR spectroscopic analysis of vibrations of functional groups shows C=C functional groups peak at 1636.18 cm^{-1} and O-H group peaks observed at 3347.30 cm^{-1}. The peaks observed at 2123.81 and 1074.15 cm^{-1} showed the presence of C-H and C-O-C groups (Fig. 1B).

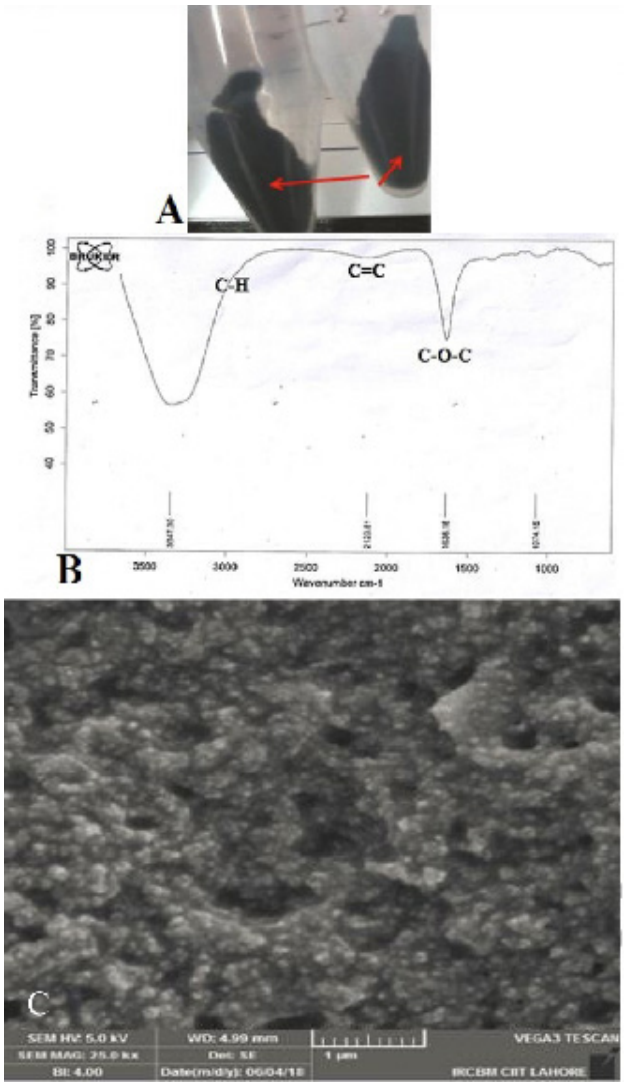

Fig. 1. Characteristics of iron nanoparticles synthesized from green tea leaves. **A**: shows the black colored pellet of nanoparticles obtained after centrifugation **B**: shows peaks of various functional groups O-H, C=C and C-O-C through FTIR spectroscopic analysis. C shows size of grains of nanoparticles as seen under Scanning Electron Microscope (SEM); **C**: shows size of green nanoparticles as seen under Scanning Electron Microscope (SEM).

Scanning electron microscopy (SEM) analysis of iron oxide nanoparticles shows round morphology grain arranged in the form of concentric rings with some pits. As per scale, a total 8460 particles were arranged in the form of layers. The mean particle size measure through image J software was 79 nm (Fig. IC).

Antimicrobial activity of iron nanoparticle

The antibacterial activity of optimized concentrations

of iron nanoparticles (50, 90, 130,170 and 250 µg/ml) on the growth of bacterial isolates in LB broth medium was recorded using Optical density (600_{nm}). Results showed that there was a general trend of increase in bacterial cell densities in the presence of nanoparticles with exception of isolate TBL, TBT and TBR (Fig. 2). Increasing concentration of Fe NPs was found to be supporting the planktonic cells growth (Fig. 2). Effect of antibiotic ciprofloxacin was also checked on the growth of bacterial isolates. There was significant inhibitory effect observed as compared to control (Fig. 2A). There was a significant reduction in growth of TFK and no effect on TFL and TFA. In Figure 2B significant inhibitory effect of nanoparticles were recorded at TFK as compared to rest of isolates.

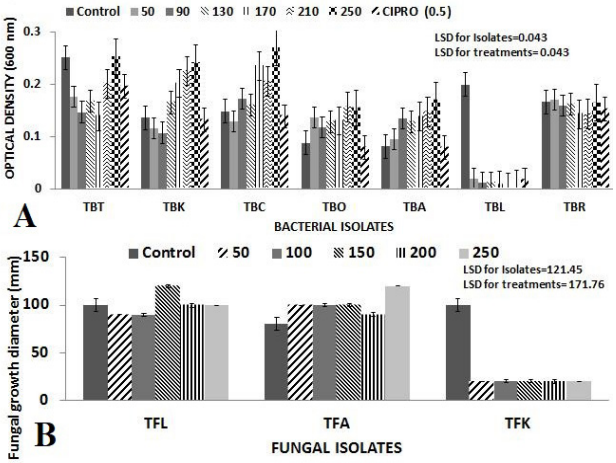

Fig. 2. Turbidity method for antibacterial analysis of iron nanoparticles on bacterial isolates B: Antifungal agar method to check effect of nanoparticles

Antibiofilm activity of iron nanoparticles against bacterial and fungal isolates

Antibiofilm effect of iron nanoparticles on pathogenic bacterial isolates showed a general trend of decrease in biofilm formation trend at increasing concentrations of nanoparticles (Fig. 3A). Biofilm formation was normalized to bacterial growth by determining the ratio of A_{570}/A_{600}. While using the two way analysis of variance (ANOVA) and recording pairwise differences (for isolates as well as treatments) results were found to be significant as compared to control. All concentrations of Fe NPs were found to be significantly ($p < 0.05$) inhibiting the biofilm formation by bacterial isolates when compared to respective control treatments. The biofilm inhibition was significantly highest at concentration 250 µg/ml (Fig. 3) as compared to control treatment. The highest biofilm inhibition was recorded by isolate TBC as compared to rest of isolates and respective control treatments (Fig. 3A).

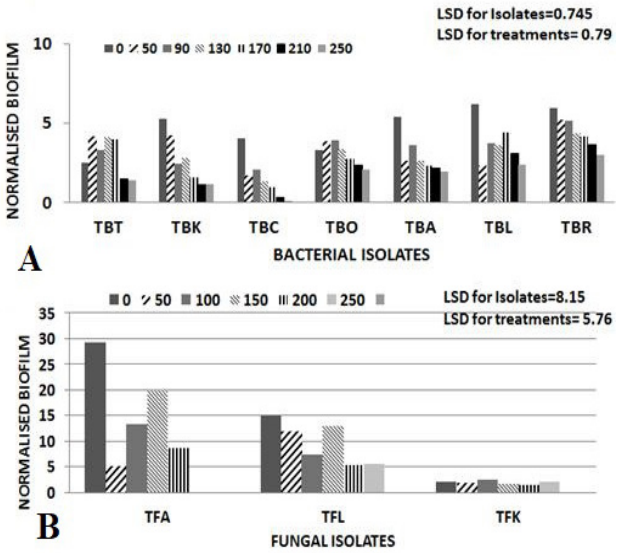

Fig. 3. Antibiofilm effect of iron nanoparticles on phyto pathogens: A, bacterial biofilm; B, fungal biofilm.

Antibiofilm effect on fungal isolates was determined using PD broth medium and biofilm was normalized to fungal growth (Fig. 3B). There were significant results recorded for all fungal isolates as compared to non-treated control. Results showed that greatest inhibitory effect of nanoparticles was significant in all treatments in case TFA isolate. Usually in all cases of the inhibition was observed a 200-250 µg/ml concentration. Isolate TFL showed significant inhibition at 50 µg /ml concentration as compared to non-treated control. While no inhibitory effect was observed at any concentration of nanoparticles on TFK fungal isolate (Fig. 3B).

Microscopy and image analysis of biofilm

Microscopy and image analysis of bacterial and fungal biofilms were done using light microscope. The images analysis showed that in case of isolate TBT the trend was corresponding to the normalized values showing inhibition of biofilm in the presence of nanoparticles (250 µg/ml concentration). Biofilm development on coupons showed dense cells deposition in non-treated control treatments by isolates TBA and TBK (Fig. 4). Inhibitory effect on TBO isolate in micrograph was not evident as there was no decrease in biofilm was observed even at highest concentration Biofilm images by TBL and TBR showed inconsistent inhibitory effects. In case of TBR no particular inhibition was observed and the results were in line with the normalized values. In case of fungal isolates, biofilm formation was reduced to a great extent in TFK as compared to other two isolates where dense fungal mass

was observed in the form of biofilm. These results trends were also in line with the normalized values.

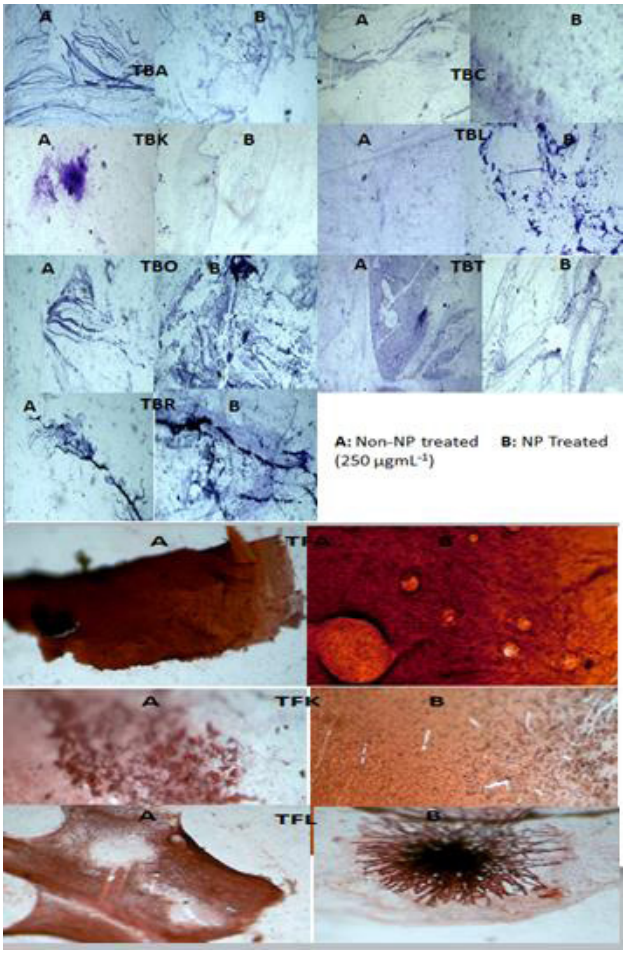

Fig. 4. Microscopic analysis of biofilm of bacterial (TBA, TBK, TBO, TBR, TBT, TBC, TBL) and fungal isolates (TFA, TFK, TFL). A, Non-NP treated; B, NPs treated (250 mgmL^{-1}).

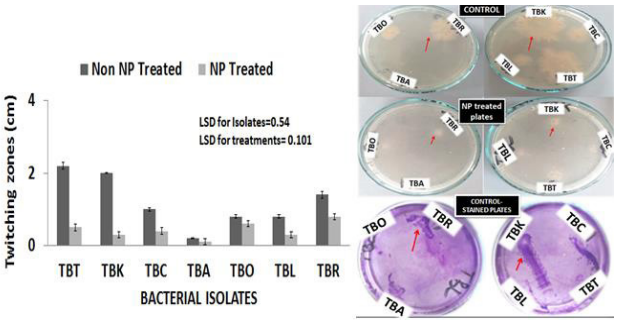

Fig. 5. Bacterial twitching assay in the presence of iron nanoparticles.

Twitching assay

Twitching assay was performed to confirm the inhibition of iron nanoparticles on the bacterial biofilm. The diameters (Fig. 5) were observed to be significantly lower (p=0.05) in the presence of iron nano-particles as compared to non-treated samples. Iron nanoparticles as compared to the control, thus indicating the positive results for biofilm inhibition. In general, twitching motility was highest in non NP treated plates as compared to NP treated plates. Plate's analysis of twitching hazy zones showed significantly stained plates especially by isolate TBR and TBK.

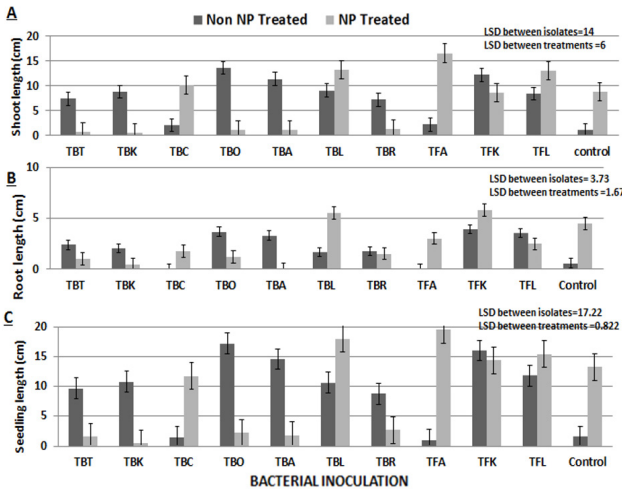

Fig. 6. Effect of inoculation and nanoparticles on length parameters of plant.

Effect of iron nanoparticles and bacterial and fungal isolates on Triticum aestivum *(wheat) growth*

Effect of nanoparticles on the seeds growth in the presence of bacterial and fungal isolates was recorded. In general, the length parameter were significantly higher in case of plants inoculated with TBL TFA and TBC (Fig. 6). In case of fungal inoculation protein contents were higher in NP treted plants while inoculation with TBT, TBK TBR resulted in significant increase in protein content as compared to other isolates (Fig. 7). The results showed that in the presence of TBT inoculum the protein content was observed to be 65 % higher than the control i.e., with no inoculum. While in the presence of inoculum and nanoparticles treatment the protein content was 95 % low showing low stress on plants in presence of nanoparticles as compared to in presence of inoculum that showed greater growth of seedling. TBK and TBO also showed same trend as TBT in both the cases of inoculum and inoculum and nanoparticles plus inoculum (Fig. 6). Unlike the trend of seed germination, fresh weight were increased

in the presence of bacterial inoculations. In general, there was a significant effect of nanoparticles treatment on plant growth and resulted in significant increment in shoot length (cm), root length (cm), fresh weight and percentage germination of seedlings. Isolates TBT and TBK showed significant increment in Protein contents of plant in the presence of nanoparticles as compared to rest of bacterial inoculation and non-inoculated control treatments. Inoculation of fungal isolates (TFK TFL) resulted in significant increment in Protein contents of plant as compared to non-inoculated control plants. However, nanoparticles treatment to plants resulted in significant (p=0.05) reduction in protein contents as compared to respective control treatments.

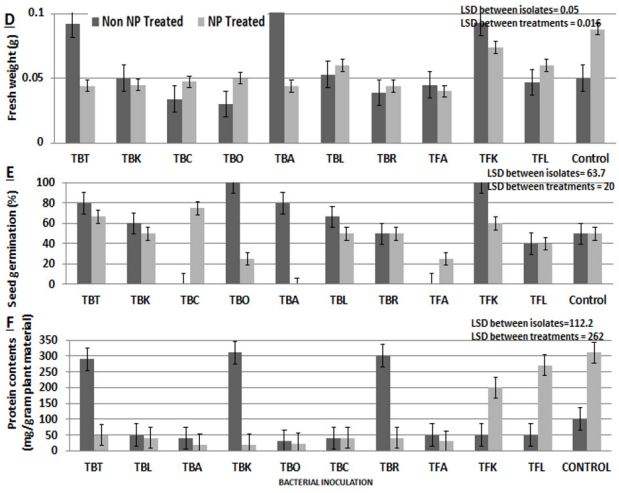

Fig. 7. Effect of inoculation and nanoparticles on seed germination, fresh weight and Protein contents of plant.

DISCUSSION

The increasing challenge to health care is of antimicrobial resistance, and the subsequent absence of access to effective antimicrobials. Antibiotic resistance is a problem of multisectors that threatens to erase decades of advancement in medicine, food security, and public health (Laxminarayan *et al*., 2016). This virulence and protection of microbes has been seen to be increased due to the biofilm formation. Infections associated with biofilms have also been reported in agricultural sector (Davey and O'toole, 2000; Prigent-Combaret et al., 2012; Li et al., 2015). Nano materials have great potential to fight against plant pathogens. Development of nanoparticles from plant extracts have been considered as an eco-friendly approach (Gottimukkala, 2017). Present study was performed to get ecofriendly iron nanoparticles using green tea plant extract. The potential of these nanoparticles to inhibit the biofilm formation of phyto pathogenic microbes was tested. Green synthesis of iron nanoparticles was done from green tea leaves extract and the net yield of the obtained nanoparticles was 25 %. Nanoparticles were black in color obtained in the form of pellet. Similar results were reported in the previous findings (Markova *et al*., 2014; Gottimukkala, 2017). The chemical characterization of obtained nanoparticles was done through FTIR analysis and size was determined by SEM. The characterization by FTIR analysis showed vibrations stretching at 1636.18 cm^{-1} for C=C and 3347.30 cm^{-1} for O-H. The C-H and C-O-C adsorption bands were also observed 2123.81 and 1074.15 cm^{-1}. The vibrations were approximately corresponding with the previously reported nanoparticles by Gottimukkala (2017) and indicates the presence of polyphenols groups that functions as reducing agent, characteristics of green tea leaves extract. Other studies such as Wei *et al*. (2016) also showed that there are various functional groups found attached to the nanoparticles, that corresponds to range of stretching for the C=C, C-H and C-O-C functional groups. This showed the significance of different reducing compounds present in the plant extract that are usually polyphenols. Hence, we can conclude that polyphenols present in the leaf extract were responsible for reduction and stabilization of iron nanoparticles. The scanning electron microscopy of synthesized nanoparticles showed spherical nano particles with mean diameter of about 79 nm. In previous reports of Smuleac *et al*. (2011) and Wang *et al*. (2011), size range of these iron nanoparticles has been reported to be 20- 80 nm. Other studies by Madhavi *et al*. (2013) and Wei *et al*. (2017) also supported the size of nanoparticles in the same range as reported to be 50-80 nm. Antibiofilm activity of iron nanoparticles were also observed on the isolated pathogens as the biofilms are the main mode of protection and virulence for the microbes. Nanoparticles tend to show positive antibiofilm effect on bacterial isolates and in line with the results of the previous studies on *Staphylococcus aureus* (Ganesh and Namasivayam, 2012; Sathyanarayanan *et al*., 2013) where positive antibiofilm effect of iron nanoparticles was reported. Other studies also indicated inhibitory effect of nanoparticles on biofilm of several bacilli thus proving antimicrobial property of iron nanoparticles (Chifiriuc *et al*., 2012; Prodan *et al*., 2013). Antibiofilm effect on fungal isolates was observed. Many previous studies have also indicated the antifungal effect of iron nanoparticles on fungal pathogens (Anghel *et al*., 2012; Grumezescu *et al*., 2012), while in case of isolates obtained, inhibition was seen at 250 µg/ml and above concentrations of nanoparticles observed in one fungal isolate. Light microscopy and image analysis of bacterial and fungal biofilms, showed more dense cells deposits

in non-treated control treatments by isolates TBA and TBK while in case of isolate TBR no particular inhibition was observed. In case of fungal biofilm, formation was greatly reduced in TFK as compared to other two fungal isolates. Bacterial twitching assay further confirmed the inhibitory effects of iron nanoparticles at bacterial biofilm. The twitching zones diameters were significantly lower (p=0.05) in the presence of iron nano-particles as compared to non-treated samples. In general, bacterial twitching was more visible in non NP treated plates while in NP treated plates, zones size were greatly reduced. Twitching is a motility behavior of bacteria to move over surfaces using hair-like type IV pili (Merz and Sheetz, 2000; Skerker and Berg, 2001). This feature of motility is more reported in pathogenic microbes like *Pseudomonas aeruginosa, Neisseria gonorrhoeae, Myxococcus xanthus* and several other species (Henrichsen, 1972). Musk *et al*. (2005) reported that presence of low concentrations of iron has been reported to favour twitching motility that in turn reduces the trend of the sessile communities development. However, when iron concentrations 1-100 µM are provided to *P. aeruginosa*, cell switch to sessile stage and form biofilm. Gloag *et al*. (2013) reported that after biofilm formation, twitching motility is facilitated with exopolysaccharides and its components. These results are in line with findings of biofilm that was reduced in the presence of nanoparticles but on the other hand the planktonic cells remained high. Reduced twitching motility in NP supplemented plates showed increased tendency of biofilm formation.

The effect of pathogenic isolates and nanoparticles were tested in influencing plant growth. In general, nanoparticles treated seeds showed significant increment in shoot length (cm), root length (cm), fresh weight and percentage germination of seedlings. Isolates TBT and TBK showed significant increment in protein contents of plant in the presence of nanoparticles as compared to rest of bacterial inoculation and non-inoculated control treatments. Inoculation of fungal isolates (TFK TFL) resulted in significant increment in Protein contents of plant as compared to non-inoculated control plants. However, nanoparticles treatment to plants resulted in significant (p=0.05) reduction in protein contents as compared to respective control treatments. These interactions showed that nanoparticle treatment showed increase in plant growth as compared to control with no treatment. In some cases (TBC, TBL, TFA and TFL) plant growth was increased in the presence of inoculum with nanoparticle treatment. The increased plant growth in the presence of nanoparticles as compared to pathogen inoculated plants might show the damage to cell membranes and enzymatic machinery of fungi as reported by for copper oxide nanoparticles (Ren *et al*., 2009). Significant increase in protein contents under inoculation shows the pathogenic stress offered to plants that results in increment in protein contents. Pathogenesis-related proteins and antimicrobial peptides (AMPs) have been reported to play a significant role in different types of biotic and biotic stresses (Ali *et al*., 2018). It could also be partly attributed to the magnetic iron nanoparticles that can increase nutrient availability and decomposition of soil organic matter (Burke *et al*., 2015; Joseph *et al*., 2015). A previous study done on the effect of iron nanoparticle concentration on the wheat seedling also indicated that smaller amounts of iron nanoparticles encouraged the seed germination of wheat (Feizi *et al*., 2013). As an agricultural country, Pakistan is facing great problems related to the plant diseases, which is mainly due to the resistance of phytopathogens occurring due to their adaptation to the polluted environment and abuse of antimicrobials, thus requiring alternative pollution free approach to cope these situations. The research conducted thus indicated the efficacy of antimicrobial and antibiofilm effect of iron nanoparticles on the phyto pathogens of significant value and their effect on plants. For this purpose a green approach of nanoparticles synthesis was used, which is not only ecofriendly, but is also beneficial in large scale production and cost effectiveness. The antibiofilm profile of nanoparticles and their interaction with plants and phytopathogens have indicated that they can be used as effective means in the agricultural field. Thus, present findings will be helpful for the exploitation of iron particles in this field of agriculture showing a promising approach in nanotechnology and plant sciences.

Despite of tremendous scope of nanotechnology in plant disease management, there are certain demerits and risks as reported by Khan and Rizvi (2014). They include deposition on aerial parts of plants that may result in physical damage to plants in the form of plugging of stomata and damage to vascular tissue resulting in impaired translocation. Moreover, inhalation by animals and humans may cause genotoxicity as well as disturbed human physiology in the use of nanoparticles in agriculture which are required to be worked out on priority and before the commercial uses of nanotechnology in agriculture.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Supplementary material

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The authors have declared no conflict of interest.

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