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# STUDY ON GROWTH AND BIOMASS OF OCIMUM SANCTUM PLANTS AND PROTEIN PROFILING OF TRICHODERMA HARZIANUM

**PADMAVATHI TALLAPRAGADA***

*Department of Microbiology, Centre for PG Studies, Jain University, Bangalore – 560011, Karnataka India.*

## ABSTRACT

The capability of the bio-control agent *Trichoderma harzianum* to trigger plant growth and improvement in biomass response was investigated by inoculating roots of medicinal plant (*Ocimum sanctum*) seedlings with *Trichoderma harzianum* in pots, under aseptic, green house environmental conditions. *Trichoderma harzianum* was isolated from ten different agro climatic zones of Karnataka. Biological and biochemical analysis revealed that inoculation with *T. harzianum* initiated increased Phosphate (P), Nitrogen (N) and soluble sugars uptake by the *O. sanctum* plants. Inoculation with *T. harzianum* increased the biomass of the plants in terms of height, number of leaves and number of branches, providing evidence that *T. harzianum* induce growth and increased biomass mechanisms in plants. The whole cell protein was extracted from all the ten isolates of *T. harzianum* and then subjected to electrophoresis (SDS-PAGE). The protein profile on the gel was scored and the dendrogram was constructed using Phylip software of *T. harzianum*. Exploiting bio-control agents to enhance the plant growth and biomass production of medicinal plants is a topic of current interest.

**Keywords*:*** **Trichoderma harzianum, Bio-control agent, Ocimum sanctum, biomass, Protein profiling**

**PADMAVATHI TALLAPRAGADA**
**Department of Microbiology, Centre for PG Studies, Jain University, Bangalore – 560011, Karnataka India.**

# INTRODUCTION

In soil, *Trichoderma* spp. is favored by the presence of high levels of plant roots. In addition to colonizing roots, these species. attacks, parasitize or otherwise gain nutrition from other fungi. Several new general methods for both bio-control and for enhancement of plant growth have recently been demonstrated[1]. *Trichoderma* has some antibiotic producing and bio-controlling properties. Isolates of *Trichoderma* including isolates from the same species produce a range of volatile antibiotics that have different effects on different test fungi[2]. The isolates may have produced the same complex of metabolites in different proportions or may have produced entirely different chemical compounds[3]. Non availability of resistant crop varieties, non desirability of applying huge quantities of fungicides to soil owing to residue problems, development of resistance in soil borne plant pathogens have lead to increased research efforts on biological control of soil plant pathogens all over the world[4]. *Ocimum sanctum*, commonly called as Tulsi is herbaceous plant throughout India, having a medicinal value in the pharmaceutical industry[5]. In the Indian system of medicine, the whole plant is used irrespective of its active ingredient properties. Hence the biomass production is of utmost significance in pharmaceutical industries. Object of the current study is isolation and identification of *T. harzianum* from soils of different agro climatic zones of Karnataka and to study the effect of *T. harzianum* on the biological and biochemical characteristics of *Ocimum sanctum* plants. Molecular techniques, which provide valuable information on the magnitude of genetic variability within and between organisms of different species, have been developed. One such method is based on proteins that can be analyzed using electrophoresis or direct amino acid sequencing. Electrophoretic analysis of proteins has long been a valuable tool in systematic and population genetic studies of bacteria, plants and fungi[6]. Electrophoretic analysis of whole cell proteins by one-dimensional protein pattern provides a rough measure of the number and physicochemical properties of gene products. One-dimensional polyacrylamide gel electrophoresis of proteins has been used extensively for identification and classification at the strain and species level[7]. The mobility of total proteins during electrophoresis has been used to characterize many organisms including fungi[8]. Therefore protein markers can be used to observe the variability of *Trichoderma harzianum* and hence one-dimensional sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (1-D SDS-PAGE) of soluble peptides is considered as an effective tool capable of giving discrimination at morphospecies level[9]. Non availability of resistant crop varieties, non desirability of applying huge quantities of fungicides to soil owing to residue problems, development of resistance in soil borne plant pathogens have lead to increased research efforts on biological control of soil plant pathogens all over the world.

# MATERIALS AND METHODS

### *(1) Isolation and identification of Trichoderma harzianum*

Soil samples of 500 g were collected from six-inch layer of soil from each agro-climatic zone of Karnataka. Each soil sample was sieved through a 1000 µ mesh and samples were used for the isolation by standard plate count method[10]. One ml of the dilutions was used for plating on Martin's Rose Bengal agar (MRBA) medium and was incubated at $30^0$C for 4 days. Based on the colony morphology, the mold colonies were selected and cultured separately to obtain pure culture. Microscopic observation was carried out in order to confirm the isolates[11]. Further, protein markers were used to compare the ten isolates.

### *(2) Response of Ocimum sanctum plants to Trichoderma harzianum isolates*

Two weeks old healthy *Ocimum sanctum* seedlings were selected and transplanted to polythene covers containing sterile sand soil mixture (1:1 w/w). *Trichoderma harzianum* isolates were grown separately, in a 250 ml

flask containing 100 ml potato dextrose broth for 2 weeks. The grown cultures were homogenized and 15 ml of each of the culture suspension was inoculated to each polythene cover transplanted with two weeks old *O. sanctum* seedlings and the covers were labeled as C for control and $Th_1$ to $Th_{10}$ for inoculated covers. Three replications were maintained for each treatment, the seedlings were regularly watered. The observation with respect to the growth parameters such as plant height, number of leaves and number of branches, plant biomass were studied to analyze the effect of *T. harzianum* on the growth of *O. sanctum* plants[12].

### *(3) Response of T. harzianum isolates on the protein, carbon & phosphorus content of Ocimum sanctum plants*

After 60 days of growth, the plants were harvested, weighed and total biomass was recorded initially and then root and shoot parts were separated and total fresh weight of root and shoot was recorded. Then all the plants were oven dried at $60^0$ C for 4 days to remove the moisture content in the plants and again the weight of dried shoot and root were recorded and expressed as grams per plant[12]. Plant phosphorus concentration was estimated calorimetrically following the vanado-molybdate yellow color method[13]. Total plant sugar was estimated colorimetrically following the phenol sulphuric acid method[14]. Nitrogen content of shoot and root was estimated by kjeldahl method[13].

### *(4) Protein profiles of T. harzianum isolates by SDS- PAGE*

Isolates were grown overnight at 37°C in 100ml potato dextrose broth under shaking condition. Soluble proteins were extracted by grinding 100mg of freeze-dried mycelium with pestle and mortar with or without liquid nitrogen and 5ml of buffer solution (0.1M Tris-HCl, pH 6.8). The mixture was centrifuged for 30 min at 7000 rpm and the supernatant was collected. The protein content was estimated as described by Lowry et al[15] with bovine serum albumin as standard protein. Protein content was adjusted to 100μg /ml of sample. SDS-PAGE was performed using bovine serum albumen as a standard. Thoroughly cleaned glass plates and spacers were assembled together with the aid of clips after greasing the spacers. The assembly was set up in upright position. Electrophoresis was carried out in 1-D polyacrylamide gel. Sufficient quantities of 10 % separating gel was prepared and poured into the space between two glass plates. Separating gel was poured to about two-third the height of the gel plate. The top of the gel was over laid with distilled water or butanol and the entire set up was left undisturbed for about 30 minutes to allow for the polymerization of the resolving gel. After polymerization the water on top was removed. The wet surface between the plates was dried using strips of blotting paper. The space above the resolving gel was then rinsed with stacking gel buffer and then filled with stacking gel solution (3.5%). The combs were inserted gently and the entire set-up was kept aside undisturbed to allow for polymerization to occur. After polymerization the gel was installed in an electrophoretic apparatus after removing the lower spacer and traces of residual grease on the lower end of the gel plate. The upper and lower tanks were filled with tris glycine electrode buffer. The combs were then gently removed without disturbing the wells. 70μl of sample was mixed with 6X loading dye and loaded into each well with a micropipette. The current was adjusted to 35mA until the samples migrated through the stacking gel and later increased to 100mA for the resolving gel until the bromophenol blue reaches the bottom of the gel. After the run, the gel was carefully disengaged from the glass plates and slipped into solution A for silver staining as described by Rabilloud *et al*[16]. The gel was allowed to stand in solution A for one hour. Then it was washed 3 times with 50 % methanol for 20 minutes and kept in solution B for 60 seconds. To remove the methanol, it was washed with double distilled water 3 times for 20 seconds each. The gel was then transferred to solution C and allowed to stand for 15 minutes. Further it was washed with double distilled water twice. Finally the gel was developed with solution D. After the bands were seen 8 or 10 % acetic acid was added after decanting solution D.

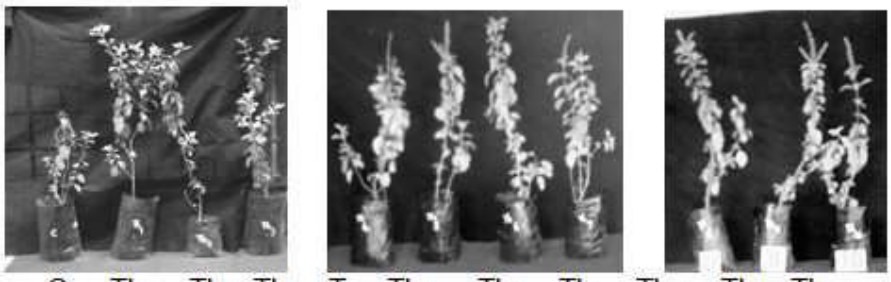
***(5) Statistical analysis***

The data obtained from the experiments were subjected to two-way analysis of variance by software package for social studies (SPSS) using ANOVA method.

## RESULTS

**(1) *Response of Ocimum sanctum plants inoculated with T. harzianum isolates*** Fungi are the major class of organism among P solubilizers. *T. harzianum* like any other plant growth promoting rhizomicroorganism's (PGPRs) have received considerable attention because they are known to occur globally in a broad range of dissimilar environments[17]. There are many reports of inter and intra specific differences in the effectiveness of fungi for plant growth and protection but the response range can be affected by host-plant or soil conditions and the physiological basis of variations[1]. The data pertaining to the influence of *Trichoderma harzianum* isolates from ten agro climatic zones on plant height, number of leaves, branches and plant biomass of *Ocimum sanctum* is represented in plate1.

***Plate 1: Response of Ocimum sanctum plants treated with T.harzianum isolates***




*C: Control plant without T. harzianum treatment*
*$Th_1$ – $Th_{10}$: Plants treated with 10 isolates of T. harzianum*

***(2) Response of T. harzianum on biomass of the plants in terms of height & number of leaves***

The plant height was found to increase steadily with time, the plants inoculated with the isolates had increased plant height compared to un-inoculated plants throughout the observation period. However, the heights differed significantly among the plants inoculated with various isolates. The least plant height (43 cms) was recorded in plants inoculated with isolate 2, and the highest (56 cms) by plants treated with isolate 1 after 60 days of transplanting (Fig 1a). The number of leaves was found to increase progressively with time. It was observed that number of leaves in inoculated plants were always higher than the control plant. The highest number of leaves (182) was observed in plant inoculated with the isolate 1 and least number of leaves (70) was found in un-inoculated plants. However, there was no significant difference in number of leaves among the inoculated treatments. Except isolate 5, which had less number of leaves (88) compared to plants treated with other isolates (Fig 1 b).

**Figure 1a**
***Response of T. harzianum on biomass of the plants in terms of height***

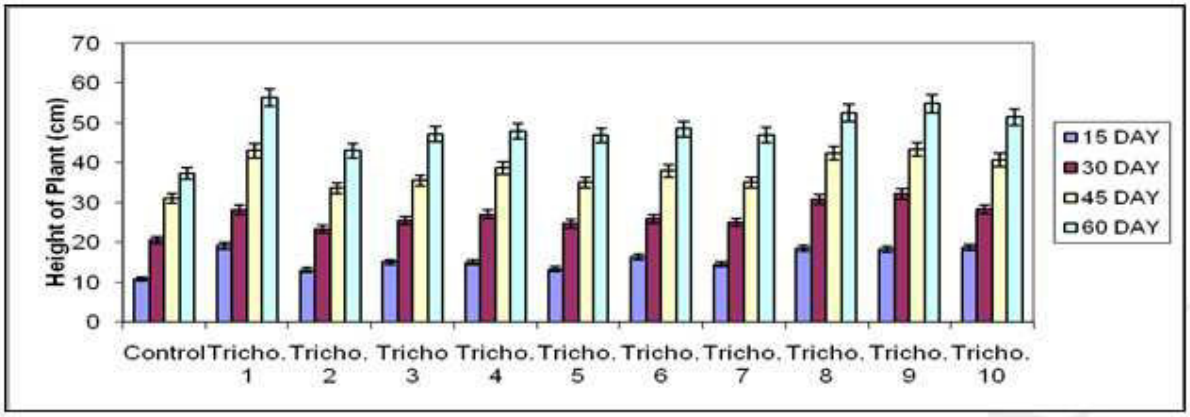
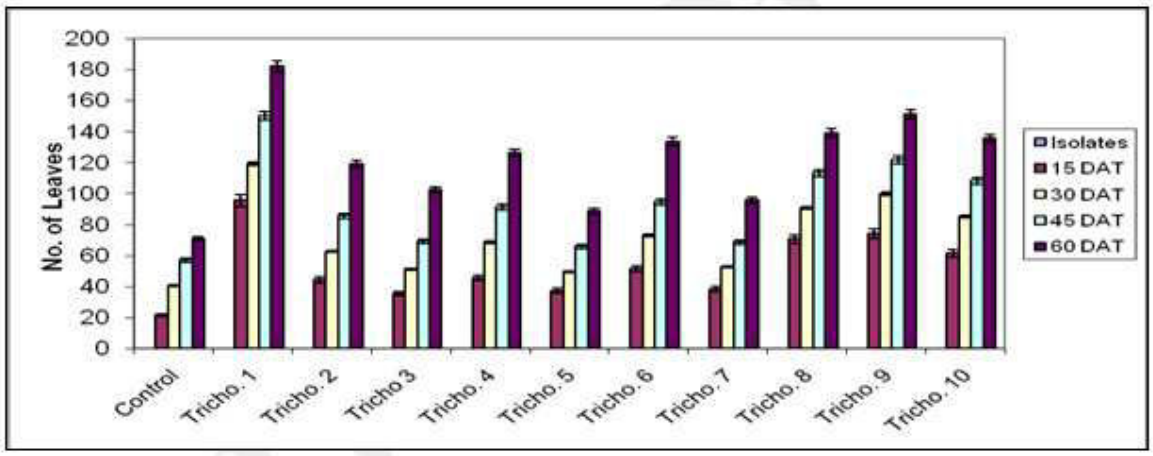


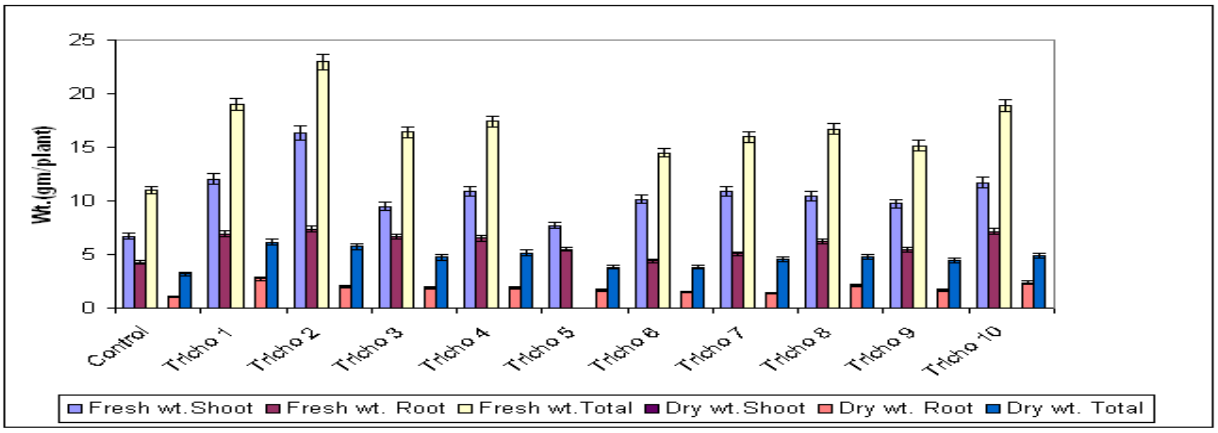

**Figure 1b**
***Response of T. harzianum on biomass of the plants in terms of number of leaves***




***(3) Response of Ocimum sanctum on plant fresh & dry weights of shoot and root after harvesting the plants***

**(4)** The fresh weight and dry weight in the plants inoculated with isolates were higher than un-inoculated plants. The highest shoot and root fresh weight (15.9 g and 8.77 g respectively) were recorded in plants inoculated with the isolate 4. It was superior to all other treatments. However, the fresh weight differed significantly among the plants inoculated with various isolates. Minimum shoot and root fresh weight (6.65 g and 4.8 g respectively) was recorded in un-inoculated plants (Fig. 2).Highest shoot and root dry weight was recorded in plants inoculated with isolate 2 and 10 (3.9 g and 2.4 g respectively), which was significantly higher over other treatments and minimum shoot and root dry weight (2 g and 1.42 g respectively) was recorded in un-inoculated plants. No significant difference in the total dry weight was observed in plants inoculated with all other isolates (Figure 2) except isolates 5, 6 and 8 that showed lower dry weight compared to other treatment.

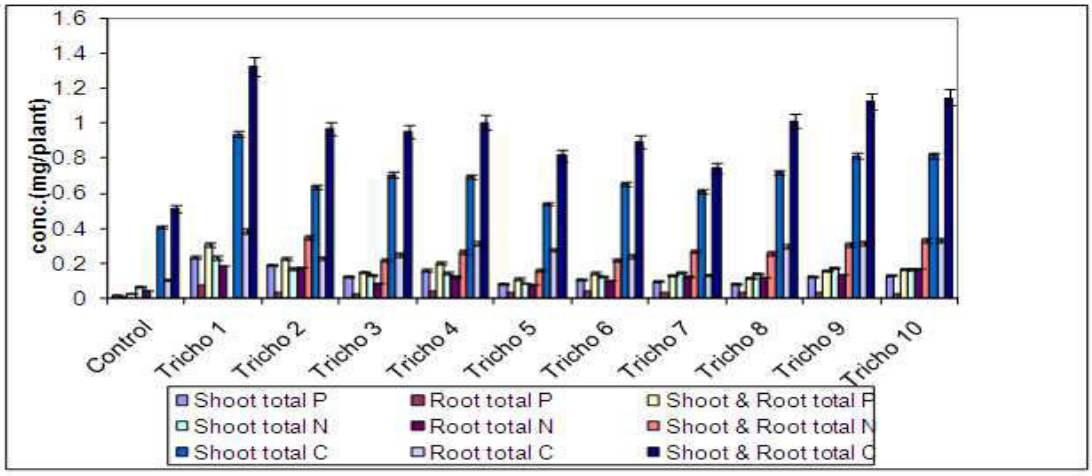
**Figure 2**
***Response of Ocimum sanctum on plants fresh & dry weights of shoot and root***




***(5) Response of T. harzianum isolates on the C, N & P concentrations of Ocimum sanctum***

The shoot P content in the plants treated with *T. harzianum* isolates was found to differ significantly from the control plants. No significant difference was observed within the treated plants. The highest shoot P content (0.188 mgs/plant) was observed in the treated with isolate 1 while minimum shoot P (0.018 mgs / plant ) was observed in the control plants (Figure 3). The total soluble sugar content of shoot was the highest in plants inoculated with isolate 1 (0.940 mg /plant). The control plants significantly differ with other treatments (0.410 mg/plant). No significant difference was observed among the treatments. Less sugar content (0.542 mg /plant) was observed in plant treated with isolate 5 (Figure 3). Total soluble sugar content of shoot was the highest in plants treated with isolate 1 (1.323 mg /plant). Control plant significantly differed over other treatment. No significant difference was observed between other treatments. Least total soluble sugar was observed in control plant (0.513 mg / plant) (Figure 3).The total nitrogen content of shoot in the plants inoculated with the isolates were represented as protein content of shoot in Figure 3. The total protein content of shoot was the highest in plants inoculated with isolate 1 (0.230 mg /plant). Control plant significantly differed with other treatments (0.063 mg/plant). No significant difference was observed among the treatments. Least protein content was observed in control plant (0.106 mg/plant) (Figure 3).

**Figure 3**
***Response of T. harzianum isolates on the C, N & P concentrations of Ocimum sanctum***

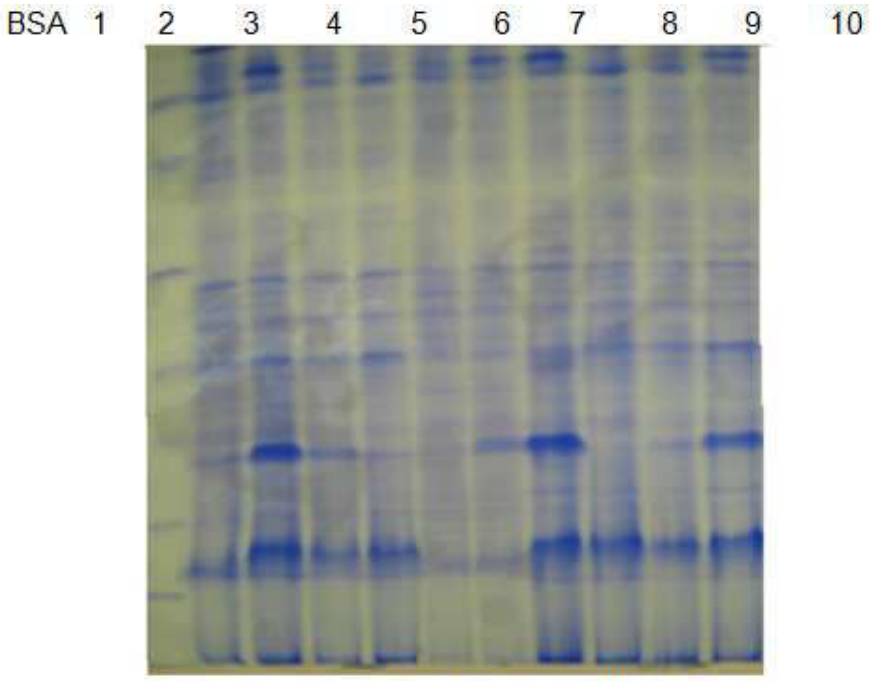



The root P content in the plants treated with isolates was found to differ significantly from the control plants. No significant difference was observed among the inoculated plants. The highest plant root P content (0.068 mg/plant) was observed in the treated with isolate 1 while minimum root P (0.005 mg/plant ) was observed in control plants (Figure 3) The total soluble sugar content of root was the highest in plants inoculated with isolate 1 (0.383mg/plant). Control plant significantly differed with other treatments (0.108 mg /plant). No significant difference was observed among the treatments. Less sugar content (0.542 mg/plant) was observed in plant treated with isolate 5 (Figure 3) The total nitrogen content of root in the plants inoculated with the isolates were represented as protein content of root in graph V. The total protein content of root was the highest in plants treated with isolate 1 (0.182 mg res./plant). Control plant significantly differed with other treatments (0.043 mg/plant). No significant difference was observed among the treatments. Control plant significantly differed with the other treatments. No significant difference was observed between other treatments. Least protein content was observed in control plant (0.106 mg / plant) (Figure 3).

***(6) Protein profiles of T. harzianum isolates by SDS- PAGE***

The data pertaining to the protein banding patterns of *T. harzianum* isolates (Plate 2) are presented based on the Relative mobility value (Rm), similarity index and intensity of the bands (Table 1 and 2). Rm value of the bands ranged from 0.006 to 0.34. Among these isolates, *T. harzianum* isolate 2 was different (one extra band in 0.026, 0.300 and 0.160) from other isolates but rather and more similar to *T. harzianum* isolate 7 as both have a common bands in intensity and occurrence, while it is absent in all other *T. harzianum* isolates. Almost common bands were observed between the isolates 1, 2, 3 and 4 except some bands, but they differed only in their intensity. Similarity index was more between *T. harzianum* isolates 2,4; 7,4; 7,9 and 4,10 (0.76) whereas it was less between *T. harzianum* isolates 1,2; 1,7; 3,4; 9,4; 8,7 and 6,4 (0.14 and 0.22).

**Plate 2**
**Protein profiles of *T. harzianum* isolates by SDS- PAGE**




*BSA: Bovine serum albumin used as a protein standard*
*1-10 Protein samples of 10 isolates of T .harzianum*

**Table 1**
***Band intensity and Relative mobility value in protein profile of* T. harzianum *isolates***

| Isolates | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Protein Bands | Rm value | | | | | | | | | | |
| 1 | 0.006 | ++ | +++ | + | 0 | + | + | +++ | 0 | 0 | + |
| 2 | 0.013 | + | + | + | ++ | + | + | + | ++ | + | + |
| 3 | 0.026 | 0 | + | 0 | 0 | 0 | 0 | + | + | 0 | + |
| 4 | 0.033 | + | + | + | + | + | + | + | + | + | + |
| 5 | 0.040 | + | + | 0 | 0 | + | + | + | + | 0 | + |
| 6 | 0.046 | + | + | + | + | + | + | + | + | + | + |
| 7 | 0.053 | + | + | 0 | 0 | + | 0 | + | + | + | 0 |
| 8 | 0.080 | + | ++ | + | ++ | + | + | +++ | ++ | + | ++ |
| 9 | 0.100 | + | + | 0 | 0 | 0 | 0 | + | 0 | 0 | + |
| 10 | 0.110 | + | + | 0 | 0 | 0 | 0 | + | + | 0 | + |
| 11 | 0.150 | + | +++ | + | + | 0 | ++ | +++ | 0 | + | +++ |
| 12 | 0.160 | 0 | + | 0 | 0 | 0 | 0 | + | + | 0 | + |
| 13 | 0.300 | + | + | 0 | 0 | 0 | 0 | + | + | 0 | 0 |
| 14` | 0.320 | 0 | +++ | + | ++ | 0 | 0 | +++ | +++ | +++ | +++ |
| 15 | 0.340 | ++ | ++ | + | + | + | + | ++ | + | 0 | 0 |

***+ Less band intensity; ++ Moderate band intensity; +++ High band intensity.***
***1-10 ⇒ T. harzianum isolates***
***1-15 ⇓ Number of Protein bands visible in SDS-PAGE***

**Table 2**
***Similarity index of T. harzianum isolates based on protein profile analysis***

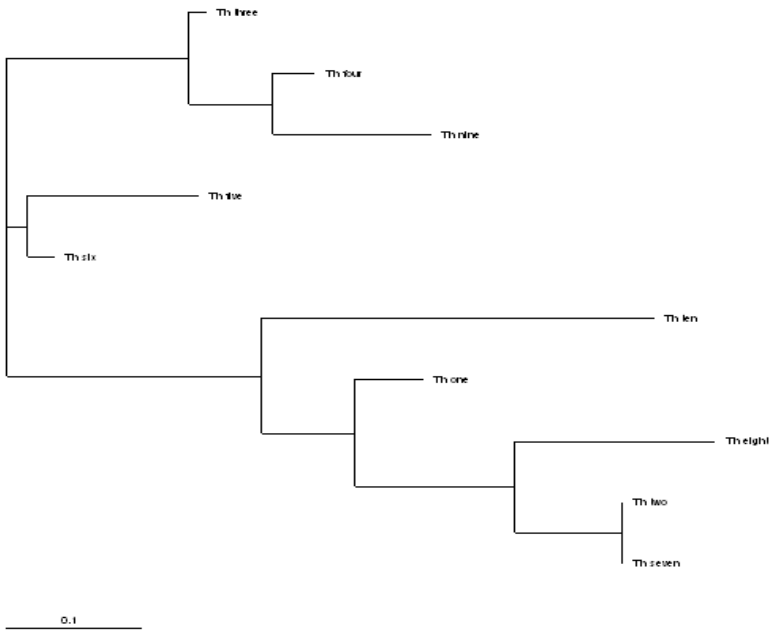
| Isolates | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 0 | | | | | | | | | |
| **2** | 0.14 | 0 | | | | | | | | |
| **3** | 0.41 | 0.63 | 0 | | | | | | | |
| **4** | 0.51 | 0.76 | 0.07 | 0 | | | | | | |
| **5** | 0.41 | 0.63 | 0.31 | 0.41 | 0 | | | | | |
| **6** | 0.41 | 0.63 | 0.14 | 0.22 | 0.14 | 0 | | | | |
| **7** | 0.14 | 0.00 | 0.63 | 0.76 | 0.63 | 0.63 | 0 | | | |
| **8** | 0.41 | 0.22 | 0.76 | 0.63 | 0.51 | 0.76 | 0.22 | 0 | | |
| **9** | 0.51 | 0.76 | 0.22 | 0.14 | 0.41 | 0.41 | 0.76 | 0.63 | 0 | |
| **10** | 0.51 | 0.31 | 0.63 | 0.76 | 0.63 | 0.40 | 0.31 | 0.63 | 0.76 | 0 |

***1-10 T. harzianum isolates***

Euclidean distances and dendrogram was constructed (Figure 4) using Phylip software. Three clusters were illustrated. The first cluster contains three groups. The first group had *T. harzianum* isolates 3, 4 and 9, while the second cluster is subdivided into 2 groups wherein *T. harzianum* isolates 5 and 6 form one group. On the other hand the third cluster had the four groups. Within that first group forms the sub group with *T. harzianum* isolate 10 separately and the other group formed by *T. harzianum* isolate 1 and sub divided to T. *harzianum* isolate 8 and one more sub group with *T. harzianum* isolates 2 and 7 placed under the same sub group.

**Figure 4**
***Dendrogram of T. harzianum isolates obtained by protein profile***

Tree diagram for 10 variables
Ward's method
Squared Euclidean distance




- ***0.1 Linkage distance,***
- ***Th one – Th ten: Ten isolates of Trichoderma harzianum***

## DISCUSSION

*Trichoderma* is a fungus that exists in almost all soils and a wide range of habitats. In soil, they are the most widespread, culturable fungi. They prefer locations with a large supply of plant roots, which they promptly colonize. Additionally, the *Trichoderma* species attack, parasitize or derive nutrition from other fungi. Because the species flourishes best when there are copious amounts of healthy roots, they have developed many mechanisms for attacking other fungi and for improving plant and root growth[1]. The potential of the phosphate solubilizers have been exploited as microbial inoculants for crops grown in Indian soils that are deficit in available P and amended with rock phosphate or tricalcium phosphate[18]. The increase in plant growth by the use of these organisms may be due to an increase in phosphorus uptake as well as other minor nutrients and plant growth promoting rhizomicroorganisms (PGPRs) activity[19]. Cucumber plants (*Cucumis sativus*) showed enhanced plant growth and increased biomass when the seedlings were treated with *T. harzianum* isolates[20]. Elongation of roots in sweet corn was observed in when they were treated with *T. harzianum* isolates[21]. The increase in height in these plants may be due to an increase in phosphorus uptake as well as other minor nutrients and plant growth promoting rhizomicrobial (PGPR) activity[19]. Many other reasons such as, role of humic acids in phosphate solubilization has been reported by Vidyasekaran et al[22].

SDS-PAGE is used because the method alleviates the need for culturing, and the samples are analyzed in a more direct manner. The results obtained by this method can discriminate the whole cell proteins at much the same level as DNA finger printing[23] (Priest and Austin, 1993) in some cases. The above results suggest that protein profile data can closely separate isolates from different zones. These results agree with the work done by several scientists such as Aly et al[24] wherein the protein profile data of *Fusarium* isolates of cotton obtained from different areas clearly separated the isolates. Protein profiles were distinct and each isolate showed a unique characteristic profile. The data obtained from protein profiles support the potential use of this experimental approach to help distinguish between different *Trichoderma* isolates. Three clusters were illustrated from the dendrogram obtained. The first cluster contains a single group. The first group had the *T. harzianum* isolates 3, 4 and 9, while the second cluster is divided into two groups wherein *T. harzianum* isolates 5 and 6 form one group. On the other hand the third cluster is divided into subgroups where *T. harzianum* isolate 10 alone forming a separate subgroup. Within that first group forms the sub group with isolate 1 and in second group also sub group was formed by isolate 8 and further subdivision with isolates 2 and 7. This result upholds the study conducted by Avio and Giovannetti[25]. The above results suggest that protein profile data can closely separate isolates from different zones. The present investigation has showed *O. sanctum* plants inoculated with *T. harzianum* isolates grew taller as compared to control plants. However, the plants differed significantly in response to some isolates within the treatments but among the parameters observed, the highest response was seen in case of *O. sanctum* plants treated with isolate 1, followed by plants treated with isolates 9, 8, 10 and 6. Plants treated with isolates 2, 3, 4 and 7 showed almost similar results. The least response was noticed in plants treated with isolate 5, but there was a better response compared to control plant where *T. harzianum* was not inoculated. The concentrations of P, N and C were also more in plants, which showed better response in total biomass. A significant increase in N and P uptake and green manure was observed during the first stage of vegetation in sunflower plants due to soil inoculation with phosphate dissolving microorganisms [26]. Intricate chemical reactions in the soil convert applied phosphate fertilizers into highly insoluble forms. However, the soil also has fungi that convert these insoluble forms into soluble forms. Such fungi are said to possess a mineral phosphate solubilizing ability that is an alternative to

chemical fertilizers[27]. Many studies on the interaction of PSMs have been conducted in different crop plants. However, the information on the capability of these organisms in localized soil conditions, in promoting plant growth and the biochemical characterization of phosphate solubilizing bacteria and fungi isolated from different soil types of different zones, is very much limited. Furthermore, screening of the isolates, either individually or in combination, is needed to select efficient isolates to improve the plant growth and biomass[28]. This proves the fungi might have played a significant role in the enhanced absorption of the nutrients and proves *T. harzianum* can be used as a bio-fertilizer to improve the biomass of the plants. Since, all the isolates belong to the same species of *T. harzianum*, their effect on growth in terms of height, number of leaves and P, N and C concentrations may not be similar as those observed in all the plants. The significance in their effect may be due to the diverse environmental conditions existing in different agro climatic zones from where they were isolated, could have contributed to the biological and physiological changes in *T. harzianum isolates*, which was evident in this.

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