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Biological Control of *Fusarium* wilt of *Chrysanthemum* with *Trichoderma* and Botanicals

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The suitable biocontrol agent and botanical for controlling *Fusarium* wilt of *Chrysanthemum* were investigated. Seven isolates of *Trichoderma harzianum* (Th) namely T1, T2, T3, T4, T5, T6 and T7 were screened for their biocontrol potential against highly virulent *Fusarium oxysporum* f. sp *chrysanthemi* (Foc) isolate FO-10. Th isolates effectively inhibited the mycelial growth of Foc and maximum inhibition was recorded with T3 (66.0%). Eight botanicals namely *Mentha arvensis* (MA), *Tagetus patula* (TP), *Eucalyptus sp* (ES), *Datura stramonium* (DS), *Calotropis procera* (CP), *Lantana sp* (LS), *Ricinus communis* (RC) and *Catharanthus roseus* (CR) were evaluated for their biocontrol potential against Foc using food poison technique. Maximum inhibition in radial growth of Foc was recorded at the concentration of 3.0% with MA (63%) and minimum with CR (42%). An increase in % mycelial growth inhibition of Foc was recorded with increasing concentration of botanicals. Th isolate T3, T4 and T5 and botanical MA, TP and DS were selected for the biological control trails in pot conditions based on their performance in *in vitro* conditions. Disease control was recorded maximum with T3 (92%) and minimum with T5 (81.0%). Soil treatments with botanicals reduced wilt disease and maximum disease control was recorded with MA (70.0%), followed by TP (61.0%) and DS (50.0%). Results of co-application of botanicals with Th reveals that there was no significantly increased in the disease control and individual applications of Th isolates was more effective in controlling Foc.

Key Words: *Fusarium oxysporum* f. sp *chrysanthemi, Trichoderma harzianum, Botanicals,* Biocontrol, *Chrysanthemum,* growth inhibition

Introduction

Chrysanthemum is one of the most leading commercial floriculture crop, grown for cut and loose flowers throughout the globe. *Chrysanthemum* plants are infected by various fungal, bacterial and viral diseases. *Fusarium* wilt of *Chrysanthemum* caused by *Fusarium oxysporum* f. sp *chrysanthemi* (Foc) is

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one of the most wide spread and destructive disease, causing infection and loss from nursery to flowering stage. Foc is difficult to control because of its persistence in the soil. Severe losses to *Chrysanthemum* crop caused by Foc are reported from various part of the world (Garibaldi *et al.,* 2009; Minuto *et al.,* 2007; Murkar *et al.,* 1994). Foc are an unique ability to change its host range and it is reported that along with *Chrysanthemum*, it also infects *Gerbera jamesonii Argyranthemum frutescens* (Paris daisy) and *Osteospermum* sp. Only few varieties of the *Chrysanthemum* were reported resistant to all strains of Foc tested. Selection and use of resistant cultivars of *Chrysanthemum* have a limited success in controlling the disease (Minuto *et al.,* 2007; Garibaldi *et al* 2009; Minuto and Garibaldi, 2008).

Various chemicals and fungicides such as Benomyl, Ethazol, Thiophanate-Methyl, Bitertanol, Triadimefon, Thiabenzadole, Carboxin etc have been used to control Foc of which Thiophanate methyl was reported to provide maximum disease control at 0.21g.a.i./liter. Benomyl was also effective but at higher doses (1.2 g.a.i./liter). An increase in the concentrations of the chemical fungicides has negative effect on the plants (Strider, 1985). Use of the chemicals and fungicides leads to severe environmental pollutions and also the population of beneficial microbes are reduced.

Reduction in the population of Foc with extracts of clove and pepper were reported by Bowers and Locke (2000). The antifungal and antimicrobial properties of several plant extracts and essential oils are investigated by many workers (Aliero *et al.,* 2000; Boulenouar *et al.,* 2009; Pizana *et al.,* 2010; Ayed *et al.,* 2007; Singh and Singh, 1980; Guddewar *et al.,* 1999; Hassannein *et al.,* 2008). Antifungal activity of botanicals against local isolate of Foc was also investigated by us in our earlier studies (Singh and Kumar, 2011). It is generally assumed that the active constituents which are contributing to these anti-fungal properties of the extracts are phyto-chemicals (Okwu, 2004).

Trichoderma sp. is an unique ability to control the plant pathogens by using the mechanism of mycoparasitism, antibiosis, competition, siderophore production, induction of systemic resistance etc (Chet, 1987; Dennis and Webster 1971, Upadhyay and Mukhopadhyay, 1986; Howell, 2003). Antagonistic ability of different isolates of *Trichoderma* on different formae speciales of *Fusarium oxysporum* is well documented (Kerkeni *et al.,* 2007; Dohroo, 1995; Orole and Adejumo, 2009; Sivan and Chet, 1989).

A critical review of the literature reveals that very little work has done on eco-friendly management of Foc. The present investigation was undertaken to find out eco-friendly methods of controlling Foc, using Th and botanicals. Attempts were also done to find out the suitability of co-application of Th with botanicals.

Material and methods

Isolation of Antagonist

The present study was carried out during 2007 to 2010. Rhizospheric soil samples were collected from nurseries and fields in fresh poly bags from Kanpur, India. Samples were dried under laminar air flow and serial dilutions up to 10^{-6} were prepared. Test tubes with soil dilutions were shaken on vortex mixer (Make: REMI) at 1500 RPM for 2 minutes and spread on the Trichoderma Selective Medium (TSM) Petri plates. The fungal colonies were subcultured on PDA and were identified as the strains of Th.

Screening of antagonist against Foc

All the 7 isolates of Th were screened for their biocontrol potential against highly virulent Foc isolate FO-10 (NCFT No-1374), using dual culture technique. Five mm disc of Th and Foc were cut from 5 days old culture and placed opposite to each other in 90 mm Petri dishes containing PDA medium. The inoculated Petri dishes were incubated at 25±2°C and colony diameter of Th and Foc were recorded periodically up to 7 days. Controls of both the fungi were inoculated separately in the center of the Petri dishes. All the treatments were taken in triplicates and the experiment was carried out twice. The formula used for calculation of the percent inhibition in the dual culture Petri plates was as follows:-

$$\% \text{ inhibition over control} = \frac{C-T}{C}$$

C = Growth of fungus in the control Petri dishes.
T = Growth of fungus in the Dual Culture Petri dishes.

Screening of botanicals against Foc

Plant extracts of MA, TP, ES, DS, CP, LS, RC and CR were tested for their antifungal potential against above Foc isolate FO-10, using poisoned food technique (Grover and Moore, 1962). Leaf samples of above plants were used for extract preparation. Fifty gram of leaf samples were taken in a mortar pestle and equal amount of warm water was added to it. After proper grinding, samples were filtered through muslin cloth followed by sintered glass (G 5) and used for the further studies. Concentration of 1.0% and 3.0% were prepared by adding requisite amount of dried extract to Potato Dextrose Agar medium. The

media containing extracts were poured in Petri dishes (90mm) and centrally inoculated with the mycelial disc (5 mm) of above Foc isolate, cut from the margin of 5 days old cultures. The plates were incubated at 25±2^{0}C and radial growth was recorded periodically upto 10 days.

Pot trials for wilt disease control using botanicals and Th

Three best *Th* isolates (T3, T4, T5) and botanicals (MA, TP, DS) were selected for the pot trials on the basis of their efficacy towards Foc isolate FO-10, under *in vitro* conditions. Selected *Th* isolates were mass multiplied on Rice Bran (RB) agro-cellulosic waste. RB agriwaste (500 gm) were moistened, sterilized and autoclaved three times at 15 lbs for 20 minutes in autoclavable polybags. Spore suspension (cfu 10^5) of *Th* isolates were prepared in sterile distilled water and inoculated at 5 ml/bag. Foc was multiplied on sand maize bran medium using the above procedure. Bags inoculated with *Th* isolates and Foc were incubated at 25±2°C for 15 days. Mass multiplied cultures were dried under shade, milled to powder and used as inoculants of antagonist and the pathogen. *Chrysanthemum* cuttings were prepared from healthy mother plants using flat earthen pots (18 inch diameter) containing sterilized sand medium.

Garden soil was sterilized and filled in earthen pots (12 inch diameter) for biocontrol trials. Foc inoculum (Cfu 10^8) was added at 10 g/kg of pot soil and mixed thoroughly. In treatment first, *Th* inoculum (cfu 10^8) was added at 10g/kg of pot soil, two days after addition of the Foc inoculum. In treatment second botanicals was added at 30 ml/kg (3.0%) of pot soil, two days after addition of the Foc inoculum. In treatment third, co-application of botanical and *Th* isolate was done at 30 ml and 10 gm per kg of pot soil respectively. Control pots were not inoculated with *Th* and botanicals and they only contained Foc. The above prepared pots were planted with 35 five days old rooted *Chrysanthemum* cuttings. Twenty four replicates were maintained for each treatments and control. The plants were observed for wilting symptoms and the data on number of wilted plants were recorded. The above trials were conducted for two successive seasons using same material and methods.

Statistical Analysis

Data of radial growth inhibition and disease control were analyzed for standard deviation followed with standard error calculation. All the data were subjected to analysis of variance (ANOVA) to find out its significance.

Results

Effect of Th in controlling Foc under invitro conditions

All the 7 isolates of *Th* effectively inhibited the mycelial growth of Foc in the dual culture. Maximum inhibition was recorded with isolate T3 (66%) and minimum with T2 (50%). Per cent growth inhibition of Foc by Th isolates in decreasing order was T3, T4, T5, T1, T7, T6 and T2 (Figure 1).

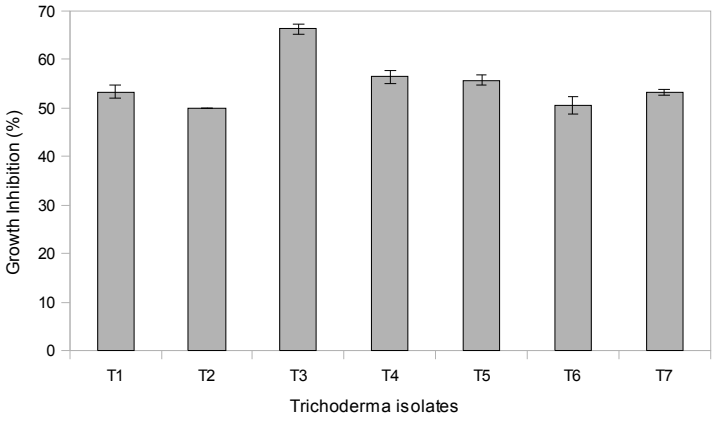

Fig 1. Percent growth inhibition of Foc with different isolates of *Th* under *in vitro* conditions. Values represent percent inhibition means with standard error (±).

Effect of botanicals against Foc in Food Poison technique

All the 8 botanicals used against Foc isolate effectively checked its mycelial growth in the Petri plate medium. An increase in the % growth inhibition of Foc was observed with increasing concentration of botanicals. For all treatments maximum inhibition in the radial growth was recorded at the concentration of 3.0%. Mycelial growth inhibition was maximum with MA (63%) followed by TP (59%), DS (56%) CP (54%), LS (51%), ES (48%), RC (44%) and CR (42%). Similar sequence of effectiveness was recorded at the concentration of 1.0% (Table 1).

Table 1. Efficacy of botanicals against Foc at concentration of 1.0% and 3.0%. Values represent the means with standard error (±)

Botanicals	Concentration (%)	Radial Growth (mm) of Foc	Growth Inhibition (%)
M.arvensis	1.0	44.3 ± 0.33	46.80
	3.0	30.6 ± 0.33	63.20
T.patula	1.0	46.0 ± 0.00	44.80
	3.0	34.0 ± 0.00	59.20
Eucalyptus sp	1.0	54.6 ± 0.33	34.40
	3.0	42.6 ± 0.33	48.80
D.stramonium	1.0	47.0 ± 0.58	43.60
	3.0	36.0 ± 0.58	56.80
C.procera	1.0	49.0 ± 0.58	41.20
	3.0	38.0 ± 0.58	54.40
Lantana sp	1.0	53.0 ± 0.58	36.40
	3.0	40.0 ± 0.00	51.90
R.communis	1.0	58.0 ± 0.58	30.40
	3.0	46.3 ± 0.33	44.40
C.roseus	1.0	59.0 ± 0.00	29.20
	3.0	48.0 ± 0.58	42.40
Control	0.0	83.3 ± 0.33	-

Effect of Th in controlling Foc in pot conditions

Th isolates T3, T4 and T5 were selected for the biological control trails based on their performance in the dual culture studies against Foc. Among the 3 isolates maximum disease control was recorded with T3 (91.0%) and minimum with T5 (81%). Disease control provided by T3 isolate was significantly higher among all the *Trichoderma* isolates. A slight variation in disease control potential was observed within isolates of *Th* as it was recorded in dual culture (Figure 2).

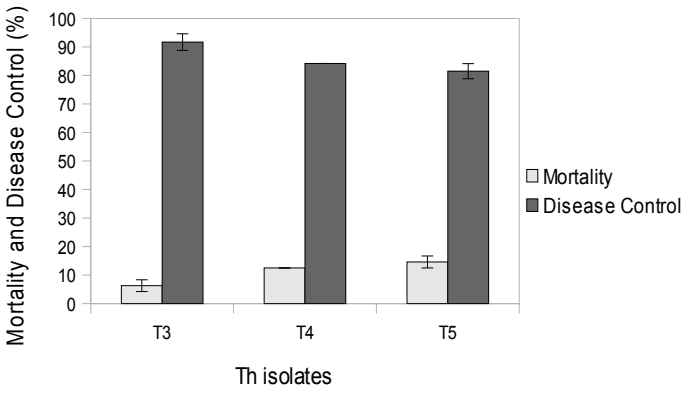

Fig 2. Effect of soil application of *Trichoderma* isolates in controlling *Fusarium* wilt disease of *Chrysanthemum* plants, caused by Foc isolate FO-10. Value represent means with standard error (±). Data are the average of two seasons.

Effect of botanicals in controlling Foc in pot conditions

Soil treatments with botanicals caused reduction in the *Fusarium* wilt disease of *Chrysanthemum* plants (Figure 3). Maximum disease control was recorded with MA (70.0%), followed by TP (61.0%) and DS (50.0%). All the 3 botanicals showed antifungal potential and successfully reduced the efficacy of Foc to cause disease in *Chrysanthemum* plants.

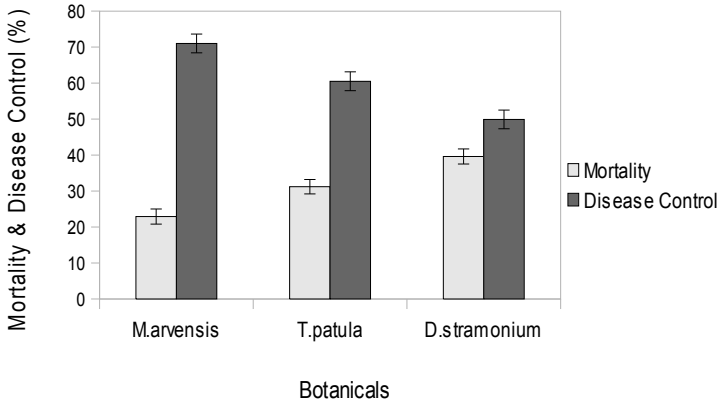

Fig 3. Effect of soil application of 3 botanicals in controlling *Fusarium* wilt disease of *Chrysanthemum* plants, caused by Foc isolate FO-10. Values represent means with standard error (±). Data are the average of two seasons.

Effect of co-application of Th and botanicals in controlling Foc in pot conditions

Results obtained from co-application trails revealed that there was no significant increase in the disease control when application of *Th* was done along with botanicals. The results were similar as it was with botanicals with a slight reduction in wilt (Figure 4) Disease control provided by *Th* isolates was highest compared to botanicals either alone or in co-application.

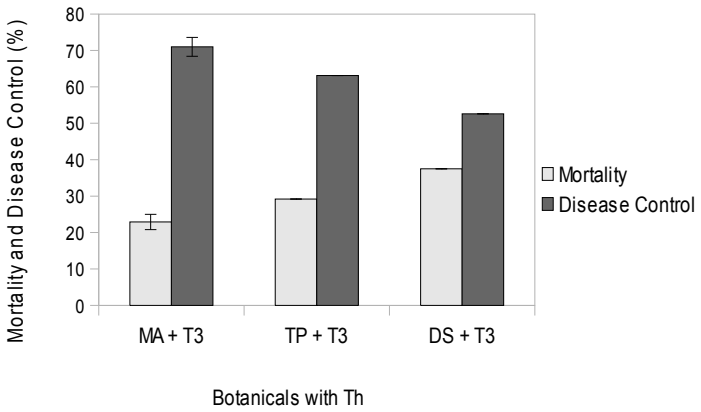

Fig 4. Effect of soil application of 3 botanicals and *Trichoderma* isolate T3 in controlling *Fusarium* wilt disease of *Chrysanthemum,* caused by Foc isolate FO-10. Values represent means with standard error (±). Data are the average of two seasons

Discussion

Biocontrol potential of *Th* isolates against Foc varied among themselves. Per cent growth inhibition of 50.0 to 66.0 was recorded by different isolates of *Th*. T3 was most promising against the wilt pathogen and registered maximum inhibition. Similar results were the findings of Orole and Adejumo (2009). They reported radial growth inhibition of *Fusarium* species from 25 to 75% by *Trichoderma*. There is variation in per cent inhibition by individual isolate of *Trichoderma* against Foc.

Results of food poison technique revealed that the botanicals used have the capability to reduce the growth of Foc isolate FO-10. Per cent inhibition of Foc, increased with the increasing concentration of botanicals which suggests that at high concentration is more suitable due to presence of high amount of antifungal compounds. Maximum percent inhibition was recorded with MA followed by TP, DS and minimum with CR. Riaz *et al.* (2008) reported 54.0 to 79.0% growth inhibition of *Fusarium oxysporum* f. sp. *gladioli* using the

extract of *Tagetus erecta* at 2.0 to 8.0% concentrations. *Tagetus* sp. has fungicidal properties due to presence of thiophenes in its tissues (Gomez-Rodriguez *et al.,* 2003). Antifungal activity of botanicals (LS, CP, CR, RC etc) have been reported by several workers against *Fusarium* sp and other plant pathogenic fungi (Bansal and Rajesh, 2000; Begum *et al.,* 2007; Abdulrahman and AlKhail, 2005).

Results obtained from pot trails revealed that the efficacy of Foc to infect the plants drastically reduced due the soil application of botanicals. Bowers and Locke (2000) reported reduction in soil population of Foc by 97.5% when soil was treated with 10% aqueous extract of clove. Decrease in the soil population of the pathogen (Foc) due to botanicals extract may be the possible reason for the wilt disease reduction. MA extracts showed highest anti-fungal activity and was most promising in controlling Foc. Ghorbany *et al.* (2010) reported *Mentha* sp extract to cause growth inhibition of *Fusarium oxysporum* f. sp. *cumini,* supports our findings. Sharma and Trivedi (2002) reported growth inhibition (72.0%) of *Fusarium oxysporum* f. sp *cumini* with the extract of *D.stramonium.* It is generally assumed that the active constituents which are contributing to these anti-fungal properties of the extracts are phyto-chemicals. Phyto-chemicals constitute on of the most widely distributed groups of substances in the plant kingdom. Woody plants and herbs are known to synthesize and accumulate a great variety of phyto-chemicals in their cells and tissues. These phyto-chemicals include low molecular weight phenolics such as hydroxybenzoic acid, hydroxycinamic acid, acetophenone, flavanoids, stilbenes and lignans) as well as oligo or polymeric forms such as hydrolysable and condensed tannins and lignins (Close and McArthur, 2002; Okwu and Omodamiro, 2005).

Results obtained from the biocontrol experiments revealed that *Th* isolates effectively controlled Foc and there was a reduction of 81.0 – 91.0% in the wilt disease of *Chrysanthemum* plants. Maximum disease control was recorded with isolate T3, followed by T4, and T5. A direct correlation was observed in the biocontrol potential of *Th* isolates in dual culture plates and in the pot trials. In both the experiments (*in vitro* and *in vivo*) *Th* isolate T3 was most effective in controlling the wilt pathogen.

Co-applications of *Th* and botanicals were not significantly affected in reducing the wilt disease as compared to *Th* and botanicals alone. The reason behind, this may be reduced biocontrol potential of *Th* due to fungi toxic nature of the botanical and therefore biocontrol effects of botanical was only observed in co-application. The present experiment clearly indicated that application of *Th* with botanicals is not an effective method of biocontrol of Foc. *Th* isolates and botanicals both gave the potential to control Foc. Potential of botanicals in

in vitro conditions was at par with the *Th* isolates but in the pot trials *Th* were superior in controlling the wilt disease caused by Foc. Therefore, application of *Th* isolates would more effective method of controlling Foc as compared to botanicals.

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