CHAPTER 5

Development of formulation of promising PGPF in large scale and test its efficacy against sunflower downy mildew
Trichoderma has gained widespread attention as resistance inducing agent and is effective against a large number of plant diseases and capable of promoting growth of crop plants. The ability of Trichoderma spp. to systemically activate plant resistance mechanism against fungal pathogens has been demonstrated in several crops against various pathogens like Rhizoctonia solani, Botrytis cinerea, Alternaria spp., Colletotrichum spp., Magnaporthe grisea and Phytophthora spp. (Woo et al., 2006).

One of the major limitations to the application of biological control agents such as Trichoderma sp. is the development of appropriately formulated products (Fravel, 2005). In recent years, considerable success in control of plant disease has been achieved by the use of various fungal formulations namely, fine clay, peat, vermiculite, alginate, wheat bran, talc, diatomaceous earth and pasteurized soil (Boyetchko et al., 1998). We propose the use of talc as a solid substrate for T. harzianum proliferation and as a carrier based on the consideration that it is harmless to the environment, inexpensive and easily available, and can be easily exploited for commercial usage (Niranjana et al., 2009). Trichoderma formulations can be applied to the seed either by dry seed treatment or by seed biopriming for control of several soil-borne diseases of some crop plants. The major aspects of successful biological control technologies include the establishment of product, formulation and delivery system for microorganism that enable them for efficient disease control. The mass production systems should be compatible with industrial and commercial development methods and field applications (Sriram and Ray, 2005).

One of the popular methods of introducing biological control agents is seed treatment. Applying microorganisms to seed is an attractive proposition because of the combination of specific effect and limited environmental impact. In the familiar adage, seed treatment has the potential to deliver agents “in the right amount, at the right place and at the right time” (McQuilken et al., 1998). With increasing public awareness of the potential environmental and health hazards of both agrochemicals and fertilizers, and the advances in biotechnology to improve the performance of microbial products, application of microorganisms to seeds is likely to increase in future. In view of this, we have evaluated the efficiency of T. harzianum PGPFYCM-14 in different formulations to promote growth and induce disease resistance.
Materials and Methods

PGPF isolate

*Trichoderma harzianum* PGPFYCM-14 that exhibited the maximum protection against *Plasmopara halstedii* both under greenhouse and field conditions was selected for mass production and bioformulations to evaluate its efficacy to promote plant growth and induce resistance in sunflower against downy mildew disease in farmer’s field conditions.

Mass production of *T. harzianum* PGPFYCM-14 and preparation of talc based formulations

*Trichoderma harzianum* PGPFYCM-14 isolate cultured on PDA plates and incubated at 25±2°C under 12/12 h alternate cycles of NUV light and darkness for seven days. After seven days of incubation, 2-5 mycelial agar discs (10 mm diameter) from actively growing margins of each PDA plates were transferred to separate 250 ml Erlenmeyer flasks each containing 50 ml of PDA and incubated at 25±2°C for 10 days. After 10 days of incubation, 30 ml of SDW was added to each of the culture flask and the flasks were shaken at 70 rpm for 30 min in an orbital shaker. The content of each flask was filtered through sterile double layered muslin cloth. Filtrate containing the conidia was collected and spore load was measured separately using haemocytometer and adjusted to 1×10^8 spores ml⁻¹ and 2.1×10^8 spores ml⁻¹ by dilution with SDW. Conidial suspension of 100 ml containing 2.1×10^8 spores ml⁻¹ was added to sterile talc mixture (1:10 v/w) bringing the final concentration to 2.1×10^7 spores ml⁻¹, packed in polyethylene bags and stored under ambient conditions of 25±2°C before being used for formulation treatments (Sudisha *et al.*, 2006) in the present study.

Seed treatment with biotic inducer *T. harzianum* PGPFYCM-14

Seeds of sunflower cv. Morden, surface sterilized with 0.02% sodium hypochlorite by shaking for 3 min, followed by three wash with SDW were treated with conidial suspension and talc formulations *T. harzianum* PGPFYCM-14 separately. Seeds were treated by mixing 400 seeds with 50 ml conidial suspension (1×10^8 spores ml⁻¹) and incubated at 25±2°C in an incubator rotary shaker at 150 rpm for 6 h. Seeds were also treated by mixing 8 g and 10 g of talc formulations (2.1×10^7 spores g⁻¹) separately to per kg of seeds. Distilled water treated seeds served as
control. After incubation, the seeds were air dried and further tested for growth promotion and disease protection under farmer’s field conditions.

**Effect of *T. harzianum* PGPFYCM-14 bioformulations on growth and development of sunflower under farmer’s field conditions**

To evaluate the ability of *T. harzianum* PGPFYCM-14 in promoting vegetative and reproductive growth parameters, treated seeds as described above along with the control sets were sown separately in downy mildew disease free plot of a farmer’s field at Alur village, Chamarajanagar taluk and district, Karnataka, India, (11.92°N 96.95°E, 662 m altitude, red loam soil) in a randomized complete block design and grown as explained in Chapter 2. Plants were watered at regular intervals and no artificial fertilizers (NPK) were provided. The number of days taken for first flowering was recorded. Treatment effects on growth parameters viz., plant height, and crop duration and earhead diameter were measured at maturity. After harvesting the earhead, seeds were separated manually for each treatment and weight of the 1000 seeds was measured using electronic balance. The experiment was conducted with four replications of four rows in each replication, having 25 plants in each row and repeated thrice.

**Effect of *T. harzianum* PGPFYCM-14 bioformulations on downy mildew disease incidence under farmer’s field conditions**

Field experiments were conducted in a farmer’s field at Alur village, Chamarajanagar taluk and district, Karnataka, India, (11.92°N 96.95°E, 662 m altitude, red loam soil) in a randomized complete block design of 20×16 m plots in four replications consisting four rows each, with 50 plants in each row and repeated thrice. The experiments were conducted in three separate plots with a gap of one month duration between the experiments. Each row was with 15 m long and 75 cm apart (between rows) with 25 cm plant spacing. Both control and inducer treated 4-day-old sunflower seedlings were provided with primary inoculum (4x10⁴ zoospores ml⁻¹) during early hours for three consecutive days. Plants were raised with recommended agronomical practices. At the end of 30th day, disease incidence was recorded and per cent protection was calculated using the formula:

\[
\text{Per cent protection: } \frac{\text{Per cent downy mildew in control plants} - \text{Per cent downy mildew in treated plants}}{\text{Per cent downy mildew in control plants}} \times 100
\]
Statistical analysis

Each experimental data was subjected to analysis of variance (ANOVA) using SPSS Inc. 16.0. Significant effects of treatments were determined by the magnitude of the F value ($P \leq 0.05$). Treatment means were separated by Tukey’s HSD test.

Results

Effect of *T. harzianum* PGPFYCM-14 bioformulations on growth and development of sunflower under farmer’s field conditions

*Trichoderma harzianum* PGPFYCM-14 showed improvement in sunflower growth parameters and the degree of growth promotion varied between the concentrations (Table 5.1). Between treatments, a significant difference was observed where PGPFYCM-14 treated at $1 \times 10^8$ spores ml$^{-1}$ showed a maximum of 141 cm plant height, followed by 8 g kg$^{-1}$ seed and 10 g kg$^{-1}$ seed treatments which showed 137.95 cm and 134 cm, respectively. However, the increase in 1000 seed weight was highest in seeds treated with 10 g kg$^{-1}$ treatments which showed that the bioformulations enhanced the crop yield when compared to all other treatments including control.

Effect of *T. harzianum* PGPFYCM-14 bioformulations on downy mildew disease incidence under farmer’s field conditions

Under farmer’s field conditions, significant downy mildew disease protection was observed in the test rows raised from seeds treated with bioformulations of the *T. harzianum* PGPFYCM-14, when compared with untreated control rows (Fig. 5.1). The results showed that there was a significant reduction in the incidence of downy mildew disease in seedlings treated with conidial suspensions followed by talcum powder formulation of *T. harzianum* PGPFYCM-14. Downy mildew disease protection in conidial suspension treated seedlings was highest (71.26%) and it was 63.51 and 58.67% in seedlings treated with 10 g kg$^{-1}$ and 8 g kg$^{-1}$ respectively (Table 5.2). Contrarily, the untreated seeds recorded a maximum downy mildew disease incidence.
Table 5.1: Effect of seed treatment with *T. harzianum* PGPFYCM-14 on growth and development of sunflower under farmer’s field conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Plant height (cm)</th>
<th>First flowering (days)</th>
<th>Earhead (dia) (cm)</th>
<th>Maturity (days)</th>
<th>1000 seeds weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGPFYCM-14</td>
<td>1×10^8 spores ml(^{-1})</td>
<td>141.07±4.47(^a)</td>
<td>46.75±0.48(^b)</td>
<td>15.12±0.21(^a)</td>
<td>78.75±0.25(^c)</td>
<td>59.5±0.07(^b)</td>
</tr>
<tr>
<td></td>
<td>8 g kg(^{-1}) seed</td>
<td>137.95±3.80(^{ab})</td>
<td>47.25±0.25(^b)</td>
<td>14.52±0.21(^{ab})</td>
<td>80.50±0.29(^b)</td>
<td>59.3±0.02(^b)</td>
</tr>
<tr>
<td></td>
<td>10 g kg(^{-1}) seed</td>
<td>134.00±2.48(^b)</td>
<td>47.00±0.00(^{b})</td>
<td>15.32±0.20(^a)</td>
<td>79.00±0.48(^{c})</td>
<td>61.5±0.05(^a)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>102.20±2.94(^c)</td>
<td>53.25±0.25(^a)</td>
<td>13.77±0.31(^b)</td>
<td>86.00±0.41(^a)</td>
<td>51.4±0.04(^c)</td>
</tr>
</tbody>
</table>

Values are means of four independent replicates with ± SE
Means followed by the same superscript(s) within the same column are not significantly different according to Tukey’s HSD at P ≤ 0.05
Fig. 5.1: Effect of *T. harzianum* PGPFYCM-14 bioformulations treatment on sunflower downy mildew disease protection under farmer’s field conditions. T1- 10 g kg\(^{-1}\) seed; T2- 8 g kg\(^{-1}\) seed; C- Control; T3- 1×10\(^8\) spores ml\(^{-1}\)

Table 5.2: Effect of *T. harzianum* PGPFYCM-14 bioformulations treatment on downy mildew disease incidence under farmer’s field conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Disease Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGPFYCM-14</td>
<td>1×10(^8) spores ml(^{-1})</td>
<td>71.26±1.11(^a)</td>
</tr>
<tr>
<td></td>
<td>8 g kg(^{-1}) seed</td>
<td>58.67±0.53(^c)</td>
</tr>
<tr>
<td></td>
<td>10 g kg(^{-1}) seed</td>
<td>63.51±0.60(^b)</td>
</tr>
</tbody>
</table>

Values are means of four independent replicates with ± SE
Means followed by the same superscript(s) within the same column are not significantly different according to Tukey’s HSD at P \(\leq\) 0.05

Discussion

*Trichoderma harzianum* is a well know biocontrol agent which has been isolated from rhizosphere and employed against various plant pathogens (Harman *et al*., 2004; Chowdappa *et al*., 2013; Sudisha *et al*., 2013; Walters *et al*., 2013; Rubio *et al*., 2014). Various reports of application of *Trichoderma* spp. in agricultural practices as biocontrol agents, biofertilizers and soil amendments for the management of
phytopathogens and crop improvement in many crop plants have been demonstrated by many workers. Mani and Hepziba (2009) reported that seed treatment with *Trichoderma* spp. effectively controlled downy mildew of pearl millet and increased the seed quality parameters to greater extent. Haggag and Amin (2001) found *Trichoderma* effective in controlling fusarium root rot and nematode disease complex in sunflower. Perazzolli *et al.* (2008) reported that *T. harzianum* T39 (Trichodex) significantly reduced downy mildew symptoms and increased the resistance in the susceptible grapevine cultivar under greenhouse conditions. Cristina *et al.* (2007) reported that *T. harzianum* stimulates a biochemical systemic-induced response against leaf blotch caused by *Septoria tritici* on wheat plants. All these investigations support the present findings of growth promotion and resistance induction to sunflower downy mildew disease by PGPF: *T. harzianum* strains.

In recent years, *T. harzianum* has been mass multiplied on number of substrates and used for soil application as a source of biofertilizer and for disease management practices (Singh *et al.*, 2001; Mukherjee *et al.*, 2013). However, still there is a challenge for researchers to find substrates apart from sugar and simpler methodologies for mass multiplication of *T. harzianum*. In the current investigation, *T. harzianum* was mass multiplied and applied as seed treatment in the form of talc based formulations and conidial suspension to test the ability of *T. harzianum* PGPFYCM-14 to promote plant growth and induce resistance in sunflower against downy mildew disease in farmer’s field conditions.

In the present research, application of bioformulations of *T.harzianum* PGPFYCM-14, as a seed treatment, offered a significant (*P* ≤ 0.05) improvement in growth and development of treated plants compared to the untreated control. Similar findings were observed with *T. harzianum* seed treatment in wheat, which enhances the plant growth and grain yield compared to the uninoculated control (Shivanna *et al.*, 1996; Salehpour *et al.*, 2005). Observed improvement in seed germination %, seedling emergence, seedling stand, plants height, early flowering, head diameter and 1,000 seed weight were all due to the treatment effect of *T.harzianum* PGPFYCM-14, where root colonization of this bio fertilizer brought all these benefits to the host plants. Root colonization by *Trichoderma* offers a great deal of benefits to host plants, like plant growth promotion, root growth promotion, increasing drought tolerance,
increasing availability and uptake of nutrients, increasing plants yield (Harman, 2000), and increasing rate of germination of seed (Bjorkman et al., 1998).

The study has also revealed that, seed treatment with bioformulations enhanced significant ($P \leq 0.05$) protection against downy mildew disease of sunflower. A maximum of 71% downy mildew disease protection was noticed in seeds treated with conidial suspension of *T. harzianum* PGPFYCM-14, followed by talc based formulations. The results obtained are in line with Perazzolli et al. (2008), where *T. harzianum* T39 treated in the form of Trichodex significantly reduced downy mildew symptoms and increased the resistance in the susceptible grapevine cultivar under greenhouse conditions. Similar experiments were conducted by Prasad et al. (2002) using *T. harzianum* as a seed treatment and soil amendment to manage fusarium wilt of pigeon pea whereas, bioformulations of *Trichoderma* and *Talaromyces* were used against sugar beet damping-off disease (Kakvan et al., 2013). Likewise, Dubey et al. (2013) mass multiplied *T. harzianum* on agricultural waste bases successfully and used dry formulations as a seed treatment against chickpea wilt disease under field conditions. It can be noted that, *T. harzianum* PGPFYCM-14 significantly induced resistance against sunflower downy mildew disease under field conditions. Based on the experimental results obtained through the current investigations the bioformulations of *T. harzianum* PGPFYCM-14 can be extended to the farmer’s field as biofertilizer to promote growth and also as a biotic inducer to stimulate resistance in sunflower against the downy mildew pathogen in particular and for others in general.