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ROLE OF TEMPERATURE, MOISTURE AND *TRICHODERMA* SPECIES ON THE SURVIVAL OF *FUSARIUM OXYSPORUM CICERI* IN THE RAINFED AREAS OF PAKISTAN

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Abstract

It has been observed in the present study that when spores of *Trichoderma harzianum (Th-2)* isolate were applied in the sandy clay loam soil and continuously incubated for 4 months at 25°C and 35°C and at three water potentials, -0.03 MPa, -0.3 MPa and <-50 MPa, it has resulted in significantly reduced ($P<0.05$), growth of *Fusarium oxysporum ciceri (Foc)* on branches of chickpea plant. The pathogen population was greatly reduced in the moist soil (-0.3 MPa) when compared with the wet soil (-0.03 MPa) at both temperatures which was indicated by greater colonization and growth of *T. harzianum-2* on the branch pieces of chickpea plants. The pathogen was completely eradicated from the chickpea branch pieces, after 6 months at 35°C in the moist soil. In air-dry soil (<-50 MPa), *Foc* survived in 100% of the branch pieces even after 6 months at both temperatures. When chickpea plant branch pieces having pathogen was sprayed with *T.h-2* antagonistic isolates of *Trichoderma* spp., the *T.h*-2 isolate killed the pathogen up to minimum level (10-12%) after 5 months at 35°C in the sandy clay loam soil. It can be concluded that in chickpea growing rainfed areas of Pakistan having sandy clay loam soil, *Foc* can be controlled by using specific *Trichoderma* spp., especially in the summer season as after harvest of the crop the temperature increased up and there is rainfall during this period which makes the soil moist. This practice will be able to reduce the inoculum of *Foc* during this hot period as field remain fallow till next crop is sown in most of the chickpea growing rainfed areas of Pakistan.

Introduction

Among *Fusarium* wilts the chickpea wilt caused by *Fusarium oxysporum f.sp. ciceri* is a very serious threat to all chickpea (*Cicer arietinum* L.) growing areas of the world (Anjaiah, 2003; Trapero-Casas & Jimenez-Diaz, 1985). The areas under the cultivation of chickpea in Pakistan are rainfed and the life activities of the farmers of those areas completely rely on the success of this crop. As no other crop can grow properly in that region as its demand for water is least and being a leguminous no fertilizer inputs are needed. In Pakistan annual yield losses due to this disease are 12 to 15% (Haq & Jamil, Personal communication). Use of fungicides is unaffordable and any cultural practices are useless as pathogen is established in those soils and can survive for many years. Currently there are few satisfactory management practices for its control but keeping in view the situation of the areas under cultivation they have less value so the option of biocontrol is left since no resistant variety is available nor chemicals are affordable by the poor people of these areas. Since total dependency is in its cultivation so crop rotation is also impracticable.

Controlling the plant diseases by pollution free biocontrol agents are desirable now a days, which are alternative to the chemical pesticides for the control of plant diseases especially the soil-borne (Roberts *et al*., 2005). Among various biocontrol agents of the soil-borne plant pathogens, the fungal bio-control agents are very prominent and reported by various researchers. Among the fungal agents, *Trichoderma* species have been reported to be very effective biological control agents against a number of soil borne diseases (Lewis *et al*., 1998; Wong *et al*., 2002; Ahmed *et al*., 2003; Roberts *et al*., 2005). The efficacy of biocontrol ability of an efficient biocontrol agent depends upon its different mechanisms have been suggested for their biocontrol activity, which include competition for space and nutrients, secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds (Haram *et al*., 1996; Zimand *et al*., 1996). But the main hindrance in their commercialization is their specificity in action against the plant pathogens. Some *Trichoderma* species have been commercialized not all of which show their efficient performance against specific diseases. But the main hindrances in their commercialization is their specificity in action against the plant pathogens which is a common problem with the use of these biocontrol agents in addition to others (Spadaro & Gullino, 2005). As the biocontrol efficacy is biological in nature so it depends upon many factors and its efficacy can be improved by adjusting those factors in favour of biocontrol agents so that they can efficiently do their job (Spadaro & Gullino, 2005).

Temperatures, soil moisture and soil type are among the important factors which can affect the efficacy of a biocontrol agent (Spadaro & Gullino, 2005). Studies were therefore conducted to know the efficacy of these factors that how these different factors may affect the efficacy on *Trichoderma* spp., and which in turn will effect on the survival of *Fusarium* pathogen which is established in the soils of rainfed areas since several years. This could be helpful in solving the problem of regular decrease in chickpea yield to such a extent where no any other crop is rarely successfully grown and economic position of the farmers can be improved without considerable inputs. The chickpea crop is regularly sown in rainfed areas of Pakistan and pathogen is getting more chances for establishment in those areas. The author found very few reports about the successful use of a biocontrol agent for specified areas like rainfed areas. Our research results are an example of such reports and will be useful for the farmers of those areas having sandy clay loam soils. Very little attention has been given towards the rain fed areas which are poor in production but are considerable parts of agricultural land in Pakistan as more emphasis is normally given to research in the irrigated areas which are high productive, rich in nutrients and organic matter.

Materials and Methods

Fungal pathogen: The fungal pathogen was isolated from the stem of naturally infected chickpea plant collected from *Fusarium* infested chickpea field area of Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan. It was cultivated on potato dextrose agar (PDA) for 7 to 10 days at 25°C until sporulation. All Petri plates were incubated in an alternating temperature regime, 25°C day/20°C night. The fungal pathogen was maintained on PDA at 25 ± 2°C and was sub cultured onto fresh PDA plates within a period of 3 months. The pathogenicity test was performed before use and pathogen was identified from infected chickpea plant with typical symptoms of *Fusarium* wilt and reported to be virulent on chickpea plants as described by Haq & Jamil (1995).

The fungal inoculum for the experiment was prepared as follows: Mature chickpea plant sticks which were harvested and were healthy when taken (other than stem) and that all should be of uniform diameter and cut in small pieces of 4-cm each. The pieces were soaked in water overnight and drained next day. They were put on tissue paper for 5 minutes for external drying and then transferred in conical flasks and steam sterilized for 1 h at 121^{o}C for three consecutive days for killing all microorganisms including those present deep in the tissues. They were then removed from autoclave. Under sterilized conditions they were inoculated with agar plugs of *Fusarium oxysporum ciceri* and incubated at 25 ± 2°C for four weeks. After four weeks the inoculum was stored at 4°C until use.

Fungal antagonists: The fungi used in the experiments are given in Table 1. The *Trichoderma* spp., were grown on the sterilized PDA and maintained at 25 ± 2°C.The antagonistic fungi were passed through a 50μm sieve to obtain mainly the spores of these antagonistic fungi for inoculation to the chickpea sterilized sticks.

Soil: As the soil in the rainfed areas is mostly sandy clay loam and clay loam with very less variance in soil pH so only these soils were selected for the experimentation. Each soil was air-dried, sieved through a 5-mm mesh and stored in plastic bags at 4°C until ready for use. The moisture characteristics of the soil, obtained by the filter paper method followed by (Deka *et al*., 1950) and three moisture level were maintained throughout in the experiment.

Role of *Trichoderma* spp., on the growth of *Fusarium oxysporum ciceri* in the soil: The experiment was designed to study the effect of applying spores of *Trichoderma harzianum-2* to chickpea plant stem pieces infested with *Foc* and following the observations on survival of the pathogen for 6 months in sandy clay loam soil under air – dry (<-50MPa), moist (-0.3 MPa) and wet (-0.03 MPa) soil conditions at 25°C and 35°C. The main treatments in the experiment were: (a) Control (*Foc* colonized sticks sprayed with sterilized distilled water, (b) *Trichoderma* was sprayed @ (10^7 cfu/ml) to the *Foc* infested sticks until the stick pieces were not completely moistened by the spray. Each replicate consisted of 10 sticks which were buried in 50g of air-dry soil in 9-cm-diameter sterilized plastic Petri plates. The condition of the soil was then changed either a) leaving it as air dry soil, b) moistened with sterilized distilled water to raise the water potential to -0.03 Mpa (wet) or -0.3 MPa (moist). Each plate was sealed with parafilm to reduce any loss of water or any type of contamination. There were 10 replicates. Half of the replicates (five) were incubated at 25°C and the other half at 35°C.They were placed in an incubator that had humidifier to reduce chances of water loss. The sticks were sampled after every two months. At the time of each sampling, 5 replicates (total of 50,including 5 of control) were washed free of soil, surface sterilized with 5% Sodium hypochlorite in 50% ethanol for 1 min, rinsed three times with sterilizes water and plated on PDA plates and after 5-7 days the number of sticks yielding the characteristic *Foc* was recorded and the percentage survival of the pathogen determined from each of the treatment (Haq & Jamil, 1995). The percent recovery of the *Trichoderma* from the sticks was also recorded. Moreover keeping in mind the moisture contents of the soil as it was opened for a while at the time of sampling, at the beginning of the experiment and at sampling time, the water contents of the soil of the 10 plates randomly selected from the treatments the soil was subjected to oven-drying at 105°C for 48h and then weighed.

Table 1. Fungal antagonists used in the experiment.

Trichoderma isolates	Source
Trichoderma harzianum-CPR-2	Dr. S.R. Gowen, Crop Protection Lab., Dept. Agriculture, University of Reading, UK.
Trichoderma viridi	Dr. D. Smith, IMI, Surrey, UK.
T. harzianum-2	Dr. J. Deacon, University of Edinburgh, UK.
T. harzianum- CPR-5	Dr. S.R. Gowen, Crop Protection Lab., Dept. Agriculture, University of Reading, UK.

Evaluation of various isolates of *Trichoderma* spp., on the survival of *Fusarium oxysporum ciceri* in the two soils: The present investigation involved comparing the effectiveness of different isolates of *Trichoderma* species in reducing the survival of *Foc* in sticks buried in the soil. Four *Trichoderma* isolates (Table 1) were cultured and were multiplied. Chickpea sticks colonized by the pathogen were sprayed with distilled water (controls) or with spore suspension of each of the antagonistic *Trichoderma* spp., as done before. Each replication included 10 sticks buried in 50g of soil in a Petri plate. The soils were moistened to a water potential of -0.3 MPa, the plates were sealed with the parafilm and placed in a humidified incubator as done before at 30°C. After every month the sticks were washed and surface sterilized and plated on PDA. After incubating for 5-7 days at 25°C, the percent survival of the pathogen and percent recovery of the *Trichoderma* spp., were determined. Determination of water contents of the soil was done at the beginning of the experiment and at each sampling time, as been previously described.

Results

Role of *T. harzianum*-2 on the survival of *Fusarium oxysporm ciceri* in clay loam soil: After incubation period of 2, 4 and 6 months in the air dry soil (<-50 MPa) keeping temperatures at 25°C and 35°C, 100% of the sticks in the both treatments (Control and *T h* treatment) at 25°C and at 35°C, yielded the pathogen when plated on the PDA agar plates. After 2 months incubation in the moist (-0.3 MPa) and in the wet (-0.03 MPa) soils, the survival of the pathogen in the sticks sprayed with *Th* compared to the controls was significantly reduced to 35°C and 25°C (Figs. 1 and 2). After 4 months, *Th* had reduced the survival of the pathogen significantly at both water potentials and both temperatures. The pathogen was completely eradicated from the sticks at 35°C but was still present on 25% of the sticks at 25°C, after 6 months incubation period in the moist soil. *Th* was yielded from all the sticks from which the *Foc* was not recovered on agar. However, it was observed that *Th* was less effective in the wet soil (Fig. 2) as compared to 100% in the moist soil and the percent recovery of *Th* from the inoculated chickpea branch sticks incubated at 35% for 6 months was only 45% in the wet soil. There were a lot of saprophytic nematodes, other unidentified aquatic fungi and bacteria present on many of the sticks that had been incubated in wet soil in all temperatures, but none was found in moist soil as has been reported earlier by Wong *et al*., (2002). The water contents of the soils remained the same as there was no significant difference in the

amount of water which was determined at the end of the experiment and at the time of taking the sample.

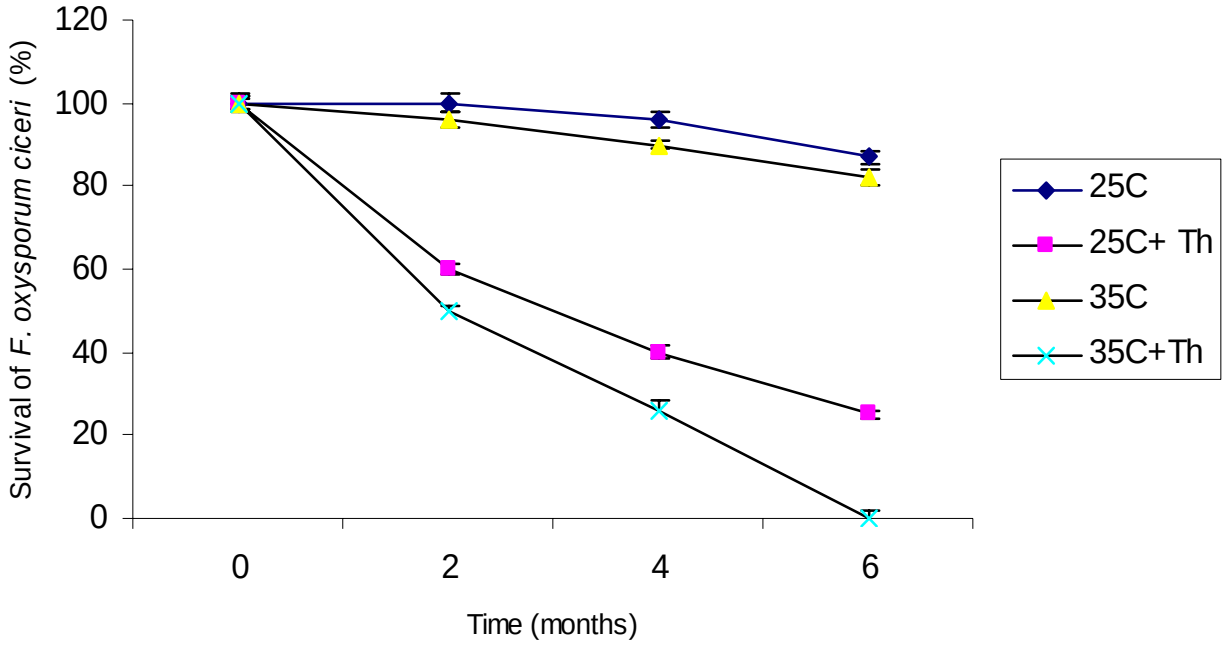

Fig. 1. Role of *Trichoderma harzianum-2* on the survival of *Fusarium oxysporum ciceri* in plant sticks incubated in moist (-0.3 MPa) sandy clay loam soil at 25°C and 35°C over 6 month period (vertical bars are standard deviations of the means).

Role of *T. harzianum*-2 on the survival of *Fusarium oxysporm ciceri* in sandy clay loam and clay loam soils: The four *Trichoderma* isolates differed considerably in their efficacy in reducing the survival of *Foc* in sticks in the moist soils at 35°C (Fig. 3). Isolates *Th-2* (*T. harzianum-2*) and isolates *Tv (T. viride)* were significantly more effective than the others in the sandy clay loam and clay loam soil. In general mostly in the barani (rainfed) areas this crop is usually being cultivated and this is the reason experimental soil was only sandy clay loam and clay loam. There was greater reduction of inoculum of *Foc* in the sandy clay loam soil as compared to clay loam. Although the pathogen's survival was significantly reduced by Trichoderma isolates, *T.h*-2 and *T.v* by 90% and 65% respectively in sandy clay loam soil and 60 and 20% respectively in clay loam after 4 months at 35°C. The water contents of the soils determined at each sampling were not significantly different ($P < 0.05$) from the original water contents and were remained the same.

Discussion

Our results showed that *Trichoderma harzianum* (isolate 2) significantly reduced the growth of *Fusarium oxysporum* when applied to chickpea plant branches infested with the pathogen and when these branches were buried in the sandy clay loam soil for a period of 4 months in moist (-0.3 MPa) or wet (-0.03 MPa) at a temperature of 25°C or 35°C. The biocontrol fungus was more effective in reducing the growth of the pathogen in moist than in wet soil but was more effective at 35°C. Greater amount of the antagonist was obtained from the sticks than the pathogen during the incubation in the moist soil, as the moist soil conditions appeared to be more supportive for the

antagonistic activity of *Th*-2 against the pathogen. The reasons for lower population of *Th*-2 in the wet soil compared to the moist soil could be due to greater antagonism by bacteria that flourished under the wet conditions (Troller & Stinson, 1981; Schnürer *et al*., 1985; Dandurand & Knudsen 1993; Zuberer & Kenerley, 1993) or parasitism by the numerous nematodes that were present on the stick pieces (Bae & Knudsen, 2001). It is reported that nematodes help in the proliferation of the pathogen and inoculum increases (Freckman & Caswell, 1985; Mai & Abawi, 1987; Powell, 1971; Bertrand *et al*., 2000; Luc *et al*., 2005; Pandey *et al*., 2005).

The pathogen was completely eliminated from the buried stick pieces in the presence of the *Th*-2 after 6 months in moist soil at 35°C, whereas about 40% of the sticks still contained the pathogen in the absence of *Th*-2. However, no decrease in the growth of the pathogen was observed in air-dry soil even after 6 months at both temperatures. The disease plant debris especially sticks on and in the soil favour the retention of the wilt pathogen and increased the amount of disease (Kaiser, 1973; Jimenez-Diaz *et al*., 1987). The evaluation of four antagonistic *Trichoderma* isolates for their efficacy in reducing the growth of the pathogen in a sandy clay loam and clay loam soil revealed that an isolate of *Th*-2 and an isolate *Tv* were the most effective in both soil types,whereas rest of all isolates reduced the growth of the pathogen to various extents. In general, *Trichoderma* spp., are favoured by acidic soil conditions (Chet & Baker, 1981; Papavizas, 1985) and the sandy clay loam are less basic than clay loam but still not very suitable for the growth and flourishing of the *Trichoderma*. Another important information can be reported from the present investigation that a few fungal isolates like *T.v* and *T.h*-2 have been identified which were highly effective in sandy clay loam but not much in clay loam soil. These isolates may be useful for controlling wilt of chickpea in the sandy clay loam rainfed areas of Pakistan.

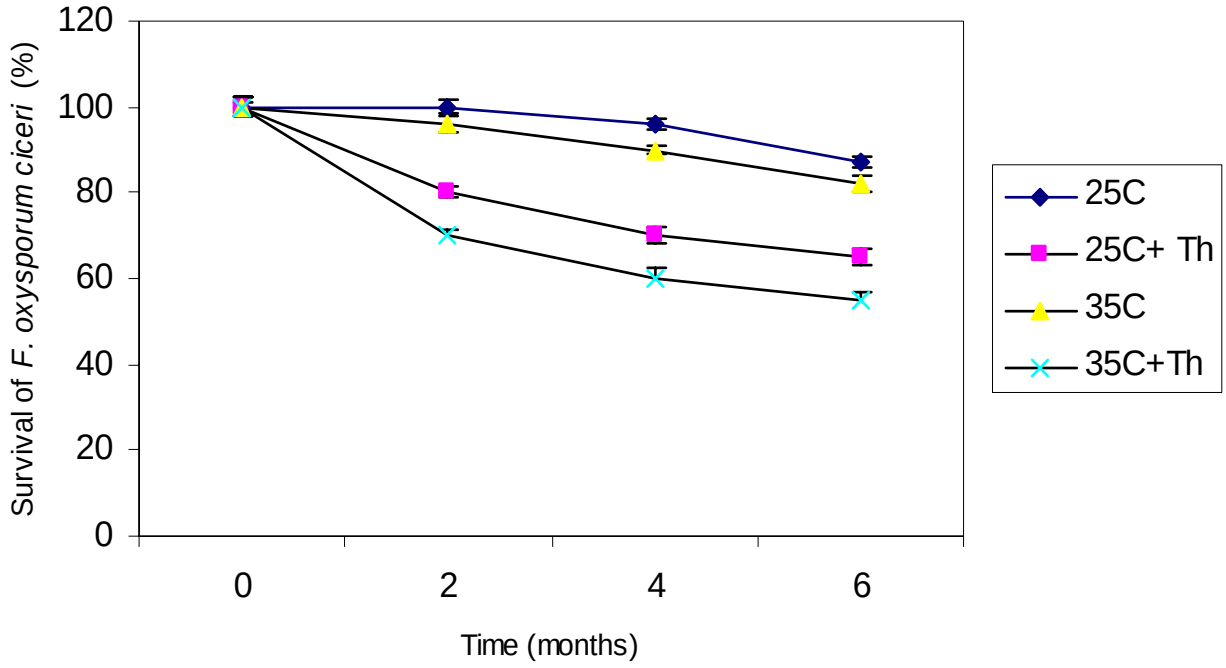

Fig. 2. Role of *Trichoderma harzianum-2* on the survival of *Fusarium oxysporum ciceri* in plant sticks incubated in moist(-0.03 MPa) sandy clay loam soil at 25°C and 35°C over 6 month period (vertical bars are standard deviations of the means).

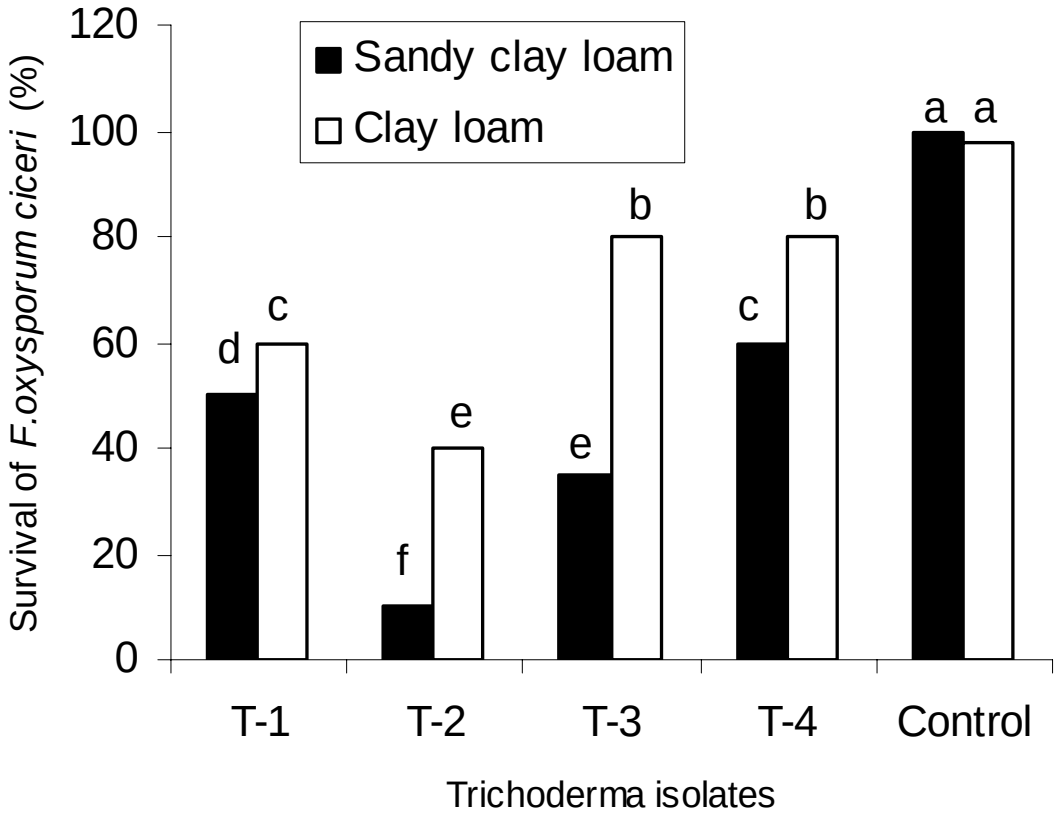

Fig. 3. Role of four *Trichoderma* isolates on the survival of *Fusarium oxysporum ciceri* in plant sticks incubated in two soil types under moist soil conditions (-0.3 MPa) after 4 months at 35°C, columns labelled by the same letter do not differ significantly at p=0.05 as per analysis of variance.

The present results revealed that the inoculum load of the *Fusarium* wilt pathogen, which in disease plant debris may be reduced significantly when applied into moist soils within 3-6 months by application of the biocontrol fungus to the plant debris before incorporation into soils in summer temperatures of 25-40°C. Our work is also supported by Wong *et al.*, (2002), who also used the two type of soil but texture was not mentioned only acidic and basic nature was mentioned. In fact under field conditions especially the soil texture is the most important as the mostly moisture retention ability varies with the texture. Moreover experimental temperatures were also different. But the moisture levels which were evaluated within the soils in the laboratory were the same and supporting to our results. Under rainfed conditions the sandy clay loam soil remains moist not wet. So this crop can be saved from the attack and loss by the wilt pathogen.

During rainy period the water leach down with salts in sandy clay loam soils and as salts leach down and making comparatively soil more acidic and internal soil is moist but in the clay loam water leaching down is very low which does not improve the acidic nature of the soil moreover internal soil is wet (Brady & Weil., 1999). For pathogen mortality moist and acidic conditions are favourable conditions as fungal antagonist love to flourish in acidic conditions. Moreover decomposition of the organic matter in the moist soil will be more which will further help in increasing the acidic conditions (Frey *et al*., 1999; Kalbitz *et al*., 2000).

However, the pathogen residing in the plant debris on the soil surface would not be as effectively controlled as the plant debris would dry out. But still after rain the debris will become wet and then soon dry due to high temperature in tropical and subtropical

countries in summer. As a result water will leach down but this alternate wet and dry condition will help in better decomposition of the organic matter. As the process of decomposition of organic matter is faster due to these wet and dry conditions of the plant debris (Birch, 1958), so plant debris on the surface can be decomposed, which can be helpful in making the soil conditions somewhat more acidic by mixing in the soil.

As far as reduction in inoculum in the soil is concerned, under moist conditions, the reduction is more as compared to wet conditions. But the choice of *Trichoderma* isolate is also important as some were ineffective under sandy clay loam soil conditions. This strategy is probably only pertinent to the summer-dominant rainfall chickpea growing rainfed sandy clay loam areas of Pakistan, where warm, moist soil conditions can be expected between the harvest and the sowing of the next chickpea crop. No crop other than chickpea is grown in those rainfed areas due to its least requirement of water, but not in irrigated and clay loam areas as irrigated areas don't remain fallow after harvest. Moreover, the internal soil conditions will remain moist after the water leaches in sandy clay loam soil and with high temperature and with *Trichoderma* the debris buried in the soil not only decomposed but the level of inoculum will also significantly decrease as compared to clay loam soils. Only debris buried in the soil will be able to reduce the inoculum and on the surface will not be destroyed, for this purpose after harvesting of the crop the debris should be buried in the soil after the spray of the selected *Trichoderma* spp., in the field area which could be helpful in reduction of inoculum potential. However, if tolerant cultivars are grown after this practice the crop can reach their full potential of yield when sown in rainfed areas. It is expected that this form of biocontrol is subsequently shown to be successful in the field, there would be good prospects for the utilization of *Trichoderma* species for chickpea wilt control in the rainfed areas having only sandy clay loam soil. Further research at the safe delivery and persistence in the soil and on the seeds of this fungal antagonist or antagonists needs further research, keeping in view the soil conditions which include temperature, moisture and pH factors principally in addition to other factors. These results are helpful for the all rainfed areas having the required temperature, soil texture and moisture prevailing during summer season and field remain fallow for one growing season.

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