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Differential biocontrol and rhizosphere competence ability in strains of *Trichodermaharzianum*

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Suitable local biocontrol agents with ability to associate and colonize the developing roots of *Chrysanthemum* plants were investigated. Seven strains of *Trichodermaharzianum* (*Th*) were isolated from the soils of Kanpur and Gorakhpur and were evaluated for their biocontrol potential against *Fusariumoxysporum*(*Fo*), a wilt pathogen of *Chrysanthemum* plant.All the 7 *Th*isolates effectively inhibited the radial growth of *Fo*, in dual culture studies and maximum significant (P<0.01) inhibition (67%) was recorded with isolate Th3 followed by Th4 while minimum with Th5 (49.2%). *Th* isolates were further screened for their rhizosphere competence; ability to colonize upper, middle and lower segments of C*hrysanthemum* roots, using root print method. All the test isolates demonstrated the capability to colonize the upper (1 to 4 cm) segment of the root. Only four (57%) out of seven isolates namely Th1, Th2, Th3 and Th6 exhibited ability to be associated with middle (5 to 8 cm) and lower or deeper root segment (9 to 12cm) of *Chrysanthemum* plant. These isolates were therefore, considered rhizosphere competent strains of *Th*for *Chrysanthemum* plant. No mycelia growth was observed from root segments between 5 to 8 cm depth inoculated with isolates Th4, Th5 and Th7, which were rated as non-competent. Results obtained revealed that the isolates which showed higher *invitro* antagonism against the pathogen were always not good rhizosphere competent. *Th* isolate Th4 showed higher inhibition in dual culture experiments than Th5, Th6 and Th7 but it failed to colonize the deeper segments of the host plant root. On the contrary isolate T6 showed low biocontrol potential in dual culture against the pathogen but demonstrated better rhizosphere competence. It is therefore, imperative to screen out the *Trichoderma* isolates with multiple potential prior to their application as agents for improving plant health at large scale.

Key words: *Trichodermaharzianum*, rhizosphere competence, *Fusarium oxysporum*, *Chrysanthemum*, biological control

Introduction

Biological control potential of a natural entity is an unique character which has been of great interest to researchers throughout the globe. Extensive work has been carried out to isolate, identify, validate, select and use various

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fungal isolates against soil borne plant pathogens. The fungal isolates which are widely studied for their biological control abilities are different species of *Trichoderma, Gliocladium, Aspergillus, Penicillium, Neurospora, Chaetomium, Fusarium, Dactylella, Arthrobotrys, Glomus* etc. Species of *Trichoderma* being a potential candidate of biological control have attracted large number of researchers throughout the globe. It has been successfully employed by several workers for biological control of fungal isolates pathogenic to various economically important crops (Jayalakshmi*et al.*, 2009; Muthukumar*et al.*, 2005; Mohamed and Haggag, 2005; Manka*et al.*, 1997; Katragadda and Murugesan, 1996; Locke *et al.*, 1985).

Several mechanisms have been suggested by researchers to explain the mode of action of *Trichoderma* species, which includes mycoparasitism, antibiosis, nutrient competition and starvation, induction of systemic resistance, etc (Dennis and Webster, 1971; Upadhyay and Mukhopadhyay, 1986; Chet, 1987; Howell, 2003). Mycoparasitism is a complex bio-logical-chemical process which involves recognition of the host, coiling, penetration and digestion of host's structures by enzymatic activity. Several researchers have opined that *Trichoderma* utilizes mycoparasitism as one of the main mechanisms for biological control of plant pathogens (Papavizas, 1985; Harman and Kuibreck, 1998; Howell, 2003; Vinale*et al.*, 2008). Starvation is another cause of inhibition of microbe in competition between antagonist and plant pathogen. *Trichoderma* have well proved its suitability as competitors for space and nutritional resources. It has been suggested that in the absence of mycoparasitism and antibiosis, starvation is the operating mechanism of biological control (Cook and Baker, 1983). *Trichoderma* species have been reported to produce various volatile and non-volatile compounds, antibiotics such as gliotoxin, glioviridin, viridin, alkyl pyrones, isonitriles, polyketides, peptailbols and some steroids which play significant role in the biological control (Dennis and Webster, 1971; Sharma and Dohroo, 1991; Tronsmo and Dennis, 1978; Upadhyay andMukhopadhyay, 1986; Howell, 1998).

The term "Rhizosphere Competence" was used initially by Ahmad and Baker (1987) to describe the ability of microorganisms to grow and function in the developing rhizosphere. Lo and Lin (2002) during their experiments in Cucumber plants observed that most of the *Trichoderma* strains do not have capability to colonize on all the parts of growing roots and their growth are mostly restricted to the upper portions. Lo and Lin (2002) and Miranda *et al.* (2005) suggested that only few *Trichoderma* isolates that are able to colonize the root segments at all the depth should be considered as competent for rhizosphere. *Trichoderma* isolates are known to colonize the plant root in U shape with higher establishments in the upper and lower portions (Miranda *et*

al., 2005, Tsahouridou and Thanassoulopoulos, 2002, Lo and Lin, 2002, Ahmad and Baker, 1987, Sivan and Harman, 1991). Rhizospheric colonization by *Trichoderma* isolates is an important parameter which contributes in the increased growth of the treated plants and biocontrol towards the pathogens (Carvajal*et al.,* 2009, Miranda *et al.,* 2005; McLean *et al.,* 2005). However, Jash and Pan (2007) suggested that antagonistic potential of *Trichoderma* isolate were independent of their rhizosphere competence ability.

Success rate of biological control of plant pathogens may be increased by selecting the superior microbial agents with multiple biocontrol potential such as high degree of mycoparasitism, fast growing, ability to proliferate in the root zone of host plant, induce systematic resistance etc. Mycelial interaction between the fungal pathogen and *Trichoderma* is a pre-requisite for achieving biological control especially through mycoparasitism, a largely applied mechanism. High degree of biological control using *Trichoderma* may be achieved by stopping the growth of soil borne pathogen and thereby eliminating their chances of attack, penetration and proliferation in the root zone of host plant. Rhizosphere competent strains of *Trichoderma* are compactly associated with the root surface and root zone of the plant and therefore it may provide higher protection against the soil borne pathogen attack and disease development. Though thousands of *Trichoderma* have been isolated but the process of isolation and screening is continuously being carried out by researchers to identify the local isolates with increased and multiple potential of biocontrol. A critical review of literature suggests that very little research work has been done to identify the rhizosphere competence ability of *Trichoderma* and main focus is given to its biocontrol potential and also the rhizosphere competence experiments have been conducted only on few selected crop species. Considering the importance of rhizosphere competence, the present study was conducted to investigate the biocontrol potential of the local isolates of *Trichoderma,* their ability to colonize and associate with the plant roots and to evaluate the possible correlation between the biological control and rhizosphere competence of *Trichoderma*. *Chrysanthemum* is an economically important and widely cultivated floriculture crop, attacked by several soil borne fungal pathogens and therefore was selected for the above study.

Materials and methods

Isolation of Antagonist from soil

Soil samples from the root zone of different plant species were collected in fresh poly bags from 15 different locations of Kanpur and Gorakhpur districts of Uttar Pradesh, India. Poly bags with samples were sealed, labeled

and brought to laboratory for the isolation of antagonists. Soil samples were dried under laminar air flow and isolation was carried out using serial dilution technique. One gram of dried sample was put in a test tube containing 9 ml of sterile distilled water (dilution 10^{-1}) and serial dilutions up to 10^{-6} were prepared by transferring 1 ml of the diluted sample to another test tube. All the test tubes with different dilutions of soil samples were shaken properly with the help of vortex mixer at 1500 RPM for 2 to 4 minutes.

Hundred micro liter (µl) samples from each dilution was taken with the help of a micro pipette and put on the Petri plates containing Trichoderma Selective Medium (TSM) (Elad*et al.*, 1981). Soil samples were spread over the medium with the help of a sterile glass spreader under laminar air flow, incubated at 25±2°C into a BOD incubator and observed periodically for the development of fungal colonies. Once the growth was visible, tips of fungal mycelium was picked up and sub-cultured repeatedly on the PDA Petri plates for the isolation of pure colonies. Purified fungal cultures were maintained on PDA medium at 4°C in refrigerator for further studies.

Petri plates containing fungal colonies of all the isolates were used to prepare the cotton blue stained slides for microscopic observations. Slides of fungal isolates were observed under microscope and were identified as the isolates of *Trichodermaharzianum*(*Th*) by comparingwith standard monographs. Confirmation of the fungal cultures identity was also carried out by sending the pure fungal cultures to National Centre for Fungal Taxonomy (NCFT), New Delhi, India.

Screening of Trichoderma against Fusarium oxysporum

Trichoderma isolates were screened for their biocontrol potential against the pathogenic *Fusarium oxysporum* isolate FO-5 (NCFT No 1369-07), under *in vitro* conditions using dual culture technique (Skidmore and Dickinson, 1976). Mycelial disc of both antagonistic and pathogenic fungi were picked up with the help of a sterilized inoculation needle and placed opposite to each other in 90 mm Petri plates containing PDA medium. Colony diameters of both antagonistic and pathogenic fungi were measured periodically up to 7 days. Controls of both the fungi were inoculated separately in the centre of the Petri plates containing PDA medium. All the Petri plates of dual culture studies were incubated at 25±2°C and data on per cent inhibition was recorded. All the treatments were in triplicates and the experiment was carried out twice and percent inhibition over control was calculated using standard method.

Rhizosphere competence ability of Trichoderma

All the seven local isolates of *Th*were screened for their ability to colonize the rhizosphere and rhizoplane of the *Chrysanthemum* roots. The antagonists which are able to colonize the rhizosphere of the plant are considered to provide better protection against the plant pathogens. The experimental protocols used for studying root colonization are defined below.

Preparation of rooted Chrysanthemum cuttings

Cuttings of approximately 9 inch length were cut from the stem of healthy mother plants of *Chrysanthemum* and were planted in flat pots (diameter 18 inch), containing sterilized mixture of sand and soil (ratio 1:3). The potted cuttings were watered at regular intervals to maintain the moisture, required for rooting process. One cutting was pulled out after every 7 days to evaluate the process and stage of rooting. After 35 days of the plantation, *Chrysanthemum* cuttings developed proper roots and were used for the above experiments to determine the rhizosphere competence of *Th* after its inoculation in soil.

Pot trials to evaluate Rhizosphere competence

Inoculum of all the seven isolates was developed on a cellulosic solid substrate Rice bran (RB), procured from local market. RB substrate was moistened, sterilized, cooled and inoculated with spore suspension of all the seven isolates of *Th*for multiplication. Once the mycelia and spores of *Th*isolates were visible after 10 to 12 days, the inoculated substrate was dried under shade conditions and grinded at low temperature to obtain powdered inoculum of all the isolates. The above prepared inoculums of all the isolates were used for the rhizospheric competence studies.

Garden soil was filled in the autoclavable poly propylene bags and sterilized in autoclave three times for 1 hour at 121ºC and 15 p.s.i pressure for two successive days. Earthen pots of 12 inch diameter were filled with sterilized garden soil and powdered inoculums of all the isolates were added separately in the pots at 10 g inoculum per kg of potting soil. The inoculated pots were loosely covered with a poly sheet and kept in shade for 6 days for proper growth and establishment of *Th*isolates in soil. After 6 days, the above pots were then planted with the rooted *Chrysanthemum* cuttings. Prior to plantation, cuttings were properly washed under the running water to remove the soil particles and surface micro-flora. In control pots, only un-inoculated sterilized rice bran powder was added and cuttings were planted after washing the roots properly with tap water. Total of 9 replicates were maintained for each

treatment, and the above experiments were repeated twice by using the same material and methods.

Evaluation of root colonization by Trichoderma

Chrysanthemum cuttings (3 cutting per treatment) were uprooted periodically at 3rd, 7th and 15th days after plantation from each treatment to evaluate the rhizosphere competence by studying colonization and association with root segments. The cuttings were brought to laboratory; root portions were separated from the shoot and then washed thoroughly under the running tap water to remove the soil particles. All the root portions were brought under laminar air flow and dried. From each treatment, root pieces of 4 cm were cut with the help of a sterile scissor by dividing in to three portions upper (up to 4 cm from top), middle (5 to 8 cm from top) and lower (9 to 12 cm from top). These root pieces were washed thrice, thoroughly with sterile distilled water and placed over Trichoderma Selective Medium (TSM) Petri plates (100 mm diameter). The Petri plates were kept at 25±2ºC for incubation in a BOD incubator under dark conditions. Mycelium growths of the antagonistic fungi from the roots of *Chrysanthemum* cuttings were observed and the root portions with *Th*colonization were recorded.

Statistical Analysis

Data of radial growth inhibition in dual culture studies were analyzed for standard deviation followed by standard error calculation. Further the data was also subjected to analysis of variance (ANOVA) to find out its significance.

Results

Isolation and Growth Characteristic of Antagonists

After 5 to 7 days of incubation, fungal colonies appeared on the TSM Petri plates spread with different dilutions of soil samples. Total seven fungal isolates were obtained from 15 different soil samples. On the basis of mycelium colour, colony morphology and sporulation the fungal isolates were identified as different strains of *Trichodermaharzianum*(*Th*), using standard manual. Identities of the *Th*strains were also re-confirmed by sending the cultures to National Centre for Fungal Taxonomy (NCFT), New Delhi, India.

All the 7 isolates of *Th* were studied for their colony characteristics on Potato Dextrose Agar (PDA) medium. Isolates of *Th*showed variations in their morphological features viz. mycelium colour, reverse colour of medium,

growth pattern, sporulation and number of days to completely fill a 90 mm Petri plate. On PDA medium *Th*isolates grew rapidly and covered the 90 mm Petri plates in 5 to 6 days after inoculation. The colonies of *Th*were initially whitish, turned light greenish followed by dark green colour after complete sporulation. As the colonies grew older on Petri plates they turned to dull green. Few isolates grew faster (Th2, Th3 and Th4) and they covered the Petri plates within 5 days with complete sporulation while few of them were slow (Th1, Th5, Th6 and Th7) in growth and sporulation (Table 1, Fig. 1).

Table 1. Colony characteristics of *Th* isolates on PDA Petri plates

Th	Front View of Fungi in Petri plate	Reverse Coloration
Th1	Light green with loose mycelium	Light pale yellow
Th2	Light green with dark green in centre	Light pale yellow
Th3	Light green with compact mycelium	Light yellow green
Th4	Light green with compact mycelium	Light pale yellow
Th5	Light green with compact mycelium	Light brown greenish
Th6	Light green with dark green in centre	Light pale yellow
Th7	Light green with dark green in centre	Light pale yellow

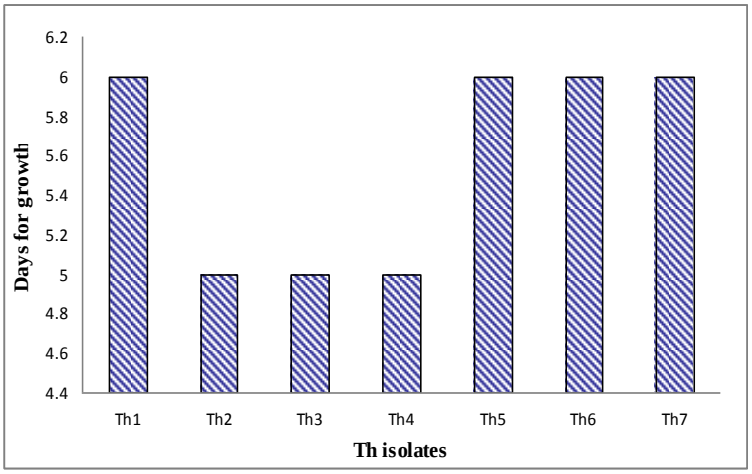

Fig. 1. Time taken to cover 90 mm PDA Petri plates by *Trichoderma*isolates
*Values are means of three replicates. Data are average of two experiments

Dual Culture Studies of Trichodermaharzianum

Trichoderma harzianum isolates effectively inhibited the radial growth of pathogen (*Fo*), in dual culture studies. Maximum significant ($P<0.01$) inhibition (67.0%) against pathogenic isolate FO-5 was recorded with antagonist isolate Th3 while minimum was registered with Th5 (49.2%). There

was no significant difference in the % growth inhibition of *Fusarium oxysporum* caused by isolate Th1, Th2, and Th4. Isolate Th4 showed significantly (P<0.01) higher inhibition (60.1%) against the pathogen as compared to isolate Th5, Th6 and Th7. Among all Th3 and Th4 were two superior isolate and they registered % inhibition higher than 60 per cent.

Per cent inhibition caused by isolate Th1, Th2 and Th6 do not differ significantly among them. Results clearly reveals that isolate Th3 was most promising in controlling growth of pathogen while as isolate Th5 showed lowest ability of biological control (Fig. 2).

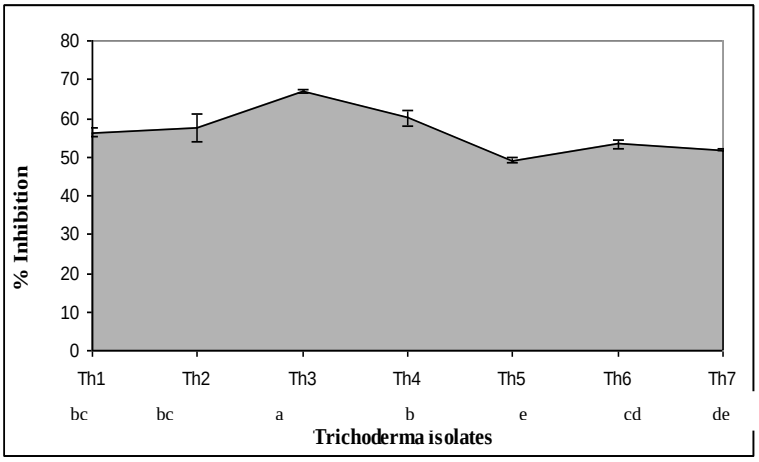

Fig. 2. Per cent mycelial growth inhibition of FO-5 by *Trichoderma* isolates.
*Values are means of three replicates with standard error (±).Data are average of two experiments. Values followed by the same letter are significantly not different from each other, according to Duncans test (P < 0.01).

Root colonization by Trichoderma harzianum

Results of root colonization experiments showed that all the 7 isolates of *Th* were re-isolated from upper root segments of *Chrysanthemum* at the depth of 1 to 4 cm. Only 57% (four out of seven) *Th* isolates namely Th1, Th2, Th3 and Th6 were found associated with the root segment of 5 to 8 cm and 9 to12 cm depth, which was evident from the mycelial growth of the above isolates on the TSM Petri plates. No mycelial growth was observed from the *Chrysanthemum* root segments of 5 to 8 cm depth which were inoculated with isolate Th4, Th5 and Th7. Data clearly reveals that out of seven only four (Th1, Th2, Th3 and Th6) of them were able to colonize and associate with the *Chrysanthemum* rhizoplane or root segments at the deeper depth of 9 to12 cm and therefore were considered rhizosphere competent strains of *Th*(Fig. 3, Photo 1).

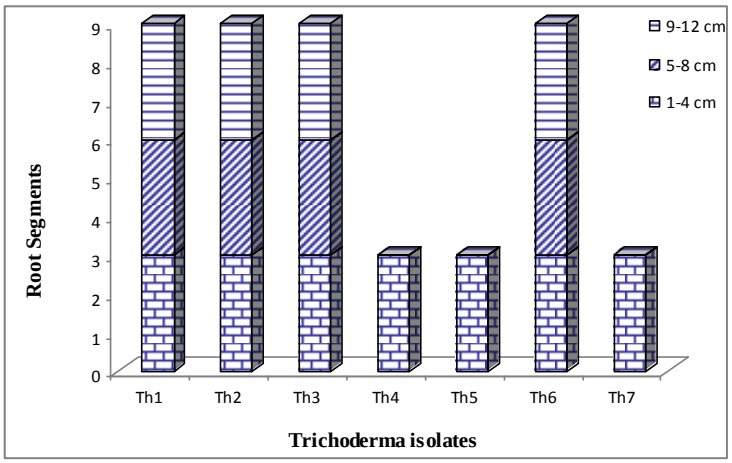

Fig. 3. Colonization of root segments of *Chrysanthemum* by *Trichodermaharzianum.* Isolate Th1, Th2, Th3 and Th6 were found associated with all the segments. Isolate Th4, Th5 and Th7 were not associated with the deeper root segments.

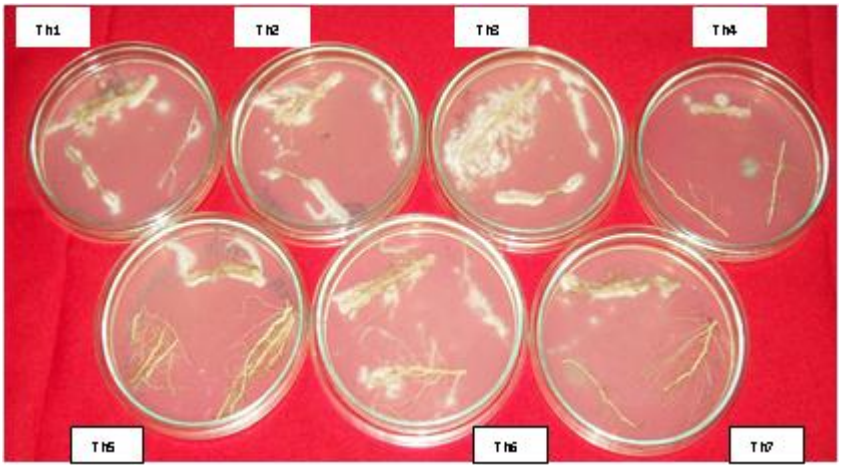

Photo .1. Growth and emergence of *Trichodermaharzianum*mycelium from *Chrysanthemum* root segments of different depth. Th1, Th2, Th3 and Th6 were re-isolated from all the root segments (1 to12 cm).

Discussion

A variation among the *Trichodermaharzianum*(*Th*) isolates was observed with respect to their colour, growth pattern and sporulation. Results of colony interaction study revealed that at early stages both the fungi (*Th* and*Fusariumoxysporum*) grew together in dual culture. At later stages fungal colonies intermingled and *Th* isolates overgrew in the areas that had been

previously occupied by the pathogen and inhibited its growth. *Trichoderma* isolate Th3 was most promising in inhibiting the pathogen growth and it registered 67% inhibition. Re-isolation from the areas where pathogen had been growing resulted in the recovery of *Th* alone. It is indicative of the fact that *Th* isolates caused mortality of *Fusarium oxysporum*, showing typical characteristics of mycoparasitism. Antagonistic interaction between *Trichoderma* species and pathogenic isolates of *Fusarium* has also been reported by several workers (Orole and Adejumo, 2009; Cho *et al.*, 1989; Ciprianoet *al.*, 1989; Berthier *et al.*, 1988). Differential biocontrol potential of *Th*isolates may be due to variable capability in the mode of action employed by different strains against the pathogenic fungi.

"Rhizosphere competence" is an unique ability of soil microbes to establish themselves on the rhizoplane and in the roots and function in the developing rhizosphere of the plants. Results obtained from rhizosphere competence trials clearly indicated that all the *Th* isolates are not capable to associate and colonize the root segments of the *Chrysanthemum* plants. Though several isolates of *Th*are found in same environmental conditions but their capability to grow, multiply, proliferate and function as biological control agent varies at great extent. It is evident from our experiments that there is a variation among the *Th*isolates with respect to there ability to colonize and compactly associate with plant rhizosphere and rhizoplane. Lo and Lin (2002) in their experiments on cucumber plants found that most of the *Trichoderma* strains colonized only the upper portions of the root and were not good rhizosphere competent. In our experiments, *Th* isolate Th1, Th2, Th3 and Th6 were found associated with the root segments at all the depth (1 to 12 cm) evaluated and these isolates should be considered as rhizosphere competent.

Colonization ability of *Th*on *Chrysanthemum* roots (middle and lower) was not found in the isolates Th4, Th5 and Th7. Researchers have suggested that *Trichoderma* species colonize the plant root segments in U shape with maximum viable count and higher establishments in the upper and lower portions (Sivan and Harman, 1991; Ahmad and Baker, 1987). Lo and Lin (2002) found similar results with their experiment on cucumber plants. They suggested that *Trichoderma* isolates which colonize all the portions (upper, middle and lower) of growing roots are only considered to be rhizosphere competent. *Trichoderma* isolates have great capability of root colonization up to the depth of 12 to 22 cm (McLean *et al*., 2005; Sivan and Chet, 1989; and Sivan and Harman, 1991). All the seven strains of *Th* were isolated from soil of different crop species other than *Chrysanthemum.* About 57% of *Th* isolates (4 out of 7) successfully colonized and associated with the root segments of *Chrysanthemum* which suggest that root colonization ability is independent of

host plant species. Rhizosphere competence is an ability of an individual isolate to establish in the rhizosphere, grow and associate with the rhizoplane of the plants. The association of *Th*with plants roots may be dependent on its integral capability to recognize and utilize the root exudates.

There was no correlation between the ability of *Th* to inhibit the growth of *Fusarium oxysporum* in dual culture and to colonize the growing root segments of *Chrysanthemum* plants. Our result clearly reveals that *Th* isolates which have higher biocontrol potential against the plant pathogens are always not good rhizosphere competent. *Th* isolate Th4 showed higher biocontrol potential than Th5, Th6 and Th7 but it was not found in the deeper segments of the *Chrysanthemum* root. Where as isolate Th6 showed low biocontrol potential against the pathogen but have characteristic of rhizosphere competence. In biological control phenomenon *Th*isolates have to mycoparasitize, secrete volatile and non-volatile compounds, and cause cell-lysis of the pathogen but in rhizosphere competence *Th*have to establish, grow and colonize in the developing root system. The process of biological control and root colonization are entirely different from each other and this may be a possible reason behind this non-correlation between biocontrol potential and rhizosphere competence.

Rhizospheric colonization by *Trichoderma*is an important parameter which contributes in the increased growth of the treated plants and provides biocontrol against the soil borne plant pathogens (Carvajal*et al*., 2009, Miranda *et al*., 2005, McLean *et al*., 2005, Harman *et al.,* 2004). Complete colonization or covering to developing roots of *Chrysanthemum* plant by *Th*may have several benefits such as higher protection against plant pathogens, mobilization of nutrients, etc. Th1, Th2 and Th3 possess high potential to colonize and associate with developing roots and were considered as rhizosphere competent strains for *Chrysanthemum* plants. In addition, these strains effectively controlled the plant pathogen in dual culture studies. Considering the presence of dual potential, above isolates could be successfully employed in the biocontrol of the *Fusarium oxysporum* under field conditions. It is clearly indicative from our experiments that *Th* isolates should be screened thoroughly for their multiple desirable biocontrol abilities under lab as well as field conditions using economically important crops. The process of initial screening of *Trichoderma* for its rhizosphere competence and biocontrol potential will be very beneficial for getting a potential isolate with an ability of increased performance in the field conditions.

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