

***In vitro* evaluation of some bioagents against tobacco wilt pathogen**

K. Sumana and N. S. Devaki

ABSTRACT

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *nicotianae* (Johnson) W. C. Snyder and H. N. Hansen. is the most destructive disease of tobacco. The present study was carried out *in vitro* conditions to assess the possible use of biocontrol agents in field conditions. *Trichoderma viride*, *Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens* were evaluated for their antagonistic activity against *F. oxysporum* f. sp. *nicotianae* under *in vitro* conditions. Different biocontrol agents showed varying degrees of antagonism. *T. viride* and *T. harzianum* showed 70 and 40% inhibition of radial growth of the *F. oxysporum* f. sp. *nicotianae* respectively in dual culture technique. Mode of parasitism between the biocontrol agents and *F. oxysporum* f. sp. *nicotianae* were studied under the microscope. Formation of several loops and coiling of hyphae by *T. viride* and *T. harzianum* around the hyphae of the pathogen forming a thick compact rope like structure was observed. The biocontrol agents produced volatile metabolites. These metabolites suppressed the growth of *F. oxysporum* f. sp. *nicotianae*. The two biocontrol agents varied in the extent of volatile metabolite production. Among bacteria, *B. subtilis* inhibited the radial growth of *F. oxysporum* f. sp. *nicotianae* by 36%. The bacterial and fungal antagonists were not mutually antagonistic as the co-inoculation did not inhibit each other. The present work is the first report on the use of *T. viride*, *T. harzianum* and *B. subtilis* against *F. oxysporum* f. sp. *nicotianae* as potential biocontrol agents.

Key words: *Bacillus subtilis*, biocontrol, *Fusarium oxysporum*, *Trichoderma* spp.

INTRODUCTION

Fusarium oxysporum f. sp. *nicotianae* (Johnson) W. C. Snyder and H. N. Hansen. is a destructive pathogen of both green house and field grown tobacco plants in the warm areas of the world (Lucas, 1975; Gopalachari, 1984; Moore *et al.*, 1999). Fungicidal application as seed or soil treatment, however, has been found to be ineffective against these pathogens as the propagules are enormously distributed in the soil and often beyond the reach of chemicals (Campbell, 1989). Biological control, therefore, holds promise as a strategy for disease management and it is environment friendly too. Antagonistic fungi especially *Trichoderma* species and the bacteria *B. subtilis* and fluorescent Pseudomonads have been widely used against a number of phytopathogens (Bell *et al.*, 1982; Rini and Sulochena, 2006; Sallam *et al.*, 2008; Morsy *et al.*, 2009; Christopher *et al.*, 2010; Pandya and Sabalpara, 2011). Mode of parasitism depends

upon the different enzymatic potential and biochemical activity of the antagonist (Papavizas, 1985; Devaki *et al.*, 1990). Mycoparasitism is the most important antagonistic association where the pathogen is killed by the lysis of the fungal mycelia, perforation and digestion of fungal hyphae. The parasitic infection is inhibited by hyphal contact or coiling around the fungal cell or by direct invasion of hyphae around fungal cells (Barnet and Binder, 1993). Antibiosis through volatile and non-volatile toxic metabolites is the next most important mechanisms where these compounds directly kill the pathogen (Denis and Webster, 1971a; 1971b). In recent years, attempts are made to use a consortium (association of two) of biocontrol agents to get persistent control of plant pathogens (Chaube and Sharma, 2002). Keeping this in view and the growing importance of biological control agents, the present study was carried out. The main objective was to evaluate the

biocontrol efficiency of *T. viride*, *T. harzianum*, ATCC cultures of *B. subtilis* 6633 and *P. fluorescens* 13525 against *F. oxysporum* f. sp. *nicotianae* and to study their nature of action.

MATERIALS AND METHODS

Maintenance of biocontrol agents

The fungal antagonist *T. viride* and *T. harzianum* strains developed at Division of Plant pathology GKVK, Bangalore was used in the present study. The pure cultures were maintained on agar slants in *Trichoderma* selective medium (TSM) (Elad and Chet, 1983). *B. subtilis* obtained from ATCC were maintained on agar slants in Bacillus Growth medium and Fluorescent pseudomonad slants on King's medium B slants.

In vitro studies

Antagonism between *T. viride*, *T. harzianum*, *B. subtilis*, *P. fluorescens* and the pathogen were studied under *in vitro* conditions following the dual culture technique (Skidmore and Dickinson, 1976). The observations were recorded on the radial growth of the pathogen after every 24 hours till the colony of the pathogen was completely overgrown. The diameter of the pathogen was also recorded in dual culture till the growth of the pathogen ceased. After contact of antagonist with the pathogen, microscopic observations were carried out every 24 hrs. The slides were prepared from overgrown intermingling zone of dual culture plate. The culture plates were observed constantly, the radial growth of the pathogen was recorded on the seventh day of inoculation and the percent inhibition worked out was as follows:

$$PI = C - T / C \times 100$$

Where PI= Percent inhibition of mycelial growth, C= Radial growth of the pathogen in control plates (cm) and T= Radial growth of pathogen in dual culture (cm)

Production of volatile and non-volatile diffusible metabolites

Trichoderma viride, and *T. harzianum* isolates were evaluated for the production of volatile inhibitory substances *in vitro* following the

technique of Devis and Webster (1971). Both the biocontrol agents (0.5 cm diam disc) were inoculated separately to the centre of the petriplates containing PDA medium in triplicates.

The petriplates were sealed at the edges and kept in polythene bags and incubated at room temperature. After 5 days, the test pathogen was inoculated on fresh PDA medium. The upper lids of both the petriplates were removed. The fully grown *Trichoderma* plate and freshly inoculated pathogen culture plates were sealed with cello tape and incubated further for 7 days. Control plates consisted of inoculated lower lid with pathogen and inverted petriplates with PDA agar plug. Growth of the pathogen was measured after 5 days of incubation and the percent inhibition was calculated as mentioned above.

Bacterial suppression of *F. oxysporum* f. sp. *nicotianae*

Isolates of *B. subtilis* and *P. fluorescens* were tested for their antagonistic activity against *F. oxysporum* following the dual culture technique. Culture discs of 0.5 cm size of the pathogen were placed at the centre of the sterilized petriplates containing sterilized media. The respective bacterial isolate was then streaked 2 cm away from the pathogen at the centre, by a parallel non overlapping pattern, inhibition zone was measured after 5 days of incubation.

Compatibility of the selected antagonistic isolates under *in vitro* conditions

Cell suspension of both the bacterial bioagents were prepared, mixed with their respective selective agar media, poured into sterile petriplates and a 5 mm disc of *T. viride* and *T. harzianum* cultures were inoculated separately on the media and observed for the inhibition of growth of *B. subtilis* and *P. fluorescens* (Rini and Sulochena, 2006).

RESULTS AND DISCUSSION

Trichoderma Vs *F. oxysporum* f. sp. *nicotianae*

The dual culture study revealed strong antagonistic activity of *T. viride* against *F. oxysporum* f. sp.

nicotianae (Figure 1). *T. viride* quickly occupied the surface of the medium limiting the space for *F. oxysporum* restricting further growth of the pathogen. The per cent inhibition was observed to be 70 %. Microscopic observation revealed prominent coiling around the hyphae of *F. oxysporum* in six-day-old culture plate (Figure 2). The observations were on par with De cal *et al.* (1995) on *F. oxysporum* f. sp. *lycopersici*. Both the species of *Trichoderma* were promising against

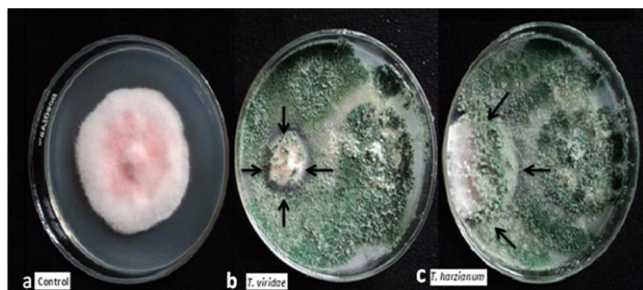


Figure 1. Dual culture plates of *F. oxysporum* f. sp. *nicotianae* alone (a), along with *Trichoderma viride* (b) and *Trichoderma harzianum* (c) on PDA (seven-day-old); arrow indicates the restricted size of the pathogen by the biocontrol agents

F. oxysporum f. sp. *nicotianae* and they reduced the growth of the pathogen by more than 70% within 6 days of incubation. These isolates completely overgrew the pathogen and suppressed it within 7 days of inoculation. Similar observations were made by Windham *et al.*, (1986); Morsy *et al.* (2009).

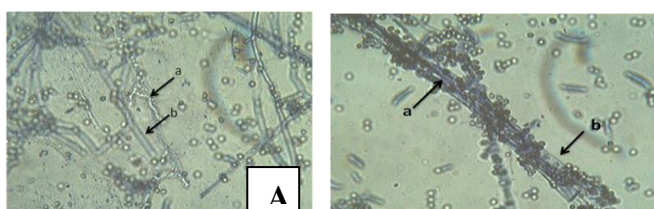


Figure 2. (A) *Trichoderma viride* and *Fusarium oxysporum* f.sp. *nicotianae* (B) *T. harzianum* Vs *F. oxysporum* f.sp. *nicotianae*. *Trichoderma* hypha (a) and *F. oxysporum* f.sp. *nicotianae* hypha (b)

T. harzianum overgrew on *F. oxysporum* and turned dark green due to sporulation over it but mycelium of the pathogen was clearly visible with slight yellow colored hyphae (Figure 1). Percentage of inhibition was observed to be 40 %. Intermixed hyphae of the pathogen and *T. harzianum* made two clear zones of green and

whitish pink till the pathogen was fully colonized. Similar observation was recorded by Morton and Strobue (1955) on *Sclerotium rolfsii*. Microscopic observation revealed *T. harzianum* formed several loops around the hyphae of the pathogen and coiling around it giving thick mycelial rope like appearance at initial stage of incubation (Upadhyay and Mukhopadhyay, 1986).

In vitro studies on the inhibitory mechanisms showed that *Trichoderma* cultures apparently produced volatile substances in the growth medium that suppressed the pathogen growth. The

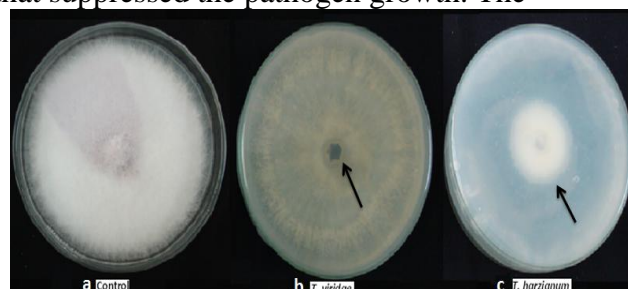


Figure 3. Effect of volatile metabolite of *Trichoderma* species on the growth of *Fusarium oxysporum* f. sp. *nicotianae*. *F. oxysporum* f. sp. *nicotianae* (a) alone, with *Trichoderma viride* (b) and *Trichoderma harzianum* (c). Arrow indicates the extent of growth of pathogen.

volatile substances derived from *T. viride* and *T. harzianum* inhibited mycelial growth of the pathogen to an extent of 72% and 56%, respectively (Figure 3). *T. viride* produced more inhibitory volatile metabolite when compared to that of *T. harzianum*. The results were on par with Pandey and Upadhyay (1997) where *T. viride* may be considered as a promising biocontrol agent with maximum production of volatile and non-volatile metabolic substances (Rajeswari and Kannabiran, 2011).



Figure 4. Dual culture plates on PDA. Control inoculated only with *F. oxysporum* f. sp. *nicotianae* (a), *F. oxysporum* f. sp. *nicotianae* Vs *Bacillus subtilis* (b), *F. oxysporum* f. sp. *nicotianae* Vs *Pseudomonas fluorescens* (c)

Bacterial Suppression of the pathogen

The inhibitory potential of both the bacteria also differed significantly. *B. subtilis* exhibited more inhibitory effect when compared to *P. fluorescens* (Figure 4). Inhibitory effect remained same on both Potato Dextrose Agar (PDA) and Nutrient Agar (NA) and in harmony with those recorded by Berg *et al.*, (1994); Saddlers, (1996) and Wang *et al.* (1999). Khalifa, (2003) reported that *Bacillus subtilis* and *B. megtila* were the best antagonistic bacteria for limiting growth of *Macrophomina* isolates.

Compatibility of the selected antagonistic isolates under *in vitro* conditions

Compatibility test carried out showed *T. viridae*, *T. harzianum*, *B. subtilis* and *P. fluorescens* were compatible to each other in dual cultures. There was no inhibition zone that was formed in all the dual culture plates. Similar observations have been reported previously by Rini and Sulochana (2006); Chaube and Sharma (2002). In conclusion, this study demonstrated the extent efficacy of *T. viride* and *T. harzianum* is more than *B. subtilis* and *P. fluorescens* in controlling *F. oxysporum* f. sp. *nicotianae*. It also showed the involvement of volatile antibiotics produced by *T. viride* and *T. harzianum*. Further the bacterial and fungal antagonists are compatible with one another and hence a consortium approach could be used in controlling the pathogens.

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