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PROTEIN PROFILING OF *TRICHODERMAHARZIANUM* ISOLATED FROM DIFFERENT AGRO-CLIMATE ZONES OF KARNATAKA AND INTERACTION WITH *COLEUS FORSKOHLII*

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Short Profile

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Short Profile

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ABSTRACT:
Biological control mainly consists of using microorganisms to control harmful microorganisms causing plant disease without disturbing the ecological balance. Weindling (1932) suggested potential use of *Trichoderma spp*, as a biocontrol agent against the soil borne plant pathogens like *Rhizoctonia solani.* The biological control of root diseases of crop plants by introduction of antagonistic microorganism has been suggested as an environmentally safer alternative to the use of fungi toxic chemicals (Baker and Cook, 1974)
In the present investigation,*Trichoderma harzianum*, a biocontrol agent was isolated from various agroclimatic zones of Karnataka. These isolates were examined for their molecular variability using protein markers. Efficiency of *Trichoderma harzianum* was studied in in vitro conditions for confirmation of the isolates and then in a green house experiment using all the isolates to identify efficient isolates using Coleus forskohlii as the indicator plant.Inoculation with *Trichoderma harzianum* increased the biomass of the plants in terms of height, number of leaves and number of branches, providing evidence that, *Trichoderma harzianum* induced growth and increased biomass mechanisms in plants. In the presents study, both qualitative and quantitative differences were observed in the protein profile of different *Trichoderma harzianum* isolates. The results suggest that, protein profile data can closely separate isolates from different zones.

KEYWORDS
Biological Control, Trichodermaharzianum, Protein Profiling, Coleus forskohlii.

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INTRODUCTION :

Trichoderma is a genus of filamentous deuteromycetes.Its members are generally found in all soil including forest humus layer as well as in agricultural & orchid soils (wardle, Parkinson & Waller, 1993).Trichoderma species are rarely reported to occur on living plants and have not been found as endophytes of living plants (Petrini, 1986). The genus comprises a great number of fungal strains that acts as biological control agents, the antagonistic properties of which are based on their activation of multiple mechanisms. Trichoderma strains exert biocontrol against fungal Phytopathogens either indirectly, by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms and antibiosis, or directly, by mechanisms such as mycoparasitism(Dennis,1971). The ancient medicinal plant Coleus (*Coleus forskohlii*), belonging to the family Lamiaccae is the source of the compound forskolin which possesses unique biological activity. While clinical results are thought to be better obtained using the whole plant versus the isolated constitutent forskolin, research on forskolin is upholding the traditional uses of the plant. Due to the unique pharmacological parameters of forskolin, *C. forskohlii* may prove to be useful in a wide range of clinical conditions. Presently, *C. forskohlii* is best suited for asthma, eczema, hypertensions, congestive heart failure (Anonymous, 2000). Hence, the biomass production is of utmost significance in pharmaceutical industries. Objective 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 *Coleus forskohlii* 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(Dodd,1996). 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 Poly Acrylamide Gel Electrophoresis(PAGE) of proteins has been used extensively for identification and classification at the strain and species level(Candance,1973), The mobility of total proteins during electrophoresis has been used to characterize many organisms including fungi(Garber,1973). 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(Brasier,1991). 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

Four soil samples of 500 grams each were collected randomly from top six-inch layer of soil from each agro climatic zone of Karnataka and packed in polyethylene bag. Each soil sample was sieved

through a 1000µ mesh to remove the bigger soil particles and debris. The sieved soil samples were used for the isolation of the organismby standard plate count method(Malloch,1997). One ml of dilutions was used for plating on Martin's Rose Bengal agar (MRBA) medium and was incubated at 300C 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(Gilman,1961). Further, protein markers were used to compare the ten isolated.

(2) Response of Coleus forskohlii plants to Trichoderma harzianum isolates.

Coleus forskohlii plants 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 100ml potato dextrose broth for 2 weeks. The grown cultures were homogenized and 15ml of each polythene cover with Coleus forskohlii and covers were labeled as C for control and Th1 to Th10 for inoculated covers. Three replicates were maintained for each treatment and were regularly watered. Observation with growth parameters such as plant height, No. of leaves and, root fresh weight, root dry weight , shoot fresh weight, shoot dry weight were studied to analyse effect of *Trichoderma harzianum* on the growth of *Coleus forskohlii* 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 600 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

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

Isolates were grown overnight at 370C 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 20 min at 17000 rpm and the supernatant was collected. The protein content was estimated as described by Lowry et al. (1951) with bovine serum albumin as standard protein. Protein content was adjusted to 100 µg/ml of sample. 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 per cent 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 then rinsed with stacking gel buffer and then filled with staking gel solution 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.70 µl 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,1988). The gel was allowed to stand in solution A for one hour. Then, it was washed 3 times with 50 per cent 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 are seen pour 8 or 10 percent acetic acid 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.

EXPERIMENTAL RESULTS

(1)Isolation of Trichodermaharzianum

Trichoderma harzianum were isolated from different agro climatic zones of Karnataka by growing in the Rose Bengal agar media, by serial dilution plate method. For isolation, preliminary identification was carried out by morphological observations of the fungal colonies such as colour, mycelia growth pattern, colour of the spores etc. All the check isolates and the standard strains formed yellowish green fungal colonies initially and turned to complete black colour after sporulation due to colour of the spores.

Microscopic examination

All the isolates exhibited the typical spore arrangement on the conidial heads as that of the standard reference strain, in which the spores were arranged linearly on the conidial head and also spores.

(2)Response of coleus forskholii to Trichodermaharzianum isolates

The data pertaining to the influence of *Trichoderma harzianum* isolates from ten agro climate zones on plant height of coleus forskholii is presented in Table 3.

The plant height was found to increase steadily with time. The plants inoculated with *Trichoderma harzianum* isolates, increased plant height compared to uninoculated plants throughout the observation period. However, the heights differed significantly among the plants inoculated with various isolates. The least plant height (30.0cms) was recorded in uninoculated control plants while maximum height (62.3 cms) was recorded in plants inoculated with isolate 5, at 60 days after transplanting, which was followed by plants inoculated with isolate 8.

Number of leaves

The data pertaining to influence of the isolates on number of leaves are given in Table 4.

The number of leaves was found to increase progressively with time. It was observed that, number of leaves in inoculated plants was always higher than the control. Maximum number of leaves (118) as observed in plants inoculated with the isolate 5 and least number of leaves (26) was found in uninoculated plants.

However, there was no significant difference in the number of leaves among the inoculated treatments except isolate 5, which had maximum number of leaves.

Table 3. Plant height of coleus forskholii as influenced by *Trichodermaharzianum*

	Plant height (cm) at different intervals	
	15 DAT	60DAT
Control	25.3	30.0
T1	27.5	40.2
T2	30.0	44.3
T3	36.2	43.5
T4	38.4	52.6
T5	45.0	62.3*
T6	35.2	42.7
T7	36.5	45.8
T8	48.0	54.0
T9	50.0	57.5
T10	42.0	47.2

DAT: Days after transplanting

*:Isolate with maximum response

Table 4. Number of leaves of Coleus forskohlii as influenced by *Trichodermaharzianum*

	Number of leaves at different intervals	
	15 DAT	60DAT
Control	20	26
T1	25	32
T2	42	52
T3	58	66
T4	84	93
T5	106	118*
T6	66	76
T7	86	94
T8	97	104
T9	63	71
T10	78	86

DAT: Days after transplanting
*:Isolate with maximum response

Biomass

The fresh weight and dry weight of the plants harvested after 60 days after transplanting are presented in table 5.

The total fresh weight and dry weight in the plants inoculated with *Trichoderma harzianum* isolated were higher than uninoculated plants. Maximum shoot and root fresh weight was recorded in plants inoculated with the isolate of zone 5 (40.65g and 65.29g). It was superior over all other treatments. However, the fresh weight differed significantly among the plants inoculated with various *Trichoderma harzianum* isolates. Minimum shoot and root fresh weight (22.42g and 3.96g) was recorded in uninoculated plants. Maximum shoot and root dry weight was recorded in plants inoculated with isolate from zone 5 (9.68a and 43.03g) respectively, which was significantly higher over all other treatments and minimum shoot and root dry weight (7.16g and 2.37g) was recorded in uninoculated plants. No significant difference in the total dry weight was observed in plants inoculated with all other isolates of *Trichoderma harzianum* except isolates 1 and 2 that showed lower dry weight compared to other treatment

Table 5. Total Biomass of Coleus forskholiias influenced by *Trichodermaharzianumisolates*

	Fresh weight (g/plant)			Dry weight (g/plant)		
Isolates	Shoot	Root	Total	Shoot	Root	Total
Control	22.42	3.96	26.38	7.16	2.37	9.54
1	30.27	2.68	32.95	8.70	0.80	9.50
2	54.02	10.33	64.35	13.75	3.32	17.07
3	46.72	31.50	78.22	12.12	20.03	32.15
4	60.70	34.88	95.58	13.59	14.07	27.66
5	40.65	65.29	105.94*	9.68	43.03	52.71*
6	28.72	38.28	67.00	10.68	19.80	30.48
7	54.78	37.16	91.94	14.49	23.52	38.02
8	67.53	36.72	104.25	19.40	17.56	35.96
9	68.30	20.97	89.27	14.20	7.05	21.25
10	50.06	28.72	78.78	15.59	17.94	33.53

(3) Protein profile of *Trichodermaharzianum*

The data on the protein banding patterns of *Trichodermaharzianum* isolates (plate 6) are presented based on the Relative mobility value (Rm), similarity index and intensity of the bands (Table 8 and 9). Rm value of the bands ranged from 0.006 to 0.54. Among these isolates, isolate 10 was different (one extra band in 0.35, 3.10 and 3.80) from other isolates but rather more similar to isolate 9 as both have a common band in 1.20,while it is absent in all other 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 isolates 1, 4; 2,5 and 4,5 (0.94) whereas, it was less between isolates 1,9; 1, 10;5,10; 6,9; 6,10 and 8, 10 (0.44). A Dendogram was constructed using phylip software showed that, the isolates from zone 3, zone 4, zone 5, zone 6, zone 7, zone 8, zone 9 were grouped together. Isolates from zone 1 and zone 2 were grouped separately and isolate from zone 10 was quite, distinct forming a separate entity.

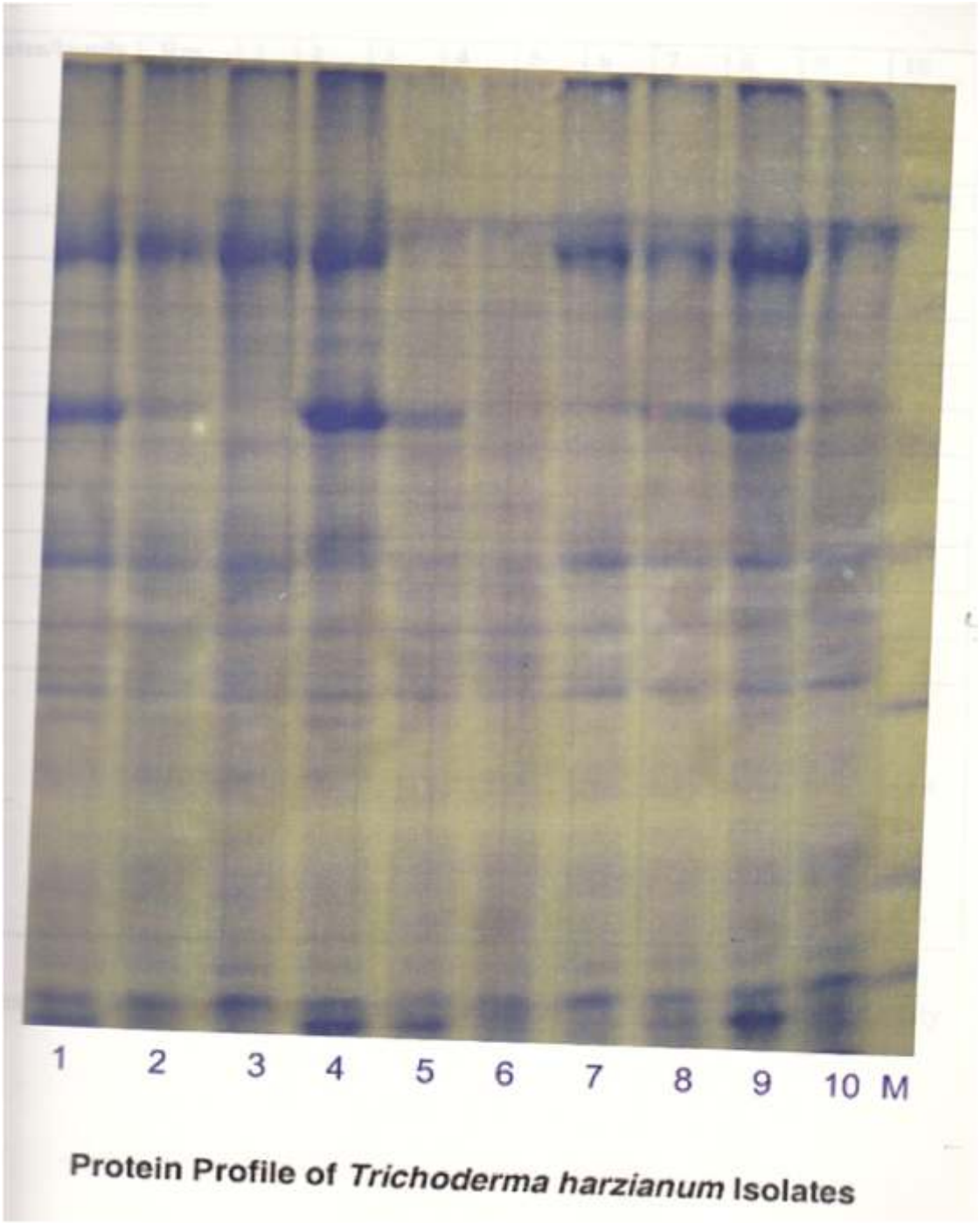


PLATE 6 (M)BSA: Bovines serum albumin used as a protein standard
1-10 Protein samples of 10 isolates of *T. harzianum*

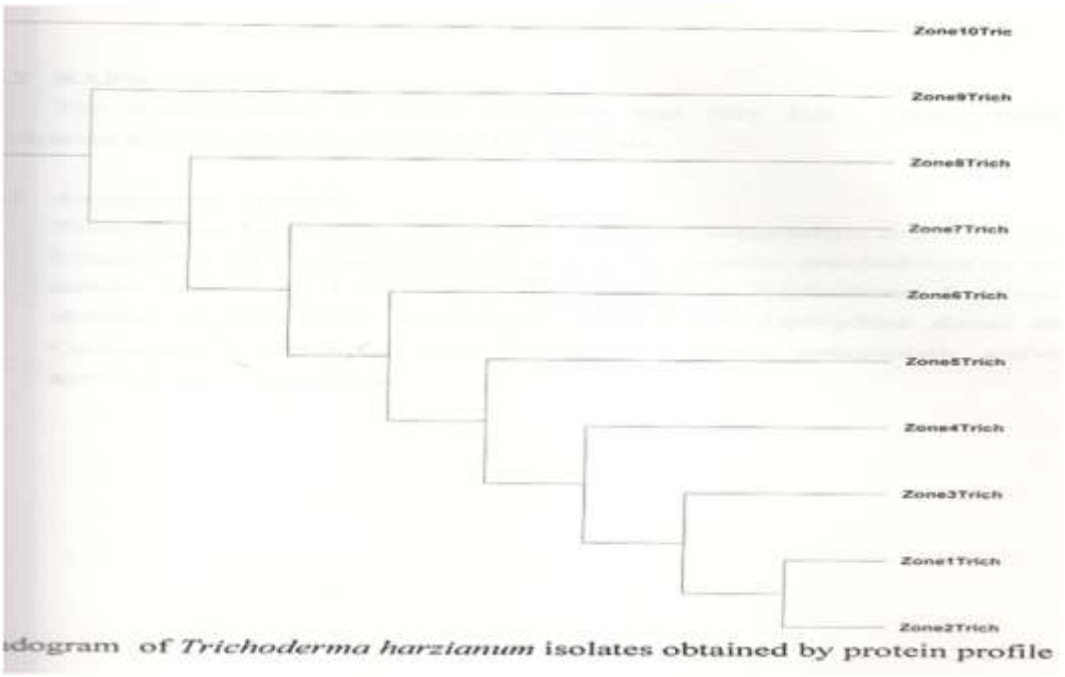
Table 8. Band intensity and Rm value in protein profile of *Trichodermaharzianum* isolates

Isolates/ Bands	Rm Value	1	2	3	4	5	6	7	8	9	10
1	0.006	+	+	+	+	+	+	+	+	++	+++
2	0.013	+	+	+	+	+		+	++	+++	+++
3	0.026			+				++	+	++	+++
4	0.033	++	++	++	+		+				++
5	0.040		++	+			+			+++	+++
6	0.046										++
7	0.053		+	+	+					+	++
8	0.080							+		+	
9	0.100				++					++	
10	0.110	++	++	++	++	+	+	+	++	+++	++
11	0.150									++	
12	0.160	-								++	+
13	0.300	++	++		++	+	+		+	+	+
14	0.320	+	+		+	+			+	+++	+++
15	0.340								++		++
16	0.410										+
17	0.500										
18	0.540										

+ Less band intensity; ++ Moderate band intensity ; +++ High band intensity

Table 9. Similarity index of *Trichodermaharzianumisolates* based on protein profile analysis

	1	2	3	4	5	6	7	8	9	10
1	-									
2	0.83	0								
3	0.88	0.83	0							
4	0.94	0.88	0.83	0						
5	0.88	0.94	0.77	0.94	0					
6	0.77	0.72	0.77	0.72	0.66	0				
7	0.66	0.72	0.66	0.72	0.77	0.55	0			
8	0.77	0.72	0.77	0.72	0.77	0.66	0.77	0		
9	0.44	0.61	0.55	0.50	0.55	0.44	0.77	0.55	0	
10	0.44	0.50	0.55	0.50	0.44	0.44	0.55	0.44	0.66	-



idogram of *Trichoderma harzianum* isolates obtained by protein profile

DISCUSSION

Trichoderma spp.is the most widely studied biocontrol agents (BCAs) against plant pathogens because of their ability to reduce the population of soil borne plant pathogens (Papavizas, 1985). In the present investigation, *Trichoderma harzianum*, a biocontrol agent was isolated from various agro climatic zones of Karnataka. These isolates were examined for their molecular variability using protein markers. Efficiency of *Trichoderma harzianum* was studied in in vitro conditions for confirmation of the isolates and then in a greenhouse experiment using all the isolates to identify efficient isolates using *Coleus forskohlii* as the indicator plant. In the present study, both qualitative and quantitative differences were observed in the protein profile of different *Trichoderma harzianum* isolates. The results suggest that, protein profile data can closely separate isolates from different zones. The in vitro and pot experiment studies varied considerably which may due to several facts such as soil conditions, environmental factors, Synergistic effect of other organisms etc.

Studies on effect of *Trichodermaharzianum* on *Coleus forskholii*

Biological variability studies were also conducted using growing *Coleus forskholii* in the sterilized soil. In this study, different geographical isolates of *Trichoderma harzianum* were used as inoculants. The biological and biochemical changes were recorded in *Coleus forskholii.* The present investigation has showed that, *Coleus forskholii* inoculated with *Trichoderma harzianum isolates* grew taller as compared to uninoculated plants. Similar result was reported by (Harlapur,1988) who worked on interactions between VA mycorrhizal fungus *Glomus fasciculatum,Trichoderma harzianum* and root pathogen, *S.rolfsii* in a pot experiment reported that, combined inoculation of VA mycorrhizal fungus and Trichoderma reduced the severity of foot rot disease of wheat due toS.rolfsii. (Sreenivasa,1994) worked on the biological control of *S. rolfsii* disease of chilli using combined inoculation of *Glomus*

macrocarpum and *Trichoderma harzianum* and found to be effective in suppressing *S.rolfsii.* Also, (Shiva Kumar ,2004) reported that, *A. awamori*, in the presence of compost in the soil significantly increased the biomass, pod weight and oil content of groundnut when compared to the plants inoculated with compost alone.

However, the plants differed significantly in height in response to some isolates within the treatment, but the height was seen in case of isolate 5.Since all the isolates belong to the same species of *Trichoderma harzianum* their effect on growth in terms of height may not be as significant as those usually observed in the plants inoculated with different species or genera or in combination with other beneficial microorganisms. (Weindling, 1932)suggested that, R.Solani was best with *T.viridae, Trichodermaharzianum* and *Trichoderma.koningii* while for control of *Fusariumudum*(Bhatnagar,1996) Padmavathi(2011)studied the phosphate solubility and biocontrol activity of *Trichoderma harzianum* and proved the efficiency of *Trichoderma harzianum* in possessing a mineral phosphate solubilizing ability, that is alternative to chemical fertilizers.

PROTEIN PROFILE ANALYSIS

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 DNAfinger printing(Priest and Austin,1993)in some cases.

In the present study ,both Qualitative and Quantitative differences were observed in the protein profile of different *Trichodermaharzianum* isolates (Plate 6).The data on the protein profile of *Trichoderma harzianum* isolates are presented on the Rm value , Similarity index and intensity of bands (Table 14 and Table15).The above results suggest that, protein profiles data can closely separate isolates from different zones.The results agree with the work done by several scientists such as (Aly,2003) wherein the protein profile data of Fusarium isolates of cotton obtained from different areas clearly separated the isolates. These results also agree with the results obtained by (Mandeel,1994) who compared SDS-PAGE patterns from eight isolates belongingto three Fusarium species.Protein profiles were distinct and each isolate showed unique characteristic profile. The data obtained from protein profiles support the potential use of this experimental approach to help distinguish between different Fusarium isolatesv.On the contrary ,(Belisario 1998) found no differences when a comparison of total mycelial protein profiles of different species of *F.oxysporum ,F.solani,F.culmorum* was done.

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