SUMMARY

The study was carried out to investigate the effectiveness of *Glomus fasciculatum*, *Trichoderma viride* and *Pseudomonas fluorescens*, alone and in combinations to control disease spread in tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici*. The three biological control agents were effective in controlling the disease; however, the success rate for inhibition varied among the different treatments. Plants inoculated with *P. fluorescens* had higher concentration of phenol and greater activities of phenylalanine ammonia lyase and catalase. Inoculation with *T. viride* led to maximum induction of anti-oxidative enzymes such as catalase and peroxidase. On the other hand, *G. fasciculatum*-inoculated plants showed improved growth and highest phosphorus uptake. A combination of all the three biological control agents together, promoted growth and inhibited disease up to 94% in tomato plants. Thus, use of multiple biocontrol agents leads to enhanced level of disease resistance than individual use of bio-inoculants through the induction of multiple defense mechanisms.

Key words: arbuscular mycorrhiza, biocontrol, *Pseudomonas fluorescens*, tomato, *Trichoderma viride*, vascular wilt.

The causal agent of wilt, *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) is responsible for important crop losses in the field and commercial greenhouses. Management of Fusarium wilt is mainly through soil fumigation and wilt-resistant cultivars (Datnoff et al., 1995). However, the broad spectrum biocides used to fumigate soil before planting, methyl bromide in particular, are environmentally damaging, and the evolution of new pathogenic races that overcomes the defense of a resistant cultivar in a short period (Fravel et al., 2003) have shown the need for new methods of controlling the disease. Plant root diseases can be controlled by manipulation of microbes or by introducing them in soil (Vazquez et al., 2000; Larkin and Fravel, 1998), representing a natural, safe, effective and durable alternative to the use of pesticides.

Biocontrol agents (BCAs) that have been reported to reduce damage by Fusarium wilt of tomato include arbuscular mycorrhizal fungi (AMF), *Trichoderma*, *Gliocladium*, *Pseudomonas* and *Bacillus* spp. All of these microorganisms are important members of the rhizosphere and are effective in increasing plant growth and rendering plant tolerant to various stress factors (Akköprü and Demir, 2005). Although much research has been conducted to investigate the biocontrol of *Fol* in tomato afforded separately by AMF, *Trichoderma* and *Pseudomonas*, there have been very few attempts to study interactions among these biocontrol agents on plant growth and disease tolerance (Martinez-Medina et al., 2009). One possible means to develop an appropriate technology is to search for combinations of BCAs that complement and enhance each other’s contribution to disease resistance of the plant.

The purpose of this study was firstly to compare the individual potential of *Glomus fasciculatum* (*Gf*), *Trichoderma viride* (*Tv*) and *Pseudomonas fluorescence* (*Psd*) in controlling *Fol* in tomato and, secondly, the interactions among these BCAs in various combinations (dual and triple), evaluating their performance in enhancing the tolerance of plants to the pathogen.

Seeds of tomato cv. Pusa Ruby were obtained from the National Seed Cooperation, Indian Agricultural Research Institute (IARI), New Delhi. An aggressive isolate of *Fol* and pure cultures of *Tv* and *Psd* were obtained from the Division of Plant Pathology, IARI, New Delhi and maintained on potato dextrose agar (Himedia, Difco Laboratories, USA) and nutrient agar (Himedia, Difco Laboratories, USA) respectively. Conidial suspensions of *Fol* and *Tv* were prepared, and spore densities were determined by haemocytometer to be 2.8 x10^6 spores ml^{-1} and 3.0 x10^5 spores ml^{-1}, respectively. Bacterial spore suspension consisted of 2.1 x10^9 spores ml^{-1}. Soil based inoculum of *Gf* was multiplied using *Sorghum balepense* as trap plant (Kapoor et al., 2007). The inoculum density was 98 spores per 10 g soil.

The experiment used plants grown in pots at the
Botanical Garden, Department of Botany, University of Delhi, and was designed in a randomized block consisting of nine different treatments, each with three plants per pot in six replicates (total 18 plants per treatment). Tomato seeds were germinated on steam-sterilized soil (15 psi, 121°C, 20 min). One-month-old seedlings were transplanted to earthenware pots filled with a mixture of sand and soil (1:1, v/v), sterilized by fumigation (0.1% formaldehyde). A soil-based Gf inoculum (10 g per pot) including root fragments (88% colonized), was added at the time of transplanting just below the tomato seedlings. Conidial suspension of Tv and spores of Psd were also added to the rhizosphere soil during transplanting by injecting a conidial suspension at the rate of 10 ml per seedling. Finally, after Gf colonization, the seedlings were challenged with Fol by following the same procedure as for the bio-inoculants.

The experiment consisted of the following nine treatments: (i) Uninoculated plantlets serving as control (C); (ii) C+Fol; (iii) C+Fol+Tv; (iv) C+Fol+Gf; (v) C+Fol+Psd; (vi) C+Fol+Tv+Gf; (vii) C+Fol+Tv+Psd; (viii) C+Fol+Psd+Gf; and (ix) C+Fol+Tv+Gf+Psd. Plants were grown outdoors in pots and were watered regularly with no application of pesticides or fertilizers. After 51 days three plants with roots were harvested at random. Dry weights of shoots and roots were recorded after drying them in an oven at 70-80°C till their weight was constant.

Disease severity was monitored visually during the growth period following inoculation with Fol. The number of leaves showing symptoms of wilt and the total number of leaves were counted. The percent disease severity was calculated as:

\[
\text{Number of leaves with symptoms} \times 100 \\
\text{Total number of leaves}
\]

Gf colonization was assessed following Phillips and Hayman (1970). The phosphorus in the digested sample was estimated by molybdenum blue method (Allen, 1989) at 700 nm using UV-visible spectrophotometer.

Phenylalanine ammonia lyase (PAL) (EC 4.1.1.5) activity was assayed according to Dunn et al. (1998) with slight modification. Absorbance was read at 290 nm and the activity was expressed as nM trans-cinnamic acid g⁻¹ h⁻¹. Total phenols were quantified using Folin and Ciocalteu's phenol reagent, following Bray and Thorpe (1954).

To know whether lipid peroxidation, catalase and peroxidase were associated with Gf, Tv and Psd, three more treatments namely (x) C+Tv; (xi) C+Gf and (xii) C+Psd, were included in the study. The number of replicates and sampling of these three treatments were the same as mentioned before. Since the oxidative burst is believed to occur during initial pathogen infection, estimations of lipid peroxidation, catalase and peroxidase were performed after three and 51 days after pathogen inoculation. The level of lipid peroxidation in leaf tissue was determined in terms of malondialdehyde (MDA) concentration by thiobarbituric acid reaction as described by Heath and Packer (1968). The amount of MDA was calculated by using molar extinction coeffi-

Table 1. Effect of different combination treatments of G. fasciculatum, T. viride and P. fluorescens on dry weight of root and shoot and phosphorus concentration in shoot of tomato plants infected with F. oxysporum f. sp. lycopersici.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dry weight (g plant⁻¹)</th>
<th>Concentration of phosphorus (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Root</td>
<td>Shoot</td>
</tr>
<tr>
<td>Control</td>
<td>0.46 e</td>
<td>4.25 f</td>
</tr>
<tr>
<td>C+FOL</td>
<td>0.33 a</td>
<td>3.00 a</td>
</tr>
<tr>
<td>C+FOL+Gf</td>
<td>0.35 b</td>
<td>3.27 bc</td>
</tr>
<tr>
<td>C+FOL+TV</td>
<td>0.36 bc</td>
<td>3.30 c</td>
</tr>
<tr>
<td>C+FOL+Psd</td>
<td>0.37 c</td>
<td>3.14 ab</td>
</tr>
<tr>
<td>C+FOL+TV+Gf</td>
<td>0.36 bc</td>
<td>3.66 d</td>
</tr>
<tr>
<td>C+FOL+Psd+Gf</td>
<td>0.38 cd</td>
<td>3.85 e</td>
</tr>
<tr>
<td>C+FOL+TV+Psd</td>
<td>0.39 d</td>
<td>4.22 f</td>
</tr>
<tr>
<td>C+FOL+TV+Psd+Gf</td>
<td>0.53 f</td>
<td>5.40 g</td>
</tr>
</tbody>
</table>

Within a column, values are mean of three replicates. Values followed by the same letter are not significantly different at P ≤ 0.05.
The activities of guaiacol peroxidase (EC 1.11.1.7) and catalase (EC 1.11.1.6) were assessed according to Kato and Shimizu (1987). Reaction mixture for peroxidase activity consisted of 0.1 M phosphate buffer pH 6.0, 44 mM \( \text{H}_2\text{O}_2 \), 45 mM tetraguaiacol and enzyme extract. Peroxidase activity was calculated using the molar extinction coefficient of tetraguaiacol (2.6 mM cm\(^{-1}\)) and enzyme extract. Peroxidase activity was expressed in units g\(^{-1}\) min\(^{-1}\). For catalase activity, the reaction mixture consisted of 0.1 M phosphate buffer pH 7.0, 264 mM \( \text{H}_2\text{O}_2 \), and enzyme extract. The decrease in \( \text{H}_2\text{O}_2 \) concentration was calculated using the molar extinction coefficient of \( \text{H}_2\text{O}_2 \) (3.94 mM cm\(^{-1}\)) and enzyme extract. Catalase activity was expressed in units g\(^{-1}\) min\(^{-1}\). For \( \text{H}_2\text{O}_2 \) decomposition was calculated using the molar extinction coefficient of \( \text{H}_2\text{O}_2 \) (3.94 mM cm\(^{-1}\)) and enzyme extract. Catalase activity was expressed in units g\(^{-1}\) min\(^{-1}\).

One-way analysis of variance was carried out for each parameter studied. Tukey’s post hoc multiple mean comparison test was used to check for significant differences between treatments (at \( P \leq 0.05 \) level). All statistical analyses were performed with the Statistical Package for Social Sciences version 10 (SPSS Inc., USA).

Tomato plants exposed to \( \text{Fol} \) were 100% infected showing typical wilt symptoms, whereas controls remained healthy. Single, dual and triple combinations of BCAs (\( \text{Tv} \), \( \text{Gf} \) and \( \text{Psd} \)) employed against \( \text{Fol} \) proved effective in pathogen inhibition (Fig. 1). The success rate for controlling disease spread varied among the different treatments. Maximum inhibition of disease up to 94% was observed in plants receiving all the three bio-inoculants. When applied singly, \( \text{Psd} \) (76%) was most effective with regard to suppressing wilt disease followed by \( \text{Tv} \) (64%) and \( \text{Gf} \) (62%). Dual inoculations were found to be more effective than single treatments when plants were inoculated by combinations of C+Fol+Gf+Tv and C+Fol+Gf+Psd. However, inoculation of C+Fol+Tv+Psd (70%) was unable to afford a better disease control, as compared with \( \text{Psd} \) applied alone.

When applied alone, \( \text{Gf} \) colonized 56.6% of tomato roots and its colonization levels were not affected by co-inoculation with \( \text{Tv} \) and/or \( \text{Psd} \) (data not shown). In all treatments without mycorrhizal inoculation, the absence of the inoculant was confirmed. Vazquez et al., (2000) reported that microbial inoculants in the rhizosphere of tomato did not influence AM colonization; similarly, \( \text{Psd} \) was neither stimulatory nor inhibitory for \( \text{Gf} \) colonization. Correspondingly, \( \text{Gf} \) had a positive influence on saprophytes such as Trichoderma in its mycorhizosphere (Kapoor et al., 2000).

Increase in plant dry weight was detected in all treatments except C+Fol as compared to control plants (Table 1). The bio-inoculants synergistically interacted with each other to result in further increase in biomass on dual inoculation in comparison with single inoculations. Dual inoculations in all combinations were significant among each other and to all mono-inoculated plants. Plants receiving all the three bioinoculants showed a higher shoot and root dry weight than that of control plants.

A decrease in phosphorus concentration was observed in \( \text{Fol} \)-infected tomato plants as compared to the control. This may be due to root damage resulting from fungal infection as revealed from a decrease in root biomass. Single inoculation of BCAs resulted in better growth and phosphorus nutrition in \( \text{Fol} \)-infected and non-infected tomato plants over the control. The phosphorus (P) concentration was higher in all mycorrhizal treatments viz C+Fol+Gf, C+Fol+Gf+Tv, C+Fol+Gf+Psd compared to their corresponding non-mycorrhizal treatments viz. C+Fol, C+Fol+Tv, C+Fol+Psd, respectively (Table 1). This is due to AMF ability to increase P uptake by various mechanism (Srivastava et al., 1996) and compensate
Synergism among biocontrol agents

The increase in P uptake in *Psd*-inoculated plants over *Fol*-challenged plants could be due to a lesser damage suffered by infected roots. A possible mechanism of increased P uptake and growth in *Tv*-inoculated plants may be due to the stimulation of nutrient transfer (also observed in the present study) from soil to roots as *Trichoderma* can colonize the interior of the roots (Kleifeld and Chet, 1992). Dual inoculation of *Tv* and *Psd* exhibited a stimulatory effect in improving plant growth similar to that of control plants. The three bio-inoculants together were not only successful in completely controlling Fusarium wilt, but were also capable of improving the growth and P uptake (in tomato) compared to control plants.

Challenging tomato plants with *Fol*, induced PAL activity which, however, was significantly lower than in plants inoculated with BCAs. This suggests that the level of PAL activity (in *Fol*-infected tomato plants) was lower.

**Fig. 2.** Effect of different combination treatments of *G. fasciculatum*, *T. viride* and *P. fluorescens* on (a) catalase, (b) peroxidase and (c) lipid peroxidation in shoot of tomato plants infected with *F. oxysporum* f. sp. *lycopersici* after 3 days. Histograms with the different letters are significantly different at P ≤ 0.05.

**Fig. 3.** Effect of different combination treatments of *G. fasciculatum*, *T. viride* and *P. fluorescens* on (a) catalase, (b) peroxidase and (c) lipid peroxidation in shoot of tomato plants infected with *F. oxysporum* f. sp. *lycopersici* after 51 days. Histograms with the different letters are significantly different at P ≤ 0.05.
not high enough to control the pathogen spread. Among the mono-inoculations, induction of PAL activity was maximum in Psd-inoculated plants. PAL activity hit the highest in tri-inoculated (C+Fol+Tv+Psd+Gf) treatment followed by dual inoculations C+Fol+Psd+Gf, C+Fol+Tv+Psd and C+Fol+Tv+Gf, in the order (Table 2). Increased PAL activity induces accumulation of phenolic compounds via the phenylpropanoid pathway (Singh et al., 2003). A positive correlation was observed between PAL activity and accumulation of total phenols in the shoots of tomatoes. Of all treatments Psd-treated plants showed the highest PAL activity and, proportionally, the maximum total phenol concentration. The co-inoculation of Psd with Tv/Gf had an additive effect on phenol concentration with an increase as much as sixfold higher in tri-inoculated plants over C+Fol-treated plants (Table 2). Accumulation of secondary metabolites including phenols on inoculation with Psd, Tv and Gf have been reported separately by Kumar et al. (2007), Harman et al. (2004) and Kapoor et al. (2007).

The peroxidation of unsaturated lipids of biological membranes is the most prominent symptom of oxidative stress in plants. The production of lipid peroxides has been proven to be induced by necrotrophic pathogens (Mandal et al., 2008). In the present investigation, maximum MDA concentration was observed in tomato roots challenged with Fol (Fig. 2). Recent studies have shown that some pathogens (necrotrophs) may induce production of ROS to their own advantage resulting in hypersensitive cell death that facilitates fungal colonization (Mandal et al., 2008; Mayer et al., 2001). A similar pathogenicity mechanism was evident in case of Fol-infected tomato plants for a decrease of MDA concentration was observed, following all treatments. Psd inoculated either with Tv or Gf induced reduced lipid peroxidation as compared with co-inoculation of Tv and Gf. Least oxidative damage, comparable with that of control, was observed in plants exposed to all three inoculants.

Increase in ROS triggers a network of signalling cascade and up-regulate the activities of anti-oxidative scavenging enzymes such as catalase, peroxidase, superoxide dismutase and ascorbate peroxidase (Able, 2003; Rolke et al., 2004). Inoculation of Tv, Gf and Psd in absence of Fol resulted in significant higher catalase and peroxidase enzyme activities compared to C+Fol treatment (Fig. 2), suggesting induction of anti-oxidative enzymes in response to the bio-inoculants. The induction of ROS-sca

venging enzymes in AMF-inoculated plants may be an efficient mechanism to attenuate plant defense responses, allowing AM to colonize the root cortical tissue (Kumar et al., 2009). The increase activity of anti-oxidative enzymes in the above mentioned treatments (except control) was inversely proportional to MDA concentration. The effective concentration of free radicals in plant tissues is a result of a dynamic equilibrium between the rates of their production and scavenging. The increase in catalase activity might be involved in the reduction of lipid peroxides thus protecting membranes of tomato plants from oxidative burst resulting in decrease in MDA concentration (Mandal et al., 2008).

Significant and progressive stimulation in the activities of both enzymes was observed with increase in number of BCAs used. Among mono-inoculations, the activity of catalase was significantly higher in Tv- and Psd-treated plants than in Gf-colonized plants. Correspondingly, plants with Tv and Psd treatment showed maximum activities of peroxidase and catalase among dual treatments. The oxidative burst (observed in C+Fol) served as a weapon for the necrotrophic pathogen (Fol). Induction of anti-oxidative enzymes by BCAs used in our study – primarily to facilitate their own colonization of tomato roots – served as an early line of defense obstructing Fol invasion and disease progression. Sustained high activities of antioxidative enzymes and low MDA concentration in tomato plants treated with BCAs prevented spread of Fol even after several weeks from inoculation (Fig. 3).

All dual or triple combinations of BCAs (except C+Fol+Tv+Psd) inhibited the pathogen more efficiently than single applications. Besides, co-inoculation of microbial inoculants also improved growth, P uptake and enhanced tolerance of plants to the pathogen. This was expressed by reduced lipid peroxidation, higher concentration of phenol and peroxidase, catalase and PAL activities. These results indicate functional synergism among BCAs employed in the present study. Tomato plants dual inoculated with C+Fol+Tv+Psd showed decreased lipid peroxidation and increased peroxidase and catalase activities compared to Tv or Psd used alone. Phenol concentration also showed additive effect of the two bio-inoculants. However, the above changes, as expected, did not result in decreased disease severity.

The use of combinations of multiple antagonist organisms provide improved disease control over the use of single organism. The results obtained in this study constitutes an important foundation towards understanding the biochemical basis of synergism among the three BCAs used for resistance of tomato plants to Fol. Multiple organisms may enhance the level and consistency of control by providing multiple mechanisms of action (Larkin and Fravel, 1998). Psd, Tv and Gf have different mechanisms to reduce plant diseases such as accumulation of phenolic compounds, increasing PAL activity (observed in the present study), pathogenesis-related proteins and lysis of the fungal pathogen cell wall by secretion of extra-cellular lytic enzymes (Saikia et al., 2004). While P nutrition was higher in mycorrhizal plants, Psd-inoculated plants showed higher phenol concentration, PAL activity, catalase activity, while Tv-inoculation resulted in induction of anti-oxidative enzymes such as catalase and peroxidase. These effects
were additive when all bio-inoculants were used together suggesting functional compatibility among them.

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