1. INTRODUCTION

The rhizosphere is one of the most diverse ecological niche with innumerable types of soil microorganisms due to rich nutrient availability from the host. This mutualistic relationship between the host and rhizosphere microbes makes it a highly competitive ecosystem in nature (Ahmad et al., 2008). Both the host plant and its concomitant microbiome gain an evolutionary benefit to survive under various adverse conditions by establishing tight interplays (Mapelli et al., 2013).

Plant diseases result in economic loss of billions of rupees, by reducing crop yield, lower production attribute and contaminating food grain with noxious chemicals in the form of pesticides. At present, management of fungal infections in agriculturally important plants has been majorly dependent on the use of chemical fungicides. However, synthetic chemical pesticides are expensive and have deleterious influence on the environment. Thus, allelopathic (synthesis of bioactive compounds known as “allelochemicals” which play a vital role as natural pesticides) root soil microorganisms, such as PGPR are incorporated into agriculture as an environmental friendly system of disease management (Saraf et al., 2014). The rich microbial flora in the rhizosphere arena provides a seemingly endless resource for this purpose. Based on the positive impact on the host plant, PGPR can be categorized as; biofertilizers, bioprotectants and biostimulants (Prashar et al., 2014). Most of the reported PGPR belongs to genera *Arthrobacter*, *Azoarcus*, *Bacillus*, *Azospirillum*, *Azotobacter*, *Paenibacillus*, *Glucanacetobacter*, *Actinomyces*, *Enterobacter*, *Clostridium*, *Serratia* and *Pseudomonas* (Hariprasad et al., 2014). Among the wide group, *Pseudomonas* and *Bacillus* are the most investigated genera and are currently in use as field application agents (Babu et al., 2015).

The foremost threat is the evolution of new pathogenic variants affecting crops, especially tomato, resulting in incessant loss (Babu et al., 2015). Several fluorescent pseudomonad strains are known to improve plant health and/or growth, are deemed as potential biocontrol agents against fungal diseases (Haas and Defago, 2005). The disease control efficiency of fluorescent pseudomonad strains depend on the number of traits, comprising substrate utilization, nitrogen dissimilation, production of siderophores, phenazine antibiotics and N-AHL. Hence, the overall
biocontrol activity is highly inconsistent in field trials because of various factors (Ghirardi et al., 2012).

*Fusarium oxysporum* is a major pathogen of tomato worldwide, which triggers inordinate crop loss in warm climates and sandy soils of temperate regions (Ignjatov et al., 2012; McGovern, 2015) causing the disease fusarium wilt which results in stunted seedlings/yellowing and defoliation of older leaves, further leading to wilt and death. Treatment of *F. oxysporum* infection in tomato plants is a tough task even with chemical fungicides. Currently chemical fungicides such as carbendazim, benomyl, thiram and thia bendazole are being used to manage the wilt fungus. But, they adversely affect other expedient soil microorganisms and also pollute the environment (Hariprasad et al., 2014). A realistic alternative, or supplement, to chemical fungicides is the use of soil-borne non-pathogenic bacteria that inhibit fungal phytopathogens (Saraf et al., 2014).

Rhizospheric microorganisms produce chemical signals that can affect plant growth and induce systemic resistance in host plant, through a signaling process called priming (Venturi and Keel, 2016). The phenomenon of conferring an augmented defensive competence to the host plant by PGPR priming is known as ISR, induced by an array of cellular, biochemical and gene expression variations. Fluorescent pseudomonads are well known agents of ISR activation through their diverse signaling mechanism (Haas and Défago, 2005; García-Gutiérrez et al., 2013; Pieterse et al., 2014; Pieterse et al., 2016). The present objective was designed to test the efficiency of shortlisted fluorescent pseudomonad isolates to induce resistance against *Fusarium* infection under green-house conditions. In addition, a secondary objective also was designed to evaluate the rhizosphere competence of the best performing fluorescent pseudomonads.
2. MATERIALS AND METHODS

2.1. Bacterial Inoculants and Test Pathogenic Strains for Green-house Study

All the eleven shortlisted fluorescent pseudomonad isolates (as described in the Chapter I) were considered as the test bacterial isolates, which were identified as *Pseudomonas* genus using specific primer as explained in Chapter II. In addition, two reference strains of PC1 and PC2 (*P. fluorescens* reference strains used in the Chapter I) were used as reference strains in the study. *F. oxysporum* MTCC1755 fungal strain was used as test pathogen in the study.

2.2. Seed Sterilization and Bacterial Treatments

Tomato seeds of variety Arka Vikas (Lot no.: 90) were used in the study. The tomato seeds were surface sterilized using sodium hypochlorite (4 % v/v) for 20 min followed by thorough washing in distilled water thrice. Tomato seeds were soaked in 72 h old revived fluorescent pseudomonad suspension (In LB broth with a bacterial cells concentration of ~ 1 × 10^8 CFU mL^{-1}) for 2 h followed by plating them in plastic seedling trays containing autoclaved soil and sand in the ratio 1:1. The trays with tomato seeds were kept under controlled conditions with regular watering and monitoring.

2.3. Fusarium Wilt Disease Severity after Seed Priming under Green-house Conditions

Twenty-day-old seedlings with uniform physical parameters were selected and transplanted into pots filled with soil as per the completely randomized design. Ten days after transplantation, the seedlings were again treated with the same pseudomonad isolates and watered regularly. Two commercial *Pseudomonas* strains (PC1 and PC2) were included in the study, along with two control sets (plants without any microbial priming called untreated control, represented as UTC and plants challenged with *F. oxysporum* MTCC1755 called challenged control, represented as CC). The first three compound leaves of the tomato plant were challenge inoculated by drop inoculation method, using a spore suspension of *F. oxysporum* (2×10^6 spores mL^{-1} in 0.85 % w/v NaCl). Throughout the study, the bacterial cell number and fungal spore concentration were maintained. Plants were observed for disease symptoms periodically following the fungal challenge.
2.4. Assay for Rhizosphere Competence under Green-house Condition

The best performing isolates were shortlisted for another set of experiment. Seeds were primed with fluorescent pseudomonads and disease incidence was assessed in seedlings grown in autoclaved and unautoclaved soils. The test soil was autoclaved thrice on three consecutive days at 121º C and 15-18 psi pressure and then transferred to the pots. All other green-house conditions were maintained similar to that of the previous experimental set. Visible wilt disease symptoms in host plants such as yellowing of leaves, spots surrounded by yellow hallo, necrotic lesions and shedding of leaves were considered for disease index scoring. In order to quantitatively measure incidence in host plants a 0-3 scale was set based on symptomatology (Bhattacharya et al., 1985). The disease incidence on test plants were calculated as per the formula

\[ DI = \frac{0(Hn) + 1(Sn) + 2(Hn) + 3(Dn)}{\text{Total number of plants examined}} \]

DI= Disease index, Hn = Healthy plants, Sn = Slightly infected plants, Hn = Heavily infected plants and Dn = Dead plants

Similarly, the percentage of disease reduction (DR) was calculated by the following formula

\[ DR = \frac{\text{Disease incidence of challenged control} - \text{Disease incidence of test}}{\text{Disease incidence of challenged control}} \times 100 \]

2.5. Molecular Identification of the Shortlisted Fluorescent Pseudomonads

The shortlisted pseudomonad isolates were further identified through 16S ribosomal gene amplification followed by sequencing. Nucleotide sequence similarity was performed using the advanced BLAST for inferring the sequence identity. Isolate 16S rRNA sequence data was submitted to the National Center for Biotechnology Information (NCBI) GenBank data base.

2.6. Statistical Analyses

All the experiments were carried out in triplicate except for the green-house studies, which were carried out in four sets as per complete randomized block design. Mean and standard error was calculated from the experimental data. All the data were
analysed using analysis of variance (Anova) to find out the significant difference among treatments and a $p$ value $\leq 0.5$ was considered significant. Pearson coefficient was calculated using Graph Pad Prism 6.1 software.
3. RESULTS AND DISCUSSION

In the present objective, eleven fluorescent pseudomonad isolates were carried to green-house conditions to study their capability to induce disease resistance mechanism against fusarium wilt. The disease incidence and percentage disease protection was calculated and the best isolates were shortlisted. This was followed by testing the rhizosphere competence of the shortlisted candidates under green-house conditions followed by molecular identification.

3.1. Test for Disease Severity under Green-house Conditions

Tomato seeds were primed with eleven fluorescent pseudomonads and were challenged with spores of Fusarium under green-house conditions and the disease incidence was recorded. Disease symptoms were appeared five days following Fusarium challenge in the control plants (CC). All the foliar lesions and physiological changes were systematically observed and recorded at regular intervals. Overall, it was observed that the level of disease suppression was pronounced in all the eleven pseudomonad primed plants compared to the challenged control plants. Nevertheless, the green-house results demonstrated that the seedlings from the seeds primed with isolates M80, M96 and T109 showed significant disease reduction after challenge inoculation with spores of F. oxysporum, disease severity was maximum in challenged control plants. Minimum disease incidence of 0.80 was recorded on M80 treatment, followed by 0.93 and 1.00 as observed on treatment with isolates M96 and T109 (Figure 25; 26). The percentage of disease reduction 71.42 ± 0.61, 66.20 ± 0.27 and 66.42 ± 2.14 was recorded on treatment with isolates M80, M96 and T109 respectively (Table 13). One way ANOVA analysis using Duncan multiple-range test showed that the reduction in disease incidence by M80, M96 and T109 were statistically significant when compared with challenged control plants.

It is almost impossible to predict the behavior of microbes in an environmental habitat which they belong to, as it is highly complex and dynamic. The outcome of such an interaction is multifaceted, which has been well studied by Cray et al. (2016). It is highly influenced by aspects such as physiological conditions, species, stress factors, biophysical and physicochemical parameters (temperature, pH, water activity and chaotropicity; motility of and/or distance between, competing microbes; and antimicrobials) etc. Despite possessing the well-established direct and indirect plant
probiotic traits, most of the time biocontrol agents fail or perform inconsistently under field conditions. In addition, the positive effect of pseudomonads on plant growth augmentation and defence depends on various factors including plant genotype, pathogen species and abiotic conditions (Hol et al., 2013; Kumar et al., 2013). Hence it is necessary to establish the functional dominance of selected biocontrol isolates under naturally competent environment.

3.2. Rhizosphere Competence of Shortlisted Fluorescent Pseudomonads against Indigenous Resident Microflora

To investigate the influence of native microflora over the selected strains, the experiments were repeated with the three shortlisted isolates (M80, M96 and T109) and were carried out under autoclaved and normal soil conditions. The disease incidence in the experiment and control plants was recorded after 20 days of challenge inoculation with spores of *Fusarium*. A reduced wilt disease incidence was observed throughout the seed priming with three pseudomonad isolates. Minimum disease incidence of 0.64 ± 0.15 was recorded as with M80-priming followed by 0.75 ± 0.02 and 0.87 ± 0.07 by priming with isolates M96 and T109 treatment respectively. Similarly, in autoclaved soil conditions, disease incidences were recorded as 0.64 ± 0.15 with M80-priming followed by 0.73 ± 0.08 and 0.73 ± 0.17 by M96 and T109 respectively, against 2.86 ± 0.08 in challenged control. Respective percentage disease reduction was calculated as 77.65 ± 5.09, 74.61 ± 5.47 and 72.87 ± 2.51 in autoclaved soil conditions, whereas in unautoclaved soil conditions, percentage disease reduction of 76.97 ± 5.18, 74.42 ± 0.75 and 68.80 ± 2.27 was observed on M80, M96 and T109 priming (Table 14). Comparative two-way Anova between autoclaved and unautoclaved treatment data showed a non-significant P value of 0.01 for disease incidence and 0.001 for percentage of disease reduction. The reduction in disease incidence on the priming with best three pseudomonad isolates significantly varied from 72-66% compared to the challenged control plants.

The results demonstrated that disease symptoms were almost similar in both autoclaved and unautoclaved soil conditions. Hence it can be concluded that the shortlisted pseudomonad isolates were efficient biocontrol agents against *Fusarium* in tomato plants under competitive rhizospheric environment. A minor non-significant rise in disease symptoms under autoclaved soil conditions must be contributed by the
natural soil beneficial bacteria. In addition, pH and abiotic factors reportedly have a considerable influence on the effectiveness of PGPR (Duca et al., 2014). Hence, number, diversity and action of rhizosphere microorganisms varies with the host plant (Vacheron et al., 2013).

A major hindrance while introducing new microorganisms into an existing niche is the type of interaction between the new and existing microflora. This reaction can be neutral, mutualistic or even antagonistic with the native microflora. In the present study the competitive behavior of fluorescent pseudomonad isolates with existing soil microflora was assessed. Recent developments in sequencing have enabled screenings of P. fluorescens genome for their defence-specific traits which could help in the selection of strains with the likely positive interactions on biocontrol (Hol et al., 2013). However, more often, studies concerning effects of introducing fluorescent pseudomonad isolates to an existing natural microflora are under-represented. In the present study, disease symptoms in host plants were almost similar in both autoclaved and unautoclaved soil conditions. Hence it can be concluded that the shortlisted pseudomonad isolates M80, M96 and T109 were efficient rhizosphere competitors in addition to its biocontrol activity against Fusarium. Minor non-significant rise in disease symptoms under autoclaved soil conditions might be contributed by natural soil beneficial bacteria. From the previous objectives it has been observed that all the three isolates were identified as potential candidates for biofilm formation, phenazine production and nitrate utilization, which visibly supports the green-house rhizosphere competence.

From the present study results, a correlation between disease incidence and percentage of disease reduction was calculated using Pearson coefficient for all eleven treatments. Two tailed linear regression of correlation analysis showed a significant $p$ value at R square value of 0.9999 and at 95 % confidence interval where $N = 90$ (Figure 27).

3.3. Molecular Identification of Fluorescent Pseudomonad Isolates

Genomic identification of shortlisted fluorescent pseudomonads was carried out by partial amplification of 16S rRNA followed by sequencing. Obtained 16S rRNA sequences were subjected to BLAST analysis to verify the identity the isolates (Table 15). Accession numbers were provided by the NCBI gene data bank on
submission of sequences. All the three isolates were identified as genus *Pseudomonas* and species *putida*. The sequence similarity assessment in BLAST analyses was recorded as 99% for M96, 98% for M80 and T109. M80 and M96 isolates were from maize rhizosphere and T109 from tomato rhizosphere.

Successful plant protection induced by the PGPR not only demands advanced knowledge of the complex regulation of disease repression by antagonists in response to biotic and abiotic factors, but also the dynamics and composition of plant-associated bacterial communities (Normander and Prosser, 2000). Here, interaction between *F. oxysporum* and its natural host tomato has become a model system for the molecular study of host specific disease resistance and susceptibility. The major criterion that makes tomato-*Fusarium* a model plant-pathogen interaction system is the virulence-resistance gene, which exactly mimics the classic gene-to-gene hypothesis for disease resistance (Takken and Rep, 2010). There are a number of factors which determines the native microbial community of a host plant such as complexity of microbial flora, natural selection, competence, succession and symbiotic relationships (Bever, *et al*., 2012).

In order to exhibit efficient biocontrol activity, fluorescent pseudomonads must have efficient colonization trait (colonization above $1 \times 10^5$ CFU gram$^{-1}$) and survive in competitive environment (Thomashow *et al*., 2007). Beneficial microbes are originally recognized as potential intruders on active host plant immune system (Pieterse *et al*., 2016). From the perspective of the host, there is always a distinction between innate and invading beneficial microflora. Therefore it is highly critical to study the competence of a new strain. Overall, the results conclude that the induction of disease resistance was not distributed equally among the treatments. Among the shortlisted eleven isolates, only three fluorescent pseudomonad isolates, M80, M96 and T109 exhibited excellent induction of resistance against fusarium wilt under green-house conditions. These three were excellent competitors under natural environment on induction of resistance. It is also important to point out that M80 and M96 were positive for phenazine antibiotic production. In addition, these three were also positive for protease, chitinase, amylase and ACC deaminase. While correlating the results of genomic diversity, it was evident that M80, M96 and T109 were placed in very close clusters in both RAPD and Rep based diversity cluster analyses as
explained in Chapter II. Presumably, these traits together might have contributed towards the better resistance and rhizosphere competency as observed in the green-house results. Moreover, in both the green-house experiments, priming with the isolates M80, M96 and T109 seed priming displayed consistent and efficient suppression of fusarium wilt in host plants, which is highly significant and promising for any biocontrol agent. Hence, these three isolates have been carried further for the biochemical and histochemical studies associated with the induction of resistance in the host plants.

The major disadvantage of following Latour’s strategy for identifying and shortlisting potential PGPR isolates is that the lower ranking isolates outperform the higher ranking ones, most of the time. Even in the present study, as observed here, the results were no different, where best performing isolates in the green-house conditions were ranked low while shortlisting (Table 3). This necessitates the need to look for an alternative strategy to find best competent biocontrol strains.