1. INTRODUCTION

As a result of the industrial revolution and modern field practices rationalised in the last century, there has been wide use of chemicals without discretion (Tonelli et al., 2011). These practices have eventually led to an adverse effect on soil fertility, environment and human health. This has redirected crop scientists to find an alternative and natural strategy of disease management against plant pathogens. One of the viable alternatives has been the use of naturally competent microorganisms in agriculture. Ultimately, biological control of phyto-pathogens has been established as a key practice in sustainable agriculture as it utilizes natural mechanism to antagonize pathogens (Beck et al., 2014; Choudhary et al., 2016). In the current scenario, sustainable and ecofriendly agriculture is a major concern of crop scientists to ensure quality and safe agricultural production.

*Fusarium* is a critical infective haustorium-forming fungal pathogen which results in crop loss in temperate regions (Ma, 2014; McGovern, 2015). Symptoms of fusarium wilt are characterized by stunted seedlings/yellowing (usually begin at one side of plant) and defoliation of older leaves, afterwards the infected plants frequently wilt leading to total plant collapse (Ignjatov et al., 2012; McGovern, 2015). Despite being an environmental threat, use of chemical insecticide is the most widely used management strategy (McGovern, 2015). However, studies have revealed that there are natural antagonistic microorganisms which can reduce fusarium wilt through biological control. The biological control strategy may work in two aspects which enable the host to manage fungal penetration i) through secretion of various mycolytic enzymes and antibiotic compounds (contributes to direct biocontrol mechanism), ii) through modifying plant’s immune system using a series of signaling pathways (contributes to indirect biocontrol mechanism). Interestingly, the plant rhizosphere associated microbiome was identified as a potential source of such microbes used as biocontrol agents.

Bioformulations are active biopesticides composed of a multiple active microbial agents such as bacteria, fungi, viruses, nematodes or extracts of plant or semiochemicals (Gasic and Tanovic, 2013). Due to the safety and reproducibility, microbe based formulations are the popular choice for the integrated pest management. *Bacillus thuringiensis* is a major biocontrol agent which has been used...
since long time in different forms. *B. thuringiensis* has been used in different forms such as dust, granules, liquid formulations, wettable powders, oil dispersions etc as per convenience of the users and the area of application (Knowles, 2005; Gasic and Tanovic, 2013). Although there are number of reports on different biocontrol strains, their performance varies based on a number of factors, such as field conditions, environment, pathogen, plant species etc (Walters et al., 2013). Most of the time the response varies under field conditions. Another great difficulty in using biocontrol agents is its commercial application by maintaining its viability. The development of bioformulations with an improved shelf-life and broad spectrum of action with consistent field performance is a critical step in the biocontrol technology application and commercialization.

The present objective was designed to develop a bio-formulation with fluorescent pseudomonad isolate M80, which was shortlisted and identified as a potential biocontrol agent against fusarium wilt, under green-house conditions. In addition, a secondary objective was designed to evaluate the response of the formulation in reduction of non-host specific disease incidence. *F. oxysporum* was selected as host specific pathogen and *Magnaporthe grisea* as non-host specific pathogen interaction system, in *Solanum lycopersicum*. 
2. MATERIALS AND METHODS

2.1. Candidate Pathogen and Formulation Agent

Potential fluorescent pseudomonad isolate M80, shortlisted from 144 rhizospheric fluorescent pseudomonads as explained in Chapter I, was the candidate isolate for bio-formulation. *F. oxysporum* MTCC1755 was used as the pathogen and *M. grisea* MTCC1477 was used as non-specific pathogen on tomato plants.

2.2. Preparation of Formulation

A glycerol stock of the fluorescent pseudomonad isolate M80 was revived in LB broth under shaking conditions (37 ± 2 °C at 120 rpm for 72 h). 400 mL of fresh M80 culture with a colony count of $5 \times 10^9$ CFU mL$^{-1}$ was mixed with 1 kg of autoclaved (thrice in three consecutive days at 121 °C and 18 PSI) industrial grade magnesium silicate, 15 g (w/v) calcium carbonate (Fischer Scientific) and 10 g (w/v) carboxy methyl cellulose (Fischer Scientific). The formulation was dried overnight under laminar airflow cabinet and the powder was sieved through mesh filter and packed in sterile bags (Ramamoorthy *et al*., 2002).

2.3. Seed Sterilization and Formulation Treatment

Tomato seeds (Variety: Arka Vikas, Lot no.: 90) were used for the study. The seeds were surface sterilized in 4 % (v/v) sodium hypochlorite for 20 min followed by thorough washing in distilled water thrice. 1 g of bio-formulation was used for 0.1 g tomato seeds (~ 40 seeds). The tomato seeds were soaked in formulation suspension for 2 h followed by plotting them in plastic trays containing autoclaved soil and sand in the ratio 1:1.

2.4. Testing of Formulation under Green-house Conditions

To evaluate the efficiency of the fluorescent pseudomonad M80 talc formulation, a green-house study was carried out. Twenty day-old seedlings with uniform physical parameters were selected and transplanted into pots (30 cm diameter) and maintained under standard green-house conditions (temp. 27 ± 3 °C, 65 ± 5 % relative humidity), as per randomized block design (Ramamoorthy *et al*., 2002). After 40 days separate treatment sets were maintained for host specific and non-host specific response assays as explained in Table 17.
Chapter 6 | Formulation using the best isolate to induce resistance in tomato

T1 - Untreated Control (UTC): Tomato plants without any treatment or fungal challenge.

T2 - Seed-Priming (SP): Primed seeds were allowed to grow in plastic trays. On the 20th day plants with uniform physical parameters were carefully uprooted and transplanted into pots. After 40 days, tomato plants challenged with F. oxysporum / M. grisea spore suspension (~ 2 × 10⁶ spores mL⁻¹ in 0.85 % w/v NaCl).

T3 - Root-Priming (RP): Seeds were allowed to grow in trays without any bacterial treatment. On 20th day plants with uniform physical parameters were carefully uprooted, washed and dipped the roots in formulation suspension for 2 h, followed by planting them into pots. After 40 days, tomato plants challenged with F. oxysporum / M. grisea spore suspension [~ 2 × 10⁶ spores mL⁻¹ in 0.85 % (w/v) NaCl].

T4 - Leaf Priming (LP): 40-day old plants were transplanted and the compound leaves were sprayed with formulation suspension. After 40 days, the tomato plants were challenged with F. oxysporum / M. grisea spore suspension.

T5 - Challenged Control (CC): Tomato plants challenged with spore suspension of F. oxysporum / M. grisea.

T6 - Pesticide Control (C+P): Tomato plants challenged with the spore suspension of F. oxysporum / M. grisea followed by pesticide treatment (Carbendazim from SDS Remedies Crop Science Pvt Ltd, at 2 gL⁻¹ dose).

In case of seed-priming and root-priming studies, soil adjacent to root region was treated again with the formulation one day prior to challenge inoculation with the fungal pathogen. In the studies using leaf-priming, leaves were sprayed with the suspension of the formulation, one day before pathogen challenge.

For the studies on the host resistance, first three compound leaves of 40-day old plants were independently challenged with the spore suspension (~ 2 × 10⁶ spores mL⁻¹ in 0.85 % w/v NaCl) of F. oxysporum by drop inoculation method (Uma and Podile, 2015). The green-house conditions were maintained as optimized earlier. For non-specific response study, compound leaves of tomato were challenge inoculated with spore suspension of M. grisea (~ 2 × 10⁶ spores mL⁻¹ in 0.85 % [w/v] NaCl). All the control and test plants were maintained under similar green-house conditions. The pots with respective treatments were arranged in a randomized manner, monitored and
watered at regular intervals. All green-house experiments were performed in three replicates and repeated once.

### 2.5. Assessment of Disease Symptoms Under Green-house Condition

Treatment effects of bioformulation on fusarium wilt in tomato plants were tested by assessing the disease symptoms on the aerial parts. On 20th day post challenge inoculation all plants were checked for the wilt disease symptoms on aerial plant parts. Visible symptoms such as yellowing of leaves, spots surrounded by yellow halo, necrotic lesions that ensue and shedding of leaves in host tomato plants were observed. The disease incidence in host plants was recorded using a 0 – 3 scale based on the severity of disease symptoms of (Bhattacharya et al., 1985) and disease incidence of each set of plants were separately calculated.

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

Hn = Healthy plants; Sn = Slightly infected plants; Hn = Heavily infected plants and Dn = Dead plants.

Similarly, percentage of disease reduction was calculated by the following formula:

\[
\text{Disease incidence in challenged control} - \text{Disease incidence in test} \times 100
\]

### 2.3. Testing for Bacterial Viability in the M80 Formulation

The prepared talc formulation was stored in the sealed air tight polyethylene bags at room temperature (24 ± 2 °C), away from direct sunlight and heat. Viability of the isolate M80 in the formulation was checked every 30 days up to 6 months, by counting the number of revived colonies (Narasimhan and Shivakumar, 2015). 0.1 g of formulation was collected from the sealed formulation bags after every 30 days, dissolved in 0.9 % (w/v) sterile saline, followed by serial dilution and inoculation on nutrient agar plates. The number of colonies were counted and expressed as:

\[
\text{CFU mL}^{-1} = \frac{\text{Total dilution factor}}{\text{Total number of colonies mL}^{-1} \text{ plated}}
\]
2.6. Data Compilation and Statistical Analyses

All the green-house studies were conducted in triplicates and the experiment was repeated once. All disease symptoms were calculated according to the formula given and results were represented as mean ± standard error. Formulation viability assay was carried out in triplicates, each sample was collected from three separate sealed bags and stored under similar conditions. Statistical significance between the treatments were determined by Tukey’s honestly significant difference (HSD) using Graph Pad Prism 6.01 software at a significant P value 0.05.
3. RESULTS AND DISCUSSION

The foremost threat in agriculture is the evolution of new pathogenic variants, especially in the case of tomato, which can instigate incessant loss in the future (Babu et al., 2015). This necessitates the development of novel disease management strategies to combat the new pathogenic strains. Identifying a potential probiotic biocontrol strain and its efficient exploitation is a key measure to this. Therefore in the present objective, an attempt has been made to develop a bioformulation using fluorescent pseudomonad M80 isolate and to check its efficiency to induce resistance against *F. oxysporum* in tomato under green-house conditions.

3.1. Influence of Bio-formulation on Disease Incidence under Green-house Conditions

Regardless of species specificity and the environment, plants are engaged in continuous encounter with the pathogens. There are natural microbial agents in root and soil, which can act as probiotics for host plants. This microbial interaction in a host-pathogen system is highly relevant as it can contribute largely to sustainable agriculture (Hibbing et al., 2010; Ofek-Lalzar et al., 2014). To ascertain the level of the disease incidence on tomato using formulation priming, symptoms were recorded after 20 days of pathogen challenge. Seed-priming resulted in reduced disease incidence (0.84 ± 0.08), whereas respective root-priming and leaf-priming resulted in a relatively high disease incidence (1.24 ± 0.16 and 2.33 ± 0.18 respectively), which was statistically significant (Figure 36). However, seed-priming and root-priming showed significant reduction in disease incidence compared to leaf-priming, with significant statistical variation. All three types of priming (seed, root and leaf) resulted in significantly reduced disease incidence on challenge inoculation with fusarium spores, compared to the CC, under green-house conditions. Nevertheless, the observed difference in disease incidence in seed-primed and root-primed plants, were not statistically significant contrasted to the pesticide control plants.

Similarly, the calculated percentage disease reduction showed that seed-priming with M80 formulation resulted in the highest disease reduction (69.63 ± 2.83 %) compared to challenged control. This high disease reduction was followed by root (55.17 ± 5.82 %) and leaf-priming (15.98 ± 6.22 %). Although all three formulation showed significant disease reduction compared to challenged control plants (CC),
seed-priming enabled better disease protection as observed in the green-house conditions (Table 18; Figure 37).

Green-house is a relatively closed system which offers a greater control over plant growth environment. This enables the researcher to restrict the environmental factors over a period of time and reduces the variations within the experimental system. The present observations showed that the M80 talc formulation was able to reduce the disease symptoms significantly in tomato plants under green-house conditions. Previous experiments have shown that the isolate M80 isolate induces systemic resistance in tomato through activation of pathogenesis-related proteins and lipoxygenase pathway as explained in the Chapter V. This signaling must be taking place through synthesis of various secondary metabolites, where the isolate M80 initiated protease, cellulase, amylase and ACC deaminase production. In addition, the isolate M80 was positive for phenazine antibiotics and AHL production. This may enable the bacteria to combat host pathogens in a highly competent manner. Furthermore, this can be considered as an additional factor to biologically manage the local fungicidal action, in addition to the systemic signaling towards induced resistance.

Non-host resistance is the most powerful and prevalent form of plant immunity which utilizes constitutive and inducible form of defence strategy. Non-host resistance enables a plant to counter the pathogen attacks which are infectious to other plant genera (Reimer-Michalski and Conrath, 2016). Host specific-resistance in plants is governed by the R gene, where a non-host resistance is mediated by multiple genes (Senthil-Kumar and Mysore, 2013).

In the present approach, the biochemical study of the ISR mechanism by the isolate M80 has been validated by the green-house studies using talc-based formulation. Ardakani et al. (2010) stated the use of P. fluorescens as bio-formulations against damping-off of cotton seedlings caused by verticillium. The highest disease control was shown by two additive combinations (bentonite - carboxy methyl cellulose and peat powder - carboxy methyl cellulose). This study compared the efficiency of formulations against fungicide carboxin-thiram which resulted in higher disease incidence, compared to bio-formulations under green-house conditions. Liquid bio-formulations based on fluorescent pseudomonads is reported to work
efficiently under different field conditions. Manikandan and Raguchander (2014) tested liquid *Pseudomonas* formulation by the addition of trehalose, polyvinylpyrrolidone and glycerol, in which formulation displayed higher shelf life. Various seed treatments increased fruit yield and simultaneously reduced disease incidence even under field conditions. Senthilraja *et al.* (2010) used talc-based formulation using *Beauveria bassiana* and *P. fluorescens* supplemented with chitin against insect pest (*Aproaerema modicella*) and blight pathogen (*Sclerotium rolfsii*) in groundnut plants. The results showed that seed, soil and foliar application of formulation efficiently reduced disease symptoms along with significant increase in growth and yield.

Interaction between a pathogen and a non-host plant is an incompatible interaction which can induce various defence-specific signaling cascades. This includes generation of ROS and hypersensitive reaction, resulting in induction and expression of PR genes. Based on the appearance of symptoms on host and non-host, resistance can be classified into type- I and II. The type-I does not generate any visible necrosis and the type-II results in localized necrosis in the host tissue, irrespective of the type of pathogen (Mysore and Ryu, 2004). Non-host resistance based studies in *Arabidopsis* against *Blumeria graminis* f. sp. *hordei*, a known barley pathogen, rapidly developed cell wall appositions and antimicrobial molecules at the site of pathogen entry. But there was a complete absence of hypersensitive reaction in the host (Jones and Dangl, 2006). The green-house observations showed that the M80 formulation enabled the plants to inhibit both host-specific and non-host pathogen infection efficiently.

High rhizosphere competence, saprophytic ability, plant growth promotion, broad range of action, environmental safety and survival against heat, UV and oxidizing agents are the major qualities of effective biocontrol agents making it an ideal formulation candidate (Nakkeeran *et al.*, 2005). A major hurdle in bio-formulation development is the efficient storage for longer durations. Most of the bio-formulation in the market uses a suitable carrier material to combat this issue. Air-drying or lyophilization are the other alternative techniques used during development of a formulation. Basically in all approaches, the water content in the product will enable the biocontrol agent to survive long against shelf life period.
3.2. Shelf-Life of Talc-Based Fluorescent Pseudomonad Formulation

The shelf-life of the fluorescent pseudomonad isolate in the talc-based M80-formulation was tested under conditions as stated above. Highest viability of the fluorescent pseudomonad in talc formulation was observed after every 30 days of storage ($5.05 \pm 0.09 \times 10^9$ CFU mL$^{-1}$) under room temperature ($24 \pm 2$ °C). After 60 days of storage a minor decline in viability was observed i.e., $4.78 \pm 0.12 \times 10^9$ CFU mL$^{-1}$, which however was not statistically significant. After 180 days of storage the viability of the isolate in formulation displayed a drastic decline ($3.68 \pm 0.16 \times 10^9$ CFU mL$^{-1}$) with a clear statistical significance (Figure 38). The present results displayed that the viability of formulation is stable up to five months at room temperature ($24 \pm 2$ °C), which is a relatively a good trait for commercial application. This can further be extended using various additives and the changing storage conditions.

Indeed, bacterial endurance in a field condition is affected by a number of factors. A key challenge in the bioformulation industry is to create an improved formulation with high shelf-life, viability, handy, easy revival against harsh environmental conditions and cost effective (Arora et al., 2010). The viability of the potential biocontrol strains in the formulation is always a critical parameter. However, the resulting protection provided by an additive to a biocontrol strain varies with the species and strains of micro-organism (Cabrefiga et al., 2014). Present study showed that the talc-based formulation was able to preserve the viability of M80 fluorescent pseudomonad up to 160 days. Furthermore, the viability can be checked with different formulations which may improve the shelf life. A study by Narasimhan and Shivakumar (2015) used the bioformulation (talc and lignite) of *Bacillus subtilis* in chilli against anthracnose caused by *Colletotrichum gloeosporioides* (Penz.). The green-house trial results showed that soil, seed, root-dip and foliar-spray treatment significantly enhanced growth parameters of chilli. The root dip application was effective in promoting growth whereas seed application was effective in anthracnose disease control. In a different approach, Cabrefiga et al. (2014) developed freeze dried formulation of fluorescent *Pseudomonas* along with lactose as lyoprotectant, which showed an increased viability up to 12 months.
Various studies (Nakkeeran et al., 2005; Arora et al., 2010) have shown that many carriers are being used to synthesise formulations which are organic/non-organic (peat, talc, lignite, pyrophyllite, kaolinite, zeolite, alginate, press-mud, sawdust, montmorillonite, vermiculite etc) through microencapsulation (using various polymers) etc. Talc (chemically magnesium silicate with formula $\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$) is a mineral composed of numerous minerals in combination with carbonate and chloride. Talc-based formulation using pseudomonad for growth stimulation of potato seed tubers was the first reported use of PGPR which resulted in significant growth promotion (Kloepper and Scroth, 1981). Since then several reports have been published on various types of formulations by using various microorganisms. However, the trend in recent studies is towards exploring consortium based formulations as they can withstand the environmental stress to a greater magnitude (Sarma et al., 2015).

Microbe-based biocontrol strategy cannot be implemented directly into field conditions. Many of them may not survive because soil is one of the most unpredictable eco-system. In addition, well known biocontrol genus such as *Pseudomonas* does not form spores naturally, as a survival mechanism under adverse conditions. Additionally, although some of the antagonistic PGPR strains reside in soil, their number is not sufficient enough to combat the pathogen stress (Arora et al., 2010). Hence, the development of bioformulation comes into play with greater significance sustainable agriculture. Present results distinctly showed that the fluorescent pseudomonad M80 formulation was vastly applicable in reducing fusarium wilt disease incidence in tomato under green-house conditions. This was supported with the results of non-host pathogen interaction under similar conditions.