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# Complexity of *Trichoderma-Fusarium* interaction and manifestation of biological control

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**Abstract**

Several *Trichoderma* isolates were screened for their biocontrol activity against *Fusarium oxysporum* f.sp. *pisi*. Eighteen of these isolates (T1 to T18) showed considerable biocontrol potential and were taken further in the study. Initial counter inhibition was observed in all the eighteen dual culture sets where *Trichoderma* and *Fusarium* both posed varying degree of inhibition on each other. In three of the sets, *Fusarium* was found extending narrow outgrowths towards *Trichoderma*. *Trichoderma* was later found extending its mycelia along these narrow outgrowths of *Fusarium* representing a chemo-attractive mechanism of interaction. Finally, parasitic interaction was noticed in all the sets except two. In these two sets, *Trichoderma* was unable to overcome inhibition posed by *Fusarium* and, therefore, could not parasitize *Fusarium*. In the interaction between *Trichoderma* and *Fusarium*, the sequence of events noticed was categorized as pre-contact antagonistic interaction, chemo-attractive intermediate phase and, finally, parasitic interaction. Most of the *Trichoderma* isolates showed considerably good antibiosis and parasitism. Therefore, the primary criterion for selection of the best potential biocontrol agent was poised on the time taken by *Trichoderma* to complete the sequence of events until parasitism of target pathogen. Among the eighteen isolates of *Trichoderma*, *T. atroviride* (Isolate T1) was found to show best activity followed by two isolates of *Trichoderma harzianum* (Isolate T8 and T11) in terms of time taken to parasitize *Fusarium*. The present study stresses on quick control on plant disease as a desired quality for popularization of biocontrol agents.

**Keywords:** Antagonist; Pathogen; Counter inhibition; Antibiosis; Parasitism; Time interval; Biocontrol.

**Abbreviations: T1** to **T18**- *Trichoderma* isolate number; **Xf-** Growth of *Fusarium* towards *Trichoderma*, **Yf-** Growth of *Fusarium* away from *Trichoderma* on dual culture plate, **Yfc-** uninfluenced growth of *Fusarium* on control plate. **Xt-** Growth of *Trichoderma* towards *Fusarium*, **Yt-** Growth of *Trichoderma* away from *Fusarium*. **Df*-** Difference in *Fusarium* growth towards and away from *Trichoderma* on the same dual culture experiment plate, **Df-** Difference in *Fusarium* growth on experiment plate and control plate. **Dt-** Difference in growth of *Trichoderma* towards and away from *Fusarium* on the same dual culture experiment plate.

## Introduction

Importance of *Trichoderma* in biological control of soil borne pathogens has been discussed over a period of time by several workers. It is stated by researchers that *Trichoderma* is a hostile myco-parasite that can control already established pathogens as well as newly entered pathogens. But, incomplete and inconsistent disease control might result unless the antagonists are used in formulations and application plans that give them a competitive advantage over the pathogens (Harman and Kubicek, 1998). *Trichoderma* have been used in a number of crops, for example, lettuce, onion, cotton, grapes, peas, apples, sweet corn, carrots and others to control various pathogens such as *Phytophthora, Pythium, Sclerotinia, Botrytis, Rhizoctonia and Fusarium* (Benítez et al., 2004). John et al., 2010, stated that *Trichoderma* showed marked enhancement in root system and also in biological nitrogen fixation besides controlling pathogens like *Fusarium oxysporum* and *Pythium* sp. *Trichoderma* is special also in the sense that it can apply a combination of mechanisms to control the growth of pathogen. The three well known mechanisms associated with pathogen control by *Trichoderma* are competition for nutrients, antibiosis, and myco-parasitism (Chet, 1987). *Trichoderma* species have a better competence to mobilize and take up soil nutrients compared to other organisms. It was noticed by Tjamos et al. (1992) that *T. harzianum* controls *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients. They observed that biocontrol became more effective as the nutrient concentration decreased. *Trichoderma* may put forth direct biocontrol by parasitizing a variety of fungi as they are capable of detecting other fungi and growing towards them. The distant sensing is partly due to the sequential expression of cell wall degrading enzymes, mostly chitinases, glucanases and proteases (Harman et al., 2004). Most *Trichoderma* strains produce volatile and nonvolatile toxic metabolites that obstruct colonization by antagonized micro organisms. Some of these metabolites have been studied and the production of harzianic acid, alamethicins, tricholin, peptaibols, 6-penthyl-alpha-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described (Vey et al., 2001). Role of *Trichoderma* is also indicated in plant growth promotion. Chang et al., 1986, observed enhanced germination, rapid flowering and increase in height and fresh weigh of plants treated with *Trichoderma*. There are reports which indicate role of *Trichoderma* in induction of defence mechanisms in plants. It was found that the addition of *Trichoderma* metabolites results in the synthesis of phytoalexins, PR proteins and other compounds in plants.

And, thereafter, may increase resistance in plants against several plant pathogens, including fungi and bacteria (Benítez et al., 2004; Jayalakshmi et al., 2009). The ease of formulation and delivery system is an advantage as *Trichoderma* can be grown on a wide range of carbon and nitrogen source. *Trichoderma* produce three types of propagules: hyphae, chlamydospores and conidia (Papavizas, 1985). Conidia have been commonly employed for use in biological control. Conidial biomass can be cultivated by submerged culture method or solid substrate culture method (Harman and Kubicek, 1998). In spite of the above listed advantages, expected market value has not yet been achieved by *Trichoderma*. Substantially rapid effect of chemical pesticide on pathogen is responsible for its high market value. Therefore, it is necessary to find more aggressive strains of *Trichoderma* which can compete with chemical pesticide in terms of quick action on pathogen.

## Results and discussion

### *Study of interaction between Trichoderma and Fusarium on dual culture plates*

Antibiosis and myco-parasitism are the well known mechanisms involved in biocontrol of pathogens by *Trichoderma*; competition for nutrition, space and dominance being equally important and mutually inclusive phenomenon. The complete course of interaction between *Trichoderma* and *Fusarium* as observed on the dual culture plates can be divided into three phases. The initial phase marked by interaction without mycelia contact in which diffusible metabolites from both the organisms decide the fate of interaction (Fig 1). The intermediate phase in which *Trichoderma* may or may not be able to overcome the inhibitory effect of *Fusarium*. In the intermediate phase, some chemo-attractive mechanisms may also be activated as observed in this research (Fig 6). And, the final phase where *Trichoderma* parasitizes *Fusarium* (Fig 7). Eighteen of twenty six *Trichoderma* isolates which showed biocontrol potential were analysed in this research. The initial interaction between *Fusarium* and *Trichoderma* seemed to be combat involving defensive and offensive mechanisms from both the organisms. The pattern of zone of inhibition (Fig 1) between the two organisms clearly indicated that *Fusarium* initially posed inhibition to the progress of *Trichoderma* towards itself by secreting deleterious metabolites but it also suffered stress which resulted from metabolites released by *Trichoderma.*

This caused inhibition of *Fusarium* growth as well (Table 1). Physiological changes in *Fusarium* were discernible by its altered cultural characteristics like waxy moist appearance, scanty or no mycelia and excessive pigmentation (Fig 6). This manifestation is believed to be under the effect of *Trichoderma* (Lorito et al., 1996a) noticed in most of the test plates as a prominent feature. On the other hand, the sporulation by *Trichoderma* seemed to be influenced by presence of *Fusarium* metabolites. In certain cases, sporulation in *Trichoderma* was hindered initially by *Fusarium* but in later stages heavy sporulation by *Trichoderma* was seen at the verge of inhibition zone marked by influence of *Fusarium*. Exceedingly fast growth rate (Table 2) and also heavy and quick sporulation activity of *Trichoderma* is an added advantage to its ability to overcome inhibition posed by *Fusarium*. Further in the course of interaction, *Trichoderma* reinforced itself after an initial halt and extended its mycelia towards *Fusarium.* This advancement was possible only when *Trichoderma* succeeded in effectively overcoming the inhibitory upshot of *Fusarium*. These characteristics were observed in most of the strains of *Trichoderma* tested against *Fusarium oxysporum*. After a period of cross signalling by pre-contact chemical interactions between the two fungi, competent strains of *Trichoderma* were able to reach and overwhelm *Fusarium* in later stage of interaction. In all, except T10 and T15, plates *Trichoderma* was able to encroach into the inhibition zone of *Fusarium* and extend mycelia towards *Fusarium* followed by heavy sporulation immediately on the colony of *Fusarium* which depicts a step in the mechanism of parasitic activity of *Trichoderma* (Brunner et al., 2005). A special feature was noticed in three of the plates, T2, T3 and T12 in the intermediate stage of interaction just before parasitic interaction began. It appeared as if *Fusarium* was being attracted towards *Trichoderma*, through specific chemical signalling. *Fusarium* was seen extending a string like channel towards *Trichoderma* which stimulated *Trichoderma* to grow towards *Fusarium* along this channel (Fig 6).

This suggests the role of chemo-attractants in driving *Fusarium* and *Trichoderma* towards each other. This phenomenon is in contrast to the one noticed previously in the initial stage of interaction where both the fungi were showing inhibitory activity towards each other. Therefore, after an initial phase of intense counter inhibition *Trichoderma* was not only able to overcome inhibitory effect of *Fusarium* but also attract *Fusarium* towards itself as observed in this research. This is a striking feature of *Trichoderma* in which specific signal transduction cascade is believed to play a role. Omann and Zeilinger (2010) mentioned the importance of G-protein signalling in myco-parasitic activity of *Trichoderma*. In T4, heavy pigmentation by *Fusarium* and heavy sporulation by *Trichoderma* around *Fusarium* colony was observed. At the same time, *Trichoderma* was also seen growing precisely on the colony of *Fusarium* and showed heavy sporulation there. In T3, the colony of *Fusarium* was completely overwhelmed and covered by *Trichoderma*. *Trichoderma* very precisely covered only the area on which *Fusarium* was growing leaving the peripheral area of the colony unoccupied. Later, *Trichoderma* extended even in this peripheral area.

This behaviour of *Trichoderma* suggests that *Fusarium* was the target towards which *Trichoderma* was specifically attracted. Similar results were shown by all the other isolates except T10 and T15 which did not cross the zone of inhibition to parasitize *Fusarium.* Yet, T10 and T15 showed a strong counter inhibition to growth of *Fusarium*. The time taken by each *Trichoderma* isolate to parasitize *Fusarium* has been mentioned in Table 3. *Trichoderma* isolate T1 was the quickest of all isolates in crossing the zone of inhibition and parasitizing *Fusarium*. On agricultural field, dominance of biocontrol agent through its high growth rate and offensive mechanisms against pathogen are decisive in manifestation of disease control. Parasitism by *Trichoderma* is its powerful weapon in destruction of pathogen. But, *Trichoderma* must overwhelm pathogen before the pathogen proliferates and infects germinating plant seeds. Therefore, a measure of speed with which biological control on pathogen is achieved is important when searching an aggressive strain of *Trichoderma*. The analysis of *Trichoderma- Fusarium* interaction has been discussed in four sections mentioned below.

**Table 1.** Measurements on *Trichoderma*- *Fusarium* interaction in terms of pre-contact antagonism.

| Source | Isolate no. | Difference in Growth of *Fusarium* towards and away from *Trichoderma* (radius, cm) | | Difference in Growth of *Trichoderma* towards and away from *Fusarium* (radius, cm) Yt-Xt=Dt | Zone of inhibition, Z (cm) | % inhibition posed by *Fusarium* on *Trichoderma* | % inhibition posed by *Trichoderma* on *Fusarium* |
|---|---|---|---|---|---|---|---|
| | | Yf-Xf= Df * | Yfc-Xf= Df | | | | |
| Rose (*Rosa* sp.) | T1 | 0.2 | 0.15 | 0.4 | 0.5 | 19 | 25 |
| Pepal (*Ficus religiosa*) | T2 | 0.15 | 0.25 | 0.85 | 0.85 | 34 | 38 |
| Guava (*Psidium guajava*) | T3 | 0.1 | 0.2 | 0.9 | 0.7 | 45 | 31 |
| Mango (*Mangifera indica*) | T4 | 0.1 | 0.25 | 0.6 | 0.6 | 29 | 38 |
| Mehndi (*Lawsonia inermis*) | T5 | 0.25 | 0.3 | 0.4 | 0.3 | 16 | 44 |
| African marigold (*Tagetes erecta*) | T6 | 0.2 | 0.15 | 0.15 | 0.25 | 6 | 25 |
| Garden pea (*Pisum sativum*) | T7 | 0.3 | 0.3 | 0.6 | 0.7 | 30 | 44 |
| Garden pea (*Pisum sativum*) | T8 | 0.1 | 0.05 | 0.45 | 0.35 | 19.5 | 13 |
| Garden pea (*Pisum sativum*) | T9 | 0.25 | 0.25 | 0.35 | 0.25 | 11.9 | 38 |
| Alpine aster (*Aster alpinus*) | T10 | 0.15 | 0.2 | 0.5 | 0.4 | 22.7 | 31 |
| Ginger (*Zingiber officinale*) | T11 | 0.2 | 0.15 | 0.3 | 0.2 | 12.5 | 25 |
| Aleo vera (*Aleo vera*) | T12 | 0.15 | 0.3 | 0.35 | 0.6 | 18.4 | 44 |
| Custard apple (*Annona squamosa*) | T13 | 0.3 | 0.05 | 0.2 | 0.2 | 9.5 | 13 |
| Bougainvillae "Buttiana" | T14 | 0.35 | 0.25 | 0.2 | 0.35 | 9 | 38 |
| Green chilly (*Capsicum frutescens*) | T15 | 0.05 | 0.5 | 1.3 | 1.5 | 76 | 69 |
| Vinca (*Vinca rosea*) | T16 | 0.3 | 0.25 | 0.45 | 0.2 | 20.9 | 38 |
| Currypatta (*Bergera Koenigii*) | T17 | 0.25 | 0.05 | 0.3 | 0.25 | 16.6 | 13 |
| French marigold (*Tagetes patula*) | T18 | 0.45 | 0.2 | 0.2 | 0.15 | 8.6 | 31 |

Table 1: Average of replicates taken, Xf= Growth of *Fusarium* towards *Trichoderma*, Yf= Growth of *Fusarium* away from *Trichoderma* on dual culture plate, Yfc= uninfluenced growth of *Fusarium* on control plate. Xt= Growth of *Trichoderma* towards *Fusarium*, Yt= Growth of *Trichoderma* away from *Fusarium*. Df*= Difference in *Fusarium* growth towards and away from *Trichoderma* on the same dual culture experiment plate, Df= Difference in *Fusarium* growth on experiment plate and control plate. Dt= Difference in growth of *Trichoderma* towards and away from *Fusarium* on the same dual culture experiment plate.

The two readings, Df* and Df, have been taken because *Trichoderma* influenced growth of *Fusarium* colony even on distal side on the dual culture plate indicating that certain diffusible factors from *Trichoderma* were showing long distance influence. But, distal side of *Trichoderma* was not influence by *Fusarium* as growth of *Trichoderma* was faster than that of *Fusarium*. The table is based on the manifestation of pre-contact interactions between *Trichoderma* and *Fusarium* and depicts antibiosis between the two fungi. The difference in growth of both the fungi on either side of the zone of inhibition clearly states that both the fungi exerted stress on each other. All the strains of *Trichoderma* show varying degree of influence on *Fusarium*. In turn, *Fusarium* also shows varying degree of influence on different strains of *Trichoderma*. These counter effects have also been represented graphically.

**Zone of inhibition analysed in relation to growth of *Fusarium* under influence of *Trichoderma***

This section was further divided into two cases:

***A. Considering difference in growth of Fusarium under direct intense influence of Trichoderma and under lesser influence of Trichoderma on dual culture plate. Df* verses Z; Fig 2:***

Due to rapid spread of *Trichoderma* on dual culture plate with the advancement of interaction, *Fusarium* was almost encircled by *Trichoderma* such that even the distal side of *Fusarium* colony came under influence of *Trichoderma* metabolites. Later, in some cases, *Trichoderma* trapped *Fusarium* from all sides (Fig 1). Therefore, no side of *Fusarium* colony was free from influence of *Trichoderma*.

***B. Considering difference in growth of Fusarium under direct intense influence of Trichoderma and under no influence of Trichoderma. Df verses Z; Fig 3:***

Difference in growth of *Fusarium* on dual culture plate and on control plate (*Fusarium* alone) was plotted against zone of inhibition. This was done to assess actual magnitude of *Trichoderma* influence on *Fusarium*. Both the plots (Fig 2 and Fig 3) showed an irregular pattern of relationship between zone of inhibition (Z) and growth differences of *Fusarium* (case I A- Df* vs Z as well as case I B- Df vs Z). This irregular pattern of relation implies that each of the eighteen *Trichoderma-Fusarium* interactions was unique in its expression. Every isolate of *Trichoderma* exerted a different degree of stress on *Fusarium*. T15 showed highest degree of antibiosis on *Fusarium* as depicted by Fig 3. Some differences were seen in case I A and case I B, although over all inference was the same. These differences are believed to result from long distance influence of *Trichoderma* metabolites on the dual culture plate. T15-*Fusarium* interaction is the best example of long distance influence of metabolites released by both the organisms on each other as seen in Fig 1 and also depicted by the difference in Fig 2 and Fig 3 plots at T15.

**Zone of inhibition analysed in relation to growth of *Trichoderma* under influence of *Fusarium***

The pattern of plot (Fig 4) suggests that the growth difference of *Trichoderma* (Dt) largely corresponds to zone of inhibition (Z) which implicates that *Fusarium* definitely exerted an inhibitory effect on the growth of *Trichoderma*. A very smooth and regular correspondence was not found between Dt and Z because each isolate of *Trichoderma* experienced varying degree of opposition from *Fusarium*. In this plot (Fig 4), despite uniqueness in each of the eighteen *Trichoderma-Fusarium* interactions, a relationship of proportionality can be drawn between Dt and Z. Fig 4 also shows an outstanding feature where *Trichoderma* isolate T15 faced severe opposition from *Fusarium*.

**Analysis of counter inhibition shown by both the organisms; *Trichoderma* and *Fusarium***

The plot (Fig 5) shows that most of the isolates of *Trichoderma* were able to overcast the inhibitory effect of *Fusarium*. Isolate T3, T8, T15 suffered strong opposition from *Fusarium*. Exceptionally intense and almost equally opposite inhibitory counteraction was seen between T15 and *Fusarium*. T13 and T17 also showed almost equally opposite counter inhibition but the intensity was much less. T2 showed similar feature but with medium intensity. All the four plots suggest that the degree of antibiosis imposed by each *Trichoderma* isolate on *Fusarium* and the level of competitiveness and opposition shown by *Fusarium* towards each *Trichoderma* isolate was uniquely different. In other words, the consequence of counter stress shown by both the organisms on each other was specifically a character of individual *Trichoderma- Fusarium* interaction for each of the 18 isolates of *Trichoderma*.

**Analysis of Parasitic phase of *Trichoderma-Fusarium* interaction**

After the phase of antibiosis, the next phase is to cross the zone of inhibition and initiate the mechanism of parasitism. The measure of efficiency of a biocontrol agent such as *Trichoderma* is determined by its ability to cross the zone of inhibition posed by the pathogen and to overwhelm the pathogen. The best biocontrol agent is the one which takes least time to perform this process. The total time taken by biocontrol agent to cross zone of inhibition and overwhelm pathogen was the prime criterion for evaluation of biocontrol potential of *Trichoderma* isolates in this research (Table 3, Fig 8). Besides *Trichoderma* isolate T1 other isolates such as T8 and T11 also showed considerable activity against *Fusarium.* While T14 was the slowest of all, T10 and T15 were unable to cross the zone of inhibition posed by *Fusarium.* Isolate T1 was identified to be *T. atroviride*; T8 and T11 as *Trichoderma harzianum* (Courtesy: ARI, Pune). Lahlali and Hijri (2010) reported *T. atroviride* as a promising biocontrol agent which can also stay in endophytic symbiosis with plants.

**Dual culture on microscopic slide**

***Macroscopic observation of Trichoderma Isolate T4 and Fusarium oxysporum interaction on slide culture***

Macroscopic observation showed that the borderlines where *Trichoderma* and *Fusarium* encountered each other offensive as well as defensive mechanisms were activated. *Fusarium* showed magenta to purple pigmentation specifically where it encountered metabolites of *Trichoderma* and *Trichoderma* showed heavy sporulation where it encountered metabolites of *Fusarium* (Fig 9). Probably, these characteristics were materialization of cross signalling before any physical interaction. These results were comparable to those found in dual culture plate experiment.

**Microscopic observations of *Trichoderma* Isolate T4 and *Fusarium oxysporum* interaction on slide culture**

The mycelia of *Fusarium* showed abnormal morphology such as constrictions in mycelia giving a bead-chain appearance. Also, cell wall appeared thick and dark in *Fusarium* mycelia. The mycelia of *Trichoderma* did not show such abnormalities but showed branching as they approached *Fusarium* (Fig 10). Marked differences were observed in morphology on comparing *Fusarium* mycelia under direct influence of *Trichoderma* and that free from *Trichoderma* influence. *Fusarium* mycelia away from *Trichoderma* showed smooth, hyaline and normal morphology. Two broad phenomena were observed in the results obtained in dual culture experiment. First, antibiosis; in terms of counter inhibition which clearly states that not only *Trichoderma* inhibits growth of *Fusarium* but *Fusarium* also exerts inhibitory activity on *Trichoderma*.

**Table 2.** Comparing growth rates of *Trichoderma sp.* (Isolate T1) and *Fusarium oxysporum*

| Time hours | Growth of *Trichoderma sp.* (T1) Radius, mm | Growth of *Fusarium oxysporum* Radius, mm |
|---|---|---|
| 0 | 0 | 0 |
| 24 | 5 | 4 |
| 48 | 17 | 7.5 |
| 72 | 33 | 12 |
| 96 | On the walls of plate, 53 | 17 |
| 120 | Overwhelming growth | 21.5 |

* In a nine cm plate, at temp 25$^{o}$C , pH 6.8, T*richoderma* (radius) = 5.3cm/96h= 550 µm/h, *Fusarium* (radius) = 1.7 cm/96h = 177 µm/h ≈180 µm/h, Growth rate ratio, *Trichoderma* / *Fusarium* = 550/180= 3.05, Growth rate of *Trichoderma* isolate T1 is about three times higher than that of *Fusarium oxysporum*. The high growth rate of *Trichoderma* is an added advantage to its dominance as a biocontrol agent over *Fusarium* or other pathogens which exhibit slow growth rate compared to that of *Trichoderma*. High growth rate is a supportive factor in the mechanism of parasitism.

**Table 3.** Evaluation of effectiveness of *Trichoderma* species on the basis of total time taken to overwhelm and parasitize *Fusarium oxysporum.*

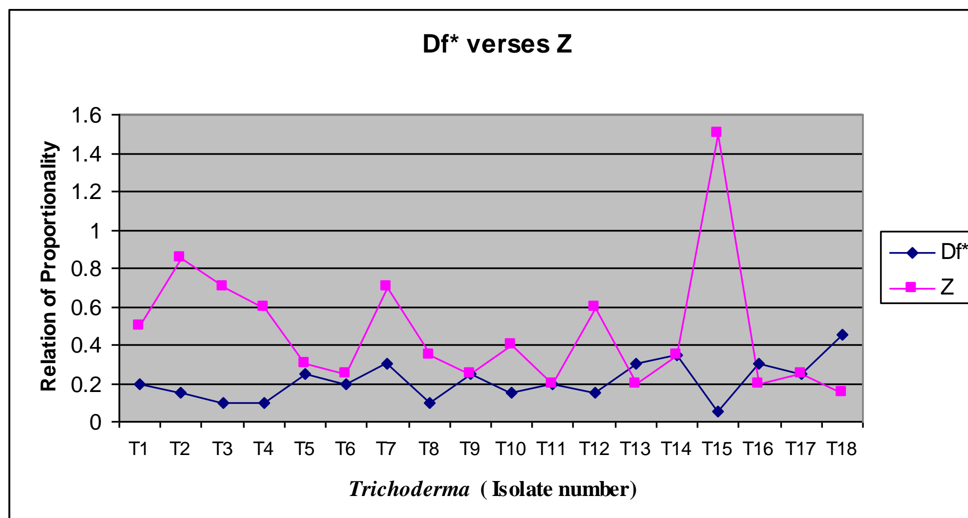
| source | Isolate no. | Time taken to cross zone of inhibition (Hours) | Time taken to overwhelm/parasitize (Hours) | Total time taken by *Trichoderma* to parasitize *Fusarium* (Hours) |
|---|---|---|---|---|
| Rose (*Rosa* sp.) | T1 | 8 | 40 | 48 |
| Pepal (*Ficus religiosa*) | T2 | 12 | 76 | 98 |
| Guava (*Psidium guajava*) | T3 | 16 | 72 | 88 |
| Mango (*Mangifera indica*) | T4 | 14 | 120 | 134 |
| Mehndi (*Lawsonia inermis*) | T5 | 12 | 42 | 54 |
| African marigold (*Tagetes erecta*) | T6 | 10 | 44 | 54 |
| Garden pea (*Pisum sativum*) | T7 | 28 | 96 | 124 |
| Garden pea (*Pisum sativum*) | T8 | 14 | 38 | 52 |
| Garden pea (*Pisum sativum*) | T9 | 12 | 70 | 82 |
| Alpine aster (*Aster alpinus*) | T10* | -- | -- | -- |
| Ginger (*Zingiber officinale*) | T11 | 14 | 38 | 52 |
| Aleo vera (*Aleo vera*) | T12 | 20 | 58 | 78 |
| Custard apple (*Annona squamosa*) | T13 | 16 | 80 | 96 |
| Bougainvillae "Buttiana" | T14 | 24 | 168 | 192 |
| Green chilly (*Capsicum frutescens*) | T15* | -- | -- | -- |
| Vinca (*Vinca rosea*) | T16 | 16 | 70 | 86 |
| Currypatta (*Bergera Koenigii*) | T17 | 10 | 70 | 80 |
| French marigold (*Tagetes patula*) | T18 | 8 | 64 | 72 |

* Did not cross the zone of inhibition, †Average of replicates taken

Each isolate of *Trichoderma* exhibits uniqueness in its response to the presence of pathogen as shown by the graphs. Second, parasitism is shown by *Trichoderma* on *Fusarium* only in case *Trichoderma* is able to overcome inhibition posed by *Fusarium*. Also, the time taken by each *Trichoderma* isolate to overcome inhibition posed by pathogen and thereafter enter parasitic phase is important in determining its efficiency as a potential biocontrol agent. Other researchers have also scrutinized these mechanisms of biocontrol shown by *Trichoderma*. Vinale et al. (2008) studied pre-contact events of the myco-parasitic interaction and divided the interaction into two phases. In the first phase, the myco-parasite (*Trichoderma*) produces specific high molecular weight compounds that reach the host (pathogen). In the second phase, low molecular weight degradation products that are released from the host cell walls reach the myco-parasite and activate the myco-parasitic gene expression cascade. A number of secondary metabolites produced by *Trichoderma* spp. are involved in pre-contact events. These belong to three categories. First, volatile antibiotics such as 6-pentyl-alpha-pyrone (6pp) and isocyanide derivatives, second, water soluble compounds such as heptelidic acid or koningic acid, and third , peptaibols which are linear peptides known to inhibit radial growth of many fungi (Fuji et al., 1978). It has also been stated that low molecular weight, non polar, volatile compounds such as 6pp have a quite long distance range of influence on the host. Production of these secondary metabolites is stated to be strain dependent (Vinale et al., 2008). In the present research, this could be one reason why each *Trichoderma* isolate showed distinct response to presence of *Fusarium*. A number of antibiotics have been isolated from *Trichoderma* spp by other workers. Harzianolide from *T. harzianum* was found to inhibit germination of *Fusarium oxysporum* conidia and chlamydospores. Viridin produced by *T. viride* also inhibits spore germination of a number of fungi. Other antimicrobial agents such as isonitrites, sesquiterpenes and diketopiperazines are released by different species of *Trichoderma* (Brian and Hemming, 1945; Harman and Kubicek, 1998). Jones and Hancock (1988) stated that secondary metabolites of *Trichoderma* can reduce uptake of amino acids and glucose in the target fungus by 85%. It was also demonstrated that trichorzianines modify membrane permeability of liposomes and disturb ionic balance in the cells of target fungus (Goulard et al., 1995).

**Fig 1.** *Trichoderma* Isolate T15 and *Fusarium oxysporum* on a dual culture plate. Strong antibiosis is shown by *Trichoderma* and *Fusarium* on each other. *Fusarium* is confined to a small area and is unable to extend its growth. On the other hand, *Trichoderma* also experiences inhibition from *Fusarium* as indicated by the pattern of inhibition zone.

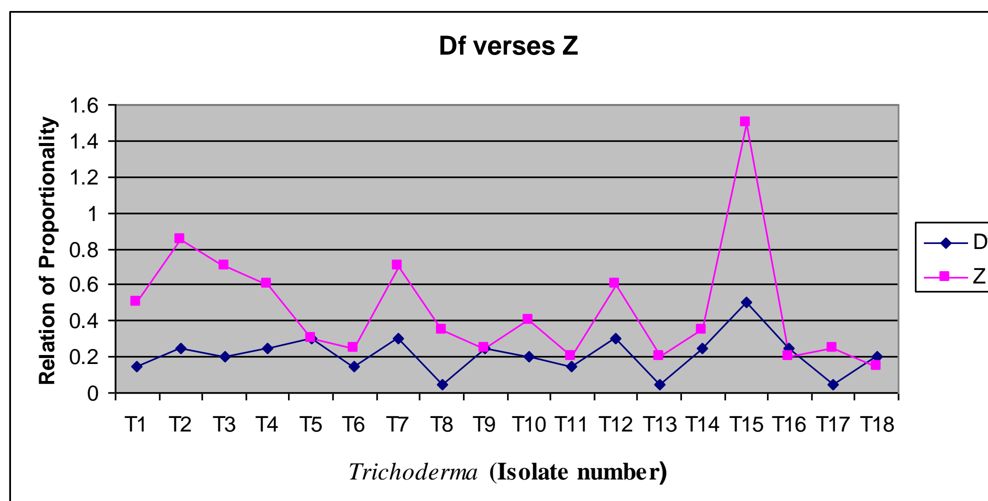
Researchers (Lorito et al., 1996b; Fogliano et al., 2002) found that peptaibols act synergistically with beta-glucanases of *Trichoderma* and inhibit beta-glucan synthase activity in the host fungus. The inhibition of this enzyme in the host prevents reconstruction of the host cell wall. Therefore, in the present research work, the waxy appearance and scanty mycelia of *Fusarium* may be a result of these synergistic activities (Fig 6). Chet (1990) interpreted that myco-parasitism by *Trichoderma* can be distinguished into four stages. The first stage is positive chemotropism which means that *Trichoderma* can detect its host from a distance. *Trichoderma* begins to branch as it approaches its target host. This phenomenon is very clearly visible in the microscopic image of dual culture on slide shown in this research (Fig 10). The next stage is molecular recognition event between parasite and host. This involves interaction between complementary molecules; likely to be lectin – carbohydrate interaction (Elad et al., 1983a). Lu et al., 2004, suggested that particular compounds released by the host cell walls were responsible for inducing activation of myco-parasitic genes in *Trichoderma*. Similarly, Zeilinger and Omann (2007) found that diffusible factors from host fungus are recognized by *Trichoderma* which activate transcription of myco-parasitism related genes. The authors also specified that cAMP signalling pathway was found to be involved in *T. atroviride* gene activation. During recognition events many alterations take place in the host cell such as plasma membrane retraction, cytoplasm aggregation, formation of numerous septae in the host and initiation of cell wall degradation (Harman and Kubicek 1998, Benhamou and Chet, 1997). Such alterations have also been notice in this research as shown in the microscopic slide dual culture (Fig 10). Further, in the third stage, the parasite attaches and coils it self on host (Elad et al., 1983b; Harman et al., 1981). The final stage is the lytic activity involving a range of enzymes to degrade fungal cell wall. The degradation activity is mainly due to chitinases, glucanases and proteases (Elad et al., 1982). 1-4-beta-N-acetylglucosaminidase is among the first enzymes induced in *Trichoderma harzianum* in early stages of host recognition (Inbar and Chet, 1995). The expression of *Trichoderma* chitinases is very specifically regulated by the host, therefore, it is thought to play an important role in antagonism against pathogens (Haran et al., 1996). Chitinolytic enzymes studied in *T. atroviride* showed inhibitory effect on the germination and hyphal elongation of several pathogenic fungi including *Fusarium* species. Also, many morphological changes were noted in the target fungus such as swelling, branching, necrosis and vacuolization by researchers (Harman and Kubicek, 1998). Cellulolytic enzymes of *T. longibrachiatum* have also been implicated in biocontrol activity (Migheli et al., 1994). The demonstration of final effect of the myco-parasite *Trichoderma* on its host fungus largely depends on synergism shown among the assortment of enzymes produced and also on synergism shown between enzymes and antibiotics produced by the attacking myco-parasite. DiPietro et al. in 1993 proved that *Trichoderma* enzymes and antibiotics showed 95% inhibition of pathogen spore germination when they acted in synergism but singly they were able to show only 20% inhibition. Schirmbock et al. (1994) noted similar phenomenon with *T. harzianum* antibiotics trichorzianines and enzymes endochitinase, chitobiosidase and glucanase which showed better results in synergism rather than singly. Synergism between hydrolytic enzymes and antibiotic peptaibols of *T. harzianum* was found effective against *F. oxysporum* (Lorito et al., 1996b). Also, *F. oxysporum* cell wall chitin is buried in beta-glucans. Thus, synergistic activity of chitinase with beta-1,3- glucanase is essential. Lipases and proteases are also required at the same time to clear hindrances in cell wall degradation. As these enzymes diffuse and reach the host, degradation activity begins even before physical contact takes place (Cherif and Benhamou, 1990). *Trichoderma* have specific mechanisms to protect their own cell wall from their own lytic enzymes. One of such mechanisms involves activity of protein Qid3 (Lora et al., 1994; Adams et al., 1993). It has been interpreted in the results of this work that *Fusarium* also exerts a deleterious effect on the approaching myco-parasite *Trichoderma* as depicted by the graphical representation in this work (Fig 4 and Fig 5). Other researchers have also noted the same phenomenon where toxins such as fusaric acid produced by *Fusarium* species can adversely affect antagonistic activity of *Trichoderma* by down regulating *Trichoderma* myco-parasitism related genes. Therefore, toxins produced by *Fusarium* give this pathogen a competitive attribute (Lutz et al., 2003). In a study conducted by El-Hasan et al. (2008) it was found that fusaric acid produced by *Fusarium oxysporum* and many other species of *Fusarium* is directly implicated in retarding mycelia growth and conidia production in *Trichoderma.* Further, in response to this effect of *Fusarium*, activity of 6- pentyl-alpha-pyrone from *Trichoderma* is generated to degrade and/or suppress synthesis of fusaric acid. In the present research, this can be one reason for initial halt in the progress of *Trichoderma* towards *Fusarium* (Fig 1) after which *Trichoderma* moved ahead to parasitize *Fusarium* only when it was able to overcome inhibitory metabolites of *Fusarium* (Fig 7). In this research work, another feature that has been marked is excessive pigmentation by *Fusarium* where it encountered presence of *Trichoderma* (Fig 6 and Fig 9). This activity is believed to be a response to the environmental changes in the vicinity of the fungus. Signal cascades are activated in fungi by such environmental changes which in turn alter their gene expressions (Xu, 2000; Idnurm and Howlett, 2001). High production of pigment could be one such response to presence of *Trichoderma* and its metabolites in the vicinity. Carlile (1956) reported that red-purple pigmentation in *F. oxysporum* was a result of carotenoids and a mixture of substituted dihydroxy naphthoquinones, the production of which was dependent on C/N ratio, light-dark conditions and pH variations. According to Naim and Sharoubeem, 1963, pigmentation in *Fusarium*




| Isolate no. | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 | T9 | T10 | T11 | T12 | T13 | T14 | T15 | T16 | T17 | T18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Df* | 0.2 | 0.15 | 0.1 | 0.1 | 0.25 | 0.2 | 0.3 | 0.1 | 0.25 | 0.15 | 0.2 | 0.15 | 0.3 | 0.35 | 0.05 | 0.3 | 0.25 | 0.45 |
| Z | 0.5 | 0.85 | 0.7 | 0.6 | 0.3 | 0.25 | 0.7 | 0.35 | 0.25 | 0.4 | 0.2 | 0.6 | 0.2 | 0.35 | 1.5 | 0.2 | 0.25 | 0.15 |

Df*- Difference in *Fusarium* growth on the same dual culture experiment plate, Z- Zone of inhibition

**Fig 2.** Zone of inhibition analysed in relation to growth of *Fusarium* under influence of *Trichoderma*.
(I A. Considering difference in growth of *Fusarium* under direct intense influence of *Trichoderma* and under lesser influence of *Trichoderma* on dual culture plate: Df* verses Z. No relation of proportionality was observed.)

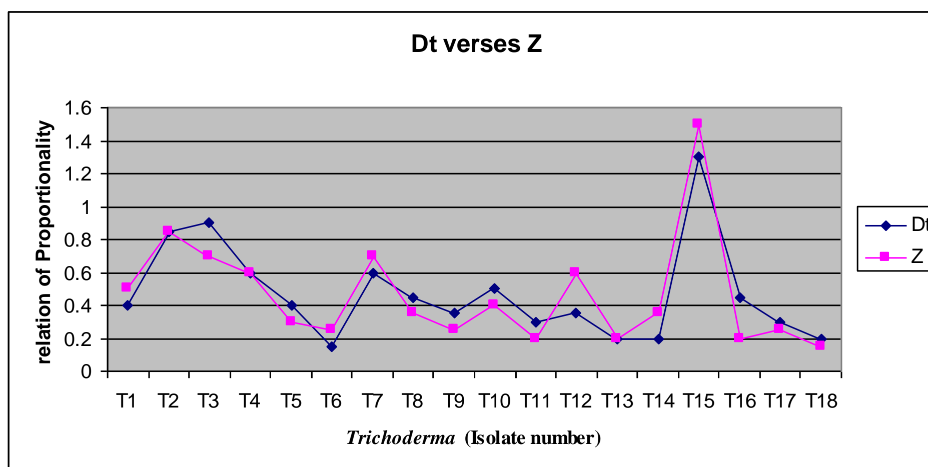



| Isolate no. | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 | T9 | T10 | T11 | T12 | T13 | T14 | T15 | T16 | T17 | T18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Df | 0.15 | 0.25 | 0.2 | 0.25 | 0.3 | 0.15 | 0.3 | 0.05 | 0.25 | 0.2 | 0.15 | 0.3 | 0.05 | 0.25 | 0.5 | 0.25 | 0.05 | 0.2 |
| Z | 0.5 | 0.85 | 0.7 | 0.6 | 0.3 | 0.25 | 0.7 | 0.35 | 0.25 | 0.4 | 0.2 | 0.6 | 0.2 | 0.35 | 1.5 | 0.2 | 0.25 | 0.15 |

Df- Difference in *Fusarium* growth on the dual culture experiment plate and control plate, Z- Zone of inhibition

**Fig 3.** Zone of inhibition analysed in relation to growth of *Fusarium* under influence of *Trichoderma*. (I B. Considering difference in growth of *Fusarium* under direct intense influence of *Trichoderma* and under no influence of *Trichoderma*: Df verses Z. No relation of proportionality was observed.)

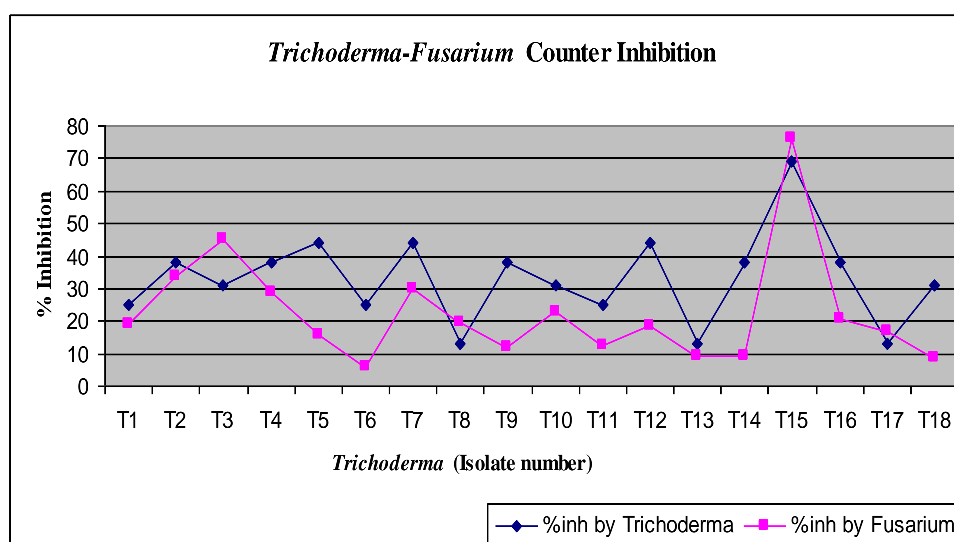
*oxysporum* was correlated to source and concentration of nitrogen, also, low concentration of phosphate helped production of colour. Researchers have given nutritional and physiological variations as a reason for pigmentation but their role in self defence or antagonism may also be possible as Son et al. (2008) indicated production of pigments under stress conditions. They also stated that Bikaverin which is a wine red pigment is produced by *Fusarium oxysporum* and possesses antifungal properties. Similar phenomenon has also been mentioned by Feng et al. in 2009. Therefore, biological control of pathogen is an outcome of bidirectional responses between the antagonist and the pathogen.




| Isolate no. | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 | T9 | T10 | T11 | T12 | T13 | T14 | T15 | T16 | T17 | T18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dt | 0.4 | 0.85 | 0.9 | 0.6 | 0.4 | 0.15 | 0.6 | 0.45 | 0.35 | 0.5 | 0.3 | 0.35 | 0.2 | 0.2 | 1.3 | 0.45 | 0.3 | 0.2 |
| z | 0.5 | 0.85 | 0.7 | 0.6 | 0.3 | 0.25 | 0.7 | 0.35 | 0.25 | 0.4 | 0.2 | 0.6 | 0.2 | 0.35 | 1.5 | 0.2 | 0.25 | 0.15 |

Dt- Difference in growth of *Trichoderma* towards and away from *Fusarium*, Z- Zone of inhibition

**Fig 4.** Zone of inhibition analysed in relation to growth of *Trichoderma* under influence of *Fusarium*. The graph clearly shows inhibitory effect of *Fusarium* on growth of *Trichoderma*. A relation of proportionality is observed in the graph.




| Isolate no. | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 | T9 | T10 | T11 | T12 | T13 | T14 | T15 | T16 | T17 | T18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| %inh by *Trichoderma* | 25 | 38 | 31 | 38 | 44 | 25 | 44 | 13 | 38 | 31 | 25 | 44 | 13 | 38 | 69 | 38 | 13 | 31 |
| %inh by *Fusarium* | 19 | 34 | 45 | 29 | 16 | 6 | 30 | 19.5 | 11.9 | 22.7 | 12.5 | 18.4 | 9.5 | 9 | 76 | 20.9 | 16.6 | 8.6 |

**Fig 5.** Analysis of counter inhibition shown by both the organisms; *Trichoderma* and *Fusarium*

## Materials and methods

### *Isolation of pathogen*

The fungal pathogen under study was isolated from brown lesions on roots of diseased pea plant obtained from severely wilt afflicted agricultural land of Ghana village, Jabalpur (India). The pathogenicity of this agent on pea *(Pisum sativum)* was confirmed by application of fungal spores and metabolites on pea seeds and seedlings. The pathogen was identified as *Fusarium oxysporum* f. sp. *pisi* (code: FP-2/G) and deposited under accession number NFCCI-2195 at Agharkar Research Institute, Pune, India.

### *Isolation and screening of potential antagonists of the pathogen under study*

### *Isolation of Trichoderma on selective medium*

Isolation of *Trichoderma* was performed on modification of *Trichoderma* selective medium defined by Williams et al. (2003). The medium consisted of 0.2g $MgSO_4.7H_2O$, 0.9g $K_2HPO_4$, 1.0g $NH_4NO_3$, 0.15g KCl, 0.15g Rose Bengal, 3g

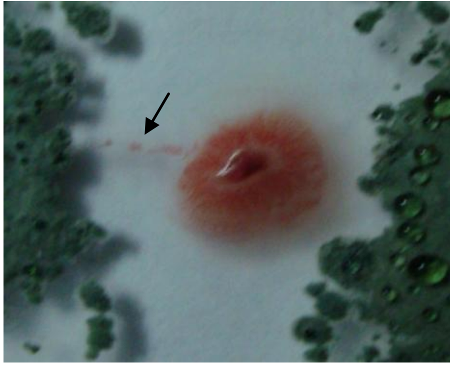


**Fig 6.** The arrow points out an extension from *Fusarium oxysporum* colony reaching *Trichoderma* sp. This strongly indicates role of chemo-attractants (positive chemotropism) between the two fungi before parasitic contact by *Trichoderma* as observed in *Trichoderma* isolate T12-*Fusarium oxysporum* dual culture plate.

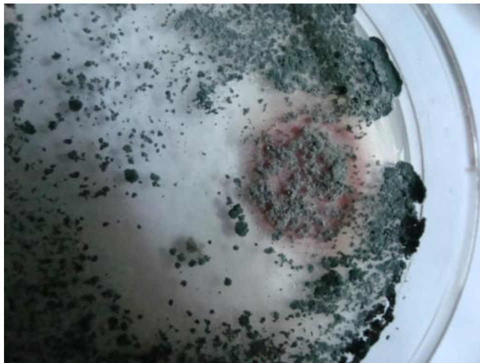


**Fig 7.** Overwhelming growth of *Trichoderma* isolate T8 on *Fusarium oxysporum* which indicates parasitic interaction. Heavy sporulation by *Trichoderma* is seen on and in vicinity of *Fusarium* colony.

Glucose and 20g agar in 950 ml distilled water. The medium was autoclaved at 121$^{o}$C for 15 min and cooled down to about 45-50$^{o}$C. 0.25g Chloramphenicol and 9.0 ml Streptomycin stock solution (1% wt/vol) added in 41 ml sterilized distilled water was mixed to this basal medium. Several healthy crop and ornamental plants from Jabalpur, India were screened to isolate *Trichoderma* from rhizosphere, rhizoplane and endophytic habitats. Eighteen of these plants have been mentioned in Table 1 and Table 3. Modified methods were adopted for isolation of *Trichoderma* from rhizosphere and rhizoplane (Hasan, 2002; Pandey and Palni, 1998).

***Isolation of Trichoderma from rhizosphere***

Roots of freshly obtained plants were taken and adhered soil was shed off. The roots were washed in minimum amount of sterilized distilled water. This washing was taken as sample suspension containing rhizosphere microbes. 0.1 ml of suspension was spread on medium.

***Isolation of Trichoderma from rhizoplane***

Roots were washed under tap water for about two minutes to remove all the adhered soil particles and cut into segments of 3-4 cm length. The segments were rinsed in sterilized distilled water several times and left in sterilized distilled water for 5 minutes. Thereafter, the segments were placed on medium.

***Isolation of endophytic Trichoderma***

Roots were washed under tap water for about two minutes and cut into segments of 4 cm length. The segments were treated with 0.05% of $HgCl_2$ for 30 seconds to one minute depending on the kind of root. The segments were rinsed several times in sterilized distilled water. About half centimetre portion was cut off from either side of each root segment and then the segment was placed on medium (modification of method by Paul et al., 2007). All isolates were maintained on Potato Dextrose Agar slants.

***Test of antagonism by dual culture technique (in 90 mm Petri-plate)***

Twenty six *Trichoderma* isolates were subjected to test of biocontrol activity against one isolate of *Fusarium oxysporum* f. sp. *pisi* by dual culture technique on Potato Dextrose Agar (PDA) medium (modification of method adopted from Morton and Strouble, 1955; Hajieghrari et al., 2008). The potential biocontrol agent *Trichoderma* spp. and the pathogen; *Fusarium oxysporum* f. sp. *pisi* (FP-2/G) were point inoculated 3cm apart on PDA medium plates. Twenty six such sets were prepared to study interaction of each of the 26 isolates of *Trichoderma* with one isolate of pathogen. Eighteen isolates of *Trichoderma* showing considerable antagonistic activity on pathogen were coded T1 to T18 and scrutinized for their pattern and mechanism(s) of biocontrol activity. The other eight isolates were slow growing or showed feeble antagonism. Therefore, they were not analysed further.

***Analysis of observations recorded in Trichoderma-Fusarium dual culture.***

1. Zone of inhibition analysed in relation to growth of *Fusarium* under influence of *Trichoderma*

A. Difference in growth (radius) of *Fusarium* towards (Xf) and away (Yf) from *Trichoderma* on dual culture plate was calculated (Yf-Xf=Df*) and plotted against zone of inhibition (Z) to study the pattern of relationship between Df* and Z.

B. *Fusarium* was more or less under influence of *Trichoderma* even on the distal side in the dual culture plate, therefore, one more comparison was made represented by plot between Df and Z where Df was the difference between Xf and Yfc (growth of *Fusarium* on control plate); Yfc-Xf=Df.

2. Zone of inhibition analysed in relation to growth of *Trichoderma* under influence of *Fusarium*

Difference in growth (radius) of *Trichoderma* towards (Xt) and away (Yt) from *Fusarium* was calculated (Yt-Xt=Dt) and plotted against zone of inhibition (Z) to study the pattern of relationship between Dt and Z.

3. Study of counter inhibition shown by the two fungi on each other

- %Inhibition by *Fusarium* on *Trichoderma* = (Dt/ unrestricted growth of *Trichoderma*) × 100
- %Inhibition by *Trichoderma* on *Fusarium* = (Df/ unrestricted growth of *Fusarium*) × 100

The counter inhibition was graphically analysed.

4. Calculation and comparison of time taken by *Trichoderma* isolates to overcome zone of inhibition and parasitize *Fusarium*.

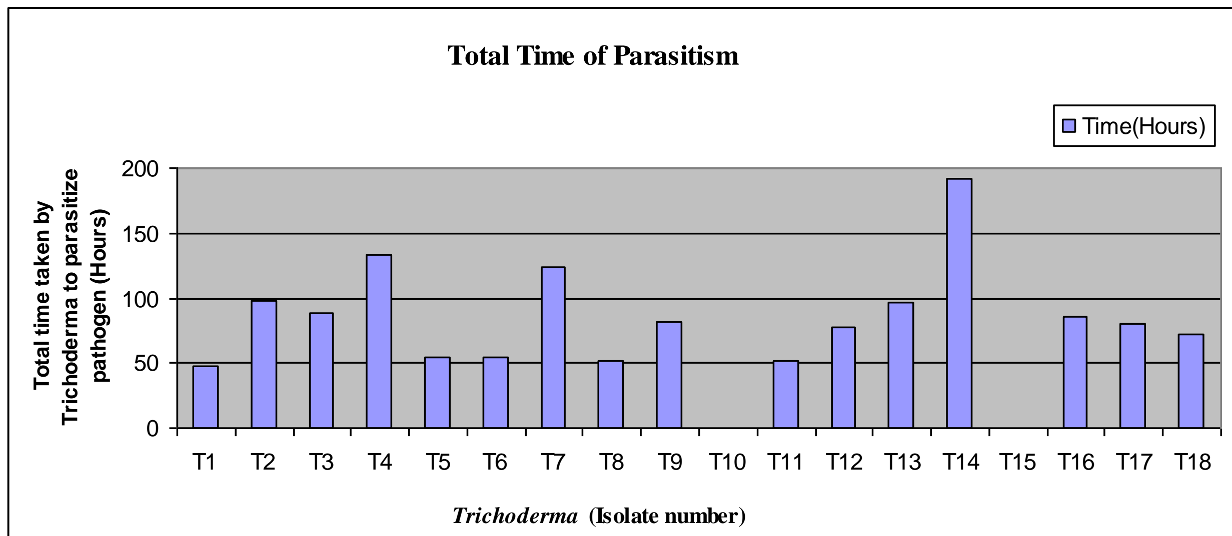



**Fig 8.** Evaluation of effectiveness of *Trichoderma* on the basis of total time taken to overwhelm pathogen *Fusarium oxysporum*

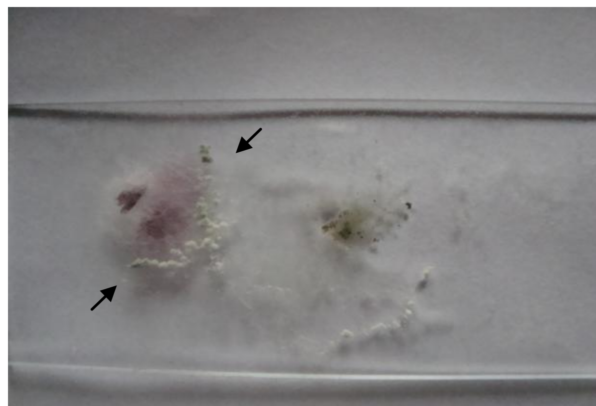


**Fig 9.** *Trichoderma* isolate T4 - *Fusarium oxysporum* dual culture on microscopic slide showing a remarkable phenomenon where interaction between *Trichoderma* and *Fusarium* is manifested very clearly. *Trichoderma* encloses *Fusarium* as indicated in the figure. Purple pigmentation by *Fusarium* is observed at the interface where the two fungi meet as discussed in the text.

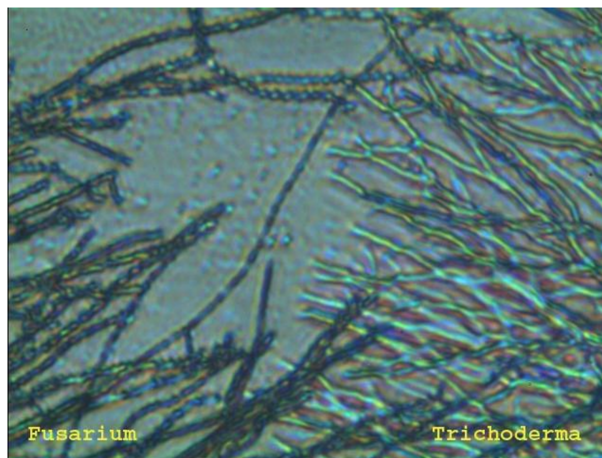



**Fig 10.** *Trichoderma* isolate T4 - *Fusarium oxysporum* dual culture (10$_{\times}$10X) , Microscopic observation of dual culture slide reconfirms the effect of both the organisms on each other. Figure shows deformed mycelia (bead chain like structure) of *Fusarium* and branching in the advancing mycelia of *Trichoderma*.

## Conclusion

The bottom line of this work is that among several criteria defined for an ideal biocontrol agent, one important criterion is a fast acting agent. An isolate of *Trichoderma* which can overcome the inhibition posed by the pathogen and parasitize the pathogen in a short span of time will be an efficient biocontrol agent. In this research *Trichoderma* isolate T1 (*T. atroviride*) showed quicker action than the others. After reaching the verge of inhibition zone posed by *Fusarium*, this agent took about two days to parasitize the pathogen. More isolates shall be screened to find a better agent in terms of rapid activity and the present isolates shall be subjected to genetic modifications. Brunner et al. (2005) reported that their isolate of *T. atroviride* took five days to show parasitism on the pathogens such as *Pythium* and *Rhizoctonia*. This also suggests that there may be variations in pattern and time duration of parasitism depending upon the pathogen encountered.

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